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Impact of harmful algal blooms (*Dinophysis acuminata*) on the immune system of oysters and mussels from Santa Catarina, Brazil

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Blooms of the harmful alga Dinophysis acuminata*, which produces okadaic acid (OA), are becoming recurrent in Santa Catarina coast, where most of the shellfish marine farms in Brazil are located. We evaluated the impact of* D. acuminata *blooms on various haemato-immunological parameters and on tissue integrity of cultivated oysters (*Crassostrea gigas*) and mussels (*Perna perna*). Animals were sampled during two natural algal blooms, one at Praia Alegre (PA: 2950 cells* l^{-1}*) and the other at Praia de Zimbros (PZ: 4150 cells* l^{-1}*). Control animals were sampled at the same sites, 30 days after the end of the bloom. The assayed parameters were: total (THC) and differential (DHC) haemocyte counts, percentage of apoptotic haemocytes (AH), phenoloxidase activity (PO), agglutinating titre (AT) and total protein concentration in haemolymph (PC). Histological analyses were carried out in oysters from PZ. The results showed that some immune parameters were modulated during the toxic blooms, but not in a consistent manner, especially in mussels that accumulated more OA (10×) than oysters. For example, mussel THC decreased significantly (54%) during the bloom at PA, whereas it augmented markedly (64%) at PZ. PO activity was significantly altered by the algal blooms in both bivalve species, while PC increased significantly (66%) only in mussels from PZ bloom. The other parameters (DHC, AH and AT) did not vary in both bivalve species. Histological analyses showed an intense haemocytic infiltration throughout the oyster digestive epithelium, particularly into the stomach lumen during the algal bloom.*

Keywords: Harmful algal bloom, *Dinophysis acuminata*, *Perna perna*, *Crassostrea gigas*, haemato-immunological parameters, haemocytic infiltration

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INTRODUCTION

Harmful algal blooms (HABs) are well-known for their ecological and economic impacts in coastal areas. They are one of the major sources of contamination of the marine environment and can cause mass mortality of wild and cultivated animals due to the potent phycotoxins they produce (see reviews of Shumway, 1990; Landsberg, 2002).

Among cultivated species, marine bivalves are of particular importance during HAB events in view of their sessile and filter-feeding habits that favour the accumulation of high levels of algal toxins in their tissues. As a consequence, the consumption of contaminated bivalves can be dangerous for human health and their commercialization is prohibited when harmful algae concentrations reach critical levels. These commercial embargos are becoming more and more frequent worldwide, including in Brazil, where they are causing important economic losses to fisheries and aquaculture-based industries (see http://www.algasnocivas.pro.br/monitoramento.php).

Most of the studies on algal toxins are focused on their impact on human health and only few of them examine their potential negative effects on bivalve physiology and survival. Even though mortalities are uncommon among bivalves during HAB events since they seem to be particularly resistant to phycotoxins, recent studies have shown that microalgal toxins may indeed have damaging or stressful effects on bivalve metabolism and make them more susceptible to infections (Hégaret *et al.*, 2007a, 2010).

Diarrheic shellfish poisoning (DSP) is a symptom caused in humans by the consumption of contaminated bivalves, which have accumulated toxins from certain species of dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum* (Yasumoto *et al.*, 1985; Lee *et al.*, 1989; Bravo *et al.*, 2001). The major DSP toxins are okadaic acid (OA) and its structural derivatives, the dinophysistoxins -1 and -2 (DTX1 and DTX2). After consumption of contaminated shellfish, these lipophilic toxins produce gastrointestinal symptoms such as nausea, vomiting, diarrhoea and abdominal pain (Yasumoto *et al.*, 1978). In mammals, OA toxins are known to inhibit serine-threonine protein phosphatases (PP-1 and especially

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PP-2A) (Biolojan & Takai, 1988), which are key components of cell signalling and cell regulation pathways that underlie a myriad of essential physiological processes. Interestingly, mussel phosphatases 1 and 2A are not apparently affected by OA (Svensson & Förlin, 1998). More recently, OA and derivatives also have been shown to be potent tumour promoters in mammals (Fujiki & Suganuma, 2009) and implicated in micronucleus formation (Carvalho Pinto-Silva *et al.*, 2003, 2005), cell apoptosis (Lago *et al.*, 2005; Prado-Alvarez *et al.*, 2013) and genotoxicity (Valdiglesias *et al.*, 2010; Gonzalez-Romero *et al.*, 2012) in mammals and bivalves.

