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# Ecotoxicology of marine biotoxins in bivalve shellfish

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**A thesis submitted in fulfilment of the requirements for the degree of**

**Doctor of Philosophy**

**National University of Ireland, Cork**

**School of Biological, Earth and Environmental Science**

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# DECLARATION

This thesis is my own work and has not been submitted for another degree at University College Cork or elsewhere.

Signed: _____

Moira McCarthy (PhD researcher)

Date: _____

**ABSTRACT**

A small proportion of marine phytoplankton species produce phycotoxins that can be harmful to co-occurring organisms. When these algae form Harmful Algal Blooms (HABs) they can have negative effects on the shellfish aquaculture industry through accumulation of phycotoxins in the tissues of shellfish species, leading to closure of harvesting grounds. Algal toxins can cause illness in humans that consume contaminated shellfish, thus monitoring the occurrence of algal toxins is important for the protection of human health. However, the effects of these biotoxins on the shellfish that accumulate them are little-studied, and further economic losses could be incurred if they are a source of physiological stress for the shellfish.

This study examined the use of Solid Phase Adsorption and Toxin Tracking (SPATT) as a tool for the passive sampling of algal biotoxins at Lough Hyne Marine Nature Reserve in West Cork, Ireland. Spatial and temporal monitoring of the incidence of a wide range of lipophilic toxins was assessed over a 4-month period and toxin occurrence was primarily in the top 20 m of the water column. Active sampling of the water column was also performed to determine whether sufficient quantities of toxin could be accumulated for use in subsequent experimentation. A submersible pump coupled with graded filtration units and two cartridges filled with adsorbent resin was deployed for 7-days and quantities of marine toxins were accumulated on the resin. In addition to commonly occurring Diarrhetic Shellfish Poisoning (DSP) toxins, Dinophysis toxin-1 (DTX1) and Pinnatoxin G were both detected in the samples from Lough Hyne. This is the first identification of these latter two toxins in Irish waters.

The effects of the DSP toxin okadaic acid (OA) were investigated on three shellfish species: *Mytilus edulis*, *Ruditapes philippinarum* and *Crassostrea gigas*. Three biological organisational levels were examined experimentally to ascertain whether OA had an impact at the tissue, cellular and subcellular levels using three concentrations. Histological examination of the gill, mantle and hepatopancreas tissues revealed varying intensity of damage depending both on the tissue type and the species involved. In all three species, significant effects were observed in the

hepatopancreas tissues of the shellfish. Effects on the mantle were observed in *Mytilus edulis* and *Ruditapes philippinarum*; no significant increase in pathology or immune response to the toxin was observed in the gill tissues of the three species.

At the cellular level, flow cytometric analysis of the differential cell population distribution was assessed in the three species. Long-term versus short-term fixation methods were evaluated and it was confirmed that samples were successfully preserved in 6% formalin: seawater for up to 8 months prior to analysis using flow cytometric analysis. No change in cell population distribution was observed in *Mytilus edulis* or *Ruditapes philippinarum*, however in *Crassostrea gigas* significant changes were observed in the granulocytes at the lower levels of toxin exposure. This indicated a chemically-induced response to OA in this shellfish species.

Cell viability and DNA fragmentation were measured in the haemolymph and hepatopancreas cells post OA-exposure in *Mytilus edulis* and *Crassostrea gigas*. Cell viabilities remained above 85% for both shellfish species. A significant increase in DNA fragmentation was observed in both species over time, even at the lowest OA concentrations, using 1-way ANOVA ($p < 0.05$) a noteworthy result for these important aquaculture species. The DNA fragmentation could be due to genotoxicity of OA and/or to the induction of apoptosis in the cells. This study highlighted that the commonly occurring biotoxin OA, can have negative impacts on bivalve shellfish, even at low levels, causing physiological stress to the animals which may lead to greater susceptibility to disease and parasitism.

# CHAPTER 1

# General Introduction

## Background Information

Phytoplankton are single-celled, microscopic plants that inhabit marine and freshwater environments. These plants are a key component of food-web structure and provide a vital source of food for co-inhabiting organisms. Of the 3,400-4,100 phytoplankton species present in the marine environment, about 300 (7%) produce red tides and only 60-80 (2%) of these species are actually harmful. These harmful algal blooms (HABs) can be separated into two groups: the first group cause harm through their physical structure or biomass, by altering food-web structure and through their decomposition deoxygenate the water; the second group are those that release dangerous toxins and metabolites. Approximately 75% (45-60) of all harmful species are dinoflagellates (Smayda 1997). The occurrence of HABs are firmly rooted in history and have been mentioned by scholars such as Giraldus Cambrensis and Shakespeare (Fogg 2002). A description in the Old Testament of the Bible is often referenced as the first written record of a toxic red tide causing fish kills (Landsberg 2002): Exodus 7:20-21: "all the water of the river was changed into blood. The fish in the river died and the river itself became so polluted that the Egyptians could not drink the water".

Rising global ocean temperatures, increasing occurrence of extreme weather events (such as El Nino), as well as growing coastal eutrophication have all been linked to an increase in the incidence of HABs worldwide (Anderson *et al.* 2002, James *et al.* 2010). The growing geographical spread of these harmful species has been attributed to ballast waters transporting encysted algae to new environments and similarly, spread of algae by aquaculture (van Dolah 2000, Anderson *et al.* 2002, Masó and Garcés 2006, Smayda 2007). Toxins produced from algal species have been found to accumulate in filter feeding animals such as sponges and ascidians, and also in bivalve shellfish (Kumagai *et al.* 1986, Campas *et al.* 2007, Reizopoulou *et al.* 2008).

The shellfish industry is of global economic importance. The increase of these toxin-producing HAB's has negative implications for these farms, causing closure for weeks at a time, particularly during the summer months (Hoagland *et al.*

2002, Sellner *et al.* 2003). Phytoplankton levels in the water and the toxin-burden of shellfish are rigorously monitored by regulatory bodies in shellfish producing countries, using multiple detection methods (James *et al.* 1997a, Draisci *et al.* 1999, Furey *et al.* 2001, Lehane *et al.* 2002) to ensure levels of toxin in the tissues remain below the limits deemed safe for human consumption (Alexander *et al.* 2008) (Table 1.1).

Table 1.1. Shellfish poisoning syndromes, the causative toxins and the E.U. limits for human consumption in shellfish (Alexander *et al.* 2008).

| **Toxic syndrome** | **Causative Toxins** | **Limits for human consumption** |
|---|---|---|
| Diarrhetic shellfish poisoning (DSP) | Okadaic acid (OA), dinophysis toxins (DTXs), pectenotoxins (PTXs) | 160 µg/kg shellfish meat |
| Paralytic shellfish poisoning (PSP) | Saxitoxin (STX), neosaxitoxin (NEO), gonyautoxin (GTX) | 800 mg/kg shellfish meat |
| Amnesic shellfish poisoning (ASP) | Domoic acid (DA) and analogues | 20 mg/kg shellfish meat |
| Neurotoxic shellfish poisoning (NSP) | Brevetoxins (PbTx) | 1 mg/kg shellfish meat |
| Azaspiracid poisoning (AZA) | Azaspiracids (AZAs) and analogues | 160 µg/kg shellfish meat |

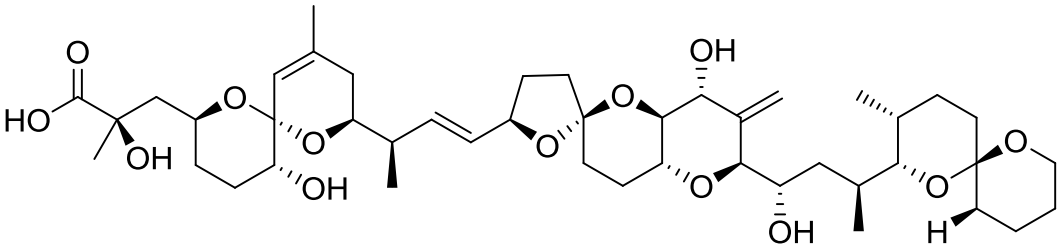
## Why do some algae produce toxins?

A frequently posed question in harmful algal research is, why do these species of algae produce such noxious chemicals? It is unclear what ecological or evolutionary advantages these toxins confer. There are a few theories expounding the reasons behind the production of these algal biotoxins. These biotoxins may be plankton 'pheromones' that promote mating of gametes (Wyatt and Jenkinson 1997), or the more commonly expressed theory explaining toxicity is that of defence against predation from zooplankton and filter feeding invertebrates (Wyatt and Jenkinson 1997, Landsberg 2002, Galimany *et al.* 2008c). In many terrestrial plant species, adaptations in morphology such as spiky leaves and the production of allelochemicals or antiherbivory compounds, which can be foul tasting and poisonous act as a deterrent to herbivores (Chen 2008). Selander *et al.* (2006) linked an increase of toxin production in the paralytic shellfish poisoning algae *Alexandrium minutum* to the presence of the copepod *Acartia tonsa*, conferring

increased resistance to grazing on the dinoflagellate. The more indirect effects of these phycotoxins produced in dinoflagellate species is their toxicity to the human consumers of contaminated bivalve shellfish. In addition to their human health effects, HABs have been linked with episodic fish kills and increased mortalities in marine mammals and birds, dependent on marine food web (van Dolah 2000).

## Toxic syndromes of Shellfish Poisoning

There are five main toxic syndromes associated with shellfish contamination from toxic phytoplankton species; these are named according to the acute symptoms they produce in human consumers (James *et al.* 2010) (Table 1.1). Gastrointestinal symptoms such as nausea, stomach cramps and diarrhoea are common to all of the toxic syndromes. In the case of **Amnesic Shellfish Poisoning (ASP)**, at high doses neurological symptoms can develop, as well as dizziness, headache and short-term memory loss. At very high concentrations exposure can be fatal, particularly in the elderly. **Neurotoxic shellfish poisoning (NSP)** causes neurological symptoms such as slurring of speech, peripheral tingling and in severe cases broncho-constriction, seizures and coma. Mild symptoms of **Paralytic shellfish poisoning (PSP)** are numbness and tingling in the lips, which spreads to the face and neck and is accompanied by a prickly sensation in fingers and toes. More serious intoxication can result in nausea, vomiting, increasing muscular paralysis and respiratory difficulty (Popkiss *et al.* 1979). **Diarrhetic shellfish poisoning (DSP)** is the most common type of shellfish poisoning and is often mistaken for bacterial food-poisoning. Symptoms include diarrhoea, nausea and vomiting. **Azaspiracid shellfish poisoning (AZA)** is the newest toxin found to accumulate in shellfish species (Satake *et al.* 1998) and is the only one not named after the symptoms produced. After ingestion, the acute response is similar to DSP- poisoning. Nausea, stomach cramps, vomiting and diarrhoea. Symptoms from acute short-term exposure are well documented, but the effects of sub-acute and chronic exposure to these marine biotoxins in humans are still under investigation (Alexander *et al.* 2008). In this study we will be focusing on the marine biotoxins from the diarrhetic shellfish poisoning group, with particular emphasis on the effects of okadaic acid (Figure 1.1) on mammalian and invertebrate models.




Okadaic Acid

Chemical Formula: $C_{44}H_{68}O_{13}$
Exact Mass: 804.46599
Molecular Weight: 805.00292
m/z: 804.46599 (100.0%), 805.46935 (47.6%), 806.47270 (11.1%), 806.47024 (2.7%), 807.47606 (1.7%), 807.47359 (1.3%)
Elemental Analysis: C, 65.65; H, 8.51; O, 25.84

Figure 1.1. The chemical structure of okadaic acid (Suganuma *et al.* 1988), its formula, molecular mass and mass to charge ratio. (ChemBioDraw version 11.0).

## Detection of DSP toxins

### *Mouse and rat bioassay*

In order to prevent the intoxication of humans through consumption of contaminated shellfish, legislation has been developed and monitoring programmes for both the toxin-producing phytoplankton and the levels of toxin in shellfish meat deemed safe for human consumption have been implemented. Up until 2012 the mouse and rat bioassay were the official methods for the detection of lipophilic marine toxins from shellfish extracts in the European Union (European Commission 2004).

The mouse bioassay involved administering samples of extract from suspected contaminated shellfish, to mice. Briefly, the toxins were extracted from tissues using acetone, followed by a liquid-liquid partitioning using dichloromethane or dichloroether. This was evaporated and reconstituted in 1% polysorbate solution. The extract is injected intraperitoneally into three male mice weighing 20 g. If at least two out of three mice died within 24 hours of injection then the samples were considered contaminated with lipophilic marine toxins (Authority 2001, Gerssen *et al.* 2010). For the rat bioassay, starved female rats (24 hours) were fed 10 g of the shellfish hepatopancreas. After 16 hours, softening of the faeces was investigated and severe diarrhoea corresponded with the presence of toxin levels at approximately the current EU regulatory limits (Gerssen *et al.* 2010) (Table 1.1). These methods were imprecise, lacking specificity and sensitivity. Thus, alternative methods have been investigated and implemented.

### *Liquid Chromatography-Mass Spectroscopy (LC-MS)*

Liquid Chromatography-Mass Spectroscopy (LC-MS), in conjunction with phytoplankton monitoring and assessment, has become the primary technique adopted throughout the E.U for the majority of marine phycotoxins, excluding Paralytic Shellfish Poisoning (PSP) toxins and Domoic acid DA) (Annex III to Regulation (EC) No 2074/2005, Chapter III) for which alternative detection procedures exist. For PSP toxins there is a Prechromatographic Oxidation and Liquid Chromatography method (AOAC 2005) and for DA there is an enzyme-linked

immunosorbent assay for detection (Kleivdal *et al.* 2007). LC-MS has been proven a sensitive and valuable analytical tool for the identification and quantification of marine biotoxins in shellfish tissues, and for the elucidation of new compounds (James *et al.* 1997a, James *et al.* 1997b, Satake *et al.* 1998). However despite its high precision as a detection method, there are some disadvantages to the method. It requires costly equipment and highly trained personnel to operate it, and a wide range of reference standards are needed for identification and quantification (Alexander *et al.* 2008).

***Biochemical and* in vitro *methods***

A number of biochemical and *in vitro* methods have also been explored for the detection of lipophilic marine toxins from shellfish and phytoplankton. Laycock *et al.* (2006) developed an assay based on lateral flow immunochromatographic test strips using antibody methods for the detection of DSP toxins, Jellett Rapid Testing Ltd. The method is accurate to 5 nM for OA, Dinophysis toxin-1 (DTX-1) and DTX-2. The Enzyme-Linked-Immunosorbent assay (ELISA) has been successfully applied to the detection of DSP toxins directly from toxic algal samples (Imai *et al.* 2003). Mouratidou *et al.* (2006) compared ELISA, high performance liquid chromatography (HLPC) methods and the mouse bioassay for the detection of OA and found significant correlation between the ELISA and HPLC methods.

A phosphate inhibition assay (PP2A) based on the mode of action of OA has also been developed (Simon and Vemoux 1994). The assay was rapid, reproducible and simple, providing an excellent method for the detection of OA in complex matrices. Tubaro *et al.* (1996) optimised the assay further and used a commercially available PP2A preparation. High sensitivity in okadaic acid detection was achieved, concentrations as low as 0.063 ng/ml in aqueous solutions and 2 ng/g in mussel digestive glands were measured. Vieytes *et al.* (1997) combined the PP2A assay with a fluorometric multiwall plate method, and achieved a detection level for OA of 12.8 µg/kg of mussel hepatopancreas, optimising the method further to allow high sample throughput.

## Passive toxin monitoring

In addition to using LC-MS techniques for the detection of toxins from shellfish extracts, a system for the passive monitoring of biotoxins directly from the water column was developed using these methods coupled with Solid Phase Adsorbent and Toxin Tracking (SPATT) (MacKenzie *et al.* 2004). The protocol was based on the observation that during HABs, quantities of toxin 'leak' from the cell and are suspended in the seawater (MacKenzie *et al.* 2003). These toxins can then accumulate passively onto adsorbent resin for subsequent extraction and analysis using LC-MS techniques. In the study performed by MacKenzie *et al.* (2004) SPATT bags were constructed using nylon mesh and filled with the adsorbent resin. Various alterations of this original design have been proposed, such as the fastening of the resin within the nylon mesh using embroidery hoops (SPATT discs) (Fux *et al.* 2008) and utilising a heat-sealer to fasten the edges of the bags as a time-efficient alternative to sewing (Wood *et al.* 2011). The bags/discs were then deployed in the aquatic environment for varying time periods. A number of trials have been completed using the SPATT technology to determine its efficacy as an early-warning detection system for marine algal toxins, with mixed results (Turrell *et al.* 2007, Rundberget *et al.* 2009, Rodríguez *et al.* 2011). However despite some questions regarding its effectiveness as an early warning tool for HAB's, SPATT has a number of advantages over the use of phytoplankton samples to determine the presence of toxic algae in the water column. Phytoplankton sampling gives a 'snapshot' of the species present at the time and depth of sampling, while the SPATT bags provide time and spatial integrated monitoring of the water column over a pre-determined period of time (Fux *et al.* 2009). Phytoplankton identification is labour-intensive and requires highly trained personnel, while the manufacturing of SPATT bags/discs is simple with a low-cost per unit (MacKenzie 2010). The adsorption of the biotoxin is direct and there is no biotransformation of the toxins, such as fatty acid esterification found in shellfish (Vale *et al.* 1999). Lack of biotransformation coupled with relatively 'clean' sample matrices simplifies the extraction and analysis of toxins accumulated using this method (MacKenzie 2010).

## Active toxin sampling

Based on the efficacy of adsorbent resin as a biotoxin accumulator, Rundberget *et al.* (2007) performed large-scale, active sampling of a water body. This was done to determine if the sampling resin found by previous studies (MacKenzie *et al.* 2004, Fux *et al.* 2008) to be the most efficient accumulator of lipophilic biotoxins (Diaion HP-20) could be used for large-scale bioharvesting of marine biotoxins, for subsequent experimentation. A machine consisting of a seawater pump, coupled with different grades of filtration units and four columns filled with 500 g of adsorbent resin was constructed. Seawater was pumped through the machine from a site in Norway with low densities of algae, and a site in Spain which had a bloom of *Dinophysis acuta*. High quantities of OA and DTX-2 were successfully harvested using this method at both sites, 2.7 mg OA and 1.3 mg DTX2 in Spain after two days of sampling and 1.8 mg OA and 0.8 mg of DTX2 after 16 days of pumping in Norway.

## Diarrhetic shellfish poisoning toxins

The main focus of this investigation was the DSP toxins. This group of toxins include okadaic acid, DTX1 and DTX2, collectively known as the OA-group of shellfish toxins. As mentioned, the main physiological impact of these toxins in humans is nausea, stomach cramps and diarrhoea (James *et al.* 2010), these symptoms appear within three hours of consumption. These toxins are lipophilic, thermostable, polyether compounds (Figure 1.1) that are produced by a number of dinoflagellates of genus *Prorocentrum* and *Dinophysis* and have been found to accumulate in filter feeding animals such as sponges and ascidians, and also in bivalve shellfish (Kumagai *et al.* 1986, Campas *et al.* 2007, Reizopoulou *et al.* 2008). DSP toxin-producing algae have been identified off of the Irish coastline in varying abundances, among them are the species *Dinophysis acuta*, *Dinophysis acuminata*, and *Prorocentrum lima* (James *et al.* 1999, Fux *et al.* 2009), and the

increase and spread of these toxins are of concern in Europe, and indeed, worldwide (Figure 1.2).

***Okadaic acid***

Okadaic acid (OA) was the primary toxin utilised in exposure experiments in this study. The toxin was first isolated from two sponge species, *Halichondria okadai* Kadota, a black sponge found on the Pacific coast of Japan and *H. melanodocia* a Caribbean sponge found in the Florida Keys (Tachibana *et al.* 1981). OA is a known inhibitor of serine/threonine protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) activity, which are important in signalling cascades and regulate different cellular processes in eukaryotic cells (Bialojan and Takai 1988, Vieytes *et al.* 1997). The diarrhoeogenic effect of okadaic acid is caused by the accumulation of phosphorylated proteins which control sodium secretion in cells, and the inhibition of dephosphorylation of cytoskeletal elements that regulate permeability to solutes, in combination these cause a passive loss of fluids (Tubaro *et al.* 1996).

***OA effects on mammalian cells***

In initial *in vivo* studies examining the effects of okadaic acid on mammalian cells, it was found to be a tumour-promoter in mouse skin and the mucosa of rat glandular stomach (Suganuma *et al.* 1988, Fujiki and Suganuma 1999). There has been some debate regarding the *in vitro* effects of this compound on mammalian cells and the results reported with regard to OA cytotoxicity and genotoxicity are often contradictory. In a study by Souid-Mensi *et al.* (2008) a number of cell lines were examined using cytotoxicity and genotoxicity assays and it was concluded that both the cytotoxic and genotoxic effects of OA are dependent on the cell-type and on the concentration of toxin present. Short-term experiments have measured genotoxic and mutagenic effects of OA on a number of mammalian cell lines (Rogers *et al.* 1994, Fessard *et al.* 1996). Fessard *et al.* (1996) discovered genotoxic impacts of OA on BHK21 C13 fibroblasts and HESV keratinocytes by measuring increased DNA adduct formation in cell lines exposed to the biotoxin. Rogers *et al.* (1994) determined that OA may function as a direct-acting mutagen on V79 Chinese hamster lung cells. A number of studies have established that OA causes apoptosis in some mammalian cell lines. This was first described by Bøe *et al.* (1991) according

to morphological criteria in GH3 cells, at very high OA concentrations (500 nM). Since then OA has been shown to induce apoptosis in many cell lines; human colonic epithelial cells, CHO K1 cells; BALB/c 3T3 cells and amniotoic FL cells (Traoré *et al.* 2001, Huynh-Delerme *et al.* 2003, Le Hégarat *et al.* 2005, Ao *et al.* 2008, Xing *et al.* 2009).

***OA effects in bivalve shellfish***

DSP algal toxins which cause harmful effects on human consumers have also been found to negatively impact on shellfish species. These effects vary greatly between species. Vast intra-species variation in both toxin accumulation and depuration rates have also been recorded (Blanco *et al.* 1999, Svensson 2003, Mafra Jr *et al.* 2010a, Mafra Jr *et al.* 2010b), as well as transformation rates of OA-group toxins into fatty-acid acyl derivatives (DTX-3) (Torgersen *et al.* 2008). Differences have been attributed to environmental conditions such as temperature, and salinity as well as the body weight of the individual animal (Blanco *et al.* 1999, Svensson and Förlin 2004) and to feeding mechanisms such as reduced filtration and pre-ingestive rejection of food particles in some species (Mafra Jr *et al.* 2010b). The mechanism of filtration is similar in all bivalves, however, the fate of the filtered food is determined by the differing arrangement of the frontal cilia, which varies between species (Morton 1983).

Blanco *et al.* (1999) tested one and two-compartment models of depuration in *Mytilus galloprovincialis*, with the model fit depending greatly on the inclusion of variables such as temperature, salinity, light transmission fluorescence and body weight. The one compartment model gave a good fit when these variables were included. However, if the two-compartment model outlined in that study was considered the most realistic, it was concluded that environmental variables have little or no importance in regulating depuration. Svensson and Förlin (2004) discovered that increased lipid breakdown did not correlate with increased depuration rates and short-term regulation of the external environment (temperature/food limitation) did not influence the rate of depuration. However, their experimental observations provided information on the depuration rates of *Mytilus edulis* with the half life of OA measured at $t_{1/2}$ = 8 days.

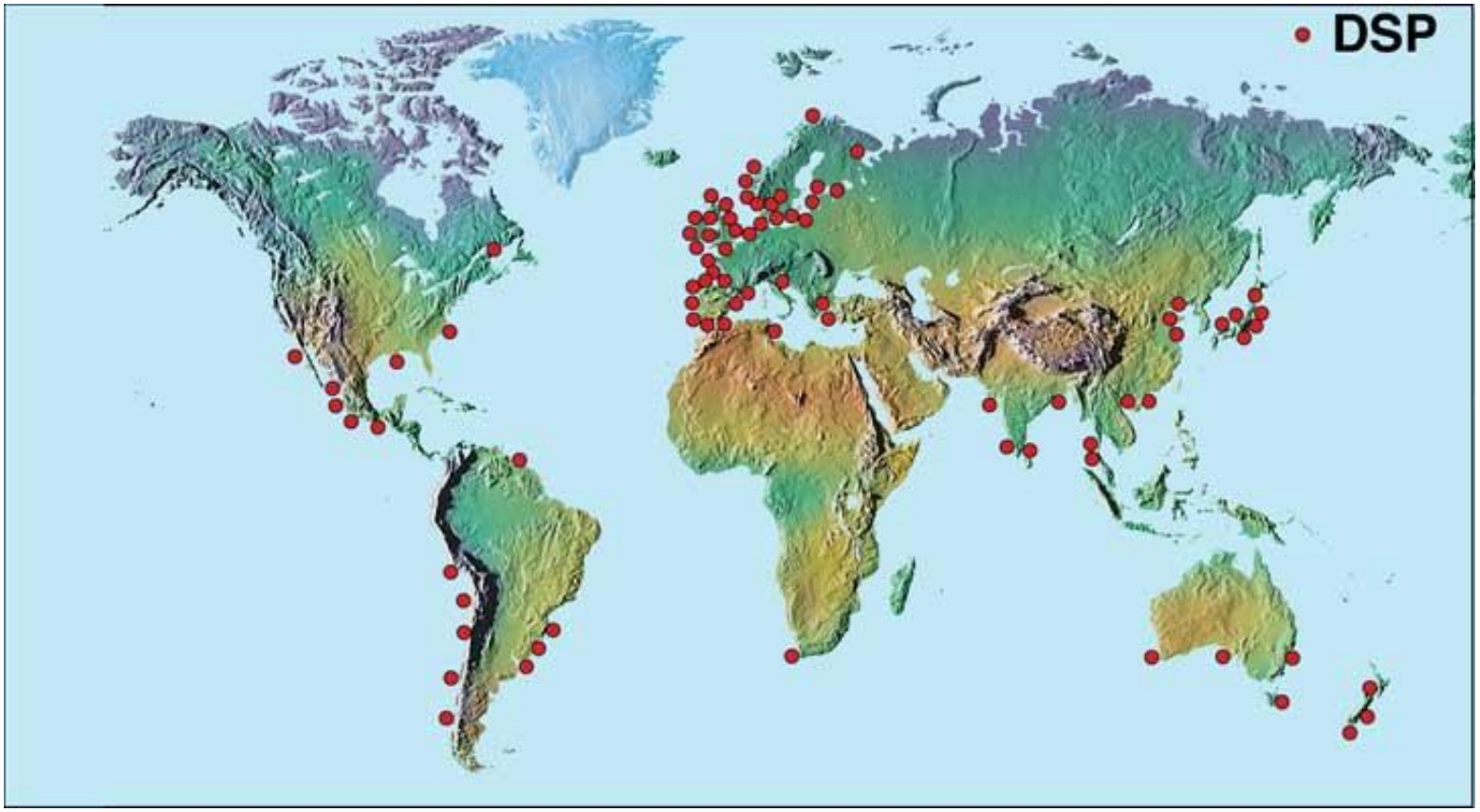



Figure 1.2. Map of Diarrhetic shellfish poisoning (DSP) toxin distribution worldwide (picture from http://www.whoi.edu)

● = DSP toxin detected in shellfish tissue.

***Phycotoxin effects at different organisational levels***

**Tissue**

Negative effects of a number of marine phycotoxins have been recorded at different levels of organisation in bivalve shellfish including DSP, PSP and vererupin toxins (Pearce *et al.* 2005, Talarmin *et al.* 2007, Galimany *et al.* 2008b, Galimany *et al.* 2008c, Flórez-Barrós *et al.* 2011). Accumulation of lipophilic shellfish toxins in bivalves has been found to occur primarily in the digestive gland (Yasumoto *et al.* 1978, Blanco *et al.* 2007). Thus, it is not unexpected in histological studies examining the effects of marine biotoxins on shellfish, that thinning of the gut wall tubules and sloughing of cells has been observed in the digestive gland (Wikfors and Smolowitz 1995, Pearce *et al.* 2005, Galimany *et al.* 2008a, Galimany *et al.* 2008c). Other tissues have also been found to respond to the presence of these marine toxins, such as gill and mantle (Bricelj *et al.* 1992, Pearce *et al.* 2005).

**Cellular**

Within the circulating haemolymph, the effects of a number of algal toxins on the immune status of different bivalve species have been investigated. A number of criteria such as differential cell population changes, cell number, phagocytosis, aggregation, and increased reactive oxygen species have been measured (Hégaret and Wikfors 2005a, b, Hégaret *et al.* 2007b, Mello *et al.* 2010). The impacts vary considerably depending on both bivalve and algal species, emphasising the necessity of examining the effects of these toxin-producing algae on multiple bivalve species. Host-parasite interactions and the immune-modulating effects of harmful algae on these relationships have also been explored (Hégaret *et al.* 2007a, da Silva *et al.* 2008, Hégaret *et al.* 2010). Hégaret *et al.* (2009) found that exposure to *Prorocentrum minimum* caused an increased susceptibility to *Perkinsus olseni* in the manila clam *Ruditapes philippinarum*. Bivalve gill, haemolymph and heart cells have been found to be relatively resilient to the cytotoxic effects of OA (Svensson *et al.* 2003, Talarmin *et al.* 2007, Flórez-Barrós *et al.* 2011).

**Subcellular**

At the subcellular level, induction of micronuclei and DNA fragmentation has been measured in bivalve species exposed to DSP toxins (Carvalho Pinto-Silva *et al.* 2003, Carvalho Pinto-Silva *et al.* 2005, Flórez-Barrós *et al.* 2011). There are a few studies connecting known genotoxic compounds and DNA fragmentation with negative impacts on aquatic species; Steinert *et al.*, (1998) linked DNA fragmentation, measured in mussels using the comet assay, at San Diego Bay with decreased growth rates and Lee *et al*. (2000) demonstrated that *Palaemonetes pugio* embryos exposed to chromium (III) chloride, sodium chromate, mercuric chloride, and 2-methyl-1,2-naphthoquinone (MNQ) showed reduced hatching rate of embryos at early developmental stages and high DNA tail moments in the exposed embryos. To date, it has not been established whether the DNA fragmentation measured post-exposure to OA in shellfish is due to a direct genotoxic effect, or whether it is due to the same apoptotic effect of OA previously measured in mammalian cells (Traoré *et al.* 2001, Huynh-Delerme *et al.* 2003, Le Hégarat *et al.* 2005, Ao *et al.* 2008, Xing *et al.* 2009).

## Gaps in knowledge

Review of the literature has revealed that there are still many gaps in our knowledge of the impact of DSP algal biotoxins, and indeed many other phycotoxins, on numerous shellfish species. In order to perform a toxicological study examining the impact of marine algal biotoxins, it is essential to have access to the toxin or the toxin-producing algae. This lack of availability is a major challenge that has been an important limitation when investigating effects of marine biotoxins on mammalian cells and bivalves. Until recently, only a small proportion of biotoxins were commercially available in quantities sufficient to enable experimentation on shellfish species. Even still, costs are prohibitive for large-scale experimentation.

The alternative route of exposure is harvesting of toxic algal species from the marine environment and culturing in the laboratory. This presents a difficulty in

itself, as toxic algae of species *Dinophysis* are very difficult to grow in the laboratory, and only in the past decade has the species *Dinophysis acuminata* successfully been cultured (Park *et al.* 2006). From a scientific standpoint, exposure regimes utilising a purified toxin or a cultured algal species both have their merits. Many DSP-producing algal species, such as *Dinophysis acuta*, *Dinophysis acuminata* and *Prorocentrum lima* have been found to produce multiple DSP-toxins (OA, DTX1, PTX2, PTX11, PTX2-SA) (Lee *et al.* 1989, Puente *et al.* 2004) these can vary in concentration, depending on the stage of growth of the algae (MacKenzie *et al.* 2005). Thus, the endpoints from exposure experiments using toxin-producing algae, examine the effects of multiple toxins on shellfish species; this is a relevant technique for the determination of the overall negative impacts of DSP-toxin producing algae. However, the use of purified toxin will provide information on the effects of one specific biotoxin and all negative responses observed can be attributed to the effects of that toxin. This is important information as it is possible that not all toxins produced from algal species have the same effects on shellfish. In addition to these challenges, it is clear from the literature that the effects of these toxins differ between species. These differences have been attributed to varying rates of accumulation and depuration between species (Blanco *et al.* 1999, Svensson 2003, Mafra Jr *et al.* 2010a, Mafra Jr *et al.* 2010b) due to environmental factors such as temperature and salinity (Blanco *et al.* 1999, Svensson and Förlin 2004) or greater selectivity regarding food intake, coupled with differing capabilities for biotransforming and excreting the phycotoxins (Torgersen *et al.* 2008). Thus, it is important to measure effects on multiple species of shellfish and not rely on results obtained from one species, and using these, infer that similar responses occur within all bivalves.

## Study outline

This project was a component of an interdisciplinary, collaborative research programme, between University College Cork and the the Mass Spectrometry Research Centre for Proteomics and Biotoxins (PROTEOBIO), Cork Institute of Technology. The main focus of this research was to assess the ecotoxicology of marine biotoxins with particular reference to marine molluscs. In addition to this

original objective, research was also performed to determine whether methods exist for the efficient harvesting of marine biotoxins from the aquatic environment, for subsequent use in scientific experimentation.

Two main questions were addressed:

*Can adequate quantities of marine biotoxins be harvested from the marine environment using existing sampling methods?*

*Does okadaic acid have a negative effect on marine bivalves when examined using a multi-species and multi-biomarker approach?*

To accumulate large quantities of biotoxin for subsequent experimentation an active sampler was constructed based on the design and methodology of Rundberget *et al.* (2007). This active sampler was deployed at Lough Hyne Marine Reserve, Co Cork, Ireland to determine whether successful accumulation of large quantities of marine phycotoxins could be achieved in this location. Passive SPATT samplers were also deployed at Lough Hyne to determine their efficacy as a monitoring tool for establishing the temporal and spatial distribution at which marine phycotoxins can occur.

In addition, the effects of one marine biotoxin, OA, were measured at different organisational levels on three commercially important bivalve species, *Mytilus edulis*, the blue mussel; *Ruditapes philippinarum*, the manila clam and *Crassostrea gigas*, the pacific oyster. Okadaic acid is a commonly occurring toxin in coastal waters worldwide. The shellfish chosen for experimentation are farmed off of the Irish coastline, and are important aquaculture species in many countries. HABs are known occur primarily in the summer months, however in the current series of experiments, many of the exposure experiments were performed from October to March, during winter and early spring. This was to facilitate extracting haemolymph samples for DNA instability analysis and cell population distribution analysis, without the presence of sperm or egg cells in the samples. Attempts to extract haemolymph during periods of late and ripe gonad development were generally unsuccessful, however successful extraction of mussel HL was performed in June 2011 and these samples were subsequently utilised in the comet assay. For the experiments carried out during the winter months, it was hypothesised that a

response during the resting stage of development when less energy is being expended on gonad development would still provide a valid measurement of the negative effects of OA on these shellfish species. In addition, the low concentration levels utilised in this series of experiments have been found in Irish mussels year-round, thus providing further validation for the time periods chosen when performing these experiments, particularly for the lower toxin concentrations (Table 1.2).

Table 1.2. Month and year when each exposure experiment was performed on the three bivalve species and the methods utilised to assess OA damage for each experiment.

| ***Histology/FCM*** | ***Mytilus edulis*** **Winter/ (Summer)** | ***Ruditapes philippinarum*** | ***Crassostrea gigas*** |
|---|---|---|---|
| **2 µg/tank** | November 2010 (June 2010) | December 2010 | January 2011 |
| **1 µg/tank** | November 2010 (June 2010) | December 2010 | January 2011 |
| **40 µg/tank** | June 2011 | | January 2012 |
| ***Comet assay/FCM**** | ***Mytilus edulis*** | ***Ruditapes philippinarum*** | ***Crassostrea gigas*** |
| **2 µg/tank** | November 2011 | Excluded | February 2012 |
| **1 µg/tank** | November 2011 | | February 2012 |
| **40 µg/tank*** | June 2011 | | January 2012 |

Three different concentrations of OA were administered over two week-long exposure regimes for each shellfish species. In toxicology an **acute exposure** is defined as a single dose of a chemical at the beginning of an exposure experiment, the effects of which are monitored over the subsequent days. **Sub-acute exposure** is a short-term exposure consisting of daily quantities of a toxic substance administered to the test organism. The exposure time period is too short to be considered chronic or sub-chronic.

In the current study the first exposure regime was a single (acute) exposure of the shellfish to 2 µg into a 15 l tank of OA, giving an approximate value of 0.1 µg/shellfish of okadaic acid. This was performed to establish whether low quantities

of OA would elicit a 'response' in the shellfish species. The second exposure regime utilised two concentrations of toxin 1 µg into a 15 l tank and 40 µg into a 15 l tank for daily (sub-acute) exposures administered over 7-days. This sub-acute exposure regime was chosen to simulate environmental conditions, where shellfish can be exposed to toxin-producing algae continuously for varying time periods.

Table 1.3 outlines the projected accumulation/depuration of OA in the tissues of three shellfish species over the exposure period. Estimated concentrations per shellfish assume an even suspension of OA in the seawater and equal uptake and distribution of the toxin in each shellfish. Quantities of toxin in the tissues over the exposure period were estimated using the depuration model proposed by Svensson and Förlin (2004) based on experiments on *Mytilus edulis*, in which OA-containing shellfish were maintained at 18$^{o}$C with food, and assumed a constant depuration rate over time.

Toxin quantities in seawater vary throughout the year, with periods of low phycotoxin levels and times of explosive growth during an algal 'bloom' (Alexander *et al.* 2008). Okadaic acid has been found to be ubiquitous at low levels (i.e. below the levels deemed safe for human consumption) in Irish mussels year round (Marine Institute 2004, 2007) thus, it is important to determine if these 'low' levels have harmful impacts on the shellfish species themselves. The toxin concentrations for mussels in this series of experiments of acute exposure to 2 µg/15 l tank and 1 µg/15 l tank sub-acute exposure, were chosen to reflect these low levels of OA (Marine Institute 2004, 2007). The high concentration of 40 µg/tank daily exposure gave 'bloom' levels of toxin in the tissues in this study, reaching an estimated 673.7 µg/kg for mussels and 215.5 µg/kg for oysters, after 7 days (Table 1.3). The mean level of toxin accumulation in Irish shellfish quoted by Alexander *et al.* (2008) was 268 µg/kg, with the median level at 120 µg/kg based on 758 samples, 41% of these samples had OA levels > 160 µg/kg, which is greater than the recommended level for human consumption (Table 1.1). Thus, these exposure concentrations inform us of the impacts of an environmentally relevant range of OA quantities to which shellfish are exposed year-round.

Table 1.3. Projected accumulation/depuration rates of OA in the tissues of the blue mussel, the pacific oyster and the manila clam after daily dosing of 20 animals in 15 l of seawater over 7 days. Estimated quantities of OA in the tissues of one animal (µg/g) after each day assume an even suspension of OA in the water and equal uptake and distribution of the toxin in each shellfish. The quantities are estimated using the exponential decay model proposed by Svensson and Förlin (2004) examining the effects of temperature and food on depuration rates of OA. The data were derived by obtaining the average wet tissue weight per animal, and calculating the toxin quantity per gram of shellfish. The exponential decay equation used: $T_t = T_0 e^{-\lambda t}$, $T_0$: toxin concentration at the start of depuration (µg OA equivalent.g-1), λ: exponential decay coefficient (% per day) and t: time (days). Using this model, Svensson and Förlin (2004) fitted their data to the exponential decay function and proposed a depuration curve based on their experimental results for depuration rates of shellfish maintained at 18°C with food: $T_t = 2.51\ e^{-0.086t}$, $r^2 = 0.82$. This equation was applied to the data in the current study.

| OA/ 15l tank (n = 20) | weight(g) /shellfish | $T_0$ | $-\lambda$ | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mussel: 1 µg/15l tank** | 1.957 | 0.026 | -0.086 | 0.023 | 0.045 | 0.065 | 0.083 | 0.099 | 0.114 | 0.128 |
| **Mussel: 40 µg/15l tank** | 1.957 | 1.021 | -0.086 | 0.937 | 1.797 | 2.585 | 3.309 | 3.973 | 4.583 | 5.142 |
| **Oyster: 1 µg/15l tank** | 10.739 | 0.005 | -0.086 | 0.004 | 0.008 | 0.012 | 0.015 | 0.018 | 0.021 | 0.023 |
| **Oyster: 40 µg/15l tank** | 10.739 | 0.185 | -0.086 | 0.169 | 0.325 | 0.467 | 0.598 | 0.728 | 0.828 | 0.929 |
| **Clam: 1 µg/15l tank** | 3.313 | 0.015 | -0.086 | 0.014 | 0.026 | 0.038 | 0.049 | 0.058 | 0.067 | 0.076 |

**Objectives**

- Assess methods for the harvesting of marine biotoxins in the marine environment by utilising two existing techniques for the passive and active sampling of phycotoxins (MacKenzie *et al.* 2004, Rundberget *et al.* 2007).
- Perform a detailed temporal and spatial analysis of the DSP toxins present at Lough Hyne Marine Reserve using Solid Phase Adsorption and Toxin Tracking (SPATT).
- Examine the pathological impacts of the marine biotoxin okadaic acid on the tissues of *Mytilus edulis, Ruditapes philippinarum* and *Crassostrea gigas.*
- Assess whether okadaic acid has an impact on the innate immune response of *Mytilus edulis*, *Ruditapes philippinarum* and *Crassostrea gigas*, by measuring changes in the differential cell populations in the circulating haemolymph.
- Measure DNA fragmentation in *Mytilus edulis* and *Crassostrea gigas*, and determine whether okadaic acid causes a significant increase using the comet assay.

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# CHAPTER 2

# Monitoring and bioharvesting of marine biotoxins using passive (SPATT) and active sampling methods

**ABSTRACT**

Solid phase adsorbent and toxin tracking (SPATT) is a method for the passive accumulation of lipophilic marine biotoxins onto adsorbent resin. The method enables temporally and spatially integrated monitoring of biotoxins in aquatic environments. This study had two main objectives: examining the use of SPATT as a method for monitoring the distribution of lipophilic marine biotoxins, both vertically and horizontally in marine coastal waters; and to determine whether an active sampling methodology using Diaion HP-20 resin could be utilised to harvest large quantities of marine phycotoxins for use in subsequent experimentation.

SPATT monitoring using two adsorbent resins was performed over a 4-month period at Lough Hyne Marine Reserve, Co. Cork, Ireland. A range of lipophilic toxins were successfully detected from the SPATT extracts throughout the study period including: okadaic acid (OA), dinophysis toxin-1 (DTX1), DTX2, pectenotoxin-2 (PTX2), pectenotoxin-2-seco acid (PTX2-SA) and the spirolide 13-desmethyl-SPX-C. The majority of biotoxins were detected in the top 20 m of the water column and an increase in toxin quantity during August 2010 was detected. Phytoplankton analysis confirmed the presence of toxin-producing species *Dinophysis acuta* and *D. acuminata* during the period of increased toxicity. Active sampling was performed continuously over 7-days and high quantities of toxins were successfully accumulated in the HP-20 resin, OA (13 mg), DTX1 (3.8 mg) DTX2 (29 mg), PTX2 (20 mg) and PTX2-SA (6 mg). In addition to these DSP toxins, the cyclic amines 13-desmethyl-SPX-C and Pinnatoxin G were also detected in the resin from the active sampler.

This study is the first to detect DTX1 and Pinnatoxin G in Irish waters and the first to isolate 13-desmethyl-SPX-C using high mass accuracy (< 2 ppm). Utilised in conjunction with biological methods, SPATT has the potential to provide useful information on phycotoxin distribution in the water column; enabling evidence-based decisions to be made regarding the appropriate depths for obtaining phytoplankton and shellfish samples in marine biotoxin monitoring programmes.

## INTRODUCTION

Harmful algal blooms are occurring with increasing frequency and their incidence is spreading geographically, primarily through anthropogenic influences such as eutrophication, ballast waters of ships and climate change (Anderson *et al.* 2002, Smayda 2007, James *et al.* 2010). The contamination of filter-feeding shellfish with phycotoxins produced from Harmful Algal Blooms (HABs) is becoming an increasing health risk for human shellfish consumers worldwide. Regulation EC No 853/2004 governs the total amount of marine biotoxins that may be present in shellfish for the protection of consumers within the E.U (European Commission 2004). Phytoplankton abundances in the water and the toxin-burden of shellfish are rigorously monitored by regulatory bodies in shellfish producing countries, using multiple detection methods (James *et al.* 1997a, Draisci *et al.* 1999, Furey *et al.* 2001, Lehane *et al.* 2002) to ensure levels of toxin in the tissues remain below the limits deemed safe for human consumption (Alexander *et al.* 2008).

Up until 2012 the mouse and rat bioassay were the official methods for the detection of lipophilic marine toxins from shellfish extracts in the European Union (Annex III to Regulation (EC) No 2074/2005, Chapter III). Liquid Chromatography-Mass Spectroscopy (LC-MS), in conjunction with phytoplankton monitoring and assessment, has become the primary technique adopted throughout the E.U for the majority of marine phycotoxins, excluding Paralytic Shellfish Poisoning (PSP) toxins and Domoic acid (DA) (European Commission 2004). The method has been proven to be a sensitive and valuable analytical tool for the confirmation of a wide range of lipophilic marine biotoxins in shellfish tissues, and for the elucidation of new compounds (James *et al.* 1997a, James *et al.* 1997b, Satake *et al.* 1998).

However, despite the efficacy and precision of phytoplankton monitoring coupled with LC-MS analysis of shellfish tissues, there are some disadvantages to both techniques (MacKenzie *et al.* 2004, Lane *et al.* 2010). Phytoplankton monitoring provides a ‘snapshot’ of the current algal species present in the marine environment and it is limited in its detection of toxic species both spatially and temporally. Furthermore, the presence of toxin-producing algae does not provide

definitive evidence that toxin accumulation is occurring in shellfish. Toxin production has been found to vary in algal species depending on the stage of growth (Anderson 1994, Caillaud *et al.* 2011). In *Alexandrium sp.* toxin content per cell was found at the highest concentrations during exponential growth of the algae, with a decline measured during the stationary phase (Wyatt and Jenkinson 1997). Identification of phytoplankton to species level can be difficult and time-consuming, and for some algae, discrimination between species requires detailed morphologic observations, and specialised tools have been explored for positive identification to species level (Miller and Scholin 1998, Lane *et al.* 2010). LC-MS analysis is a precise tool for the direct monitoring of biotoxins accumulated in shellfish tissues, however, the lack of certified standards, analytical interference from biological matrix effects, and the biotransformation of the biotoxin within the shellfish can create analogues and fatty acid esters of the toxin (Vale *et al.* 1999, Suzuki *et al.* 2004, Fast *et al.* 2006), thus increasing the difficulty of detection of both the principle toxins and their metabolites.

