



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



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ABSTRACT

Two bivalve species of global economic importance: the blue mussel, *Mytilus edulis* and the pacific oyster, *Crassostrea gigas* were exposed *in vivo*, to the diarrhoeic shellfish toxin okadaic acid (OA), and impacts on DNA fragmentation were measured.

Shellfish were exposed using two different regimes, the first was a single (24 h) exposure of 2.5 nM OA (~0.1 µg/shellfish) and algal feed at the beginning of the trial (T_0), after which shellfish were only fed algae. The second was daily exposure of shellfish to two different concentrations of OA mixed with the algal feed over 7 days; 1.2 nM OA (~0.05 µg OA/shellfish/day) and 50 nM OA (~2 µg OA/shellfish/day). Haemolymph and hepatopancreas cells were extracted following 1, 3 and 7 days exposure. Cell viability was measured using the trypan blue exclusion assay and remained above 85% for both cell types. DNA fragmentation was examined using the single-cell gel electrophoresis (comet) assay.

A significant increase in DNA fragmentation was observed in the two cell types from both species relative to the controls. This increase was greater in the pacific oyster at the higher toxin concentration. However, there was no difference in the proportion of damage measured between the two cell types, and a classic dose response was not observed, increasing toxin concentration did not correspond to increased DNA fragmentation.

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1. Introduction

The marine biotoxin okadaic acid was first isolated from two sponge species, *Halichondria okadaei* Kadota, a black sponge found on the Pacific coast of Japan and *Halichondria melanodocia*, a Caribbean sponge found in the Florida Keys (Tachibana et al., 1981). Okadaic acid (OA) and its analogues, the dinophys 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 (Campas et al., 2007; Kumagai et al., 1986; Reizopoulou et al., 2008). Human consumption of these contaminated shellfish causes Diarrhoeic Shellfish Poisoning (DSP) (James et al., 2010). The symptoms

of DSP appear within 3 h 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* (Fux et al., 2009; James et al., 1999). Blooms of these toxin-producing algae regularly cause closure of aquaculture farms off the Irish coast during the summer months.

To date, there have been many toxicological investigations to determine the impact of OA on mammalian cells, both *in vivo* and *in vitro*. *In vivo* studies have found it to be a tumour-promoter in

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mouse skin and the mucosa of rat glandular stomach (Fujiki and Suganuma, 1999; Suganuma et al., 1988). Several *in vitro* studies have been performed to determine OAs genotoxic, mutagenic and cytotoxic effects on a number of mammalian cell lines (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. A number of *in vitro* studies have established that OA also causes apoptosis in some mammalian cell lines (Ao et al., 2008; Bøe et al., 1991; Huynh-Delorme et al., 2003; Le Hégarat et al., 2005; Traoré et al., 2001; Xing et al., 2009) and in salmon hepatocytes (Fladmark et al., 1998).

Thus, while the focus of much research has been on the impact of OA in humans, it is only relatively recently that the pathological effects of marine biotoxins on filter-feeding bivalve species have begun to be fully investigated. Cytotoxicity, DNA fragmentation, apoptosis, immunological effects, mRNA modulation in expression of genes involved in cell cycle regulation and the immune system, and gross physiological damage detected using histopathology have all been examined in a number of shellfish species (Carvalho Pinto-Silva et al., 2003; da Silva et al., 2008; de Jesús Romero-Geraldo et al., 2014; Galimany et al., 2008a; Hégaret and Wikfors, 2005; Prado-Alvarez et al., 2013, 2012; Svensson et al., 2003). Studies on bivalves indicate that the gill, haemolymph and heart cells are relatively resistant to cytotoxic effects (Flórez-Barrós et al., 2011; Svensson et al., 2003; Talarmin et al., 2007), but DNA fragmentation has been observed in different cell types exposed to OA (Carvalho Pinto-Silva et al., 2005; Carvalho Pinto-Silva et al., 2003; Flórez-Barrós et al., 2011).

