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Effects of the toxic dinoflagellates *Prorocentrum lima* and *Ostreopsis* cf. *ovata* on immune responses of cultured oysters *Crassostrea gasar*

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Abstract :

Oyster production in Brazil has been highlighted as an important economic activity and is directly impacted by the quality of the environment, which is largely the result of human interference and climate change. Harmful algal blooms occur in aquatic ecosystems worldwide, including coastal marine environments which have been increasing over the last decades as a result of global change and anthropogenic activities. In this study, the native oysters Crassostrea gasar from Northeast of Brazil were exposed to two toxic benthic dinoflagellate species, Prorocentrum lima and Ostreopsis cf. ovata. Their respective effects on C. gasar physiology and defense mechanisms were investigated. Oyster hemocytes were first exposed in vitro to different concentrations of both dinoflagellate species to assess their effects on hemocyte functions, such as phagocytosis, production of reactive oxygen species, as well as mortality. Results highlighted an alteration of hemocyte phagocytosis and viability in presence of O. cf. ovata, whereas P. lima did not affect the measured hemocyte functions. In a second experiment, oysters were exposed for 4 days in vivo to toxic culture of O. cf. ovata to assess its effects on hemocyte parameters, tissues damages and pathogenic Perkinsus spp. infection. An increase in hemocyte mortality was also observed in vivo, associated with a decrease of ROS production. Histopathological analyses demonstrated a thinning of the epithelium of the digestive tubules of the digestive gland, inflammatory reaction and a significant increase in the level of infection by Perkinsus spp. in oysters exposed to O. cf. ovata. These results indicate that oysters C. gasar seem to be pretty resilient to an exposure to P. lima and may be more susceptible to O. cf. ovata. Furthermore, the latter clearly impaired oyster physiology and defense mechanisms, thus highlighting that harmful algal blooms of O. cf. ovata could potentially lead to increased susceptibility of C. gasar oysters to parasite infections.

Highlights

► *Ostreopsis* cf. *ovata* is potentially more toxic to oyster hemocytes than *Prorocentrum lima* ► *Ostreopsis* cf. *ovata* affects immune and cell parameters of *C. gasar* ► *Ostreopsis* cf. *ovata* favors *Perkinsus* spp. proliferation in oyster tissues ► Digestive gland of *C. gasar* is the organ affected by *O*. cf. *ovata*

Keywords : bivalve, harmful algal blooms, histopathology, hemocytes, toxic dinoflagellate, benthic, oyster

1. INTRODUCTION

Oyster production in Brazil has been highlighted as an important economic activity, but also for its social nature, bringing diverse benefits to the coastal community. In farms from the southern region of Brazil it stands out for its high productivity and use of specialized technology. The oyster *Crassostrea gigas* was introduced in Brazil in the 1990s and is farmed only in the State of Santa Catarina (South), which is the largest national bivalve producer in Brazil (95%). In 2019, Santa Catarina state alone, has accounted for 2,760 of *C. gigas* but also12,294 tons of mussels *Perna Perna*, 5.2 tons of scallops *Nodipecten nodosus*, 96.8 tons of *C. gasar* (https://www.infoagro.sc.gov.br). In contrast, farms in the North and Northeast regions of Brazil, use production methods that are extensive and artisanal, and the production of oysters is focused on native species *C. gasar* and *C. rhizophorae*. According to the data of the Brazilian Institute of Geography and Statistics (IBGE, https://sidra.ibge.gov.br/tabela/3940), the production of native oysters *C. rhizophorae* and *C. gasar*, in the North and Northeast was around 86.6 tons and 120.6 tons in 2019, respectively, however it is a consensus that they are underestimated.

Oyster production is directly impacted by the quality of the environment, which is largely the result of human interference and climate change. One of the impacts is the increased frequency of harmful algal blooms (HABs) (Bindoff et al., 2019). Moreover, one of the main problems in oyster production is the occurrence of disease outbreaks caused by pathogens, such as protozoans *Perkinsus* spp. (Villalba et al., 2011). These parasites were reported in several states of Northeast Brazil (Dantas-Neto et al., 2015; Luz et al., 2018; Queiroga et al., 2015; Sabry et al., 2013; Silva et al., 2018) and recently in Santa Catarina state in Southern Brazil (Luz Cunha et al., 2019).

HABs occur in aquatic ecosystems worldwide, including coastal marine environments. More recently, an increase in the intensity and frequency of marine HABs have been noticed which consequently affects the aquatic organisms and marine resources (Anderson, 2009; Hallegraeff, 2010). Even low abundances of toxic dinoflagellate species may be deleterious to aquatic species, ecosystems, and human health (Landsberg, 2002). However, in the Brazilian coast, routine monitoring programs for HABs occurrence is still scarce and limited to the state of Santa Catarina (Castro et al., 2016; Mafra et al., 2019). An increasing interest in benthic HABs is related to the

remarkable toxicity of benthic dinoflagellates from the genera *Prorocentrum*, *Ostreopsis* and *Gambierdiscus*.

The cosmopolitan *Prorocentrum lima* species complex is widely distributed in tropical and temperate regions (Nishimura et al., 2020). In the Brazilian coast, *P. lima* was found epiphytically on macroalgae and coral reefs in Pernambuco, Bahia, Rio de Janeiro, and Paraná states (Moreira-González et al., 2019; Nascimento et al., 2016, 2017). *P. lima* produces okadaic acid and its analogues (Hoppenrath et al., 2013), which presents considerable cytotoxicity and selective inhibitory activity against two essential enzymes responsible for protein dephosphorylation in eukaryotic cells: type 1 protein phosphatase (PP1) and 2A (PP2A) (Hambright et al., 2014; Hu et al., 2010; Valdiglesias et al., 2013). The toxic benthic dinoflagellate *Ostreopsis* cf. *ovata* was found in tropical and temperate regions (reviewed in Accoroni and Totti, 2016; Nascimento et al., 2020). Massive blooms of *O.* cf. *ovata* form a biofilm that covers macroalgae, corals, sponges, and other marine invertebrates in the Mediterranean Sea (Accoroni et al., 2015) and in the South Atlantic Ocean (Nascimento et al., 2008). *O.* cf. *ovata* was found in the states of Rio Grande do Norte, Bahia, Rio de Janeiro, São Paulo, Paraná, and in the oceanic islands of Fernando de Noronha, Trindade, and São Pedro and São Paulo Archipelago (Gómez et al., 2017; Nascimento et al., 2020; Tibiriçá et al., 2019).

