



HAL
open science

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

Fernando Ramos Queiroga, H el ene Hegaret, Wanderson Fernandes Carvalho, Clarissa Naveira, Nath alia Rodrigues, Fernanda Silva Dos Santos, Silvia Mattos Nascimento, Raquel A.F. Neves

► To cite this version:

Fernando Ramos Queiroga, H el ene Hegaret, Wanderson Fernandes Carvalho, Clarissa Naveira, Nath alia Rodrigues, et al.. In vitro effects of the harmful benthic dinoflagellates *Prorocentrum hoffmannianum* and *Ostreopsis cf. ovata* on immune responses of the farmed oyster *Crassostrea gasar*. *Marine Environmental Research*, 2024, 198, pp.106503. 10.1016/j.marenvres.2024.106503 . hal-04589537

HAL Id: hal-04589537

<https://hal.univ-brest.fr/hal-04589537v1>

Submitted on 27 May 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destin ee au d ep ot et  a la diffusion de documents scientifiques de niveau recherche, publi es ou non,  emanant des  tablissements d'enseignement et de recherche fran ais ou  trangers, des laboratoires publics ou priv es.

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

4
5 Fernando Ramos Queiroga^a, Hélène Hegaret^a, Wanderson Fernandes de Carvalho^b,
6 Clarissa Naveira^c, Nathália Rodrigues^c, Fernanda Silva dos Santos^c, Silvia Mattos
7 Nascimento^d and Raquel A. F. Neves^c.

8
9 ^a Laboratory of Environmental Marine Sciences (UMR6539 CNRS/UBO/IFREMER/IRD),
10 European Institute for Marine Studies. Rue Dumont D'urville, Technopôle Brest-Iroise,
11 Plouzané, CP: 29280, France.

12
13 ^b Department of Ecology and Marine Resources, Federal University of the State of Rio de Janeiro
14 (UNIRIO). Avenida Pasteur, 458 - 307, Urca, Rio de Janeiro, CEP: 22.290-240, Brazil.

15
16 ^c Research Group of Experimental and Applied Aquatic Ecology, Federal University of the State
17 of Rio de Janeiro (UNIRIO). Avenida Pasteur, 458 - 307, Urca, Rio de Janeiro, CEP: 22.290-
18 240, Brazil.

19
20 ^d Marine Microalgae Laboratory, Federal University of the State of Rio de Janeiro (UNIRIO),
21 Avenida Pasteur, 458 - 307, Urca, Rio de Janeiro, CEP: 22.290-240, Brazil.

28 **ABSTRACT**

29 Oyster culture is a sustainable solution to food production. However, this activity
30 can be severely impacted by the presence and proliferation of harmful microalgae such
31 as the benthic dinoflagellates *Prorocentrum hoffmannianum* and *Ostreopsis cf. ovata*.
32 This study aimed to evaluate the *in vitro* effects of *P. hoffmannianum* and *O. cf. ovata* on
33 immune system cells (hemocytes) of the native cultured oyster *Crassostrea gasar*. The
34 direct toxicity of both dinoflagellates was first evaluated assessing hemocyte viability
35 exposed to eight concentrations of each HAB species. No reduction in hemocyte viability
36 was found with the exposure to cell culture or the crude extract of *P. hoffmannianum*, but
37 *O. cf. ovata* culture induced hemocyte death in a concentration-dependent manner.
38 *Ostreopsis cf. ovata* concentration that promoted half of maximal reduction in hemocyte
39 viability (EC₅₀) was 779 cells mL⁻¹. Posteriorly, hemocytes were exposed to both
40 dinoflagellate cells and crude extracts to investigate their effects on hemocyte functional
41 parameters. Despite no direct toxicity of the dinoflagellate cells, *P. hoffmannianum*
42 extract caused a threefold increase in ROS production and decreased the phagocytosis
43 rate by less than half. *Ostreopsis cf. ovata* cells and crude extracts also triggered an
44 increase in ROS production (two-fold), but the phagocytosis rate was reduced (by half)
45 only in response to the two lower cell concentrations. These results indicate a harmful
46 potential of both dinoflagellates through a direct toxicity (only for *O. cf. ovata*) and
47 functional impairment of hemocytes (both species) which could expose *C. gasar* oyster
48 to opportunistic infections.

49

50

51

52 **KEYWORDS:** Harmful algae; Hemocytes; Intracellular compounds; Phagocytosis;
53 Reactive oxygen species; Toxicity.

54 1. INTRODUCTION

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

64 To develop aquaculture, we must understand the physiological aspects of the
65 organisms and how they interact with the surrounding environment. For example, their
66 immune system performance, which, in bivalves, is strongly associated with hemolymph,
67 circulating liquid where cells called hemocytes are contained (Song et al., 2011; Wang et
68 al., 2018). Hemocytes have several functions, but most of the studies about this cells are
69 focused on their remarkable role as defenders against potential pathogens (Soudant et al.,
70 2013).