Bivalve immune responses against pathogens and/or toxicants are carried out by the circulating blood cells or haemocytes and by a variety of soluble molecules found in their haemolymph (Hine, 1999; Roch, 1999). Recent reports have shown that some immune functions might be affected in bivalves upon experimental exposure to toxic microalgae or their purified toxins (Hégaret & Wikfors, 2005a; da Silva *et al.*, 2008; Galimany *et al.*, 2008a, b; Malagoli *et al.*, 2008; Haberkorn *et al.*, 2010a; Bricelj *et al.*, 2011; Hégaret *et al.*, 2011; Mello *et al.*, 2013; Prado-Alvarez *et al.*, 2013). However, very few studies (Hégaret & Wikfors, 2005b; Mello *et al.*, 2010; Prado-Alvarez *et al.*, 2012) have examined the effect of *natural* harmful algal blooms on the immune functions of cultivated or wild shellfish.

The coastal area of Santa Catarina (southern Brazil) is by far the major producer of cultured bivalves in Brazil (more than 95% of the total national production). In the last few decades, blooms of harmful algae have been reported along the Brazilian coast, such as diatoms of the genus *Pseudo-nitzschia* that cause amnesic shellfish poisoning (ASP) and the dinoflagellates *Alexandrium tamarense* and *Dinophysis acuminata* Claperede & Lachman (1859) that cause paralytic shellfish poisoning (PSP) and diarrhoeic shellfish poisoning (DSP) respectively (Proença *et al.*, 2007; Schramm & Proença, 2008). In Santa Catarina coast, blooms of *D. acuminata* are currently becoming more and more frequent (Proença *et al.*, 2007) and besides the ecological impact and risk to human health, these toxic blooms are also threatening the malacoculture activity.

Recently, Mello *et al.* (2010) reported that natural blooms of *D. acuminata* may affect some haemato-immunological parameters in oysters (*Crassostrea gigas* Thunberg, 1793), clams (*Anomalocardia brasiliana* Gmelin, 1791) and especially mussels (*Perna perna* Linnaeus, 1758) cultivated in the south bay of Santa Catarina Island. The aim of this study was to expand the observations of Mello *et al.* (2010) to other regions of Santa Catarina coast, where bivalves are being intensely cultivated, in order to validate the results of these authors and to better understand the potential toxic effects of *D. acuminata* toxins on bivalve immunity. In this study, we report the modulation of different haemato-immunological parameters in *C. gigas* and *P. perna* during two blooms of *D. acuminata* that took place in different localities of Santa Catarina coast.

MATERIALS AND METHODS

Animals and experimental design

Adult Pacific oysters *Crassostrea gigas* (shell height, 90–100 mm, N = 60) and brown mussels *Perna perna* (60–70 mm, N = 80) were obtained from commercial marine farms in Santa Catarina coast during two natural blooms of *D. acuminata* (lasting from 6–9 days). The first (2950 microalgal cells l^{-1}) occurred in November 2009 at *Praia Alegre* (PA – 26°46′S; 48°39′08″W) and the second (4150 cells l^{-1}) in March 2010 at *Praia de Zimbros* (PZ – 27°11′S; 48°32′31″W) (Figure 1). At PZ both bivalves were collected, whereas at PA only mussels were obtained because oysters were not available. Animals were sampled and used in different analyses (immunological and histological assays) during the algal blooms and also after 30 days from the end of the bloom (reference animal group).

Seawater samples were preserved in lugol (1%) for algal cell counts. Water salinity, temperature and dissolved oxygen were measured during the samplings.

Fig. 1. Sites of oysters and mussels samplings at Santa Catarina coast during blooms of *Dinophysis acuminata*. Bar: 20 km.

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Microalgal cell counts, concentration of okadaic acid (OA) and mouse bioassays

The number of *D. acuminata* cells in seawater samples was estimated under an inverted phase-contrast microscope according to the protocol of Utermöhl (1958).

The concentration of OA was determined in mussel and oyster digestive glands (N = 12, for each animal group) by liquid chromatography (LC) coupled to mass spectrometry (LC-MS/MS). The gland extracts (2 g) were prepared by alcohol extraction (absolute methanol), centrifuged and filtered (0.2 µM nylon filters). Chromatography was performed on Agilent 1200 LC system RR equipped with a fast Zorbax Eclipse XDB-C18, 4.6 × 50 mm chromatography column at 35°C. The identification and quantification of OA and its derivatives was performed using an API 3200 QTrap MS/MS detector calibrated with pure standards from NRC Canada, following the settings obtained from Villar-González *et al.* (2008).

Mouse bioassays (MB) were carried out by injecting 1 ml of the digestive gland extracts of both bivalves in three mice (weight 18–20 g) intraperitoneally. A reaction was considered positive when at least two mice died within 24 h.