The benthic dinoflagellate *Ostreopsis* cf. *ovata* synthesizes palytoxins (PLTXs) analogues, which are a large and very complex molecule with both lipophilic and hydrophilic regions (Wang, 2008). Toxicity mechanism are related to PLTXs alteration of ion homeostasis mechanisms in the cells (Rossini and Bigiani, 2011), directly or indirectly related to their interaction with Na^+/K^+-ATPase and its consequent conversion into a non-specific cation channel (Bellocci et al., 2011).

The increasing magnitude and severity of HABs makes them a matter of great concern due to their negative effects on marine invertebrates and vertebrates causing cellular disruption, physiological impairment, altered behavior and mass mortalities (reviewed in Neves and Rodrigues, 2020). A wide variety of physiological responses can result from bivalve exposure to harmful microalgae. Among them, the immunological responses are one of the most studied (reviewed in Lassudrie et al., 2020). The phycotoxins accumulated in bivalve tissues reduces their health status and fitness, which leads to physiological damage and immunological impairment. Histopathological changes in bivalve exposed to harmful microalgae have been reported

(Carella et al., 2015; García-Lagunas et al., 2015; Haberkorn et al., 2010; Neves et al., 2019).

Some studies have demonstrated that biological toxins can alter the expression of ABC transporters in marine bivalves tissues (Buratti et al., 2013; Huang et al., 2014, 2015). ABC transporters are transmembrane proteins with two ATP binding sites. Some of these proteins confer a multixenobiotic resistance phenotype (MXR) in aquatic organisms, which is analogous to the multidrug resistance phenotype in mammalian tumor cells. This phenotype (MXR) acts by decreasing intracellular concentrations of xenobiotics below their toxic level (Bard, 2000). The presence of ABC transporters has been demonstrated in several bivalves, such as the oysters *C. virginica* (Keppler and Ringwood, 2001), *C. gigas* and *C. gasar* (Marques-Santos et al., 2017), mussels *Mytilus californianus*, *M. edulis* and *M. galloprovincialis* (Della Torre et al., 2014; Eufemia and Epel, 2000; Minier et al., 1993).

Due to great concern regarding HABs adverse effects on aquatic biota and shellfish production, the present study aimed to fully evaluate *in vitro* and *in vivo* effects of both toxic dinoflagellates *P. lima* and *O.* cf. *ovata* on defense responses of the native oyster *C. gasar* cultured in the North and Northeast of Brazil and the related histopathological effects. The present study disclosed for the first time the immune and histopathological response of native oysters infected by *Perkinsus* to toxic benthic dinoflagellates, as well as the activity of ABC transporters in oyster hemocytes after toxic exposure.

2. MATERIALS AND METHODS

2.1. Sampling and culture of oysters

Adult oysters *Crassostrea gasar* (> 7 cm in shell length) were sampled from a commercial farm located at Mamanguape River estuary (Marcação municipality, Paraíba State, 06°47’08.2” S, 34°59’46.7” W). The oysters were transported to laboratory facility where they were cleaned of incrustations and maintained in tanks (40 L) with 20 L of sea water at 27 salinity, under constant aeration. The oysters were acclimated under laboratory conditions for 5 days.

2.2. Dinoflagellate cultures

Clonal cultures of the benthic dinoflagellates *P. lima* (UNR-09 strain) and *Ostreopsis* cf. *ovata* (UNR-05 strain) used in this study were isolated from Armação dos Búzios, Rio de Janeiro state (22°45'18" S, 41°54'07" W) (Nascimento et al., 2016, Neves et al., 2018). The toxicity of the *P. lima* strain (UNR-09) was evaluated using *Artemia salina* bioassay (as described in Neves et al., 2017), in which 100% of brine shrimp mortality was recorded after 4h of exposure to 200 cells mL^{-1}.

Both microalgae were grown in culture flasks (75 cm^2 / 250 mL) in F2/2 medium (Sigma, Guillard's F/2) prepared using filterer sterilized seawater (FSSW; 0.22 µm) at 27 salinity and cultured in a temperature-controlled BOD (Bio-Oxygen Demand) incubator at 24 ± 1 ºC, and 12:12 h, dark-light cycle.

2.3. *In vitro* hemocyte exposure to the toxic dinoflagellates *P. lima* and *O.* cf. *ovata*

Hemolymph was withdrawn from the adductor muscle of oysters with 1 mL syringe attached to a needle (21 G) through a hole made in the dorsal region of the shell. Hemolymph was checked for impurities under an optical microscope. Impure hemolymph samples were discarded, and the clean hemolymph samples were immediately deposited in microtubes kept on ice to prevent cell aggregation.

The assay consisted of hemolymph samples from five individual oysters exposed separately to the toxic microalgae suspensions *P. lima* and *O.* cf. *ovata* (1:1, v:v) for 3 h at 24 ± 1 ºC. Two concentrations of *P. lima* (10^3 and 10^2 cells mL^{-1}) and three doses of *O.* cf. *ovata* (10^4, 10^3 and 10^2 cells mL^{-1}) were tested. Microalgae densities were chosen according to abundances of these dinoflagellate species in Brazilian natural environments (our data, not published). Controls were hemolymph in FSSW and F2/2 medium in the same proportion as treatments.

