In Vitro Analysis of Early Genotoxic and Cytotoxic Effects of Okadaic Acid in Different Cell Types of the Mussel Mytilus galloprovincialis

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IN VITRO ANALYSIS OF EARLY GENOTOXIC AND CYTOTOXIC EFFECTS OF OKADAIC ACID IN DIFFERENT CELL TYPES OF THE MUSSEL *Mytilus galloprovincialis*

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Okadaic acid (OA) is the predominant biotoxin responsible for diarrhetic shellfish poisoning (DSP) syndrome in humans. While its harmful effects have been extensively studied in mammalian cell lines, the impact on marine organisms routinely exposed to OA is still not fully known. Few investigations available on bivalve molluscs suggest less genotoxic and cytotoxic effects of OA at high concentrations during long exposure times. In contrast, no apparent information is available on how sublethal concentrations of OA affect these organisms over short exposure times. In order to fill this gap, this study addressed for the first time in vitro analysis of early genotoxic and cytotoxic effects attributed to OA in two cell types of the mussel *Mytilus galloprovincialis*. Accordingly, hemocytes and gill cells were exposed to low OA concentrations (10, 50, 100, 200, or 500 n*M*) for short periods of time (1 or 2 h). The resulting DNA damage, as apoptosis and necrosis, was subsequently quantified using the comet assay and flow cytometry, respectively. Data demonstrated that (1) mussel hemocytes seem to display a resistance mechanism against early genotoxic and cytotoxic OA-induced effects, (2) mussel gill cells display higher sensitivity to early OA-mediated genotoxicity than hemocytes, and (3) mussel gill cells constitute more suitable systems to evaluate the genotoxic effect of low OA concentrations in short exposure studies. Taken together, this investigation provides evidence supporting the more reliable suitability of mussel gill cells compared to hemocytes to evaluate the genotoxic effect of low short-duration exposure to OA.

Harmful algal blooms (HAB) are oceanographic phenomena characterized by the proliferation and occasional dominance of particular species of toxic or harmful algae (Anderson, 2005). Examples of HAB species include *Dynophysis* and *Prorocentrum* dinoflagellates, which are the predominant producers of the biotoxin okadaic acid (OA) (Yasumoto et al., 1985), responsible for diarrhetic shellfish poisoning (DSP). OA is generally accumulated by marine organisms (especially shellfish) due to its thermostable lipophilic nature, entering the human food chain and producing the DSP syndrome, which is characterized by nausea, abdominal pain, and diarrhea (Dominguez et al., 2010). The frequency, geographical area, intensity, and magnitude of HAB have increased during the last few decades (Van Dolah, 2000; Díaz et al., 2013), especially in coastal areas dependent upon the economic input of aquaculture and fisheries (Rodríguez-Rodríguez et al., 2011).

Altogether, the economic impact of HAB on European coasts is in the order of 177 million Euro/yr (Eriksson, 2011).

The molecular basis underlying the harmful effects of OA was discovered by Bialojan and Takai et al. (1988), demonstrating the biotoxin's ability to inhibit several types of serine/threonine protein phosphatases. Since then, a large number of studies contributed to better understand the influence of OA (Valdiglesias et al., 2012a, 2012b), showing the role played in promoting apoptosis, cytoskeleton disruption, and cell cycle alterations in mammalian cell lines (Valdiglesias et al., 2013). Unfortunately, the assessment of OA-induced toxicity in marine organisms, which constitute an important economic and ecological relevance, is still limited (Prego-Faraldo et al., 2013). However, the few studies that are available on bivalve molluscs indicated that OA produces the most dramatic genotoxic and cytotoxic effects at low concentrations after short exposure duration, whereas high concentrations and longer exposures initiate resistance mechanisms (Carvalho Pinto-Silva et al., 2003, Flórez-Barrós et al., 2011). Such resistance behavior was ascribed to frequent and ongoing contact of bivalves with this toxin in the marine environment (Svensson and Forlin, 1998; Flórez-Barrós et al., 2011).