A method for the passive accumulation of the toxins directly from the water-body was proposed by MacKenzie *et al.* (2004) to circumvent difficulties in the detection and prediction of toxic algal bloom events. The protocol was based on the observation that during HABs quantities of toxin 'leak' from the cell and are suspended in the seawater (MacKenzie *et al.* 2003). Solid Phase Adsorption and Toxin Tracking (SPATT) analysis involves the deployment of adsorptive resins, enclosed in a polyester mesh bag, into the marine environment. MacKenzie *et al.* (2004) utilised a number of resins and successfully detected a wide range of lipophilic toxins including the OA-group of toxins and the pecteno-toxins. Based on this original design, a number of additional trials have been completed using the SPATT technology to determine its efficacy as an early-warning detection system for marine algal toxins, with mixed results (Turrell *et al.* 2007, Rundberget *et al.* 2009, Rodríguez *et al.* 2011). Further optimisation of the toxin-extraction protocol has been performed (Fux *et al.* 2008) and additional studies have confirmed that Diaion HP-20 is the most efficient resin for the accumulation of lipophilic toxins (Turrell *et al.* 2007, Fux *et al.* 2008, Fux *et al.* 2009). Identification of the most effective resin for the accumulation and detection of more polar toxins, such as domoic acid and saxitoxin, is ongoing (Lane *et al.* 2010). SPATT bags provide

temporal and spatial integrated monitoring of the water column. Toxin accumulation in SPATT has also been examined in conjunction with accretion rates in bivalves, and phytoplankton abundance in the water column to determine whether the toxin quantities on the SPATT can be placed in a biologically relevant context (Turrell *et al.* 2007, Fux *et al.* 2009, Rundberget *et al.* 2009, Lane *et al.* 2010). For a comprehensive review of current advances and research performed using SPATT analysis see MacKenzie (2010).

Using the information obtained from the passive SPATT samplers designed by MacKenzie *et al.* (2004) a method for the active sampling of biotoxins from the marine environment was devised (Rundberget *et al.* 2007). Large-scale, active sampling of the water body was performed to determine if the sampling resin found by previous studies to be the most efficient accumulator of lipophilic biotoxins (Diaion HP-20) could be used for large-scale bioharvesting of marine biotoxins, for subsequent experimentation (MacKenzie *et al.* 2004, Turrell *et al.* 2007, Fux *et al.* 2008). High quantities of okadaic acid (OA) and dinophysis toxin-2 (DTX-2) were successfully harvested using this method. In order to perform experiments using marine phycotoxins it is necessary to have adequate quantities of the toxin, both from a chemical and a biological standpoint. It is only recently that reference standards for many of the commonly occurring marine biotoxins such as Azaspiracids (AZAs), DTX1 and DTX2 have been made available through the Certified Reference Materials Programme, Halifax, Canada (Canada 2012). This enables accurate quantification of toxins from environmental samples using analytical methods. However, toxin metabolites are not readily available and isolation must still be performed from bulk shellfish tissues. Furthermore, for acquisition of phycotoxins in the quantities required for large-scale *in vivo* toxicological testing, costs are still prohibitive.

Lough Hyne marine reserve was chosen as our study site as a comprehensive investigation of the phytoplankton profile of the marine reserve was performed from January 2008 – June 2009 (Jessopp *et al.* 2011) and a number of toxin-producing algal species were detected (*Dinophysis acuminata, Dinophysis acuta, Dinophysis fortii, Dinophysis norvegica, Dinophysis sacculus, Dinophysis tripos, Dinophysis caudata, Prorocentrum lima, Prorocentrum balticum/minimum, Protoperidinium*

*crassipes/curtipes* and *Alexandrium sp.*). Lough Hyne was also selected as it provides an opportunity to examine the occurrence of these algal toxins in an enclosed and highly sheltered system (Maughan and Barnes 2000) to further investigate the use of the SPATT technology as a biomonitoring tool, in addition to its previously explored role as an early-warning system. Due to Lough Hyne's classification as a Marine Reserve, transplantation of animals into Lough Hyne from locations outside of the Nature Reserve is strictly prohibited. Thus, examining the accumulation of toxins within shellfish tissues in conjunction with the SPATT analysis was not performed.

The main objectives of the current study were two-fold. First, a temporal and spatial analysis of the marine biotoxins present at Lough Hyne Marine Nature Reserve, West Cork, Ireland was performed over a 4-month period using SPATT analysis. Two resins were utilised in the study, the well-established Diaion HP-20 which has been proven effective for the accumulation of lipophilic toxins and Amberlite ® XAD761 for which there are only limited data available on its efficacy. In conjunction with the SPATT analysis, phytoplankton samples were taken at discrete depths using a closing phytoplankton net. The second objective of this study was the construction and testing of a seawater pumping machine modelled after the unit devised by Rundberget *et al.* (2007). This was performed in order to determine whether high quantities of lipophilic toxin could be harvested from the water column and used for subsequent experimentation.

## MATERIALS AND METHODS

### Construction and deployment of SPATT bags

SPATT bags were constructed from 95 µm polyester mesh purchased from John Staniar and Co., Whitefield, Manchester, UK. The bags had dimensions of 100 mm x 100 mm and sealed at the top with velcro to allow removal of the resin and bags to be reused. A loop made of nylon fishing line was sewed on the top corner of the bag to enable attachment to the mooring line using cable ties. Two types of resin were placed in the bags, Amberlite ® XAD761 (Supelco, 10356) an adsorbent resin for the removal of proteins, high MW colorants, organic impurities, useful for the

purification of pharmaceuticals; and Diaion HP-20 (Supelco, 13607) a polyaromatic adsorbent resin for hydrophobic compounds: antibiotics and biomolecules; useful for desalting and has broad application base. The resins were activated by soaking in methanol according to manufacturer's instructions and rinsed using deionised water, 5 g dry weight or 6.1 g wet weight of activated XAD761; and 5 g dry weight or 8.8 g wet weight of HP-20 were added to each SPATT bag. The bags were stored in airtight ziplock bags at 4$^{o}$C and kept from drying out until deployment, as per Rundberget *et al.* (2009).

The bags were attached to mooring lines at different depth intervals for two weeks at a time over a 4-month period from May 2010 to August 2010 (Figure 2.1). After removal from the marine environment, bags were transported back to the laboratory in airtight ziplock bags and frozen at -20$^{o}$C until extraction.

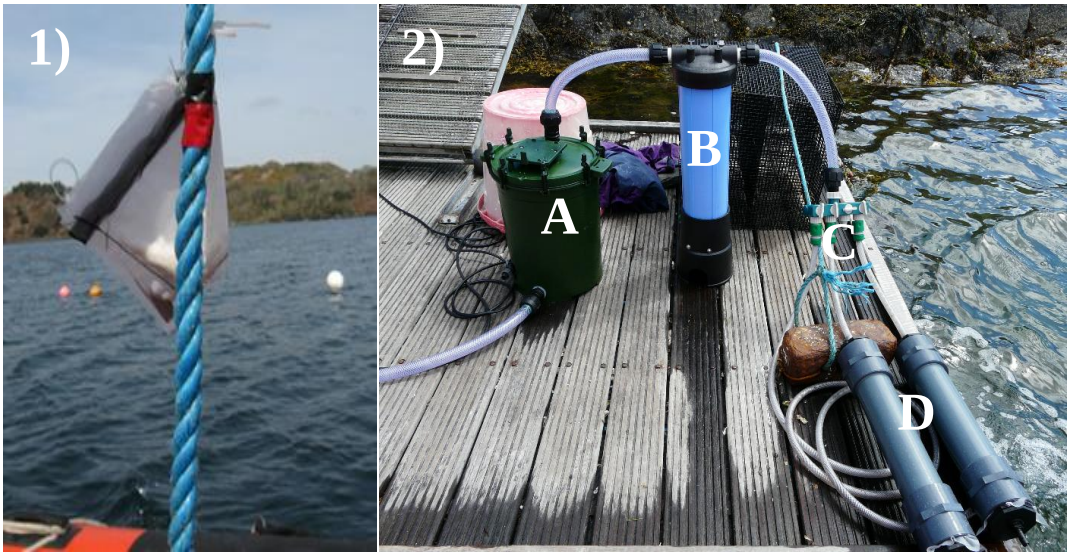



Figure 2.1. 1) Photograph of Solid Phase Adsorption and Toxin Tracking (SPATT) bag attached to mooring line prior to submersion in Lough Hyne.
2) Submersible pumping device for the active accumulation of marine biotoxins on adsorptive resin. A: Pre-filter; B: 50 µm filter; C: 4-way connector with taps; D: Resin-filled polyethylene columns.

**Construction and deployment of the active toxin sampler**

Construction of the active toxin sampler was modelled on Rundberget *et al.* (2007). The machine consisted of a KSB Ama-Drainer 301 C submersible seawater pump and float switch (KSB Ltd, Leicestershire, England), this was attached to two filters. The pre-filter was an Eheim 2260 External Canister Filter (Aquatics Online, Manchester, England) filled with wadding. The second filtration unit was an Amtek 1" I/O 20" Big Blue Bag filter housing with pressure gauge and a 50 µm bag filter (Cat no: 150236; Big Brand Water Filter Inc, CA 91311, USA). Hose and polygrip attachments were purchased from Irish International Trading Corporation, Cork, Ireland. Two polyethelene columns were constructed from 90 mm (diameter) pipe, with male hose adaptors attached to each (Delta Valves and Plastics, Cork, Ireland). A 500 g quantity of HP-20 resin was sewed into bags made of 95 µm polyethylene mesh and one was placed in each column. The two columns were attached to a garden hose 4-way connector to allow water flow to be regulated using taps (Figure 2.1).

The submersible pumping machine was deployed for 7 consecutive days at Lough Hyne Marine Reserve (51°29' 58"N 9°17' 49"W) (Figure 2.1), from the August 24$^{th}$ – August 31$^{st}$, 2010 and remained pumping continuously over the period, apart from 30 minutes on the 25/08/10 when the 50 µm bag filter in the Amtek housing was replaced. The rate of water flow through the entire unit was measured at 6.67 l min$^{-1}$ and the seawater pump was submerged 1 m below the surface.

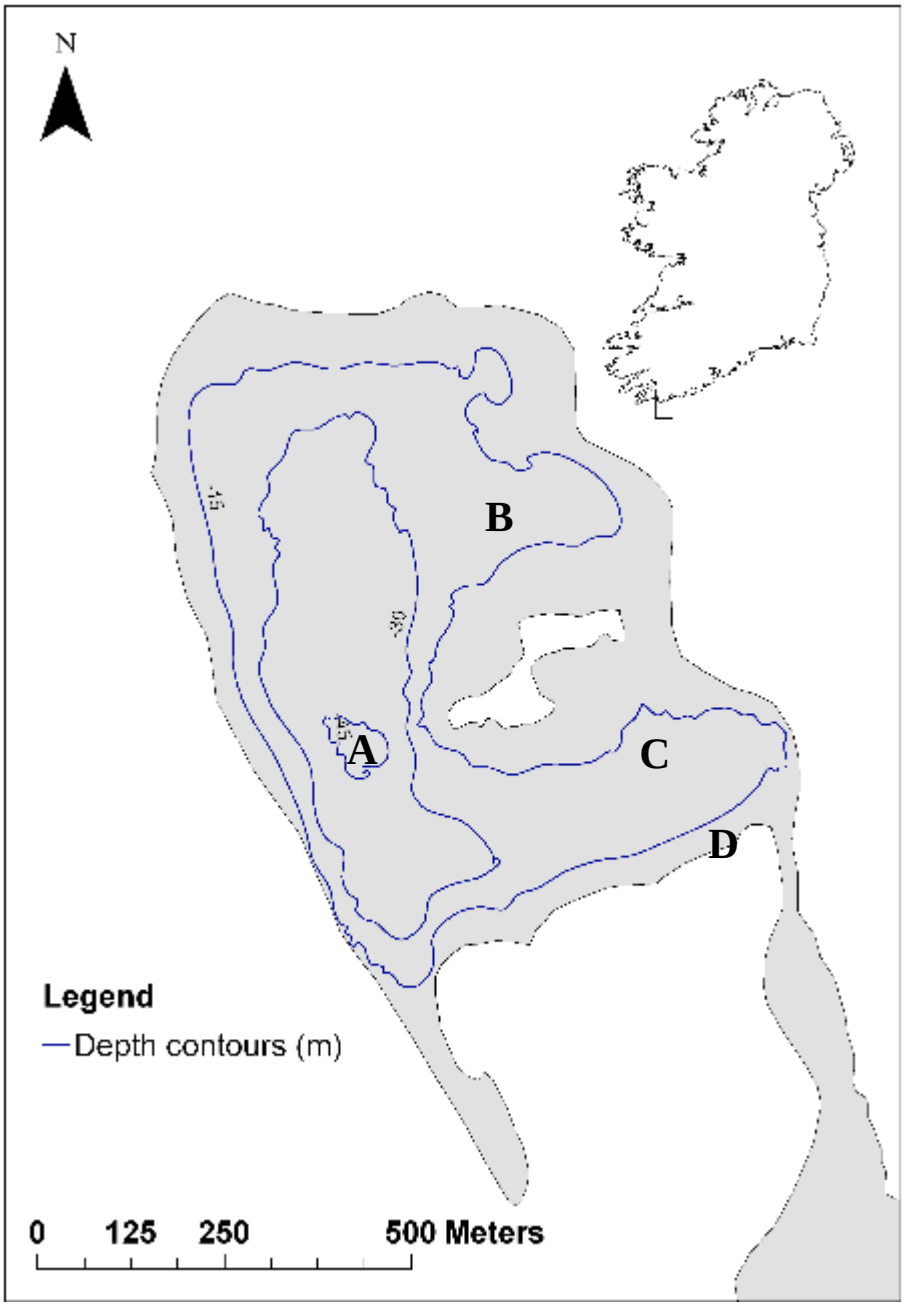



Figure 2.2. Map of Lough Hyne Marine Reserve, with relevant field sites labelled as follows: sampling sites for SPATT analysis; A = Western Trough (WT); B = North Basin (NB); C = South Basin (SB). Active toxin accumulation; D = sampling site for pump deployment.

(Map courtesy of Dr. Stefanie Brozeit, School of Biological, Earth and Environmental Sciences, University College Cork.)

**Field study**

Lough Hyne Marine Reserve, Co. Cork, Ireland was chosen as the study location for this investigation. Three sites were chosen within the Lough, two sites of 20 m depth, the North Basin (NB) (51°30'81"N 9°18'81"W) and the South Basin (SB) (51°30'77"N 9°30'78"W) and one 50 m deep site in the Western Trough (WT) (51°30'48"N 09°18'271"W) (see Figure 2.2). The SPATT bags were attached at the surface, 5 m, 10 m and from then on at 10 m intervals until the sea-bed. This was done to establish a spatial and temporal pattern of marine biotoxins at Lough Hyne.

In addition to deploying bags every two weeks, vertical phytoplankton hauls were taken with a closing phytoplankton net (Aquatic Research Instruments, U.S.A) at the depths the bags were deployed. The net was deployed in the water, pulled vertically up through one metre of water and closed, giving a concentrated sample from approximately 70 l of water, at discrete depths. The phytoplankton samples were preserved in Lugol's acidified iodine solution for subsequent microscopic analysis and identification. Identification of the phytoplankton was performed to determine the causative algal species. This was done in August 2010 where a significant increase in some phycotoxin quantities was detected in the passive filters. This identification was performed by Dunmanus Seafood Ltd, Goleen, Co. Cork, Ireland.

Temperature and oxygen concentrations were measured at the WT at the surface and 1 m intervals to the sea-bed. Unfortunately the oxygen probe malfunctioned on the June 2nd 2010, thus data on oxygen and temperature was not available after that period. However, these data did enable monitoring of the impact a developing oxy-thermocline had on the distribution of the marine biotoxins**.**

**Solvents and reagents**

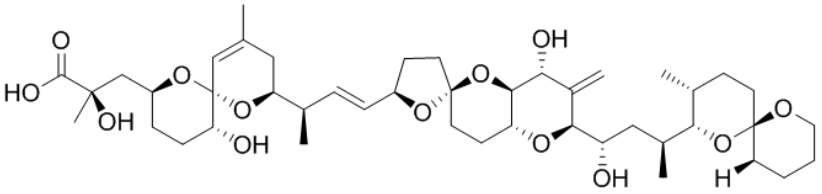
Chemicals used for liquid chromatography coupled to mass spectroscopy (LC-MS), were obtained from Fisher Scientific, Dublin, Ireland. Methanol, HPLC-grade, for extraction of SPATT was purchased from Sigma-Aldrich, Germany. Water was obtained from MilliQ water dispenser. Certified Reference Material (CRM) Standards of OA, DTX1 DTX2 and pectenotoxin-2 (PTX2) (Figure 2.3) were

purchased as certified calibration solutions from the National Research Council (NRC), Halifax, Canada. These were used to verify and quantify OA, DTX1, DTX2, and PTX2 from the passive and active samplers. The CRM for PTX2 was used to quantify PTX2-SA an analogue of PTX2. Standards of biotoxins (OA, DTX1, DTX2 and PTX2) in solvent (Methanol) were prepared from the CRM by serial dilution. Mixtures of the compounds were analysed at 1 ppm, 0.5 ppm, 0.1 ppm, 0.05 ppm and 0.01 ppm concentrations.

**Sample extraction methods**

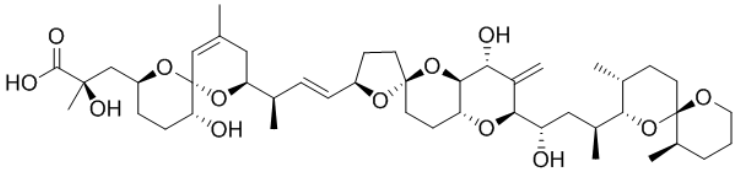
The extraction protocol as per Fux *et al.* (2008) was utilised for removal of toxins from the resin. The samples were rinsed twice in 500 ml of MilliQ water to remove salts and shaken to remove excess water. The resins were removed from the bags using a metal spatula, with the remaining resin rinsed from the bag using MilliQ water and placed into empty glass SPE cartridges (Supelco, 504394) on a manifold. Remaining water was removed by applying a vacuum. For every 3 g (dry weight) of resin 25 ml of methanol was used for resin elution at ca 1 ml/min flow rate. The extracts were transferred to amber glass jars and stored at -20$^{o}$C until subsequent LC-MS/MS analysis. The same extraction process was performed for both resins with one exception; for XAD761, the resin was eluted with 80:20 methanol: water. This was done to ensure the removal of more polar toxins (e.g. domoic acid) for possible future analysis (Vale and Sampayo 2002). Samples from each depth were pooled with the corresponding depths from May, June and July for the three sites due to low quantities of toxin present. In August SPATT samples were examined individually for each site.

To ensure samples fit the calibration curve for accurate quantitation, a number of samples were concentrated using a rotary evaporator and were made up in methanol (10 ml). Aliquots were placed in HPLC cartridges and re-analysed. For the resin used in the active sampler, levels of toxin accumulated were greater than those utilised for the standard curve, therefore samples were diluted and re-analysed to ensure accurate quantitation.



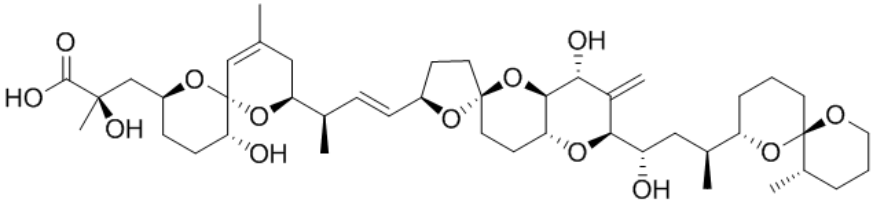
**Okadaic Acid**

Chemical Formula: $C_{44}H_{68}O_{13}$
Exact Mass: 804.46599
Molecular Weight: 805.00292
m/z: 804.46599 (100.0%), 805.46935 (47.6%), 806.47270 (11.1%), 806.47024 (2.7%), 807.47606 (1.7%), 807.47359 (1.3%)
Elemental Analysis: C, 65.65; H, 8.51, O, 25.84



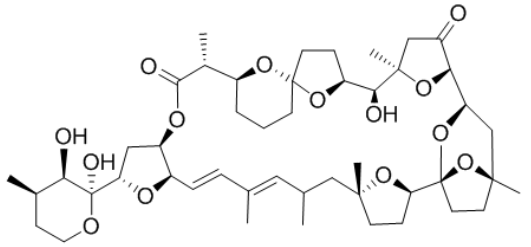
**DTX1**

Chemical Formula: $C_{45}H_{70}O_{13}$
Exact Mass: 818.48164
Molecular Weight: 819.02950
m/z: 818.48164 (100.0%), 819.48500 (48.7%), 820.48835 (11.6%), 820.48589 (2.7%), 821.49171 (1.8%), 821.48924 (1.3%)
Elemental Analysis: C, 65.99; H, 8.61; O, 25.39



**DTX2**

Chemical Formula: $C_{44}H_{68}O_{13}$
Exact Mass: 804.46599
Molecular Weight: 805.00292
m/z: 804.46599 (100.0%), 805.46935 (47.6%), 806.47270 (11.1%), 806.47024 (2.7%), 807.47606 (1.7%), 807.47359 (1.3%)
Elemental analysis: C, 65.65; H, 8.51; O, 25.84



**PTX2**

Chemical Formula: $C_{47}H_{70}O_{14}$
Exact Mass: 858.47656
Molecular Weight: 859.05030
m/z: 858.47656 (100.0%), 859.47991 (50.8%), 860.48327 (12.6%), 860.48080 (2.9%), 861.48662 (2.1%), 861.48416 (1.5%)
Elemental Analysis: C, 65.71; H, 8.21; O, 26.07

Figure 2.3. Chemical structures of DSP toxins (ChemBioDraw version 11.0).

**Liquid Chromatography-Mass spectrometry**

***Diarrhetic shellfish toxins***

LC-MS/MS analysis was conducted on a Thermo Scientific Quantum Discovery Max triple quadropole mass spectrometer, equipped with a heated electrospray ionization source, hyphenated to a Thermo Scientific Accela LC system. The Accela LC system is comprised of a quaternary pump, thermostat autosampler, degasser and column heater. The isocratic LC conditions utilised were as per the optimised method of Carey *et al.* (2012), 8% HPLC grade water with a 1.0 mM of ammonium acetate and 0.005% TFA (A), and 92% HPLC grade acetonitrile and water (85:15) with 1.0 mM ammonium acetate and 0.005% TFA (B) over 5 minutes. Machine calibration and method re-optimisation was performed with the assistance of Caroline Griffin, Cork Institute of Technology.

The isocratic flow rate was 30% (A) and 70% (B) at a rate of 250 µl/min. The analytical column used was an Agilent Zorbax Eclipse XDB-C8 column, (2.1 x 150 mm, 3.5 µm) and the pre-column was an Aligent Eclipse XDB-C8 (4.6 x 30 mm, 3.5 µm; Aligent Technologies Ireland Ltd., Cork, Ireland) maintained at 35$^{o}$C. The autosampler tray temperature was kept at 4$^{o}$C and the injection volume was 5 µl, the analysis time was 14 mins per sample. Optimisation of the method was carried out by Carey *et al.* (2012). Briefly, source parameters were: capillary temperature, 340$^{o}$C; vaporiser temperature, 220$^{o}$C; sheath gas ($N_2$) flow rate: 10 (arbitrary units); and spray voltage 3kV. For the mass transitions and corresponding collision energies for each of the DSP toxins see Table 2.1. DTX1 was added to the existing method of Carey *et al* (2010). DTX1 tuning was performed using CRM, the parent mass of m/z 842 was broken down into fragments of m/z 824.4 and m/z 806.3 at a ratio of 100:40. These fragmentation m/z coupled with the ion ratios were utilised to confirm DTX1 in the environmental samples.

***Spirolides and cyclic amines***

Confirmation of 13-desmethyl-SPX-C and Pinnatoxin G from the active sampler was carried out by María García Altares Pérez, IRTA, Reus Area, Spain using a Thermo Scientific LTQ Orbitrap Discovery Fourier transform mass spectrometer.

Confirmation of these compounds in environmental samples was performed using CRM for 13-desmethyl-SPX-C and Pinnatoxin G; mass accuracy (< 2 ppm) for precursors and fragments. Confirmation and quantitiation of 13-desmethyl-SPX-C in the SPATT samples was performed by Vaishali Bane, Cork Institute of Technology.

Table 2.1. The selective mass transitions (parent and fragment) and corresponding collision energies (CE) for each of the compounds examined using LC-MS/MS.

| Compound | Parent | Fragment | Isolation Width | Time (s) | CE (%) | Q1 | Q3 | Tube Lens |
|---|---|---|---|---|---|---|---|---|
| **DTX2** | 827.456 | 429.225 | 0.010 | 0.100 | 60 | 0.70 | 0.70 | 190 |
| **OA** | 827.456 | 443.241 | 0.010 | 0.100 | 60 | 0.70 | 0.70 | 190 |
| **OA** | 827.456 | 571.325 | 0.010 | 0.100 | 50 | 0.70 | 0.70 | 190 |
| **DTX2** | 827.456 | 581.309 | 0.010 | 0.100 | 55 | 0.70 | 0.70 | 190 |
| **OA (DTX2)** | 827.456 | 595.325 | 0.010 | 0.100 | 55 | 0.70 | 0.70 | 190 |
| **OA (DTX2)** | 827.456 | 723.408 | 0.010 | 0.100 | 45 | 0.70 | 0.70 | 190 |
| **PTX2** | 881.466 | 539.300 | 0.010 | 0.100 | 62 | 0.70 | 0.70 | 210 |
| **PTX2** | 881.466 | 837.500 | 0.010 | 0.100 | 50 | 0.70 | 0.70 | 210 |
| **PTX2-SA** | 899.477 | 557.300 | 0.010 | 0.100 | 62 | 0.70 | 0.70 | 210 |
| **PTX2-SA** | 899.477 | 855.500 | 0.010 | 0.100 | 50 | 0.70 | 0.70 | 210 |
| **DTX1** | 842.0 | 824.4 | 0.010 | 0.100 | 35 | 0.70 | 0.70 | 160 |
| **DTX1** | 842.0 | 806.3 | 0.010 | 0.100 | 43 | 0.70 | 0.70 | 160 |

**Validation**

Linearity: the linear range of this method was investigated for OA, DTX1, DTX2 and PTX2 by construction of a serial dilution of analyte compounds. The range of the analytical method was established by confirming an acceptable degree of linearity and precision within the specified range.

Precision of the method was evaluated using both intra-day and inter-day studies. Sample numbers of three and six were used for intra-day and interday repeatability respectively. Precision was evaluated using five concentrations over the linear ranges.

### Data analysis

Data from SPATT were tested for normality using the Kolmogorov- Smirnov test. Square root and log transformation of non-normal data was performed, however skewed distribution could not be normalised. Consequently, data were analysed using non-parametric tests. Spearman's Rank Order Correlation was performed between the different toxins to determine whether a relationship existed between their occurrences over time. Toxin distribution in the water column was assessed using Kruskal-Wallis non-parametric ANOVA, followed by Mann-Whitney U tests between categories to determine where differences in distribution occurred. Chi-square tests using Yate's correction where 1 degree of freedom was present were performed between resin types, to determine which provided the greater frequency of positive samples. Data were analysed using Predictive Analytics SoftWare Statistics (PASW) 17 for Windows.

## RESULTS

### Linearity and precision

The linear range for each of the DSP toxins was investigated using methanol spiked with known quantities of CRM for OA, DTX1, DTX2 and PTX2 at different concentrations. These samples were analysed daily six times over a two day period prior to testing environmental samples. The calibration curves were also assessed during the environmental sample runs and repeated again six times daily over two days at the end of the experimental period. The data were analysed graphically and found to be linear between concentrations of 1 - 0.01 µg/ml. The validation data showed excellent linearity for each of the toxins from a curve constructed of five data points ($r \geq 0.99$) (see Table 2.2).

Table 2.2. Validation data for the determination of OA, DTX1, DTX2 and PTX2 dissolved in methanol

| Algal Toxin (CRM) | Linear range (µg/ml) | Equation of line | Correlation coefficient (r) |
|---|---|---|---|
| **OA** | 1 – 0.01 | y = 495368x - 6002.5 | r = 0.9997 |
| **DTX1** | 1 – 0.01 | y = 184873x - 6124.7 | r = 0.9988 |
| **DTX2** | 1 – 0.01 | y = 529295x - 8617.3 | r = 0.9999 |
| **PTX2** | 1 – 0.01 | y = 1E+06x - 19114 | r = 0.9999 |

Method precision was assessed in terms of both intra-day and inter-day repeatability of detection. Five concentrations (0.01 – 1 µg/ml) of the CRM for each of the DSP toxins were spiked in methanol and analysed for intra-day samples (n = 3) and inter-day samples (n = 6) using tandem LC-MS/MS on the Thermo TSQ Quantum Discovery Max. Precision results were good for this method, Table 2.3 shows a list of the relative standard deviations (RSD) for the concentrations analysed. Apart from the lowest concentration of DTX1 (0.01 µg/ml) all %RSD were < 10 for each of the toxins (Table 2.3).

Table 2.3. Precision assessment data for OA, DTX1, DTX2 and PTX2 from methanol spiked with CRM of each toxin.

| | Repeatability (Intra-day) | | Repeatability (Inter-day) | | |
|---|---|---|---|---|---|
| **OA** | **SD** | **%RSD** | **OA** | **SD** | **%RSD** |
| **µg/ml** | **n = 3** | **n = 3** | **µg/ml** | **n = 6** | **n = 6** |
| **0.01** | 0.001 | 3.7 | **0.01** | 0.001 | 3.6 |
| **0.05** | 0.003 | 7.4 | **0.05** | 0.003 | 7.6 |
| **0.1** | 0.002 | 2.6 | **0.1** | 0.002 | 2.4 |
| **0.5** | 0.009 | 1.8 | **0.5** | 0.026 | 5.1 |
| **1** | 0.044 | 4.3 | **1** | 0.042 | 4.2 |
| **DTX1** | **SD** | **%RSD** | **DTX1** | **SD** | **%RSD** |
| **µg/ml** | **n = 3** | **n = 3** | **µg/ml** | **n = 6** | **n = 6** |
| **0.01** | 0.002 | 6.8 | **0.01** | 0.004 | 10.2 |
| **0.05** | 0.004 | 8.4 | **0.05** | 0.004 | 7.1 |
| **0.1** | 0.009 | 9.9 | **0.1** | 0.007 | 7.6 |
| **0.5** | 0.028 | 6.8 | **0.5** | 0.030 | 7.0 |
| **1** | 0.063 | 6.6 | **1** | 0.090 | 9.1 |
| **DTX2** | **SD** | **%RSD** | **DTX2** | **SD** | **%RSD** |
| **µg/ml** | **n = 3** | **n = 3** | **µg/ml** | **n=6** | **n=6** |
| **0.01** | 0.001 | 2.7 | **0.01** | 0.001 | 3.2 |
| **0.05** | 0.003 | 7.8 | **0.05** | 0.003 | 7.1 |
| **0.1** | 0.007 | 7.7 | **0.1** | 0.007 | 8.0 |
| **0.5** | 0.017 | 3.4 | **0.5** | 0.018 | 3.7 |
| **1** | 0.040 | 3.9 | **1** | 0.050 | 4.9 |
| **PTX2** | **SD** | **%RSD** | **PTX2** | **SD** | **%RSD** |
| **µg/ml** | **n = 3** | **n = 3** | **µg/ml** | **n=6** | **n=6** |
| **0.01** | 0.001 | 6.2 | **0.01** | 0.001 | 7.8 |
| **0.05** | 0.001 | 2.5 | **0.05** | 0.004 | 9.3 |
| **0.1** | 0.001 | 1.0 | **0.1** | 0.006 | 6.3 |
| **0.5** | 0.008 | 1.7 | **0.5** | 0.023 | 5.0 |
| **1** | 0.015 | 1.6 | **1** | 0.048 | 5.3 |

**Detection and quantitation of DSP toxins using optimised method**

Using the optimised method from Carey *et al.* (2012) successful detection of DSP toxins OA, DTX1, DTX2, PTX2 and PTX2-SA from resin samples was accomplished. Chromatographs generated gave clear indication of the presence of the biotoxins present on HP-20 and XAD761 from the passive SPATT samplers (Figures 2.4 and 2.5). Sub-samples of HP-20 were analysed from the active sampler and the presence of DSP toxins was confirmed using the Thermo TSQ Quantum Discovery Max (Figure 2.6). Chromatograms of DTX1 from both resins and both sampling methods show that further analyses of this toxin are required (Figures 2.4, 2.5 and 2.6). The fragmented ion ratios present were correct for DTX1 (m/z: 827/429); however, the retention time of DTX1 was the same as that of PTX2, thus a full-scan and further separation of the toxin is required. In addition, the signal from DTX1 was less clear in a mixture of samples than when it is examined in isolation as was observed when examining the CRM (Figure 2.7).

**Detection and quantitation of SPX-C and Pinnatoxin G**

Chromatograms and spectrograms of 13-desmethyl-SPX-C and Pinnatoxin G confirm the presence of these toxins in the samples (Figures 2.8 and 2.9). Both toxins were detected in the environmental samples with high mass accuracy (< 2 ppm) (Table 2.4). (Data courtesy of María García Altares Pérez, IRTA, Reus Area, Spain). SPATT samples were quantified by Vaishali Bane, Cork Institute of technology. Pinnatoxin G has not yet been quantified in the samples.

**Comparison of resins**

Good recovery of DSP toxins from both resins was achieved using the extraction method from Fux *et al.* (2008). A comparison was made between resin types to determine which provided a higher quantity of positive samples over the study period. Chi-square tests with Yate's correction were performed on the frequency of positive samples, and they revealed that only for the spirolide, 13-desmethyl-SPX-C was there a difference between the resins, with significantly more positive samples detected using the HP-20 resin ($X^2 = 17.78$, d.f. = 1, $p < 0.001$). The ratio of toxin accumulation per gram of resin was compared between the two resins for each toxin type. HP-20 was found to be more effective in accumulating the majority of

lipophilic phycotoxins than XAD761 (e.g. OA 2.5:1; DTX1 3.5:1). The only exception was PTX2-SA in which a higher quantity per gram of resin was accumulated in the XAD761 (2:1).

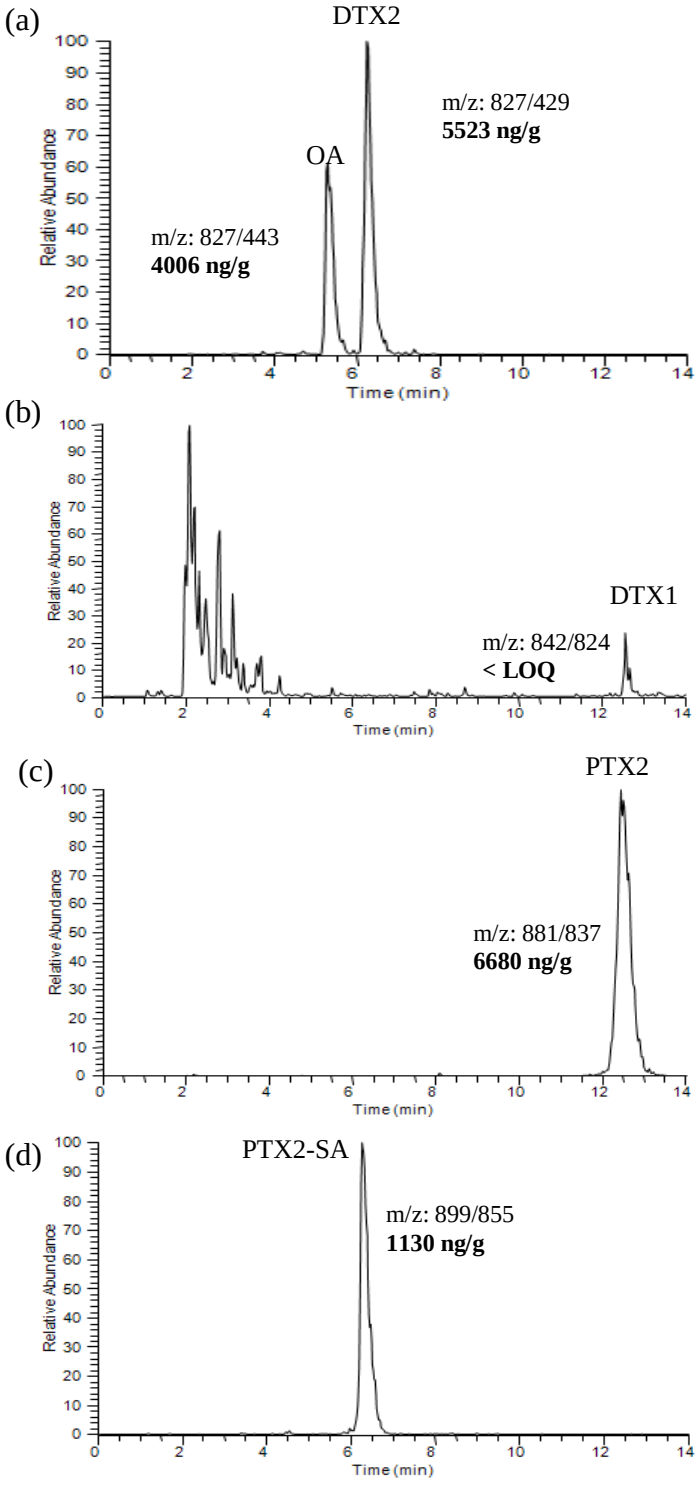



Figure 2.4. Ion chromatograms from Thermo TSQ Quantum Discovery Max triple quadropole mass spectrometer, for the DSP toxins found in the passive SPATT samplers using Diaion HP-20 resin; chromatograms show the m/z values, retention times (RT) and quantities (ng/g) for each toxin (a) OA (m/z 827/443; RT 5.28; 4006 ng/g) and DTX2 (m/z 827/429; RT 6.23; 5523 ng/g); (b) DTX1 (m/z: 842/824; RT 12.55; < LOQ (limit of quantitation)); (c) PTX2 (m/z: 881/837; RT 12.45; 6680 ng/g) (d) PTX2-SA (m/z: 899/855; RT 6.25; 1130 ng/g).

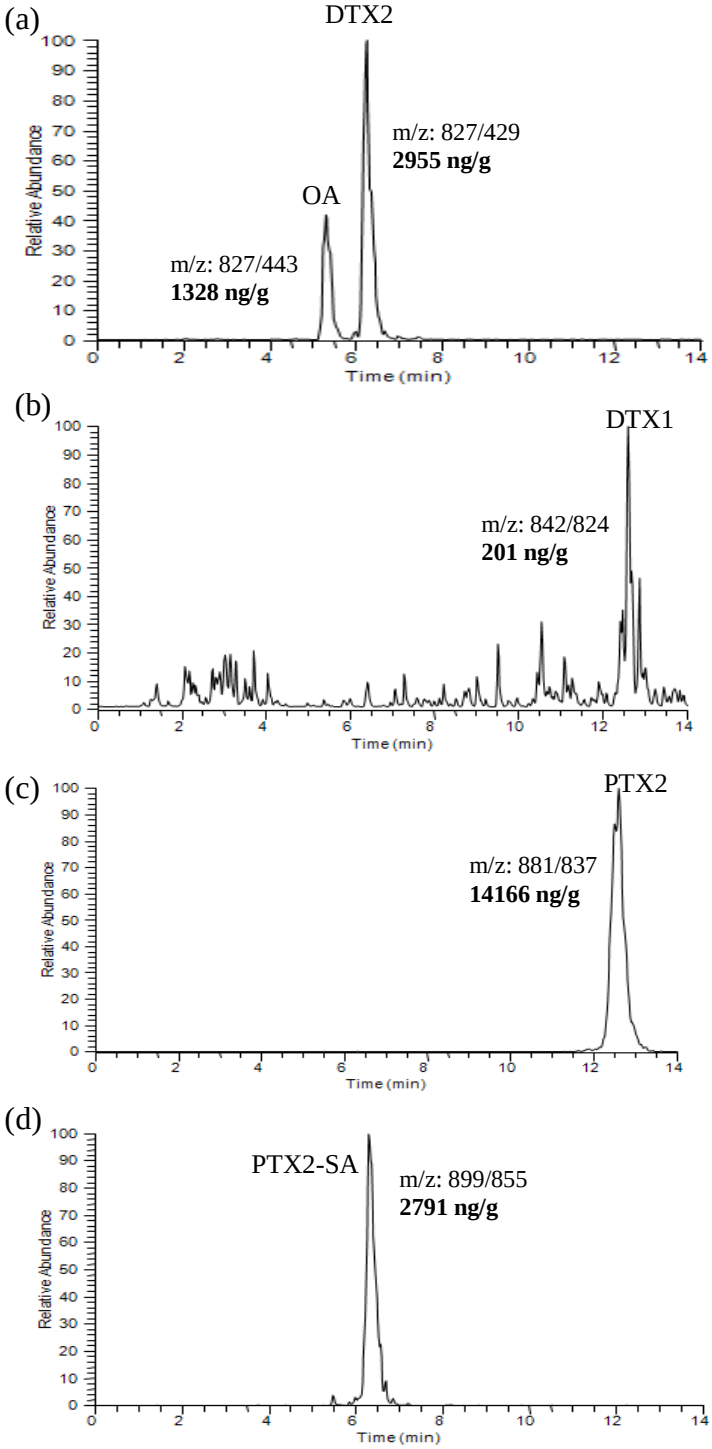



Figure 2.5. Ion chromatograms from Thermo TSQ Quantum Discovery Max triple quadropole mass spectrometer for the DSP toxins found in the passive SPATT samplers using Amberlite ® XAD761 resin; chromatograms show the m/z values, retention times (RT) and quantities (ng/g) for each toxin (a) OA (m/z 827/443; RT 5.3; 1328 ng/g) and DTX2 (m/z 827/429; RT 6.25; 2955 ng/g); (b) DTX1 (m/z: 842/824; RT 12.59; 201 ng/g); (c) PTX2 (m/z: 881/837; RT 12.59; 14166 ng/g) (d) PTX2-SA (m/z: 899/855; RT 6.29; 2791 ng/g).

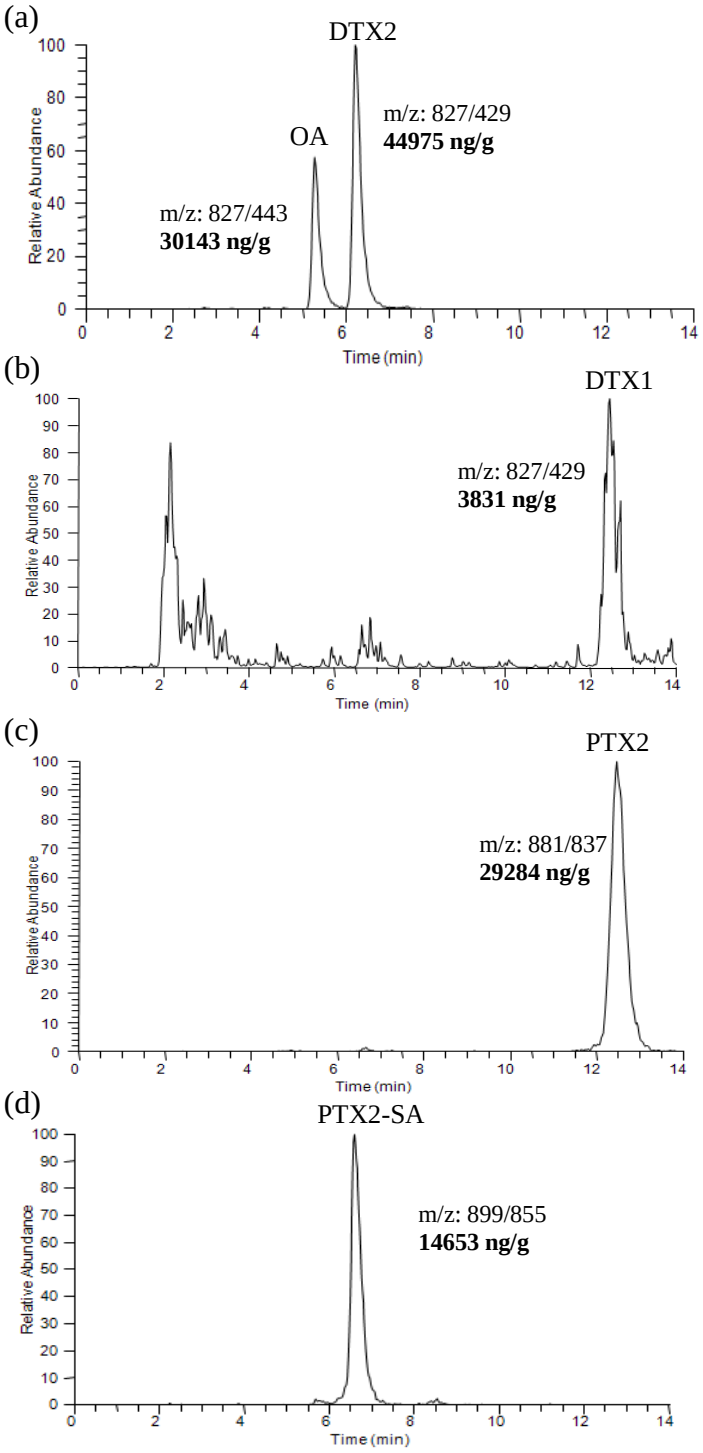



Figure 2.6. Ion chromatograms from Thermo TSQ Quantum Discovery Max triple quadropole mass spectrometer for the DSP toxins found in the active sampler using Diaion resin; chromatograms show the m/z values, retention time (RT) and quantities (ng/g) for each toxin (a) OA (m/z 827/443; RT 5.27; 30143 ng/g) and DTX2 (m/z 827/429; RT 6.23; 44975 ng/g); (b) DTX1 (m/z: 842/824; RT 12.61; 3831 ng/g); (c) PTX2 (m/z: 881/837; RT 12.49 29284 ng/g) (d) PTX2-SA (m/z: 899/855; RT 6.29; 14653 ng/g).