Mortality, phagocytosis and reactive oxygen species (ROS) production of hemocytes were measured by flow cytometry as detailed below (section 2.4.2). FACSCalibur and FACSCanto II (BD Biosciences, San Jose, California, USA) were used for these analyses. Data obtained were analyzed with Flowing software (version 2.5.1).

2.4. *In vivo* oyster exposure to the toxic dinoflagellate *O.* cf. *ovata*

2.4.1. Experimental design

Assays were performed in a static system, with constant aeration, in 3 L tanks having 2 L of seawater (in triplicate), containing 7 oysters for each of the two conditions, (a) oysters exposed to a daily suspension of toxic *O.* cf. *ovata* culture (60-200 cells mL^{-1}, further called "treatment") for 4 days or (b) not exposed (further called "control"). No water changes were made during the assay in order to prolong the effect of the bloom. The benthic dinoflagellate *O.* cf. *ovata* was chosen for these assays because *P. lima* did not affect hemocytes on *in vitro* exposure.

Oyster mortality was monitored daily. The assay was repeated twice in order to sample tissues for different analyses: hemocyte parameters, histopathology and *Perkinsus* spp. infection.

2.4.2. Hemocyte responses to *O.* cf. *ovata*

Hemolymph was randomly sampled from 5 oysters from each replicate, as previously described, and deposited in flow cytometry tubes, which received the corresponded probes for each specific analysis. Tubes were incubated for 1 h at 24 ± 1 ºC before flow cytometry measurements.

Hemocyte mortality was estimated by double staining with SYBR@Green I (Molecular Probes, Molecular Probes, final concentration of 10^{-4} from the original solution) and propidium iodide (Sigma-Aldrich, final concentration 10 μg mL^{-1}) (Hégaret et al., 2003a). Results are presented as percentage of dead hemocytes.

Total (THC) and differential hemocyte counts (DHC) were estimated using side scatter (SSC), which measures internal complexity, and forward scatter (FSC), a proxy of cell size. Sample dilution, cytometer flow (medium, 35 ± 5 μL min^{-1}) and acquisition time (30 s) were considered to estimate cell concentration (THC), which was expressed as cells mL^{-1}. For DHC, hemocyte subpopulations were discriminated in dot plot according to the relative flow cytometric morphological parameters (SSC *vs* FSC) and expressed as percentage of granulocytes, hyalinocytes and blast-like cells (Hégaret et al., 2003b).

Reactive oxygen species (ROS) production was estimated using 2′7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, final concentration 10 μM) (Hégaret et al., 2003a; Queiroga et al., 2013).

Phagocytic activity was estimated using fluorescent latex beads (Fluoresbrite® Yellow Green Microspheres 2 μm, Polysciences, final concentration 0.1%, 4.5×10^{7}

cells mL^{-1}). Phagocytic rate was expressed as the percentage of hemocytes that had engulfed two or more fluorescent beads (Hégaret et al., 2003a; Queiroga et al., 2013).

The ABC transporters activity of hemocyte was assessed using the calcein-AM (Sigma-Aldrich, final concentration 200 nM). Calcein-AM crosses the cell plasma membrane and accumulates in the cytoplasm as fluorescent calcein after undergoing the action of esterases that remove the AM group. Calcein-AM is a substrate for ABC transporters, which expel it from the cell; therefore, its accumulation in the cytoplasm is inversely proportional to the activity of the transporters (Marques-Santos et al., 2017). A total of 10,000 cells per sample were analyzed. The activity of ABC transporters was also evaluated in hemocyte subtypes. The results were expressed in arbitrary units (A.U.) of fluorescence (geometric mean).

ABCB1 and ABCC1 isoforms' activity was evaluated using the specific blockers, Reversin 205 (Sigma-Aldrich, final concentration 10 µM) and MK571 (Sigma-Aldrich, final concentration 10 µM), respectively. The positive control for this analysis was hemocytes that received only calcein-AM. The activity of the ABCB1 and ABCC1 proteins was expressed in fold change of the fluorescence of the positive control in relation to fluorescence of samples with the blockers.

2.4.3. Histopathological analysis

Histological sections were performed on oysters exposed to *O.* cf. *ovata* in order to detect possible tissue disruption induced by exposure to the toxic dinoflagellate.

After hemolymph withdrawn, oysters were opened by breaking shell hinge and the animal body was carefully removed from the shell. A cross-section of approximately 5 mm was made including gills, intestine, stomach, gonads and digestive gland and immediately fixed in Davidson's solution (Howard et al., 2004) for 24 h, at 4 ºC. Subsequently, the samples were processed by conventional histological procedures for inclusion in paraffin. Sections (5 µm) were cut and stained with Mayer's Hematoxylin and Eosin (Howard et al., 2004). Slides were analyzed with an Olympus BX41 optical microscope, and the images were obtained with Olympus Q-Color 5TM camera.

In the histological slide, all organs were analyzed for possible changes induced by the treatment. In the digestive gland region, a more careful analysis was performed, as follows: Digestive gland of 5 oysters from each replicate (treatment and control) were submitted to a morphometrical analysis in order to estimate the digestive tubule (DT) lumen diameter and DT epithelium width (**Figure 1**). On each oyster slide, thirty

DT were randomly selected per oyster for measurements (around 450 tubules analyzed per treatment). Considering that the DT is not a perfect sphere, two measurements of the lumen diameter were taken, a larger (d1) and a smaller (d2), perpendicularly to each other (**Figure 1**). The area (A) of the DT lumen were calculated using the following formula: $A = r1 * r2 / \pi$, where r1 and r2 are the radii from the diameters ($r = d / 2$) and $\pi = 3.14$. Four measurements of the epithelium width were taken from each DT (**Figure 1**).