Haemolymph preparation

Haemolymph was withdrawn from animal adductor muscle with the aid of a needle (21 G) coupled to a 1 ml syringe (kept on ice). Haemolymph pools (3 pools of 10 animals from each species and from each locality) were separated in two subgroups. The first was fixed in 4% formaldehyde diluted in modified Alsever solution or MAS (27 mM sodium citrate, 336 mM sodium chloride, 115 mM glucose, 9 mM EDTA, pH 7.0) (2:1 v/v) and used to determine the haemograms and the percentage of apparent apoptotic cells. The second subgroup was used to prepare total haemolymph (TH). TH was obtained by lysing the haemocytes through sonication (3 cycles of 7 s each, at 22.5 kHz/50 W, at 4°C). The disrupted cell suspension was centrifuged (12 000 ***g*** for 30 min at 4°C) and the supernatant or TH (exocytosed cell products + plasma) was separated and stored at −20°C until use (determination of AT, PO and PC).

Haemograms: total (THC) and differential (DHC) haemocyte counts

Total haemocyte counts (THC) were determined from fixed haemolymph pools with the aid of a Neubauer chamber (in duplicates). The relative percentage of the different haemocyte populations (DHC) was estimated by counting 200 cells from each fixed blood sample under a phase-contrast microscope. Results were expressed as the relative percentage of granular haemocytes (GHs). The remaining percentage (complementary) corresponds to the hyaline haemocytes (HHs).

Percentage of apoptotic haemocytes (AH)

The percentage of apparent apoptotic haemocytes was determined by using Hoechst 33258 staining (SIGMA). Fixed haemocyte smears were immersed in McIlvane buffer (0.1 M citric acid, 0.4 M disodium hydrogen phosphate, pH 5.5) for 5 min and then incubated for 5 min in a solution of bisbenzimida fluorophore (1 mg ml^{-1}) in McIlvane buffer for 5 min. The slides were then mounted with coverslips and observed under a fluorescence microscope (365 nm). The percentage of apoptotic cells was estimated by examining 200 cells per sample and counting the morphologically altered nuclei characteristic of apoptotic cells.

Haemagglutinating activity

Samples of 50 µl of TH from the different pools were serially diluted in TBS-1 (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.4) in 96-well microplates (U-shaped bottom) and incubated with the same volume of a suspension of dog erythrocytes (2% in TBS-1) for 2 h at 20°C in a humid chamber. In controls, TH was replaced by TBS-1. The agglutinating titre (AT) was expressed as the reciprocal of the highest TH dilution showing positive agglutination. The titres were converted to $\log_2$ and the assays were performed in triplicate.

Determination of phenoloxidase (PO) activity

PO activity was determined spectrophotometrically through the formation of DOPA-chrome (red pigment) by the oxidation of the enzyme substrate, L-dihydroxyphenylalanine (L-DOPA). TH samples (50 µl) from the different pools were diluted (v/v) in TBS-2 (50 mM Tris, 400 mM NaCl) and incubated with 50 µl of L-DOPA (3 mg ml^{-1}) in 96-well plates (flat-shaped bottom) at 20°C. The reaction was carried out at pH 9.0, since alkaline pH is a strong enzyme inducer. The formation of DOPA-chrome was recorded on a microplate reader (A_{490}), every 30 s, for 20 min. In controls, TH was replaced by TBS-2. One enzyme unit (1 U) corresponded to an increase of 0.001 in the absorbance per min, per mg of protein at 20°C (Söderhäll & Häll, 1984). All assays were carried out in triplicate.

Total protein concentration (PC)

PC was determined in the different TH pools according to the method of Bradford (1976), using bovine serum albumin (BSA) as a standard. Assays were carried out in triplicate.

Histopathological analysis

Whole oysters from PZ (N = 15 for each group) were removed from the shells and preserved in a Davidson solution for 24 h. Mussels were not preserved for histology. The fixed animals were then transferred to 70% ethanol and sectioned diagonally to enable the exposure of the mantle (and gonads), gills and digestive gland. Tissues were embedded in paraffin, sectioned to 5 µm and stained in haematoxylin-eosin (HE). Permanent slides were examined under a light microscope and the histological lesions were recorded with digital micrographs.

Statistical analyses

The results were first subjected to Bartlett's test to evaluate variance homogeneity. Then, the results of each immune parameter were compared by one-way ANOVA followed by Tukey's post-test (mean comparison). For DHC and AH (percentages), the data were arcsin transformed. The results were considered significant at $P < 0.05$. Statistical analysis was performed using GraphPad Prism® software, version 5.0.

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RESULTS

Abiotic parameters

Water salinity, temperature and concentration of dissolved oxygen (DO) were very similar in both sampling sites during the toxic algal blooms (PA: November; PZ: March) and 30 days after the bloom-end (reference groups) (Table 1). The sole exception was the water temperature at PZ (reference, 20°C) that was considerably lower than during the algal bloom (27°C), probably due to the beginning of autumn.