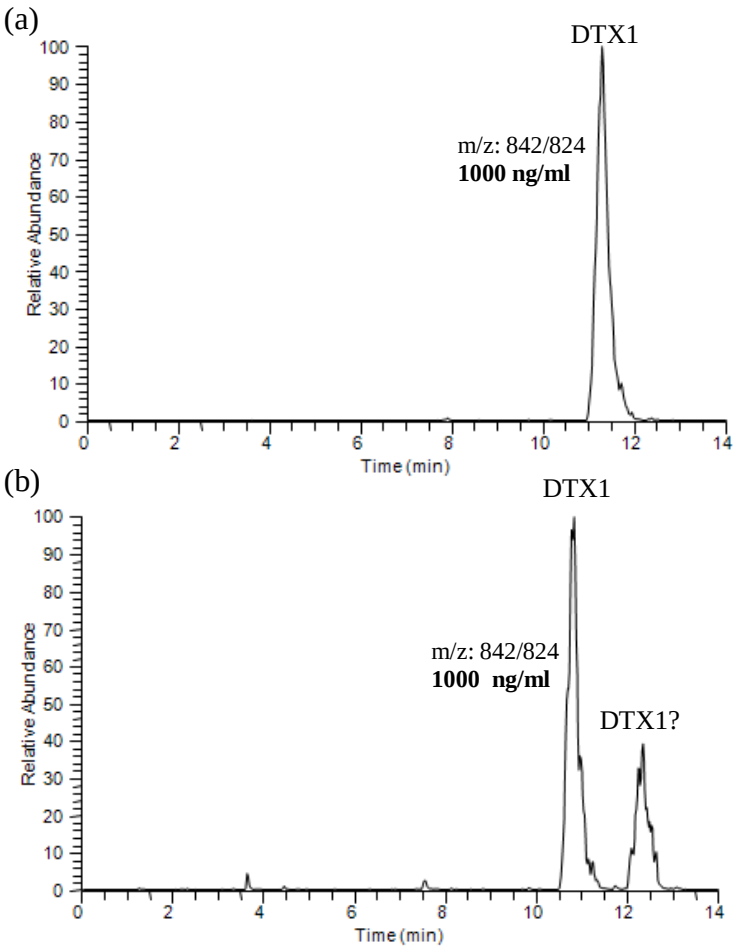



Figure 2.7. Chromatograms of DTX1 generated from analysis of CRM using the Thermo TSQ Quantum Discovery Max triple quadropole mass spectrometer; chromatograms show the m/z values, retention time (RT) and quantities (ng/ml) (a) DTX1 standard spiked in methanol (m.z: 884/824; RT 11.28; 1000 ng/ml) (b) DTX1 standard spiked in methanol with CRM of OA, DTX2 and PTX2 (DTX1: m.z: 884/824; RT 10.82; 1000 ng/ml, DTX1?: m.z: 884/824; RT 12.15).

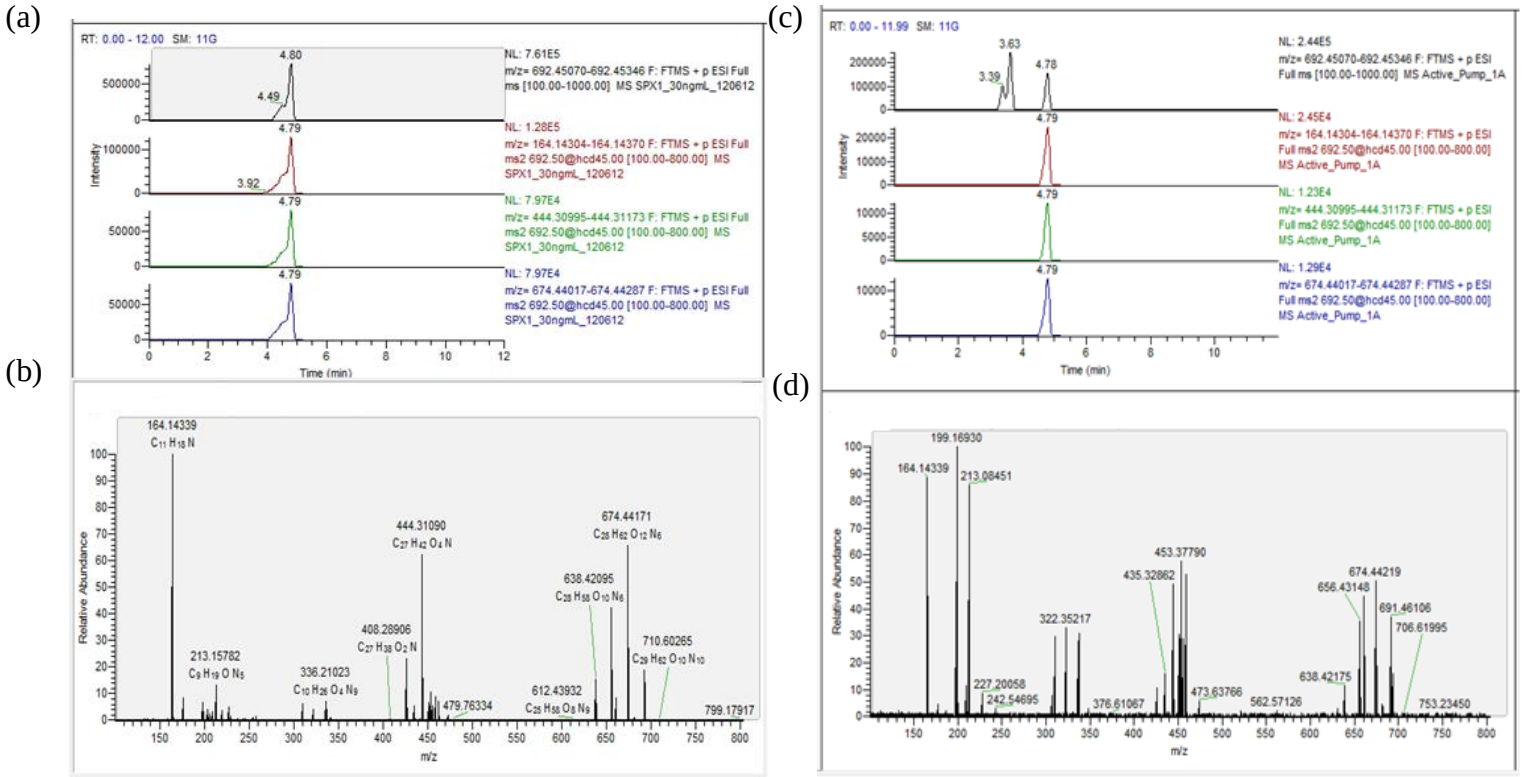



Figure 2.8. Chromatograms and spectrograms of 13 desmethyl-SPX-C analysed using a Thermo Scientific LTQ Orbitrap Discovery Fourier transform mass spectrometer. (a) Chromatogram of CRM active sampling extracts (m/z 164/444/674, Retention time 4.79); (b) spectrogram of CRM; (c) Chromatogram of SPX-C confirmed in resin from active sampler using high mass accuracy (< 2 ppm) (m/z 164/444/674, Retention time 4.79); (d) spectrogram of resin sample. (Figures courtesy of María García Altares Pérez, IRTA, Reus Area, Spain)

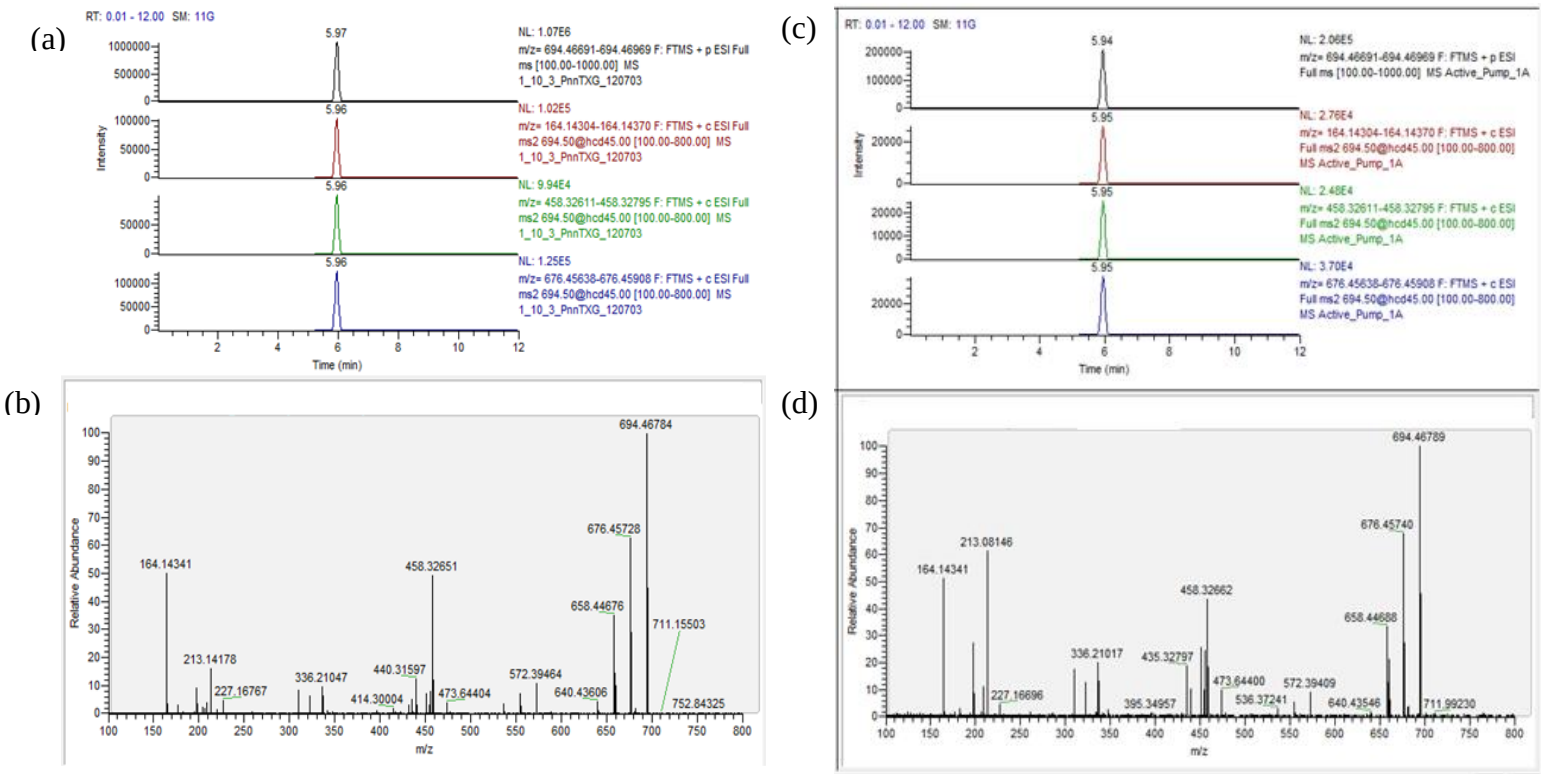



Figure 2.9. Chromatograms and spectrograms of Pinnatoxin G analysed using a Thermo Scientific LTQ Orbitrap Discovery Fourier transform mass spectrometer. (a) Chromatogram of CRM active sampling extracts (m/z 164/458/676, Retention time 5.96); (b) spectrogram of CRM; (c) Chromatogram of Pinnatoxin G confirmed in resin from active sampler using high mass accuracy (< 2 ppm) (m/z 164/458/676, Retention time 5.95); (d) spectrogram of resin sample. (Figures courtesy of María García Altares Pérez, IRTA, Reus Area, Spain).

Table 2.4. The ppm error of the theoretical mass of parent and fragmented ions versus those in the environmental samples is displayed. Acceptable confirmation of a compound is < 5 ppm, this analysis provided a high mass accuracy of < 2 ppm in the environmental samples analysed using the LTQ Orbitrap Discovery.

Data courtesy of María García Altares Pérez, IRTA, Reus Area, Spain.

| | *Theoretical* | | *Found* | | *Δ ppm* | |
|---|---|---|---|---|---|---|
| | **13desmethyl-SPXC** | **PinnatoxinG** | **13desmethyl-SPXC** | **PinnatoxinG** | **13desmethyl-SPXC** | **PinnatoxinG** |
| **[M-H]+** | 692.45208 | 694.4683 | 692.45147 | 694.46837 | -0.88 | 0.1 |
| **Cyclic imine** | 164.14338 | 164.14338 | 164.14339 | 164.14341 | 0.09 | 0.21 |
| *** | 444.31084 | 458.32703 | 444.31105 | 458.32662 | 0.47 | -0.9 |
| **H2O loss** | 674.44152 | 676.45774 | 674.44216 | 676.4574 | 0.95 | -0.5 |
| | | | | | **Confirmed** | **Confirmed** |

**Oxygen and temperature data**

Oxygen and temperature levels in the water were monitored from the surface to 50 m depth. Figures 2.10 and 2.11 illustrate the alteration in oxygen levels coupled with low temperatures (7.5-9$^{o}$C) from 30 - 50 m that occurred in Lough Hyne from March 15$^{th}$ 2010 to June 2$^{nd}$ 2010 providing evidence of an oxygen-thermocline that develops each summer in Lough Hyne marine reserve. A marked decrease in oxygen from 20 – 30m depth in the Western Trough occurred during April 2010.

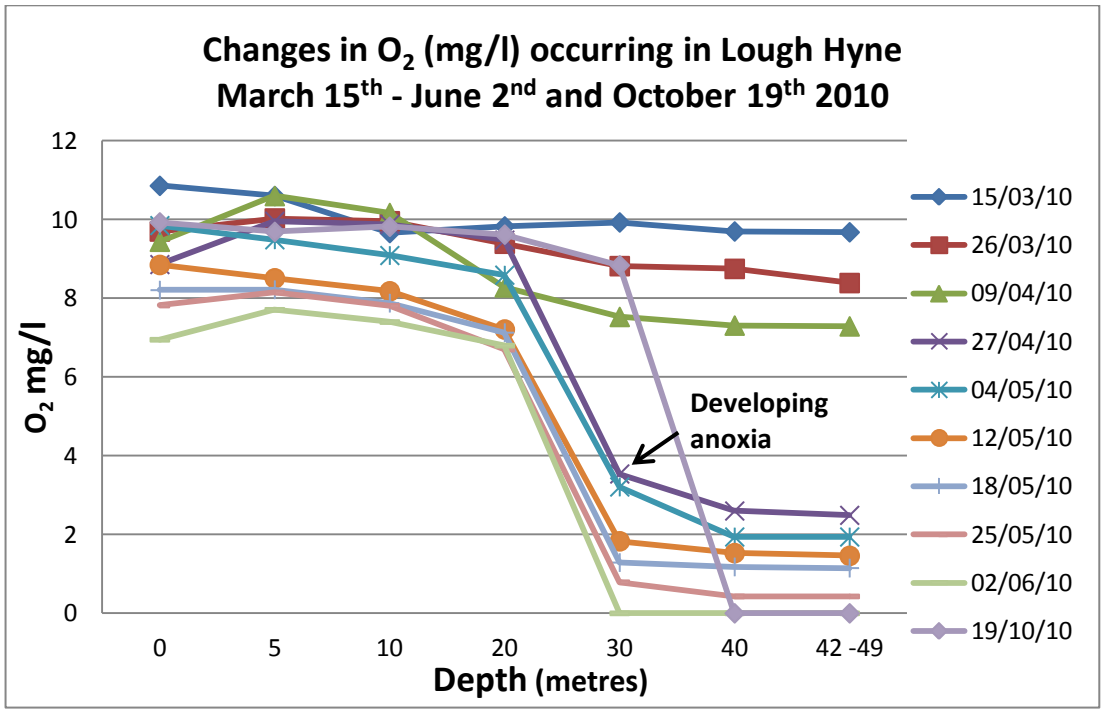



Figure 2.10. Development of an anoxic layer at Lough Hyne Marine Reserve from March 15th 2010 to June 2nd 2010. Oxygen levels in the water between 20 and 30 m drop precipitously on April 27th, with an anoxic layer of water present on the bottom 20 m of water. Data from October 19th 2010 shows some recovery with oxygen levels increasing at 30 m.

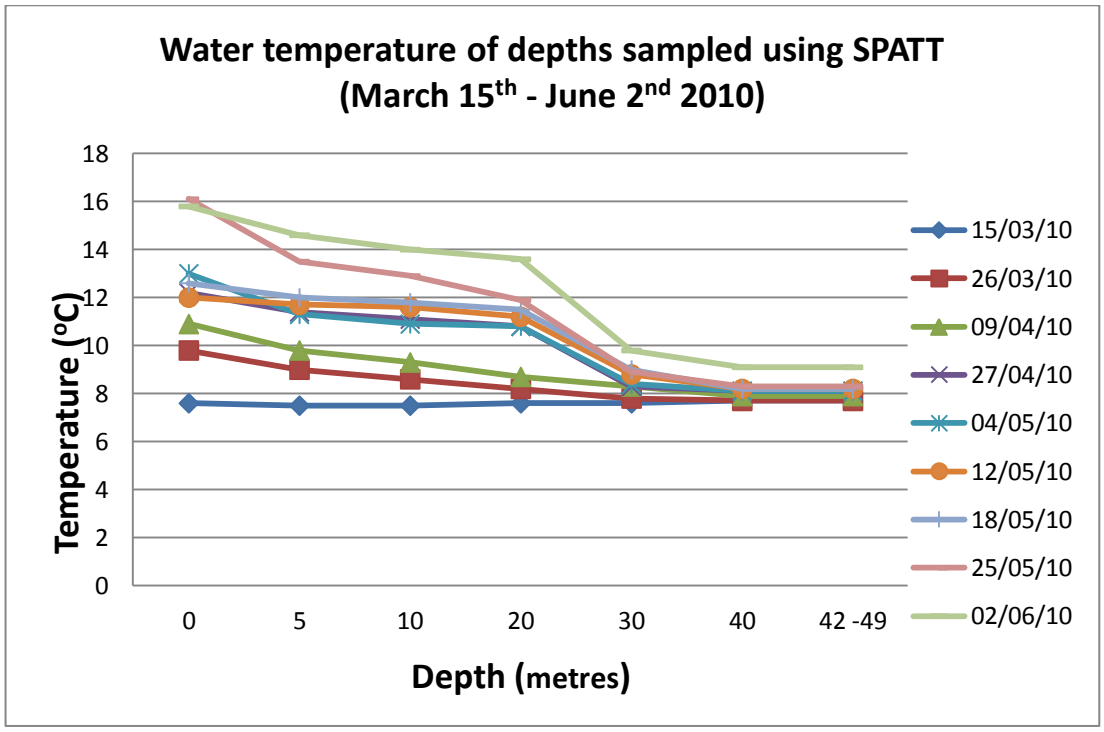



Figure 2.11. Reduction in water temperature due to the development of an oxygen-thermocline at Lough Hyne Marine Reserve from March 15th 2010 to June 2nd 2010. Temperature of the water between 20 and 30 m remains at 7.5 - 9°C despite an increase in temperatures from the surface to 20 m from the 26th of March.

**Toxin profile from SPATT samples at Lough Hyne**

Biomonitoring of Lough Hyne Marine Reserve for the occurrence of marine biotoxins using SPATT revealed a definite pattern in the presence of DSP toxins both between sites and within sites at the discrete sampling depths analysed. Spearman's correlation on the data found there was significant co-occurrence of DSP toxins over the sampling period, at the depths and sites sampled, for both resin types. However, no significant correlation was observed between the occurrence of DSP toxins and SPX-C for HP-20 resin. XAD761 resin extracts showed a significant correlation between the occurrence of OA and SPX-C (Table 2.5).

Table 2.5. Association of the occurrence of DSP toxins (OA, DTX1, DTX2, PTX2, PTX2-SA) and spirolide (SPX-C) extracted from Diaion and Amberlite containing SPATT bags, over depth and sampling period calculated using Spearman's Rank Order Correlation.

| **Diaion HP-20** | **DTX1** $R_S$ (77) = | **DTX2** $R_S$ (77) = | **PTX2** $R_S$ (77) = | **PTX2-SA** $R_S$ (77) = | **SPX-C** $R_S$ (77) = |
|---|---|---|---|---|---|
| **OA** | 0.301 ** | 0.845 ** | 0.845 ** | 0.721 ** | -0.200 |
| **DTX1** | | 0.193 | 0.240 * | 0.408 ** | -0.175 |
| **DTX2** | | | 0.866 ** | 0.680 ** | -0.167 |
| **PTX2** | | | | 0.730 ** | -0.106 |
| **PTX2-SA** | | | | | -0.134 |
| **Amberlite® XAD761** | **DTX1** $R_S$ (81) = | **DTX2** $R_S$ (81) = | **PTX2** $R_S$ (81) = | **PTX2-SA** $R_S$ (81) = | **SPX-C** $R_S$ (81) = |
| **OA** | 0.364 ** | 0.871 ** | 0.792 ** | 0.807 ** | -0.218 * |
| **DTX1** | | 0.329 ** | 0.196 | 0.279 * | -0.174 |
| **DTX2** | | | 0.825 ** | 0.825 ** | -0.205 |
| **PTX2** | | | | 0.899 ** | -0.028 |
| **PTX2-SA** | | | | | -0.002 |

'**' Correlation is significant at the 0.01 level (2-tailed).

'*' Correlation is significant at the 0.05 level (2-tailed).

**Vertical and temporal distribution**

For HP-20 resin there were significant differences found in toxin distribution for OA ($H_6 = 21.89$, $p < 0.01$); DTX2: ($H_6 = 17.14$, $p < 0.01$); PTX2: ($H_6 = 25.79$, $p < 0.01$) and PTX2SA: ($H_6 = 21.26$, $p < 0.01$). For XAD761, differences in distribution were found in OA: ($H_6 = 12.71$, $p < 0.05$) and PTX2: ($H_6 = 25.79$, $p < 0.01$). Mann-Whitney U test was performed to ascertain where these significant differences occurred. As can be seen in Table 2.6, the majority of differences were between the top 30 m of the water column and the bottom 20 m sampled. Tables 2.7 and 2.8 and Figures 2.12 and 2.13 outline the quantities of OA, DTX1, DTX2, PTX2, PTX2-SA and SPX-C that were detected per gram of resin at each depth from May – July, and for the three sites at each depth in August 2010. For the toxins compared using the

Mann-Whitney U test (Table 2.6), it is clearly evident that the majority of toxin adsorbed onto SPATT bags was from the top 30 m of the water column. SPX-C was detected at very low quantities throughout the water column over the dates and sites sampled, with no significant differences found in its distribution over the sampling period (Kruskal-Wallis) (Figures 2.12 (f) and 2.13 (f)). In contrast, no DSP toxins were detected in quantifiable levels at depths greater than 30 m for HP-20; this was also true of XAD761 apart from one sampling date. On August 4$^{th}$ 2010, OA, DTX2, PTX2 and PTX2-SA were extracted from XAD761 resin from 40 m and 50 m depths from the Western Trough (WT) (Figure 2.13). On this date there was a spike in the quantities of DSP toxins in both resin types. When examined using non-parametric ANOVA there was a significant increase in DTX2 accumulated in the HP-20 resin over the three sites for the month of August ($H_8 = 16.6$, $p < 0.05$) (Figure 2.12 (c)). For XAD761 there was a significant increase in OA: ($H_8 = 22.6$, $p < 0.01$); DTX1: ($H_8 = 21.7$, $p < 0.01$); DTX2: ($H_8 = 27.4$, $p < 0.01$); PTX2: ($H_8 = 18.4$, $p < 0.05$) and PTX2-SA: ($H8 = 30.7$, $p < 0.01$) during this period.

Table 2.6. Mann-Whitney U tests were performed to determine at what depths toxin accumulation was significantly different for each resin utilized for the SPATT analysis.

| **Diaion HP-20 SPATT** | | | | | | |
|---|---|---|---|---|---|---|
| **OA** | 5 m | 10m | 20m | 30m | 40m | 50m |
| **Surface** | X | X | X | X | * | * |
| **5 m** | | X | X | * | * | * |
| **10 m** | | | X | X | * | * |
| **20 m** | | | | X | * | * |
| **30 m** | | | | | * | * |
| **40 m** | | | | | | X |
| **DTX2** | 5 m | 10m | 20m | 30m | 40m | 50m |
| **Surface** | X | X | X | X | X | X |
| **5 m** | | X | X | X | * | * |
| **10 m** | | | | * | * | * |
| **20 m** | | | | * | * | * |
| **30 m** | | | | | X | X |
| **40 m** | | | | | | X |
| **PTX2** | 5 m | 10m | 20m | 30m | 40m | 50m |
| **Surface** | X | X | X | X | * | * |
| **5 m** | | X | X | X | * | * |
| **10 m** | | | X | X | * | * |
| **20 m** | | | | X | * | * |
| **30 m** | | | | | * | * |
| **40 m** | | | | | | x |
| **PTX2-SA** | 5 m | 10m | 20m | 30m | 40m | 50m |
| **Surface** | X | X | X | X | * | * |
| **5 m** | | X | X | X | * | * |
| **10 m** | | | X | X | * | * |
| **20 m** | | | | * | * | * |
| **30 m** | | | | | X | X |
| **40 m** | | | | | | X |
| **Amberlite ®XAD761 SPATT** | | | | | | |
| **OA** | 5 m | 10m | 20m | 30m | 40m | 50m |
| **Surface** | X | X | X | X | * | * |
| **5 m** | | X | X | X | * | X |
| **10 m** | | | X | X | * | X |
| **20 m** | | | | X | X | X |
| **30 m** | | | | | X | X |
| **40 m** | | | | | | X |
| **PTX2-SA** | 5 m | 10m | 20m | 30m | 40m | 50m |
| **Surface** | X | X | X | * | * | * |
| **5 m** | | X | X | X | * | * |
| **10 m** | | | X | X | * | * |
| **20 m** | | | | X | * | * |
| **30 m** | | | | | * | X |
| **40 m** | | | | | | X |

'X' denotes no significant difference occurred between these two depths.

'*' difference is significant at the $p < 0.05$ level (2-tailed).

Table 2.7. Quantities of toxin extracted from Diaion HP-20 resin, from SPATT and the active sampler over the study period. Toxin quantities are expressed as nanograms per gram of resin.

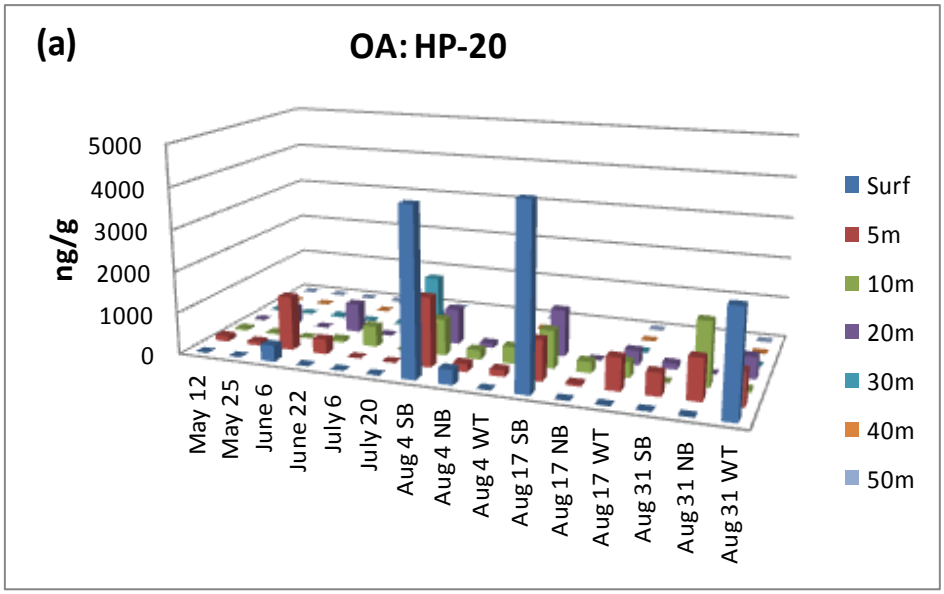
'-'denotes no toxin found in this sample.

Toxin levels colour coded according to concentration: Green = 0 – 100 ng/g; Red = 101-500 ng/g; Blue = 501 -1000 ng/g; Black = 1001 – maximum ng/g.

| Diaion (ng/g) | Depth | OA | DTX1 | DTX2 | PTX2 | PTX2-SA | SPX-C |
|---|---|---|---|---|---|---|---|
| May 12 | Surf | - | - | - | - | - | - |
| | 5m | 130.11 | - | - | 90 | 24.57 | - |
| | 10m | 44.09 | - | - | 160 | 70 | 1.43 |
| | 20m | - | - | - | 250 | 150 | 1.67 |
| | 30m | - | - | - | - | - | 2.88 |
| | 40m | - | - | - | - | - | 1.98 |
| | 50m | - | - | - | - | - | 2.60 |
| May 25 | Surf | - | - | - | - | - | 0.47 |
| | 5m | 90.22 | - | - | 10 | - | 1.37 |
| | 10m | 30.31 | 358.33 | 61.04 | 380 | 70 | - |
| | 20m | 427.45 | 161.86 | - | 190 | 30 | - |
| | 30m | - | - | - | - | - | 2.40 |
| | 40m | - | - | - | - | - | 1.77 |
| | 50m | - | - | - | - | - | 0.36 |
| June 9 | Surf | 392.37 | - | - | 310 | 20 | 2.67 |
| | 5m | 1300.09 | 64.67 | 203.99 | 40 | 142.64 | - |
| | 10m | - | - | - | 80 | - | 1.08 |
| | 20m | - | - | - | - | - | 0.88 |
| | 30m | 29.39 | 178.07 | - | 308.04 | 131.95 | - |
| | 40m | - | - | - | - | - | 0.68 |
| | 50m | - | - | - | - | - | 1.58 |
| June 22 | Surf | - | - | - | - | - | 2.88 |
| | 5m | 354.49 | - | - | 50 | - | 1.31 |
| | 10m | 66.63 | 241.56 | 166.78 | 30 | 144.52 | - |
| | 20m | 702.75 | - | 56.95 | 50 | 261.21 | - |
| | 30m | - | - | - | - | - | 0.71 |
| | 40m | - | - | - | - | - | 1.27 |
| | 50m | - | - | - | - | - | 1.04 |
| July 6 | Surf | - | - | - | - | - | 1.66 |
| | 5m | - | - | - | 90 | - | 1.21 |
| | 10m | 502.23 | - | 470.16 | 30 | 124.65 | - |
| | 20m | - | - | - | 30 | - | 0.54 |
| | 30m | 12.55 | - | - | 19 | 30 | - |
| | 40m | - | - | - | - | - | 0.79 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **50m** | - | - | - | - | - | 0.93 |
| **July 20** | **Surf** | - | - | - | - | - | 1.22 |
| | **5m** | - | - | - | - | - | - |
| | **10m** | - | - | - | - | - | 0.09 |
| | **20m** | 72.70 | - | 673.75 | 580 | 100 | - |
| | **30m** | 1294.45 | - | 0.23 | 170 | 10 | - |
| | **40m** | - | - | - | - | - | 0.07 |
| | **50m** | - | - | - | - | - | 0.09 |
| **Aug 4 SB** | **Surf** | 4006.95 | - | 5522.82 | 6680 | 660 | 0.83 |
| | **5m** | 1665.59 | - | 2422.05 | 39820 | 380 | - |
| | **10m** | 866.27 | - | 1345.64 | 3890 | 450 | 0.79 |
| | **20m** | 851.13 | - | 1416.01 | 4030 | 830 | 1.28 |
| **Aug 4 NB** | **Surf** | 359.84 | - | 623.96 | 1420 | - | 0.28 |
| | **5m** | 191.62 | - | 281.11 | 1390 | 20 | - |
| | **10m** | 222.32 | - | 263.37 | 1190 | - | 0.12 |
| | **20m** | 99.19 | - | 300.88 | 1370 | 210 | 0.86 |
| **Aug 4 WT** | **5m** | 157.08 | 61.85 | 497.97 | 1580 | 80 | 0.18 |
| | **10m** | 402.05 | 40.56 | 614.79 | 2930 | 240 | 0.54 |
| | **20m** | 323.34 | - | 440.67 | 2110 | 290 | 0.66 |
| | **30m** | - | - | - | 180 | - | - |
| | **40m** | - | - | - | - | - | 0.70 |
| | **50m** | - | - | - | - | - | 1.17 |
| **Aug 17 SB** | **Surf** | 4321.07 | 259.20 | 5494.08 | 10950 | 630 | 1.07 |
| | **5m** | 972 | 133.71 | 1008.71 | 7710 | 670 | 4.90 |
| | **10m** | 906.40 | - | 1152.58 | 5400 | 300 | 0.60 |
| | **20m** | 1120.56 | - | 1791.55 | 9260 | 1130 | 2.86 |
| **Aug 17 NB** | **5m** | 53.27 | - | 15.16 | 1130 | - | 0.50 |
| | **10m** | 269.93 | - | 548.93 | 1960 | 70 | 0.38 |
| **Aug 17 WT** | **5m** | 780.92 | - | 1216.38 | 1890 | 70 | 0.20 |
| | **10m** | 374.92 | - | 541.40 | 1850 | 30 | 0.39 |
| | **20m** | 344.99 | - | 442.73 | 2090 | 320 | 1.34 |
| | **30m** | 16.91 | - | - | 350 | - | 0.76 |
| | **40m** | - | - | - | - | - | 0.62 |
| | **50m** | - | - | - | 20 | - | 1.04 |
| **Aug 31 SB** | **5m** | 547.36 | - | 546.02 | 630 | - | 0.11 |
| | **20m** | 139.88 | - | 209.90 | 430 | - | 0.36 |
| **Aug 31 NB** | **5m** | 991.52 | - | 1421.18 | 1590 | - | 0.47 |
| | **10m** | 1521.02 | - | 1894.36 | 3040 | - | 1.98 |
| **Aug 31 WT** | **Surf** | 2486.75 | - | 3390.33 | 1460 | 170 | - |
| | **5m** | 812.75 | - | 911.80 | 920 | 20 | - |
| | **10m** | 64.80 | - | 263.33 | 530 | - | - |
| | **20m** | 524.15 | - | 426.89 | 1010 | 200 | - |
| | **30m** | - | - | - | 20 | - | - |
| | **40m** | - | - | - | - | - | - |
| | **50m** | - | - | - | - | - | - |

| | | OA | DTX1 | DTX2 | PTX2 | PTX2-SA | SPX-C |
|---|---|---|---|---|---|---|---|
| **Aug 24-31st** | **Cartridge 1a** | 30617.28 | _ | 44975.44 | 29283.55 | 14652.59 | 3.52 |
| | **Cartridge 1b** | 16663.79 | 3831.08 | 35393.40 | 21042.27 | 7188.98 | 2.71 |
| | **Cartridge 2a** | 3574.75 | _ | 9568.75 | 5463.99 | 1638.55 | 6.61 |
| | **Cartridge 2b** | 4387.75 | _ | 27763.17 | 28058.42 | 1869.18 | 12.03 |
| **Average** | **ng/g** | 13811 | 958 | 29425 | 20962 | 6337 | 6.22 |
| **Total toxin/1kg** | **mg** | 13.81 | 3.83 | 29.43 | 20.96 | 6.34 | 0.01 |
| **Average /litre** | **ng** | 205.52 | 57.01 | 437.87 | 311.94 | 94.31 | 0.09 |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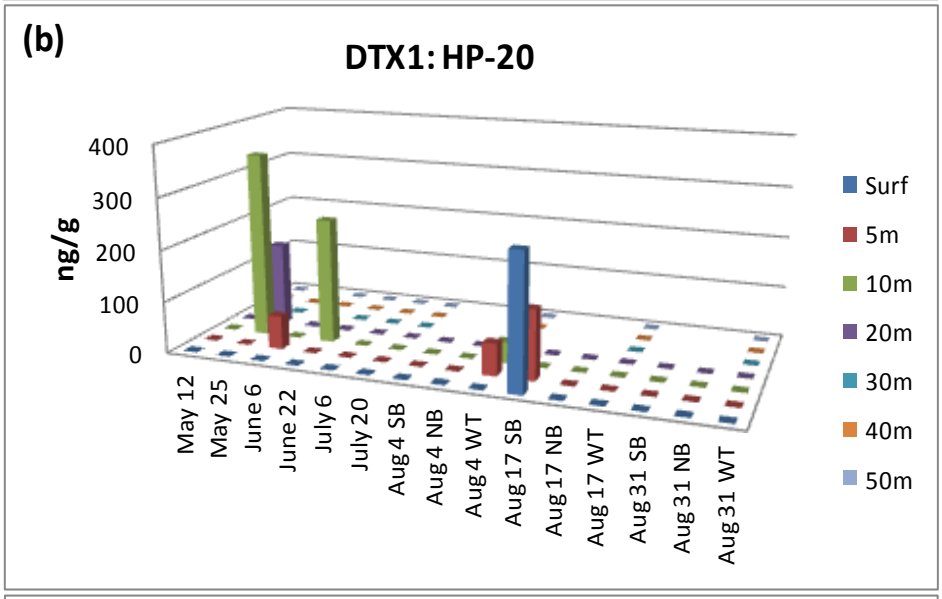
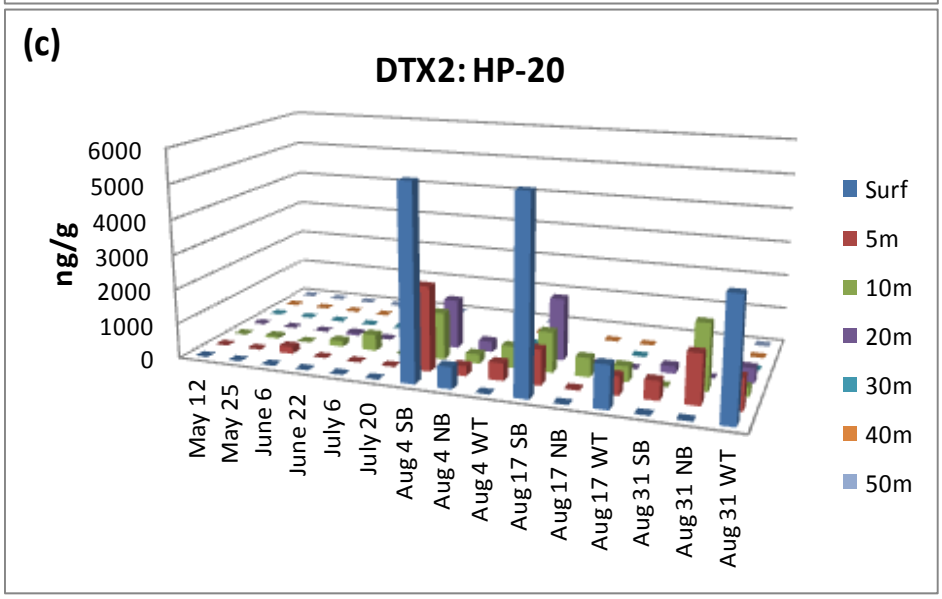
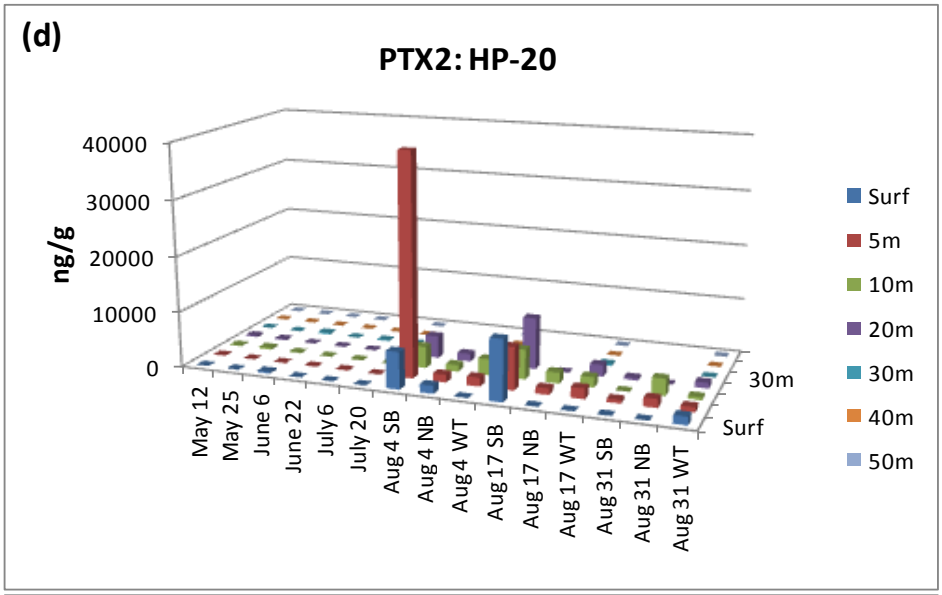
ng/g
5000
4000
3000
2000
1000
0
May 12
May 25
June 6
June 22
July 6
July 20
Aug 4 SB
Aug 4 NB
Aug 4 WT
Aug 17 SB
Aug 17 NB
Aug 17 WT
Aug 31 SB
Aug 31 NB
Aug 31 WT
Surf
5m
10m
20m
30m
40m
50m


(b)
DTX1: HP-20
ng/g
400
300
200
100
0
May 12
May 25
June 6
June 22
July 6
July 20
Aug 4 SB
Aug 4 NB
Aug 4 WT
Aug 17 SB
Aug 17 NB
Aug 17 WT
Aug 31 SB
Aug 31 NB
Aug 31 WT
Surf
5m
10m
20m
30m
40m
50m


(c)
DTX2: HP-20
ng/g
6000
5000
4000
3000
2000
1000
0
May 12
May 25
June 6
June 22
July 6
July 20
Aug 4 SB
Aug 4 NB
Aug 4 WT
Aug 17 SB
Aug 17 NB
Aug 17 WT
Aug 31 SB
Aug 31 NB
Aug 31 WT
Surf
5m
10m
20m
30m
40m
50m


(d)
PTX2: HP-20
ng/g
40000
30000
20000
10000
0
May 12
May 25
June 6
June 22
July 6
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Aug 4 SB
Aug 4 NB
Aug 4 WT
Aug 17 SB
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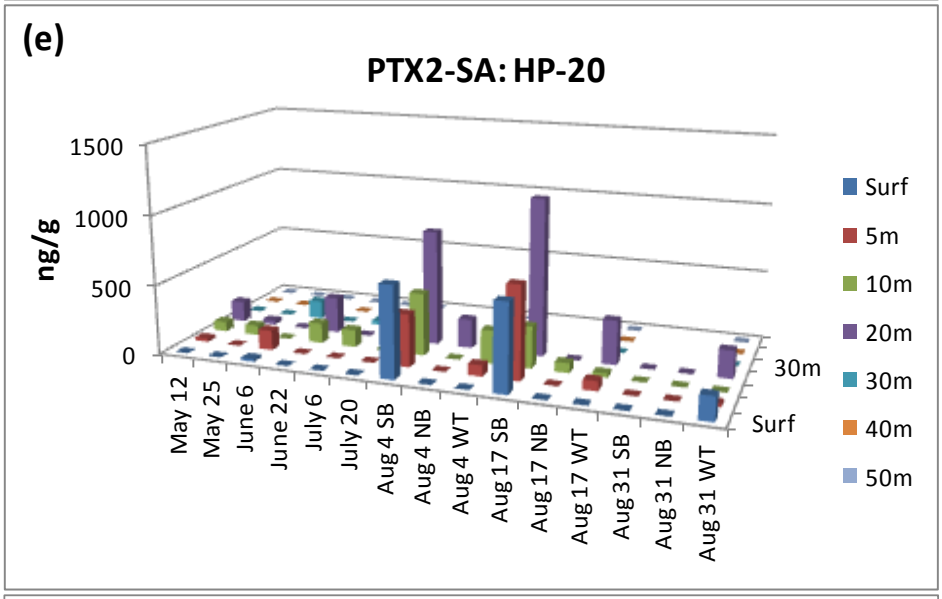
20m
30m
40m
50m


(e)
PTX2-SA: HP-20
ng/g
1500
1000
500
0
May 12
May 25
June 6
June 22
July 6
July 20
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Aug 4 NB
Aug 4 WT
Aug 17 SB
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20m
30m
40m
50m

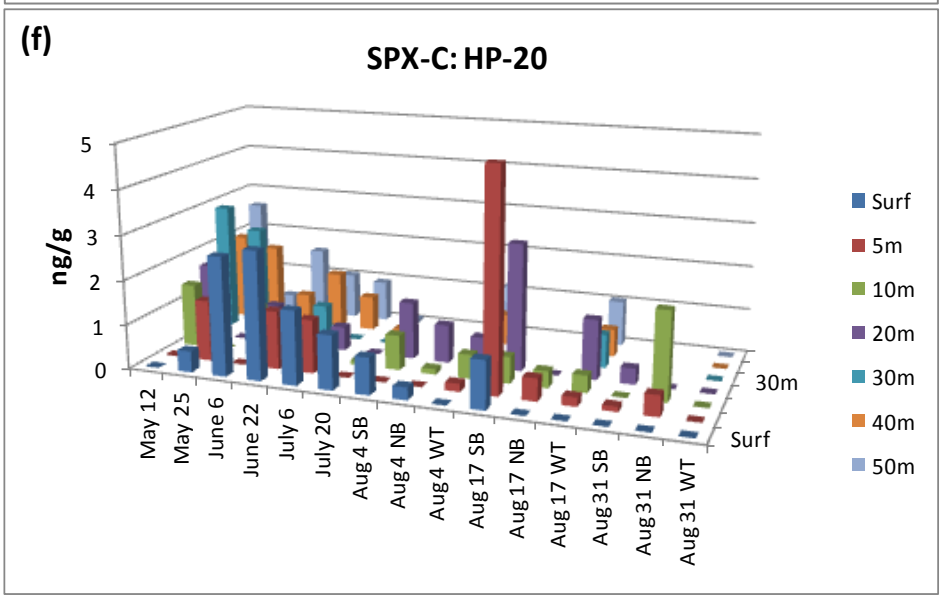

(f)
SPX-C: HP-20
ng/g
5
4
3
2
1
0
May 12
May 25
June 6
June 22
July 6
July 20
Aug 4 SB
Aug 4 NB
Aug 4 WT
Aug 17 SB
Aug 17 NB
Aug 17 WT
Aug 31 SB
Aug 31 NB
Aug 31 WT
30m
Surf
Surf
5m
10m
20m
30m
40m
50m

Figure 2.12. Quantities of toxin detected from SPATT bags containing Diaion HP-20 resin (ng/g) over the 4-month sampling period at Lough Hyne for each of the depths sampled (surface – 50m). Data for August 2010 is separated according to site: SB = South Basin; NB = North Basin; WT = Western Trough. (a) OA; (b) DTX1; (c) DTX2; (d) PTX2; (e) PTX2-SA; (f) 13-desmethyl-SPX-C.