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

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

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

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

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

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

116 Both benthic dinoflagellate species demonstrated toxicity against different
117 mammalian cells (Neves et al., 2020). No studies are available regarding the potential
118 impact of *P. hoffmannianum* in marine organisms, but some works point harmful effects
119 of its cogenders, as *P. lima* in the brine shrimp *Artemia salina* (Neves et al., 2017) and *P.*
120 *minimum* in hemocytes of the Manila clams *Ruditapes philipinarum* (Hégaret et al.,
121 2009). However, this sensitivity was not observed in hemocytes of the oysters *C. virginica*
122 and *C. gasar* exposed to *P. minimum* (Hégaret et al., 2011) and *P. lima* (Faustino et al.,
123 2021), respectively. *Ostreopsis* cf. *ovata*, also showed toxicity against invertebrates as
124 the shrimp *Litopenaeus vannamei* (Cen et al., 2019), the moon jellyfish *Aurelia* sp.
125 (Giussani et al., 2016), as well as in immunological system of *Mytilus galloprovincialis*
126 (Gorbi et al., 2013). *Ostreopsis* cf. *ovata* strain (UNR-05) obtained from Arraial do Cabo

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

129 Considering the impacts of HABs on bivalve farming and the relevance of the
130 mangrove oyster for aquaculture, the present study aimed to evaluate the effects of *P.*
131 *hoffmaniannum* and *O. cf. ovata* cells and extracts on hemocytes of *C. gasar*. Hemocytes
132 viability was assessed in response to the exposure to these two dinoflagellate species, as
133 well as their physiological responses (phagocytosis and ROS production), which may
134 impact defense against invaders. This research enriches the effort to guarantee seafood
135 quality and safety, and line up with the scope of Sustainable Development Goal 14 (SDG
136 14 – *Conserve and sustainably use the oceans, seas, and marine resources for sustainable*
137 *development*) and SDG 02 (*end hunger, achieve food security and improved nutrition and*
138 *promote sustainable agriculture*) of the United Nations (UN, 2021).

139

140 **2. MATERIAL AND METHODS**

141 **2.1 Dinoflagellate strains, cultures, and crude extracts**

142 Two dinoflagellate species were used in the present study: *Prorocentrum*
143 *hoffmannianum* (strain UNR-45; Fig. S1A) and *Ostreopsis cf. ovata* (strain UNR-119;
144 Fig. S1B), both isolated from Fernando de Noronha Archipelago (3°50'52.3" S,
145 32°26'31.8" W), Northeastern Brazil, on 19th June 2016 and 5th June 2022, respectively.
146 These two benthic dinoflagellates were collected and isolated as described in Nascimento
147 et al. (2020). Samplings were conducted in accordance with the Chico Mendes Institute
148 for Biodiversity Conservation (ICMBio N°. 35192-3) and the National System for the
149 Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN N°.

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

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

160 Crude extracts of dinoflagellates were used in the present study to evaluate the
161 effects of the toxic compounds produced by each dinoflagellate species. To the production
162 of crude extracts, cellular density from each dinoflagellate culture was assessed before
163 cells were harvested in exponential growth phase by a series of three to four
164 centrifugations (20 min, 5,000 g), and cell pellets were stored at -80 °C and then freeze-
165 dried. Lyophilized cells from each strain were suspended in 700 μL of DMSO ($\geq 99\%$,
166 Sigma-Aldrich D2650) to achieve a nominal concentration of 1×10^6 cells mL^{-1} in crude
167 extracts. Experimental conditions (i.e., dinoflagellate concentrations and extraction of
168 intracellular compounds) were determined based on a previous study (Neves et al., 2020).
169 DMSO was chosen as anhydrous solvent considering its application as a suitable
170 extraction vehicle for *in vitro* cytotoxicity tests using mammalian cells (ISO 10993-5
171 2009). Cell lysis was performed using a 2.0 mm diameter probe sonicator (Vibra cell,
172 Sonics) at 500 W. The sonication was conducted in an ice bath for 15 min in pulse mode
173 (6s: ON, 1s: OFF). DMSO extracts (cells mL^{-1}) were centrifuged at 10 °C and 4,000 g for
174 10 min and supernatants were filtered through a 0.22 μm sterile syringe filter (Millex GV

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

178

179 **2.2 Animals and hemolymph sampling**

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

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

195

196 **2.3 Step 1: Dinoflagellate toxicity and EC₅₀**

197 To evaluate the toxicity of both species, eight different cellular densities of the
198 harmful dinoflagellates were established by successive dilutions (factor of 2) of stock
199 cultures in filtered artificial seawater (FASW) at salinity 31. Considering the maximum
200 concentrations achieved in each stock culture, the ranges of cellular concentrations were
201 234 to 30000 cells mL⁻¹ for *P. hoffmannianum*, and 140 to 17950 cells mL⁻¹ for *O. cf.*
202 *ovata*. These solutions were mixed with the pools of hemolymph (1:1) and incubated for
203 2 h at 22 °C; incubation of hemolymph with FASW was used as negative control
204 following the same procedures.

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

213 The toxicity was evaluated as a measure of the hemocyte viability (as detailed
214 below, section 2.5) using flow cytometry. When toxicity was observed, the effective
215 concentration that induced 50% of the maximal reduction in hemocyte viability observed
216 (EC₅₀) was calculated as detailed below (section 2.6).

217

218 **2.4 Step 2: Physiological effects of dinoflagellates**

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

221 hemocyte ROS production and phagocytosis rate. Incubations were performed for both
222 dinoflagellate species using its cell cultures and its equivalent cell concentration in crude
223 extracts. Three cellular concentrations of dinoflagellates were prepared based on results
224 obtained in step 1; 1) the concentration equivalent to the EC₅₀, 2) the minimal
225 concentration tested, and 3) the maximal concentration tested. When the dinoflagellate
226 species showed no significant toxicity, step 2 was performed comparing only negative
227 control and the maximal concentration tested in step 1.

228

229 **2.5 Hemocyte parameters**

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

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

236 The phagocytic rate of *C. gasar* hemocytes (Freire et al., 2023) was assessed using
237 a solution of fluorescent latex beads (2 µm in diameter) (Polysciences, final concentration
238 1% in Milli-Q water) added 1 h prior to flow cytometry analysis. The phagocytic rate was
239 calculated as the percentage of hemocytes that phagocytized two or more particles
240 (Hégaret et al., 2003).

