In vitro effects of the harmful benthic dinoflagellates Prorocentrum hoffmannianum and Ostreopsis cf. ovata on immune responses of the farmed oyster Crassostrea gasar

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ABSTRACT

Oyster culture is a sustainable solution to food production. However, this activity can be severely impacted by the presence and proliferation of harmful microalgae such as the benthic dinoflagellates *Prorocentrum hoffmannianum* and *Ostreopsis* cf. *ovata*. This study aimed to evaluate the *in vitro* effects of *P. hoffmannianum* and *O.* cf. *ovata* on immune system cells (hemocytes) of the native cultured oyster *Crassostrea gasar*. The direct toxicity of both dinoflagellates was first evaluated assessing hemocyte viability exposed to eight concentrations of each HAB species. No reduction in hemocyte viability was found with the exposure to cell culture or the crude extract of *P. hoffmannianum*, but *O.* cf. *ovata* culture induced hemocyte death in a concentration-dependent manner. *Ostreopsis* cf. *ovata* concentration that promoted half of maximal reduction in hemocyte viability (EC_{50}) was 779 cells mL^{-1}. Posteriorly, hemocytes were exposed to both dinoflagellate cells and crude extracts to investigate their effects on hemocyte functional parameters. Despite no direct toxicity of the dinoflagellate cells, *P. hoffmannianum* extract caused a threefold increase in ROS production and decreased the phagocytosis rate by less than half. *Ostreopsis* cf. *ovata* cells and crude extracts also triggered an increase in ROS production (two-fold), but the phagocytosis rate was reduced (by half) only in response to the two lower cell concentrations. These results indicate a harmful potential of both dinoflagellates through a direct toxicity (only for *O.* cf. *ovata*) and functional impairment of hemocytes (both species) which could expose *C. gasar* oyster to opportunistic infections.

KEYWORDS: Harmful algae; Hemocytes; Intracelullar compounds; Phagocytosis; Reactive oxygen species; Toxicity.

1. INTRODUCTION

Aquaculture has increased around the world and is clearly recognized as a significant activity. Molluscan aquaculture represents 25.8 % of the total aquaculture production, with 17.5 million tons in 2020, reaching the amount of USD 29.8 billion (FAO, 2022). In Brazil, as other main producers, the Pacific oyster *Crassostrea gigas* is the most cultivated species (around 2122 tons in 2020; Souza et al., 2022). However, there are an effort to production of native species like the mangrove oyster *Crassostrea gasar*. The mangrove oyster production is more relevant in the North and Northeast regions of Brazil (147 tons), representing most of the local bivalve production and an important source of income for local populations and artisanal fishermen (Suplicy, 2022a).

To develop aquaculture, we must understand the physiological aspects of the organisms and how they interact with the surrounding environment. For example, their immune system performance, which, in bivalves, is strongly associated with hemolymph, circulating liquid where cells called hemocytes are contained (Song et al., 2011; Wang et al., 2018). Hemocytes have several functions, but most of the studies about this cells are focused on their remarkable role as defenders against potential pathogens (Soudant et al., 2013).

Hemocytes act by identifying particles of potential parasites through receptors known as “Pattern Recognition Proteins” (PRPs) that bind to chemical components, typically found in invading organisms, known as Pathogen Associated Molecular Patterns (PAMPs) (Song et al., 2011; Wang et al., 2018). Once hemocytes bind to a potential invader, they must phagocyte it and, once internalized, destroy the invader through lytic mechanisms in the phagosome (Soudant et al., 2013). In addition to lysosomal enzymes and stored antimicrobial peptides, hemocytes can produce reactive oxygen species (ROS), highly reactive chemical species that act in the digestion of the phagocytosed particle

(Schmitt et al., 2011; Soudant et al., 2013). Some types of invaders, especially the larger ones, can be enveloped by hemocytes via encapsulation and destroyed by the lytic compounds released into the capsule (Soudant et al., 2013).

Various environmental factors can affect the immune system of invertebrates in different ways (Coates and Söderhäll, 2021). Harmful microalgae and their toxins are included between these factors as stressful biotic components. Their toxins are widely involved in human poisonings that occur through the consumption of contaminated marine organisms, especially bivalves (Bagnis et al., 1979; Randall, 2005). From the point of view of bivalve health, the phycotoxins from harmful microalgae can cause several negative effects on individuals when exposed to harmful microalgal blooms (HABs), such as damages in the reproductive system, changes in clearance and growth rates, in the condition index, and even mortality (Aguilar-Trujillo et al., 2017; Carella et al., 2015; Landsberg, 2002; Neves et al., 2021). Specifically, regarding the immune system, the effects of harmful microalgae can be diverse depending on the species of bivalve and microalgae and which interaction must be evaluated (Lassudrie et al., 2020; Tan et al., 2023). The greatest concern is associated with an immunosuppression that is observed in some bivalve-algae models (Hégaret and Wikfors, 2005; Mello et al., 2010; Prego-Faraldo et al., 2016) and the consequent exposure of the animal to opportunistic pathogens (Lassudrie et al., 2020; Soudant et al., 2013).

Despite marine organisms being common food items along the extensive Brazilian coast (more than 8 thousand km), there are no official programs for monitoring marine harmful microalgae in Brazil, except in the coast of Santa Catarina state (Suplicy, 2022b). However, some studies have been conducted to isolate and characterize different harmful species (Mafra et al., 2023). Two benthic dinoflagellate species have been recently

identified and isolated from Fernando de Noronha Archipelago: *Prorocentrum hoffmannianum* (UNR-45) and *Ostreopsis* cf. *ovata* (UNR-119).

The benthic dinoflagellate *P. hoffmannianum*, as other cogenders, can produce okadaic acid and derivatives (Rodríguez et al., 2018), as well as some complex and bioactive macrolides, such as hoffmanniolide (Hu et al., 1999), belizeanolide (Napolitano et al., 2009) and belizentrin (Domínguez et al., 2014); and large polyoxygenated polyketides such as belizeanolic acid (Napolitano et al., 2009). Other recently studied compounds, such as the neuroactive super-carbon-chain prorocentroic acid (Domínguez et al., 2020) are also produced by this species. *Ostreopsis* cf. *ovata* is also a benthic dinoflagellate that forms frequent blooms in tropical to temperate shallow coastal seas that can produce potent toxins, the ovatoxins, that are palytoxin analogues (Accoroni et al., 2017). Both *P. hoffmannianum* and *O.* cf. *ovata* have its toxins associated human intoxication (see review by Louzao et al., 2022).