Among all marine species affected by OA, the mussel *Mytilus galloprovincialis* offers a dual benefit for assessing the harmful effects of this biotoxin: (1) This species is the aquaculture resource most severely affected by HAB, leading to drastic economic losses in coastal areas; and (2) the wide geographical distribution of mussels and their sessile and filter-feeding lifestyle (Viarengo and Canesi, 1991) make this organism an appropriate sentinel organism widely used in pollution biomonitoring studies (Goldberg, 1986). The choice of mussels as sentinel organisms is further supported by the suitability of their tissues for experimental procedures. Hemolymph cells are easily extracted and separated to readily examine critical roles in the immune response to biotoxins (Hégaret et al., 2007, 2011; Haberkorn et al., 2010). Gill cells are the first part of the organism to enter into contact with biotoxins and other compounds dissolved in the water, and are thus often used as model systems in bivalve ecotoxicological studies (Venier et al., 1997; Rank and Jensen, 2003; Akcha et al., 2004; Talarmin et al., 2008; Flórez-Barrós et al., 2011; Hanana et al., 2012).

The aim of this study was to further our understanding of the harmful effects of OA on marine invertebrates using mussels as model organisms. Indeed, for the first time the early effects of this biotoxin were determined at genetic and cytological levels in different tissues.

MATERIAL AND METHODS

Sample Collection

Mussels (*Mytilus galloprovincialis*) were collected in 2014 from a commercial mussel raft from Lorbé in the Ria of Ares-Betanzos (Galicia, northwestern Spain, Figure 1). This location was selected based upon the presence of low OA levels as reported by the Galician aquaculture administration (www.intecmar.org). Mussels were acclimated to lab conditions for 48 h in highly aerated tanks with filtered sea water in a chamber with a photoperiod (18°C, 12-h light–dark cycle), and were fed daily with a 1:1 mixture of two microalgae species (*Isochrysis galbana* and *Tetraselmis suecica*).

Sample Preparation: Isolation of Hemolymph and Gill Cells

Hemolymph was extracted from the posterior adductor muscle of each mussel with a sterilized syringe. Samples (1.5 ml) were mixed simultaneously with precooled anticoagulant solution (modified Alsever´s: NaCl 382 m*M*, glucose 115 m*M*, sodium citrate 27 m*M*, ethylenediamine tetraacetic acid [EDTA] 11.5 m*M*), 1:5 (hemolymph:Alsever). Samples (20 mussels) were pooled to eliminate interindividual variations and the resulting pool was filtered using a nylon mesh (55 μm diameter). The number of hemocytes was determined
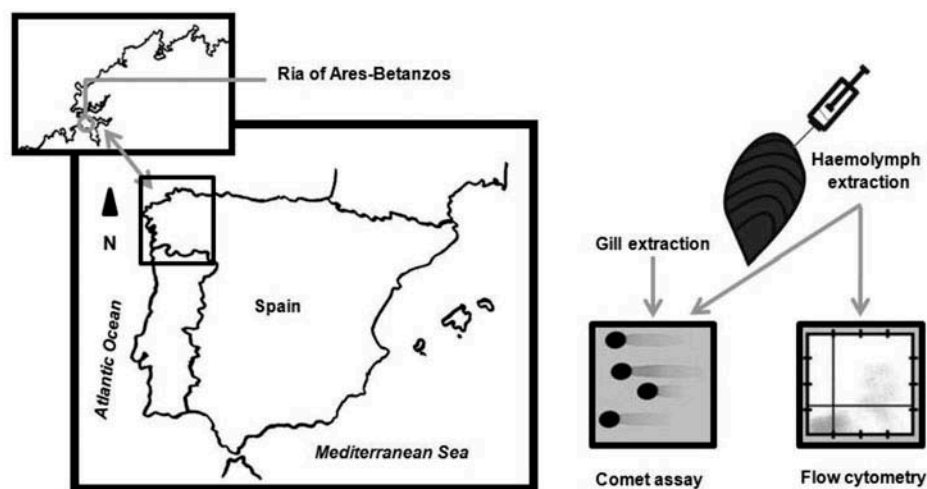
FIGURE 1. Sampling location of mussel specimens (*M. galloprovincialis*) in the Ria of Ares-Betanzos (Galicia, northwestern Spain). Hemolymph and gill cells were extracted, individualized, and subsequently exposed to short pulses of low OA concentrations. The resulting DNA damage and apoptosis/necrosis levels were quantified using the comet assay and flow cytometry, respectively.