Number of algal cells, okadaic acid (OA) concentration and mouse bioassays

Dinophysis acuminata concentration in seawater reached 2950 cells l^{-1} at PA in November 2009, and 4150 cells l^{-1} at PZ in March 2010. In Santa Catarina coast, a concentration of 500 cells l^{-1} of *D. acuminata* in seawater is already considered critical and bivalve consumption is unsafe. Mouse bioassays gave positive results in both natural microalgal blooms. Bivalve mortalities were not recorded during both HABs. Following 30 days of the bloom period the concentration of *D. acuminata* in seawater dropped to 0 cells l^{-1} at both sites.

The concentration of OA in the digestive gland extracts was measured only in animals from PZ blooms and was 10× higher in mussels (60.1 μg kg^{-1}) than in oysters (5.9 μg kg^{-1}). Following 30 days of the end of the blooms there was a marked decrease of the OA concentration in mussel tissue extracts (0.9 μg kg^{-1}), whereas no traces of toxins were found in oysters.

Haemograms

The total haemocyte count (THC) of mussels varied significantly during the algal blooms when compared with the reference groups, even though in a contrasting manner (Figure 2A). At PA, there was a decrease of THC (56%) during the *D. acuminata* bloom (2.9 ± 0.5 × 10^6 cells ml^{-1}) when compared with the reference group (5.2 ± 1.8 × 10^6 cells ml^{-1}), whereas at PZ it increased about 44% (algal bloom: 6.9 ± 1.2 × 10^6 cells ml^{-1} and reference group: 4.8 ± 0.4 × 10^6 cells ml^{-1}). On the other hand, in oysters from PZ, the THC was similar in both algal-exposed and reference animals (about 2.5 × 10^6 cells ml^{-1}).

The differential haemocyte count (DHC) was presented here as the percentage of granular haemocyte populations (GHs). The complementary percentage represented by the hyaline haemocyte (HHs) population is not shown. The percentage of GHs did not change significantly in both bivalve species during the algal blooms (Figure 2B). This haemocyte population (GHs) was always predominant (more than 80%) over the hyaline haemocytes (HH) in both bivalve species.

Table 1. Abiotic parameters at the sampling sites during blooms of *Dinophysis acuminata*.

Sites of sampling	Salinity (‰)	Temperature (°C)	DO (mg l^{-1})
PA: algal bloom	32	25.6	6.1
PA: reference	33	27.8	6.8
PZ: algal bloom	33	27.7	6.9
PZ: reference	32	20.3	6.8

DO, dissolved oxygen; PA, Praia Alegre; PZ, Praia de Zimbros.

Percentage of apoptotic haemocytes (AH)

The number of haemocytes showing altered nuclei suggesting apoptosis was very low in both bivalves (less than 1%) during both algal blooms and no significant differences were observed with the reference groups.

Total protein concentration (PC) in haemolymph

The PC of mussel haemolymph increased significantly (63%) only during the algal bloom at PZ (3.1 ± 0.5 mg ml^{-1}) when compared with the reference group (1.9 ± 0.1 mg ml^{-1}) (Figure 2C). No variation was observed on the PC of oyster haemolymph. Curiously, the PC of oyster haemolymph was considerably lower (less than 1 mg ml^{-1}) than that of mussels.

Phenoloxidase activity (PO)

The PO activity varied significantly in both mussels and oysters during the algal blooms, but in contrasting manners (Figure 2D). In mussels, the enzyme activity increased strongly (about 100%) during the dinoflagellate bloom at PA (bloom: 575.4 ± 66.7 U min^{-1} mg^{-1}, reference group: 287.2 ± 6.7 U min^{-1} mg^{-1}) but remained unaltered at PZ and the values were about half (120–140 U min^{-1} mg^{-1}) of those found at PA (reference group). On the other hand, PO activity in oysters dropped significantly (about 60%) during the algal bloom at PZ (algal bloom: 298.0 ± 8.1 U min^{-1} mg^{-1}, reference: 478. 8 ± 68.0 U min^{-1} mg^{-1}).

Haemagglutinating activity

The agglutinating titres (AT) of the TH of both bivalves against dog erythrocytes did not change significantly during both toxic algal blooms (Figure 2E). However, there was a tendency to higher values in mussels at PA (AT = 512) when compared with the reference group (AT = 256). The AT of both bivalve species was very similar.