Both benthic dinoflagellate species demonstrated toxicity against different mammalian cells (Neves et al., 2020). No studies are available regarding the potential impact of *P. hoffmanninanum* in marine organisms, but some works point harmful effects of its cogenders, as *P. lima* in the brine shrimp *Artemia salina* (Neves et al., 2017) and *P. minimum* in hemocytes of the Manila clams *Ruditapes philipinarum* (Hégaret et al., 2009). However, this sensitivity was not observed in hemocytes of the oysters *C. virginica* and *C. gasar* exposed to *P. minimum* (Hégaret et al., 2011) and *P. lima* (Faustino et al., 2021), respectively. *Ostreopsis* cf. *ovata*, also showed toxicity against invertebrates as the shrimp *Litopenaeus vannamei* (Cen et al., 2019), the moon jellyfish *Aurelia* sp. (Giussani et al., 2016), as well as in immunological system of *Mytilus galloprovincialis* (Gorbi et al., 2013). *Ostreopsis* cf. *ovata* strain (UNR-05) obtained from Arraial do Cabo

(Rio de Janeiro, Brazil) has also demonstrated remarkable toxicity against hemocytes of *C. gasar* (Faustino et al., 2021).

Considering the impacts of HABs on bivalve farming and the relevance of the mangrove oyster for aquaculture, the present study aimed to evaluate the effects of *P. hoffmaniannum* and *O.* cf. *ovata* cells and extracts on hemocytes of *C. gasar*. Hemocytes viability was assessed in response to the exposure to these two dinoflagellate species, as well as their physiological responses (phagocytosis and ROS production), which may impact defense against invaders. This research enriches the effort to guarantee seafood quality and safety, and line up with the scope of Sustainable Development Goal 14 (SDG 14 – *Conserve and sustainably use the oceans, seas, and marine resources for sustainable development*) and SDG 02 (*end hunger, achieve food security and improved nutrition and promote sustainable agriculture*) of the United Nations (UN, 2021).

2. MATERIAL AND METHODS

2.1 Dinoflagellate strains, cultures, and crude extracts

Two dinoflagellate species were used in the present study: *Prorocentrum hoffmannianum* (strain UNR-45; Fig. S1A) and *Ostreopsis* cf. *ovata* (strain UNR-119; Fig. S1B), both isolated from Fernando de Noronha Archipelago (3º50'52.3" S, 32º26'31.8" W), Northeastern Brazil, on 19th June 2016 and 5th June 2022, respectively. These two benthic dinoflagellates were collected and isolated as described in Nascimento et al. (2020). Samplings were conducted in accordance with the Chico Mendes Institute for Biodiversity Conservation (ICMBio N°. 35192-3) and the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN N°.

AA02660). These two dinoflagellate species are maintained in the Marine Microalgae Collection of UNIRIO-Brazil (SISGEN N°. CD23B79).

Dinoflagellate cells were kept in optimum culture conditions (exponential growth phase) in a temperature-controlled cabinet at 24 ± 2 ºC, with a 12:12 h dark-light cycle and photon flux density of 60 µmol $m^{-2}s^{-1}$ provided by cool-white fluorescent tubes in seawater supplemented with enrichment medium (Guillard, 1995) modified by omitting silicate, nickel, vanadium and chromium. The culture of *P. hoffmannianum* was kept in filtered (Millipore AP40) and sterilized seawater at salinity 30 and enrichment medium L1, and *O.* cf. *ovata* was maintained in filtered and sterilized seawater at salinity 34 and L2/2 enrichment medium.

Crude extracts of dinoflagellates were used in the present study to evaluate the effects of the toxic compounds produced by each dinoflagellate species. To the production of crude extracts, cellular density from each dinoflagellate culture was assessed before cells were harvested in exponential growth phase by a series of three to four centrifugations (20 min, 5,000 *g*), and cell pellets were stored at -80 ºC and then freeze-dried. Lyophilized cells from each strain were suspended in 700 µL of DMSO (≥ 99%, Sigma-Aldrich D2650) to achieve a nominal concentration of 1×10^6 cells mL^{-1} in crude extracts. Experimental conditions (i.e., dinoflagellate concentrations and extraction of intracellular compounds) were determined based on a previous study (Neves et al., 2020). DMSO was chosen as anhydrous solvent considering its application as a suitable extraction vehicle for *in vitro* cytotoxicity tests using mammalian cells (ISO 10993-5 2009). Cell lysis was performed using a 2.0 mm diameter probe sonicator (Vibra cell, Sonics) at 500 W. The sonication was conducted in an ice bath for 15 min in pulse mode (6s: ON, 1s: OFF). DMSO extracts (cells mL^{-1}) were centrifuged at 10 ºC and 4,000 *g* for 10 min and supernatants were filtered through a 0.22 µm sterile syringe filter (Millex GV

SLGV033RS) and stored at 4 ºC. The *P. hoffmannianum* extract was applied in the hemocyte toxicity and functional assays, while *O.* cf. *ovata* extract was only used in the hemocyte functional assays.

2.2 Animals and hemolymph sampling

Adult oysters *C. gasar* (> 6 cm in the shell length) were obtained from a commercial farm located in the South Bay of Florianópolis island (Ribeirão da Ilha) at Santa Catarina state (Brazil) and maintained for five and twelve days, respectively, prior to the step 1 (section 2.3) and step 2 (section 2.4) of experiments, both performed independently at the UNIRIO facilities (Rio de Janeiro, Brazil). Oysters were maintained in glass aquariums (40 L; Boyu ZJ-401) with artificial seawater at salinity 31, under constant aeration (290 L h^{-1}) and fed *ad libitum* with the microalga *Tetraselmis* sp. (Chlorophyta). No food was added 48 h before the experiments.

For the obtention of hemolymph, a notch in the posterior side of the shell was made to insert a syringe coupled to a needle (25 G) in the adductor muscle of oysters. The quality of hemolymph was evaluated under light microscope to observe characteristic hemocytes (Freire et al., 2023) and impure samples (containing bacteria, feces, tissue fragments, etc.) were discarded. For the *in vitro* assay, pools were made mixing hemolymph of 3-4 animals in a conic tube in the ice right before the exposure. A total of four pools were used as experimental independent replicates for each assay.