241 The reactive oxygen species (ROS) content in the hemocytes was determined
242 using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma Saint Louis, Missouri, EUA;
243 final concentration of 10 µM) added 1 h prior to flow cytometry analysis. Upon entering
244 the cell, DCFH-DA is cleaved by intracellular esterases, and converted in 2',7'-
245 dichlorofluorescein (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):

$$269 \quad \% \text{ FE} = \text{SE Log } EC_{50} * \text{Ln}10 * 100$$

270 Where SE is the standard error.

271

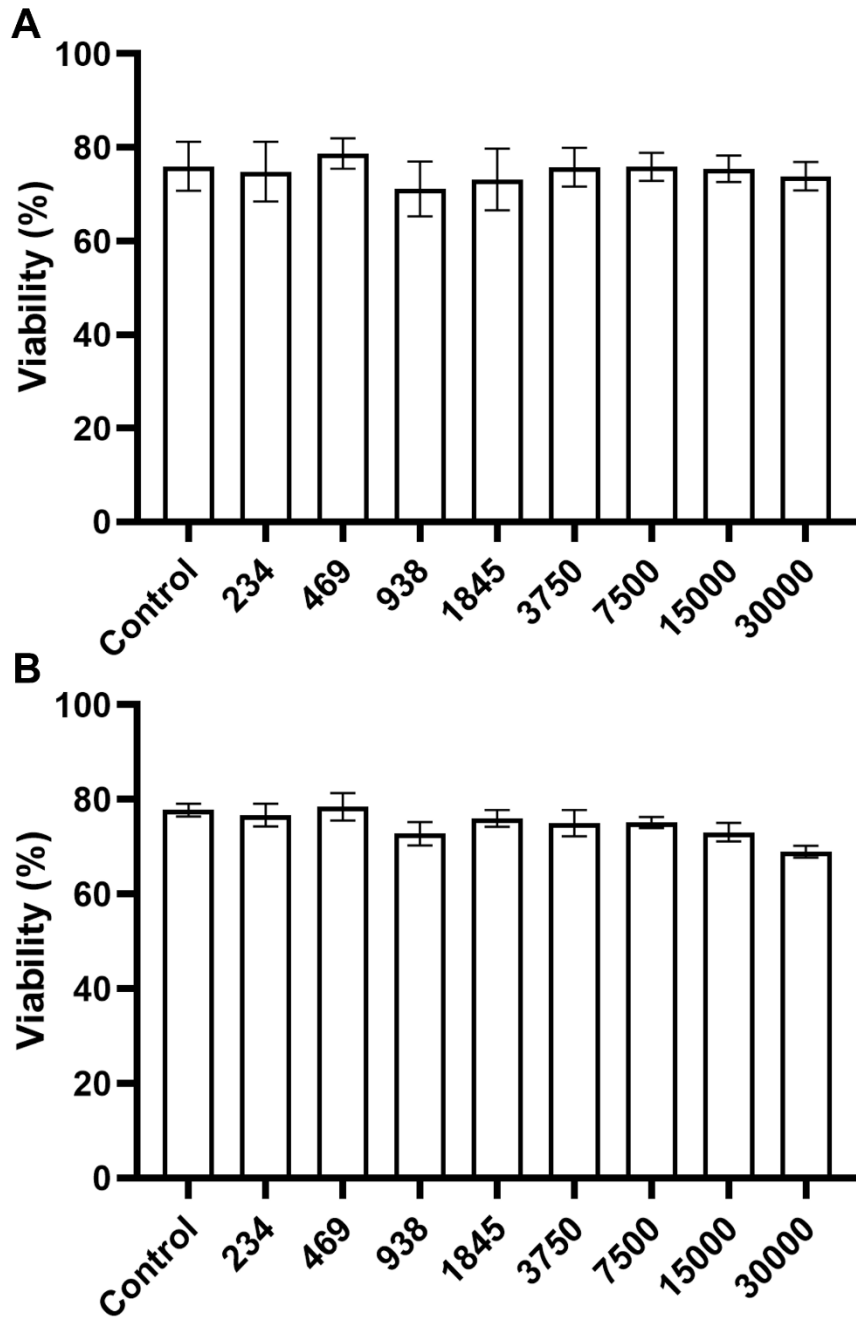
272 **3. RESULTS**

273 **3.1 Microalgal toxicity**

274 The viability of hemocytes obtained from *C. gasar* oysters after *in vitro* exposure
275 to the harmful dinoflagellates *P. hoffmannianum* (Fig. 1) and *O. cf. ovata* (Fig. 2) was
276 successfully analyzed through flow cytometry. Hemocyte viability in the negative and
277 solvent controls, respectively FASW (Fig. 1A) and DMSO (Fig. 1B), reached 75.97 % (\pm
278 5.2) and 77.77% (\pm 1.4). In addition, no significant difference was observed between
279 negative and solvent controls (t-test, $p = 0.7596$).