postfiltering by counting in a Thoma chamber under the microscope. Gill cells were isolated from the same 20 mussel individuals following a modified protocol described by Pérez-Cadahía et al. (2004). After dissection at room temperature, gills were washed 3 times in 2 ml ice-cold calcium magnesium-free saline solution (CMFS: 20 mM HEPES, 500 mM NaCl, 12.5 mM KCl, 5 mM EDTA in RPMI medium, pH 7.5). Gills were shredded after the last wash and cell suspension was added to 6 ml CMFS and shaken gently for 1 h at 4°C in the dark. The entire suspensions were subsequently distributed (1 ml per tube), filtered through a nylon mesh (55 μm diameter), and centrifuged at 500 × g for 5 min. The resulting pellet was resuspended in 1 ml Kenny´s salt solution (KSS: 0.4 M NaCl, 9mM KCl, 0.7 mM K$_2$HPO$_4$, 2 mM NaHCO$_3$, pH 7.5) and kept on ice. Gill cell number was determined using microscopy. In both tissues, cell viability was determined by the trypan blue exclusion method, obtaining viability values above 80% in all samples.

In Vitro Exposure to Okadaic Acid

Hemolymph and gill cells were incubated in vitro with OA (Sigma, CAS no. 78111-17-8) for 1 or 2 h. Temperature was kept between 15–18°C during each exposure. OA was diluted in dimethyl sulfoxide (DMSO) to obtain the final concentrations of 10, 50, 100, 200, or 500 nM. Final volumes of 10 μl of each OA solution were added to cell suspension. DMSO was used as negative control solution, whereas hydrogen peroxide (100 μM, 10 min) and camptothecin (4 μM, 4 h) were used as positive controls in comet assay and flow cytometry experiments, respectively.

Assessment of DNA Damage Using Comet Assay

The comet assay, also known as the single-cell gel electrophoresis (SCGE) assay, is a sensitive, easy, rapid, and quantitative technique providing detection of DNA damage in individual cells. The comet assay capacity to detect damage in cells of marine organisms was previously established (Michelmore and Chipman, 1998; Wilson et al., 1998; Lee and Steinert, 2003), and its application for the study of biotoxin effects has increased dramatically in recent years (Juhel et al., 2007; da Silva et al., 2011; Flórez-Barrós et al., 2011; McCarthy et al., 2014). In this investigation, the comet technique used was based on the alkaline assay described by Wilson et al. (1998), with minor modifications. Accordingly, hemolymph and gill cells were centrifuged for 5 min at 250 × g and

3 min at 1000 × g, respectively. The resulting pellet was resuspended in 90 μl 0.5% low-melting-point agarose (Invitrogen) in KSS; each sample was divided in two and placed on a slide precoated with a layer of 0.5% normal-melting-point agarose (Intron Biotechnology). After 25 min at 4°C, the slides were placed in a Coplin jar with lysis solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 250 mM NaOH, 10 mM Tris-HCl, 1% sarcosyl, pH 10, with 1% Triton X-100 added just before use) for 1 h at 4°C. From this point on, all steps were conducted in the dark to prevent additional DNA damage. After lysis, slides were placed in alkaline solution (0.3 M NaOH, 1 mM Na$_2$EDTA, pH > 13) for 20 min for DNA unwinding, followed by electrophoresis in the same solution for 20 min (0.83 V/cm). After electrophoresis, slides were washed with neutralization buffer (0.4 M Tris-HCl, pH 7.5) and stained with 4,6-diamidino-2-phenylindole (DAPI). Image capture and analysis were performed using the Comet IV Software. Fifty cells were scored from each replicate slide (100 cells in total) and percent DNA in the tail (%tDNA) was used as the DNA damage parameter.

Assessment of Cytotoxicity Using Flow Cytometry

Annexin V-phycoerythrin (PE) and 7-amino-actinomycin (7-AAD) staining were used in flow cytometry experiments to evaluate apoptosis/necrosis resulting from OA exposure. BD Pharmingen Annexin V-PE Apoptosis Detection Kit I was used according to the manufacturer´s instructions with minor modifications. After OA exposure, hemolymph samples were centrifuged at 250 × g for 5 min at 4°C. Cell pellets were resuspended in 200 μl Annexin binding buffer (0.5×) previously diluted in saline solution (NaCl, 500 nM), yielding a final concentration ranging between 4×10^5 and 6×10^5 cells/ml. Samples were incubated with Annexin V-PE and 7-AAD (1 mg/ml) for 15 min at room temperature in the dark. Cells were then analyzed by flow cytometry. Hemocytes population was fixed in a dot-plot according to