2.3 Step 1: Dinoflagellate toxicity and EC_{50}

To evaluate the toxicity of both species, eight different cellular densities of the harmful dinoflagellates were established by successive dilutions (factor of 2) of stock cultures in filtered artificial seawater (FASW) at salinity 31. Considering the maximum concentrations achieved in each stock culture, the ranges of cellular concentrations were 234 to 30000 cells mL^{-1} for *P. hoffmannianum*, and 140 to 17950 cells mL^{-1} for *O.* cf. *ovata*. These solutions were mixed with the pools of hemolymph (1:1) and incubated for 2 h at 22 °C; incubation of hemolymph with FASW was used as negative control following the same procedures.

When microalgal cell suspensions did not induce any significant toxicity to hemocytes, additional assays using the crude extracts of dinoflagellates were performed to test the toxicity of their intracellular compounds. Similarly, eight solutions were prepared by successive dilutions (factor of 2) of crude extracts in FASW (corresponding to the equivalent cell concentration used in previous incubations with dinoflagellate cells) to mix with the hemolymph (1:1) and incubated at 22 °C. A DMSO solution in FASW, in the maximum solvent percentage tested for crude extracts (1%), was used as solvent control following the same procedures.

The toxicity was evaluated as a measure of the hemocyte viability (as detailed below, section 2.5) using flow cytometry. When toxicity was observed, the effective concentration that induced 50% of the maximal reduction in hemocyte viability observed (EC_{50}) was calculated as detailed below (section 2.6).

2.4 Step 2: Physiological effects of dinoflagellates

When microalgal toxicity was observed (i.e. hemocyte viability affected), an additional assay was conducted to evaluate the effects of dinoflagellate strains on

hemocyte ROS production and phagocytosis rate. Incubations were performed for both dinoflagellate species using its cell cultures and its equivalent cell concentration in crude extracts. Three cellular concentrations of dinoflagellates were prepared based on results obtained in step 1; 1) the concentration equivalent to the EC_{50}, 2) the minimal concentration tested, and 3) the maximal concentration tested. When the dinoflagellate species showed no significant toxicity, step 2 was performed comparing only negative control and the maximal concentration tested in step 1.

2.5 Hemocyte parameters

Hemocyte parameters were measured using flow cytometry (FACSCalibur, BD Biosciences, San Jose, California, USA).

The hemocyte viability was estimated by the percentage of unstained cells by propidium iodide (Sigma-Aldrich, final concentration 10 μg mL^{-1}) (adapted from Hégaret et al., 2003) - a fluorescent DNA intercalator unable to cross the intact membrane of live cells. Propidium iodide was added 30 min prior to flow cytometry analysis.

The phagocytic rate of *C. gasar* hemocytes (Freire et al., 2023) was assessed using a solution of fluorescent latex beads (2 μm in diameter) (Polysciences, final concentration 1% in Milli-Q water) added 1 h prior to flow cytometry analysis. The phagocytic rate was calculated as the percentage of hemocytes that phagocytized two or more particles (Hégaret et al., 2003).

The reactive oxygen species (ROS) content in the hemocytes was determined using 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma Saint Louis, Missouri, EUA; final concentration of 10 μM) added 1 h prior to flow cytometry analysis. Upon entering the cell, DCFH-DA is cleaved by intracellular esterases, and converted in 2',7'-diclorofluorescein (DCFH), which emits fluorescence when reacting with ROS produced

246 by the cell (Hégaret et al., 2003; Lambert et al., 2003). Results are expressed as arbitrary
247 units of fluorescence (AUF).

248

249 **2.6 Statistical analyses**

250 Normality of the data was verified by the D'Agostino & Pearson test before all
251 analyses. Percentage data (viability and phagocytosis rate) were transformed before
252 analysis by dividing the percentage by 100 (i.e., proportion) and performing the arcsine
253 of the square root of proportion data. Differences between three or more conditions
254 (treatments and control) were evaluated by one-way analysis of variance (ANOVA)
255 followed by LSD post-hoc test. Differences only between treatment and negative control
256 were analyzed by unpaired t-test with Welch's correction (for normal data set) or Mann
257 Whitney test when data were not normally distributed. These analyses were performed in
258 Statgraphics Centurion Software, version XVI. Differences were considered significant
259 when $p \leq 0.05$.

260 The EC_{50} (i.e., the microalgal concentration providing half-maximal response and
261 95% of confidence intervals) was determined using the inhibition data of replicates
262 corrected by the mean of each control data. A four-parameter logistic equation (variable
263 slope) with the least squares fitting method was applied after log-transformation of x-axis
264 values (dinoflagellate concentrations). Adjustments were made to find the best R^2 and
265 nonlinear regressions and graphics were conducted using the software GraphPad Prism
266 8. EC_{50} results were validated if the fitted concentration-response curves had a $R^2 \geq 0.75$
267 and if the percent fitting error of the EC_{50} (FE, in percentage) was < 40%. The % FE was
268 calculated by the equation (Beck et al., 2004):

269 $$\% FE = SE\ Log\ EC_{50} * Ln10 * 100$$

Where SE is the standard error.