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

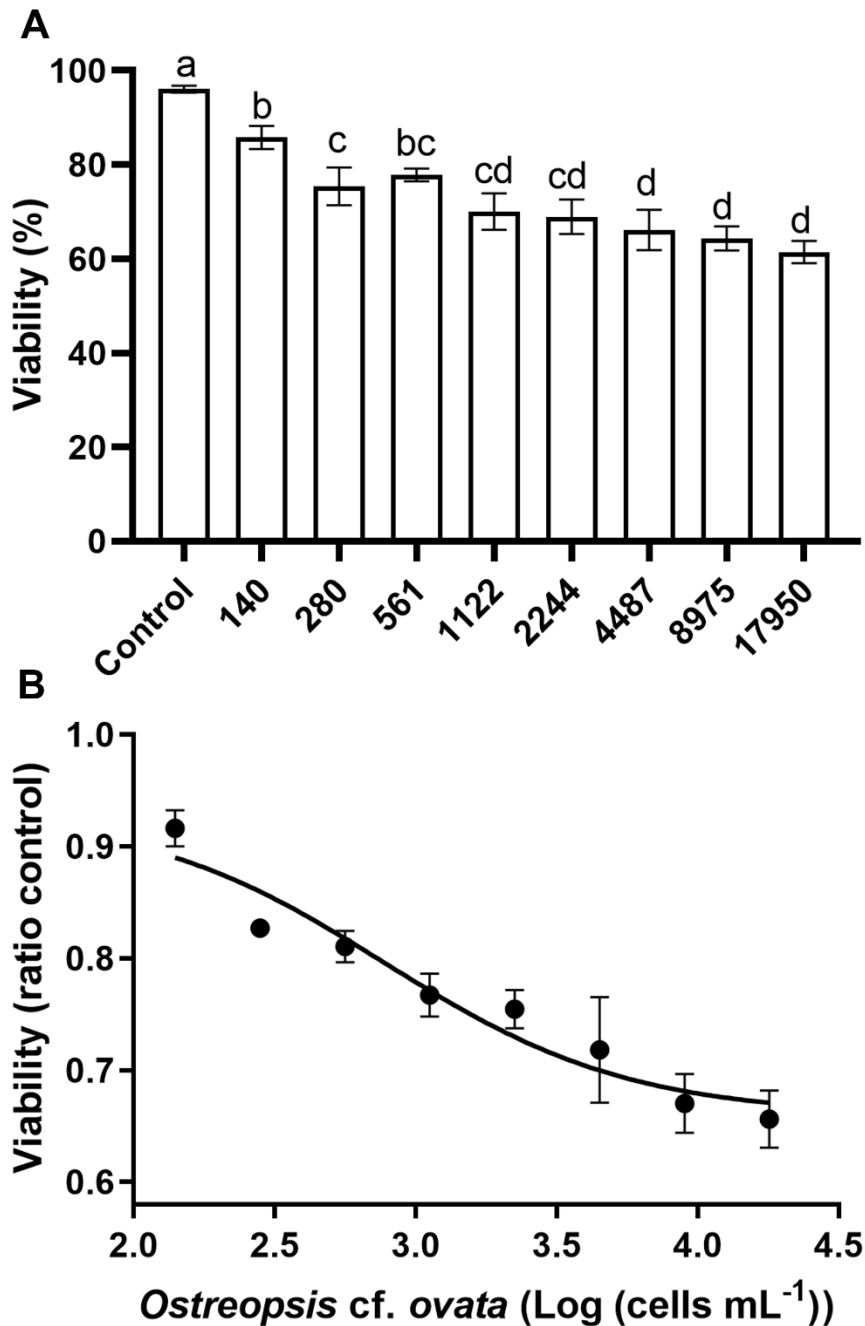
289 As a significant toxicity of *O. cf. ovata* cells was observed on oyster hemocytes,
290 the EC₅₀ was calculated (Fig. 2B). A valid dose-response curve was obtained for
291 hemocytes exposed to *O. cf. ovata* cells with 0.74% of coefficient of variation of control
292 replicates, R²= 0.7938, and EC_{50,2h} (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 \pm SE (N = 4 pools of 3-4 oysters). Control comprises the FASW
 307 (Filtered Artificial Seawater) as negative control.

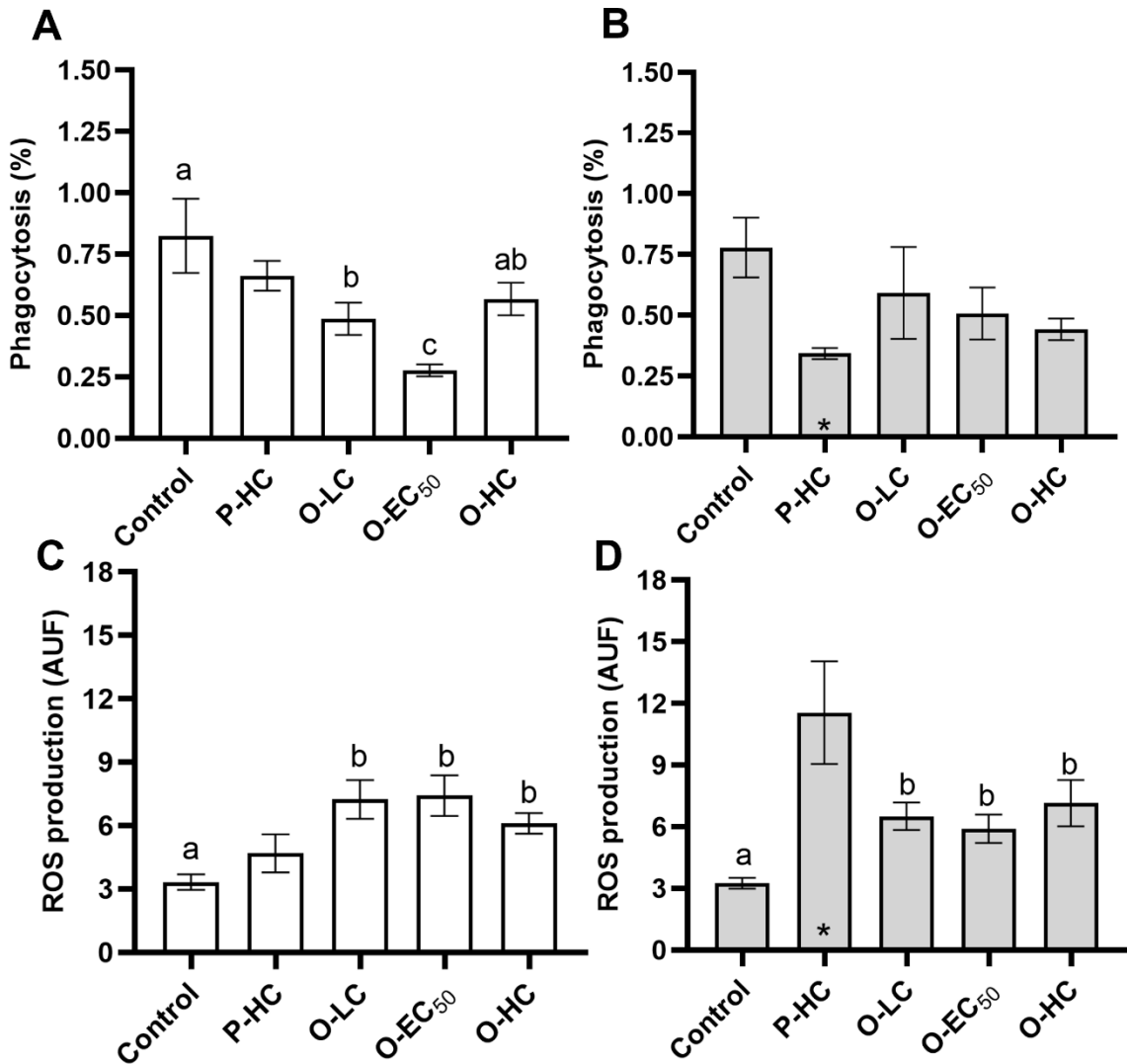
308 3.2 Physiological effects of dinoflagellates