The aim of this investigation was to determine whether exposure to the marine biotoxin OA caused reduction in cell viability through cytotoxicity and also to measure changes in DNA fragmentation in two bivalve species. The blue mussel, *Mytilus edulis*, and the pacific oyster, *Crassostrea gigas*, were the species chosen for this study. These shellfish are farmed off of the Irish coastline and are important aquaculture species in many countries. It is important to determine whether there are differences in the susceptibility to marine biotoxins between shellfish species, in order to perform adequate assessment of the risks of Harmful Algal Blooms.

Cytotoxicity was measured using the Trypan Blue Exclusion assay, this assay is based on the principle that viable (live) cells possess intact membranes which can exclude this dye while membrane damaged non-viable cells cannot. DNA fragmentation was measured using the single cell gel electrophoresis (comet) assay. This assay can measure single strand breakages in the DNA of eukaryote cells. It does this by uncoiling of DNA loops through cell lysis, followed by electrophoresis which separates damaged DNA from the nucleus forming a distinctive comets tail (Supplementary Figure). This has been successfully utilised on a number of bivalve species measuring the impact of a wide range of toxins, both anthropogenic and biological (Çavas and Könen, 2008; Coughlan et al., 2002; Hartl et al., 2006; Juhel et al., 2007; Rank et al., 2005).

2. Materials and methods

2.1. Toxins and reagents

The biotoxin okadaic acid (OA) sodium salt (L.C. Laboratories, 05857) dissolved in methanol (HPLC grade) (1 mg ml^{-1} OA: in methanol) was utilised for bivalve exposure. 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 quadrupole 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.

2.2. 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 °C. Shellfish were acclimated 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; 40% *Isochrysis* sp., 15% *Pavlova* sp., 25% *Tetraselmis* sp. and 20% *Thalassiosira weissglogii* (Grunow); 52% protein, 16.1% lipid, 22.0% carbohydrate and 9.9% ash). The bivalves were not fed for 24 h prior to exposure. Mussels of shell length 50–60 mm and oysters of shell length 60–80 mm were chosen for experimentation.

2.3. Experimental design

Experimental and control plastic aquaria (polypropylene) were set up, with 20 shellfish and 15 l of aerated seawater placed in each tank. Two replicate tanks for each OA exposure concentration were used. Seawater was changed daily in each of the tanks for all experiments and addition of test chemical and algal feed was performed post water-change at the same time on each day.

2.3.1. Two treatment regimes were used

The first was a single (acute) 24 h exposure to one concentration of OA. On Day 1 (T_0) of the experiment 2.5 nM OA (2 μl OA stock solution in 98 μl MeOH suspended in 100 μl algal feed) was administered to the test animals and for the remaining six days of the trial, exposure animals were provided only with algal feed (100 μl). This gave a maximum exposure of approximately 0.1 μg OA/shellfish.

The second treatment regime was daily (sub-acute) exposure of animals to two concentrations of OA. Concentration 1: 1.2 nM OA (1 μl OA stock solution in 99 μl MeOH suspended in 100 μl algal feed). This was administered daily over the 7 days, which gave a maximum exposure of 0.05 μg OA/shellfish/day. Concentration 2: 50 nM OA (40 μl OA in 60 μl methanol; 100 μl algal feed) was also administered daily to the shellfish. This gave a maximum exposure of approximately 2 μg OA/shellfish/day.

Five animals were removed from each replicate tank and sacrificed for experimentation after 1 day (24 h), 3 days (72 h) and 7 days (168 h) exposure, except for mussels exposed to the 40 μg concentration, when three animals were removed at each sampling time. 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, at 5 μM per day for mussels and at 10 μM per 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. These sample sizes are comparable to previous *in vivo* studies on bivalve shellfish

(Coughlan et al., 2002; Hartl et al., 2004; Galimany et al., 2008a; Prado-Alvarez et al., 2012).

2.4. Isolation of single cell suspensions

Approximately 300 μ l of haemolymph (HL) was removed from the posterior adductor sinus using a sterile 23-gauge 1½ inch needle and 2.5 ml syringe. Aliquots of 30 μ l 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 g l^{-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 °C (Hartl et al., 2010).