Prado-Alvarez et al. (2012). Flow cytometry analyses were performed in a FACScalibur flow cytometer (Bencton Dickinson). A minimum of 20,000 events was acquired in each case and fluorescence signals for Annexin V-PE and 7-AAD were measured using the FL-2 and FL-3 detectors, respectively. The percentages of apoptotic and dead cells were analyzed using Cell Quest Pro software (Bencton Dickinson). Early apoptosis and late apoptosis/necrosis were expressed as percent Annexin V+/7-AAD- and Annexin V+/7-AAD+ cells, respectively.

Statistical Analyses

Three independent experiments were performed for each OA exposure condition tested. Experimental data were expressed as mean ± standard error and tested for normality using the Kolmogorov–Smirnov test. Since data obtained did not fit a normal distribution, analyses were performed using nonparametric tests. Differences between groups were tested using the Kruskal–Wallis test and the Mann–Whitney U-test. Dose-response relationships for apoptotic/necrotic rates were initially analyzed using Pearson´s correlation coefficient and Spearman's rank correlation coefficient (r). Since both methods yielded similar results, Spearman´s coefficient was selected as the most appropriate approach for testing monotonic relationships between variables that are not normally distributed. Values of $p < .05$ were considered significant. Statistical analyses were performed using the IBM SPSS software package V. 20.

RESULTS

The early effects of OA exposure on mussel DNA integrity were studied by exposing cells to low concentrations of OA (10, 50, 100, 200, or 500 nM) for short periods of time (1 or 2 h). DNA damage and cytotoxicity were evaluated using the comet assay and flow cytometry, respectively (Figure 1). Experiments were developed in two different tissues based

on their role during early responses to tox-
ins: (a) hemolymph, which is in close contact
with toxins in the open circulatory system of
mussels, and (b) gills, which constitute the first
tissue to enter into contact with OA-producing
dinoflagellates during HAB episodes.

Evaluation of DNA Damage After In Vitro Exposure to OA

Quantification of DNA damage in
hemolymph cells resulting from OA expo-
sure is shown in Figure 2. No significant
differences were observed between OA-
treated hemocytes for 1 versus 2 h with the
exception of 10 nM OA at 2 h. In contrast,
incubation of gill cells with OA for 1 h (Figure 3)
produced significant increases in DNA damage
at 50, 100, 200, or 500 nM, with a marked
concentration-response correlation. A possible
explanation for the lack of significant damage
after 2 h might reside in the progressive rise in
basal DNA damage over time. In addition, it is
important to bear in mind that the mechanical
procedures employed in the isolation of gill
cells, together with tissue-specific features, may
be contributing to enhanced basal DNA dam-
age. To test this hypothesis, basal DNA damage
levels were compared between negative con-
trols in hemolymph and gill cells. Our results
seem to refute this hypothesis, as no significant
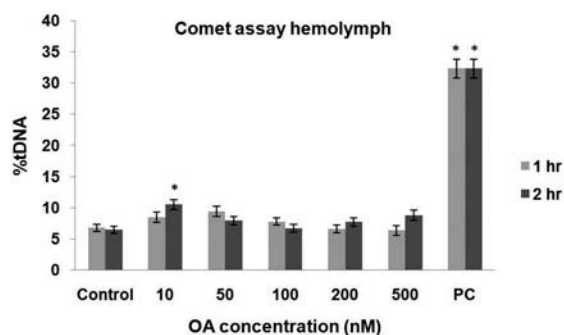DNA damage differences were found between

FIGURE 3. DNA damage quantified using comet assay in gill
cells after *in vitro* exposure to OA. %tDNA represents percent-
age of DNA in the comet tail. Control and PC represent negative
and positive controls, respectively. Asterisk indicates significant
differences at $p < .05$ from negative control in Mann–Whitney
U-test.