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 EC₅₀ 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 ($0.66 \pm 0.06\%$) 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 ($0.34 \pm$
324 0.02% ; DMSO = $0.77 \pm 0.12\%$; Fig. 3B).

325 Phagocytic rate of oyster hemocytes was reduced by the lower tested
326 concentration of *O. cf. ovata* cells ($0.48 \pm 0.06\%$; FASW = $0.83 \pm 0.14\%$), and more
327 strongly by the cellular concentration achieved by EC₅₀ ($0.27 \pm 0.02\%$; $p = 0.0017$; 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 ($0.56 \pm 0.06\%$). No significant difference was detected for the phagocytosis of



332

333 Figure 3: Physiological responses of *C. gasar* hemocytes after *in vitro* exposure to the
 334 dinoflagellates *P. hoffmannianum* and *O. cf. ovata* cells (white bars) and crude extracts
 335 (grey bars). (A and B) Phagocytosis rate (%) and (C and D) reactive oxygen species
 336 (ROS) production (AUF). P-HC: Highest concentration of *P. hoffmannianum*; O-LC:
 337 Lower concentration of *O. cf. ovata*; O-EC₅₀: EC₅₀ concentration of *O. cf. ovata*; O-HC:
 338 Highest concentration of *O. cf. ovata*. Distinct letters indicate statistically significant
 339 differences between the concentrations of *O. cf. ovata* and control (LSD post-hoc test, P
 340 < 0.05). Asterisks indicate a statistically significant difference between P-HC and control
 341 (t-test, $p < 0.05$). Data are shown as means \pm SE (N = 4 replicates by treatment).

342

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

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

369 Crude extract of *P. hoffmannianum* used in the present study (strain UNR-45)
370 induced a concentration-response cell growth inhibition in mammalian cells with EC_{50} ,
371 $_{24h}$ varying from 152 to 783 cells mL^{-1} of *P. hoffmannianum* (Neves et al., 2020). However,
372 in the present study, none of the eight tested concentrations with cells nor crude extract
373 of *P. hoffmannianum* (234 - 30000 cells mL^{-1}) induced hemocyte mortality compared to
374 control. Similarly, its congener *P. lima* did not promote hemocyte mortality in *C. gasar*
375 (Faustino et al., 2021), nor did *P. minimum* on hemocytes of oysters *C. virginica* and other
376 bivalves, such as the mussel *Mytilus edulis* (Galimany and Sunila, 2008), the cockle *Mya*
377 *arenaria*, and the clam *Mercenaria mercenaria* (Hégaret et al., 2011, 2010). These
378 observations, including results shown by the present study, resulted from analyses of
379 hemocyte death by necrosis (i.e., viability) and can contribute to the general concept that
380 dinoflagellates of the genus *Prorocentrum* cause no direct lethality to this invertebrate
381 cell model (hemocyte). However, hemocyte death through apoptosis, a less harmful cell
382 death mechanism to animals, was already detected in *M. galloprovincialis* after 48 h of
383 *in vitro* exposure to *P. lima* (Prego-Faraldo et al., 2016).

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

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

401 The toxicity of the *O. cf. ovata* strain UNR-119 on hemocytes of *C. gasar* was
402 less intense, causing a reduction in hemocyte viability to 61.41%, compared to the *O. cf.*
403 *ovata* strain UNR-05 tested by Faustino et al. (2021), which promoted a reduction to
404 13.1% in hemocyte viability of the same oyster species. This difference in toxicity may
405 be a consequence of the shorter incubation time used in our assays (1 h less than the
406 previous study). In addition, in the present study it was used a *O. cf. ovata* strain isolated
407 from the Northeastern region of Brazil, while Faustino et al. (2021) used a strain isolated
408 from the Southeast region; thus, we cannot rule out the possible genetic and physiological
409 differences that may arise from the strains of distinct geographical origin. The amount
410 and profile of toxins synthesized by *O. cf. ovata* can differ in strains isolated from
411 different geographical locations and environmental conditions (Accoroni et al., 2017;
412 Pistocchi et al., 2011), although the dinoflagellate cultures used in both studies were used
413 at exponential growth phase and were kept in the same growth medium and abiotic
414 conditions (e.g., salinity, light, and temperature). The present study is the first carried out
415 with the UNR-119 strain of *O. cf. ovata*, so the first to evaluate its toxicity.