2.4.4. *Perkinsus* spp. diagnosis

The recommended method for surveillance of *Perkinsus* spp. infections in bivalves, the Ray's fluid thioglycollate medium (RFTM) culture assay, was used in order to evaluate the effect of *O.* cf. *ovata* on oyster's immunological response to the infection by *Perkinsus* that affect this oyster population (Queiroga et al., 2015). Two demibranchies of 5 randomly collected oysters per tank (15 per group) were excised and incubated for 5 days in tubes containing RFTM with added penicillin, streptomycin (final concentrations 100 U mL^{-1} and 0.1 mg mL^{-1}, respectively), and nystatin (100 U mL^{-1}), in the dark at ambient temperature (around 25ºC). Gills were macerated with a scalpel blade on a slide and stained with Lugol's iodine solution. The preparation was examined under a light microscope, and parasite infection intensities were ranked based on the scale of Mackin (Ray, 1954) adapted by da Silva et al. (2013), as follows: Level 1: Very Light, Level 2: Light, Level 3: Moderate and Level 4: Heavy. The mean of the infection level was estimated as the sum of the infection intensity levels (1-4) divided by the number of infected oysters at each sampling time (Bush et al., 1997).

2.5. Statistical analysis

Normality of the data was verified by the D'Agostino & Pearson test before all analyses. Percentage data (phagocytosis rate and mortality) were transformed before analysis by dividing the arcsine of the square root of the percentage by 100.

For *in vitro* assays, differences in immunological parameters between groups (treatment and control) were evaluated by one-way analysis of variance (ANOVA) followed by LSD post-hoc test. For *in vivo* assays, differences between groups (treatment and control) were analyzed by Unpaired *t*-test with Welch's correction (for normal data set) or Mann Whitney test when data set were not normally distributed.

Differences were considered significant when $P \leq 0.05$, for all analyses, except for *Perkinsus* infection level which considered significant when $P \leq 0.06$.

Data were expressed as mean ± standard error. Statistical analyses were performed using the Statgraphics Centurion XV software and graphs were plotted using the software GraphPad Prism 7.0 and R package.

3. RESULTS

3.1. *Ostreopsis* cf. *ovata* is potentially more toxic to oyster hemocytes than *P. lima*

The toxic dinoflagellates studied affected hemocytes in different ways. Microalgal growth medium (F2/2) did not cause any significant cell alteration (in comparisons with hemocytes in FSSW).

Ostreopsis cf. *ovata* exposure induced an increase in hemocyte mortality (P = 0.0000). This effect was observed at the lowest concentration tested (10^2 cells mL^{-1}: 50.8 ± 6.65%), however it was more expressive in the highest concentrations, 81.2 ± 1.51% (10^3 cells mL^{-1}) and 86.9 ± 1.50% (10^4 cells mL^{-1}), without significant differences between them. In contrast, *P. lima* exposure did not cause any significant hemocyte mortality in any of the tested concentrations (10^2 cells mL^{-1}: 5.6 ± 1.42% and 10^3 cells mL^{-1}: 5.1 ± 0.73%). Unexposed oysters showed low mortality when incubated with FSSW and F2/2, respectively 3.8 ± 0.67% and 3.7 ± 0.80% (**Figure 2A**).

Hemocyte phagocytosis rate was reduced after hemocyte exposure to *O.* cf. *ovata* (P = 0.0001), regardless of the dinoflagellate concentration (10^2 cells mL^{-1}: 2.1 ± 0.44%, 10^3 cells mL^{-1}: 1.6 ± 0.35% and 10^4 cells mL^{-1}: 2.5 ± 0,55%). Conversely, *P. lima* exposure did not significantly affect hemocyte phagocytosis at any concentration tested (**Figure 2B**). Controls with FSSW and F2/2 showed phagocytic rates of 4.4 ± 0.50% and 3.8 ± 0.30%, respectively.

Total hemocyte ROS production was not affected by any toxic dinoflagellate exposure (P = 0.5583, **Figure 3A**). Similarly, ROS production of granulocytes (P = 0.4121), hyalinocytes (P = 0.8214), and blast-like cells (P = 0.8546) were not significantly different among treatments and controls (**Figures 3B-D**).

3.2. *Ostreopsis* cf. *ovata* affects immune and cell parameters of *C. gasar*

Immunological and cell responses of oysters after 4 days of exposure to the toxic dinoflagellate *O.* cf. *ovata* are shown in **Tables 1** and **2**. THC, percentage of hemocyte

subpopulations, phagocytosis rate and ABC proteins activity did not significantly change. On the other hand, hemocyte mortality doubled in treated group when compared with control group. ROS production strongly decreased in hemocytes of treated oysters. This reduction was more pronounced in blast-like cells and hyalinocytes (3.4x and 3.8x, respectively), than in granulocytes (2.3x) (**Table 1**).

Calcein intracellular accumulation in hemocytes from treated and control oysters were similar. The same result was observed after using specific blockers for ABCB1 and ABCC1 transporters (**Table 2**).

3.3. *Ostreopsis* cf. *ovata* favors *Perkinsus* spp. proliferation in oyster tissues

Twenty-nine oysters were infected by *Perkinsus* spp., only one from exposed group was uninfected after 4 days of exposure. The mean level of infection was significantly higher in oysters exposed to *O.* cf. *ovata* (3.1 ± 0.31) than in control oysters (2.3 ± 0.29) (Mann Whitney test, $P = 0.0651$) (**Figure 4**).

3.4. Digestive gland of *C. gasar* oyster is the organ affected by *O.* cf. *ovata*

A thorough histological evaluation was performed on tissues of the *C. gasar* oysters, both exposed and unexposed to *O.* cf. *ovata*. The analyses revealed that the digestive gland was the only organ that was morphologically altered after 4 days of exposure to the toxic dinoflagellate (**Figure 5**). The DT epithelium suffered a significant reduction in oysters exposed to the dinoflagellate, although the DT area was not modified (**Table** 3). Additionally, hemocytic infiltration was observed in connective tissue among DT of exposed oysters (**Figure 5**).