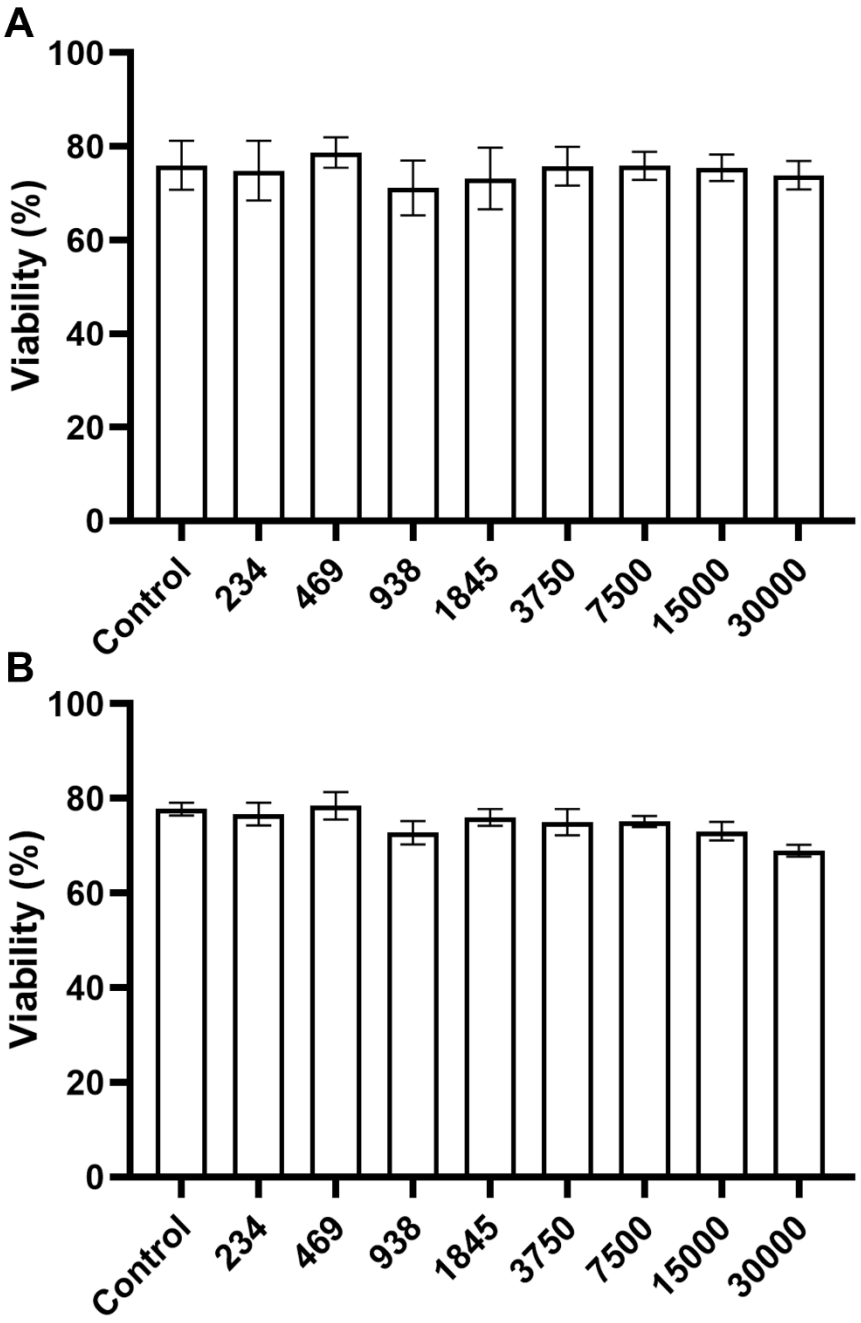
3. RESULTS

3.1 Microalgal toxicity

The viability of hemocytes obtained from *C. gasar* oysters after *in vitro* exposure to the harmful dinoflagellates *P. hoffmannianum* (Fig. 1) and *O.* cf. *ovata* (Fig. 2) was successfully analyzed through flow cytometry. Hemocyte viability in the negative and solvent controls, respectively FASW (Fig. 1A) and DMSO (Fig. 1B), reached 75.97 % (± 5.2) and 77.77% (± 1.4). In addition, no significant difference was observed between negative and solvent controls (t-test, $p = 0.7596$).

No significant effect on hemocyte viability was observed after exposure to *P. hoffmanianum* cells (Fig. 1A, ANOVA, $p = 0.9915$), nor its crude extract (Fig. 1B ANOVA, $p = 0.1044$). Conversely, exposure to *O.* cf. *ovata* cells significantly reduced hemocyte viability (ANOVA, $p < 0.0001$) compared to control (95.95 ± 1.43%), even for the lowest cellular concentration of *O.* cf. *ovata* (86.45 ± 4.91 %; Fig. 2A). Hemocyte viability was significantly reduced to 72.16 ± 7.75% when exposed to *O.* cf. *ovata* at concentration of 1222 cells mL^{-1}. Higher concentrations of *O.* cf. *ovata* caused a similar (non-statistically different compared to 1222 cells mL^{-1}) reduction in hemocyte viability, reaching 61.69 ± 4.73 % at the highest concentration tested (17950 cells mL^{-1}).

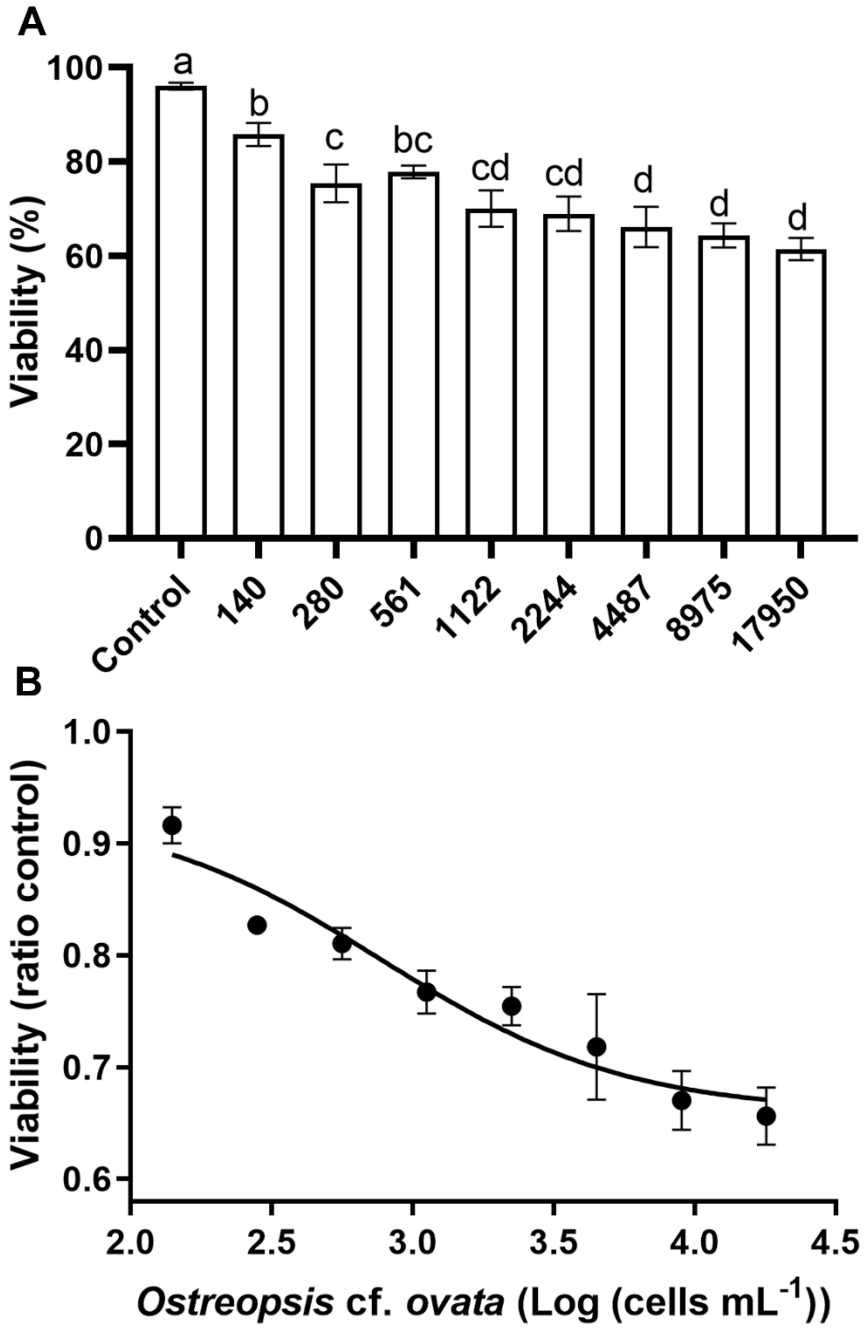
As a significant toxicity of *O.* cf. *ovata* cells was observed on oyster hemocytes, the EC_{50} was calculated (Fig. 2B). A valid dose-response curve was obtained for hemocytes exposed to *O.* cf. *ovata* cells with 0.74% of coefficient of variation of control replicates, $R^2 = 0.7938$, and $EC_{50,2h}$ (95% CI) of 779 (253 - 2400) cells mL^{-1}.