Hepatopancreas (HP) cells were isolated using a method adapted from Coughlan et al. (2002). 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 solution (conc: 0.05% w/v). The suspension was gently rocked and incubated for 10 min at room temperature. HBSS (10 ml) was added to the tube and the sample was filtered through a 40 μ m cell filter (BD Biosciences) to remove larger tissue and debris. After centrifuging at 800 \times g for 10 min the supernatant was discarded and the pellet resuspended in 0.5 ml of HBSS.

The trypan blue exclusion method was used to assess cell viability, as this method has previously been used in conjunction with the comet assay for bivalve *in vivo* exposure experiments (Coughlan et al., 2002; Flórez-Barrós et al., 2011; Hartl et al., 2004). Equal quantities of cell suspension and trypan blue (20 μ l: 20 μ l) were mixed, 20 μ l of this solution was pipetted onto a haemocytometer and examined under a microscope at 40 \times magnification. Fifty cells per slide were counted and percentage viability recorded.

2.5. Comet assay

The comet assay was performed using a protocol developed by Woods et al. (1999). Briefly, 30 μ l of haemolymph/hepatopancreas cell suspension was mixed with 70 μ l 1% Low Melting Point Agarose. The cell/agar mix (70 μ l) was gently pipetted onto the 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 °C for 2 h. The samples were submerged in an alkaline solution (0.3 M NaOH, 1 mM EDTA; pH > 12) in an electrophoresis tank for 30 min at 4 °C; this unwound the DNA. A current (25 V, 300 mA) was then applied for exactly 25 min. 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). A total of 50 randomly chosen nuclei per slide were assessed (Supplementary Figure).

2.6. 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 were arcsine transformed. *T*-tests were performed between replicate groups to establish whether there were any differences; none were found so 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 these data were used to compare the

amount of DNA fragmentation recorded in HL and HP cells and between species using independent *T*-tests. Data were analysed using Predictive Analytics SoftWare Statistics (PASW) 17 for Windows.

3. Results

3.1. Cell viability

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

3.2. Comet assay results expressed as %tDNA

3.2.1. Negative and positive controls

Negative control mussels showed no significant difference in %tDNA between T_0 and T_7 sampling periods. There was a significant difference between the negative and positive controls in each of the mussel exposure experiments (Table 1). For oysters, there were no significant differences between the negative controls at T_0 and T_7 (Figs. 1–3). There was a significant difference between the negative and positive controls in each of the experiments (Table 1).

3.2.2. Single exposure: 2.5 nM (~0.1 μ g/shellfish)

Mussels and oysters subjected to a single (24 h) exposure of 2.5 nM OA (~0.1 μ g/shellfish) showed similar trends in the occurrence of DNA fragmentation detected using the comet assay (Fig. 1). 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.

3.2.2.1. Mussels. After a single 24 h exposure of 2.5 nM OA, %tDNA in the HL showed a significant increase from negative control levels of 11.4%–20.5%; this decreased to 19.4% after 3 days exposure, which was still significantly greater than negative control levels. After 7 days, levels of %tDNA dropped to non-significant levels (Table 1). This pattern of an initial increase in DNA fragmentation after 1 day, followed by a reduction was also observed in the HP cells. After 1 day's exposure, %tDNA increased significantly by 58% from negative control levels of 21.1%–33.4%. %tDNA levels decreased after 3 days and 7 days to non-significant levels (Table 1). In both HL and HP cells there was a significant difference between the levels of %tDNA after 1 day and 7 days exposure, showing a

Table 1

Analysis results of Haemolymph (HL) and Hepatopancreas (HP) cells exposed to differing concentrations of OA: ¹24 h exposure; ² daily exposure for 7 days). Data were compared to the negative controls using 1-way ANOVA followed by Tukey's pairwise comparison on data. * = $p < 0.05$; ND = no difference.