4. DISCUSSION

The present study evaluated the potential noxious effects of two toxic benthic dinoflagellates *Prorocentrum lima* and *Ostreopsis* cf. *ovata* using two different approaches, (a) hemocytes (*in vitro*) exposure and (b) oysters *Crassostrea gasar* (*in vivo*) exposure; the latter representing a more realistic approach to what happen in the natural environment. Results showed that *C. gasar* oysters responded differently to the tested toxic microalgae; *P. lima* exposure had no effect on the hemocytes, while *O.* cf. *ovata* had negatively affected hemocyte function and viability. The benthic

dinoflagellate *O.* cf. *ovata* also induced a significant impact on oyster tissue and immune responses, including an increase in infection rate by *Perkinsus* spp.

The main impact of *O.* cf. *ovata* on oyster hemocytes was indeed an increase in cell mortality. Cell mortality, in general, can result from injuries triggered by necrotic process or can be induced by programmed cell death, such as apoptosis. A wide variety of biotic and abiotic factors are capable of inducing apoptosis in bivalve hemocytes (Goedken and DeGuise, 2002; Renault, 2015; Romero et al., 2015), including harmful microalgae (Hégaret et al., 2009; Lassudrie et al., 2020). In the present study, *P. lima* did not cause any hemocyte mortality following *in vitro* exposure, despite being a toxin producer. A *P. lima* strain simultaneously isolated from the same sampling location as the one used in the present study synthesized 38.5 pg of okadaic acid (OA) cell^{-1} (Nascimento et al., 2016). Hégaret and Wikfors (2005) investigated the effect of *P. minimum* and found a similar result: low mortality of *C. virginica* hemocytes after 2 days of *in vivo* exposure. Similarly, *M. edulis* mussels exposed to *P. minimum* for 9 days did not experienced hemocyte mortality (Galimany et al., 2008). Therefore, the overall findings suggest that *Prorocentrum* spp. might have low or no direct cytotoxicity to oyster hemocytes and, consequently, to *C. gasar* hemocytes.

In contrast, *O.* cf. *ovata* caused a significant increase in hemocyte mortality (average of 73%), especially in the two highest microalgal concentrations (10^3 and 10^4 cells mL^{-1}), which corresponded to microalgae: hemocytes ratio of 1:400 and 1:40, respectively. Hemocyte mortality was again observed in the *in vivo* assay, albeit much less remarkably (9.3% increase). One explanation is a likely higher toxin uptake by hemocytes in *in vitro* assay than in *in vivo* experiments. Cytotoxic effect on hemocytes induced by *O.* cf. *ovata* exposure might be due to palytoxins (PLX) analogues (Nascimento et al., 2020; Pelin et al., 2016). The *O.* cf. *ovata* UNR-05 strain produces palytoxins (PLTXs) analogues, mostly ovatoxin-a (68.2 pg cell^{-1}) and -b (31.7 pg cell^{-1}) (Nascimento et al., 2020). Currently, more than 25 analogues have been reported, including PLTX, 42-hydroxy PLTX, homoPLTX, bis-homoPLTX, deoxyPLTX, neoPLTX, isoPLTX, ovatoxins-a, ovatoxins-k ostreocins-b, ostreocins-d (Brissard et al., 2015; Pelin et al., 2016). The mechanism of action of these toxins has been related to the interruption of the Na^+/K^+ pump function (Bellocci et al., 2011), which, after binding to palytoxin, behaves like a cationic channel, abolishing the ion gradient and triggering adverse biological effects (Pelin et al., 2011). In fact, a study by Gorbi et al. (2012) revealed that exposure to *O.* cf. *ovata* toxins resulted in inhibition of Na^+/K^+

pump activity in the mussels *Mytilus galloprovincialis*, suggesting a direct involvement of PLTX-group compounds in the activity of this protein. An inhibition of the initial development and mortality of eggs and embryos of the sea urchin *Lytechinus variegatus* was detected in the first hours (1 to 3 h) of exposure to the same strain of *O.* cf. *ovata* used in the present study (UNR-05) at concentrations of 400 and 4,000 cells mL^{-1} (Neves et al., 2018). Moreover, very low concentration of this strain of *O.* cf. *ovata* (42 cells mL^{-1}) was able to cause a strong toxic effect on *Artemia salina* in acute toxicity assays (Neves et al., 2017). Differential cytotoxicity induced by extracts of benthic dinoflagellate species was also detected using seven distinct mammalian cell lines; highest toxic effects were detected for *O.* cf. *ovata* ($EC_{50,\ 24\ h}$= 3.3–40 cells mL^{-1}) compared to the effects induced by *P. lima* ($EC_{50,\ 24\ h}$= 191-1,027 cells mL^{-1}) (Neves et al., 2020). Further investigation of the effect of the individual toxins on hemocyte response of the oyster *C. gasar* will enhance the understanding of the causative effect of this cytotoxic mechanism.