FIGURE 4. Comparison between basal DNA damage levels in
negative controls from hemolymph and gill cells after 1 h and
2 h of exposure of OA. %tDNA represents percentage of DNA
in the comet tail. Asterisk indicates significant differences at
$p < .05$ from negative control in Mann–Whitney U-test.

gill controls at 1 and 2 h (Figure 4). However,
significant differences between controls from
different tissues were observed, with gill cells
displaying higher basal damage levels. Taken
together, our comet results suggest that (a) OA
exerts different genotoxic potential in different
cell types, and (b) gill cells constitute a more
appropriate tissue to assess DNA damage in
mussel after 1 h of exposure.

Evaluation of Apoptosis/Necrosis After In Vitro Exposure to OA

The cytotoxic effect of early OA expo-
sure was studied in mussels in parallel to
DNA damage experiments using the same
biotoxin concentrations and exposure times.

FIGURE 2. DNA damage quantified using comet assay in mussel
hemolymph cells after *in vitro* exposure to different OA concen-
trations for 1 and 2 h. Control and PC represent negative and
positive controls, respectively. %tDNA represents percentage of
DNA in the comet tail. Asterisk indicates significant differences at
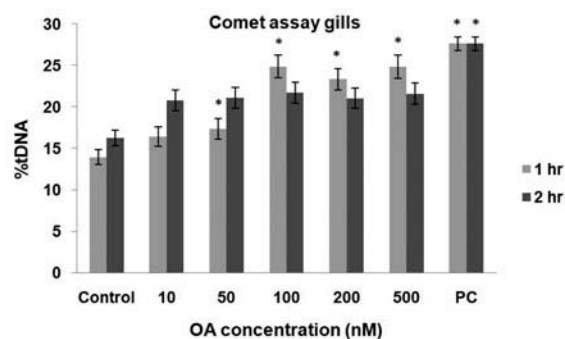$p < .05$ from negative control in Mann–Whitney U-test.

Apoptosis and necrosis rates were measured using flow cytometry analyses of Annexin V/7-AAD staining. While this approach has multiple advantages (high efficiency, clear distinction between apoptotic and necrotic cells, high degree of automation) it is also conditioned by the specific characteristics of the tissue under study, limiting the present analyses to hemolymph cells. In that specific tissue, our results showed that OA lacks cytotoxic potential (Figure 5a), with the exception of high levels of necrosis at 500 nM OA after 2 h (Figure 5b). Still, significant concentration-response relationships were obtained for necrosis after 1 and 2 h (Figures 5a and 5b). In summary, flow cytometry results suggest that (a) although OA exposure did not produce significant cytotoxic damage at low concentrations, the contribution of apoptosis to the overall cytotoxicity is substantially higher than of necrosis, and (b) flow

cytometry may only be useful to study OA-mediated cytotoxicity in hemolymph after long exposure periods.

DISCUSSION

Early Genotoxic Effect of OA in Hemolymph and Gill Cells in Mussels

Comet assay results revealed an overall absence of significant DNA damage in hemolymph cells after exposure to OA. However a significant increase in DNA damage was noted at 10 nM OA after 2 h of exposure. These results are in agreement with the lack of DNA damage observed in hemocytes from the clam *Ruditapes decussatus* after in vivo exposure to different concentrations of the OA-producing dinoflagellate *Prorocentrum lima* (Flórez-Barrós et al., 2011). Similarly, low levels