	OA conc	Exposure time			
		1 day (24 h)	3 days (72 h)	7 days (168 h)	Positive control
Mussel HL	2.5 nM ¹	*	*	ND	*
	1.2 nM ²	*	*	*	*
	50 nM ²	ND	*	*	*
Mussel HP	2.5 nM ¹	*	ND	ND	*
	1.2 nM ²	ND	*	*	*
	50 nM ²	*	*	*	*
Oyster HL	2.5 nM ¹	*	ND	ND	*
	1.2 nM ²	ND	*	*	*
	50 nM ²	*	*	*	*
Oyster HP	2.5 nM ¹	*	*	*	*
	1.2 nM ²	ND	*	*	*
	50 nM ²	*	*	*	*

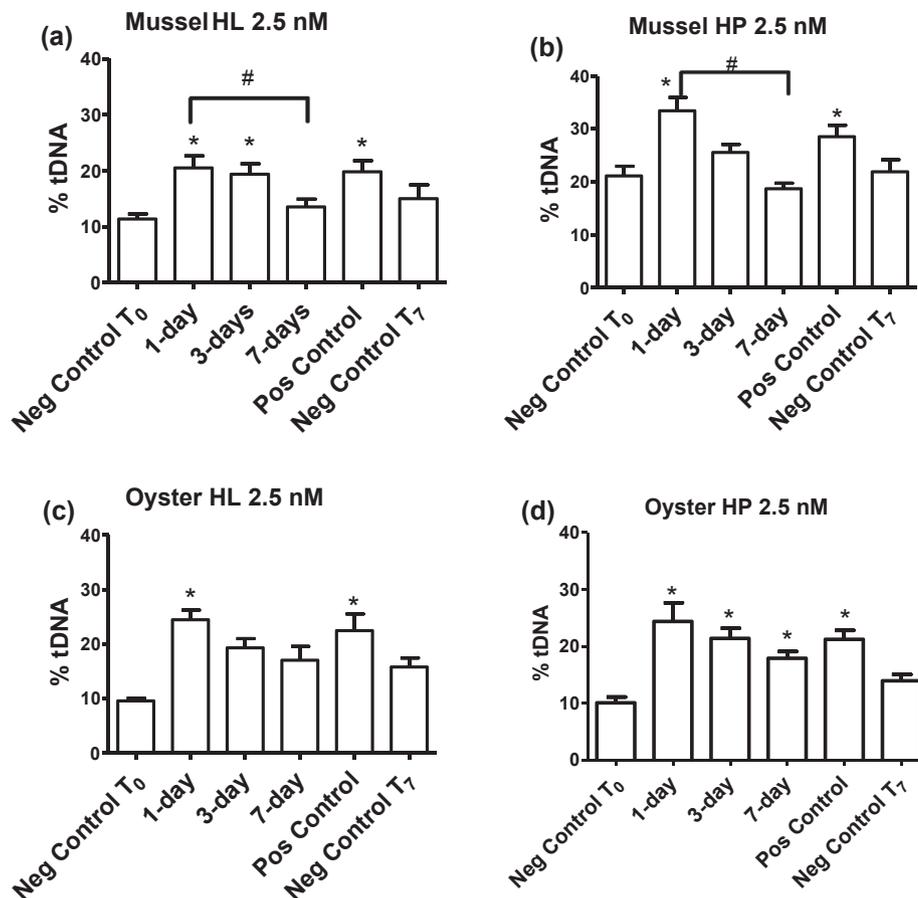


Fig. 1. Changes in DNA fragmentation that occurred after acute exposure to 2.5 nM OA ($\sim 0.1 \mu\text{g}/\text{shellfish}$) at T_0 ; "*" denotes significant difference from the negative controls; '#' denotes a significant difference between individual sampling days, ($p < 0.05$). (a) Mussel haemolymph (HL); (b) Mussel hepatopancreas (HP); (c) Oyster haemolymph (HL); (d) Oyster hepatopancreas (HP).

noteworthy decrease in %tDNA over the experimental period (Fig. 1b). There was no significant difference in DNA fragmentation measured between HL and HP cells from exposed shellfish.

3.2.2.2. Oysters. Oyster HL cells showed a similar trend to that of mussels (Fig. 1c). An initial significant elevation in %tDNA to 24.4% from negative control levels of 9.6% after 1 day was 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 a 142% increase in fragmentation from control levels after 1 day (Fig. 1d) followed by a decrease to non significant levels after 3 days exposure. 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).