Histopathological analysis

Histological analysis was carried out only in oysters. Animals exposed to the bloom of *D. acuminata* displayed an intense migration and infiltration of haemocytes into the lumen of the stomach, intestine, and to a lesser extent into the digestive primary or secondary tubules (diapedesis through the epithelium) (Figure 3). Tissue alteration was observed in the stomach epithelium. Oysters from the reference group did not exhibit such abnormalities.

DISCUSSION

In the last two decades, blooms of *Dinophysis acuminata* have been increasing dangerously in the Santa Catarina coast and the presence of okadaic acid (OA) and derived toxins have

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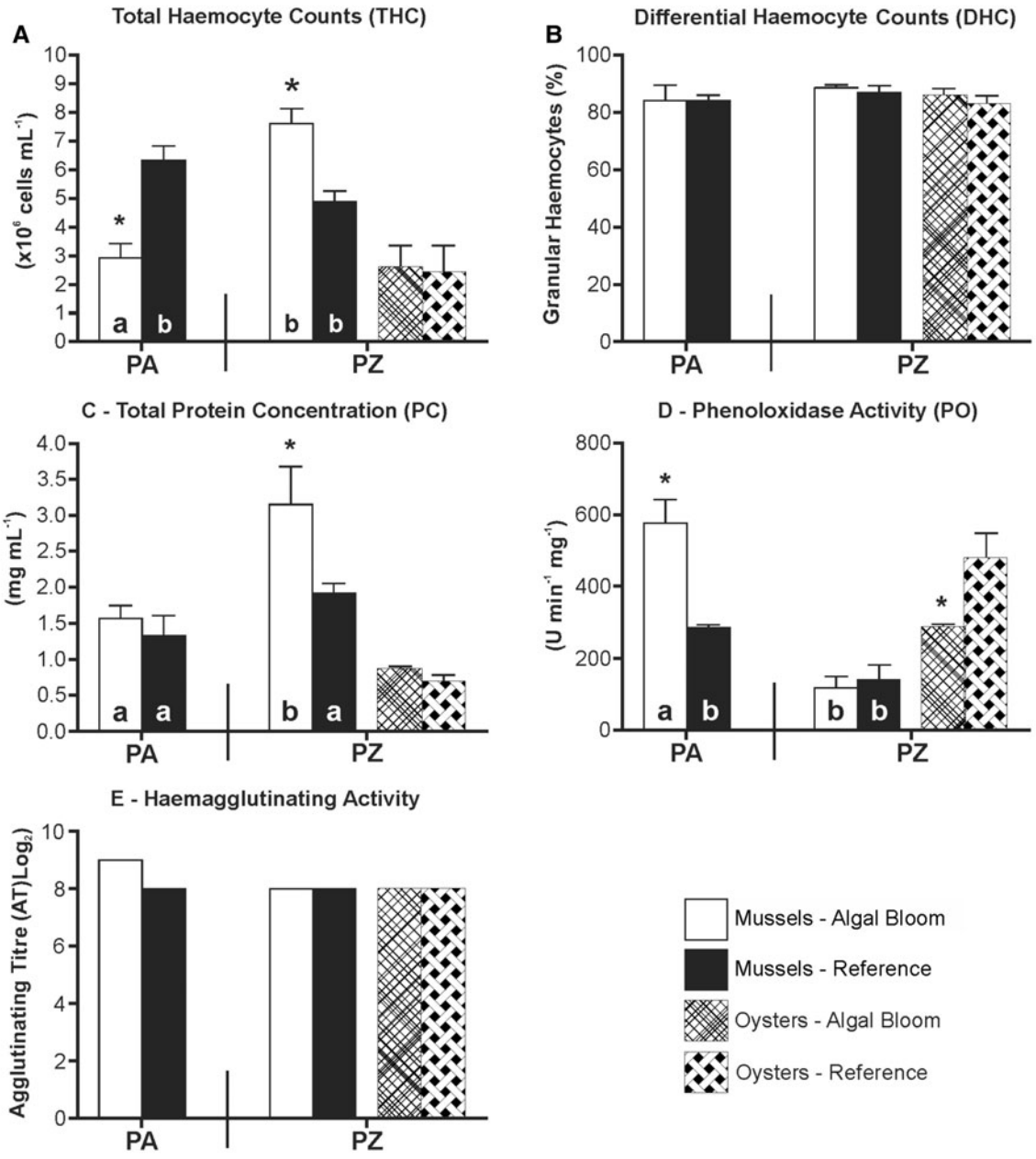

Fig. 2. Immunological parameters of mussels and oysters sampled during (algal bloom) and after (reference) the bloom of *Dinophysis acuminata*. PA: Praia Alegre; PZ: Praia de Zimbros. *Represent significant differences ($P < 0.05$) between the exposed (HAB) and reference group at the same site. Letters represent significant differences ($P < 0.05$) between sites (mussels).