In addition to the high hemocyte mortality, *O.* cf *ovata* was also associated with impaired defense responses in the oyster *C. gasar*. Phagocytosis is one of the main cellular defense responses in bivalves performed by hemocytes (Allam and Raftos, 2015). Hemocyte phagocytosis inhibition was observed after *in vitro* exposure to *O.* cf. *ovata* in all tested concentrations. This reduction could be partially explained by the high cytotoxicity previously described. In contrast, no significant change in this parameter was observed when oysters *C. gasar* were exposed *in vivo* to *O.* cf. *ovata*, which is consistent with low hemocyte mortality observed after *in vivo* assay. Some studies suggested that reduced phagocytosis could be due to low concentration of granulocytes in the hemolymph, since oyster granulocytes are more efficient in the engulfment of particles than hyalinocytes (Donaghy et al., 2009; Hégaret and Wikfors, 2005b; Hong et al., 2013; Lambert et al., 2007). In fact, a decrease in granulocytes was observed in mussels *M. galloprovincialis* after 7 and 14 days of exposure to *O.* cf. *ovata* (Gorbi et al., 2013). However, our data did not show any significant variation in the proportion of hemocytes populations in response to *O.* cf. *ovata* exposure. Further studies need to be carried out to investigate the inhibitory effect on *C. gasar* hemocyte phagocytosis when exposed to this toxic dinoflagellate.

Malagoli et al. (2008) observed a relationship between okadaic acid (OA) effects on phagocytosis activity and temperature. At 20 ºC, OA (82.7 ng mL^{-1}) did not change hemocyte phagocytosis of mussels *M. galloprovincialis*, while at 25 ºC, it increased

phagocytic activity. In the present study, *in vitro* assays were carried out at 24 ºC, and it does not seem to have significantly interfered with the phagocytosis of the hemocytes. Like other oyster species, *C. gasar* may be more resilient to microalgal toxins than mussels (Lassudrie et al., 2020). Corroborating this hypothesis, a previous study with the mussel *Perna perna* using the same strain of *P. lima* used in the current study (UNR-09) revealed an increase in phagocytic activity of hemocytes after 48 h of exposure (Neves et al., 2019). In our study, we did not evaluate the *in vivo* effect of *P. lima* since hemocyte toxicity was not observed when this microalgal species was tested *in vitro*.

Total and differential hemocyte counts are widely used parameters to assess health status of bivalves (Lassudrie et al., 2020; Mello et al., 2010; Vargas-Albores and Barracco, 2001). In the present study, these parameters did not change in oysters after *in vivo* exposure to *O.* cf. *ovata*. The opposite was observed in mussels *M. galloprovincialis* exposed to 10^5 cells mL^{-1} of *O.* cf. *ovata* for 7 and 14 days, which was associated with a decrease in granulocytes (Gorbi et al., 2013). A similar study on *M. galloprovincialis* naturally exposed to *O.* cf. *ovata*, in two locations, differently impacted at estimated concentrations of $1.3 \sim 1.6 \times 10^6$ cells g^{-1}, revealed an increase in granulocytes after the first month of exposure (Gorbi et al., 2012). However, THC has not been evaluated in studies above-mentioned.

Studies on hemograms of bivalves exposed to microalgae of the genus *Prorocentrum* showed different patterns of THC responses. In *C. virginica* oysters exposed to *P. minimum* (10^4 cells mL^{-1}) for 2 days, the increase in THC was mainly influenced by an increase in granulocytes (Hégaret and Wikfors, 2005). While in *P. perna* mussels exposed to *P. lima* (900 cells mL^{-1}) for 4 days, the decrease in THC was possibly associated with a reduction of hyalinocytes (Neves et al., 2019). *Prorocentrum minimum* triggered changes in the general function of all hemocytes types and *P. lima* has induced migration of the defense cells to damage tissues (Neves et al., 2019), which explains the differences in THC. A recent review of the effects of harmful microalgae on hemocytes of marine bivalves (Lassudrie et al., 2020) highlights the variations in responses among studies performed on the same bivalve / microalga biological model. This fact indicates that there are likely other factors, such as environmental conditions and the physiological state of the animal that can directly affect the defense response of the bivalve to the microalga species. This also suggests that each biological interaction model (bivalve-microalga) and its environment (habitat) should be studied and

understood independently. Moreover, the high variation in intracellular toxin content and profile within the same microalgal species must to be considered (Guerrini et al., 2010; Moreira-González et al., 2019; Neves and Rodrigues, 2020), which may directly influence its toxicity to marine organisms.

ROS production of *C. gasar* hemocytes was not altered after *in vitro* exposures to both dinoflagellates *P. lima* and *O.* cf. *ovata*, which is consistent with the fact that phagocytosis was not induced as well. ROS production is a lytic mechanism activated after phagocytosis of infection agents (Allam and Raftos, 2015; Cheng, 1996) or in mitochondrial complex III (Donaghy et al., 2012). Surprisingly, a decrease in ROS production was observed in *C. gasar* exposed to *O.* cf. *ovata* without altering phagocytic rate. This reduction could be a result of the oyster antioxidant defense response orchestrated by antioxidant and detoxifying enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione (GSH), glutathione peroxidase (GPx), against the microalgal toxins damages (Liu et al., 2020). ROS reduction has been reported in other studies on bivalves exposed to harmful microalgae (Fabioux et al., 2015; Lassudrie et al., 2014; Mello et al., 2013). Specifically, *O.* cf. *ovata* was able to promote an activation of antioxidant enzymes in cells of the digestive gland of *M. galloprovincialis* (Gorbi et al., 2012, 2013). It is possible that the passage of microalgal cells through the bivalve digestive tract caused the release of its intracellular toxins (Neves et al., 2019) and toxins absorption (Landsberg, 2002; Tibiriçá et al., 2019) , which might have induced a potential antioxidant response. Direct exposure of the hemocytes to *O.* cf. *ovata* through *in vitro* assays did not affect the production of ROS by hemocytes, which is probably related to the short-term exposure time (4h) or mild exposure to intracellular toxins.