Table 2.8. Results of Amberlite ® XAD761 resin from SPATT over the sites and depths sampled during the study period. Toxin quantities are expressed as nanograms per gram of resin.

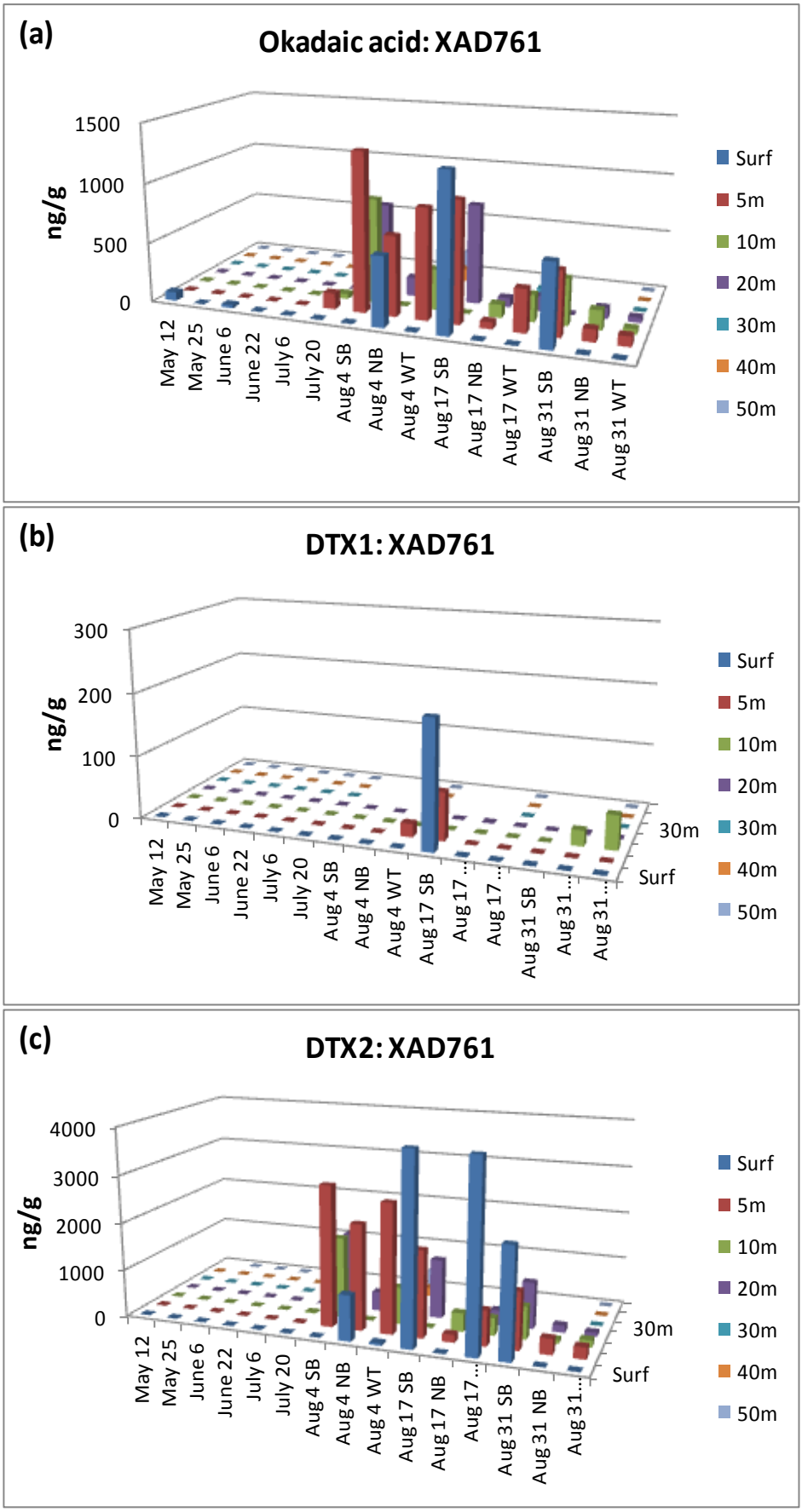
'-' denotes no toxin found in this sample.

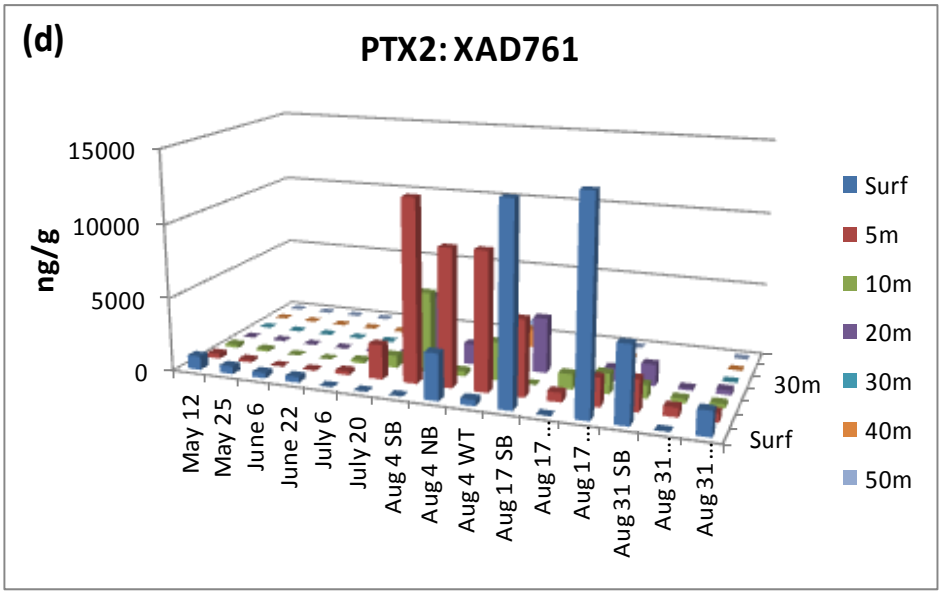
Toxin levels colour coded according to concentration: Green = 0 – 100 ng/g; Red = 101-500 ng/g; Blue = 501 -1000 ng/g; Black = 1001 – maximum ng/g.

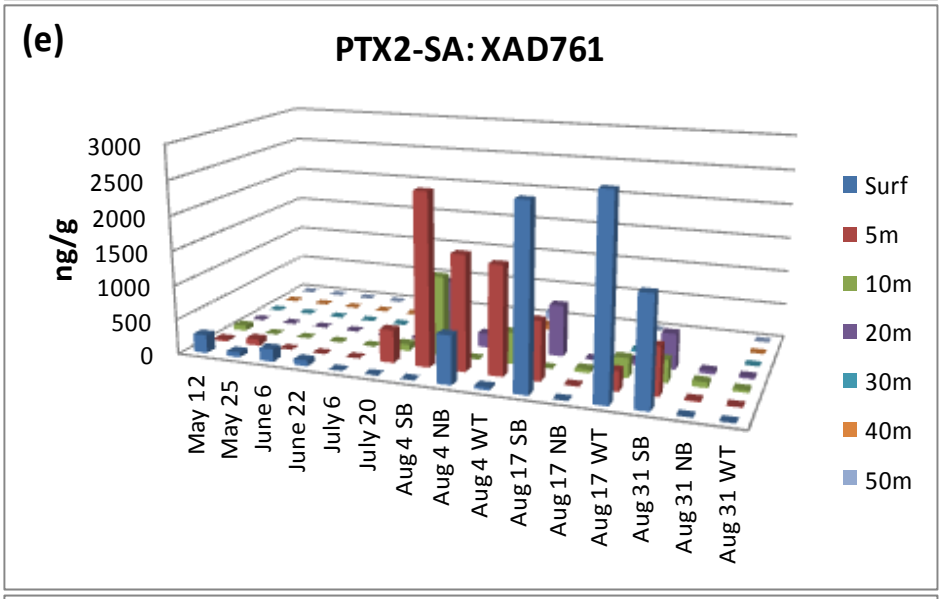
| Amberlite (ng/g) | Depth | OA | DTX1 | DTX2 | PTX2 | PTX2-SA | SPX-C |
|---|---|---|---|---|---|---|---|
| May 12 | Surf | 72.91 | - | - | 926.80 | 265.05 | 0.77 |
| | 5m | - | - | - | 316.70 | 29.30 | 0.77 |
| | 10m | - | - | - | 222.49 | 75.77 | 0.40 |
| | 40m | - | - | - | - | - | 0.34 |
| May 25 | Surf | - | - | - | 511.67 | 72.58 | 1.38 |
| | 5m | - | - | - | 166.78 | 93.31 | 0.34 |
| | 10m | - | - | - | 126.06 | - | 0.26 |
| | 20m | - | - | - | 20.30 | - | - |
| | 30m | - | - | - | - | - | - |
| | 40m | - | - | - | - | - | - |
| | 50m | - | - | - | - | - | - |
| Jun 09 | Surf | 25.23 | - | - | 374.20 | 182.69 | - |
| | 5m | - | - | - | - | - | - |
| | 10m | - | - | - | - | - | - |
| | 20m | - | - | - | - | - | 0.32 |
| | 30m | - | - | - | - | - | - |
| | 40m | - | - | - | - | - | - |
| | 50m | - | - | - | - | - | - |
| Jun 22 | Surf | - | - | - | 390.61 | 83.47 | 0.72 |
| | 5m | - | - | - | 46.21 | - | - |
| | 10m | - | - | - | - | - | - |
| | 20m | - | - | - | - | - | - |
| | 30m | - | - | - | - | - | - |
| | 40m | - | - | - | - | - | - |
| Jul 06 | Surf | - | - | - | - | - | 0.57 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 5m | - | - | - | 263.82 | - | 0.38 |
| | 10m | - | - | - | 198.33 | - | - |
| | 20m | - | - | - | 107.19 | - | - |
| | 30m | - | - | - | 6.87 | - | - |
| | 40m | - | - | - | - | - | - |
| | 50m | - | - | - | - | - | - |
| Jul 20 | Surf | - | - | - | - | - | 0.64 |
| | 5m | 133.99 | - | - | 2367.81 | 473.05 | - |
| | 10m | 43.25 | - | 16.64 | 830.18 | 94.35 | - |
| | 20m | - | - | - | 160.56 | - | 0.36 |
| | 30m | - | - | - | 264.16 | - | 1.05 |
| | 40m | - | - | - | - | - | - |
| | 50m | - | - | - | - | - | 0.72 |
| Aug 4 SB | Surf | - | - | - | - | - | 1.38 |
| | 5m | 1328.95 | - | 2955.19 | 12371.21 | 2467.44 | - |
| | 10m | 880.26 | - | 1698.58 | 5450.76 | 1145.32 | - |
| | 20m | 766.04 | - | 1612.90 | 4502.23 | 923.01 | - |
| Aug 4 NB | Surf | 583.38 | - | 953.77 | 3151.65 | 692.86 | 0.93 |
| | 5m | 668.91 | - | 2217.62 | 9322.71 | 1648.41 | - |
| | 10m | - | - | - | 336.02 | 5.31 | - |
| | 20m | 158.97 | - | 406.51 | 1407.16 | 200.93 | - |
| Aug 4 WT | Surf | - | - | 46.32 | 414.68 | 39.90 | - |
| | 5m | 921.34 | 21.05 | 2711.17 | 9357.68 | 1557.74 | - |
| | 10m | 343.01 | - | 779.46 | 2615.81 | 454.08 | - |
| | 20m | 148.27 | - | 219.95 | 1191.10 | 125.74 | - |
| | 30m | 126.73 | - | 219.92 | 1172.94 | - | - |
| | 40m | 106.10 | - | 185.58 | 1245.28 | 68.03 | - |
| | 50m | 186.65 | - | 389.66 | 1078.57 | 104.03 | - |
| Aug 17 SB | Surf | 1295.58 | 201.38 | 3943.79 | 13359.10 | 2582.32 | - |
| | 5m | 1008.84 | 76.39 | 1818.96 | 5160.61 | 848.17 | - |
| | 10m | 0.86 | - | 1.57 | 3.68 | 0.69 | - |
| | 20m | 834.91 | - | 1247.68 | 3771.65 | 740.91 | - |
| Aug 17 NB | Surf | - | - | - | - | - | - |
| | 5m | 57.34 | - | 163.50 | 636.96 | 6.52 | - |
| | 10m | 110.54 | - | 390.08 | 1038.13 | 52.37 | - |
| | 20m | 76.87 | 1.11 | 143.34 | 113.95 | 9.22 | - |
| Aug 17 WT | Surf | - | - | 3917.67 | 14166.87 | 2791.11 | - |
| | 5m | 359.27 | - | 743.53 | 1938.28 | 285.90 | - |
| | 10m | 225.12 | - | 366.14 | 1383.97 | 281.87 | - |
| | 20m | 123.82 | - | 309.55 | 895.02 | 68.62 | - |
| | 30m | 98.73 | - | 132.67 | 157.26 | 26.04 | - |
| | 40m | - | - | - | - | - | - |
| | 50m | - | - | - | - | - | - |
| Aug 31 SB | Surf | 681.61 | - | 2290.60 | 5168.22 | 1541.12 | 2.40 |
| | 5m | 535.14 | - | 1200.97 | 2161.05 | 687.76 | 0.70 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **10m** | 388.64 | - | 686.46 | 929.25 | 321.43 | - |
| | **20m** | - | - | 978.52 | 1457.94 | 513.53 | 0.39 |
| **Aug 31 NB** | **Surf** | - | - | - | - | - | - |
| | **5m** | 103.90 | - | 332.61 | 636.13 | 17.66 | - |
| | **10m** | 162.44 | 24.70 | 79.24 | 305.37 | 79.56 | - |
| | **20m** | 90.97 | 2.58 | 149.64 | 122.34 | 46.06 | - |
| **Aug 31 WT** | **Surf** | - | - | - | 1664.03 | - | - |
| | **5m** | 84.01 | - | 244.29 | 616.19 | 8.02 | - |
| | **10m** | 44.42 | 54.24 | 151.30 | 301.21 | 37.22 | - |
| | **20m** | 42.01 | - | 80.79 | 328.13 | 23.41 | - |
| | **30m** | - | - | - | - | - | 0.56 |
| | **40m** | - | - | - | - | - | - |
| | **50m** | - | - | - | - | - | - |


(a)
Okadaic acid: XAD761
ng/g
1500
1000
500
0
May 12
May 25
June 6
June 22
July 6
July 20
Aug 4 SB
Aug 4 NB
Aug 4 WT
Aug 17 SB
Aug 17 NB
Aug 17 WT
Aug 31 SB
Aug 31 NB
Aug 31 WT
Surf
5m
10m
20m
30m
40m
50m
(b)
DTX1: XAD761
ng/g
300
200
100
0
30m
Surf
(c)
DTX2: XAD761
ng/g
4000
3000
2000
1000
0
30m
Surf


(d)
PTX2: XAD761
15000
10000
5000
0
ng/g
May 12
May 25
June 6
June 22
July 6
July 20
Aug 4 SB
Aug 4 NB
Aug 4 WT
Aug 17 SB
Aug 17 ...
Aug 17 ...
Aug 31 SB
Aug 31 ...
Aug 31 ...
30m
Surf
Surf
5m
10m
20m
30m
40m
50m


(e)
PTX2-SA: XAD761
3000
2500
2000
1500
1000
500
0
ng/g
May 12
May 25
June 6
June 22
July 6
July 20
Aug 4 SB
Aug 4 NB
Aug 4 WT
Aug 17 SB
Aug 17 NB
Aug 17 WT
Aug 31 SB
Aug 31 NB
Aug 31 WT
Surf
5m
10m
20m
30m
40m
50m

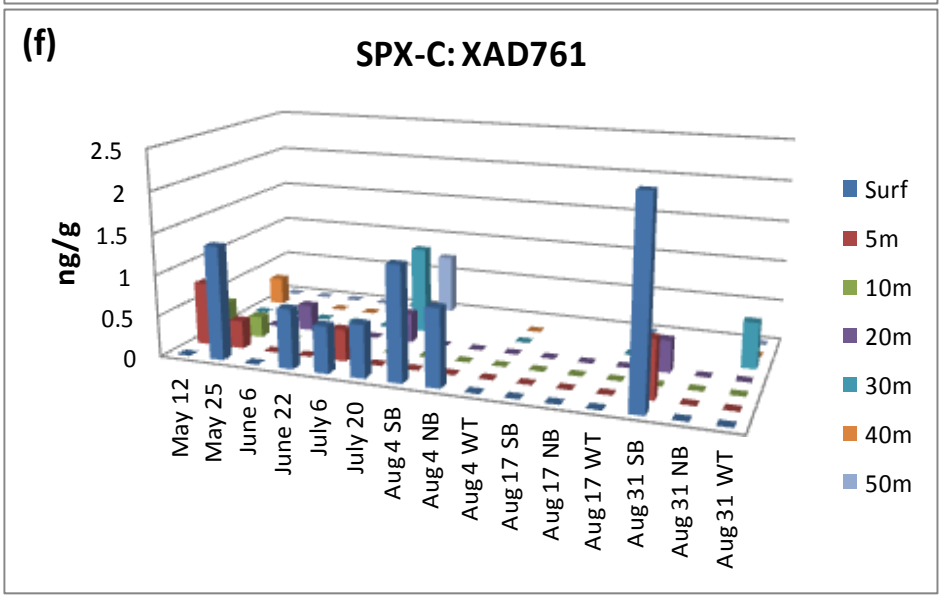

(f)
SPX-C: XAD761
2.5
2
1.5
1
0.5
0
ng/g
May 12
May 25
June 6
June 22
July 6
July 20
Aug 4 SB
Aug 4 NB
Aug 4 WT
Aug 17 SB
Aug 17 NB
Aug 17 WT
Aug 31 SB
Aug 31 NB
Aug 31 WT
Surf
5m
10m
20m
30m
40m
50m

Figure 2.13 Quantities of toxin detected from SPATT bags containing Amberlite ® XAD671 resin (ng/g) over the 4-month sampling period at Lough Hyne for each of the depths sampled (surface – 50m). Data for August 2010 is separated according to site: SB = South Basin; NB = North Basin; WT = Western Trough. (a) OA; (b) DTX1; (c) DTX2; (d) PTX2; (e) PTX2-SA; (f) 13-desmethyl-SPX-C

**Site differences**

The increase in toxin accumulation observed during August 2010 is particularly marked in the South Basin (SB); this site is located near to the rapids, which are fed by tidal currents (Figure 2.2). The three sites (from surface to 20 m) from August were compared using non-parametric ANOVA for each resin type. HP-20 showed differences between sites for OA: ($H_2 = 7.3$, $p < 0.05$); PTX2: ($H_2 = 6.9$, $p < 0.05$) and PTX2-SA: ($H_2 = 10.9$, $p < 0.01$). These differences were between the SB and the Western Trough (WT); and the SB and the North Basin (NB). There were no differences between the WT and the NB for OA or PTX2, however there was for PTX2-SA (Mann-Whitney U-test, $p < 0.05$).

XAD761 showed differences between sites for OA: ($H_2 = 8.8$, $p < 0.05$); DTX2: ($H_2 = 9.9$, $p < 0.01$); PTX2: ($H_2 = 8.3$, $p < 0.05$); PTX2-SA: ($H_2 = 9.3$, $p < 0.01$) and SPX-C: ($H_2 = 6.5$, $p < 0.05$). These differences occurred between the SB and the NB for all toxins, excluding SPX-C, and between the SB and the WT for all toxins excluding PTX2. There were no differences between the NB and the WT using XAD761 (Mann-Whitney U-test, $p < 0.05$).

**Active toxin sampler**

A high accumulation of toxin was measured per gram of resin for OA (13811 ng/g), DTX2 (20962 ng/g), PTX2 (20962 ng/g) and PTX2-SA (6337 ng/g), however DTX1 was only detectable in one sample and based on this quantity an average of 958 ng/g of this toxin was detected (Table 2.7). Additional analysis is still required to confirm the presence of DTX1 from the active sampler, as there was possible interference from the presence of the other toxins, particularly PTX2, which has the same retention time as DTX1. SPX-C was also accumulated in higher quantities than were

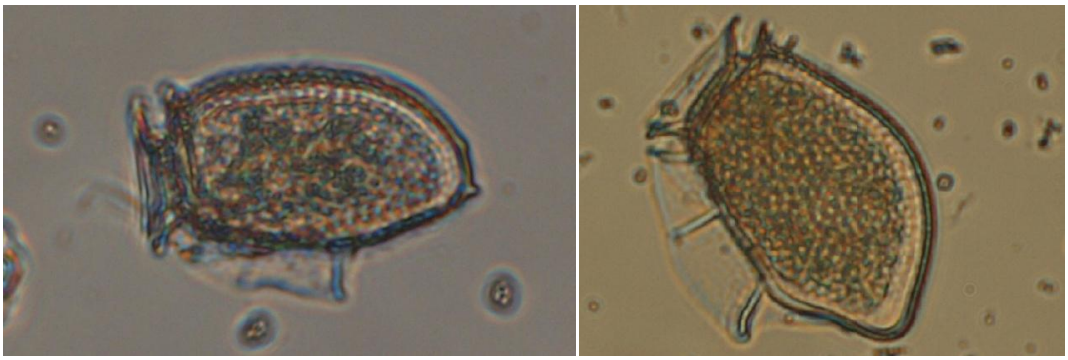
detected in the SPATT bags, with an average of 6.22 ng/g extracted. Pinnatoxin G was confirmed but not quantified in these samples.

The flow rate was measured as 400 l $hr^{-1}$, thus assuming a constant flow rate over the sampling period approximately 6.7 x $10^{-4}$ l of water was pumped through the cartridges and sampled continuously by the resin. Using these data the toxin content (nanograms) per litre of water was estimated for each of the toxins OA: 206 ng/l; DTX1: 57 ng/l; DTX2: 438 ng/l; PTX2: 312 ng/l; PTX2: 94 ng/l and SPX-C 0.09 ng/l (Table 2.7).

**Algal species detected**

Six samples from the South Basin (see Figure 2.2 for site location) were chosen as representatives for the determination of the toxin-producing algal species present in Lough Hyne. This site was chosen because during the month of August, 2010 levels of DSP toxins increased substantially from the quantities per gram of resin detected in the previous months. Table 2.9 gives a full list of algal species identified in samples examined, including the potential DSP-toxin producing algae: *Dinophysis acuta*, *Dinophysis acuminata*, and *Prorocentrum* sp. Also identified in the samples was *Alexandrium* sp. which was suspected as a possible producer of SPX-C, as this toxin was found at low levels in the SPATT bags over the sampling period (Figure 2.14).

*Dinophysis acuminata* *Dinophysis acuta*



*Prorocentrum* sp. *Alexandrium* sp

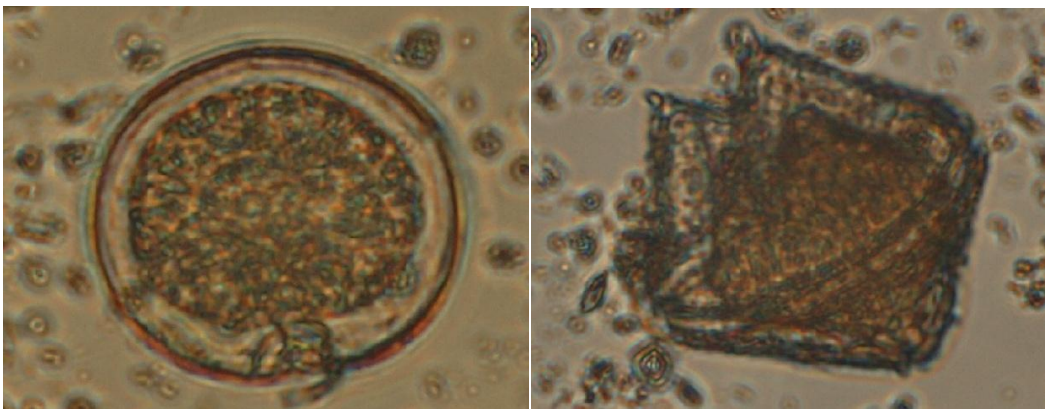


Figure 2.14. Examples of potential toxin-producing algal species isolated from Lough Hyne marine reserve. Photographs taken by Dunmanus Seafood Ltd. Goleen, Co. Cork.

Table 2.9. Full list of phytoplankton species identified from the South Basin at different depths on the 4th August 2010 and the 17th August 2010.

* Potentially toxin-producing species

'X' denotes the presence of an algal species

| Algal species | 04/08/10 (surf) | 04/08/10 (5m) | 17/08/10 (surf) | 17/08/10 (5m) | 17/08/10 (10m) | 17/08/10 20m |
|---|---|---|---|---|---|---|
| *Actinoptychus spp.* | - | - | - | X | X | X |
| *Alexandrium type spp** | - | X | - | - | - | - |
| *Bacteriastrum spp* | - | - | - | - | X | - |
| *Ceratium fusus* | X | X | X | X | X | X |
| *Ceratium horridum* | - | X | - | - | - | - |
| *Ceratium lineatum* | X | X | - | - | - | - |
| *Ceratium longipes* | - | - | - | - | - | X |
| *Ceratium tripos* | - | - | - | - | X | - |
| *Ceratiun candelabrum* | - | - | - | - | X | - |
| *Ceratiun tripos* | - | - | - | - | - | X |
| *Ceratulina pelagica* | - | - | X | - | - | X |
| *Ceratulina spp.* | - | - | - | X | - | - |
| *Cillates* | - | X | - | - | - | - |
| *Dictyocha speculum* | - | - | X | - | - | - |
| *Dinobryon spp.* | - | - | - | X | - | - |
| *Dinoflagellate spp type 30um* | - | - | - | - | - | X |
| *Dinophysis acuminata** | X | X | X | X | X | X |
| *Dinophysis acuta** | X | X | X | X | X | X |
| *Dinophysis dens* | - | X | - | - | X | - |
| *Dinophysis rotundata* | - | X | - | - | - | - |
| *Dissodium asymmetricum* | - | - | - | - | - | X |
| *Ditylum brightwelli* | - | X | - | - | - | - |
| *Eucampia zoodiacus* | - | - | - | - | X | - |
| *Favella spp* | - | - | X | X | - | X |
| *Gonyaulax spinifera* | - | X | - | - | - | - |
| *Gonyaulax spp* | X | - | - | - | - | - |
| *Guinardia delicatula* | - | - | - | - | - | X |
| *Guinardia flaccida* | - | - | - | X | X | - |
| *Haptophytes* | X | - | - | - | - | - |
| *Karenia mikimotoi** | X | X | X | - | - | - |
| *Karenia spp.* | - | X | - | - | - | - |
| *Lauderia confervacea* | - | - | - | - | X | - |
| *Lauderia spp* | - | - | X | X | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Leptocylindrus danicus* | - | - | X | - | - | - |
| *Leptocylindrus minimus* | - | X | X | X | X | - |
| *Meuniera membranacea* | - | - | X | - | - | - |
| *Muineria membranacea* | - | X | - | X | - | - |
| *Navicula spp* | X | - | - | - | - | - |
| *Navicula spp <10um* | - | - | - | - | - | - |
| *Navicula spp <20um* | - | - | - | X | - | X |
| *Nitzschia single cells<20 um* | - | - | - | X | - | - |
| *Oxytoxum spp* | - | - | X | X | - | - |
| *Paralia sulcata* | - | X | X | X | - | X |
| *Pleurosigma angulatum* | - | - | - | - | X | - |
| *Polykrikos* | - | - | X | - | - | - |
| *Prorocentrum balticum* | - | - | - | X | - | - |
| *Prorocentrum gracile* | - | X | - | - | - | - |
| *Prorocentrum micans* | X | X | - | X | X | - |
| *Prorocentrum minimum/balticum** | X | X | - | - | - | X |
| *Protoperidinium pallidum* | - | X | - | - | - | X |
| *Protoperidinium spp<50um* | - | - | - | X | - | - |
| *Pseudo nitzscha seriata** | - | - | - | - | X | - |
| *Pseudo nitzschia delicatissima* | X | - | - | X | X | X |
| *Rhizosolenia setigera* | - | X | X | X | - | - |
| *Rhizosolenia spp (broken)* | - | - | X | - | X | X |
| *Rhizosolenia styliformis* | - | - | - | X | - | - |
| *Scrippsiella troichoideum* | - | X | - | - | - | X |
| *Striatella unipunctata* | - | X | X | - | - | - |
| *Thalassionema nitzschoides* | - | X | - | - | - | - |
| *Tintinnids* | - | - | X | - | X | X |

## DISCUSSION

The current study had two main objectives; the first, utilising SPATT technology as a monitoring tool for assessing the distribution of lipophilic marine phycotoxins at varying depths within the water column over a 4–month period; and the second, determining whether an active sampler designed by Rundberget *et al.* (2007) was an effective method of harvesting toxins from this site for subsequent use in laboratory experimentation. Using the Thermo Scientific Quantum Discovery Max, successful application of a previously optimised method for the detection of DSP toxins (Carey *et al.* 2012) was applied to environmental samples from passive and active samplers. Good precision and linearity was achieved with the method, enabling sensitive measurement of marine biotoxins at low quantities from samples. From this research, a suite of lipophilic toxins were detected, DSP toxins OA, DTX1, DTX2, PTX2 and PTX2-SA and the spirolide 13-desmethyl-SPX-C were all found using both sampling methods. The cyclic amine Pinnatoxin G was detected using the active sampling method. SPX-C has previously been detected in Irish waters (Fux *et al.* 2009, Touzet *et al.* 2010); however this is the first confirmation of this toxin using high mass accuracy. This is the first report of DTX1 and Pinnatoxin G detected in Irish waters. DTX1 is a member of the DSP toxins that cause symptoms of food poisoning in exposed humans. Pinnatoxins E, F and G have not been found to cause harmful effects in humans that consume contaminated shellfish (Munday *et al.* 2012), thus the detection of Pinnatoxin G does not impact on food safety. However, these results do highlight the ongoing global expansion of HABs that has previously been reported in the literature (Anderson *et al.* 2002, Smayda 2007, James *et al.* 2010).

### Resin analysis

Of the two resins utilised for SPATT analysis in this research, Diaion HP-20 has been previously validated for use in the adsorption of lipophilic phycotoxins (Turrell *et al.* 2007, Fux *et al.* 2008, Lane *et al.* 2010). Preliminary work on Amberlite ® XAD761 has suggested this resin may be useful for the detection of more polar toxins in the water column such as Domoic Acid, with 100% binding of the toxin

observed in laboratory trials (Marine Institute 2009). Additional validation of the resins prior to deployment in the marine environment was not performed in this study. Comparison of the two resins for their efficacy in adsorption of toxins, found that for the DSP toxins the numbers of positive samples detected in the SPATT were not significantly different from each other; however HP-20 accumulated a higher quantity of these toxins, apart from PTX2-SA, an ester of PTX2. The less toxic PTX2-SA was originally thought to form within the digestive gland of filter-feeding shellfish from PTX2, through hydrolysis of the lactone moiety (Miles *et al.* 2004), however it has since been detected in other SPATT studies within the water column (Fux *et al.* 2009, Rundberget *et al.* 2009). It was postulated that PTX2 from lysed *Dinophysis* cells can also be transformed within the water column to PTX2-SA by enzymes released from planktonic organisms (Pizarro *et al.* 2008, Fux *et al.* 2009). Possibly due to its transformation, the toxin is more readily adsorbed onto XAD761 than HP-20, however further research is necessary to confirm this. SPX-C was found at comparatively lower levels in the SPATT than the DSP toxins. For SPX-C a higher proportion of HP-20 SPATT bags tested positive than the XAD761 and higher quantities of toxin were found in the HP-20 bags. Where quantifiable, toxin was found from 0.2 – 4.9 ng/g, while for the XAD761 resin, the highest quantity found was 2.4 ng/g.

In the current investigation SPATT bags were deployed at two-week intervals throughout the study period. This was in contrast to previous studies where one-week exposure regimes were utilised, in order to determine the efficacy of SPATT as an early-warning system for HABs (Turrell *et al.* 2007, Fux *et al.* 2009, Lane *et al.* 2010). Previous research examining the saturation point of HP-20 resin for OA and DTX1 stated that the resin can hold a maximum of 1639 and 2934 μg/g respectively (Li *et al.* 2011). Comparing results of the active sampler with these saturation points, OA or DTX1 did not reach levels higher than 30 μg/g in this study. Comparison of the quantities of other toxins (DTX2, PTX2, PTX2SA and SPX-C) detected in this study from the active sampler versus the SPATT, it is clear that at no point in the SPATT study did the HP-20 resin reach saturation point. Therefore, during the two-week period constant temporal and spatial monitoring of the water column was successfully performed. Unfortunately, to date, there is no published validation data

available on the maximum saturation of XAD761 for comparison with the current study.

**SPATT as a monitoring tool**

The use of SPATT as a tool for the monitoring of marine phycotoxins was assessed. Significant co-occurrence of DSP toxins was measured in the SPATT samplers; this is not surprising as many DSP-producing algal species have been found to produce multiple toxins simultaneously (MacKenzie *et al.* 2005, Fux *et al.* 2010). Vertical and horizontal distribution of toxins were evaluated over the monitoring period, with depths from surface to 50 m examined, to determine where toxin occurrence was dominant. Daily vertical migration has been observed in *Dinophysis* species, consequently it was important to measure multiple depths within the water column (Villarino *et al.* 1995). For the DSP toxins, the majority of phycotoxin was accumulated on SPATT bags positioned in the top 20 m of the water column; this could be due to the formation of an oxygen-thermocline layer in April 2010 (Figures 2.10 and 2011) resulting in a decline in oxygen levels from 30 m to the seabed with temperatures remaining between 7.5 and 9$^{o}$C at these depths. The development of this anoxic layer has been well-established at Lough Hyne (McAllen *et al.* 2009, Jessopp *et al.* 2011) and its formation has been applied in a broader context as a model for assessing the impact of anoxic/hypoxic conditions on vertical species distribution (Ballard and Myers 1996, 1997). However, despite this anoxic layer, quantities of DSP toxins (except for DTX1) were measured from SPATT bags containing XAD761 from surface to the seabed removed on the 4$^{th}$ of August, 2010. This date coincides with an increase in toxin accumulation at all sites using both resins, indicating an increase in harmful algae in the water column (Tables 2.7 and 2.8). It is unclear why the HP-20 resin did not adsorb the toxins at these depths during this sampling period. The effects of anoxia and other environmental conditions on these adsorptive resins has not been assessed, and may provide an explanation for this lack of consistent accumulation in the two resin types (Lane *et al.* 2010). However, it should be noted that a similar effect was not observed with SPX-C, HP-20 resin successfully accumulated this toxin at 40 and 50 m on this sampling date. Over the duration of the study, SPX-C distribution occurred throughout the water column at low levels. This toxin has been detected at low

quantities in previous SPATT studies (Fux *et al.* 2009, Rundberget *et al.* 2009). The frequent detection of phycotoxins at depths of 20 and 30 m over this study, while on occasion being absent at the surface, highlights the contribution this method can make in providing vital information to policy makers on current practices in phytoplankton monitoring. In Ireland, current monitoring protocols recommend sampling either the surface or integrated sampling of the top 5 m of the water column (Food Safety Authority Ireland 2012), and due to the migratory capacity of these dinoflagellates key toxic species may be missed. Similarly, in Australia integrated phytoplankton sampling to 6 m depth is performed (Victoria 2009). The standard operating protocol for phytoplankton sampling in the United Kingdom does not state a recommended maximum depth for collection, and instead advises taking of the water sample as close to the shellfish bed as possible (WG 2006).

Temporal changes in the occurrence of phycotoxins were also examined using SPATT. As was previously mentioned, an increase in the quantities of DSP toxins was detected in bags removed on August 4$^{th}$ 2010, for some toxins this increase was significant. Before this date, low levels of toxin were accumulated. Comparison with other SPATT studies shows that the quantities detected were equivalent to those with correspondingly low levels/absence of causative algae present in the water column (Turrell *et al.* 2007, Fux *et al.* 2009, Rundberget *et al.* 2009). The increase of DSP toxins was particularly marked in the South Basin. This site was located near to the 'Rapids', the narrow, shallow constriction through which all tidal waters enter and leave Lough Hyne. The Rapids are the cause of a unique asymmetrical tide in Lough Hyne, with a 4 h flood and 8.5 h ebb (Jessopp *et al.* 2011). Thus the South Basin is located nearer to the tidal influences and influx of oceanic waters. The quantities of toxin detected at this site were significantly greater than those detected from the other two sites during the month of August. No differences were detected between the North Basin and the Western Trough apart from PTX2-SA measured using HP-20. The more uniform distribution between these two sites may be due to the lengthy water retention times in the Lough, which has been suggested to be as long as 41 days for complete flushing through the tidal restriction from the Rapids (Jessopp *et al.* 2011). Thus changes in the phytoplankton assemblage at sites located further from the rapids may occur more slowly. No

significant changes in the occurrence of SPX-C were detected over the study period, or between sites.

In conjunction with SPATT analysis, phytoplankton sampling was also performed. Samples were chosen from the South Basin from the 4th and the 17th of August, as these dates corresponded to an increase in the accumulation of DSP toxin in the SPATT bags. DSP-toxin producing species *Dinophysis acuta* and *Dinophysis acuminata* were confirmed in the samples and *Prorocentrum* were confirmed to genus level (Table 2.9). *Alexandrium* sp was also detected in the samples. *A. peruvianum* was detected in a previous study in Irish waters, and was found to produce 13-desmethyl-SPX-C (Touzet *et al.* 2011).

The data acquired in this study confirms that SPATT can be applied as a tool for monitoring the occurrence of marine phycotoxins in an enclosed system. The method allowed integrated temporal sampling and was successfully applied to track the vertical distribution of toxin-producing algae at discrete depths within the water column and the horizontal distribution of toxins through monitoring of multiple sites. The data acquired can be utilised to make informed decisions on the monitoring of toxin-producing phytoplankton by regulatory bodies. The efficacy of the method at detecting toxins from algal species was confirmed through identification of key species from phytoplankton samples taken during a period of increased toxin-occurrence.

However it should be noted that SPATT analysis is a semi-quantitative method for determining toxin concentrations in the environment, as toxin loads in resin cannot be directly related to environmental concentrations. Comparison within and between SPATT studies can be performed allowing internal comparison of toxin levels and subsequent classification of the toxin load as ‘low’ or ‘high’ (Lane *et al.* 2010). Two studies have looked at the accumulation of toxins on SPATT bags and within mussels (Fux *et al.* 2009, Rundberget *et al.* 2009). Both studies found that an increase in toxin concentration on the SPATT disks did not always correspond to an increase in the toxin concentration detected in the shellfish. Rundberget *et al.* (2009) hypothesised that this may be due to the density of non-toxic algae present in the water, hence even though the toxic algal levels increased, they accounted for only a small percentage of potential food for the bivalves. It would seem that a combination

of methods utilised together, may provide the most effective means for monitoring the occurrence of lipophilic biotoxins that cause shellfish poisoning in human consumers.

**Active toxin sampling**

Original application of the active sampling method (Rundberget *et al.* 2007) found that even at low quantities of algae in the water (< 1000 cells/l), active pumping of the water column over 16 days provided a high toxin yield. The active sampling methodology used in the current study successfully harvested large quantities of OA (13 mg), DTX2 (29 mg), PTX2 (20 mg) and PTX2-SA (6 mg) during the 7-day deployment. This method can provide high quantities of marine phycotoxins to enable large-scale toxicological studies to be performed examining the impacts of these toxins on mammalian and invertebrate systems. Active sampling also successfully accumulated detectable levels of the cyclic amine Pinnatoxin G, which was previously undetected in Irish waters.

In addition to providing a source of toxin for experimentation, active sampling was also used to give an estimate of toxin levels per litre of water present at Lough Hyne, these concentrations assumed both a constant rate of water-flow through the system (i.e. no clogging from debris within the water) and that the resin adsorbed all toxin that passed through the cartridges. Comparison of the quantity of OA/litre (0.2 µg/l) with our *in vivo* exposure studies on bivalve shellfish (2.6, 0.13 and 0.07 µg/l) suggest that at the quantities present negative impacts on shellfish may be observed (see Chapters 3, 4 and 5). Previous published *in vivo* studies examining the effects of similar low-levels of pure OA and OA-producing algae on shellfish also measured negative response at the cellular and sub-cellular level (Carvalho Pinto-Silva *et al.* 2003, Carvalho Pinto-Silva *et al.* 2005, Flórez-Barrós *et al.* 2011). The effects of the other DSP toxins and SPX-C on bivalve shellfish are yet to be determined.

**Conclusions**

The SPATT technology was successfully applied to monitor the distribution of marine phycotoxins over a 4-month period in an enclosed system. Utilised in conjunction with biological methods, SPATT has the potential to provide useful information on phycotoxin distribution in the water column; enabling informed decisions to be made regarding the appropriate depths for obtaining phytoplankton and shellfish samples in marine biotoxin monitoring programmes. Successful accumulation of high quantities of biotoxins using the active sampling method devised by Rundberget *et al.* (2007) was also achieved, proving the method a useful means for the harvesting of large amounts of biotoxin for future experimentation.

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# CHAPTER 3

## Can histological techniques detect impacts of okadaic acid on *Mytilus edulis, Ruditapes philippinarum* and *Crassostrea gigas*?

**ABSTRACT**

Okadaic acid is a marine biotoxin produced by harmful algal blooms (HABs) that occur worldwide, and is known to accumulate in the tissues of filter feeding bivalves. Information is still lacking regarding the effects this toxin has on many important shellfish species. Significant impacts in the mantle and hepatopancreas tissues of *Mytilus edulis*, the blue mussel, *Crassostrea gigas*, the pacific oyster and *Ruditapes philippinarum*, the manila clam were observed in this study using histology. Three exposure regimes were utilised. An acute exposure of two replicate tanks to 2 µg/15 l tank mixed with algal feed. A sub-acute exposure of the replicate tanks to daily quantities of 1 µg/15 l tank mixed with algal feed and a sub-acute exposure to 40 µg/15 l tank of okadaic acid mixed with algal feed given daily over the 7 days. Shellfish were sampled after 1 day, 3 days and 7 days exposure.

Mantle tissues displayed a significant response in the blue mussel and manila clam, with limited damage evident in the pacific oysters over the exposure time. In all species exposed to daily quantities of okadaic acid, injury significantly increased in the hepatopancreas over the exposure period. Gill tissues showed no response to the biotoxin in any of the shellfish species. Recovery of tissues was observed in the single feed of OA with effects decreasing towards the end of the study period. This is the first time these three species have been examined for the effects of okadaic acid using histology. These results show that physiological response to okadaic acid can be detected using histology after a short exposure time, and that the effects may vary between bivalve species.

## INTRODUCTION

Rising global ocean temperatures, increasing occurrence of extreme weather events (such as El Niño), as well as growing coastal eutrophication have all been linked to an increase in the occurrence of Harmful Algal Blooms (HABs) worldwide (Anderson *et al.* 2002, James *et al.* 2010). Shellfish species are regularly exposed to these harmful algae and negative responses to the phycotoxins produced have been observed. Tissue damage, which can amplify susceptibility to bacterial infiltration and disease (Parry *et al.* 1989, Pearce *et al.* 2005); changes in differential cell counts (Galimany *et al.* 2008a), elevated phagocytosis and haemocyte count (Hégaret and Wikfors 2005a, Malagoli *et al.* 2008) and intracellular effects such as DNA fragmentation (Carvalho Pinto-Silva *et al.* 2003, Çavas and Könen 2008) and DNA lesions (Dizer *et al.* 2001) (Chapter 5). In addition, the complex host-parasite relationship in marine bivalves can be affected by these toxins causing an increase in the hosts susceptibility to the parasite (Hégaret *et al.* 2007), or a suppression of the parasite within the host (da Silva *et al.* 2008). HABs also cause negative economic effects through closure of aquaculture farms due to accumulation of marine biotoxins in the tissues of filter-feeding bivalves. Aquaculture and farming of marine bivalve molluscs is an economically important industry world-wide (Hoagland *et al.* 2002, Sellner *et al.* 2003). Shellfish and water quality are rigorously monitored by regulatory bodies in shellfish producing countries, using multiple detection methods (James *et al.* 1997, Draisci *et al.* 1999, Furey *et al.* 2001, Lehane *et al.* 2002) to ensure levels of toxin in the tissues remain below the limits deemed safe for human consumption (Alexander *et al.* 2008).

Okadaic acid (OA) was the phycotoxin chosen for these exposure experiments. This toxin is a member of the OA-acid group of marine biotoxins which includes OA, dinophysis toxin-1 (DTX1) and DTX2. These toxins are diarrhetic shellfish poisoning (DSP) toxins (James *et al.* 2010) and cause illness after ingestion of contaminated fish and shellfish. Filter-feeding shellfish in particular, can accumulate these toxins in their tissues and research has revealed that this accumulation occurs primarily in the digestive gland (Yasumoto *et al.* 1978, Blanco *et al.* 2007). Algae from the genus *Prorocentrum* and *Dinophysis* produce this

biotoxin and species such as *D. acuta*, *D. acuminata* and *P. lima* have regularly been sampled from northern European waters (Foden *et al.* 2005, Rundberget *et al.* 2007, Fux *et al.* 2009). This study examined the impact of okadaic acid on three commercially important bivalve species which are commonly farmed worldwide, *Mytilus edulis*, the blue mussel, *Ruditapes philippinarum*, the manila clam and *Crassostrea gigas*, the pacific oyster, using histological techniques. These techniques have proven useful for the determination of pathological effects of pollutants, both anthropogenic and biological, on many marine bivalve species (Howard and Smith 1983, Aarab *et al.* 2004, Galimany *et al.* 2008a, Galimany *et al.* 2008b, Watermann *et al.* 2008). Hepatopancreas (HP), gill and mantle tissue were examined to determine any changes in morphology or evidence of immunological response. HP tissues are known to accumulate DSP toxins; gill tissues in mussels have been previously suggested as having non-specific responses to anthropogenic pollutants which could be used as a biomarker (Micic *et al.* 2001, Au 2004, Gómez-Mendikute *et al.* 2005); mantle tissues were inspected as these are the first to be exposed to any pollutants and are a key structure involved in the sorting of food particles (Purchon 1977).