293

294 Figure 1: Viability of hemocytes (%) exposed to increasing concentrations of *P.*
295 *hoffmannianum* cells (A) and its crude extract (B). Numbers in the x-axis indicate the
296 cell concentration in cell mL^{-1} (A) or its equivalent in crude extract (B). Controls
297 comprised the use of FASW (Filtered Artificial Seawater, A) as negative control or
298 DMSO (B) as solvent control. Data are shown as mean ± SE (N = 4 pools of 3-4 oysters).

299



300

301 Figure 2: Viability of hemocytes (%) exposed to increasing concentration of *O.* cf. *ovata*
302 cells (A). Numbers in the x-axis indicate dinoflagellate concentration in cells mL^{-1}.
303 Different letters above the bars indicate statistical differences among concentrations (LSD
304 post-hoc test, $p < 0.005$). (B) Dose-response curve of hemocyte viability (normalized by
305 negative controls) to increasing concentrations of *O.* cf. *ovata* cells (Log-transformed).
306 Data are shown as mean ± SE (N = 4 pools of 3-4 oysters). Control comprises the FASW
307 (Filtered Artificial Seawater) as negative control.

308 **3.2 Physiological effects of dinoflagellates**

309 Considering that only the exposure to *O.* cf. *ovata* showed a significant
310 concentration-response on hemocyte viability (step 1), its physiological effects on oyster
311 hemocytes (phagocytosis rate and ROS production) were tested using three conditions:
312 the EC_{50} concentration (779 cells mL^{-1}), the lowest (140 cells mL^{-1}) and the highest
313 (17950 cells mL^{-1}) concentrations tested for hemocyte viability. While *P. hoffmannianum*
314 effects on the physiological conditions of oyster hemocytes was only tested using the
315 highest concentration reached by this dinoflagellate culture (30000 cells mL^{-1}).

316 The negative and solvent controls (respectively, FASW and DMSO) did not differ
317 statistically for both analysis of phagocytosis rate (t-test, $p = 0.8226$) and ROS production
318 (t-test, $p = 0.8786$). Distinct physiological responses were observed for hemocyte
319 parameters comparing the exposure to each dinoflagellate specie (Fig. 3).

320 The phagocytic rate of hemocytes did not show significant difference after
321 exposure to *P. hoffmannianum* cells (0.66 ± 0.06%) in relation to the control (0.82 ± 0.15
322 %; Fig. 3A). However, exposure to *P. hoffmannianum* crude extract induced a reduction
323 to less than half of hemocyte phagocytosis observed in the solvent control group (0.34 ±
324 0,02 %; DMSO = 0.77 ± 0.12 %; Fig. 3B).

325 Phagocytic rate of oyster hemocytes was reduced by the lower tested
326 concentration of *O.* cf. *ovata* cells (0.48 ± 0.06 %; FASW = 0.83 ± 0.14 %), and more
327 strongly by the cellular concentration achieved by EC_{50} (0.27 ± 0.02 %; $p = 0.0017$; Fig.
328 3A). However, the phagocytic rate observed after exposure to the maximal concentration
329 of *O.* cf. *ovata* cells reached an intermediate value between the rates observed in
330 incubations with the lower cellular concentration tested and the negative control with
331 FASW (0.56 ± 0.06 %). No significant difference was detected for the phagocytosis of