Regarding the ROS production by hemocyte subpopulations, hyalinocytes and blast-like cells were the ones that suffered the largest reduction (approximately 70%). Granulocytes are considered the “professional phagocytes”, and accordingly had the lowest reduction in ROS production. It is known that hemocytes perform other physiological functions such as tissue repair, shell production, and nutrition (Cheng, 1996) and that hyalinocytes are less participative in immune response than granulocytes, with lower phagocytic capacity and ROS production (Andreyeva et al., 2019; Hong et al., 2013; Wang et al., 2017). Thus, it is possible that hyalinocytes can participate more directly in other functions than immune response, such as tissue repairs or detoxification processes by inducing antioxidant mechanisms, which could explain

the decrease in ROS production in *O.* cf. *ovata* exposed oysters. In fact, a recent study in flat oysters *O. edulis* showed differences in proteomic profile between hyalinocytes and granulocytes. Hyalinocytes had some proteins related to oxidative stress metabolism, giving this cell type the ability to tolerate and eliminate ROS molecules. However, glutathione S transferase 3 was only presented in granulocytes (de la Ballina et al., 2020). Therefore, further studies are needed to unravel the functional mechanisms of hemocyte types in response to toxic dinoflagellates and their toxins.

Almost all oysters (97%) of the current study were infected by *Perkinsus* spp., which was not unexpected, since the prevalence of *Perkinsus* spp. in oysters from the farm located at Mamanguape Estuary is frequently high (> 80%). The current study revealed that the *Perkinsus* spp. infection levels increased in oysters exposed to *O.* cf. *ovata.* Control oysters had an infection level of 2.3, corresponding to a light parasite burden, whilst exposed oysters had an intensity level of 3.1, corresponding to a moderate infection. Although we did not have preliminary parasitic indexes of the oysters used in the experiment, we have been monitoring perkinsosis in oysters from that farm since 2011. The average of *Perkinsus* spp. intensity of infection has remained consistently around 2 (light intensity), as observed in the studies by Farias et al. (2017), 2.2, and by Queiroga et al. (2013 and 2015), 2.0. Additionally, a most recent monitoring of *Perkinsus* ssp. in 625 oysters sampled in 2015 showed a mean of parasite intensity of 2.3 (unpublished data), which is exactly the same as that observed in control oysters from the current study. Therefore, the parasitic infection status of the control certainly represents the status of the entire population before the assay.

This is the first study that evaluated the impact of harmful algae on an oyster infected by *Perkinsus* spp.. Only four studies were carried out on clams (*Ruditapes philippinarum*) naturally infected by *Perkinsus olseni* and exposed to simulated blooms of *Karenia selliformis, K. mikimotoi, P. minimum* and *Alexandrium ostenfeldii* (da Silva et al., 2008; Hégaret et al., 2009, 2007; Lassudrie et al., 2014). Contrary to our findings, the intensity of infection decreased after short-term *A. ostenfeldii* and long-term *K. selliformis* exposures and it was not changed by the other microalgae. Thus, the current result suggests for the first time a potential impact which increases the sensitivity of *C. gasar* to pathogenic parasite. This observation may be associated with the deleterious effect on the immune response of oysters as a consequence of the toxic dinoflagellate *O.* cf. *ovata*, even under a short-term exposure (4 days) and a low microalgae concentration.

The activity of ABC-type transporters was studied in hemocytes of *C. gasar* by evaluating the intracellular accumulation of calcein, including the participation of two specific subtypes (ABCB1 and ABCC1). These proteins were associated to MXR phenotype, conferring resistance to several pollutants, from anthropogenic contaminants to biological toxins (Corsi and Marques-Santos, 2018). The activity of these transporters was characterized in the hemocytes of *C. gasar* (Marques-Santos et al., 2017), but little is known about the role of phycotoxin in the activity of ABC transporters in hemocytes. To date, few studies have been conducted to assess the role of ABC transporters in bivalves subjected to microalgae exposure (Huang et al., 2014, 2015; Xu et al., 2014). Buratti et al. (2011) showed a decrease in the expression of ABCB1 and ABCC1-like transporters in *M. galloprovinciallis* naturally exposed to algal toxins. This work is the first study evaluating the activity of ABC transporters in hemocytes after exposure to a toxic microalga. Our results indicated that bioactive molecules of *O.* cf. *ovata* did not alter ABCB1 and ABCC1 activity and consequently the MXR phenotype in hemocytes of *C. gasar*. This result explains the ability of the putative toxins produced by *O.* cf. *ovata* to promote cell mortality and reduce ROS in hemocytes.

Atrophy of the digestive tubule epithelium and inflammatory reaction were observed in oysters exposed to *O.* cf. *ovata*. Similar damages were reported in the mussel *M. galloprovincialis* after exposure to *O.* cf. *ovata* (Carella et al., 2015). The authors observed that the toxic dinoflagellate induced an increase in the area and perimeter of the lumen of digestive tubules and atrophy of this organ. Other studies also reported similar histopathological effects on mussels *P. perna* exposed to *P. lima* (Neves et al., 2019) and *C. gigas* exposed to *Gymnodinium catenatum* (García-Lagunas et al., 2015). In oysters *C. gigas* exposed to *Alexandrium minimum*, hemocyte infiltration were observed in intestine and digestive tubules epithelia (Haberkorn et al., 2010, Castrec et al. 2018). In a long-term exposure, the retraction of the epithelium can cause a deficit in the absorption of nutrients, which would weaken the health of oysters exposed to *O.* cf. *ovata*.