3.2.3. Daily exposure 1.2 nM ($0.05 \mu\text{g OA}/\text{shellfish}/\text{day}$)

A significant increase in %tDNA was evident in both cell types of mussels and oysters after daily exposure to 1.2 nM OA (Fig. 2).

3.2.3.1. Mussels. In mussel HL, there was significant elevation from the negative control after 1 day's exposure from 11.4% to 20.9% (Table 1). Levels of %tDNA plateaued and remained significantly different from controls for the duration of exposure (Fig. 2a). Mussel HP cell %tDNA increased to significant levels after 3 days. Similarly to HL cells, fragmentation then plateaued at this level and did not increase further over the exposure period (Fig. 2b). No

significant difference in DNA fragmentation was observed between the HP cells and the HL cells from toxin-exposed shellfish.

3.2.3.2. Oysters. Oyster HL %tDNA was significantly different from control levels after 3 days exposure and reached 26.3% after 7 days exposure to OA, an increase in fragmentation of 174% from control levels of 9.5% (Table 1) (Fig. 2c). As it can be seen in Fig. 2d, this trend was repeated in oyster HP cells, with an elevation from control levels of 10.1%–16.9% after 1 day. %tDNA increased significantly after 3 days and after 7 days reached 24.1%, an increase of 140% from the percentage fragmentation recorded in the controls. In both HL and HP 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.

3.2.4. Daily exposure 50 nM ($2 \mu\text{g}/\text{shellfish}/\text{day}$)

The trend observed for daily exposure to 1.2 nM OA was repeated in the daily exposure to 50 nM OA. A significant increase in DNA fragmentation was detected for both cell types in both species over the exposure period (Fig. 3).

3.2.4.1. Mussels. The same pattern in %tDNA was observed in both mussel HL and HP cells, with a significant increase in %tDNA from negative control levels after 1 day's exposure (Table 1). The levels of %tDNA plateaued and remained significantly different from the