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

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

440 Exposure to *O. cf. ovata*, even at the lowest cell concentration (140 cells mL⁻¹),
441 induced a reduction in phagocytosis rate, which decreased even more at the concentration
442 of EC₅₀ (779 cells mL⁻¹). These findings corroborate the observations of Faustino et al.
443 (2021) that also observed a decrease (55% of control) in phagocytosis at a concentration
444 as low as 100 cells mL⁻¹. Interestingly, in the present study, only an intermediate
445 phagocytic rate was observed after hemocytes exposure to the highest tested
446 concentration of *O. cf. ovata* (17950 cells mL⁻¹). This pattern is coincident with the ones
447 previously obtained through *in vivo* assay exposure of oysters *C. gasar* to *O. cf. ovata*
448 cells after four days, when no changes on phagocytosis was observed (Faustino et al.,
449 2021). A possible explanation could be that some cellular components of the
450 dinoflagellate *O. cf. ovata* that initially inhibit phagocytosis in hemocytes may trigger the
451 activation of some restimulation pathway (like a negative feedback mechanism), once the
452 cell is submitted to an exposure to higher concentration or for a prolonged incubation
453 time (e.g., *in vivo* exposure). As the crude extract of *O. cf. ovata* did not significantly
454 affect the phagocytosis rate, the involvement of intracellular compounds in the activation
455 of this cellular mechanism appears less likely.

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

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

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

490 Considering previous evaluations of oyster hemocytes exposed to *O. cf. ovata*,
491 results obtained in the present study for ROS production were unexpected. In the study
492 conducted by Faustino et al. (2021), no effect was observed on this parameter after 3 h of
493 *in vitro* exposure of *C. gasar* hemocytes to 10^2 , 10^3 and 10^4 cells of *O. cf. ovata* mL⁻¹,
494 and a reduction in ROS production was detected after *in vivo* exposure of oysters to *O.*
495 *cf. ovata* cells (60-200 cells mL⁻¹) for 4 days. Again, it is possible that these contrasting
496 results may be explained by differences between *O. cf. ovata* strains, oyster origin, and
497 laboratory procedures (e.g., type of exposure, incubation time). It is known that the
498 exceeding ROS produced can be removed in hemocytes through enzymatic systems, as
499 superoxide dismutase (Boutet et al., 2004), glutathione peroxidase (Jo et al., 2008)
500 peroxiredoxin (David et al., 2007), and different glutathione-S-transferases (Boutet et al.,
501 2004), that can prevent cellular damages promoted by ROS excesses. It is possible to
502 consider that an acute exposure to *O. cf. ovata* cells can induce an initial increment of
503 oxidative species amount (as shown here), but these ROS produced could be reduced later
504 through the activation of mechanisms of cellular protection.

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

515 **5. CONCLUSION**

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

528

529 **6. ACKNOWLEDGMENTS**

530 Authors are grateful to the Clube da Ostra that provided live oysters from farms
531 to our laboratory facilities for the assays. F.R. Queiroga was supported by a fellowship
532 provided by European Union's Horizon 2020 research and innovation program under the
533 Marie Skłodowska-Curie grant agreement N° 899546, from the Brittany BIENVENÜE
534 program. This study was financially supported by Foundation Carlos Chagas Filho
535 Research Support of the State of Rio de Janeiro (FAPERJ) (E-26/201.283/2021; E-
536 26/210.024/2024) and by Brazilian National Council for Scientific and Technological
537 Development (CNPq) PQ2; 306212/2022-6) through the Research Grants attributed to
538 R.A.F. Neves, and by the French Research Network GdR PHYCOTOX: From microalgae

539 to the risks for human and ecosystems. This study was financed in part by the
540 Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) –
541 Finance Code 001 (scholarships).

542

543 7. REFERENCES

544 Accoroni, S., Ceci, M., Tartaglione, L., Romagnoli, T., Campanelli, A., Marini, M.,
545 Giulietti, S., Dell’Aversano, C., Totti, C., 2018. Role of temperature and nutrients on
546 the growth and toxin production of *Prorocentrum hoffmannianum* (Dinophyceae) from
547 the Florida Keys. *Harmful Algae* 80, 140–148.
548 <https://doi.org/10.1016/j.hal.2018.11.005>

549 Accoroni, S., Tartaglione, L., Dello Iacovo, E., Pichierri, S., Marini, M., Campanelli, A.,
550 Dell’Aversano, C., Totti, C., 2017. Influence of environmental factors on the toxin
551 production of *Ostreopsis cf. ovata* during bloom events. *Mar Pollut Bull* 123, 261–
552 268. <https://doi.org/10.1016/J.MARPOLBUL.2017.08.049>

553 Aguilar-Trujillo, A.C., Okolodkov, Y.B., Herrera-Silveira, J.A., Merino-Virgilio, F. del C.,
554 Galicia-García, C., 2017. Taxocoenosis of epibenthic dinoflagellates in the coastal
555 waters of the northern Yucatan Peninsula before and after the harmful algal bloom
556 event in 2011–2012. *Mar Pollut Bull* 119, 396–406.
557 <https://doi.org/10.1016/J.MARPOLBUL.2017.02.074>

558 Allam, B., Raftos, D., 2015. Immune responses to infectious diseases in bivalves. *J*
559 *Invertebr Pathol* 131, 121–136. <https://doi.org/10.1016/J.JIP.2015.05.005>

560 Bagnis, R., Kuberski, T., Laugier, S., 1979. Clinical Observations on 3,009 Cases of
561 Ciguatera (Fish Poisoning) in the South Pacific. *Am J Trop Med Hyg* 28, 1067–1073.
562 <https://doi.org/10.4269/AJTMH.1979.28.1067>

563 Ballina, N.R., Maresca, F., Cao, A., Villalba, A., 2022. Bivalve Haemocyte
564 Subpopulations: A Review. *Front Immunol* 13, 1–28.
565 <https://doi.org/10.3389/fimmu.2022.826255>

566 Beck, B., Chen, Y., Dere, W., Devanarayan, V., Eastwood, B., Farmen, M., Iturria, S.,
567 Iversen, P., Kahl, S., Moore, R., Sawyer, B., Weidner, J., Xu, X., 2004. Assay
568 operations for SAR support, in: *Assay Guidance Manual*. Eli Lilly & Company and
569 the National Center for Advancing Translational Sciences, Indianapolis.