CONCLUSION

Defining harmful algal effects on hemocytes of cultured *C. gasar* oysters in Northeast Brazil is important to verify the following issues: identifying possible adaptations mechanisms in oysters, potential risks for the cultured oysters, and ultimately preventing future economic losses due to mortality and consequent reduction

in oyster productivity. The results obtained here corroborate the findings of the review published by Lassudrie et al. (2020) that shows a widely differed responses among microalgae and bivalve species, and its infection condition (herein *C. gasar* - *O.* cf. *ovata* - *Perkinsus* spp.). We concluded that *C. gasar* was resilient to *P. lima* but was affected by *O.* cf. *ovata* and this effect may play a negative role promoting the growth of *Perkinsus* infection. We suggest that the digestive tubules epithelium damages could be used as a histopathological marker for harmful algal blooms. Despite the lack of observed animal mortality, the impairment of hemocytes suggests an immunosuppression that can lead to susceptibility towards opportunistic infections or increased damages caused by pathogens already infecting the oysters.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CAPTIONS

Figure 1. Schematic representation of the morphometrical analysis of the digestive gland (DG) of *Crassostrea gasar*. **A**. Illustration of a histological cross-section of an oyster, showing different organs (adapted from Howard et al. (2004)). Three microscopy fields were randomly selected in DG (red circles). **B**. Histological image of a microscopy field positioned under the DG. 9-10 digestive tubules (DT) were randomly selected for analysis. **C**. A DT viewed at high magnification. Dashed lines show the measures taken of lumen diameters (d1 and d2) and double-headed arrows the measures taken of the width epithelium of the DT.

Figure 2. *Crassostrea gasar* hemocyte responses after *in vitro* exposure to the toxic dinoflagellates *P. lima* and *O.* cf. *ovata*. **A**. Hemocyte mortality (%). **B**. Phagocytosis rate (%). Dinoflagellate concentrations (cells mL^{-1}) are indicated below the columns. FSSW: filtered sterile seawater. Different letters denote significant differences among conditions (LSD post-hoc test, $P < 0.05$, n = 5).

Figure 3. *Crassostrea gasar* hemocyte reactive oxygen species (ROS) production after *in vitro* exposure to the harmful dinoflagellates *P. lima* and *O.* cf. *ovata*. **A**. Total hemocytes (all subpopulations together). **B**. Granulocytes. **C**. Hyalinocytes **D**. Blast-like cells. Dinoflagellate concentrations (cells mL^{-1}) are indicated below the columns. FSSW: filtered sterile seawater (n = 5).

Figure 4. *Perkinsus* spp. infection level in oysters *C. gasar* hemocytes after 4 days of exposure to *O.* cf. *ovata*. Mann-Whitney test, $P = 0.0651$. n = 15 for control, n = 14 for treatment.

Figure 5. Micrographs of digestive gland of *C. gasar* oysters. **A**. Control group. **B**. Treated group. Observe the reduced epithelium width (arrows), the larger diameter and bigger lumen space (#) of the digestive tubules and hemocytic infiltration (*) in oysters exposed to *O.* cf. *ovata* compared with the control oysters. Bars = 50 µm.

Table 1. Immune responses of oysters *C. gasar* after 4 days of exposure to *O.* cf. *ovata*.

Parameters	**Control**	**Treatment**	***P***
THC (x 10^5 cells mL^{-1})	3.8 ± 0.51	4.4 ± 0.77	0.5949
Granulocytes (%)	7.8 ± 1.41	6.41 ± 1.84	0.3453
Hyalinocytes (%)	46.0 ± 2.39	48.6 ± 3.09	0.5063
Blast-like cells (%)	46.2 ± 3.05	45.0 ± 3.14	0.7765
Phagocytosis rate (%)	8.2 ± 0.91	8.7 ± 0.83	0.6275
Mortality (%)	5.1 ± 0.85	13.5 ± 2.05	0.0003*
ROS - Total hemocytes (A.U.)	175.3 ± 19.79	65.4 ± 5.50	0.0000*
ROS - Granulocytes (A.U.)	323.0 ± 32.54	138.4 ± 13.12	0.0000*
ROS – Hyalinocytes (A.U.)	149.5 ± 23.82	43.6 ± 3.94	0.0005*
ROS - Blast-like cells (A.U.)	53.2 ± 6.74	14.1 ± 1.43	0.0000*

THC: Total Hemocyte Count. ROS: Reactive Oxygen Species production. Asterisks (*) denote significant differences between groups (*t*-test or Mann Whitney test, $P < 0.05$, n = 15).

Table 2. Activity of ABC transporters (fluorescence levels, A.U.), ABCB1 and ABCC1 isoforms of oysters *C. gasar* hemocytes after 4 days of exposure to *O.* cf. *ovata*.

Hemocyte population	Control	Treatment	*P*
	ABC (A.U.)		
Total	1684 ± 222	1583 ± 186	0.7810
Granulocytes	3355 ± 626	5474 ± 1133	0.1315
Hyalinocytes	2910 ± 393	2226 ± 321	0.1598
Blast-like cells	1906 ± 240	1727 ± 201	0.5604
	ABCB1 (FC)		
Total	0.8 ± 0.16	1.1 ± 0.17	0.1598
Granulocytes	1.9 ± 0.72	2.8 ± 1.08	0.8201
Hyalinocytes	1.5 ± 0.28	1.3 ± 0.31	0.6371
Blast-like cells	1.5 ± 0.24	1.2 ± 0.23	0.5004
	ABCC1 (FC)		
Total	1.3 ± 0.20	2.0 ± 0.27	0.0825
Granulocytes	2.9 ± 1.09	4.1 ± 1.09	0.1598
Hyalinocytes	1.5 ± 0.28	2.2 ± 0.35	0.3217
Blast-like cells	1.5 ± 0.25	2.2 ± 0.31	0.0968

FC: fold change. Mann Whitney test (n = 12 for control, n = 14 for treatment).

Table 3. Morphometric data of digestive tubules (DT) of oysters *C. gasar* after 4 days of exposure to *O.* cf. *ovata*.

Digestive tubule	Control	Treatment	*P*
Epithelium width (µm)	19 ± 0.5	15 ± 0.8	0.0006*
Area (μm^2)	914 ± 41.8	1034 ± 56.5	0.1004

Asterisks (*) denote significant differences between groups (*t*-test or Mann Whitney test, $P < 0.05$, n = 14 for control (420 DT), n = 15 for treatment (450 DT).