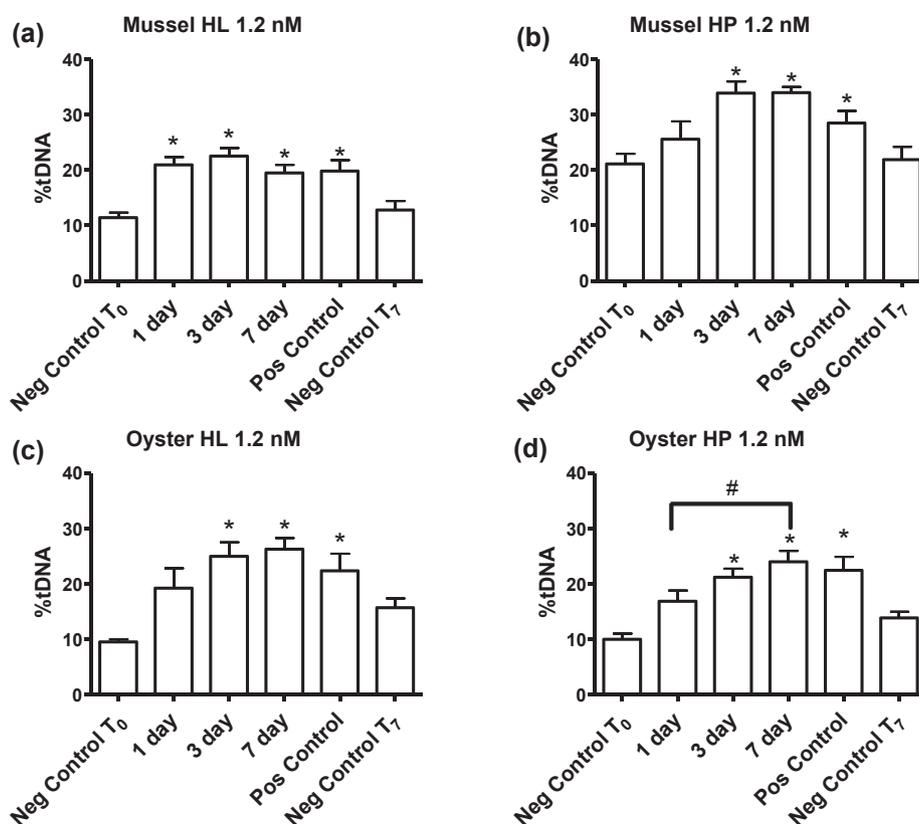


Fig. 2. Changes in DNA fragmentation that occurred after sub-acute exposure to 1.2 nM of OA (0.05 μg OA/shellfish/day) over a 7-day exposure period. *** denotes significant difference from the negative controls ($p < 0.05$); '#' denotes a significant difference between individual sampling days, ($p < 0.05$). (a) Mussel haemolymph (HL); (b) Mussel hepatopancreas (HP); (c) Oyster haemolymph (HL); (d) Oyster hepatopancreas (HP).

controls throughout the exposure period for both cell types (Fig. 3a, b). There was no significant difference in the increase of %tDNA from control levels measured between the two cell types.

Comparison between daily treatments of 1.2 nM and 50 nM OA, showed no significant difference in the increase of %tDNA within HL cells for OA-exposed mussels. However, within the HP cells there was a greater increase in DNA fragmentation from control levels in mussels exposed to 1.2 nM than those exposed to 50 nM OA (T -test: $t_{46} = -3.37$; $p < 0.01$).

3.2.4.2. Oysters. Oyster HL %tDNA increased significantly from control levels of 14.9%–24.1% after 1 day (Fig. 3c), a 62% elevation in fragmentation. Fragmentation plateaued and remained significantly different from controls after 3 days and 7 days exposure (Table 1). In HP cells there was a significant elevation in %tDNA from the controls of 11.4%–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. 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.

Comparison between daily exposure experiments of 1.2 nM and 50 nM OA showed no significant difference in the increase of %tDNA from control levels in exposed oysters for either cell type.

3.2.5. Species variation

A comparison between the percentage increase in damage, relative to the negative controls at T₀ was performed between

species, to determine whether there was variation in OA susceptibility in exposed shellfish.

A significant difference between species occurred in the HP cells exposed to a single 24 h dose of 2.5 nM OA, with oyster HP cells showing a greater overall increase in DNA fragmentation relative to the controls ($t = -3.968$, $p < 0.05$) compared to mussel HP. There was no difference between HL cells at this concentration. No significant differences occurred in the proportion of DNA fragmentation relative to the negative controls at T₀ between mussels and oysters exposed to 1.2 nM OA for either HL or HP cells.

At 50 nM OA exposure there were significant differences between species in both HL ($t = -1.375$, $p < 0.01$) and HP ($t = -3.309$, $p < 0.05$) cells relative to the controls, with oyster cells showing greater increase in DNA fragmentation relative to controls than mussel cells.

4. Discussion

This research presents new and original data for *M. edulis* and *C. gigas*. Both shellfish species showed significant increases in DNA fragmentation after short-term *in vivo* exposure to differing concentrations of OA and no substantial cytotoxic impact, indicating that DNA fragmentation was not secondary to cytotoxicity.

In this study cell viability remained at >85%; these levels were comparable with previous studies examining cell viability using the trypan blue exclusion assay in conjunction with DNA fragmentation as a biomarker of pollution in bivalves (Coughlan et al., 2002; Flórez-Barrós et al., 2011; Hartl et al., 2004). Previous research