Negative effects of OA have been investigated in shellfish species, through exposure to the algal-producers, or the purified toxin (Svensson and Förlin 1998, Carvalho Pinto-Silva *et al.* 2003, Carvalho Pinto-Silva *et al.* 2005), only a few studies have examined the impacts on shellfish tissues using histology (Auriemma and Battistella 2004, Pearce *et al.* 2005, Escoffier *et al.* 2007). Detrimental effects were recorded post-exposure in these experiments; with the digestive gland particularly affected. As this organ is known to accumulate DSP lipophilic toxins (Yasumoto *et al.* 1978), it is not surprising that there were higher levels of damage observed in this tissue. *Mytilus galloprovincialis* showed digestive gland alteration, with an increase of lipid vesicles in both digestive and basophilic cells a week after OA toxin exposure on alternate days (Auriemma and Battistella 2004). Degeneration of mussel digestive cells was noted from the second week of treatment. Pearce *et al.* (2005) examined *Crassostrea gigas* spat that had been exposed for 21-days to *Prorocentrum rhathymum*, a dinoflagellate that produces a haemolytic toxin and has recently been discovered to produce OA (An *et al.* 2010). No mortalities were noted during exposure, however thin, dilated gut tubules and sloughing of gut cells was

observed. Gill damage also occurred in laboratory exposed oysters; this had not previously been seen in field-exposed oysters. Escoffier *et al.* (2007) determined the effects of pure OA purchased from a manufacturer and extracts from *Prorocentrum arenarium* on *Oryzias latipes,* medaka fish embryos. Differing impacts were noted in the treatments of pure OA and the extract. Pure OA caused significant effects on liver and digestive tracts while the extract had a significant impact on the global bodies and vitellus areas.

Variation in temperature, salinity and oxygen occurs year-round in marine and freshwater environments. In addition to these changing abiotic factors, internal physiological dynamics in shellfish, such as gonad development and metabolism, ensure an ever-changing internal and external condition. Seasonal variation in the occurrence of DNA instability, oxidative stress and biochemical markers in a number of bivalve species has previously been recorded (Wootton *et al.* 1996, Kagley *et al.* 2003, Hartl *et al.* 2004). In the case of marine biotoxins, in temperate climates large-scale algal 'blooms' tend to occur primarily in the summer months, however in Irish waters a number of samples have tested positive for the presence of DSP toxins perennially (Marine Institute 2004, 2007). Thus, it is important to determine whether season increases susceptibility to the negative effects of these toxins; and in addition, whether season has a confounding impact on the biomarkers chosen to indicate a response to OA in the current study (Kagley *et al.* 2003). A few studies have examined whether gender of a fish or shellfish is a factor in increased manifestation of disease, or susceptibility to anthropogenic or biological toxins (Brosseau and Baglivo 1994, Livingstone *et al.* 1995, Barber 2004) and have found differing impacts of pollutants and incidence of disease in males and females. Thus, whether these phycotoxins cause more of a measurable response or 'damage' in males or females is of great import, as it may influence the fecundity and homeostasis of the population as a whole.

A study performed by Galimany *et al.* (2008c) highlighted the need to examine the effects of HABs on different bivalve species rather than using one species and inferring similar responses occur in all other bivalves. The current study is the first to examine the pathological impacts of OA on the tissues of these three

different bivalve species. Farmed rather than wild shellfish were chosen for this study, as they are commonly consumed in Ireland and exported world-wide.

## MATERIALS AND METHODS

### Toxins and reagents

The biotoxin Okadaic acid (OA), Sodium Salt (L.C. Laboratories, 05857) was utilised for exposure experiments. Okadaic acid was standardised against a reference standard using Liquid Chromatography-Mass Spectroscopy (LC-MS) analysis. This was conducted on a Thermo Scientific Quantum Discovery Max triple quadropole mass spectrometer, equipped with a heated electrospray ionization source, hyphenated to a Thermo Scientific Accela LC system by the Mass Spectrometry Research Centre for Proteomics and Biotoxins (PROTEOBIO), Cork Institute of Technology (Carey *et al.* 2012). The toxin was dissolved in methanol (HPLC-grade) (1 mg OA in 1 ml MeOH) and was vortexed with phytoplankton feed prior to shellfish exposure.

### Experimental animals

Shellfish were obtained from certified aquaculture producers off the West coast of Ireland. All animals were acclimatised for one week in aerated seawater prior to experimentation at 12 ± 0.5$^{o}$C. Water was changed daily and the animals were given algal feed every two days (Shellfish Diet 1800, Reed Mariculture Inc, USA) to ensure optimal condition prior to experimentation. Bivalves were not fed for 24h prior to exposure. The size ranges of the animals chosen for experimentation were: Mussels 50 – 60 mm; clams 45 – 55 mm and oysters 60 – 80 mm.

### Experimental design

Experimental tanks were set up with 20 animals and 15 l of seawater in each tank. Two replicate tanks were used for each concentration of OA, over the three exposure experiments. Water in the tanks was changed daily, prior to feeding/exposure. Five animals were sampled after 1 day, 3 days and 7 days for the sub-acute exposure regime, except for the mussels exposed to 40 µg/15 l OA, when after 7-days

exposure 3 animals were sampled per tank, this was due to additional assays being performed. Extra assays being carried out simultaneously with histology also decreased the sample size in the acute exposure experiments. For the acute exposure, 2 animals per tank were sampled at 3 days and 3/15 l tank removed at 7 days. Experimental animals removed were replaced with the same number, and size range, of marked animals from the stock population, to avoid shellfish-density effects (Juhel *et al.* 2007).

**Okadaic acid exposure**

Three exposure experiments were carried out on each shellfish species, and each experiment was 7 days in duration. See Table 1.2, Chapter 1, for time-periods when each experiment was performed.

***Acute (single) exposure***

In the first experiment, there was an acute exposure of two replicate tanks to 2 µg /15 l tank OA of mussels/oysters/clams mixed with the algal feed on day 1 of the trial (2 µl OA stock solution in 98µl MeOH suspended in 100 µl algal feed) and from then on, animals were given the algal feed (100 µl).

***Sub-acute (daily) exposure***

Sub-acute exposure was performed in the following two experiments. 1 µg /15 l tank OA mixed with the algal feed fed daily over the 7 days and 40 µg /15 l tank OA mixed with algal feed administered every day.

**Mussels:** For the blue mussel two experiments were performed at the lower OA toxin concentrations of 1 µg/15 l tank and 2 µg/15 l tank; one in summer (summer mussels) and one in winter (winter mussels), and one experiment at 40 µg/15 l tank. **Clams:** In clams, experiments were completed at 1 µg and 2 µg/15 l tank. **Oysters:** For oysters one series of experiments was performed at each toxin concentration: 1 µg, 2 µg and 40 µg OA/15 l tank **(See Table 1.1).**

**Controls**

Water was changed daily in the control tanks and negative control animals were given 100 µl of algal feed daily. Positive control animals were fed cadmium chloride mixed with 100 µl algal feed daily for 7-days. Mussels and clams were given 5 µM $CdCl_2$/day and oysters were fed 10 µM $CdCl_2$/day to ensure a response, due to their larger size. Cadmium chloride was chosen as the positive control, as it is a known toxicant and has previously been found to cause a tissue-level response in *Mytilus edulis* (Sheir and Handy 2010). Ten animals were sampled from the stock population prior to the start of the experiment ($T_0$) and ten animals were taken from each of the control tanks after 7 days ($T_7$). Positive controls were not run for the summer mussels at the 1 µg and 2 µg OA concentrations.

**Histological analysis of samples**

A cross-section of bivalve tissue including digestive diverticulum, gills, mantle, gonad and foot was placed in histocassettes and fixed for 48h in Davidson's fixative (Howard and Smith 1983). Tissues were then placed in 70% ethanol until processing. Standard histological processing methods were performed on tissues (Howard and Smith 1983). Briefly, the processed samples were embedded in wax using a Tissue-tek TEC (Saukura) and afterwards stored at 4$^{o}$C. Using a Leica RM2235 microtome, the samples were sectioned at 5 µm. Tissue sections were stained with haematoxylin-eosin using a Leica Autostainer XL and slides were screened using a Nikon Eclipse 80i microscope at 4 x 10 magnification.

Histological changes compared to the controls over the okadaic acid exposure period, were used as markers to determine any impact on health status and to indicate a response to the biotoxin. Three tissue types were examined in this study: mantle, hepatopancreas and gill. Based on initial screening of the slides the detection of the following changes were deemed a measure of tissue damage, in the mantle tissues: lipofuscin granule formation and sloughing of cells; in the hepatopancreas: haemocyte infiltration, thinning of the tubule epithelium, dialated lumens as well as cellular debris within the digestive tubules and oedema and atrophy between the tubules. In the gill the presence of lipofuscin granules, haemocyte infiltration, epithelial lifting and gill lesions were assessed. Tissue damage was not quantified

merely denoted as present or absent and the HP and mantle tissues were analysed statistically.

**Data analysis**

Slides were scored and categorised according whether the tissues were damaged or undamaged the sex of the individual was recorded. The Chi-Squared Goodness of Fit test was performed to determine whether this damage was significant. Where there was only 1 degree of freedom present, Yate's correction for continuity was applied. Expected frequencies of damage occurring were determined using the negative control information and exposed animals were compared to these calculated expected frequencies. To determine seasonal and sex variation, Data collected on exposed individuals from the three sampling periods were combined, and an overall proportion of damaged and undamaged individuals from each sex were calculated from each exposure experiment. Fisher's Exact Test was performed on data with only 1 degree of freedom and Pearson's Chi-Square Test was performed on data with greater than 1 degree of freedom. The statistical software package Predictive Analytics SoftWare (PASW) version 17 was used for data analyses.

**RESULTS**

**Histopathology**

Mantle deterioration was denoted where lipofuscin granules were evident at its edge; this was determined by presence of brown cells at the edge of the mantle and is indicative of oxidative stress (Figure 3.1b). Sloughing of cells from the mantle edge and haemocyte infiltration was also taken as a response to the okadaic acid exposure (Figure 3.1c). Damage to HP was classed by haemocyte infiltration (Figure 3.2c); functional atrophy of the digestive diverticula, which was characterised by thinning of the tubule epithelium and dilated lumens (Figure 3.2b); cellular debris observed within the digestive tubules and oedema and atrophy/necrosis between the tubules. In the gill tissues there was no specific increase in immune response evident for the three species, nor was there any evidence of pathology in the gill cells, thus these tissues were considered to be unaffected by the toxin over the study period, at the

concentrations utilised. The numbers of individuals expressing each of the biomarkers chosen to indicate a negative response to okadaic acid exposure in the mantle and hepatopancreas tissues were recorded and these data were combined and utilised to determine the overall change in health of the shellfish over the exposure period (Table 3.1).

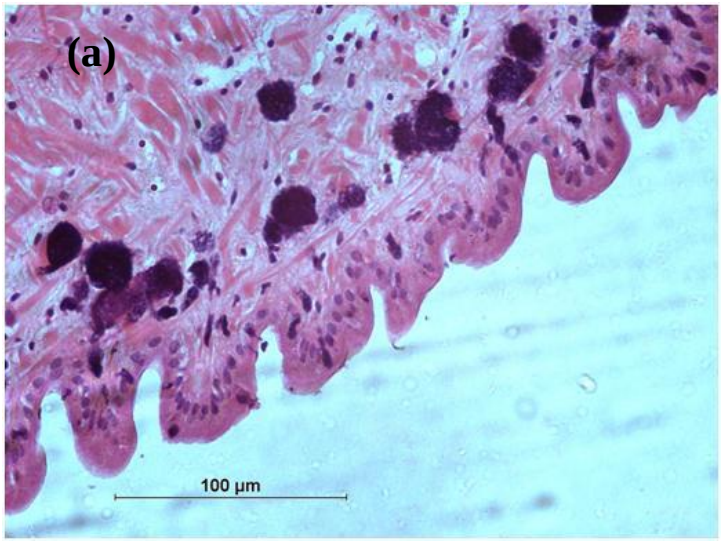
**Positive and negative controls**

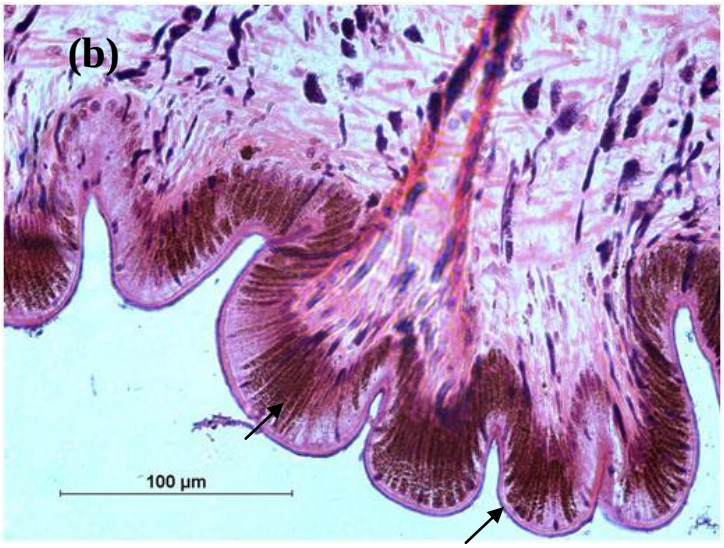
*Mantle*

For the mantle tissues, the negative controls were significantly different from the positive controls in the winter mussels and the clams, however for the summer mussels 'damage' was already observed in the negative controls prior to experimentation. In the blue mussel a significant difference in the levels of damage occurred between the mantle tissues examined in summer and winter, with the summer samples having significantly more evidence of baseline damage in unexposed shellfish (Fishers exact test, two-tailed: 1 d.f.; $p < 0.01$). In the pacific oyster there was no significant effect of either Cadmium Chloride or OA on the mantle of this species.

*Hepatopancreas*

In the hepatopancreas tissues there was a significant difference between the positive and negative controls for all species at all concentrations except for oysters exposed to 40 µg/15 l tank OA. In this experiment there was evidence of 'damage' present in the negative controls prior to commencing OA exposure. There was no significant difference between the controls for the HP tissues from summer and winter mussels.


(a)
100 µm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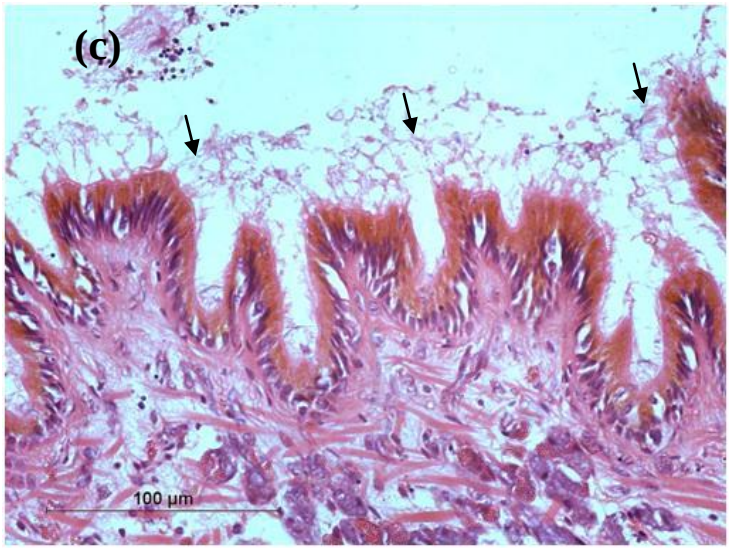
(c)
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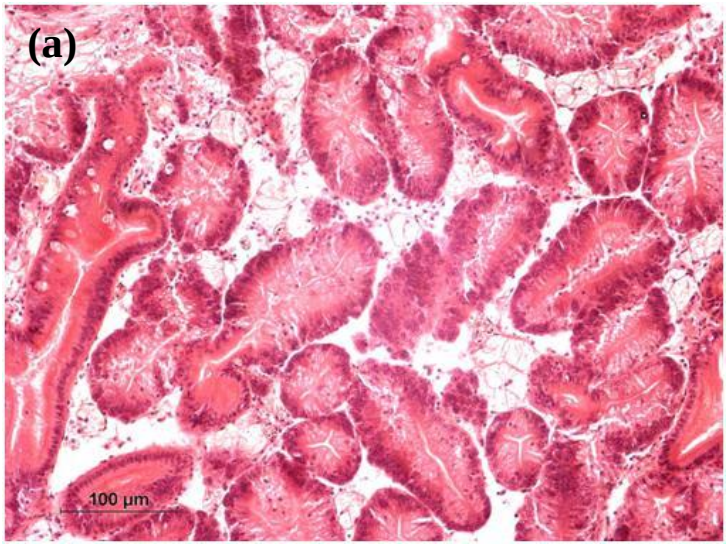
Figure 3.1. Photographs of mantle tissues (a) example of healthy tissue from *Ruditapes philippinarum;* control animal from stock population, (b) arrows point to examples of lipofuscin granules at the edges of the mantle of *Ruditapes philippinarum;* 7-days daily exposure to 1 µg/15 l tank OA, (c) arrows point to ceroidosis and sloughing of cells evident on the mantle edge of *Mytilus edulis;* 24h exposure to 40 µg/15l tank OA. All photographs at 400x magnification.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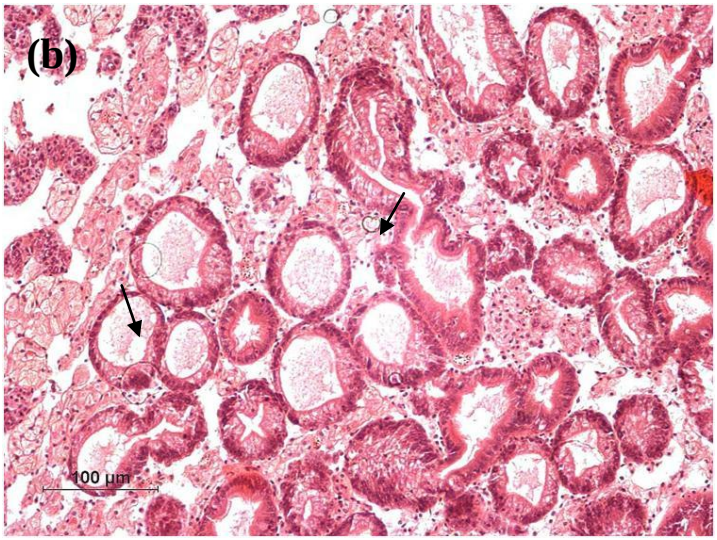
(b)
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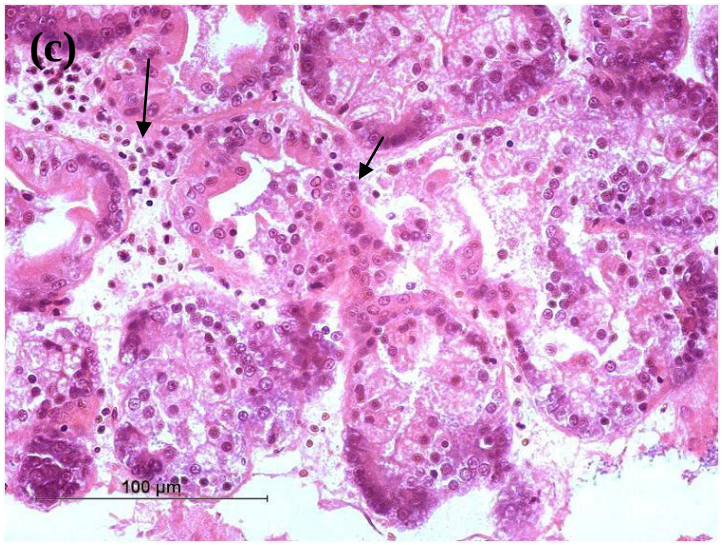
(c)
100 µm

Figure 3.2. Photographs of hepatopancreas (HP) tissues from experimental animals (a) healthy HP tissue from *Crassostrea gigas* control at $T_0$, 200x magnification (b) *C. gigas* HP tissues, arrows point to thinning of tubule walls a sign of functional atrophy; 7-days daily exposure to 1 µg/15 l tank OA, 200x magnification (c) *Ruditapes philippinarum* HP tissues after 3 days exposure to 1 dose of 2 µg/15 l tank OA at $T_0$ 400x magnification; arrows point to aggregation of haemocytes in the connective tissues and infiltration in the digestive tubules.

Table 3.1. The numbers of individuals expressing each of the biomarkers chosen to indicate a negative response to okadaic acid, data are for each species and each exposure regime.

| | | *Hepatopancreas (HP)* | | | | *Mantle* | | |
|---|---|---|---|---|---|---|---|---|
| | | **Thinning tubule** | **Sloughing/ Necrosis** | **Odema** | **Haemocyte infiltration** | **Lipofuscin** | **Necrosis** | **Haemocyte infiltration** |
| **Summer Mussels 1 µg exposure** | Control | 0 | 0 | 0 | 0 | 5 | 2 | 0 |
| | 24 h | 0 | 1 | 2 | 1 | 4 | 3 | 0 |
| | 72 h | 1 | 0 | 2 | 3 | 6 | 2 | 0 |
| | 7-days | 3 | 0 | 1 | 3 | 2 | 3 | 0 |
| | Control T7 | 0 | 0 | 0 | 0 | 5 | 2 | 0 |
| **Summer Mussels 2 µg/tank** | Control | 0 | 0 | 0 | 0 | 5 | 2 | 0 |
| | 72 h | 1 | 0 | 2 | 0 | 1 | 0 | 0 |
| | 7-days | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | Control T7 | 0 | 0 | 0 | 0 | 5 | 2 | 0 |
| **Winter Mussels 1 µg/tank** | Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 24 h | 0 | 0 | 0 | 0 | 2 | 3 | 2 |
| | 72 h | 1 | 1 | 2 | 3 | 4 | 3 | 0 |
| | 7-days | 1 | 3 | 3 | 5 | 3 | 3 | 0 |
| | Control T7 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| | CdCl T7 | 8 | 3 | 1 | 3 | 4 | 3 | 0 |
| | Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Winter Mussels** | 72 h | 1 | 1 | 1 | 1 | 4 | 1 | 1 |
| **2 µg/tank** | 7-days | 0 | 0 | 0 | 2 | 0 | 1 | 0 |
| | Control T7 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| | CdCl T7 | 8 | 3 | 1 | 3 | 4 | 3 | 0 |
| | | | | | | | | |
| | Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 24 h | 3 | 1 | 5 | 1 | 5 | 3 | 0 |
| **Mussels** | 72 h | 9 | 1 | 7 | 5 | 8 | 7 | 0 |
| **40 µg/tank** | 7-days | 6 | 0 | 3 | 1 | 5 | 3 | 2 |
| | Control T7 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| | CdCl T7 | 8 | 3 | 1 | 3 | 4 | 3 | 0 |
| | | | | | | | | |
| | Control | 0 | 0 | 0 | 0 | 2 | 1 | 0 |
| | 24 h | 0 | 0 | 0 | 1 | 3 | 1 | 2 |
| **Clams** | 72 h | 0 | 2 | 1 | 3 | 5 | 6 | 2 |
| **1 µg/tank** | 7-days | 0 | 2 | 6 | 8 | 5 | 5 | 4 |
| | Control T7 | 0 | 0 | 0 | 0 | 4 | 1 | 0 |
| | CdCl T7 | 3 | 0 | 3 | 4 | 1 | 5 | 1 |
| | | | | | | | | |
| | Control | 0 | 0 | 0 | 0 | 2 | 1 | 0 |
| **Clams** | 72 h | 0 | 1 | 2 | 0 | 4 | 0 | 1 |
| **2 µg/tank** | 7-days | 0 | 2 | 5 | 3 | 0 | 4 | 2 |
| | Control T7 | 0 | 0 | 0 | 0 | 4 | 1 | 0 |
| | CdCl T7 | 3 | 0 | 3 | 4 | 1 | 5 | 1 |
| | | | | | | | | |
| | Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 24 h | 3 | 0 | 4 | 0 | 0 | 0 | 0 |
| **Oysters** | 72 h | 5 | 3 | 5 | 2 | 0 | 0 | 0 |
| **1 µg/tank** | 7-days | 3 | 2 | 5 | 5 | 0 | 2 | 0 |
| | Control T7 | 2 | 1 | 2 | 1 | 0 | 1 | 0 |
| | CdCl T7 | 3 | 3 | 3 | 4 | 0 | 3 | 1 |
| | | | | | | | | |
| | Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Oysters** | 72 h | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **2 µg/tank** | 7-days | 3 | 1 | 2 | 1 | 0 | 0 | 0 |
| | Control T7 | 2 | 1 | 2 | 1 | 0 | 1 | 0 |
| | CdCl T7 | 3 | 3 | 3 | 4 | 0 | 3 | 1 |
| | | | | | | | | |
| | Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 24 h | 3 | 0 | 4 | 2 | 0 | 0 | 0 |
| **Oysters** | 72 h | 6 | 1 | 3 | 4 | 0 | 2 | 0 |
| **40 µg/tank** | 7-days | 5 | 2 | 6 | 2 | 0 | 0 | 0 |
| | Control T7 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| | CdCl T7 | 1 | 3 | 3 | 3 | 0 | 2 | 1 |

**Acute exposure (2 µg/ 15 l tank)**

***Mantle***

Acute exposure of 2 µg/15 l tank OA at $T_0$ showed differing impacts between the species for the two tissue types. Due to small sample sizes, the data for the acute exposure experiments was pooled; frequency of damaged and undamaged tissues in exposed animals was compared to the controls. In the mantle tissues, there was a significant difference between the frequency of damaged versus undamaged tissue in the summer mussels and controls ($X^2$ = 12.4, d.f. = 1, $p < 0.001$) (Figure 3.2), however this difference was probably due to the elevated damage observed in the controls at $T_0$ (60%) and $T_7$ (50%). In the winter mussels there was a significant decrease in the health of the mantle tissues relative to controls, with 100% of animals damaged after 3 days, this decreased to 16.7% of the population after 7 days, overall there was a significant difference in exposed versus control winter mussels ($X^2$ = 52.6, d.f. = 1, $p < 0.001$). In the manila clam, 20% of the mantle in the negative controls at $T_0$ and 40% at 7 days was described as damaged. This increased post OA-exposure, with 100% of the population showing negative effects on the mantle after 3 days exposure this decreased to 66.7% after 7 days and overall the frequency of damage was significantly different from the controls ($X^2$ = 3.8, d.f. = 1, $p < 0.05$). For the pacific oyster no mantle damage or immune response was observed after the 2 µg single dose of OA.

***Hepatopancreas***

As was done for the mantle tissue data, frequencies were pooled for HP data and damaged and undamaged tissues in exposed animals were compared to controls. After 3 days exposure 33.3% of summer mussels displayed negative impacts ($X^2$ = 32.4, d.f. = 1, $p < 0.001$), this damage decreased to 10% after 7 days. In the winter mussels and the manila clam, damage was observed in the HP after 3 days. 50% of the population was affected in both species, showing evidence of thinning of tubule walls and/or haemocyte infiltration. The injury decreased in the winter mussels to 33% after 7 days and in the clams, damage increased further with 60% of the population displaying a response to the biotoxin. In the case of both the winter

mussels and the manila clam, the response was significantly different from the controls over the time sampled; winter mussels: ($X^2$ = 19.7, d.f. = 1, $p < 0.001$); clams: ($X^2$ = 295.7, d.f. = 1, $p < 0.001$). In the pacific oyster damage was not observed until day 7 of exposure with 43% of individuals displaying evidence of a response to OA, characterised primarily by atrophy of the tubule epithelium, overall the frequency of damaged and undamaged exposed oysters was not significantly different from the controls (Figure 3.4).

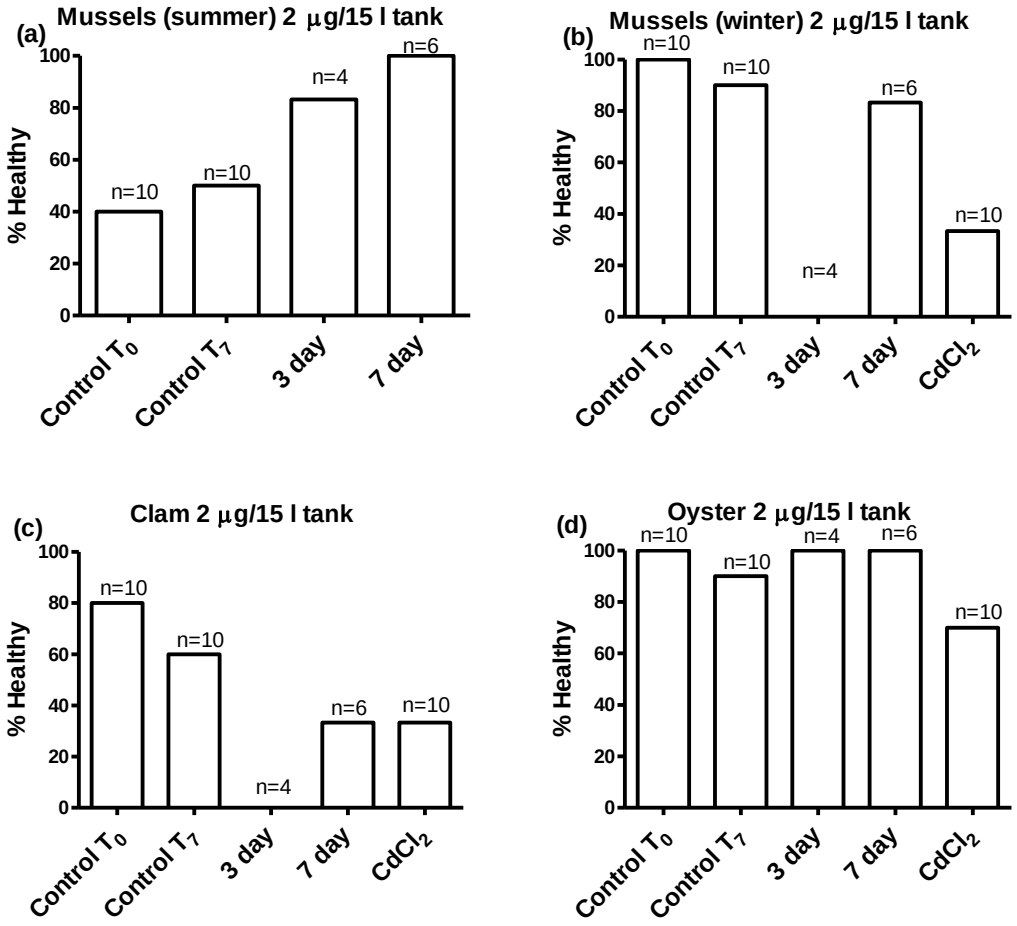


Figure 3.3. Changes in levels of damage observed in the of mantle tissues of (a) summer mussels; (b) winter mussels; (c) clams and (d) oysters, after acute exposure to 2 µg/15 l tank of okadaic acid at $T_0$. 100% = no damage present. Data from exposed shellfish were pooled and compared to controls due to small sample sizes.

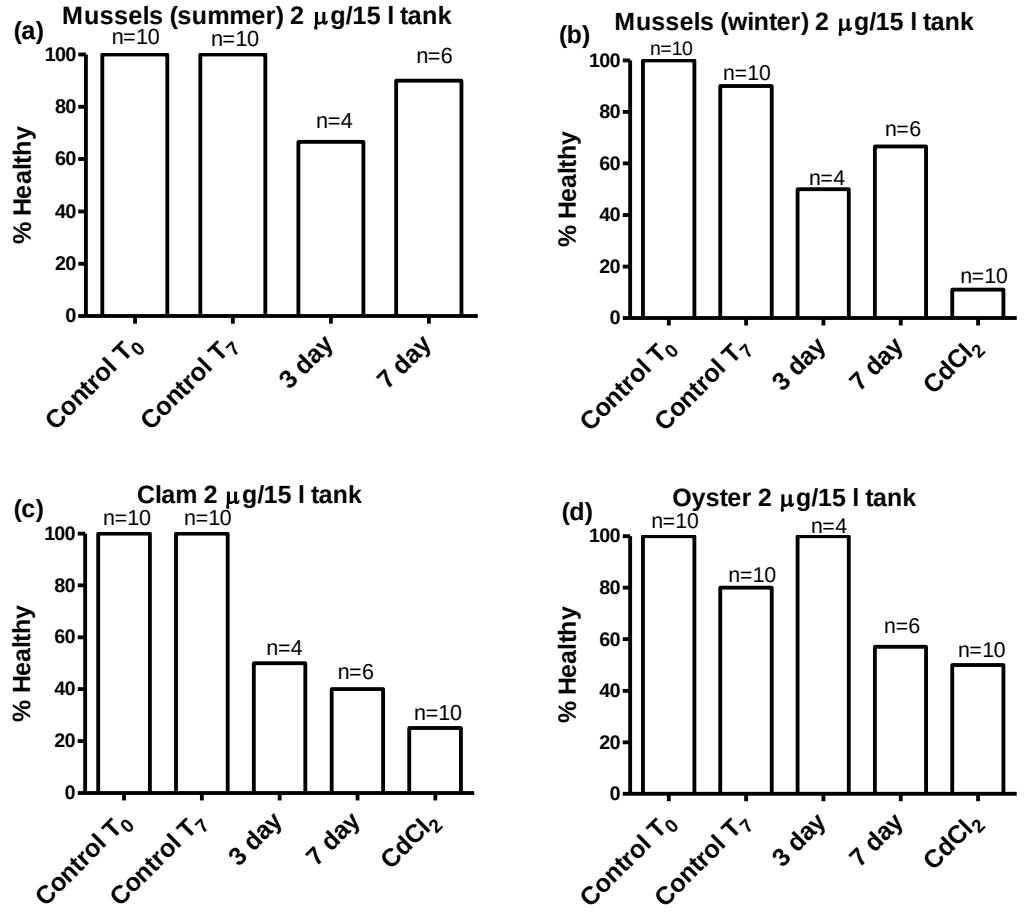


Figure 3.4. Changes in the health of hepatopancreas tissues from (a) summer mussels; (b) winter mussels; (c) clams and (d) oysters, after acute exposure to 2 µg/15 l tank okadaic acid at $T_0$. 100% = no damage present. Individual chi-squared tests comparing each time period from the control was not performed due to small sample sizes.

**Sub-acute exposure: 1 µg/15 l tank**

***Mantle***

Sub-acute exposure of summer mussels to 1 µg/15 l tank OA mixed with algal feed displayed lipofuscin granule formation and sloughing of cells in the mantle tissues. However, these effects were also observed in the control samples, thus no significant differences between exposed and negative control animals were observed over the exposure period (Figure 3.5). Whereas in the winter mussels, there was a significant

decrease in the health of the mantle tissues observed over time, with 100% of control animals at $T_0$ showing no damage, and after 7 days exposure 30% of animals showing alterations in the tissues ($X^2 = 136.5$, d.f. = 1, $p < 0.001$). Comparison of exposed mussels showed no difference between the levels of damage measured in the summer and winter mussels. The mantle of the manila clam had low levels of cellular injury present, even in the control samples at $T_0$ (20%) and $T_7$ (40%), however there was still a decrease in the health of these tissues over the exposure period, with a significant percentage of the population affected after 3 days (80%) and 100% of individuals showing a reaction to OA exposure after the 7 days ($X^2 = 22.1$, d.f. = 1, $p < 0.001$) (Figure 3.5). The pacific oyster showed low response to OA in mantle tissues over the exposure period. 100% of the animals were healthy in the $T_0$ controls and after 7 days exposure there was a non-significant drop in the proportion of healthy individuals to 80% for the daily 1µg amounts of OA.

***Hepatopancreas***

A decrease in the health of the HP was noted over time in all species for the daily 1 µg/15 l tank OA administered, with thinning of tubule walls particularly evident in mussels and oysters (Figure 3.2b), and haemocyte infiltration present in the clams (Figure 3.2c).

For the summer mussels exposed to 1 µg/15 l tank OA this response was immediate, with a 37.5% drop in healthy tissue after 1 day which was a significant deterioration in health over a short period from the control levels of 100% 1 µg: ($X^2 = 21.6$, d.f. = 1, $p < 0.001$) (Figure 3.6). The response in the winter mussels was observed after 3 days exposure to OA, with 40% of the population showing negative effects ($X^2 = 34.6$, d.f. = 1, $p < 0.001$). By 7 days the effects were more marked in the winter mussels than the summer mussels, with 90% of the population damaged after 7 days compared to 50% of the summer mussels. There was no significant difference in damage levels measured between the summer and winter mussel HP tissues. The HP of the manila clam showed a marked dose response for the sub-acute exposure of 1 µg OA (Figure 3.6); with 100% of animals displaying healthy HP tissues for the negative controls and a decrease in the health of these tissues occurring over the 7 days, with 55.5% of animals healthy after 3 days and only 10% healthy after the full 7 days exposure ($X^2 = 714.5$, d.f. = 1, $p < 0.001$). Pacific oyster

HP tissues displayed a very noticeable response to daily OA exposure. The 1 µg daily feed showed a 40% decrease in the health of the population from 100% of individuals after 1 day ($X^2 = 7.6$, d.f. = 1, $p < 0.01$), 50% after 3 days ($X^2 = 23.6$, d.f. = 1, $p < 0.001$) and 60% after 7 days exposure to the toxin.

**Sub-acute exposure: 40 µg/15 l tank**

***Mantle***

Mussels exposed to 40 µg/15 l tank OA daily showed a dose response over time in mantle tissues, with no damage observed at $T_0$. After 1 days exposure to OA 83.4% of the population displayed a negative reaction to the biotoxin ($X^2 = 140.6$, d.f. = 1, $p < 0.001$), this increased further to 95.9% after 3 days and 100% of individuals demonstrated negative effects to the biotoxin after 7 days exposure (Figure 3.5). In the pacific oyster the daily 40 µg exposure elicited a non-significant elevation in mantle damage after 3 days of 25%, however after 7 days, 100% of the animals examined showed no impact of the biotoxin on these tissues (Figure 3.5).

***Hepatopancreas***

In mussels exposed to 40 µg/15 l tank OA, the HP showed a significant reduction from 100% of animals healthy in the controls to 50% after 1 day ($X^2 = 43.1$, d.f. = 1, $p < 0.001$). All individuals had succumbed to the effects of the biotoxin after 7 days (Figure 3.6). In the pacific oyster, the control animals for the daily 40 µg exposure showed high levels of pre-existing damage present in the hepatopancreas at $T_0$ and $T_7$ (29%) this damage increased significantly in the exposed animals, with 88.9% of the oysters sampled displaying effects after 3 days ($X^2 = 13.4$, d.f. = 1, $p < 0.001$). 75% of the population sampled showed injury after 7 days exposure ($X^2 = 15.125$, d.f. = 1, $p < 0.01$).

**Sex variation**

There was no significant difference in the levels of damage measured between male and female exposed shellfish over the study period, neither within the different species nor at the different toxin concentrations.

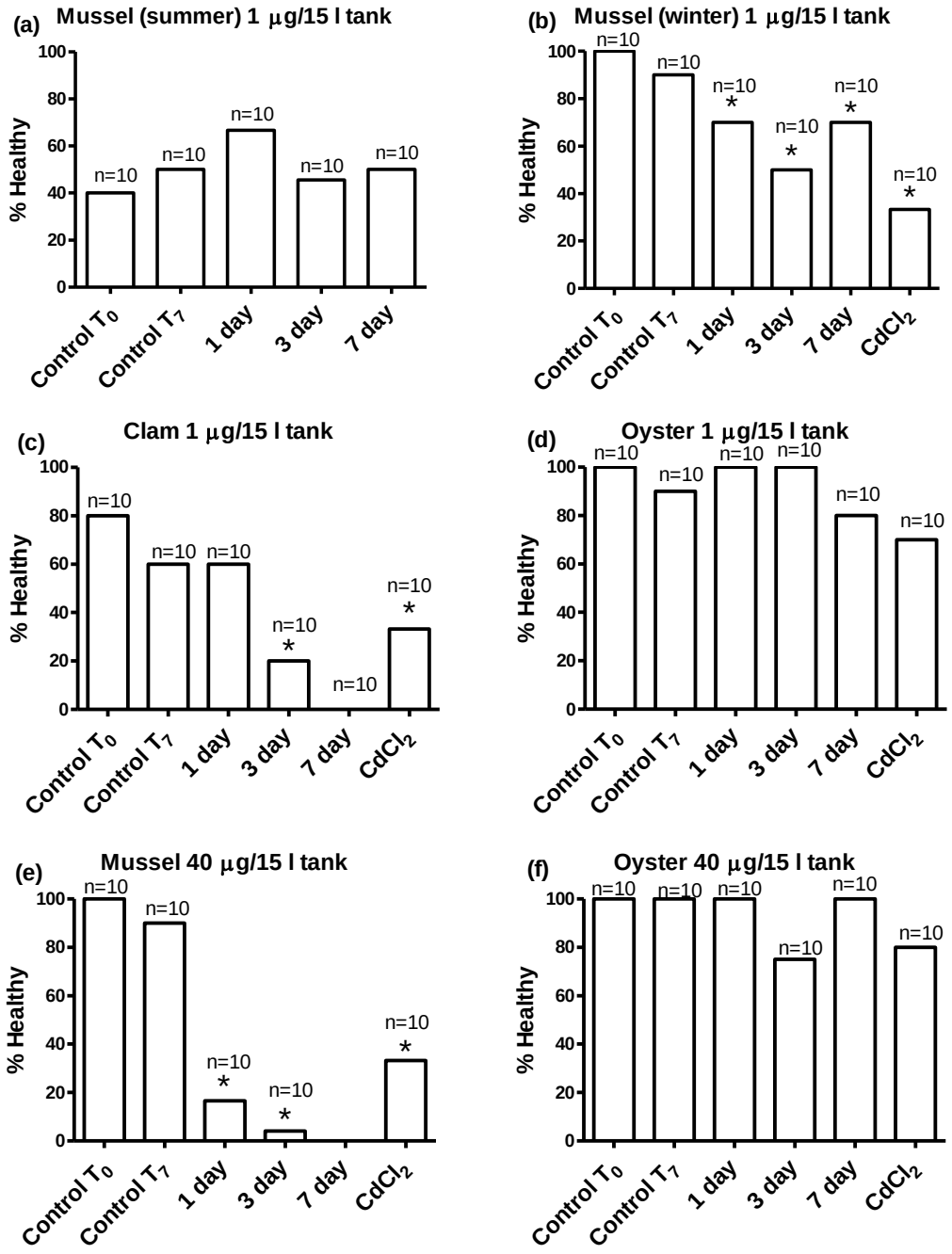


Figure 3.5. Daily exposure of OA to shellfish and its effects on mantle tissue health in (a) summer mussels 1 µg/15 l tank; (b) winter mussels, 1 µg/15 l tank; (c) clams, 1 µg/15 l tank; (d) oysters, 1 µg/15 l tank; (e) mussels, 40 µg/15 l tank; (f) oysters, 40 µg/15 l tank. 100% = no damage present.

'*' denotes where the change in health is significantly different from the control.

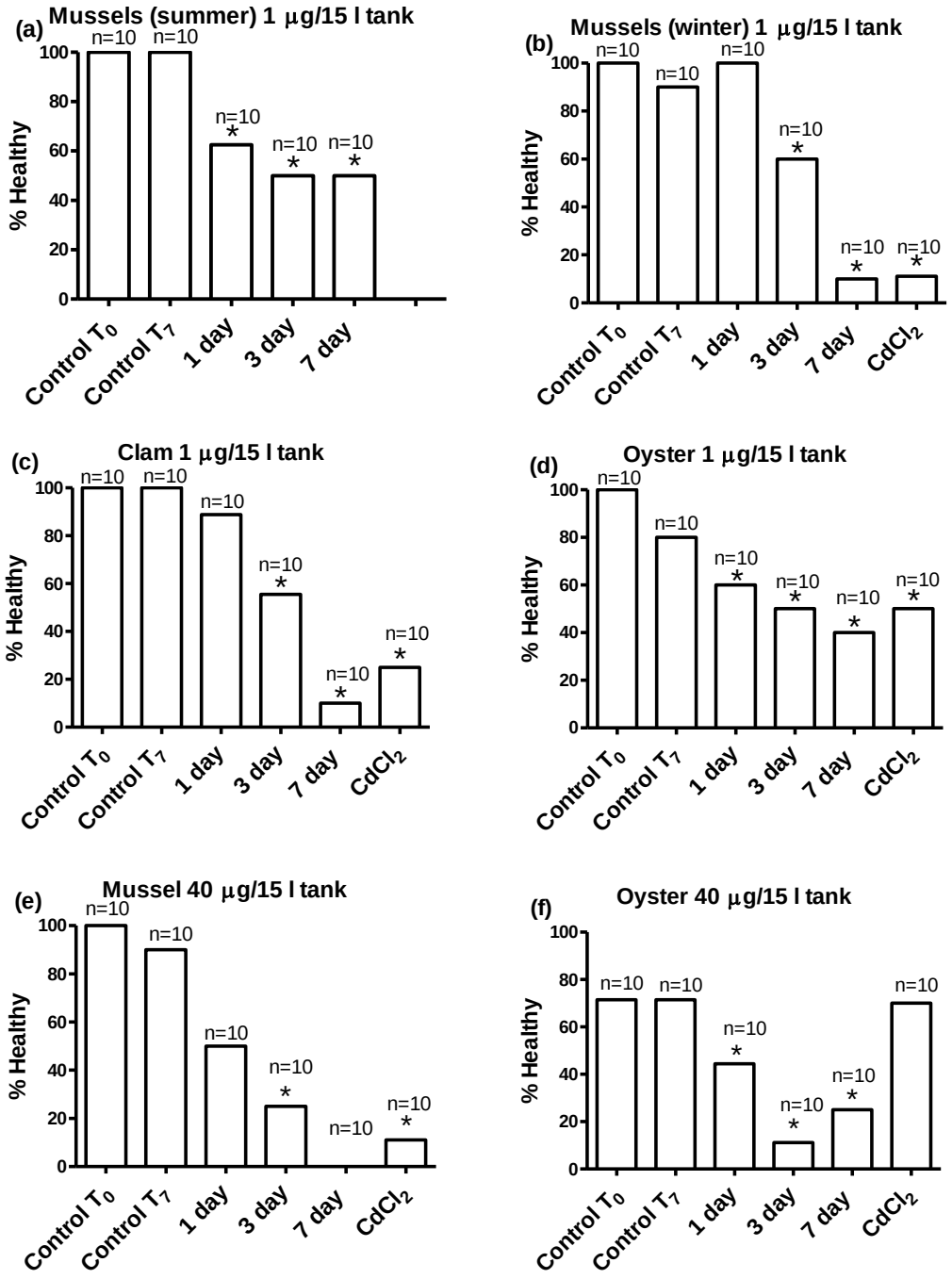


Figure 3.6. Daily exposure of OA to shellfish and its effects on hepatopancreas tissue health in (a) summer mussels, 1 µg/15 l tank; (b) winter mussels, 1 µg/15 l tank; (c) clams, 1 µg/15 l tank; (d) oysters, 1 µg/15 l tank; (e) mussels, 40 µg/15 l tank; (f) oysters, 40 µg/15 l tank. 100% = no damage present.