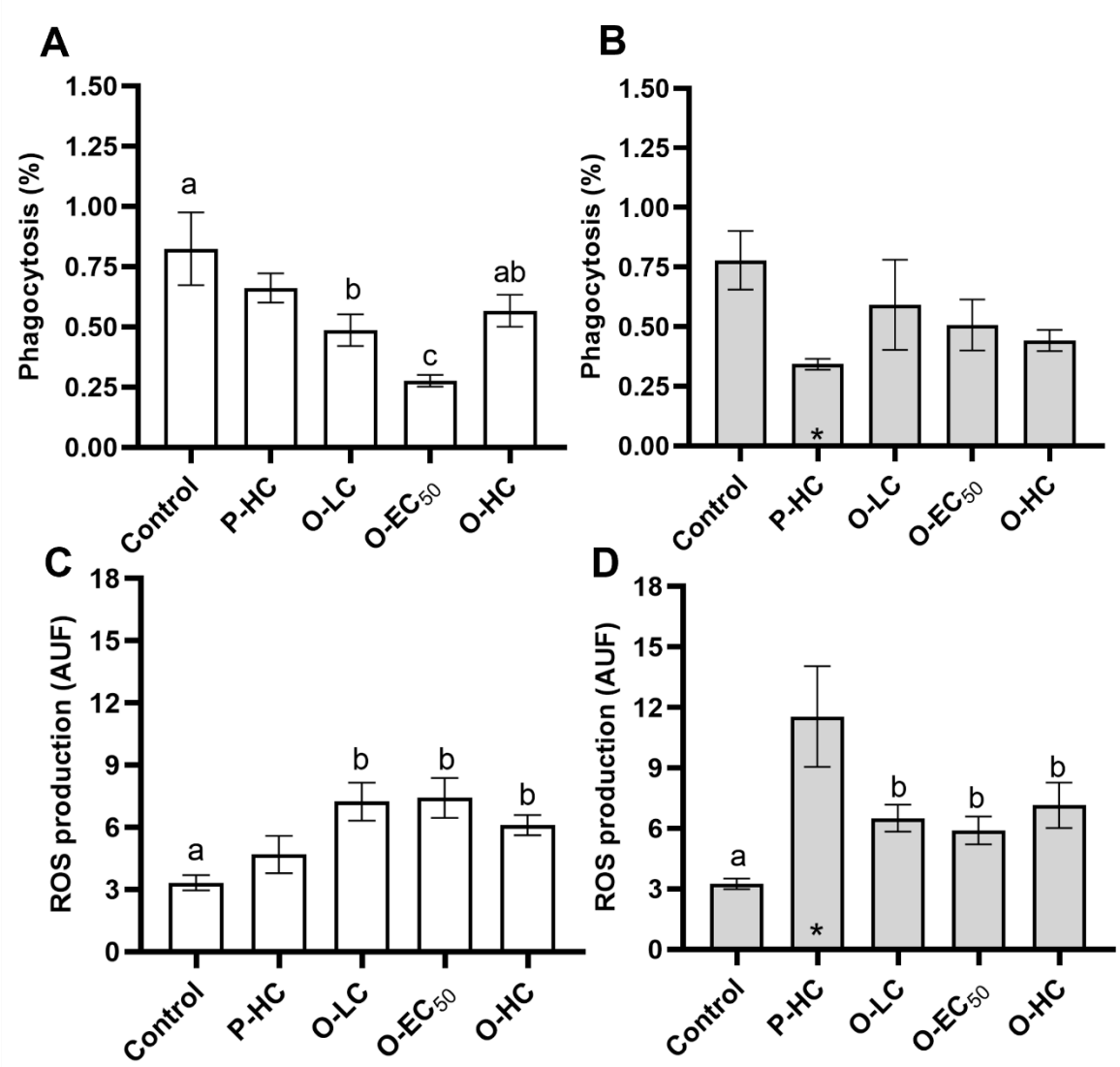


Figure 3: Physiological responses of *C. gasar* hemocytes after *in vitro* exposure to the dinoflagellates *P. hoffmannnianum* and *O.* cf. *ovata* cells (white bars) and crude extracts (grey bars). (A and B) Phagocytosis rate (%) and (C and D) reactive oxygen species (ROS) production (AUF). P-HC: Highest concentration of *P. hoffmanianum*; O-LC: Lower concentration of *O.* cf. *ovata*; O-EC_{50}: EC_{50} concentration of *O.* cf. *ovata*; O-MC: Highest concentration of *O.* cf. *ovata*. Distinct letters indicate statistically significant differences between the concentrations of *O.* cf. *ovata* and control (LSD post-hoc test, $P < 0.05$). Asterisks indicate a statistically significant difference between P-HC and control (t-test, $p < 0.05$). Data are shown as means ± SE (N = 4 replicates by treatment).

343 hemocytes (p = 0.5402) exposed to any of the crude extract concentrations of *O.* cf. *ovata*
344 (Fig. 3B).

345 The ROS produced by oyster hemocytes (Fig. 3C and D) did not differ after
346 exposure to *P. hoffmannianum* cells (4.68 ± 0.9 AUF, Fig 3C) in comparison to the
347 negative control (FASW; 3.32 ± 0.36 AUF). However, *P. hoffmannianum* crude extract
348 induced an increase of more than three-fold in ROS production (11.55 ± 2.49 AUF, Fig
349 3D) compared to solvent control (DMSO; 3.25 ± 0.25 AUF).

350 All the concentrations of *O.* cf. *ovata* cells promoted an increase (ANOVA; p =
351 0.0065) of approximately two-fold in hemocyte ROS production (means between 6.5 and
352 7.14 AUF) when compared to the control group (3.33 ± 0.37 AUF; Fig. 3C). A similar
353 result was observed for oyster hemocytes exposed to *O.* cf. *ovata* crude extract (Fig. 3D).
354 All the concentrations of *O.* cf. *ovata* crude extract promoted an increase (ANOVA; p =
355 0.0162, Fig. 3D) around two-fold in hemocyte ROS content (means between 5.9 and 7.14
356 AUF; p = 0.0162) when compared to the control group (3.26 ± 0.26 AUF; Fig. 3D).

357

358 **4. DISCUSSION**

359 The present study demonstrated the effects of two harmful benthic dinoflagellates,
360 *P. hoffmaniannum* (UNR-45 strain) and *O.* cf. *ovata* (UNR-119 strain), on the viability
361 and function *C. gasar* hemocytes through an *in vitro* approach. This is the first
362 characterization of the harmfulness of *P. hoffmannianum* and the first study carried out
363 with an isolate of *O.* cf. *ovata* obtained from the Fernando de Noronha Archipelago in a
364 commercial interest oyster in Brazil. Both dinoflagellates are HAB species regularly
365 occurring on Brazilian coasts (Borsato et al., 2023; Mafra et al., 2023). Hemocyte
366 responses can be used as primary indicators of bivalve health, as hemocytes are involved

in numerous functions, notedly defense against pathogens (Cheng, 1996; Hine, 1999; Soudant et al., 2013; Wang et al., 2018).

Crude extract of *P. hoffmannianum* used in the present study (strain UNR-45) induced a concentration-response cell growth inhibition in mammalian cells with $EC_{50,\ 24h}$ varying from 152 to 783 cells mL^{-1} of *P. hoffmanianum* (Neves et al., 2020). However, in the present study, none of the eight tested concentrations with cells nor crude extract of *P. hoffmaniannun* (234 - 30000 cells mL^{-1}) induced hemocyte mortality compared to control. Similarly, its congener *P. lima* did not promote hemocyte mortality in *C. gasar* (Faustino et al., 2021), nor did *P. minimum* on hemocytes of oysters *C. virginica* and other bivalves, such as the mussel *Mytilus edulis* (Galimany and Sunila, 2008), the cockle *Mya arenaria,* and the clam *Mercenaria mercenaria* (Hégaret et al., 2011, 2010). These observations, including results shown by the present study, resulted from analyses of hemocyte death by necrosis (i.e., viability) and can contribute to the general concept that dinoflagellates of the genus *Prorocentrum* cause no direct lethality to this invertebrate cell model (hemocyte). However, hemocyte death through apoptosis, a less harmful cell death mechanism to animals, was already detected in *M. galloprovincialis* after 48 h of *in vitro* exposure to *P. lima* (Prego-Faraldo et al., 2016).