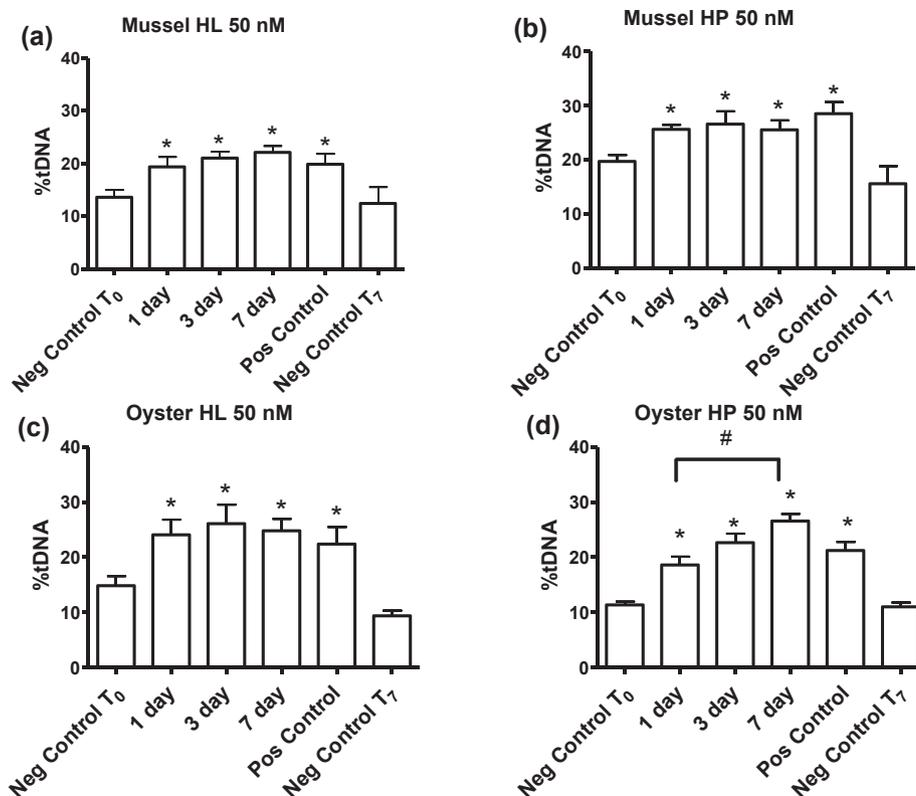


Fig. 3. DNA fragmentation that occurred after daily exposure to 50 nM of OA (2 µg OA/shellfish/day) tank measured over 7-days. *** denotes significant difference from the negative controls, ($p < 0.05$); # denotes a significant difference between individual sampling days, ($p < 0.05$). (a) Mussel haemolymph (HL); (b) Mussel hepatopancreas (HP); (c) Oyster haemolymph (HL); (d) Oyster hepatopancreas (HP).

found the cytotoxic effects of OA on bivalve cells to be low using the Eosin Y and MTT assay respectively (Svensson et al., 2003; Talarmin et al., 2007). A few suggestions have been made to explain this lack of cytotoxic effect, such as storage of toxin in the lysosomal compartments within cells (Svensson et al., 2003), and a capacity to quickly metabolise the OA into less active forms (Flórez-Barrós et al., 2011).

Mussels and oysters were exposed to the same three concentrations of OA, with significant DNA fragmentation measured in both species post-exposure (Figs. 1–3). This was in spite of the oyster's larger mass, which reduced the toxin concentration per gram of tissue (Average wet weight per mussel 1.96 g; $n = 70$; average wet weight per oyster 10.68 g; $n = 70$). Pearce et al. (2005) recognised a link between seasonal spat mortalities in *C. gigas* and an increase in *Procentrum rathymum* density. Histopathological examination of experimentally exposed spat showed thin, dilated gut tubules and sloughing of cells (Pearce et al., 2005). *P. rathymum* has been discovered to produce OA (An et al., 2010), thus *C. gigas* may be quite sensitive to OA toxicity even in adult form. Comparison of sensitivity to OA between species, showed that at the higher OA concentration there was a significantly greater impact on oyster HP and HL cells relative to the controls than that observed in the mussel cells. This greater impact was also seen in the oyster HP cells at the 24 h single OA exposure (2.5 nM). No differences were observed between species at the lower toxin concentration (1.2 nM OA). In mammalian cells, varying responses have been measured in different cells exposed to OA (Fessard et al., 1996; Soud-Mensi et al., 2008; Valdiglesias et al., 2010), and previous investigation on shellfish exposed to marine phycotoxins have found differing sensitivities and effects between species (Galimany et al., 2008b; Mello et al., 2010), as well as fluctuating

ability to metabolise and depurate biotoxins (Lindegarh et al., 2009; Mafra et al., 2010).