'*' denotes where the change in health is significantly different from the control.

## DISCUSSION

Detrimental effects were observed in mussels, clams and oysters, upon exposure to OA, a commonly occurring marine phycotoxin worldwide. Negative impacts from phytoplankton blooms elevate the stress on bivalve physiology and immune systems which could potentially increase chances of morbidity and mortality. Pearce *et al.* (2005) noted that some of the sloughed gut masses in oyster spat exposed to *Prorocentrum rhathymum* showed bacterial infiltration, and where gut epithelial erosion was complete, deep tissue permeation by bacteria. Increased mortalities in fish and shellfish have been recorded during HABs (Parry *et al.* 1989, Simon *et al.* 1997). Studies have also found that there is a link between exposure to toxic algal species and effects on host-parasite interactions in shellfish species (Hégaret *et al.* 2007, da Silva *et al.* 2008, Hégaret *et al.* 2009, Hégaret *et al.* 2010). Malagoli *et al.* (2008) discovered that differing temperatures, in conjunction with biotoxin exposure, modified the impacts on mussel phagocytosis. It is likely that there are cumulative and synergistic effects of toxic algal exposure and environmental conditions on already stressed shellfish.

Mantle, gill and digestive systems were the tissues studied for the presence of biomarkers of exposure and effect in this series of exposure experiments. Within bivalve species, these systems are inextricably linked to one and other, in the selection or rejection of food particles, and in respiration. In the three species chosen for these experiments, the mantle is fused (Purchon 1977) and stomach structure is comparatively uniform, with intracellular digestion and endocytic absorption (Morton 1983). The filtering mechanism is similar in all bivalves, however, the fate of the filtered food is determined by the differing arrangement of the frontal cilia, and this varies among species (Morton 1983). The gill structure of each species is also different in the experimental species. The blue mussel has a homorhabdic filibranch gill structure, which is considered to be the most structurally simple gill type (Cannuel *et al.* 2009). The manila clam possesses lamellibranch gills (Morton 1983) and the pacific oyster has pseudolamellibranch gills, one of the most complex gill structures (Cannuel and Beninger 2006). However, despite these structural differences no negative effects were observed in the gill tissues in any of the three

species over the study period, that were not also seen to a similar degree in the controls; normal levels of haemocytes were present, there was no evidence of epithelial lifting or gill lesions, which have been found in other histological studies of fish and shellfish exposed to chemical pollutants (Sunila 1987, Auffret 1988, Bricelj *et al.* 1992, Wepener *et al.* 2011). As there was no increase from background levels of 'damage' further analyses of these tissues were not performed. However, different degrees of detrimental effect were observed on the physiology of the mantle and HP tissues of the three bivalve species examined.

In the mussel and clam populations susceptibility in mantle tissues to the effects of the toxins was observed in both the acute and sub-acute exposure studies. Very little impact was observed in the oyster mantle tissues. The markers chosen as a measurement of response to OA was sloughing of dead or necrotic cells from the mantle edge which occurs post-contact to toxic substances (Pearce *et al.* 2005) (Figure 3.1c) and haemocyte infiltration. The primary response observed in the mantle tissues of mussels and clams was the presence of lipofuscin granules on the mantle edge. Lipofuscin granules are identifiable as a yellow-brown granular pigment in haematoxylin and eosin staining (Wood and Yasutake 1956); these granules are a byproduct in oxidation of unsaturated fatty acids and may be symptomatic of membrane or lysosomal damage. They are considered 'wear and tear' pigments and occur in mammals, fish and shellfish. In bivalve molluscs increased lipofuscin formation has been indicated as a response to contaminant exposure and a general response to pollution (Kagley *et al.* 2003) and has been found in shellfish exposed to harmful algae (Galimany *et al.* 2008c). Elevated mantle lipofuscin content was particularly evident in the manila clam post OA-exposure. Control animals from the summer mussels and clams showed elevated lipofuscin levels in the mantle at the start of the experiment and after 7 days which may have impacted on the result of the study (Figure 3.1b) (Table 3.1). Comparison between the summer and winter control mussels showed significant differences with 'damage' present in the summer control mussels prior to OA-exposure. Seasonal variation in the presence of lipofuscin has been observed in clam and mussel species (Walsh and O'Halloran 1997, Kagley *et al.* 2003, Banni *et al.* 2009), with higher levels of lipofuscin present in the summer and autumn. Kagley *et al.* (2003) hypothesised that this variation was due to the reproductive status, with times of ripe

and spawning gonadal development coinciding with periods of increased 'damage' observed through the cytochemical testing. Thus, this variation in the presence of lipofuscin granules found in these studies, and in the current investigation suggests that it is not an appropriate indicator of the impact of OA on these shellfish species. This evidence of pre-existing stress could also be an artefact from the transport and storage of the animals.

An observable, noteworthy impact on the HP tissues was recorded in all three bivalve species over the study period in the acute and sub-acute OA-exposure experiments, particularly at the 40 µg/15 l tank sub-acute exposure. In all species the effects of OA increased over time for the daily, sub-acute exposure and after 7 days, high levels of damage were observed in the digestive gland (Figure 3.6). Impacts of OA on the digestive gland of fish and shellfish have been observed previously (Auriemma and Battistella 2004, Pearce *et al.* 2005, Escoffier *et al.* 2007) and it is not surprising that there is a measurable effect in the current study as OA is known to accumulate in the digestive gland (Yasumoto *et al.* 1978, Blanco *et al.* 2007). Haemocyte infiltration of the HP was observed in all species and was particularly prevelant in the manila clam (Table 3.1); this response has been noted in experiments determining the effects of other marine biotoxins on shellfish species. It is considered to be an immunological response to the presence of a harmful substance in the tissues (Wikfors and Smolowitz 1995, Galimany *et al.* 2008a, Galimany *et al.* 2008c).

Thinning or 'atrophy' of the digestive tubule was also detected and has been observed previously as a response to toxic algae exposure (Parry *et al.* 1989, Wikfors and Smolowitz 1995). This physiological reaction has been also been linked with starvation prior to experimentation, or cessation of filtering upon exposure to the toxic algal cells (Pearce *et al.* 2005). In the pacific oysters administered the 40 µg/15 l tank feed (Figure 3.6f) there was evidence of thinning of digestive tubules already present in the controls at $T_0$. Lack of food in the environment prior to sampling may have contributed to the empty digestive tubules in the controls, as the animals were kept only a week prior to commencing experimentation and this may not have been enough time to allow complete recovery, despite regular feeding. This may also have caused increased susceptibility to OA through decreased fitness from lack of

adequate nutrition. A longer time period to acclimatise and increase condition would be advisable for the oysters prior to experimentation. However, throughout the week-long exposure experiment, the proportion of individuals that were noted as having thinning of the digestive tubules and oedema of the surrounding tissues increased significantly from the controls, and cessation of filtering during exposure seems unlikely to be the sole cause, an effect of OA-exposure seems probable. The duration of previous studies looking at the impact of starvation were carried out for far longer periods, in the case of Liu *et al.* (2010) the first samples to measure starvation effects in the pacific oyster were taken 30 days after the beginning of the experiment, and the duration of the current experiment was only 7 days with animals fed daily during experimentation. No evidence of digestive tubule atrophy was observed in HP tissues of control clams and mussels in this study.

In the acute exposure to OA, injury to the mantle was observed occurring after 3 days in mussels and clams; rapid recovery followed, particularly in the mussels exposed to the biotoxins. For HP tissues, the acute exposure to OA showed an increase in damage after 3 days and in the case of the mussel, recovery was observed after 7 days. No recovery was seen in the clam and a slower reaction to the biotoxins effects was seen in the oyster with damage observed only after the 7 days. A study done by Carvalho Pinto-Silva *et al.* (2003) examining micronucleus (MN) formation in haemolymph cells measured rapid recovery in the *Perna perna* mussel post-exposure, with visibly reduced MN from 24h to 48h. High depuration rates in shellfish have been linked with the presence of larger quantities of non-toxic food (Blanco *et al.* 1999, Marcaillou *et al.* 2010), thus daily feeding with non-toxic algae post-exposure may have assisted in rapid recovery.

No significant sex-mediated response was observed in this study for any of the three shellfish species. However, this was a preliminary study examining sex-effects using small sample sizes and additional experimentation using a larger sample sizes should be performed to confirm these results. Despite this lack of a sex-dependent effect, there may still be an indirect effect on a specific sex of shellfish species exposed to harmful algal blooms. Brosseau and Baglivo (1994) found that in *Mya arenaria* more males suffered disseminated neoplasia while Barber (2004) found that gonadal neoplasia was detected more in female than male *Mya arenaria*.

The main impact of this disease is damage to the gonad and a reduction in reproductive output. Landsberg (1996) determined a possible geographical correlation with the incidence of HABs and neoplasia in a number of bivalve species. This study was based on published data examining the prevalence of neoplasia, and correlating this information with data on the occurrence of HABs from measurement of toxin concentrations in bivalve tissues. However, the data presented was circumstantial and further field and laboratory trials are required to fully elucidate a definite cause and effect. It is very probable that HABs cause increased susceptibility of bivalve molluscs to pathogens and parasites, as many toxins have been found to have deleterious impacts on pathology and immune-response (Pearce *et al.* 2005, Galimany *et al.* 2008), and have also been found to influence the host-parasite interactions (Hégaret *et al.* 2007, Hégaret *et al.* 2009). Thus, while the effect of the biotoxin may not be mediated by the sex of the species, it may increase susceptibility to infection in the population as a whole, or to diseases that that affect one gender more than another. This may lead to negative effects on the overall health of a population and cause reduced growth and reproductive fitness; with negative consequences for their population dynamics.

Throughout the study differing impacts were observed occurring between species, particularly in the mantle, with oysters being particularly resistant to the toxic effects of OA on these tissues. Mello *et al.* (2010) examined the immunological effects of the DSP toxin-producing algae *Dinophysis acuminata* on three bivalve species, *Crassostrea gigas, Perna perna* and *Anomalocardia brasiliana* and found differences occurring between species, with the *Perna perna* mussel showing the most susceptibility to the biotoxins and the pacific oyster *Crassostrea gigas* showing the least susceptibility. Galimany *et al.* (2008c) advocated examining multiple species for the effects of harmful algae as differing effects were found in *Mytilus edulis* exposed to *Prorocentrum minimum* than the responses measured in other studies (Wikfors and Smolowitz 1993, Hégaret and Wikfors 2005b). These differences between species could be due to diverse sorting mechanisms in the gills and cilia (Morton 1983), or may also be due to the various metabolic processes occurring in each of the shellfish species. Differences in accumulation and depuration of toxins have been found to occur between bivalve species (Suzuki and Mitsuya 2001, Lindegarth *et al.* 2009) and a study done by Wootton *et al.* (2003)

found vast differences in the immune function of *Mytilus edulis, Cerastoderma edule* and *Ensis siliqua* despite the three species sharing similar cell types. These results highlight the necessity of studying the impact of toxic algae on many shellfish species and not use the results obtained from one species to infer impacts on others.

This study and additional research performed to date, emphasizes the importance of examining the impact of toxic algae on bivalve species, as little is known of the effects of many algal strains. It is vital to establish whether these biotoxins have more long-term implications on the health and fecundity of the shellfish, through direct damage caused by the toxin, or indirectly through effects on parasites infecting the shellfish, or perhaps may cause increased susceptibility to other diseases and environmental stresses. The occurrence of harmful algal blooms is on the increase and this has been linked to a number of factors such as ballast waters transporting algae to new environments, eutrophication and global climate change (van Dolah 2000, Anderson *et al.* 2002, Masó and Garcés 2006, Smayda 2007), hence it is essential we gather information on the impacts of these algae on shellfish species both to monitor their wellbeing and also more crucially, for the health of human consumers.

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# CHAPTER 4

## Effects of okadaic acid exposure on differential cell distribution in the circulating haemolymph of the blue mussel, the manila clam and the pacific oyster

**ABSTRACT**

This study examined the impact of the diarrhetic shellfish poisoning (DSP) toxin okadaic acid (OA), on the differential cell population distribution in the circulating haemolymph of three commercially important bivalve species: *Mytilus edulis*, the blue mussel; *Ruditapes philippinarum*, the manila clam and *Crassostrea gigas*, the pacific oyster. Shellfish were exposed to three different concentrations of OA over 7-days, a 2 µg/15 l tank acute exposure of OA at the beginning of the experiment followed by daily quantities of algal feed; and sub-acute exposures of 1 µg/15 l tank and 40 µg/15 l tank mixed with algal feed administered daily to shellfish. Haemolymph samples were collected after 1-day, 3-days and 7-days exposure to the phycotoxin. Viability of cells was measured prior to fixation. Long-term versus short-term fixation methods were evaluated and it was confirmed that samples were successfully preserved in 6% formalin: seawater for up to 8 months prior to analysis using flow cytometric analysis.

Cells were isolated from debris using SYBR green DNA stain. Changes in differential cell populations were measured. Two distinct populations were separated using size and granularity for the blue mussel and the manila clam; three populations were distinguished using cell granularity/complexity for the pacific oyster. The pacific oyster was the most sensitive to the effects of OA during the experiments. Significant changes in the proportions of small granulocytes and large granulocytes were detected at the lower levels of toxin exposure, indicating a possible chemical-induced response to OA. No significant changes in the proportions of granulocytes or hyalinocytes were detected in the blue mussel and manila clam. At the higher level of toxin exposure (40 µg/15 l tank) there was no significant impact on the exposed shellfish. This study demonstrates that okadaic acid has differing effects on the differential cell populations in these three bivalve species over a short-term exposure period.

## INTRODUCTION

Diarrhetic shellfish poisoning (DSP) toxins occur worldwide and are produced by algae of the genus *Prorocentrum* and *Dinophysis*. Suspension feeders such as bivalve molluscs are the primary vectors of these phycotoxins, accumulating the toxins in their tissues and causing illness to human consumers (Food Safety Authority Ireland 2001, James *et al.* 2010). Incidence of Harmful Algal Blooms (HABs) are increasing worldwide due to a range of anthropogenic activities (van Dolah 2000, Anderson *et al.* 2002), thus contamination of many economically important shellfish is occurring more frequently.

Okadaic acid (OA) is a lipophilic DSP toxin that occurs world-wide. The biotoxin is an inhibitor of serine/threonine protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) activity, these are essential in signalling cascades and regulate different cellular processes in eukaryotic cells (Bialojan and Takai 1988), this inhibitory effect has been found in both mammals and invertebrate shellfish (Takai and Mieskes 1991, Tubaro *et al.* 1996). Further investigation in mammals has found the biotoxin to be a tumour-promoter and to have cytotoxic, genotoxic, mutagenic and aneugenic effects (Suganuma *et al.* 1988, Fessard *et al.* 1996, Huynh-Delerme *et al.* 2003, Le Hégarat *et al.* 2005, Souid-Mensi *et al.* 2008), however, these negative impacts have been found to be cell-line dependant and differing susceptibility to concentration and toxicity has been recorded in different cell-lines. OA has also been found to induce apoptosis in a number of mammalian cell lines (Bøe *et al.* 1991, Traoré *et al.* 2001, Ao *et al.* 2008).

OA-exposure has been found to elicit a number of negative responses in marine bivalves. Examination of OA exposure at the cellular level in bivalves has revealed evidence of DNA instability in the *Perna perna* mussel (Carvalho Pinto-Silva *et al.* 2003, Carvalho Pinto-Silva *et al.* 2005); and pathological effects at the tissue and cellular levels (Auriemma and Battistella 2004, Pearce *et al.* 2005) (Chapter 3). DNA damage has been linked to reduced growth rates in mussels, and tissue damage caused by OA exposure has been shown to cause an increase in bacterial infiltration in affected tissues (Steinert *et al.* 1998, Pearce *et al.* 2005).

Despite these negative reactions, haemolymph (HL) cells of *Mytilus edulis*, the blue mussel; heart cells of *Crassostrea gigas*, the pacific oyster and gill and HL cells of *Ruditapes decussatus*, the manila clam have been found quite resistant to the cytotoxic effects of OA (Svensson *et al.* 2003, Talarmin *et al.* 2007, Flórez-Barrós *et al.* 2011). Further research is required to conclusively establish the effects of OA in marine bivalves and determine the accumulation and depuration process of the phycotoxin.

Bivalves have no acquired immunity, but have an innate immune response to injury, pathogens and parasites. Within this immune response the circulating haemocytes play a major role in defence against foreign invaders (Bayne 1983). Two main haemocyte cell types have been identified in marine bivalves; hyalinocytes: these are cells with a clear cytoplasm containing few or no granules. The other group of cells are granulocytes, these are larger with a well-developed cytoplasm containing granules that range from scarce to numerous (Auffret 1988). Within these two types, sub-populations have been observed in numerous bivalve species, and these are differentiated based on size and granularity, with vast diversity occurring among families and species of the same family. This is probably due in large part to interspecies and seasonal variation; however it is possible that this also occurs through a lack of consistency in both methodology, and classification schedules, for determination of the sub-populations (Sun *et al.* 2006).

The effects of a number of algal toxins on the haemocyte response of different bivalve species have been investigated. A number of criteria such as differential cell population changes, cell number, phagocytosis, aggregation, and increased reactive oxygen species have been measured (Hégaret and Wikfors 2005a, b, Hégaret *et al.* 2007b, Mello *et al.* 2010). The impacts reported vary considerably depending on both bivalve and algal species, emphasising the necessity of examining the effects of these toxin-producing algae on multiple bivalve species. Host-parasite interactions and the immune-modulating effects of harmful algae on these relationships have also been explored (Hégaret *et al.* 2007a, da Silva *et al.* 2008, Hégaret *et al.* 2010). These studies include important biological parameters and information on more than just the circulating haemocyte response to an algal bloom. In the case of Hégaret *et al.* (2009) exposure to *Prorocentrum minimum* caused an

increased susceptibility to *Perkinsus olseni* in the manila clam *Ruditapes philippinarum*. Temperature has also been linked with changing cell populations, increasing percentages of dead haemocytes and decreasing phagocytosis in *Crassostrea virginica* (Hégaret *et al.* 2003a, b); and Hégaret and Wikfors (2005a) related elevated temperature with increasing toxin accumulation in *Crassostrea gigas*. In any natural environment, inhabitants will be exposed to a host of biological, chemical and climatic stressors that could potentially increase the impact of marine biotoxins.

The current study describes the separation and characterisation of haemocytes from three commercially important bivalve species using flow cytometry: the blue mussel; the manila clam and the pacific oyster. Shellfish were exposed to differing concentrations of okadaic acid over 7 days and the proportions of differential cell populations in the circulating haemolymph were measured to investigate whether okadaic acid elicits chemically-induced changes in cell proportions in these bivalves after exposure to this commonly occurring marine phycotoxin.

## MATERIALS AND METHODS

### Experimental animals

*Mytilus edulis*, *Ruditapes philippinarum* and *Crassostrea gigas*, were purchased from certified aquaculture producers farming off of the West coast of Ireland. The size ranges of the animals chosen for experimentation were: blue mussels 50 – 60 mm; manila clams 45 – 55 mm and pacific oysters 60 – 80 mm. Animals were acclimatised for 1-week prior to experimentation at 12 ± 0.5$^{o}$C. Seawater was changed daily and animals were given algal feed every two days (Shellfish Diet 1800, Reed Mariculture Inc, USA).

### Toxins and reagents

The phycotoxin okadaic acid (OA), Sodium Salt (L.C. Laboratories, 05857) was utilised for exposure experiments. OA was standardised against a reference standard

using Liquid Chromatography-Mass Spectroscopy (LC-MS) analysis. This was conducted on a Thermo Scientific Quantum Discovery Max triple quadropole mass spectrometer, equipped with a heated electrospray ionization source, hyphenated to a Thermo Scientific Accela LC system by the Mass Spectrometry Research Centre for Proteomics and Biotoxins (PROTEOBIO), Cork Institute of Technology, Rossa Avenue, Cork (Carey *et al.* 2012). The biotoxin was dissolved in methanol (1 mg OA in 1 ml MeOH) (HPLC-grade) and was vortexed with phytoplankton feed prior to mussel exposure.

**Exposure to okadaic acid**

Week-long exposure experiments were performed for each species. Two replicate tanks of 20 animals in 15 l of seawater were used for each OA concentration. See Table 1.2, Chapter 1, for time-periods when each experiment was performed.

Three different concentrations of OA were utilised, an acute (single) exposure of 2 µg/15 l tank of OA at the beginning of the experiment ($T_0$) mixed with 100 µl of algal feed (0.1 µg/shellfish), and from then on daily quantities (100 µl) of just the algal feed. Sub-acute (daily) exposure to 1 µg and 40 µg/15 l tank OA mixed with algal feed were given to shellfish for 7 consecutive days.

Seawater was changed daily and shellfish were fed the appropriate concentrations of algal feed mixed with OA. Five animals per replicate tank were sampled after 1 day, 3 days and 7 days exposure for each toxin concentration, except for mussels exposed to the 40 µg concentration, when three animals were removed at each sampling time. This lower number was due to simultaneous sampling from the experimental tanks for subsequent LC-MS analysis of the animals (results not shown here). Animals that were removed were replaced with an equal number of marked shellfish in the same size range to prevent any shellfish density effects. Estimated concentrations per shellfish for the daily sub-acute exposure studies were based on the accumulation/depuration study of Svensson and Förlin (2004) and assumed an even suspension of OA in the seawater with complete and equal uptake and distribution of the toxin in each shellfish (Chapter 1, Table 1.2)

Ten negative control animals were taken from the stock population at $T_0$ and analysed. A control tank was set up for each experiment, animals were given daily

amounts of 100 µl algal feed and water was changed daily. Ten animals were sampled from this tank after 7 days ($T_7$).

**Haemolymph collection and staining**

A minimum of 300 µl of haemolymph (HL) was removed from the posterior adductor sinus of the shellfish using a sterile 23-guage 1½ inch needle and 2.5 ml syringe. HL cell suspensions were fixed in equal quantities of 6% formalin: seawater solution for further experimentation as per Hégaret *et al.* (2003a). Samples were stored at 4$^{o}$C until examination (up to 8 months). SYBR green I (Sigma Aldrich) 10, 000 x in DMSO, was the stain chosen for experimentation. This is a fluorescent nucleic acid stain that binds to double-stranded DNA. This was chosen to enable separation of haemolymph cells from 'debris' and non biological material within the samples. Confirmation of the efficacy of the long-term fixation method was performed prior to staining using SYBR green for the experimental HL samples. A visual microscopic comparison was completed between the fresh and long-term fixed cells to ensure that cell structure had not deteriorated during long-term storage. Optimisation of fixation and staining was performed on the flow cytometer using fresh haemolymph samples obtained from oysters and mussels. Multiple fixation and staining times were tested (15 mins, 30 mins, 1 h, 1 h 30 mins, and 2 hrs). In addition, two different concentrations of SYBR green were also tested, 0.1 µl/ml (1:10,000) and 1 µl/ml of sample (1:1000). HL sampled during OA-exposure from each individual was examined using flow cytometry for each of the phycotoxin concentrations utilised and for the negative controls at $T_0$ and $T_7$.

**Flow cytometry**

Flow cytometry analysis was performed using Becton Dickinson FACSCanto, a six-colour benchtop analyser capable of high sample throughput. 10,000 events were examined per sample. SYBR Green I fluorescence was measured at 500-530 nm emission wavelength (green) (Hégaret *et al.* 2003a, Haberkorn *et al.* 2010). Haemocytes were readily differentiated from other particles and debris in samples based on SYBR green staining. Control samples were used to optimise the gating strategy and these settings were applied to all subsequent OA-exposed shellfish samples.

### Data analysis

Data were in percentage form and were arcsine transformed prior to analysis. Normality of data was tested using the Kolmogorov-Smirnov test. Pearsons's correlation was performed on the two cell populations isolated from mussels and clams to determine whether the distribution of 'small' and 'hyaline' cells were significantly correlated. To compare whether any changes in the proportion of hyalinocytes and granulocytes occurred in the three species over the exposure period, one-way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test were performed on the data ($p < 0.05$). Data were analysed using Predictive Analytics SoftWare Statistics (PASW) 17 for Windows.

## RESULTS

### Optimisation of fixation and staining

Deterioration of cells was visually assessed using microscopy. A comparison of fresh, short and long-term fixed samples confirmed that cells remained intact after up to 8 months of storage in 6% formalin: seawater (Figure 4.1). In addition, staining of newly-fixed cells was assessed at two concentrations. The optimum SYBR green concentration was determined based on this analysis was 1 µl/ml of sample (1:1000). Binding of SYBR-green to DNA was examined using newly-fixed and long-term samples. The staining time that provided the clearest peak of staining intensity for samples stored long-term was 30 minutes.

### Gating of cell populations

Gating of cells was performed by isolating SYBR green-bound particles and using forward scatter (FSC) to determine size, and side scatter (SSC) to determine the complexity, i.e. granularity of the cells. The FSC and SSC were set to log scale, and stained cell populations were isolated from non-staining debris primarily between logs 3 and 4. In the blue mussel and the manila clam, cells were isolated from non-staining particles and within those isolated cells; two distinct populations were distinguished using both the size and granularity of the cells (Figures 4.2 and 4.3). In

the pacific oyster three populations were discerned using granularity, however only two were readily isolated using cell size (see Figure 4.4). This is due to the similarity and in some cases, overlap in cell sizes measured in the different cell populations of oyster species (Allam *et al.* 2002, Wootton *et al.* 2003, Sun *et al.* 2006).

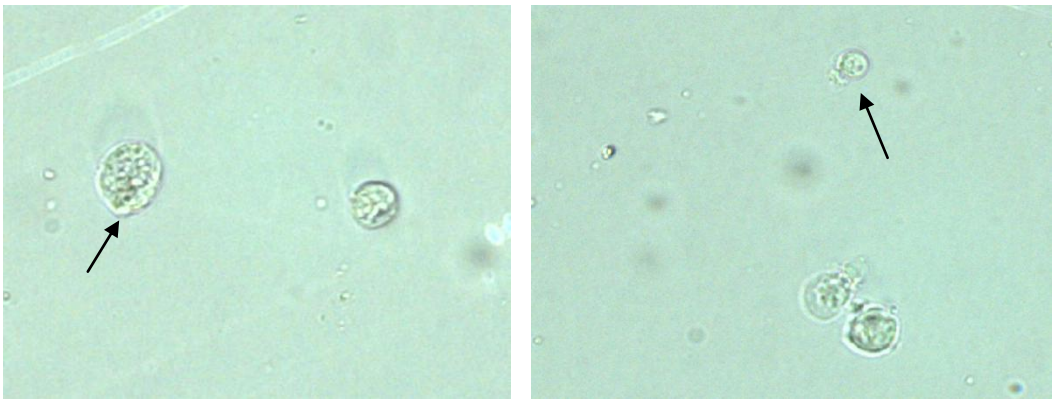

Figure 4.1. Photographs of intact haemolymph cells after long-term storage in 6% formalin;seawater from *Crassostrea gigas* as observed using microscopy at 400x magnification.

**Cell size versus granularity for distinguishing cell populations**

Two distinct cell populations were isolated using both the FSC (size) and SSC (granularity) in the blue mussel and the manila clam (Figures 4.2 and 4.3). Pearson's correlation was performed on each toxin concentration for the 'small' and less complex hyaline cell populations distinguished in the blue mussel and the manila clam. This was done to confirm that the gating strategy chosen gave related distributions of cell populations based on both size and granularity. All correlations performed at each toxin concentration were significant (Table 4.1). These analyses were completed to determine which measurement (size/granularity) would be appropriate for further evaluation of cell populations in these bivalves and using the results, choose the appropriate criteria for determining the number of cell populations that occurred in the pacific oyster. Two populations were successfully isolated using cell size in the oyster and three distinct populations were observed when using granularity to separate cell type (Figure 4.4). Based on the results, cell

complexity (i.e. granularity, SSC) was chosen as the main criterion for distinguishing cell populations in each bivalve species.

Table 4.1. Correlation results from analysis of the relatedness of cell populations separated using 'size' and 'granularity'. Analyses show significant correlation using these two gating strategies.

| **Correlation between 'small' and 'hyaline' cell populations** | | |
|---|---|---|
| **Conc OA/tank** | ***Mytilus edulis*** | ***Ruditapes philippinarum*** |
| **1µg** | r = 0.53, p < 0.01 | r = 0.74, p < 0.01 |
| **2µg** | r = 0.46, p < 0.01 | r = 0.62, p < 0.01 |
| **40µg** | r = 0.57, p < 0.01 | N/A |

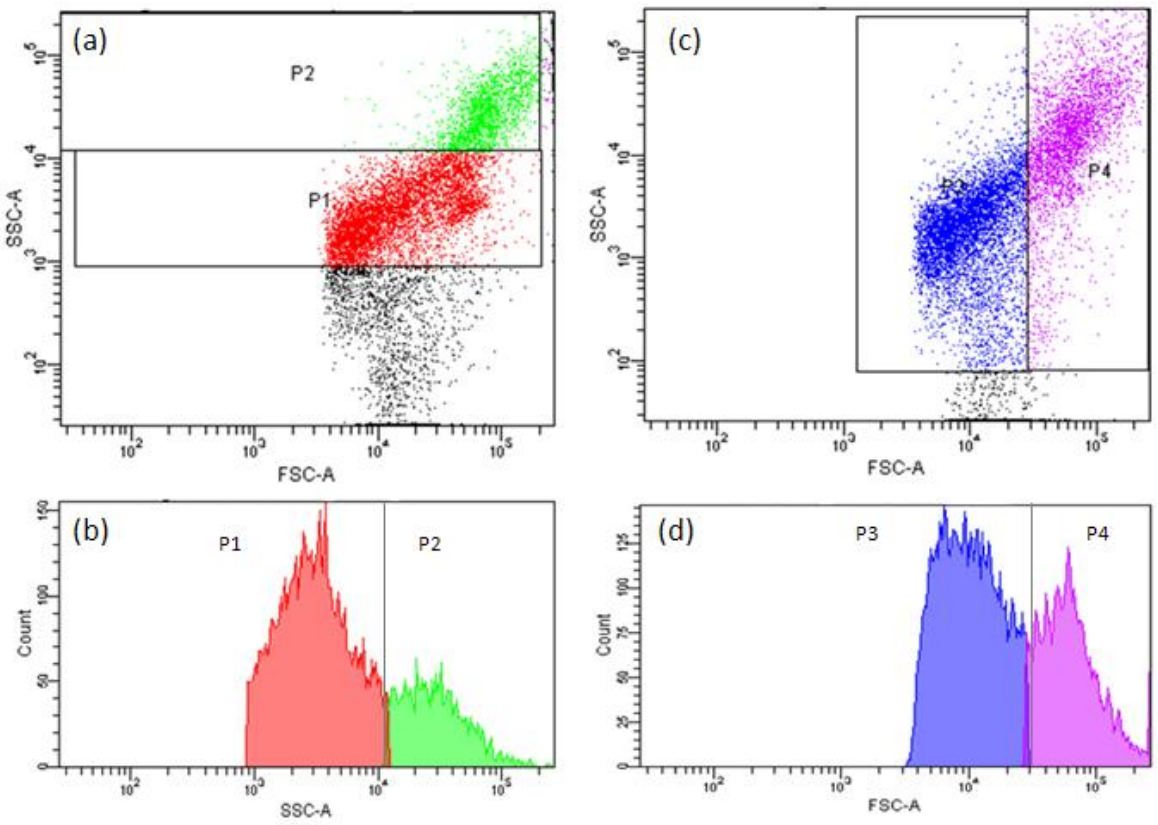


Figure 4.2. Graphs (a) and (b) from top to bottom, show *Mytilus edulis* haemolymph separated into two distinct populations by granularity (SSC); (a) scatter gram depicting two distinct populations; (b) histogram showing two separate populations and Figures (c) and (d) show the same sample with two distinct populations separated by cell size (FSC). (P1 hyaline cells; P2 = granular cells; P3 = small cells and P4 = large cells).

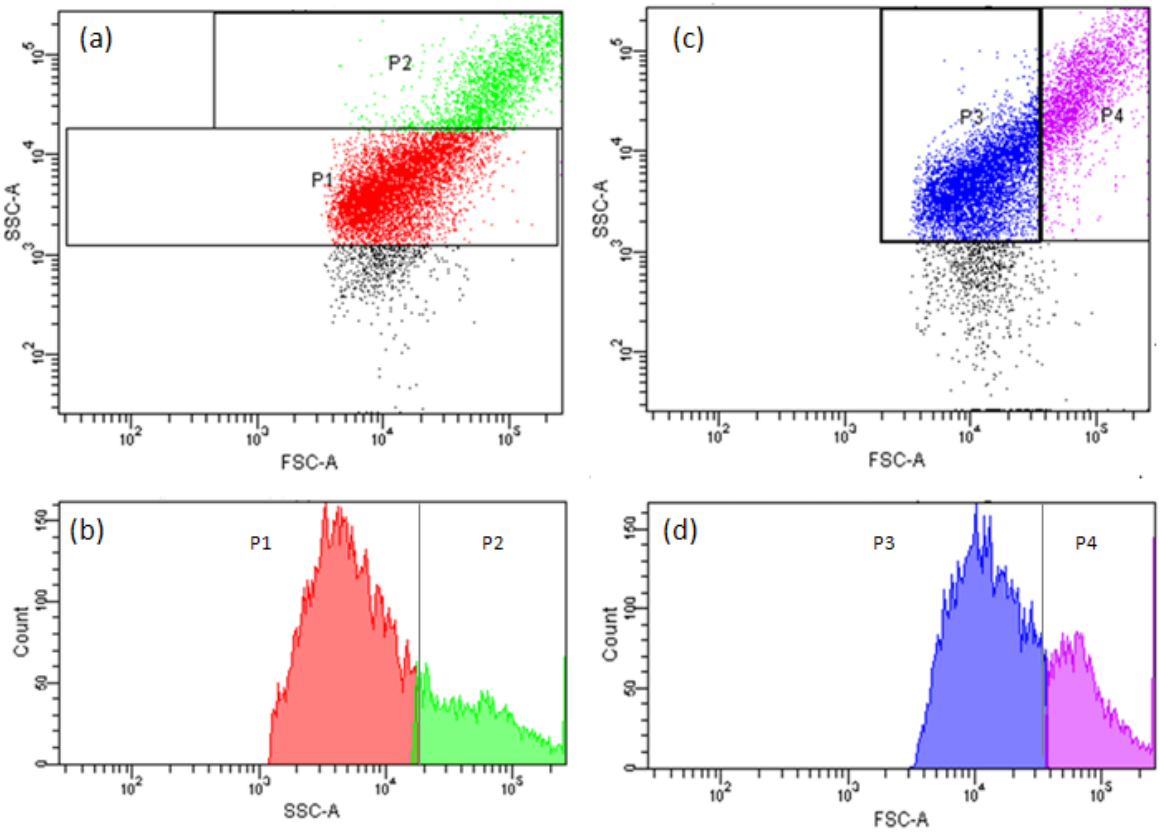


Figure 4.3. Graphs (a) and (b) from top to bottom, *Ruditapes philippinarum* haemolymph separated into two distinct populations by granularity/complexity (SSC); (a) scatter gram depicting two distinct populations; (b) histogram showing two populations separated using SSC; and Figures (c) and (d) show the same sample with two populations separated by cell size (FSC). (P1 hyaline cells; P2 = granular cells; P3 = small cells and P4 = large cells).

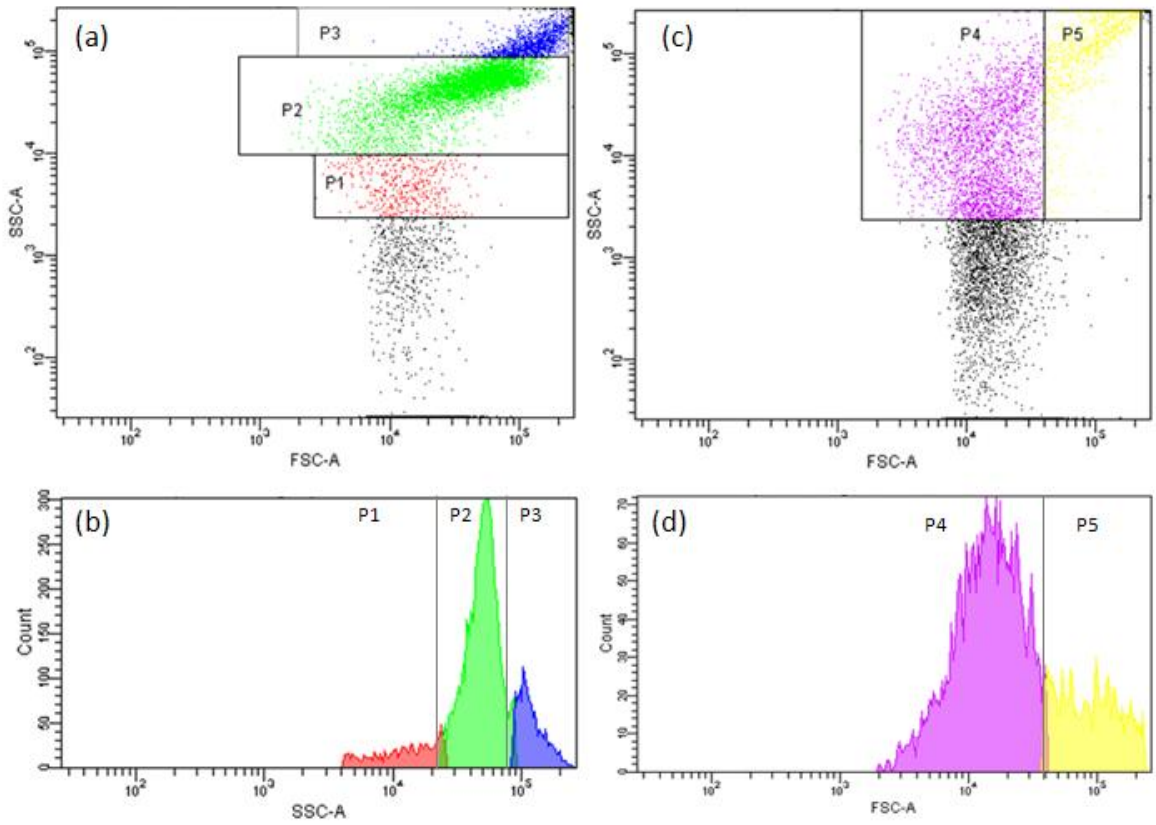


Figure 4.4. *Crassostrea gigas* haemolymph was separated into three populations using cell complexity/granularity (SSC); (a) scatter gram depicting three populations and (b) histogram showing three distinct cell populations. Oyster haemolymph was separated into two populations using cell size (FSC), graphs (c) and (d) show the separation of two populations using the FSC function**.** (P1 hyaline cells; P2 = small granular cells; P3 = large granular cells; P4 = small cells and P5 = large cells).

**Controls**

No significant differences occurred between the controls sampled prior to exposure and the negative controls sampled after 7 days. Some non-significant variation was observed, these differences could be attributable to a 'tank effect' and also to the intraspecies variation that occurs in all three species of bivalve, as evidenced by varying SEM values (Table 4.2).

**Acute exposure: 2 µg/15 l tank**

For the acute exposure of 2 µg/15 l tank administered at $T_{0,}$ no significant changes in the proportion of hyalinocytes or granulocytes were detected using flow cytometry in either the manila clam or the blue mussel over the exposure period. In the pacific oyster a significant change in the number of small granulocytes (P2) was detected (ANOVA: $F_{4, 45} = 3.5$; $p < 0.05$), post hoc testing determined that these changes occurred between the control at $T_7$ and 3 days exposure with a spike in small granulocytes from 41%/42% at control levels ($T_7/T_0$) to 50% after 3 days exposure, this decreased significantly back to control levels of 40% after 7 days (Tukey: $p < 0.05$) (Figure 4.5). There were no significant changes detected in the hyalinocyte or large granulocyte populations of the pacific oyster (see Table 4.2). In addition to analysing each of the three cell types individually for the pacific oyster, small and large granulocytes were pooled and using these data no significant changes occurred in the populations of granulocytes and hyalinocytes over the 7 days.

Table 4.2. Percentages of granulocytes, small granulocytes and hyalinocytes (mean ± standard error of the mean) isolated in the circulating haemolymph of *Mytilus edilus*, *Ruditapes philippinarum* and *Crassostrea gigas*. NA: not assayed. n = 10 individuals per sampling day. 'Ψ' denotes significant difference from the negative control, '*' denotes significant difference from subsequent exposure day (ANOVA, Tukeys post-hoc: $p < 0.05$). (Original percentage data, not arcsine transformed).

| | ***Mytilus edulis*** | | ***Ruditapes philippinarum*** | | ***Crassostrea gigas*** | | |
|---|---|---|---|---|---|---|---|
| | Granulocytes | Hyalinocytes | Granulocytes | Hyalinocytes | Granulocytes | Small granulocytes | Hyalinocytes |
| **2µg/tank OA** | | | | | | | |
| **Control T0** | 30.55 ± 5.75 | 69.45 ± 5.75 | 23.33 ± 1.30 | 76.67 ± 1.30 | 27.46 ± 3.60 | 42.33 ± 2.48 | 30.22 ± 5.23 |
| **Control T7** | 20.11 ± 2.70 | 79.89 ± 2.70 | 26.65 ± 2.73 | 73.35 ± 2.73 | 20.50 ± 2.30 | 40.98 ± 2.57 Ψ | 38.51 ± 3.85 |
| **1 day OA** | 29.37 ± 4.97 | 70.62 ± 4.97 | 26.69 ± 2.39 | 73.31 ± 2.39 | 24.21 ± 3.39 | 41.85 ± 3.47 | 33.94 ± 5.76 |
| **3 day OA** | 24.70 ± 1.67 | 75.30 ± 1.67 | 21.20 ± 1.63 | 78.79 ± 1.63 | 20.85 ± 4.12 | 49.85 ± 1.02 Ψ* | 29.34 ± 3.46 |
| **7 day OA** | 20.53 ± 2.21 | 79.47 ± 2.21 | 22.38 ± 2.01 | 77.62 ± 2.01 | 20.87 ± 2.45 | 40.35 ± 2.53* | 38.78 ± 4.19 |
| **1µg/tank OA** | | | | | | | |
| **Control T0** | 30.55 ± 5.75 | 69.45 ± 5.75 | 23.33 ± 1.30 | 76.67 ± 1.30 | 27.46 ± 3.60 | 42.33 ± 2.48 | 30.22 ± 5.23 |
| **Control T7** | 20.11 ± 2.70 | 79.89 ± 2.70 | 26.65 ± 2.73 | 73.35 ± 2.73 | 20.50 ± 2.30 | 40.98 ± 2.57 | 38.51 ± 3.85 |
| **1 day OA** | 20.72 ± 2.02 | 79.28 ± 2.02 | 25.83 ± 2.13 | 74.17 ± 2.29 | 24.14 ± 2.67 | 39.02 ± 2.79 | 36.83 ± 5.07 |
| **3 day OA** | 21.58 ± 2.89 | 78.42 ± 2.89 | 28.42 ± 3.24 | 71.58 ± 3.24 | 27.16 ± 3.57* | 39.59 ± 2.18 | 33.26 ± 4.64 |
| **7 day OA** | 18.24 ± 3.21 | 81.79 ± 3.21 | 22.63 ± 2.65 | 77.37 ± 2.65 | 16.89 ± 2.90* | 44.07 ± 1.82 | 39.04 ± 4.39 |
| **40µg/tank OA** | | | | | | | |
| **Control T0** | 21.52 ± 4.11 | 78.48 ± 4.11 | NA | NA | 15.37 ± 1.93 | 45.33 ± 4.51 | 39.29 ± 5.45 |
| **Control T7** | 28.19 ± 5.13 | 71.81 ± 5.13 | NA | NA | 20.04 ± 6.56 | 35.97 ± 3.99 | 43.98 ± 9.78 |
| **1 day OA** | 26.53 ± 6.03 | 73.47 ± 6.03 | NA | NA | 21.47 ± 3.14 | 45.18 ± 2.79 | 33.35 ± 3.42 |
| **3 day OA** | 23.85 ± 6.35 | 76.15 ± 6.35 | NA | NA | 18.15 ± 5.83 | 41.84 ± 4.06 | 40.00 ± 5.87 |
| **7 day OA** | 35.48 ± 8.14 | 64.51 ± 8.14 | NA | NA | 20.65 ± 3.07 | 38.72 ± 2.49 | 40.62 ± 5.45 |

**Sub-acute exposure: 1 µg/15 l tank**

Sub-acute exposure to OA in daily quantities of 1 µg/15 l tank produced no significant changes in the proportion of hyalinocytes and granulocytes measured in the manila clam or in the blue mussel. In the pacific oyster there were no significant changes in the proportion of hyalinocytes or small granulocytes, however there was a significant change in the proportion of large granulocytes, after 3 days the proportion of large granulocytes was at 27% and this decreased significantly to 16% after 7 days exposure (ANOVA: $F_{4, 44} = 3.1$; $p < 0.05$). There was no significant difference from the controls (Figure 4.5). Pooled granulocyte populations showed no significant change in proportion over the exposure period for the pacific oyster.

**Sub-acute exposure: 40 µg/15 l tank**

No statistically significant changes in the proportions of haemocyte populations were detected using flow cytometry at the highest concentration of OA exposure for the blue mussel or the pacific oyster, neither when granulocytes were assessed individually or pooled for the oyster samples. The experiment was not performed for the manila clam at this concentration. A definite trend in increasing proportion of granular cells was observed in the blue mussel, at the $T_0$ control granulocyte proportion was at 21% and this increased to 35% after 7 days exposure.