In contrast, the current study demonstrated a clear toxicity of *O.* cf. *ovata* on *C. gasar* hemocytes. Faustino et al. (2021) already observed an important decrease in hemocyte viability after exposure to 100 cells mL^{-1}, which appeared stronger at 1000 cells mL^{-1} and 10000 cells mL^{-1} of *O.* cf. *ovata* (strain UNR-05). Similarly, in the present study, a significant reduction was detected in the viability of *C. gasar* hemocyte from the lower tested concentration (140 cells mL^{-1}) to the concentration of 1122 cells mL^{-1}. Although the strain of *O.* cf. *ovata* (UNR-119) used in our assays has not yet been characterized in terms of toxin production, it is well known that this benthic dinoflagellate species

produces palytoxin analogues (e.g., ovatoxins) (Brissard et al., 2015, 2014; Chomérat et al., 2022; Nascimento et al., 2020; Soliño et al., 2020). These palytoxins act binding to Na/K ATPase, inhibiting the pumping of ions and allowing the free passage of Na^+ cations, breaking the cell electrolyte balance (Patocka et al., 2018). Such mechanisms and their effects may explain the mortality observed in the present study, as well as in other models. Indeed, significant mortality under direct exposure to *O.* cf. *ovata* cells were already described after exposure to 400 cells mL^{-1} in other invertebrate models such as the crustaceans *A. salina*, *Tigriopus fulvus*, *Amphibalanus amphitrite* (Faimali et al., 2012), and larvae of the sea urchin *Lytechinus variegatus* (Neves et al., 2018).

The toxicity of the *O.* cf. *ovata* strain UNR-119 on hemocytes of *C. gasar* was less intense, causing a reduction in hemocyte viability to 61.41%, compared to the *O*. cf. *ovata* strain UNR-05 tested by Faustino et al. (2021), which promoted a reduction to 13.1% in hemocyte viability of the same oyster species. This difference in toxicity may be a consequence of the shorter incubation time used in our assays (1 h less than the previous study). In addition, in the present study it was used a *O.* cf. *ovata* strain isolated from the Northeastern region of Brazil, while Faustino et al. (2021) used a strain isolated from the Southeast region; thus, we cannot rule out the possible genetic and physiological differences that may arise from the strains of distinct geographical origin. The amount and profile of toxins synthesized by *O*. cf. *ovata* can differ in strains isolated from different geographical locations and environmental conditions (Accoroni et al., 2017; Pistocchi et al., 2011), although the dinoflagellate cultures used in both studies were used at exponential growth phase and were kept in the same growth medium and abiotic conditions (e.g., salinity, light, and temperature). The present study is the first carried out with the UNR-119 strain of *O.* cf. *ovata*, so the first to evaluate its toxicity.

Another source of variability could be attributed to different immune (hemocytes) responses of geographically distinct populations of *C. gasar* (South *vs* Northeast regions of Brazil). Even though there is no previous study on the effect of geographical origin of *C. gasar* on hemocytes responses, some variations in expression of genes involved in the immune system were detected in hemocytes of *C. virginica* cultured in different locations of the Atlantic coast of the United States (Furr et al., 2021). Therefore, the observed difference in *O.* cf. *ovata* toxicity to hemocytes could also be due to different geographical origins of the oysters.

The effects of benthic dinoflagellates on hemocyte phagocytosis were also evaluated, considering that is the initial response of hemocytes once they recognize a potential pathogen (Allam and Raftos, 2015). In the present study, no change in phagocytosis rate was detected in hemocytes directly exposed to *P. hoffmannianum* cells. Similarly, *in vitro* assays carried out with other dinoflagellates of the same genus, as *P. lima* (Faustino et al., 2021) and *P. minimum* (Hégaret et al., 2011), did not detect any variation in this parameter. However, a significant reduction in phagocytosis rate was found when hemocytes were exposed to *P. hoffmannianum* crude extract, suggesting the action of dinoflagellate intracellular compounds. Despite the evidence of cytotoxicity of *P. hoffmannianum* strain UNR-45 on mammalian cell lines (Neves et al., 2020), bioactive compounds produced by this strain have not yet been analyzed. In other *P. hoffmannianum* strains, the presence of okadaic acid is well characterized (Accoroni et al., 2018; Morton et al., 1994), in addition to different uncharacterized secondary metabolites (Rodríguez et al., 2018). Thus, an intracellular stock of okadaic acid and/or other secondary metabolites could be responsible for the observed drop in phagocytic capacity.

Exposure to *O.* cf. *ovata*, even at the lowest cell concentration (140 cells mL^{-1}), induced a reduction in phagocytosis rate, which decreased even more at the concentration of EC_{50} (779 cells mL^{-1}). These findings corroborate the observations of Faustino et al. (2021) that also observed a decrease (55% of control) in phagocytosis at a concentration as low as 100 cells mL^{-1}. Interestingly, in the present study, only an intermediate phagocytic rate was observed after hemocytes exposure to the highest tested concentration of *O.* cf. *ovata* (17950 cells mL^{-1}). This pattern is coincident with the ones previously obtained through *in vivo* assay exposure of oysters *C. gasar* to *O.* cf. *ovata* cells after four days, when no changes on phagocytosis was observed (Faustino et al., 2021). A possible explanation could be that some cellular components of the dinoflagellate *O.* cf. *ovata* that initially inhibit phagocytosis in hemocytes may trigger the activation of some restimulation pathway (like a negative feedback mechanism), once the cell is submitted to an exposure to higher concentration or for a prolonged incubation time (e.g., *in vivo* exposure). As the crude extract of *O.* cf. *ovata* did not significantly affect the phagocytosis rate, the involvement of intracellular compounds in the activation of this cellular mechanism appears less likely.

ROS production has an enormous importance in cell physiology as cell signaling mediator (Matsumoto et al., 2021; Zhang et al., 2022, 2016), metabolic activity and reflect some cell stress (Ballina et al., 2022; Donaghy et al., 2012). In hemocytes, the ROS production can also be modulated in response to the recognition of potential pathogens that must be destroyed by its action and other lithic molecules (Allam and Raftos, 2015; Destoumieux-Garzón et al., 2020; Soudant et al., 2013). When hemocytes were exposed to *P. hoffmannianum* cells, no variation was observed in their ROS production, similarly to the findings for oyster hemocytes exposed to congeneric dinoflagellates, as *P. lima* (Faustino et al., 2021) and *P. minimum* (Hégaret et al., 2011; Hégaret and Wikfors, 2005).

Only the crude extract of *P. hoffmaniannum* was able to promote an increase in ROS content, suggesting an oxidative effect induced by the intracellular compounds synthesized by this dinoflagellate. Okadaic acid can promote an increase in the amount of ROS produced by hemocytes of the scallop *Argopecten irradians* (Chi et al., 2016). In this case, authors associated this effect with an inhibition in the expression of a superoxide dismutase, a component of hemocyte defense against oxidative stress also present in oysters (Boutet et al., 2004). A similar process might have occurred in the hemocytes of *C. gasar* in response to crude extract of *P. hoffmannianum* (UNR-45 strain).