570 Borsato, G.T., Salgueiro, F., De’Carli, G.A.L., Morais, A.M., Goulart, A.S., de Paula, J.C.,
571 Nascimento, S.M., 2023. Taxonomy and abundance of epibenthic *Prorocentrum*

572 (Dinophyceae) species from the tropical and subtropical Southwest Atlantic Ocean
573 including a review of their global diversity and distribution. *Harmful Algae* 127,
574 102470. <https://doi.org/10.1016/J.HAL.2023.102470>

575 Boutet, I., Tanguy, A., Moraga, D., 2004. Characterisation and expression of four mRNA
576 sequences encoding glutathione S-transferases pi, mu, omega and sigma classes in the
577 Pacific oyster *Crassostrea gigas* exposed to hydrocarbons and pesticides. *Mar Biol*
578 146, 53–64. <https://doi.org/10.1007/S00227-004-1423-6/METRICS>

579 Brissard, C., Herrenknecht, C., Séchet, V., Hervé, F., Pisapia, F., Harcouet, J., Lémée, R.,
580 Chomérat, N., Hess, P., Amzil, Z., 2014. Complex toxin profile of French
581 Mediterranean *Ostreopsis* cf. *ovata* strains, seafood accumulation and ovatoxins
582 prepurification. *Mar Drugs* 12, 2851–2876. <https://doi.org/10.3390/md12052851>

583 Brissard, C., Hervé, F., Sibat, M., Séchet, V., Hess, P., Amzil, Z., Herrenknecht, C., 2015.
584 Characterization of ovatoxin-h, a new ovatoxin analog, and evaluation of
585 chromatographic columns for ovatoxin analysis and purification. *J Chromatogr A*
586 1388, 87–101. <https://doi.org/10.1016/J.CHROMA.2015.02.015>

587 Carella, F., Feist, S.W., Bignell, J.P., De Vico, G., 2015b. Comparative pathology in
588 bivalves: Aetiological agents and disease processes. *J Invertebr Pathol* 131, 107–120.
589 <https://doi.org/10.1016/j.jip.2015.07.012>

590 Cen, J., Cui, L., Duan, Y., Zhang, H., Lin, Y., Zheng, J., Lu, S., 2019. Effects of palytoxins
591 extracted from *Ostreopsis ovata* on the oxidative stress and immune responses in
592 Pacific white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol* 95, 670–678.
593 <https://doi.org/10.1016/J.FSI.2019.11.001>

594 Cheng, T.C., 1996. Hemocytes: forms and functions, in: Kennedy, V.S., Newell, R.I.E.,
595 Eble, A.F. (Eds.), *The Eastern Oyster: Crassostrea Virginica*. Maryland Sea Grant,
596 College Park, MD, USA, pp. 299–333.

597 Chi, C., Giri, S.S., Jun, J.W., Kim, H.J., Yun, S., Kim, S.G., Park, S.C., 2016. Marine toxin
598 okadaic acid affects the immune function of bay scallop (*Argopecten irradians*).
599 *Molecules* 21. <https://doi.org/10.3390/MOLECULES21091108>

600 Chomérat, N., Antajan, E., Auby, I., Bilien, G., Carpentier, L., Casamajor, M.N. de,
601 Ganthy, F., Hervé, F., Labadie, M., Méteigner, C., Paradis, C., Perrière-Rumèbe, M.,
602 Sanchez, F., Séchet, V., Amzil, Z., 2022. First characterization of *Ostreopsis* cf. *ovata*
603 (Dinophyceae) and detection of ovatoxins during a multispecific and toxic ostreopsis
604 bloom on french atlantic coast. *Mar Drugs* 20. <https://doi.org/10.3390/MD20070461>

605 Coates, C.J., Söderhäll, K., 2021. The stress–immunity axis in shellfish. *J Invertebr Pathol*
606 186, 107492. <https://doi.org/10.1016/J.JIP.2020.107492>