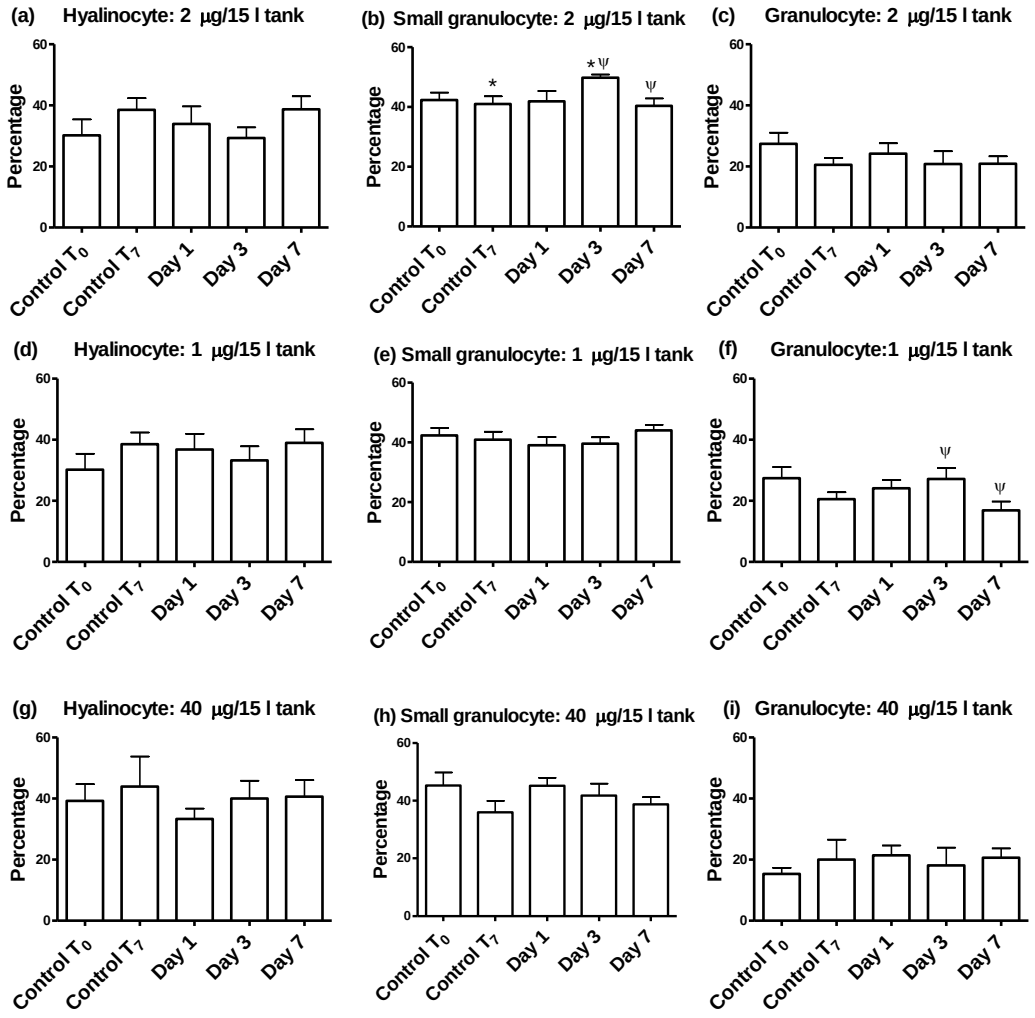


Figure 4.5. Proportions of the three oyster cell populations isolated using flow cytometry; graphs (a)-(c) acute exposure: 2 µg/15 l tank of OA; graphs (d)-(f) sub-acute exposure: 1 µg/15 l tank of OA; graphs (g)-(i) sub-acute exposure: 40 µg/15 l tank of OA.

'*' denotes significant difference from the control at $T_7$

'ψ' Denotes significant difference between the proportion of granulocytes measured in day 3 versus day

## DISCUSSION

As was previously discussed, HABs are increasing worldwide, and it is essential to determine whether these HABs have negative impacts on commercially important bivalve species. The current study examined the impact of OA, a commonly occurring DSP toxin on the differential cell population structure in the circulating haemolymph of three bivalve species: *Mytilus edulis,* the blue mussel; *Ruditapes philippinarum,* the manila clam and *Crassostrea gigas,* the pacific oyster. It is important to establish whether these algal blooms elicit a change in the normal haemolymph cell profile in these species and thus stress the organism, leading to increased susceptibility to pathogens and parasites (Pearce *et al.* 2005, Hégaret *et al.* 2009, Mello *et al.* 2010).

This research demonstrated successful separation and characterisation of haemocytes from the blue mussel, the manila clam and the pacific oyster using flow cytometry. As observed in previous studies using flow cytometry, two distinct populations were isolated in the manila clam and three populations were distinguished in the pacific oyster (Allam *et al.* 2002, Delaporte *et al.* 2003, Hégaret *et al.* 2003a). From examination of the available literature, we concluded that this was the first study to use flow cytometry to examine the differential cell populations of *Mytilus edulis.* Previous studies on *Mytilus sp*. using light microscopy have isolated two/three subpopulations within the circulating haemolymph (Carballal *et al.* 1997, Dyrynda *et al.* 1997, Wootton *et al.* 2003), in this investigation two populations were detected in the blue mussel using flow cytometry. In addition, successful long-term storage of bivalve haemolymph in 6% seawater: formalin solution was achieved. This will enable flow cytometric analysis of differential cell populations in bivalves as a component of more complex experiments, because analysis can be performed at a later stage. Cells were stored in the fixative for up to 8 months at 4$^{o}$C, these were compared using microscopy with fresh cells, and cells that were fixed according to Hégaret *et al.* (2003a) and it was confirmed that no deterioration of cell structure occurred (Figure 4.1).

The blue mussel and manila clam showed similar proportions of hyalinocytes versus granulocytes, with hyalinocytes present at higher quantities than granulocytes (65-75%, versus 25-35%, see Table 4.2). In the pacific oyster, large granulocytes were the smallest population (15-27%), small granulocytes were the most abundant (40-50%) and the remaining 30-40% of the population were hyaline cells. Similar cell population proportions have been measured previously in these species (Oubella *et al.* 1996, Barracco *et al.* 1999, Allam *et al.* 2002). Table 4.3 provides a detailed review of the proportions of cell types found in bivalve species measured using multiple methods. The proportions of each cell type vary considerably depending on both the species, and the method used to differentiate cells. Method standardisation and a more defined classification system for the identification of the sub-populations within the circulating haemolymph of marine bivalves are needed, to allow direct comparison between different studies. Another possible explanation for differences between experiments is seasonal variation within bivalve species. Seasonal variation has been observed when measuring the health status of a number of parameters in bivalve species such as baseline levels of DNA fragmentation, presence of lipofuscin granule formation, as well as the morphology and complexity of bivalve haemocytes (McCormick-Ray and Howard 1991, Kagley *et al.* 2003, Hartl *et al.* 2004, Soudant *et al.* 2004).

Table 4.3. Review of current literature examining the proportion of granulocytes and hyalinocytes in marine bivalve species using multiple methods. Table displays the cell types isolated, the proportions present in each species and where information was available the cell size measured for each cell type.

* Cell population of greater than 100% is due to antibody staining overlap among the different population subtypes.

| ***Species*** | **Cell type** | **Proportion (%)** | **Cell size (um)** | **Method** | **Reference** |
|---|---|---|---|---|---|
| ***Ruditapes philippinarum*** | Granulocytes<br>Hyalinocytes<br>Small basophilic/ multinucleated cells | 39<br>55<br>6 | | Light microscopy and monolayers | Oubella (1996) |
| ***Mytilus galloprovincialis*** | Granulocytes<br>Hyalinocytes | >50<br><50 | 10.95<br>8.33 | Light and electron microscopy | Carballal (1997) |
| ***Mytilus edulis*** | Large granulocyte<br>Small granulocyte<br>Hyalinocyte | 30-40*<br>40-60*<br>60-80* | | Light microscopy and antibody staining | Dyrynda (1997) |
| ***Perna perna*** | Granulocyte<br>Hyalinocyte | 40<br>60 | 6-14<br>5-12 | Light microscopy | Barracco (1999) |
| ***Tapes philippinarum*** | Granulocyte<br>Hyalinocyte<br>Other | 48·05<br>32.18<br>20 | 3-16<br>4-12<br>3-5 | Light and fluorescent microscopy | Cima (2000) |
| ***Ostrea edulis*** | Large granulocyte<br>Small granulocyte<br>Hyalinocyte | 9.2<br>23.5<br>65 | | Light and electron microscopy | Xue (2001) |
| ***Ruditapes philippinarum***<br>***Mercenaria mercenaria***<br><br>***Crassostrea virginica*** | Granulocyte<br>Hyalinocyte<br>Granulocyte<br>Hyalinocyte<br>Granulocyte<br>Hyalinocyte | 63.1<br>37<br>65.4<br>35<br>33.4<br>67 | 8.2<br>6.8<br>10.8<br>7.7<br>10.5<br>6.7 | Flow cytometry and light microscopy | Allam (2002) |

| | | | | | |
|---|---|---|---|---|---|
| ***Chlamys ferreri*** | Granulocytes | 66 | 14.43 | Flow cytometry and light microscopy | Xing (2002) |
| | Hyalinocytes | 34 | 5.93 | | |
| ***Mytilus edulis*** | Large granulocyte | 40 | 10-12 | Light microscopy | Wootton (2003) |
| | Small granulocyte | 50 | 7-8 | | |
| | Hyalinocytes | 10 | 3-4 | | |
| ***Cerastoderma edule*** | Large granulocyte | 45 | 4-7 | | |
| | Small granulocyte | 30 | 4-6 | | |
| | Hyalinocytes | 15 | 3-4 | | |
| | Granulocyte (Type III) | 10 | 10-12 | | |
| ***Ensis silqua*** | Large granulocyte | 45 | 4-7 | | |
| | Small granulocyte | 47 | 8-10 | | |
| | Hyalinocytes | 6 | 3-4 | | |
| ***Crassostrea gigas*** | Granulocyte | 16.8 | | Flow cytometry | Delaporte (2003) |
| | Small agranular cells | 13.3 | | | |
| | Hyalinocyte | 70 | | | |
| ***Crassostrea virginica*** | Granulocyte | 35 | | Flow cytometry | Hegaret (2003a) |
| | Small granulocyte | 5 | | | |
| | Hyalinocyte | 60 | | | |
| ***Argopectin irradians*** | Large granulocyte | 20-30 | | Light microscopy, scanning and transmission electron microscopy | Zhang (2005) |
| | Small granulocyte | 20-25 | | | |
| | Large hyalinocyte | 15-20 | | | |
| | Small hyalinocyte | 30-35 | | | |
| ***Crassostrea ariakensis*** | Granulocyte | 68.4 | 6.7 | Light and electron microscopy | Sun (2006) |
| | Large hyalinocyte | 21.6 | 3.7 | | |
| | Small hyalinocyte | 9 | 2 | | |
| ***Perna perna*** | Granulocyte | 95 | | Light microscopy | Mello (2010) |
| | Hyalinocyte | 5 | | | |
| ***Crassostrea gigas*** | Granulocyte | 90 | | | |
| | Hyalinocyte | 10 | | | |
| ***Anomalocardia brasiliana*** | Granulocyte | 70 | | | |
| | Hyalinocyte | 30 | | | |

Previous research investigating the impact of algal toxins on the immune-response and cell proportions in circulating HL of bivalves has reported varying effects, depending on both the species of bivalve and the algal toxin (Hégaret and Wikfors 2005a, b, Hégaret *et al.* 2007b, Galimany *et al.* 2008b, Galimany *et al.* 2008c, Mello *et al.* 2010). In the current study, analyses of changes in cell populations in the circulating haemolymph of the blue mussel, and manila clam showed minimal impact of OA on the differential cell-proportions of these shellfish at the concentrations utilised. At the lower concentrations acute exposure to 2 µg/15 l tank and sub-acute exposure of 1 µg/15 l tank, there were no changes measured in these species, however in the pacific oyster significant changes in the small granulocytes and large granulocytes were observed respectively for each concentration. When granulocyte populations were pooled, no effects were observed in pacific oyster hyaline or granular cell populations over the exposure period. Thus, the chemically-induced effects of OA on the differential populations in the pacific oyster occurred only within the small and large granulocyte populations. Mello *et al.* (2010) looked at the effects of *Dinophysis acuminata*, an OA-producing algae, on *C. gigas* during a bloom event. The concentration of OA reached 6.8 µg/kg of shellfish meat during the period of highest toxicity and no changes in differential cells were recorded. In this study at the 2 µg/15 l tank acute exposure (0.1 µg/ shellfish) there was a significant increase in the proportion of small granulocytes relative to the control at $T_7$ after 3 days exposure, which decreased to non-significant levels after 7-days. Based on the weight of an individual oyster (10.74 g) the initial tissue concentration of OA after exposure (assuming 100% uptake of OA) was ~4.2 µg/kg of shellfish meat. The rate of depuration coupled with the estimated 8-day half-life of OA would influence the tissue concentrations after 3 days exposure (Svensson and Förlin 2004). At 1 µg/15 l tank there was a significant decrease in the proportion of granulocytes measured between 3 days (OA: ~11.6 µg/kg of shellfish meat) and 7 days exposure (~23.2 µg/kg) (see Table 1.3, Chapter 1). Differences observed between the current study and Mello *et al.* (2010) could be due to the higher OA concentrations at the 1 µg/15 l tank exposure and also the differing methods utilised. Mello *et al.* (2010) examined 200 cells per individual using light microscopy, in comparison to 10,000 cells or 'events' per individual using flow cytometry in this study. Histopathological examination of the impacts of OA-producing algae on

pacific oyster spat have recorded negative effects (Pearce *et al.* 2005) and in our own work, histological examination of OA exposure at the same concentrations utilised in this study showed significant effects (see Chapter 3).

The changes in cell populations observed in this research may occur through alteration in the production of granular haemocytes in response to environmental stress, with a higher proportion of one cell type being produced (Delaporte *et al.* 2003, Mello *et al.* 2010), or reduction of total haemocyte number through migration of cells from the circulating haemolymph to affected tissues within the shellfish. Histological examination of tissues affected by HABs has shown haemocyte infiltration, particularly in the hepatopancreas tissues (Wikfors and Smolowitz 1995, Galimany *et al.* 2008a, Galimany *et al.* 2008c), this was confirmed in our histological investigation of OA effects (Chapter 3). Chemically-induced modulation of the immune response in bivalve species has previously been recorded in pollutant-exposed shellfish (Auffret *et al.* 2002, Gagnaire *et al.* 2006), with an increase in haemocyte populations measured in the presence of pollutants (Pipe and Coles 1995, Hine 1999). Within the circulating haemolymph, granulocytes are considered to be the main 'defenders' in the innate immune response of bivalve shellfish and are responsible for phagocytosing non-self particles such as bacteria, zymosan, protozoan parasites and algae (Cheng 1981, Moore and Gelder 1983, Hine 1999). Gagnaire *et al.* (2006) measured an increase in granulocyte proportions after exposure to polycyclic aromatic hydrocarbons and fungicides. Thus, significant changes in the populations of these cells may occur as a response to the presence of the phycotoxin OA. The increase in small granulocytes sampled at 3-days following acute exposure of 2 μg/15 l tank in the pacific oyster could be due to a chemical-induced stress-response to the toxin, the levels of small granulocytes drop again after 7 days and the proportion of hyalinocytes increases as a result, showing a short-term response to the toxin-exposure followed by a swift recovery. Galimany *et al.* (2008b) found *Mytilus edulis* showed rapid recovery after a significant haemocyte-mediated response to *Alexandrium fundyense*, a paralytic shellfish poison-producing algae. Three days post-exposure there was no difference between exposed and control animals.

The 1 µg sub-acute exposure of OA produced no significant changes from the controls; however there was a significant drop in large granulocytes measured from day 3 (27%) to day 7 (17%), possibly indicating migration of the large granulocyte cells to surrounding tissues. Increased cell mortality is not a likely cause, as cell viability analyses show no significant increase in the numbers of dead cells occurring over the exposure period in the circulating haemolymph of all three species (Chapter 5). This decrease in granulocytes corresponded to a non-significant increase in both the small granulocytes and hyaline cells after 7 days. A similar pattern was observed by Galimany *et al.* (2008a) in the blue mussel, increased proportions of granular cells were recorded after exposure to the toxic alga *Karlodinium veneficum*, levels increased significantly after three days exposure and decreased again at 6 days**.** At the higher concentration of 40 µg /15 l tank no significant trend was observed in the three oyster cell populations. Studies examining the effects of OA on marine bivalves have found that there is no apparent classical dose response post-exposure on the cells of these animals, increasing damage does not correspond to increasing concentration (Carvalho Pinto-Silva *et al.* 2005, Prado-Alvarez *et al.* 2012). In the blue mussel experiment at 40 µg /15 l tank, the numbers of granulocytes increased non-significantly after 7 days exposure from controls levels of 21/28% at $T_0/T_7$ to 35%, it is possible if the experimental exposure time was extended, a similar pattern to that observed in the pacific oyster may be observed. The lack of any significant changes in the cell proportions within the circulating HL of the blue mussel and the manila clam in this study could be due to the fact that the majority of OA accumulates in the hepatopancreas of bivalve species (Yasumoto *et al.* 1978, Blanco *et al.* 2007) possibly reducing the exposure of other tissues, including the circulatory system.

In this study, higher sensitivity to the toxicity of OA was observed in the pacific oyster, with a significant change in the proportion of circulating granulocytes and small granulocytes measured at low concentrations of the toxin (Table 4.2) indicating a chemically-induced response to the toxin. The manila clam and blue mussel showed no significant changes in the differential cell populations over the exposure period at the concentrations utilised, emphasising the necessity for examining the effects of these harmful algal toxins on multiple bivalve species. Further exposure experiments performed over a longer time period, measuring

additional cellular-response parameters is recommended, to provide a full suite of markers for assessing the reaction to OA in these shellfish species.

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# CHAPTER 5

## Does the marine biotoxin okadaic acid cause DNA fragmentation in the blue mussel and the pacific oyster?

**ABSTRACT**

*Prorocentrum* and *Dinophysis* algae produce the okadaic acid group of toxins which can accumulate in filter feeding bivalves, causing Diarrhetic Shellfish Poisoning in human consumers. Bloom events of these algal species regularly cause closure of aquaculture farms during the summer months. Little is known about the impact of these toxins on the shellfish that accumulate them. This study investigates the impact of okadaic acid (OA) on DNA fragmentation in two bivalve species of global economic importance; the blue mussel, *Mytilus edulis* and the pacific oyster, *Crassostrea gigas*.

The shellfish were exposed to three concentrations of OA. Acute exposure of two replicate tanks of shellfish to 2 µg/15 l tank (~0.1 µg/shellfish) mixed with algae on day 1 of the trial, after that shellfish were fed only algae. Sub-acute exposure of two replicate tanks to daily quantities of 1 µg OA/15 l tank, and two replicate tanks were administered 40 µg/15 l tank mixed with algal feed over each of 7 days. Haemolymph and hepatopancreas cells were extracted following 1 day, 3 days and 7 days exposure. Cell viability was measured and remained above 85% for both cell types. DNA fragmentation was examined using the single- cell gel electrophoresis (comet) assay. Similar patterns in DNA fragmentation were found in the two species. A significant increase in DNA fragmentation was observed in both species over time, even at the lowest OA concentrations, using 1-way ANOVA ($p < 0.05$) a noteworthy result for these important aquaculture species. The DNA fragmentation could be due to genotoxicity of OA and/or to the induction of apoptosis in the cells. This is the first study examining the impact of OA on DNA fragmentation in these bivalve species and the first examining the cytotoxicity of OA in *Crassostrea gigas* haemolymph and hepatopancreas cells.

## INTRODUCTION

The marine biotoxin okadaic acid was first isolated from two sponge species, *Halichondria okadai* Kadota, a black sponge found on the Pacific coast of Japan and *H. melanodocia*, a Caribbean sponge found in the Florida Keys (Tachibana *et al.* 1981). Okadaic acid (OA) and its analogues, the dinophysis toxins (DTX1, DTX2, and DTX3) together form the group of OA-toxins. These toxins are fat-soluble, thermostable, polyether compounds that are produced by a number of dinoflagellates of genus *Prorocentrum* and *Dinophysis* and have been found to accumulate in filter feeding animals such as sponges and ascidians, and also in bivalve shellfish (Kumagai *et al.* 1986, Campas *et al.* 2007, Reizopoulou *et al.* 2008). Human consumption of these contaminated shellfish causes Diarrhetic Shellfish Poisoning (DSP) (James *et al.* 2010). The symptoms of DSP appear within three hours of ingestion and include nausea, abdominal cramps, severe vomiting and diarrhoea. The DSP toxin OA is a known inhibitor of serine/threonine protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) activity, which are important in signalling cascades and regulate different cellular processes in eukaryotic cells (Bialojan and Takai 1988, Vieytes *et al.* 1997). The diarrhoeogenic effect of OA is caused by the accumulation of phosphorylated proteins which control sodium secretion in cells, and the inhibition of dephosphorylation of cytoskeletal elements that regulate permeability to solutes, in combination these cause a passive loss of fluids (Tubaro *et al.* 1996). DSP toxin-producing algae have been identified in Irish coastal waters at varying concentrations, among them are the species *Dinophysis acuta*, *Dinophysis acuminata*, and *Prorocentrum lima* (James *et al.* 1999, Fux *et al.* 2009). Blooms of these toxin-producing algae regularly cause closure of aquaculture farms off of the Irish coast during the summer months.

To date, the majority of toxicological investigations to determine the impacts of OA have been on mammalian cells, both *in vivo* and *in vitro*. *In vivo* studies have found it to be a tumour-promoter in mouse skin and the mucosa of rat glandular stomach (Suganuma *et al.* 1988, Fujiki and Suganuma 1999). Several *in vitro* studies have been performed to determine OAs genotoxic, mutagenic and cytotoxic effects on a number of mammalian cell lines (Rogers *et al.* 1994, Fessard *et al.* 1996, Souid-

Mensi *et al.* 2008, Valdiglesias *et al.* 2010). There has been some debate regarding the effects of this compound on mammalian cells, the results reported with regard to OA cytotoxicity and genotoxicity are often contradictory and overall, the findings from these *in vitro* studies are inconclusive.

Souid-Mensi *et al.* (2008) and Valdiglesias *et al.* (2010) examined the *in vitro* genotoxicity of OA on several cell lines using the DNA damage detection 3D assay and the comet assay respectively. They concluded that its genotoxicity varied depending on the cell-type and on the concentration of toxin present. The cytotoxicity was also measured in these experiments, the effects on cell viability varied depending on cell type, and a clear dose response was not observed. Fessard *et al.* (1996) reported that OA had a genotoxic impact on BHK21 C13 fibroblasts and HESV keratinocytes, by measuring increased DNA adduct formation in cell lines exposed to the biotoxin; cytotoxicity to the cell lines was also measured and the effects varied depending on cell type. A much lower $LC_{50}$ value was calculated for BHK21 C13 (15 nM) than HESV (50 nM) (Fessard *et al.* 1996). A number of *in vitro* studies have established that OA also causes apoptosis in some mammalian cell lines (Bøe *et al.* 1991, Traoré *et al.* 2001, Huynh-Delerme *et al.* 2003, Le Hégarat *et al.* 2005, Ao *et al.* 2008, Xing *et al.* 2009).

Thus, while the focus of much research has been on the impact of OA on humans, it is only relatively recently that the pathological effects of marine biotoxins on filter-feeding bivalve species have begun to be investigated. Cytotoxicity, DNA fragmentation, immunological effects and gross physiological damage detected using histopathology have all been examined in a number of shellfish species (Carvalho Pinto-Silva *et al.* 2003, Svensson *et al.* 2003, Hégaret and Wikfors 2005, da Silva *et al.* 2008, Galimany *et al.* 2008a). Studies on bivalves indicate that the gill and haemolymph and heart cells are relatively resilient to the cytotoxic effects of OA (Svensson *et al.* 2003, Talarmin *et al.* 2007, Flórez-Barrós *et al.* 2011), but DNA fragmentation has been measured in different cell types exposed to OA (Carvalho Pinto-Silva *et al.* 2003, Carvalho Pinto-Silva *et al.* 2005, Flórez-Barrós *et al.* 2011).

Although there are numerous different toxins produced by these harmful algae and a large number of shellfish species impacted by their presence, as yet there are limited data available on many important shellfish species. The aim of this

investigation was to examine whether exposure to environmentally relevant concentrations of the marine biotoxin OA (Chapter 1, Table 1.2), caused reduction in cell viability through cytotoxicity, and measure changes in DNA fragmentation. It was also determined whether this response was dose and/or time dependent. The blue mussel, *Mytilus edulis,* and the pacific oyster, *Crassostrea gigas,* were the species chosen for this study. These shellfish are farmed off the Irish coastline and are important aquaculture species in many countries. Fragmentation was measured using the single cell gel electrophoresis (comet) assay. This has been successfully utilised on a number of bivalve species measuring the impact of a wide range of toxins, both anthropogenic and biological (Coughlan *et al.* 2002, Rank *et al.* 2005, Hartl *et al.* 2006, Juhel *et al.* 2007, Çavas and Könen 2008).

## MATERIALS AND METHODS

### Toxins and reagents

The biotoxin okadaic acid (OA) sodium salt (L.C. Laboratories, 05857) dissolved in methanol (HPLC grade) (1 mg OA: 1 ml methanol) was utilised for exposure to bivalves. OA was standardised against a reference standard using Liquid Chromatography-Mass Spectroscopy (LC-MS) analysis. This was conducted on a Thermo Scientific Quantum Discovery Max triple quadropole mass spectrometer, equipped with a heated electrospray ionization source, hyphenated to a Thermo Scientific Accela LC system by the Mass Spectrometry Research Centre for Proteomics and Biotoxins (PROTEOBIO), Cork Institute of Technology (Carey *et al.* 2012). OA was mixed with phytoplankton feed prior to mussel exposure. All other experimental reagents were purchased from Sigma-Aldrich Ireland Ltd.

### Experimental animals

The blue mussels and pacific oysters were obtained from certified aquaculture producers rearing shellfish off the Irish coast and kept at 12 ± 0.5$^{o}$C. Shellfish were acclimatised for 1 week prior to exposure. Water was changed daily and the animals were given algal feed every two days (Shellfish Diet 1800, Reed Mariculture Inc,

USA). The bivalves were not fed for 24h prior to exposure. Mussels 50 – 60 mm and oysters 60 – 80 mm were chosen for experimentation.

**Experimental design**

Experimental and control tanks were set up, with 20 shellfish and 15 l of seawater placed in each tank. Seawater was changed daily in each of the tanks for all experiments and algal feed and OA were added to the tanks post water-change. See Table 1.2, Chapter 1, for time-periods when each experiment was performed.

Three concentrations of OA were tested in this series of experiments, two replicate tanks of 20 animals per tank for each concentration of phycotoxin. Acute (single) exposure of replicate tanks to 2 µg /15 l tank (2.5 nM) of OA mixed with the algal feed on day 1 of the trial (2 µl stock solution in 98 µl MeOH: 100 µl algal feed) and from then on, only the algal feed (100 µl), this gives a maximum exposure of approximately 0.1 µg OA/shellfish. Sub-acute (daily) exposure of replicate tanks of shellfish to OA of concentration 1 µg/15 l tank/day (1.2 nM) mixed with the algal feed. Sub-acute (daily) exposure of replicate tanks to 40 µg/15 l tank (50 nM) mixed with algal feed. Estimated concentrations per shellfish for the daily sub-acute exposure studies were based on the accumulation/depuration study of Svensson and Förlin (2004) and assumed an even suspension of OA in the seawater with complete and equal uptake and distribution of the toxin in each shellfish (Chapter 1, Table 1.3)

Five animals were removed from each replicate tank and sacrificed for experimentation after 1 day, 3 days and 7 days exposure; except for mussels exposed to the 40 µg concentration, when three animals were removed at each sampling time. This lower number was due to simultaneous sampling from the experimental tanks for subsequent LC-MS analysis of the animals (results not shown here). Experimental animals that were sampled were replaced with the same number of marked animals from the stock population, to avoid any shellfish-density effects (Juhel *et al.* 2007).

For the positive control, animals were exposed to cadmium chloride, $CdCl_2$ a known genotoxicant (Juhel *et al.* 2007), at 5 µM/day for mussels and at 10 µM/day for oysters, mixed with the algal feed (100 µl), daily for the duration of the experiment. Oysters were given a higher concentration of $CdCl_2$ to ensure a positive

response due to their larger size. For the negative control, the animals were fed 100 µl of algal feed each day. Ten animals were sampled from the stock population on the first day of the experiment ($T_{0)}$ to act as a negative control and after 7 days ($T_7$), 10 animals were sampled from the positive and negative control tanks.

**Isolation of single cell suspensions**

Approximately 300 µl of haemolymph (HL) was removed from the posterior adductor sinus using a sterile 23-guage 1 ½ inch needle and 2.5 ml syringe. 30 µl aliquots were utilised for the single-cell gel electrophoresis assay. HL cells were placed in equal quantities of Hanks' Balanced Saline Solution (HBSS) ($Ca^{2+}$, $Mg^{2+}$ free) with a corrected osmolarity of 990 mOsm $l^{-1}$ (22.2 $gl^{-1}$) (Coughlan *et al.* 2002), as recent studies have shown that mussel HL remains viable for at least 7-days in this solution at 4$^{o}$C (Hartl *et al.* 2009).

Hepatopancreas (HP) cells were isolated using a method adapted from Wilson *et al.* (1998). Briefly, the organ was excised and chopped 10 times using a razor blade on a glass plate in 0.2 ml of HBSS and the tissue was rinsed into a 50 ml centrifuge tube using 2.5 ml of HBSS and 0.3 ml of trypsin (conc: 0.005%). The suspension was gently rocked and incubated for 10 minutes at room temperature. 10 ml of HBSS was added to the tube and the sample was filtered through a 40 µm cell filter (BD Biosciences) to remove larger tissue and debris. After centrifuging at 800 x g for 10 minutes the supernatant was discarded and the pellet resuspended in 0.5 ml of HBSS.

The trypan blue exclusion method was used for the viability assay. Equal quantities of cell suspension and trypan blue were mixed and examined using a haemocytometer under a microscope at 40 x magnification. Fifty cells per slide were counted and percentage viability recorded. The remaining HL/HP cell suspensions were fixed in 6% formalin: seawater solution for further experimentation (Chapter 4

**Comet assay**

The comet assay was performed using a protocol developed by Woods *et al.* (1999). Briefly, haemolymph cells were sandwiched in 1% Low Melting Point Agarose on slides. The slides were placed in a lysis buffer (2.5 M NaCl, 10 mM Tris, 100 mM

EDTA, 1% (v/v) Triton X-100 and 10% (v/v) DMSO and pH 10.0) in the dark at 4$^{o}$C for 2 hours. The samples were submerged in an alkaline solution (0.3 M NaOH, 1 mM EDTA; pH>12) in an electrophoresis tank for 30 minutes at 4$^{o}$C, this unwound the DNA. A current (25 V, 300 mA) was then applied for exactly 25 minutes. Tris buffer (0.4 M Tris–HCl, pH 7.4) was added dropwise to neutralise the pH. DNA was stained with ethidium bromide and examined using an epifluorescence microscope (Nikon EFD-3) (Figure 5.1).

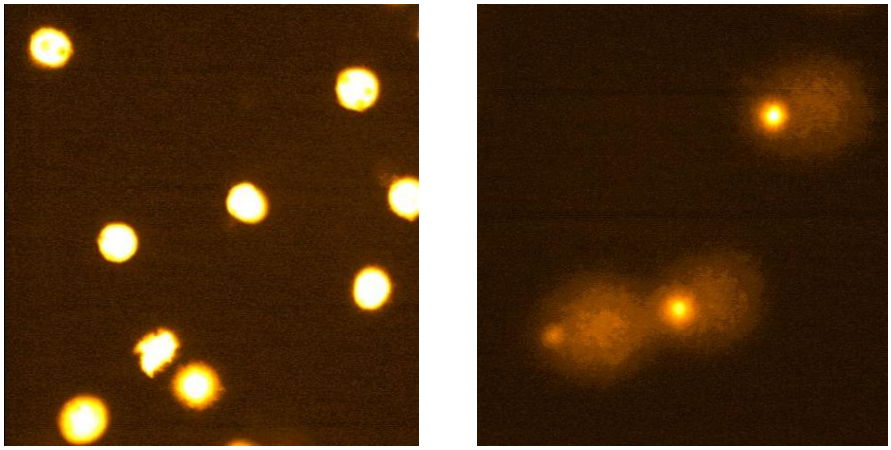

Figure 5.1. Undamaged, healthy haemolymph cells stained with ethidium bromide on the left and on the right, damaged haemolymph cells with the broken DNA being pulled away from the nucleus forming a distinct 'comets tail'.

**Data Analysis**

DNA damage was recorded as percentage tail DNA (%tDNA) and determined using the imaging analysis software package Komet 4.0. (Kinetic Imaging Ltd). Normality was tested using the Kolmogorov-Smirnov test. Non-normal data was arcsine transformed. T-tests were performed between replicate groups to establish whether there were any differences, and where none were found, replicate groups were pooled. One-way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test were performed on the data ($P < 0.05$) to determine significant differences between the sampling periods. The increase in %tDNA from control levels was calculated and this data was used to compare the amounts of DNA fragmentation recorded in HL and HP cells using independent T-tests. This was done at each toxin concentration for each species. Data were analysed using Predictive Analytics SoftWare Statistics (PASW) 17 for Windows.

## RESULTS

### Cell viability

Cell viabilities in the circulating HL and the HP remained above 85% for the duration of exposure for the blue mussel and the pacific oyster (Table 5.1).

Table 5.1. Percentage cell viabilities (± standard error of the mean) for haemolymph (HL) and hepatopancreas (HP) samples from mussels and oysters stained with trypan blue for each of the okadaic acid concentrations tested

| **Tissue type (conc/shellfish)** | | | | | | |
|---|---|---|---|---|---|---|
| **Mussel viabilities** | **Control $T_0$** | **1 day** | **3 days** | **7 days** | **$CdCl_2$ $T_7$** | **Control $T_7$** |
| **HL 1 µg/15 l tank** | 93.37 ± 1.87 | 93.01 ± 2.13 | 94.97 ± 1.36 | 85.33 ± 2.89 | 96.76 ±0.62 | 91.92 ± 3.00 |
| **HL 2 µg/15 l tank** | 93.37 ± 1.87 | 97.80 ± 0.79 | 95.60 ± 1.08 | 85.03 ± 1.09 | 96.76 ±0.62 | 91.92 ± 3.00 |
| **HL 40 µg/15 l tank** | 89.54 ± 6.98 | 88.22 ± 5.16 | 85.11 ± 0.81 | 86.84 ± 1.17 | 85.60 ± 1.51 | 84.03 ± 1.42 |
| **HP 1 µg/15 l tank** | 96.48 ± 1.07 | 93.29 ± 1.55 | 93.33 ± 0.92 | 85.26 ± 1.83 | 93.05 ± 1.84 | 91.74 ± 1.14 |
| **HP 2 µg/15 l tank** | 96.48 ± 1.07 | 95.01 ± 1.41 | 94.08 ± 1.70 | 90.22 ± 2.19 | 93.05 ± 1.84 | 91.74 ± 1.14 |
| **HP 40 µg/15 l tank** | 89.78 ± 2.47 | 88.50 ± 2.41 | 86.97 ± 2.89 | 90.13 ± 3.44 | 89.70 ± 3.48 | 89.87 ± 2.04 |
| **Oyster viabilities** | **Control $T_0$** | **1 day** | **3 days** | **7 days** | **$CdCl_2$ $T_7$** | **Control $T_7$** |
| **HL 1 µg/15 l tank** | 93.67 ± 2.29 | 93.12 ± 0.91 | 95.10 ± 1.44 | 96.42 ± 0.92 | 95.09 ± 1.73 | 96.14 ± 1.99 |
| **HL 2 µg/15 l tank** | 93.67 ± 2.29 | 92.18 ± 1.42 | 94.58 ± 1.79 | 97.22 ± 0.95 | 95.09 ± 1.73 | 96.14 ± 1.99 |
| **HL 40 µg/15 l tank** | 98.08 ±0.52 | 93.17 ± 1.02 | 95.18 ± 1.18 | 94.56 ± 1.11 | 93.10 ± 1.26 | 95.76 ± 1.15 |
| **HP 1 µg/15 l tank** | 86.77 ± 1.58 | 91.12 ± 1.41 | 93.04 ± 1.04 | 90.71 ± 1.46 | 87.76 ± 1.83 | 92.74 ± 1.94 |
| **HP 2 µg/15 l tank** | 86.77 ± 1.58 | 91.73 ± 1.45 | 91.94 ± 1.05 | 95.03 ± 1.09 | 87.76±1.83 | 92.74 ± 1.94 |
| **HP 40 µg/15 l tank** | 92.46 ± 1.33 | 90.61 ± 1.55 | 85.42 ± 2.37 | 86.13 ± 1.94 | 92.04 ± 2.25 | 90.58 ± 1.47 |

### Impact of Okadaic acid on %tDNA

### Negative and positive controls

Negative controls for mussels showed no significant difference in %tDNA between $T_0$ and $T_7$ sampling periods (ANOVA: $F_{5, 37} = 1.3$; $p > 0.05$). There was a significant difference between the negative and positive controls for each of the experiments; 2

µg/15 l tank and 1 µg/15 l tank (ANOVA: $F_{2, 15} = 4.1$ ; $p < 0.05$); 40 µg/15 l tank (ANOVA: $F_{2, 13} = 6.5$; $p < 0.05$);

For oysters, there were no significant differences between the negative controls at $T_0$ and $T_7$ (ANOVA: $F_{5, 37} = 3.9$; $p > 0.05$) (Figures 5.2 - 5.4). There was a significant difference between the negative and positive controls for the oyster samples; 2 µg/15 l tank and 1 µg/15 l tank (ANOVA: $F_{2, 12} = 5.02$; $p < 0.05$); 40 µg/15 l tank: (ANOVA: $F_{2, 18} = 4.2$; $p < 0.05$).

**Acute exposure: 2 µg/15 l tank**

Mussels and oysters subjected to an acute exposure of 2 µg/15 l tank of OA showed similar trends in the occurrence of DNA fragmentation detected using the comet assay (Figure 5.2). After 1 days exposure both species showed significant elevation in %tDNA from the control levels in both HL and HP cells, with a gradual decrease over the remainder of the experimental period.

***Mussels***

After acute exposure of 2 µg/15 l tank OA, %tDNA in the HL increased by 80% from control levels of 11.4% to 20.5%; this decreased to 19.4% after 3 days exposure (ANOVA: $F_{4, 49} = 6.5$; $p < 0.001$), which was still significantly greater than negative control levels (Tukey: $p < 0.05$). After 7 days exposure, levels of %tDNA dropped to non-significant levels, (13.6%). There was a significant difference between the %tDNA after 1 days exposure and after 7 days exposure (Tukey: $p < 0.05$) showing a noteworthy decrease in %tDNA occurred over the experimental period (Figure 5.2a). This pattern of an initial increase in DNA fragmentation after 1 day, followed by a reduction was repeated in the HP cells. Significant increases in %tDNA occurred in these cells (ANOVA: $F_{4, 51} = 7.3$; $p < 0.01$), after 1 days exposure; %tDNA increased significantly by 58% from control levels of 21.1% to 33.4%. %tDNA levels decreased at 3-days and 7-days to non-significant levels. As was found in the HL cells there was a significant difference between the levels of %tDNA after 1 day and 7 days exposure (Figure 6.2b). There was no significant difference in the DNA fragmentation measured between the HL and HP cells from exposed shellfish (T-test).

***Oysters***

Oyster HL cells showed a similar trend to that of mussels (Figure 5.2c). An initial significant elevation in %tDNA to 24.4% from $T_0$ control levels of 9.6% after 1 day is observed, a 155% increase in fragmentation. This decreased to non-significant levels after 3-days exposure. In the HP cells this pattern was repeated, with acute OA-exposure causing a significant increase in %tDNA (ANOVA: $F_{4, 48} = 8.7$; $p < 0.001$). %tDNA increased from control levels of 10.1% to 24.4% after 1 day (Figure 5.2d), a 142% increase in fragmentation. The increase was significantly different from the controls at 1 day, 3 days and 7 days exposure to the toxin, the overall trend showed a decrease in %tDNA over time, after the initial increase after 1 days exposure (Tukey: $p < 0.05$). Comparison of the increase in %tDNA for exposed HL and HP cells showed no significant difference in DNA fragmentation between the two cell types (T-test).

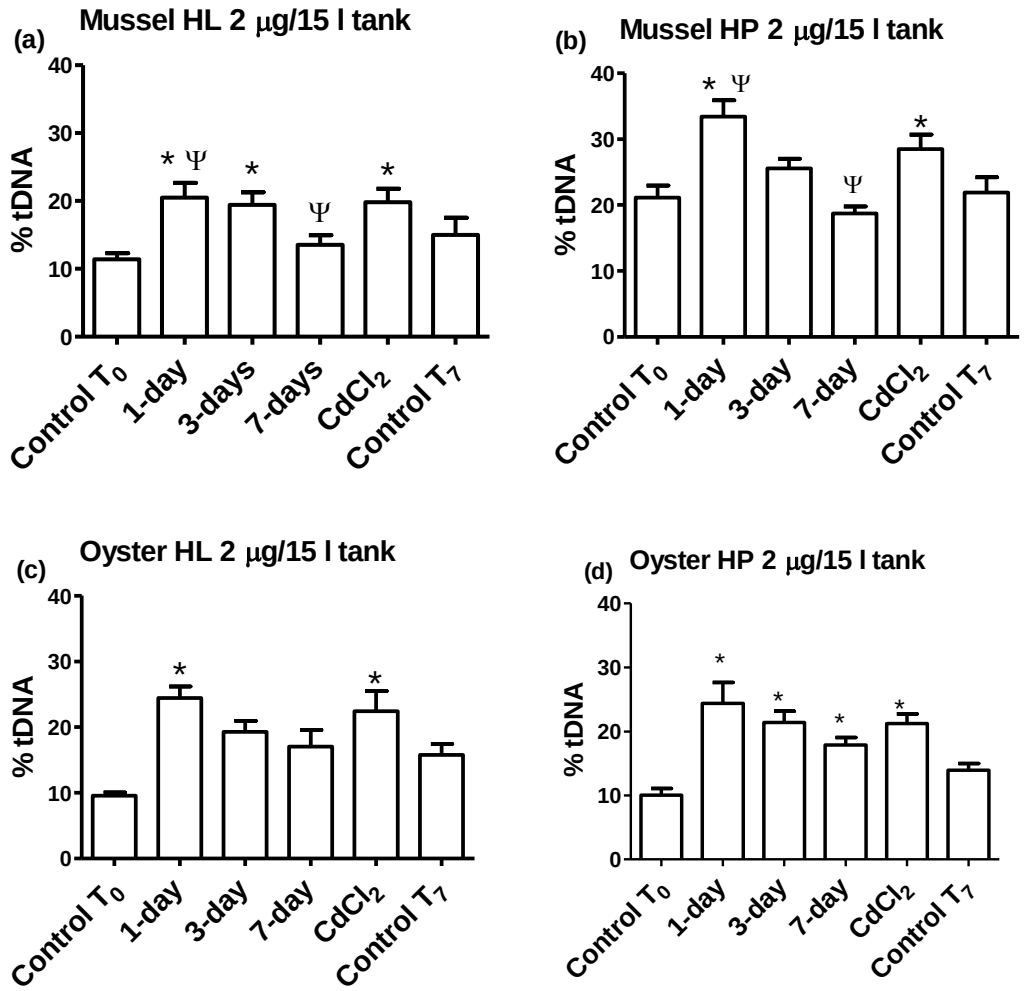


Figure 5.2. Changes in DNA fragmentation occurring after acute exposure to 2 µg/ 15 l tank OA (0.1µg/shellfish) at $T_0$; '*' denotes significant difference from the negative controls; 'ψ' represents when two sampling days are significantly different from each other, ($p < 0.05$). (a) Mussel haemolymph (HL); (b) Mussel hepatopancreas (HP); (c) Oyster haemolymph (HL); (d) Oyster hepatopancreas (HP).

**Sub-acute exposure: 1 µg/15 l tank**

A significant increase in %tDNA was evident in both cell types of mussels and oysters after sub-acute exposure to OA at 1 µg/15 l tank (Figure 5.3).

***Mussels***

In mussel HL there was a significant elevation from the $T_0$ controls after 1 days exposure from 11.4% to 20.9%, an 83% increase in %tDNA. This increase remained significantly different from controls for the duration of exposure, with %tDNA at 22.5% after 3-days exposure and 19.5% after 1 week (ANOVA: $F_{4, 49} = 7.4$; $p < 0.001$) (Figure 5.3a). Mussel HP cells showed a non-significant increase from control levels of 21.1% after 1 day to 25.6%, this further increased to significant level of 33.9% after 3 days, a 60% increase in fragmentation from control levels. Fragmentation plateaued at this level and did not increase further remaining at 33.9% after 7 days exposure to OA (ANOVA: $F_{4, 47} = 9.9$; $p < 0.01$) (Figure 5.3b). No significant difference in DNA fragmentation was observed between the HP cells and the HL cells from toxin-exposed shellfish (T-test).

***Oysters***

Oyster HL displayed a pattern similar to that observed in mussels, with a significant increase from control levels at $T_0$ of 9.5% to 19.3% after 1 day's exposure, which was a 100% elevation from the controls (Figure 5.3c). %tDNA continued to increase over the exposure period, and was at 25% after 3 days and reached 26.3% after 7 days exposure to OA an increase in fragmentation of 174% (ANOVA: $F_{4, 43} = 6.49$; $p < 0.01$). There was a significant difference between the damage observed after 1 day and 7 days exposure (Tukey: $p < 0.05$). As can been seen in Figure 5.3d, this trend was repeated in oyster HP cells, with a significant elevation in %tDNA from control levels of 10.1% to 16.9% after 1 day, this is an increase of 68% from the negative control. %tDNA increased further after 3 days and again after 7 days to 24.1%, an increase of 140% from the percentage fragmentation recorded in the controls (ANOVA: $F_{4, 49} = 8.12$; $p < 0.001$). As noted in the HL cells, there was a significant elevation in fragmentation from 1 day to 7 days exposure (Tukey: $p < 0.05$). There was no significant difference in the increase of %tDNA observed between the two cell types for exposed shellfish (T-test).