been recorded in mussel and oyster tissues (Proença *et al.*, 2007). In 2010, Mello *et al.* reported that a severe natural bloom of *D. acuminata* in Santa Catarina Island resulted in the modulation of some haemato-immunological parameters in oysters (*C. gigas*), clams (*Anomalocardia brasiliana*) and especially in mussels (*P. perna*). The aim of this study was to extend these previous observations to other localities of the Santa Catarina coast, where shellfish are intensely cultivated, in order to validate the potential noxious effect of DSP toxins on bivalve immunity. As stated before, two natural blooms of *D. acuminata* occurred in Santa Catarina coast during this study: one at PA (November 2009–2950 cells l^{-1}) and a more intense one at PZ (March 2010–4150 cells l^{-1}). Both algal blooms lasted about 6–9 days and bivalve commercialization was prohibited until natural depuration was ascertained.

Various haemato-immunological parameters were examined in farmed *P. perna* and *C. gigas* during both dinoflagellate blooms as well as the presence of histological lesions in oysters. The number and type of circulating haemocytes (haemograms) are major immune parameters in the assessment of bivalve health status. In this study, the number of total circulating haemocytes (THC) was indeed altered in mussels, but not in oysters, during both *D. acuminata* blooms. Curiously, at PA mussel THC decreased significantly (56%), but at PZ it augmented greatly (44%) (more intense bloom). Possibly,

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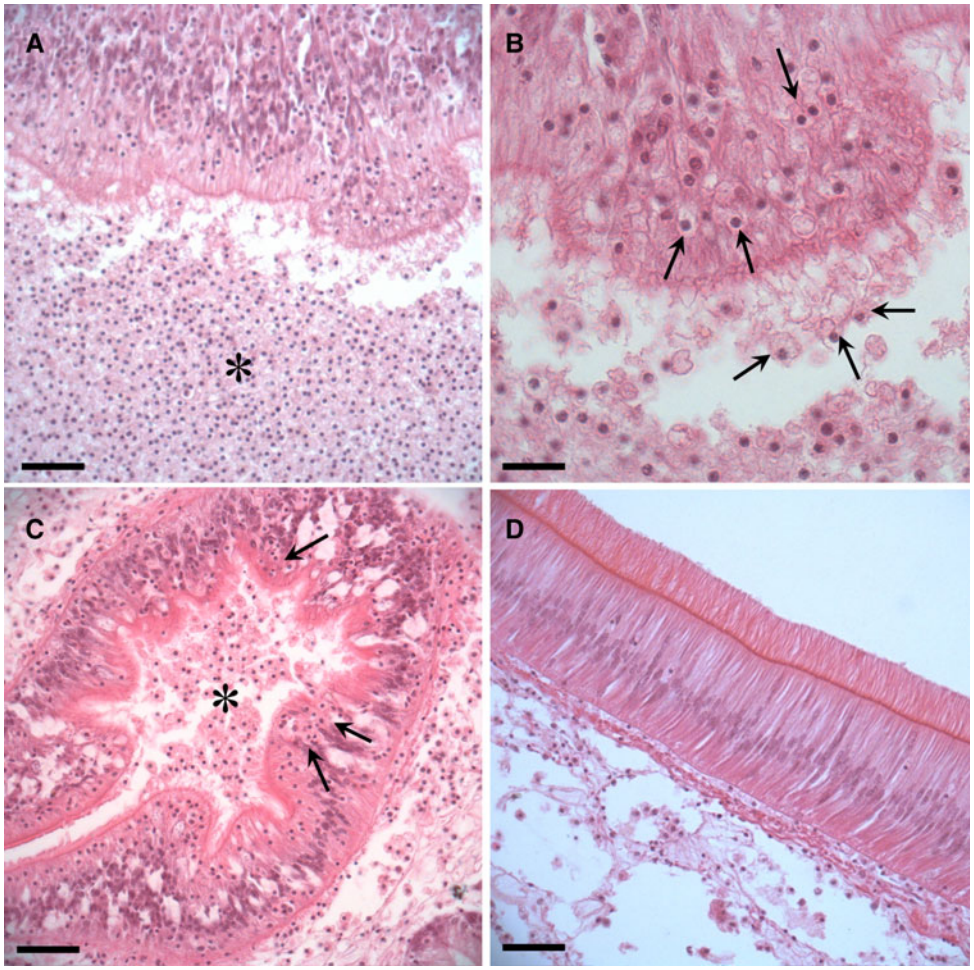