After a single exposure to 2.5 nM OA (0.1 µg/shellfish), a similar trend was noted between mussels and oysters for both cell types. An initial increase in %tDNA was recorded after only 24 h exposure with a decrease after 7 days (Fig. 1). A similar outcome was observed in a study performed on the *Perna perna* mussel (Carvalho Pinto-Silva et al., 2003), with increased micronucleus formation observed after 24 h and a decrease 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 was possibly due to this repair mechanism, or through tissue regeneration.

OA is known to accumulate almost exclusively in the HP of shellfish species (Blanco et al., 2007; Mafra et al., 2010; McCarron et al., 2008) 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 %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 1.2 nM toxin levels. Due to the ubiquitous nature of OA in Irish waters (Marine Institute, 2004, 2007), finding changes in %tDNA at the lower experimental toxin levels

was unexpected. What was also unanticipated is that for higher concentrations of the biotoxin (50 nM), 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 recorded in mussels exposed to 1.2 nM, versus those exposed to the higher concentration of 50 nM OA.

This lack of a classic dose response in DNA fragmentation post-exposure to OA-producing algae, has been previously observed in bivalves, both *in vivo* and *in vitro* (Carvalho Pinto-Silva et al., 2005; Flórez-Barrós et al., 2011). In those studies, 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. At the higher concentrations of phytoplankton cells, there was less DNA fragmentation measured than at the lower concentrations. Post-OA exposure there was a significant increase in fragmentation after 24 h, this plateaued and remained at the same level after 48 h (Carvalho Pinto-Silva et al., 2005) and 4 days exposure (Flórez-Barrós et al., 2011). A similar result was observed in the current study, with fragmentation plateauing after one day or three days exposure in mussel HL, HP and oyster HL cells. In oyster HP cells, a significant increase occurred between 24 h and 7 days, indicating a time effect and a greater sensitivity to OA-exposure in HP cells of this species (Figs. 2 and 3). This rapid, initial increase in fragmentation, followed by a plateau effect, could be due to the transformation of OA into a less active metabolite after incubation, or that damage was induced in cells until a certain threshold was reached, at which point defence and repair mechanisms were stimulated enabling repair of the damage (Carvalho Pinto-Silva et al., 2005; Flórez-Barrós et al., 2011).

Another proposal by Flórez-Barrós et al. (2011) was the induction of an apoptotic effect at increased exposure time, with the more damaged cells no longer present for detection. Studies done on apoptotic impacts of OA in bivalve cells show the same lack of a classic dose–response observed in DNA fragmentation. A recent study on *Mytilus galloprovincialis* reported there was an initial increase in apoptosis, but at increasing concentrations of OA and DSP-producing algae, the levels of apoptotic or membrane-damaged cells decreased, both *in vivo* and *in vitro* (Prado-Alvarez et al., 2012). An investigation on *R. decussatus* haemocytes found that there was an initial increase in apoptosis relative to controls post-OA exposure (24 h), but this difference was non-significant after 48 h exposure (Prado-Alvarez et al., 2013). However, investigation of the apoptotic impacts of OA and DSP producing algae on bivalves have found no significant impacts in *C. gigas*, *Perna perna* and *Anomalocardia brasiliana* (Mello et al., 2010; Talarmin et al., 2007), emphasising the importance of measuring the impacts of OA on multiple species.

This study highlights the impact that OA, a commonly occurring biotoxin, has on DNA fragmentation in two economically important aquaculture species. A significant increase in levels of DNA fragmentation was measured in HL and HP cells of mussels and oysters, even at low exposure concentrations of OA. Variation between species was observed with the pacific oyster showing a greater increase in DNA fragmentation at higher toxin concentrations. Theories have been put forward to explain the observed lack of a classic dose–response in this study and in previous publications on bivalve shellfish exposed to OA, however further research is needed to fully describe these effects.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.marenvres.2014.09.009>.

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