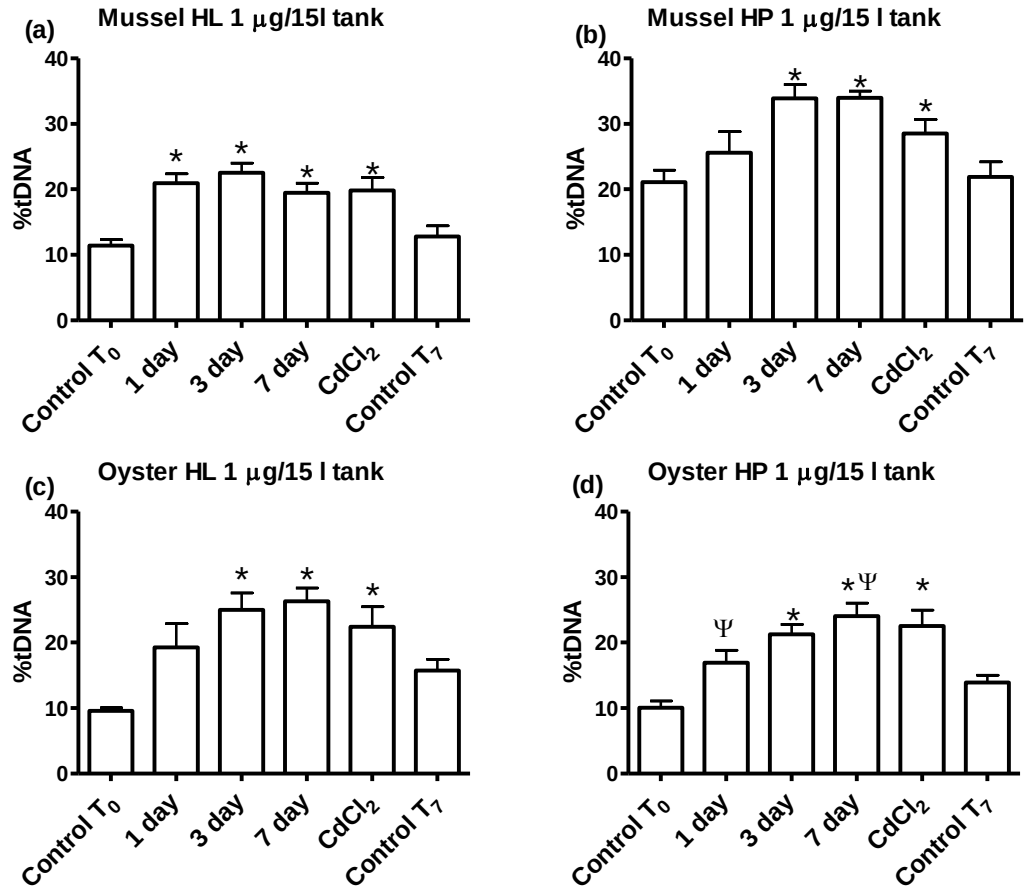


Figure 5.3. Changes in DNA fragmentation occurring after sub-acute exposure to 1 µg/15 l tank OA over a 7-day exposure period. '*' denotes significant difference from the negative controls; 'ψ' denotes when two sampling days are significantly different from each other, ($p < 0.05$). (a) Mussel haemolymph (HL); (b) Mussel hepatopancreas (HP); (c) Oyster haemolymph (HL); (d) Oyster hepatopancreas (HP).

**Sub-acute exposure: 40 µg/15 l tank**

The trend observed for sub-acute 1 µg/15 l tank exposure to OA was repeated in the sub-acute 40 µg/15 l tank exposure. A significant increase in DNA fragmentation was detected for both cell types in both species over the exposure period (Figure 5.4).

***Mussels***

In mussel HL there was a significant increase in %tDNA from $T_0$ control levels of 13.6% to 19.4% after 1 day's exposure. This trend continued and %tDNA increased to 22.1% after 7 days, a 62% increase from the control levels (ANOVA: $F_{4, 34} = 6.43$;

$p < 0.01$) (Figure 5.4a). In mussel HP cells, a significant increase of %tDNA from control levels of 19.7% to 25.6% occurred after 1 day, a 30% increase from controls. %tDNA plateaued for this treatment and remained at approximately this level after 3 and 7 days exposure (ANOVA: $F_{4, 35} = 7.49$; $p < 0.01$) (Figure 5.4b). There was no significant difference in the increase of %tDNA from control levels measured in the two cell types (T-test).

Comparison between sub-acute treatments of 1 µg/15 l tank and 40 µg/15 l tank showed no significant difference in the increase of %tDNA within HL cells for OA-exposed mussels (t-test). However, within the HP cells there was a greater increase in DNA fragmentation from control levels in mussels exposed to 1 µg/15 l tank than those exposed to 40µg/15 l tank (T-test: $t_{46} = -3.37$; $p < 0.01$). It should be noted however, that the 40µg/15 l tank experiment was conducted in June 2011, while the 1 and 2 µg exposure experiments were performed in November 2011.

***Oysters***

Oyster HL cell %tDNA increased significantly from control levels of 14.9% to 24.1% after 1 day (Figure 5.4c), a 62% elevation in fragmentation. This degree of fragmentation levelled off after 3 days and 7 days exposure with %tDNA at 26.1% and 24.8% (ANOVA: $F_{4, 47} = 6.89$; $p < 0.001$). In the HP cells there was a significant elevation in %tDNA from the controls of 11.4% to 18.6% after 1 day, a 64% increase. This level of fragmentation continued to increase and %tDNA reached 26.6%, 87% greater than control levels after 7 days (ANOVA: $F_{4, 51} = 22.87$; $p < 0.001$). There was a significant increase in fragmentation from 1 day to 7 days exposure to the toxin (Tukey: $p < 0.05$). No difference occurred in the increase of %tDNA from control levels between the HL and HP cells from exposed shellfish (T-test).

Comparison between sub-acute exposure experiments of 1 µg/15 l tank and 40 µg/15 l tank in oysters showed no significant difference in the increase of %tDNA from control levels in exposed oysters between concentrations for either cell type (T-test).

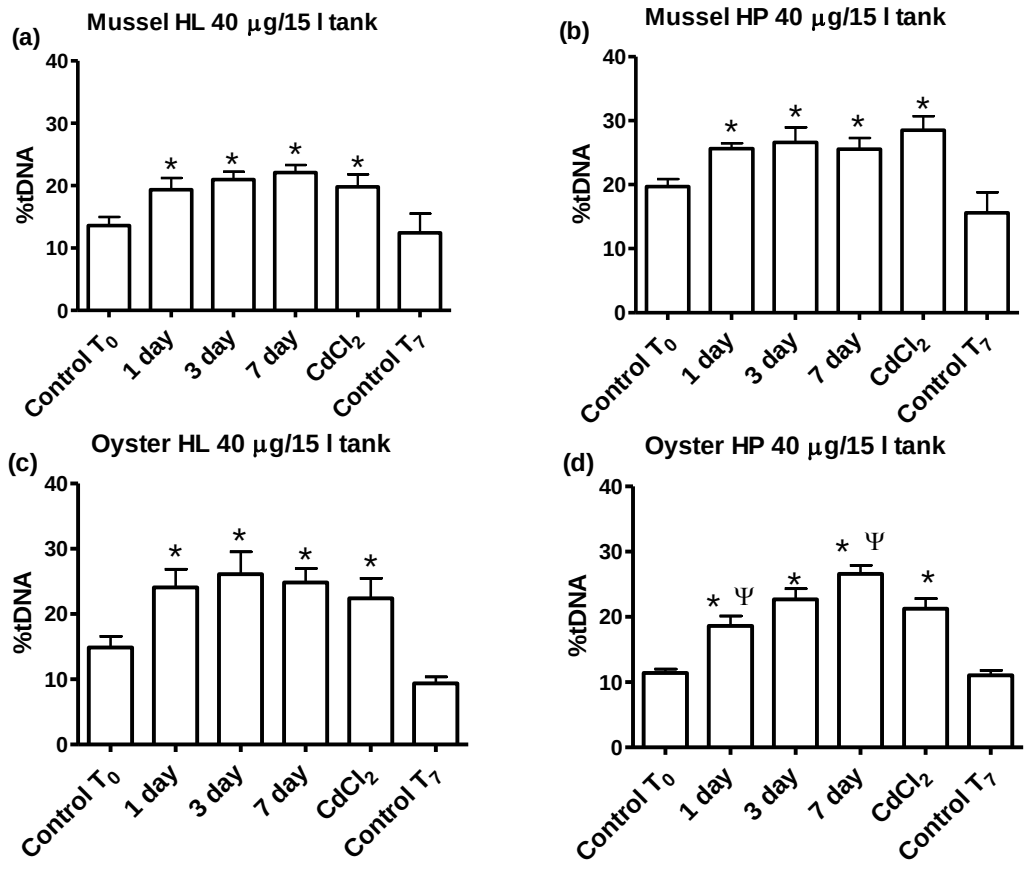


Figure 5.4. DNA fragmentation occurring after sub-acute exposure to OA at 40µg/15 l tank measured over 7-days. '*' denotes significant difference from the negative controls; 'ψ' denotes when two sampling days are significantly different from each other, ($p < 0.05$). (a) Mussel haemolymph (HL); (b) Mussel hepatopancreas (HP); (c) Oyster haemolymph (HL); (d) Oyster hepatopancreas (HP).

## DISCUSSION

This research presents new and original data for *Mytilus edulis* and *Crassostrea gigas*, which showed significant increases in DNA stand breakage after short-term exposure to differing concentrations of OA. There are a few studies connecting known genotoxic compounds and DNA fragmentation with negative impacts on aquatic species; Lee *et al.* (2000) demonstrated that *Palaemonetes pugio* embryos exposed to chromium(III) chloride, sodium chromate, mercuric chloride, and 2-methyl-1,2-naphthoquinone (MNQ) showed reduced hatching rate of embryos at early developmental stages and high DNA tail moments in the exposed embryos;

Steinert *et al.* (1998) linked DNA fragmentation, measured in mussels using the comet assay, at San Diego Bay with decreased growth rates.

Research is ongoing to determine whether OA is truly genotoxic to either mammalian or bivalve cells. However it should be noted that the biotoxin can induce apoptosis in a number of mammalian cell lines (Traoré *et al.* 2001, Huynh-Delerme *et al.* 2003, Le Hégarat *et al.* 2005, Ao *et al.* 2008, Xing *et al.* 2009). There are two possible reasons for the significant levels of DNA fragmentation observed in our data: the observed effect may be due to genotoxic effects of OA, or it may be due to the same apoptotic effects that have been described in human cell lines, and these were also observed in the bivalve molluscs. However, in this study OA did not cause substantial cytotoxic effects, with cell viabilities remaining at >85% throughout the experiment, these levels are comparable with previous studies examining cell viability in conjunction with DNA fragmentation as a biomarker of pollution in bivalves (Coughlan *et al.* 2002, Hartl *et al.* 2004); this implies that DNA fragmentation was not secondary to cytotoxicity. However, if OA exposure did cause apoptosis then the stage of apoptosis within the cells may have influenced the results of both the cytotoxicity assay and comet assay, as apoptosis has been found to interfere with comet assay results (Le Hégarat *et al.* 2004). A breach in cell membrane integrity occurs relatively late in apoptosis (Kaufmann 1997); thus, staining with the trypan blue exclusion method for viability would not detect the apoptotic cells if they were in the early stages apoptosis. If the cells had already undergone apoptosis prior to testing, then they would not be present during the viability or subsequent comet assay. Previous studies have found the cytotoxic effects of OA on bivalve cells to be low (Svensson *et al.* 2003, Talarmin *et al.* 2007), particularly *in vitro* (Flórez-Barrós *et al.* 2011). A few reasons have been suggested by these authors to explain this lack of cytotoxic effect, such as storage of toxin in the lysosomal compartments within cells and a capacity to quickly metabolise the OA into less active forms. Investigation of the apoptotic effects of OA and DSP-producing algae have found no significant impact in *C. gigas*, *Perna perna* and *Anomalocardia brasiliana* (Talarmin *et al.* 2007, Mello *et al.* 2010). A recent study on *Mytilus galloprovincialis* reported that at increasing concentrations of OA and DSP-producing algae the levels of apoptotic or membrane-damaged cells decreased (Prado-Alvarez *et al.* 2012). Further study is required to fully ascertain the

mechanisms behind this tolerance to the cytotoxic effects of OA and to precisely determine any apoptotic impacts of OA on bivalve cells.

Both mussels and oysters were exposed to the same three concentrations of OA. Significant DNA fragmentation was measured in both species after exposure to each of the OA concentrations (Figures 5.2-5.4). This is in spite of the oyster's larger mass, which reduced the toxin concentration per gram of tissue. Pearce *et al.* (2005) recognised a link between seasonal spat mortalities in *Crassostrea gigas* and an increase in *Prorocentrum rhathymum* density. Histopathological examination of experimentally exposed spat showed thin, dilated gut tubules and sloughing of gut cells (Pearce *et al.* 2005), these negative responses were also observed in our exposure experiments on pacific oysters (see Chapter 3). *Prorocentrum rhyathymum* has recently been discovered to produce OA (An *et al.* 2010), thus *Crassostrea gigas* may be quite sensitive to OA toxicity even in adult form. In the acute exposure of 2 µg/15 l tank of OA (0.1 µg/shellfish), a similar trend was noted between the two species for both cell types. An initial increase in %tDNA was recorded after only 1 day. The %tDNA gradually decreased near to control levels after 7 days. In mussels, this decrease was significant from 1 day to 7 days exposure for both cell types. A similar outcome was observed in the *Perna perna* mussel (Carvalho Pinto-Silva *et al.* 2003), when animals were exposed to a single dose of 0.3 µg/500 ml tank (1 shellfish/tank) and increased micronucleus formation was observed after 1 day, this decreased over the subsequent two days. OA has not been found to inhibit DNA repair, studies done on DNA repair activity have shown PP1 rather than PP2A participated in DNA-repair, and OA primarily inhibits PP2A activity (Herman *et al.* 2002, Le Hégarat *et al.* 2004). The detection of strand breaks in the comet assay is through interaction of two main processes, DNA damage and DNA repair (Juhel *et al.* 2007), so the observation of decreasing %tDNA over the 7 days is possibly due to this repair mechanism, or through tissue regeneration. Additionally, as was previously suggested, if cells that were in early apoptosis were detected using the comet assay, they may have disappeared by the end of the study, thus causing a decrease in the overall %tDNA recorded.

OA is known to accumulate almost exclusively in the HP of shellfish species (Blanco *et al.* 2007, McCarron *et al.* 2008, Mafra Jr *et al.* 2010) so it was

hypothesised that there would be more of an impact observed on these cells than in the circulating HL. Coughlan *et al.* (2002) observed higher %tDNA in HP cells versus HL cells when investigating sediment toxicity using the manila clam as an indicator species. However, in the current study there was no statistically significant increase between the %tDNA measured in the two cell types for either species relative to the controls. In both species there was an increase in %tDNA after the addition of OA in the two cell types, and in both this increase was significant at all concentrations, even the low toxin levels of 1 µg/15 l tank, with a significant increase in %tDNA observed over time. Due to the ubiquitous nature of OA in Irish waters, finding changes in %tDNA at the lower experimental toxin levels was unexpected. What was also unanticipated is that for higher concentrations of the biotoxin (40 µg/15 l tank) the %tDNA measured was not significantly greater than that observed at the lower levels of toxicity in oyster cells and in mussel HL. Conversely, within mussel HP cells, there was a significantly greater elevation in DNA fragmentation relative to the controls recorded in mussels exposed to 1 µg/15 l tank, versus those exposed to the higher concentration of 40 µg/15 l tank. These results correspond to an investigation of the apoptotic effects of OA on *Mytilus galloprovincialis* haemocytes, with increasing concentration of the phycotoxin, there were decreasing effects (Prado-Alvarez *et al.* 2012).

This lack of a classic dose response in DNA fragmentation post *in vivo*-exposure to OA-producing algae has been previously observed in bivalves (Carvalho Pinto-Silva *et al.* 2005, Flórez-Barrós *et al.* 2011). In those studies, sub-acute, daily exposure to DSP-producing algae was performed *in vivo* in *Perna perna* and *Ruditapes decussatus* respectively using the micronucleus assay and the comet assay. The higher concentrations of phytoplankton cells did not elicit a response in these studies, while lower concentrations, 0.005 µg OA/g and 0.01 µg OA/g of tissue (Carvalho Pinto-Silva *et al.* 2005), and 0.0018 µg OA/g of tissue (Flórez-Barrós *et al.* 2011) caused an initial increase in DNA fragmentation post-exposure. This was followed by a reduction in %tDNA over the exposure period. In the current study concentrations utilised were higher in mussels for the sub-acute exposure experiments (Chapter 1, Table 1.3: **1 µg/15 l tank**: 0.023 µg/g (day 1) - 0.13 µg/g (day 7); **40 µg/15 l tank**: 0.94 µg/g (day 1) - 5.14 µg/g (day 7)) and in oysters, the lower concentration was comparable to the previous investigations (Carvalho Pinto-

Silva *et al.* 2005, Flórez-Barrós *et al.* 2011) (Chapter 1, Table 1.2: **1 µg/15 l tank**: 0.004 µg/g (day 1) - 0.023 µg/g (day 7); **40 µg/15 l tank**: 0.17 µg/g (day 1) - 0.93 µg/g (day 7)). Contrary to previous studies, in our work sub-acute exposure to OA elicited a significant increase in fragmentation at all concentrations and the %tDNA measured increased over time, or plateaued and remained at the same level, no decrease in fragmentation was observed. Interspecies and intracellular variation could account for the different effects recorded between these studies. In mammalian cells varying responses have been measured in different cells exposed to OA (Rogers *et al.* 1994, Fessard *et al.* 1996, Souid-Mensi *et al.* 2008, Valdiglesias *et al.* 2010), and previous investigation on shellfish exposed to marine phycotoxins have found different interspecies effects (Galimany *et al.* 2008b, Mello *et al.* 2010) as well as fluctuating ability to metabolise and depurate biotoxins between species (Lindegarth *et al.* 2009, Mafra Jr *et al.* 2010).

This study highlights the impact that OA, a commonly occurring biotoxin, has on DNA fragmentation in two economically important aquaculture species. After only one day of toxin exposure, an increase in levels of tail DNA were measured in HL and HP cells of mussels and oysters, even at low concentrations of OA. Additional exposure experiments are required to determine whether the observed effects on DNA in these species is due to a genotoxic, or an apoptotic effect of the biotoxin in the haemolymph and hepatopancreas cells. It is important to promote awareness of the consequences these widespread marine biotoxins can have on aquaculture farms, both from a human-health perspective and on long-term fecundity and health of the shellfish species themselves.

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# CHAPTER 6

# General Discussion

A small proportion of Harmful algal blooms (HABs) produce phycotoxins which can accumulate in the tissues of filter-feeding bivalves, causing illness and occasionally, death in the humans that consume contaminated shellfish (James *et al.* 2010). Regulation EC No 853/2004 governs the total amount of marine biotoxins that may be present in shellfish for the protection of consumers within the E.U (European Commission 2004). Accordingly, rigorous monitoring programmes to determine both quantities of toxin in shellfish tissues and also abundance of key causative phytoplankton species are present in many countries (Alexander *et al.* 2008). In 2012 Liquid Chromatography-Mass Spectroscopy (LC-MS) replaced the mouse and rat bioassay as the primary technique for detection of the majority of marine biotoxins in shellfish tissues in the E.U., excluding Paralytic Shellfish Poisoning (PSP) toxins and Domoic acid (DA) for which alternative detection methods exist (Annex III to Regulation (EC) No 2074/2005, Chapter III). For PSP toxins there is a Prechromatographic Oxidation and Liquid Chromatography method (AOAC 2005) and for DA there is an enzyme-linked immunosorbent assay for detection (Kleivdal *et al.* 2007). LC-MS analysis is a precise tool for the direct monitoring of biotoxins accumulated in shellfish tissues, however analytical interference can occur through biological matrix effects, and the biotransformation of the biotoxin within the shellfish can create analogues and fatty acid esters of the toxin (Vale *et al.* 1999, Suzuki *et al.* 2004, Fast *et al.* 2006), thus increasing the difficulty of detection.

Solid phase adsorption and toxin tracking was a technology originally devised by MacKenzie *et al.* (2004) as an additional tool for monitoring the presence of algal toxins, based on the principle that these algae 'leak' toxin from their cells into the surrounding water. The method was suggested as a possible early warning system for HABs as the resins in the SPATT bags are highly efficient at adsorbing toxins present at extremely low levels in the water column. The phycotoxins are then extracted and analysed using LC-MS. Additional optimisation of the method has lead to some authors questioning its efficacy as an early warning system, but the technique may prove a supplementary procedure for acquiring information on the presence of toxins, and thus toxin-producing algae, missed through phytoplankton sampling. Furthermore, the extraction method for removing toxins from the resins is

faster, easier and cheaper than the isolation of toxin from shellfish tissues (Fux *et al.* 2009, Rundberget *et al.* 2009, Lane *et al.* 2010).

In the first section of this study, SPATT technology was applied as a monitoring tool to track the distribution of lipophilic marine biotoxins on a spatial and temporal scale in an enclosed marine lake (Chapter 2). A 4-month study was performed over the summer of 2010 at three sites and seven depths at Lough Hyne marine reserve in West Cork. This is the first study examining the presence of algal toxins at depths of 20-50 m over a 4-month period. Two resins were tested for their efficiency in accumulating toxins, Diaion HP-20 and Amberlite ® XAD761. Both resins accumulated detectable levels of Diarrhetic Shellfish Poisoning (DSP) toxins (Okadaic acid (OA), dinophysis toxin-1, -2 (DTX1, DTX2), pectenotoxin-2, -2 seco acid (PTX2, PTX2-SA)) and the spirolide 13-desmethyl-SPX-C, over the exposure period, with no difference in the numbers of samples testing positive when resins were compared. HP-20 was found to accumulate a higher quantity of toxin per gram than XAD761. DTX1 has not been previously isolated in Ireland using LC-MS techniques, however due to overlapping retention times in the column for DTX1 and PTX2, a full-scan of the extracts is necessary to isolate and confirm the presence of this toxin.

Application of SPATT as a monitoring tool for tracking toxin distribution over time and within discrete depths showed that the majority of toxin was detected in the top 20-30 m of the water column. This was attributed to an oxygen-thermocline that forms each year at Lough Hyne during the summer months between 20 and 30 m to the bottom (McAllen *et al.* 2009), and it is hypothesised that in the absence of an oxygen-thermocline, the algae may migrate to greater depths in the water column (Pizarro *et al.* 2008). A previous study by Fux *et al.* (2010) detected toxin-producing *Dinophysis acuta* at a depth of 81 m, in the euphotic zone. The frequent detection of toxins from 10 to 30 m depth over the study period coupled with their migratory capacity, highlights the necessity of evaluating standard sampling protocols for phytoplankton, current monitoring practices recommend sampling the surface or the top 5 m of the water column in Ireland and the top 6 m in Australia (Victoria 2009, Food Safety Authority Ireland 2012).

Lack of phycotoxins in sufficient quantities is a principal limitation in conducting comprehensive toxicology experiments, to examine their impacts on mammalian and invertebrate systems. It is only recently that reference standards for many of the commonly occurring marine biotoxins such as Azaspiracids, DTX1 and DTX2 have been made available through the Certified Reference Materials Programme, Halifax, Canada. This enables quantification of toxins from environmental samples using analytical methods. However, for toxins in the quantities required for large-scale *in vivo* toxicological testing, costs are still prohibitive. To address this issue, an active sampler modelled on the device tested by Rundberget *et al.* (2007) was constructed. Application of this sampling device involved large scale pumping of seawater through a series of filtration devices, to remove debris and particulates that would cause clogging. The water was then pumped through two cartridges containing 500 g each of HP-20 resin. The sampler was deployed at Lough Hyne in August 2010 and was run continuously for 7 days. The fine filter (50 µm) was changed half-way through sampling to reduce accumulation of debris and ensure rapid water-flow. This was performed to accumulate large quantities of phycotoxin for utilisation in subsequent experimentation. High quantities of DSP toxins were successfully harvested on the HP-20 resin during the experiment: OA (13 mg), DTX1 (3.8 mg) DTX2 (29 mg), PTX2 (20 mg) and PTX2-SA (6 mg). This is the first report of DTX1 and Pinnatoxin G detected in Irish waters. DTX1 is a member of the DSP toxins that cause symptoms of food poisoning on exposed humans. Pinnatoxins E, F and G have not been found to cause harmful effects in humans that consume contaminated shellfish (Munday *et al.* 2012), thus the detection of Pinnatoxin G does not impact on food safety. However, these results do highlight the ongoing global expansion of HABs that has previously been reported in the literature (Anderson *et al.* 2002, Smayda 2007, James *et al.* 2010).

In addition to the sampler's role as a toxin-accumulator, the toxin quantity per litre of water was estimated based on the quantity of toxin in the resin and the approximate volume of water pumped through the system over the sampling period (Table 6.2). Comparison of the quantity of OA/litre (0.2 µg/l) with our *in vivo* exposure studies on bivalve shellfish (2.6, 0.13 and 0.07 µg/l) suggest that at the quantities present, negative impacts on exposed shellfish may be observed (see

Chapters 3, 4 and 5). The effects of the other DSP toxins and SPX-C on bivalve shellfish are yet to be determined.

As previously mentioned, there are comprehensive monitoring programmes in place within the E.U. to quantify marine biotoxins in the tissues of filter-feeding bivalves (Alexander *et al.* 2008). Monitoring programmes provide important protection for human consumers against the wide range of toxic syndromes caused by phycotoxin-contaminated shellfish, the effects of which can be harmful, and in the case of some biotoxins, occasionally fatal (Isbister and Kiernan 2005). At toxin levels above recommended E.U. limits, shellfish farms are closed, sometimes for long periods, resulting in heavy economic losses for shellfish farmers (Anderson *et al.* 2000). To date, the effects of many toxins on shellfish accumulators are yet to be determined. It is important to ascertain whether these biotoxins are a source of physiological stress for bivalves, as this may further increase the losses incurred on farmers by the occurrence of HABs and also may impact on wild populations of shellfish species. Exposure experiments have measured a multitude of harmful impacts of many different marine biotoxins on a number of shellfish species; measured responses included alteration in behaviour, such as reduction in feeding (Mafra Jr *et al.* 2010b); pathology within the gill, mantle and hepatopancreas tissues (Bricelj *et al.* 1992, Wikfors and Smolowitz 1995, Pearce *et al.* 2005, Galimany *et al.* 2008a, Galimany *et al.* 2008b); alteration in the proportions of circulating haemocytes and immune status (Hégaret and Wikfors 2005a, b, Hégaret *et al.* 2007, Mello *et al.* 2010) and induction of micronuclei and DNA fragmentation (Carvalho Pinto-Silva *et al.* 2003, Carvalho Pinto-Silva *et al.* 2005, Flórez-Barrós *et al.* 2011). Within these studies a vast amount of inter-species variation was observed, highlighting the necessity of examining the effects of these toxins on multiple species.

In this study a comprehensive series of exposure experiments were performed on three bivalve shellfish species of economic importance in Ireland, *Mytilus edulis*, the blue mussel, *Ruditapes philippinarum*, the manila clam, and *Crassostrea gigas*, the pacific oyster (Chapters 3, 4 and 5). These species were chosen due to their worldwide significance in the aquaculture industry and the occurrence of wild populations in many countries. Thus, negative impacts post-exposure to HABs are of

both economical and ecological significance. The DSP toxin Okadaic Acid (OA) was chosen for these investigations. This phycotoxin occurs frequently in European waters and worldwide (Tachibana *et al.* 1981, Kumagai *et al.* 1986, MacKenzie *et al.* 2005). *In vitro* and *in vivo* examination of the toxins impacts on mammalian and bivalve species have measured negative effects (Suganuma *et al.* 1988, Rogers *et al.* 1994, Fessard *et al.* 1996, Fujiki and Suganuma 1999, Traoré *et al.* 2001, Carvalho Pinto-Silva *et al.* 2003, Huynh-Delerme *et al.* 2003, Svensson *et al.* 2003, Le Hégarat *et al.* 2004, Carvalho Pinto-Silva *et al.* 2005, Le Hégarat *et al.* 2005, Talarmin *et al.* 2007, Ao *et al.* 2008, Xing *et al.* 2009, Flórez-Barrós *et al.* 2011). The effects of three OA concentrations were measured at different levels of organisation, in a sequence of acute and sub-acute exposure experiments. Okadaic acid has been found to be ubiquitous at low levels in Irish mussels year round (Marine Institute 2004, 2007) thus, it is important to determine if low levels of this toxin have harmful impacts on shellfish accumulators. The toxin concentrations for mussels in this series of experiments of acute exposure to 2 µg/15 l tank and 1 µg/15 l tank sub-acute exposure, were chosen to reflect these low levels of environmental OA (Marine Institute 2004, 2007). 40 µg/15 l tank sub-acute exposure was chosen to provide 'bloom' levels of toxin within the tissues in this study. Shellfish were sampled for analysis after 1 day, 3 days and 7 days exposure.

At the tissue level, pathology of the gill, mantle and hepatopancreas was measured post OA-exposure using histology (Chapter 3). No changes were observed in the gill tissues in any of the three species over the study period, which were not also seen to a similar degree in the controls. A response was observed in the mantle and hepatopancreas (HP) tissues, the manifestation and severity of these effects varied between species. In the mussel and clam populations susceptibility in mantle tissues to the effects of the toxins was observed in both the acute and sub-acute exposure studies. Little or no impact was observed in the oyster mantle tissues. The sub-acute daily exposure of mussels and clams to OA showed a gradual increase in the percentage of 'damaged' individuals when compared with controls. The acute exposure to 2 µg/15 l tank showed a significant increase in the response indicators chosen; followed by rapid recovery, particularly in the mussels. Sloughing of necrotic cells (Figure 3.1 (c)) and haemocyte infiltration were used as indicators of toxic-effect on the mantle tissues (Pearce *et al.* 2005). Haemocyte infiltration

occurred with greater frequency in the manila clam mantle tissues than blue mussel (Chapter 3: Table 3.1). The primary biomarker for determining a response in the mantle was lipofuscin granule formation, these granules are a byproduct in oxidation of unsaturated fatty acids and may be symptomatic of membrane or lysosomal damage (Figure 3.1 (b)). Both mussels and clams showed high incidence of lipofuscin granule formation in the mantle tissues, with necrosis of the mantle edge often observed in conjunction with granule incidence. In bivalve molluscs increased in lipofuscin formation has been indicated as a response to contaminant exposure and a general response to pollution (Kagley *et al.* 2003) and has been found in shellfish exposed to harmful algae (Galimany *et al.* 2008b). Seasonal variation of lipofuscin formation has been observed in clam and mussel species (Walsh and O'Halloran 1997, Kagley *et al.* 2003, Banni *et al.* 2009), with higher levels of lipofuscin present in the summer and autumn. In the current study significant seasonal variation of lipofuscin granule formation between control groups in the summer and winter mussels was observed, with significantly more damage measured in the mantle tissues of the summer control mussels. A comparison between summer and winter exposed mussels showed no difference in the levels of damage recorded, however the increased damage in the controls of the summer mussels meant that statistical analysis showed no increase of damage occurring over the exposure period. This suggests that lipofuscin granule formation is not a suitable biomarker of exposure for mussels exposed to OA, particularly in the summer months.

Examination of the HP tissues revealed an observable, noteworthy impact in all three bivalve species over the study period, particularly at the highest exposure concentration of 40 µg/15 l tank sub-acute exposure (Chapter 3). When examining the markers used to determine a negative OA-response, differences were observed between the species (Chapter 3, Table 3.1), particularly between oysters and clams. In the pacific oyster a high incidence of tubule thinning coupled with odema was observed over the exposure period, with some evidence of haemocyte infiltration. In the manila clam HP tissues, little or no tubule thinning and atrophy was observed, however a high incidence of haemocyte infiltration within the digestive tissues was noted. In the manila clam, tubule atrophy was only recorded in individuals exposed to cadmium chloride. This shows the impact of OA differs significantly within the HP tissues of these two species. Evidence of all markers were recorded to some

degree in the blue mussel, however despite some tubule thinning, this was present at a much lower frequency than that observed in the pacific oyster. So while the blue mussel and the manila clam showed a significant increase in haemocyte infiltration and some evidence of necrosis and odema it was only in the pacific oyster that thinning of the tubule walls is so highly prevelant.

In all species, the effects of OA increased over time for the daily, sub-acute exposure and after 7 days, high levels of damage were observed in the digestive gland (Figure 3.6). As was observed in the mantle tissues, recovery was seen in the mussel HP tissues after the acute OA exposure at 7 days. However this recovery was not observed in the clam and a slower response to the biotoxins exposure was noted in the oyster HP with damage observed only on day 7. The indicators of 'damage' in the hepatopancreas were: thinning of the gut wall tubule, sloughing of cells and haemocyte infiltration (Parry *et al.* 1989, Wikfors and Smolowitz 1995, Pearce *et al.* 2005, Galimany *et al.* 2008a, Galimany *et al.* 2008b). OA is known to accumulate primarily in the digestive gland of shellfish, thus this significant observable response is not surprising (Yasumoto *et al.* 1978, Blanco *et al.* 2007). Tissue damage has been found to amplify susceptibility to bacterial infiltration in the pacific oyster (Pearce *et al.* 2005), and increase mortality in mussels, scallops and flat oysters (Parry *et al.* 1989, 1997).

Due to the global expansion of HABs it is important to determine whether phycotoxins cause more of a measurable response or 'damage' in males or females, as it may influence the fecundity and population dynamics of the population as a whole. Using the histological data accumulated analysis was performed to determine whether the sex of the individual played a role in susceptibility to the effects of the toxin OA (Chapter 3). Some differences in disease manifestation between male and female shellfish species have previously been measured (Brosseau and Baglivo 1994, Livingstone *et al.* 1995, Barber 2004). In this study, no differences in susceptibility to the effects of OA were measured between males and females. It should be noted however, that this was a small-scale study for this type of analysis; a larger sample size is required to fully conclude whether gender does play a role in OA effects on bivalves.

Examination of the effects of OA at the cellular level was also performed in this series of experiments. Haemolymph was extracted from the posterior adductor sinus and 300 µl was preserved in 6% formalin: seawater (Chapter 4). Successful separation and characterization of haemocytes from the blue mussel, the manila clam and the pacific oyster was demonstrated using flow cytometry. Two distinct sub-populations were isolated in the blue mussel and the manila clam and three in the pacific oyster (Allam *et al.* 2002, Delaporte *et al.* 2003, Hégaret *et al.* 2003). A review of the literature to date suggests that this was the first investigation of differential cell populations in the blue mussel, *Mytilus edulis* using flow cytometry. A cell fixation method previously utilised on bivalve cells prior to flow cytometric analysis (Hégaret *et al.* 2003), was confirmed as suitable for long-term (up to 8 months) storage of bivalve haemolymph in 6% formalin: seawater at 4$^{o}$C. Microscopy was utilised to determine whether cells remained intact after storage period. This will enable flow cytometric analysis of differential cell populations in bivalves as a component of more complex experiments, by allowing analysis to be performed at a later stage.

A review of the literature for information on the expected cell population distribution of bivalves revealed that there are vast differences in the proportions of granular and agranular cells within the circulating haemolymph detected between species, which is not surprising, but also between studies of the same species. This suggests that standardisation between methods and a more defined classification system for the identification of the sub-populations within the circulating haemolymph of marine bivalves are needed to allow direct comparison between different studies (Sun *et al.* 2006). The cell proportions of the three species measured in the current study were comparable to some previously published in the literature (Oubella *et al.* 1996, Barracco *et al.* 1999, Allam *et al.* 2002), but differed from some others (Carballal *et al.* 1997, Cima *et al.* 2000). However, the primary purpose of the study was to measure *changes* that occurred in within these sub-populations over the exposure period when compared to the controls. A number of criteria within the circulating haemolymph have been measured in shellfish exposed to algal toxins; differential cell population changes, cell number, phagocytosis, aggregation, and increased reactive oxygen species, with differing degrees of impact depending on the species studied (Hégaret and Wikfors 2005a, b, Hégaret *et al.* 2007, Mello *et al.*

2010). It is important to establish whether these algal blooms elicit a change in the normal haemolymph cell profile in these species and thus stress the organism, leading to increased susceptibility to pathogens and parasites (Pearce *et al.* 2005, Hégaret *et al.* 2009, Mello *et al.* 2010).

No significant changes in the proportions of granulocytes or hyalinocytes were detected in the blue mussel and manila clam over the exposure period. The pacific oyster was the most susceptible to the effects of OA during the experiments. Significant changes in the proportions of small granulocytes and large granulocytes were detected at the lower levels of toxin exposure, indicating a possible chemical-induced response to OA. When granulocyte populations were pooled, no effects were observed in pacific oyster hyaline or granular cell populations over the exposure period. Thus, the chemically-induced effects of OA on the differential populations in the pacific oyster occurred only within the small and large granulocyte populations. These changes in cell populations may occur through alteration in the production of granular haemocytes in response to environmental stress, with a higher proportion of one cell type being produced (Delaporte *et al.* 2003, Mello *et al.* 2010), or reduction of total haemocyte number through migration of cells from the circulating haemolymph to affected tissues within the shellfish (Chapter 3, Figure 3.2 (c)). At the higher level of toxin exposure (40 µg/15 l tank) there was no significant impact on the three species of exposed shellfish. This lack of a classic dose response to OA-exposure has previously been observed in shellfish species. A recent study on *Mytilus galloprovincialis* reported that at increasing concentrations of OA and DSP-producing algae the levels of apoptotic or membrane-damaged cells decreased (Prado-Alvarez *et al.* 2012). Investigations of OA-impact on DNA fragmentation found that at the higher concentrations the toxin did not elicit a response, while at lower levels a significant increase in fragmentation was observed (Carvalho Pinto-Silva *et al.* 2005, Flórez-Barrós *et al.* 2011). As yet, the reason behind this lack of a dose-response is yet to be discovered. This research demonstrated that OA has differing effects on the differential cell populations in these three bivalve species over a short-term exposure period. Effects of algal toxins have been previously found to differ between species. These differences have been attributed to varying rates of accumulation and depuration between species (Blanco *et al.* 1999, Svensson 2003, Mafra Jr *et al.* 2010a, Mafra Jr *et al.* 2010b) due to

environmental factors such as temperature and salinity (Blanco *et al.* 1999, Svensson and Förlin 2004) or greater selectivity regarding food intake, coupled with differing capabilities for biotransforming and excreting the phycotoxins (Torgersen *et al.* 2008).

A portion of the extracted haemolymph from the shellfish posterior adductor sinus and HP cells were utilised to determine cell viability and measure effects on DNA fragmentation over the exposure period (Chapter 5). Previous studies on bivalves indicate that the gill, haemolymph and heart cells are relatively resilient to the cytotoxic effects of OA (Svensson *et al.* 2003, Talarmin *et al.* 2007, Flórez-Barrós *et al.* 2011). Cell viabilities remained above 85% for mussels and oysters and above 80% for clams. It is commonly accepted that cell viability should be greater than 85% to ensure that the observed effect is due to the genotoxicity of the test compound and not a secondary effect of cytotoxicity (Coughlan *et al.* 2002, Hartl *et al.* 2004). Thus for the purposes of ensuring a valid measurement of DNA fragmentation for this study, the manila clam was excluded from analysis.

DNA fragmentation has been measured in different cell types exposed to OA (Carvalho Pinto-Silva *et al.* 2003, Carvalho Pinto-Silva *et al.* 2005, Flórez-Barrós *et al.* 2011). In this study a significant increase in the percentage tail DNA (%tDNA) was measured in the blue mussel and the pacific oyster over time, even at the lowest OA concentrations, a noteworthy result for these important aquaculture species. OA has been found to induce apoptosis in a number of mammalian cell lines (Traoré *et al.* 2001, Huynh-Delerme *et al.* 2003, Le Hégarat *et al.* 2005, Ao *et al.* 2008, Xing *et al.* 2009), thus in the current study the observed effect may be due to genotoxic effects of OA, or it may be due to the same apoptotic effects that have been described in human cell lines, which were also observed in the bivalve molluscs. Apoptosis has been found to interfere with comet assay results (Le Hégarat *et al.* 2004), furthermore a breach in cell membrane integrity occurs relatively late in apoptosis (Kaufmann 1997); thus, the trypan blue exclusion method for viability would not detect early apoptotic cells. If the cells had already undergone apoptosis prior to testing, then they would not be present during the viability or subsequent comet assay.

Direct investigation of the apoptotic effects of OA and DSP producing algae found no significant impact on *C. gigas*, *Perna perna* and *Anomalocardia brasiliana* (Talarmin *et al.* 2007, Mello *et al.* 2010). A recent study on *Mytilus galloprovincialis* reported that at increasing concentrations of OA and DSP-producing algae the levels of apoptotic or membrane-damaged cells decreased (Prado-Alvarez *et al.* 2012). This decreasing sub-cellular impact with increasing OA concentration was also measured in two sub-acute exposure studies on *Perna perna* and *Ruditapes decussatus* using the micronucleus assay and the comet assay respectively (Carvalho Pinto-Silva *et al.* 2005, Flórez-Barrós *et al.* 2011). In our investigation, a decrease in DNA fragmentation was not observed at either OA concentration utilised in the sub-acute exposure studies, however %tDNA measured for both exposure-concentrations was not significantly different in oyster cells and in mussel HL. Interspecies and intracellular variation could account for the different effects recorded between these studies (Galimany *et al.* 2008b, Mello *et al.* 2010); fluctuating ability to metabolise and depurate biotoxins has been observed between bivalve species (Lindegarth *et al.* 2009, Mafra Jr *et al.* 2010a).

The tissue level responses observed in Chapter 3 are analogous to the DNA fragmentation trends for the two exposure regimes utilised in Chapter 5. For the acute exposure (2 µg/15 l tank) there is an initial increase in the measured DNA fragmentation and in histological changes followed by a rapid recovery. This indicates that despite the surprising and significant susceptibility of these bivalves to low concentrations of OA, they appear to be resilient to the after-effects of the toxin and can return to homeostasis once the toxin levels in the water decrease. However, during the period of time for which the shellfish are exposed, it is likely that their susceptibility to disease is increased (Pearce *et al.* 2005), immune-modulating effects of HAB exposure coupled with parasite load have been measured in a number of bivalve species (Hégaret *et al.* 2009, Hégaret *et al.* 2010). An investigation by Carvalho Pinto-Silva *et al.* (2003) looking at DNA micronucleus formation in haemolymph cells found rapid recovery in the *Perna perna* mussel post-exposure, with visibly reduced MN from 24h to 48h. High depuration rates in shellfish have been linked with the presence of larger quantities of non-toxic food (Blanco *et al.* 1999, Svensson 2003, Marcaillou *et al.* 2010), thus daily feeding with non-toxic algae post-exposure, may have assisted in rapid recovery.

For the daily sub-acute exposure regimes a significant increase in the markers chosen to indicate a negative response were observed in both Chapter 3 and Chapter 5 suggesting that these two methods utilised in parallel provide substantial evidence that the biotoxin, OA, can cause a measurable negative response at the tissue, cellular and sub-cellular levels in the blue mussel and the pacific oyster. Little work has been performed examining the implications of increased DNA fragmentation on the tissues and overall health of bivalve species. Some studies have linked increased DNA fragmentation with reduced growth in mussels (Steinert *et al.* 1998) and reduced hatching rate in *Palaemonetes pugio* embryos (Lee *et al.* 2000). The analogous results of increasing DNA fragmentation and coupled with increasing incidence of the markers chosen for histological analysis of the shellfish post-OA exposure indicate a connection between these two responses. However, histological analysis showed differing responses within the tissues occurring between mussel and oyster species. Oysters displayed increased thinning of HP tubules at all toxin concentrations and no impact on mantle tissues, while in the blue mussel significant impacts were observed in the mantle tissues and in the HP tissues high incidence of tubule thinning was only observed at the highest toxin exposure (40 µg/ 15 l tank). Cadmium chloride, the positive control chemical chosen for these exposure experiments is a known genotoxic substance that has been found to induce tissue level responses in mussel species (Sheir and Handy 2010). In this study cadmium chloride had a similar impact on the blue mussel as OA at the highest toxin concentration, with increased HP tubule thinning and elevated lipofuscin granule formation and necrosis in the mantle tissues. This suggests that it would be unwise to connect increasing DNA fragmentation with only one physiological marker, as both OA and cadmium chloride caused increased occurrence of a number of biomarkers indicating a complex, and species-specific impact on the different tissues examined using histology.

The increase in both DNA fragmentation and histological damage occurred at high 'bloom' OA concentrations and at environmentally low levels of toxin (Marine Insitute 2004, 2007). This has important ecological significance for these shellfish species as it indicates that the low levels of toxin that have been found to occur almost ubiquitously in Irish mussels year round, may have significant long-term impacts on both these species and other filter-feeding bivalves. This increased

morbidity may have negative implications on the overall health, growth and fecundity of both farmed and wild shellfish populations in both Irish waters and worldwide.

**General conclusions**

- Successful application of the passive SPATT technology as a monitoring tool for toxin distribution of the water column was achieved. The results obtained can be utilised to inform policy-makers on the necessity of re-evaluating current phytoplankton monitoring practices within Ireland.
- A bio-harvesting methodology was effectively applied at Lough Hyne, and high quantities of lipophilic marine biotoxins were accumulated on adsorbent resin for use in subsequent experimentation.
- This was the first detection of DTX1 and Pinnatoxin G in Irish waters and the first isolation of 13-desmethyl-SPX-C using high mass accuracy (< 2 ppm).
- This was the first study to examine the effects of OA on the blue mussel, the manila clam and the pacific oyster at three different levels of organization, tissue, cellular and sub-cellular.
- Histopathology revealed a significant damage in the hepatopancreas tissues of all three species. In the mantle tissues, the blue mussel and manila clam showed a greater response to OA with little effect observed in the pacific oyster.
- Flow cytometric analysis of the differential cell populations in the circulating haemolymph of the three species showed no changes in the blue mussel and the manila clam. In the pacific oyster significant changes were observed in the small and large granulocyte populations.
- No significant increase in cytotoxicity was measured in the blue mussel or the pacific oyster with levels remaining above 85% for the duration of OA-exposure.
- Increased DNA fragmentation was observed in both the HL and HP cells in both the blue mussel and the pacific oyster. No dose response was evident,

higher concentrations of OA did not induce greater damage than low concentrations.

- The data accumulated in this thesis will add valuable information to the current knowledge on the effects of marine biotoxins in shellfish species and highlights the impacts of a commonly occurring marine biotoxin on important commercial aquaculture species.

**Future study**

There is large scope for examining the effects of HABs on multiple species of shellfish, as there are many shellfish-toxin combinations yet to be explored. From the outputs of this investigation, long-term examination of the effects of OA on the blue mussel, manila clam and pacific oyster is recommended to determine whether the responses measured in the current work will continue to be observed and the shellfish deteriorate further, or whether homeostasis is reached and the shellfish remain at the same level of morbidity measured in this investigation.

Additional examination of the genotoxic/apoptotic effects of OA on the circulating HL and HP cells of bivalve shellfish is recommended to fully ascertain the sub-cellular impacts of this toxin on bivalve shellfish species.

Large quantities of DTX1, DTX2, PTX2 and PTX2SA were harvested in this study and can be utilised individually and in combination to determine their effects on shellfish species. Exposures experiments looking at the effects of these toxins at environmentally relevant concentrations on important commercial shellfish species is recommended. Currently there is little or no information on the effects of these toxins currently available.

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