Fig. 3. Histological alterations of oysters *Crassostrea gigas* sampled during the bloom of *Dinophysis acuminata* at Praia de Zimbros. (A) Intense haemocytic infiltration at the stomach lumen (asterisk). Bar: 50 μm. (B) Magnification of figure A showing the altered stomach epithelium with haemocytes undergoing diapedesis (arrows). Bar: 20 μm. (C) Haemocytic infiltration at the lumen (asterisk) and epithelia (arrows) of the digestive tubule. Bar: 50 μm. (D) Undamaged stomach epithelium from oysters of reference group. Bar: 20 μm.

the rise of THC was related to the activation of the mussel immune system triggered by the microalgal phycotoxins. On the other hand, the reduction of the circulating haemocyte number could be related to the migration of these cells to the tissues in contact with the microalgae in order to assist dinoflagellate clearance (Galimany *et al.*, 2008b; Estrada *et al.*, 2010; Escobedo-Lozano *et al.*, 2012). Alternatively, these contrasting responses could be related to the different concentration/lasting of the algal blooms or to different environmental variables underlying field experiments. Interestingly, the significant increase of THC observed in *P. perna* at PZ was very similar to that reported by Mello *et al.* (2010) during the severe *D. acuminata* bloom (17 600 cells l^{-1}) at the south bay of Santa Catarina Island.

The THC of *C. gigas* was determined only during the second bloom (PZ), since this species was not available at PA. In contrast to the mussels, oyster THC did not vary during the algal bloom and this result was in agreement with the report of Mello *et al.* (2010). Similar results were also described in *C. virginica* exposed to *A. fundyense* and *A. catenella* (which cause PSP), where the THC remained unchanged (Hégaret *et al.*, 2007b).

The proportion of blood cell types or differential haemocyte counts (DHC) did not vary in both bivalves during both toxic blooms. This result differed from the observations of Mello *et al.* (2010) where a decrease (12%) in the percentage of granulocytes (GHs) was observed in mussels, but not in oysters, during the toxic bloom. These differences might be due to the much higher *D. acuminata* concentration (4× higher than at PZ) that occurred in the study of Mello *et al.* (2010). Changes in the percentage of haemocyte populations may result from the recruitment of a specific cell type, such as granulocytes, into the tissues in direct contact with the harmful microalgae, as are the digestive system and gills. Haberkorn *et al.* (2010a) reported a drastic increase of GHs in *C. gigas* fed *Alexandrium minutum*. On the other hand, the DHC of *C. virginica* and *C. gigas* exposed to *A. catenella* and *A. fundyense* did not vary (Hégaret *et al.*, 2007b).

The exposure of bivalves to toxic compounds may stimulate cell death through apoptosis (Sokolova *et al.*, 2004, 2011; Marcheselli *et al.*, 2011). In this study, however, the percentage of altered haemocyte nuclei exhibiting morphological features of apoptosis was very low (<1%) in both bivalves during both algal blooms. Mello *et al.* (2010) also found insignificant levels of apoptosis in clam, oyster and mussel haemocytes during the *D. acuminata* bloom. These results suggest that OA and derivatives do not trigger cell apoptosis in bivalve haemocytes. Galimany *et al.* (2008b) also found very

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low levels of apoptosis in the haemocytes of *M. edulis* exposed to *Prorocentrum minimum*. Curiously, Prado-Alvarez *et al.* (2012) reported an unexpected decrease in haemocyte apoptosis in *Mytilus galloprovincialis* (from field) highly contaminated by OA and other DSP toxins. The authors observed the same effect after incubating mussel haemocytes *in vitro* with high concentrations of purified OA toxin. They suggested that OA may inhibit the apoptosis pathway in haemocytes (e.g. inhibition of caspases). In contrast, in a more recent study, Prado-Alvarez *et al.* (2013) observed an increase in haemocyte apoptosis when the clam *Ruditapes decussatus* was exposed to *Prorocentrum lima* (OA producer) and when its haemocytes were incubated *in vitro* to the OA toxin. It should be emphasized that the recent genome sequencing of *C. gigas* (Zhang *et al.*, 2012) revealed that this bivalve has a particularly powerful anti-apoptosis system with more than 45 genes encoding for protein apoptosis inhibitors (IAPs), which is much higher than in other organisms such as sea urchins and humans that possess only 7 and 8 IAP coding genes, respectively.