FIGURE 5. Flow cytometry evaluation of cytotoxicity in hemolymph cells treated with OA for (a) 1 h and (b) 2 h. Control and PC represent negative and positive controls, respectively. Asterisk indicates significant differences at $p < .05$ from negative control in Mann–Whitney U-test.
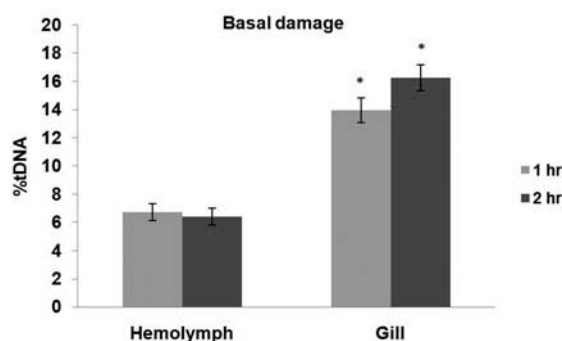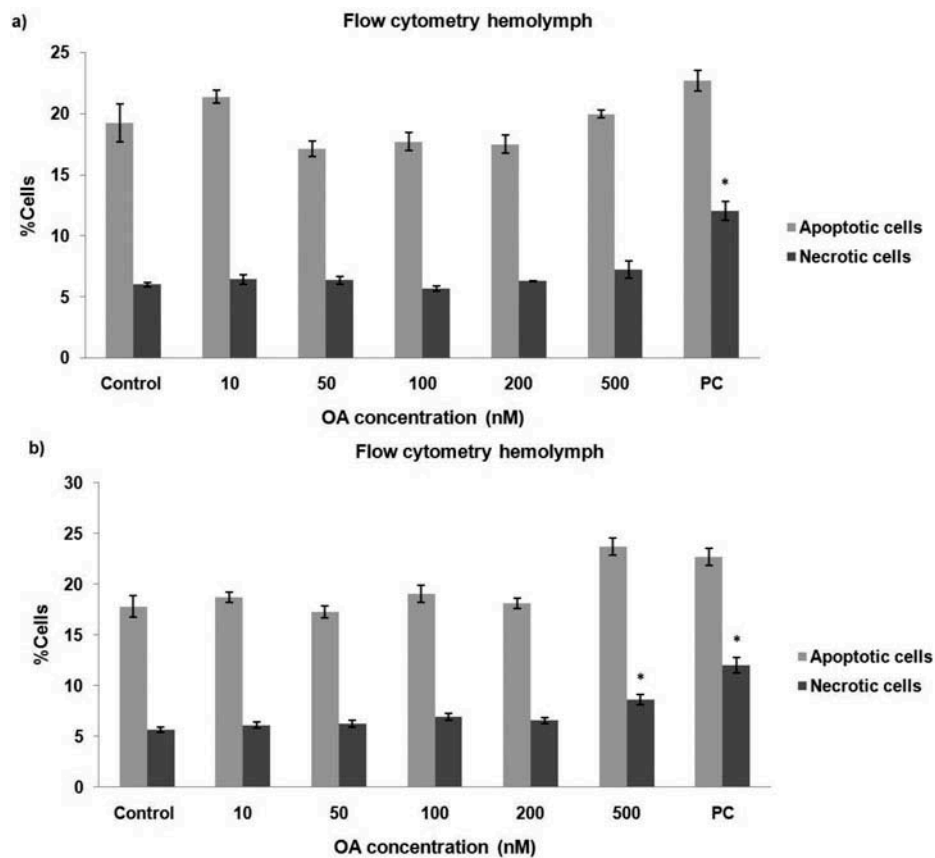
of micronucleus formation were also reported for mussel hemocytes after exposure to OA (Carvalho Pinto-Silva et al., 2003). Flórez-Barrós et al. (2011) noted the absence of DNA damage from exposure to a less active metabolite of OA in circulating cells, which might be attributed to hydrolysis and acylation of parent OA. These metabolic pathways were proposed as the main mechanisms by which *M. galloprovincialis* might metabolize OA and diminish toxicity (Rossignoli et al., 2011). Carvalho Pinto-Silva et al. (2003) suggested that the apparent lack of damage might have resulted from either exposure to excessive quantities of OA, the short life span of hemocytes, or a chronic OA effect. Our results support a rapid genotoxic effect mediated by OA on hemocytes, suggesting the presence of an immediate cellular protective response against OA in those organisms frequently exposed to this biotoxin (Svensson and Förlin, 1998; Svensson et al., 2003). These results need to be considered with caution, as at least one study has demonstrated increased damage levels at high OA concentrations in mussels and oysters (McCarthy et al., 2014).

In contrast to hemocytes, gill cells displayed a significant increase in DNA damage at all OA concentrations studied with the exception of the lowest, 10 nM. In addition, a clear positive concentration-response correlation was found after 1 h of exposure, disappearing at 2 h. As in the previous case, these results are in agreement with those described for gill cells in the clam *R. decussatus* (Flórez-Barrós et al., 2011), where higher levels of DNA damage occurred at low OA concentrations and short exposure duration. The observed stability in DNA damage after 2 h is likely due to greater basal damage at these specific conditions. Consequently, this may account for differences detected with regard to control at 1 h, falling after 2 h of OA treatment, thus leading to a false negative response under this second condition. A similar picture emerges when comparing digestive gland and hemolymph with higher levels of basal damage in the former (McCarthy et al., 2014). An alternative explanation for the maintenance of genetic damage might be induction of apoptosis by OA (Flórez-Barrós et al., 2011).