Exposures *O.* cf. *ovata*, both cells and crude extract, also induced an increase in the ROS production of *C. gasar* hemocytes. This suggests a pro-oxidative activity induced by bioactive compounds synthesized by *O.* cf. *ovata* that can be released to the extracellular medium. An increase in oxidative damage markers was detected in the hepatopancreas of the white leg shrimp *L. vannamei* exposed to palytoxins from *O.* cf. *ovata* (Cen et al., 2019). It is not possible to affirm that this increase in ROS production by hemocytes reflects activity of recognition pathogen-associated particles (Allam and Raftos, 2015; Soudant et al., 2013), since the phagocytosis rate was reduced in parallel, and an opposite pattern would be more coherent. In human keratinocytes, the palytoxins are associated with mitochondria effects by reversing mitochondrial transport chain (Pelin et al., 2013). Moreover, *in vitro* exposure of mitochondria isolated from rat liver cells to crude extract of *O.* cf. *ovata* (UNR-03 strain) negatively affected mitochondrial function by decreasing ATP synthesis-related membrane potential variations and led to failure (i.e., swelling) (Varela et al., 2021). These findings suggest a possible action mechanism of *O.* cf. *ovata*, but further studies are necessary to elucidate cellular and subcellular mechanisms activated by direct exposure to cells and intracellular compounds produced by this dinoflagellate species.

Considering previous evaluations of oyster hemocytes exposed to *O.* cf. *ovata*, results obtained in the present study for ROS production were unexpected. In the study conducted by Faustino et al. (2021), no effect was observed on this parameter after 3 h of *in vitro* exposure of *C. gasar* hemocytes to 10^2, 10^3 and 10^4 cells of *O.* cf. *ovata* mL^{-1}, and a reduction in ROS production was detected after *in vivo* exposure of oysters to *O.* cf. *ovata* cells (60-200 cells mL^{-1}) for 4 days. Again, it is possible that these contrasting results may be explained by differences between O. cf. *ovata* strains, oyster origin, and laboratory procedures (e.g., type of exposure, incubation time). It is known that the exceeding ROS produced can be removed in hemocytes through enzymatic systems, as superoxide dismutase (Boutet et al., 2004), glutathione peroxidase (Jo et al., 2008) peroxiredoxin (David et al., 2007), and different glutathione-S-transferases (Boutet et al., 2004), that can prevent cellular damages promoted by ROS excesses. It is possible to consider that an acute exposure to *O.* cf. *ovata* cells can induce an initial increment of oxidative species amount (as shown here), but these ROS produced could be reduced later through the activation of mechanisms of cellular protection.

Even though an evident higher toxicity of *O.* cf. *ovata* was observed in hemocytes of the oyster *C. gasar*, the harmful potential of *P. hoffamannianum* to *C. gasar* hemocytes cannot be ignored, mainly associated with its intracellular compounds. Both dinoflagellates were able to, at some point, induce an increase in ROS production, as previously discussed. However, this cannot be directly associated with a stimulation of hemocytes since phagocytosis was inhibited by exposure to both dinoflagellate species. Phagocytosis is the first direct action of destruction of a potential pathogen by hemocytes (Allam and Raftos, 2015). The inhibition of phagocytosis observed here indicates that exposure to these dinoflagellates represent a stressful agent for hemocytes that can weaken to the defense of the animal towards pathogens (Harvell, 1999).**5.**

5. CONCLUSION

Based on the responses of *in vitro* hemocytes exposure to cells and intracellular compounds (crude extracts) of the benthic dinoflagellates, both species can be considered harmful to the immunological system of the oyster *C. gasar*. Although the cells of *P. hoffmannianum* did not show any direct toxicity, its crude extract demonstrated potential to promote changes in the immunological system of *C. gasar*, suggesting a greater toxicity induced by its several intracellular compounds. Furthermore, our results demonstrated the toxicological risks of a new strain of *O.* cf. *ovata* isolated from Fernando de Noronha Archipelago highlighting its direct cellular toxicity and impacting hemocyte physiology in these farmed oysters. In conclusion, exposure to both *P. hoffmannianum* and *O.* cf. *ovata* show potential for immune system inhibition in the cultured oyster *C. gasar*, which could be worrying due to the possibility of exposure to opportunistic pathogens or other stressful conditions (e.g., pollutants).

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8. SUPLEMENTARY MATERIAL

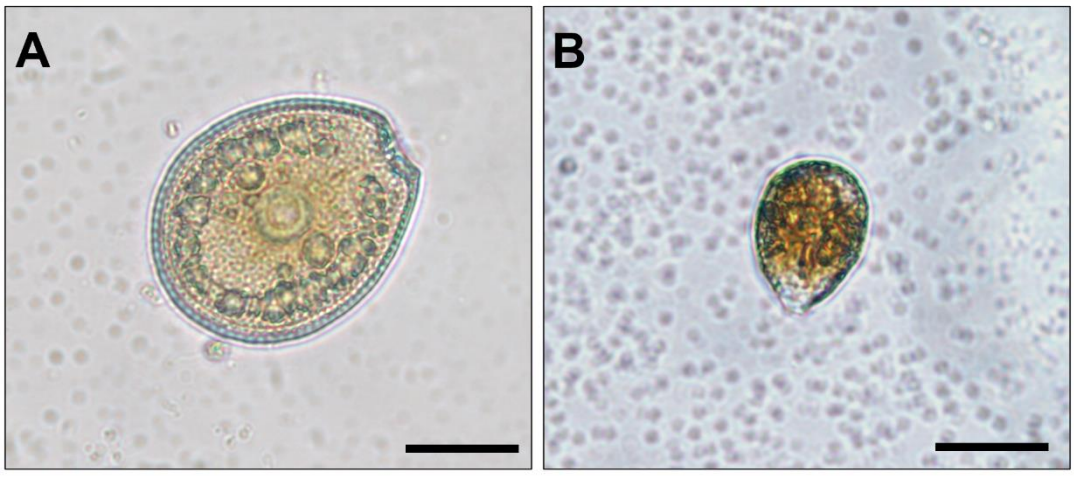

Figure S1: Benthic dinoflagellates cells in culture. (A) *Prorocentrum hoffmannianum* UNR-45 strain. (B) *Ostreopsis* cf. *ovata* UNR-119 strain. Bars = 20 µm.