Phenoloxidase (PO) activity is also an important immune parameter commonly used to assess invertebrate health, particularly in crustaceans and insects (Liu *et al.*, 2007; Cerenius *et al.*, 2010). In bivalves, however, the activity of this enzyme has not yet been clearly related to the immune system. Nonetheless, several authors have been using this enzyme activity to express shellfish health status (Thiagarajan *et al.*, 2006; Aladaileh *et al.*, 2007; Schleder *et al.*, 2008). In this study, the level of PO activity was altered in both species during the algal blooms, but in contrasting manners. In mussels, PO activity increased by about 50% during the toxic bloom at PA but did not vary at PZ. In contrast, in oysters, PO activity decreased by about 40% in oysters during the PZ bloom. These results differ from those of Mello *et al.* (2010), where PO activity decreased (30%) in *P. perna*, but did not vary in *C. gigas*. Haberkorn *et al.* (2010b) also referred to contrasting results in *C. gigas* fed *A. minutum* (which causes PSP). In a first experiment, the authors observed a reduction on PO activity, but in a second one, the enzyme activity augmented. The authors suggested that these differences could be due to the different gonad maturation stages of the animals from both experiments.

Naturally occurring lectins may function as pattern recognition proteins (PRPs) that recognize molecular sugars on pathogen surface. It was already shown that the concentration of lectins may be modulated under stressful conditions (Schleder *et al.*, 2008; Chikalovets *et al.*, 2010; Song *et al.*, 2010). In this study, the agglutinating titre (AT) of mussel and oyster haemolymph did not vary during both *D. acuminata* blooms. Only a tendency to higher values was observed in *P. perna* during the PA bloom. These results are consistent with those of Mello *et al.* (2010) who also did not observe significant differences in the AT of both bivalves during the intense dinoflagellate bloom. Similarly, Haberkorn *et al.* (2010b) did not find changes in the AT of *C. gigas* fed *A. minutum*, as well as da Silva *et al.* (2008) and Hégaret *et al.* (2009) in the cockle *Ruditapes philippinarum* exposed to different harmful microalgae. Altogether these results, although still limited, suggest that the concentration of lectins from bivalve haemolymph is not influenced by toxic microalgae blooms.

The total protein concentration (PC) in bivalve haemolymph may be affected during stress situations such as physiological changes (Schleder *et al.*, 2008) or in the presence of xenobiotics (Auffret *et al.*, 2006). In this study, only the PC of mussels from PZ increased significantly (63%) during the toxic bloom. Interestingly, the increase of PC was not the result of an increase of immune proteins, such as agglutinins and phenoloxidase. Similar results were also reported by Mello *et al.* (2010) in *P. perna*.

Histopathological analysis was carried out only in oysters. The results revealed an intense migration and diapedesis of haemocytes through the digestive epithelium of intestine, digestive tubules and especially stomach, whose epithelium was clearly altered in oysters exposed to the algal bloom. Curiously, the THC and DHC did not fall in this oyster group. Maybe, when the animals were sampled, the production of new haemocytes had already been stimulated and the new cells replaced the infiltrated ones. Several studies pointed out the occurrence of lesions in different tissues of bivalves exposed to HABs. Juvenile scallops *Nodipecten subnodosus* (Ascencio *et al.*, 2007) and *Argopecten ventricosus* (Escobedo-Lozano *et al.*, 2012) exposed to the dinoflagellate *Gymnodinium catenatum* (PSP producer) exhibited epithelial melanization (gills and mantle), and haemocyte infiltration and aggregation in several scallop tissues. Also in *C. gigas* seeds, abnormalities such as scrubs and erosions of the digestive tubules were reported after exposure to *P. minimum* (Imojen *et al.*, 2005). Similarly, in mussels *M. edulis* exposed to *P. minimum*, haemocytes migrated through diapedesis into the stomach and intestine (Galimany *et al.*, 2008b).

As previously pointed out by Mello *et al.* (2010) in accordance with this study, the immune parameters of *P. perna* were more affected than those of *C. gigas* during the blooms of *D. acuminata*. These results might be due to the higher toxin accumulation (OA and derivatives) in mussel (10× more) than in oyster tissues. Other authors have also established that mussels accumulate larger quantities of phycotoxins than oysters and scallops (Reizopoulou *et al.*, 2008; Lindegarth *et al.*, 2009; Kacem *et al.*, 2010). In addition to the higher toxin accumulation, mussels may also have a lower toxin clearance rate than oysters and other bivalves. In effect, Kacem *et al.* (2010) showed that OA clearance in *C. gigas* was faster than in *M. galloprovincialis*. Similarly, Vale (2004) reported that the clam *Donax* spp. has a higher OA clearance rate than *M. edulis*.

In conclusion, even though the obtained results did not express a consistent pattern of immunological response to harmful algal bloom, they support the observations of Mello *et al.* (2010). We can thus infer that mussels are indeed more immunologically susceptible to blooms of *D. acuminata* than oysters. It is also of particular significance to determine if DSP toxins, even though non-lethal to bivalves, may work synergistically with other stress factors and reduce their resistance to infections, thus putting the local shellfish production at risk.

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