Accordingly, only cells more resistant to OA (and therefore less damaged) would prevail, producing false results in comet assays. Within this scenario, mussel gill cells would be only useful for the assessment of OA-induced genotoxicity at short exposure duration. However, usefulness of gill cells continues to be hampered by damage increase created during the cell isolation procedure.

Overall, our results suggest that in the case of mussels, hemolymph is more resistant than gill cells to OA-mediated genotoxicity. This notion is consistent with earlier reports showing heterogeneous levels of OA-induced genotoxicity depending on the specific cell type in mammals (Souid-Mensi et al., 2008; Valdiglesias et al., 2010), as well as in oysters (Talarmin et al., 2008; Hanana et al., 2012) and clams (Coughlan et al., 2002; Flórez-Barrós et al., 2011). Recently, McCarthy et al. (2014) described similar OA-mediated genotoxic effects in hemolymph and digestive-gland cells from blue mussel and pacific oyster. This behavior may be attributed to the roles played by these tissues during immune and detoxification responses, which are different from the respiratory role of gills. Further, gills constitute the first tissue to enter into contact with biotoxins in the external environment, thus experiencing higher genotoxic potential. Finally, it was suggested that differential resistance to DNA damage across different tissues might in fact be dependent on the specific type of genotoxic compounds (Venier et al., 1997). Given the considerable resistance of mussel hemolymph to OA-induced genotoxicity, gill cells seem more appropriate to assess genotoxicity at low OA concentrations and short exposure times.

Early Cytotoxic Effect of OA in Hemolymph Cells

Flow cytometry experiments did not reveal marked differences in OA-mediated cytotoxicity in hemolymph cells exposed to different OA concentrations after 1 h. However, a significant positive concentration-response relationship was found after 2 h for both

apoptosis and necrosis, supporting previous results obtained in mussels (Prado-Alvarez et al., 2012). An absence of cytotoxic effects after short exposure times (1 h) to different OA concentrations was also reported by Talarmin et al. (2008) in cultured oyster heart cells. Indeed, data suggested that lack of cytotoxicity might be due to the presence of caspase inhibitors (Rossini et al., 2001; Flórez-Barrós et al., 2011). In contrast, Prado-Álvarez et al. (2013) found a significant rise in percent of apoptotic/necrotic cells when hemolymph cells of the clam *R. decussatus* were exposed to OA for 2 and 4 h. To a lesser degree, this elevation was also observed in our results. Therefore, consistent with our DNA damage results, our cytotoxicity analyses suggest that hemolymph cells appear more resistant to OA compared to other cells in bivalve molluscs, as suggested by Hégaret et al. (2011).

Findings reported in the present study resemble those obtained by Ferraz-Mello et al. (2010), showing that mussels are more immunologically active than other bivalves when exposed to a natural bloom of *Dinophysis acuminate,* a prominent producer of OA. The stress induced by OA may also influence the mussel´s immune system as well as phagocytic activity (Malagoli et al., 2008), since hemocytes are mainly responsible for the phagocytic process in bivalve molluscs. However, the significant concentration-response correlation findings suggest that hemolymph cytotoxic resistance to OA is reduced over time, which might be due to the role hemocytes play in early response to OA-mediated cytotoxicity. Overall, our observations suggest that the effectiveness of hemocytes might not be sufficient at the time of response to a natural HAB episode that lasts for a long period of time.

CONCLUSIONS

This study constitutes the first research effort assessing the early effects of OA in different mussel cell types using the comet assay and flow cytometry. Our results significantly contribute to expanding our current understanding regarding genotoxic and cytotoxic consequences following exposure to sublethal levels of this biotoxin. Several key conclusions may be drawn from this study: (1) Our results suggest the presence of a resistance mechanism against early genotoxic and cytotoxic OA-induced effects in mussel hemocytes, (2) mussel gill cells display higher sensitivity to early OA-mediated genotoxicity than hemocytes, and (3) mussel gill cells constitute more suitable systems to evaluate genotoxic effect of low OA concentrations for short exposure durations. Overall, the present investigation provides a framework for future molecular cytogenetic studies aimed at developing rapid, sensible, and efficient biomonitoring mechanisms to assess sublethal effects of OA in marine invertebrates.

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