- 607 David, E., Tanguy, A., Moraga, D., 2007. Peroxiredoxin 6 gene: A new physiological and
608 genetic indicator of multiple environmental stress response in Pacific oyster
609 *Crassostrea gigas*. *Aquat Toxicol* 84, 389-398.
610 <https://doi.org/10.1016/J.AQUATOX.2007.06.017>
- 611 Destoumieux-Garzón, D., Canesi, L., Oyanedel, D., Travers, M., Charrière, G.M., Pruzzo,
612 C., Vezzulli, L., 2020. *Vibrio*-bivalve interactions in health and disease. *Environ*
613 *Microbiol* 22, 4323–4341. <https://doi.org/10.1111/1462-2920.15055>
- 614 Domínguez, H.J., Cabrera-García, D., Cuadrado, C., Novelli, A., Fernández-Sánchez,
615 M.T., Fernández, J.J., Daranas, A.H., 2020. Prorocentric acid, a neuroactive super-
616 carbon-chain compound from the dinoflagellate *Prorocentrum hoffmannianum*. *Org*
617 *Lett* 23, 13–18. <https://doi.org/10.1021/ACS.ORGLETT.0C03437>
- 618 Domínguez, H.J., Napolitano, J.G., Fernández-Sánchez, M.T., Cabrera-García, D.,
619 Novelli, A., Norte, M., Fernández, J.J., Daranas, A.H., 2014. Belizentrin, a highly
620 bioactive macrocycle from the dinoflagellate *Prorocentrum belizeanum*. *Org Lett* 16,
621 4546–4549. <https://doi.org/10.1021/ol502102f>
- 622 Donaghy, L., Kraffe, E., Le Goïc, N., Lambert, C., Volety, A.K., Soudant, P., 2012.
623 Reactive oxygen species in unstimulated hemocytes of the pacific oyster *Crassostrea*
624 *gigas*: a mitochondrial involvement. *PLoS One* 7, e46594.
625 <https://doi.org/10.1371/journal.pone.0046594>
- 626 Faimali, M., Giussani, V., Piazza, V., Garaventa, F., Corrà, C., Asnaghi, V., Privitera, D.,
627 Gallus, L., Cattaneo-Vietti, R., Mangialajo, L., Chiantore, M., 2012. Toxic effects of
628 harmful benthic dinoflagellate *Ostreopsis ovata* on invertebrate and vertebrate marine
629 organisms. *Mar Environ Res* 76, 97–107.
630 <https://doi.org/10.1016/J.MARENRES.2011.09.010>
- 631 FAO, 2022. The State of World Fisheries and Aquaculture 2022, The State of World
632 Fisheries and Aquaculture 2022. FAO. <https://doi.org/10.4060/cc0461en>
- 633 Faustino, L.S., Queiroga, F.R., Hégaret, H., Marques-Santos, L.F., Neves, R.A.F.,
634 Nascimento, S., da Silva, P.M., 2021. Effects of the toxic dinoflagellates
635 *Prorocentrum lima* and *Ostreopsis cf. ovata* on immune responses of cultured oysters
636 *Crassostrea gasar*. *Aquat Toxicol* 236, 105846.
637 <https://doi.org/10.1016/J.AQUATOX.2021.105846>
- 638 Freire, J. M. S., Farias, N. D., Hégaret, H., da Silva, P. M., 2023. Morphological and
639 functional characterization of the oyster *Crassostrea gasar* circulating hemocytes: Cell
640 types and phagocytosis activity. *Fish Shellfish Immunol Rep*, 4, 100089.
641 <https://doi.org/10.1016/J.FSIREP.2023.100089>
- 642 Furr, D., Ketchum, R.N., Phippen, B.L., Reitzel, A.M., Ivanina, A. V., 2021. Physiological
643 variation in response to *Vibrio* and hypoxia by aquacultured Eastern Oysters in the

- 644 Southeastern United States. Integr Comp Biol 61, 1715–1729.
645 <https://doi.org/10.1093/ICB/ICAB176>
- 646 Galimany, E., Sunila, I., 2008. Several cases of disseminated neoplasia in mussels *Mytilus*
647 *edulis* (L.) in Western Long Island Sound. J Shellfish Res 27, 1201–1207.
648 <https://doi.org/10.2983/0730-8000-27.5.1201>
- 649 Giussani, V., Costa, E., Pecorino, D., Berdalet, E., De Giampaulis, G., Gentile, M., Fuentes,
650 V., Vila, M., Penna, A., Chiantore, M., Garaventa, F., Lavorano, S., & Faimali, M.
651 (2016). Effects of the harmful dinoflagellate *Ostreopsis* cf. *ovata* on different life cycle
652 stages of the common moon jellyfish *Aurelia* sp. Harmful Algae, 57(Pt A), 49–58.
653 <https://doi.org/10.1016/J.HAL.2016.05.005>
- 654 Guillard, R.R.R., 1995. Culture Methods, in: Hallegraeff, G.G., Anderson, D.M.,
655 Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae - IOC Manual and
656 Guides . UNESCO, France, pp. 45–56.
- 657 Harvell, C.D., 1999. Emerging marine diseases—Climate links and anthropogenic factors.
658 Science (1979) 285, 1505–1510. <https://doi.org/10.1126/science.285.5433.1505>
- 659 Hégaret, H., Da Silva, P.M., Wikfors, G.H., Haberkorn, H., Shumway, S.E., Soudant, P.,
660 2011. *In vitro* interactions between several species of harmful algae and haemocytes
661 of bivalve molluscs. Cell Biol Toxicol 27, 249–266. [https://doi.org/10.1007/S10565-](https://doi.org/10.1007/S10565-011-9186-6/METRICS)
662 [011-9186-6/METRICS](https://doi.org/10.1007/S10565-011-9186-6/METRICS)
- 663 Hégaret, H., Da Silva, P. M., Sunila, I., Shumway, S. E., Dixon, M. S., Alix, J., Wikfors,
664 G. H., & Soudant, P. (2009). Perkinsosis in the Manila clam *Ruditapes philippinarum*
665 affects responses to the harmful-alga, *Prorocentrum minimum*. J. Exp. Mar. Bio. Ecol.,
666 371(2), 112–120. <https://doi.org/10.1016/j.jembe.2009.01.016>
- 667 Hégaret, H., Smolowitz, R.M., Sunila, I., Shumway, S.E., Alix, J., Dixon, M., Wikfors,
668 G.H., 2010. Combined effects of a parasite, QPX, and the harmful-alga, *Prorocentrum*
669 *minimum* on northern quahogs, *Mercenaria mercenaria*. Mar Environ Res 69, 337–
670 344. <https://doi.org/10.1016/J.MARENVRES.2009.12.008>
- 671 Hégaret, H., Wikfors, G.H., 2005. Time-dependent changes in hemocytes of eastern
672 oysters, *Crassostrea virginica*, and northern bay scallops, *Argopecten irradians*
673 *irradians*, exposed to a cultured strain of *Prorocentrum minimum*. Harmful Algae 4,
674 187–199. <https://doi.org/10.1016/j.hal.2003.12.004>
- 675 Hégaret, H., Wikfors, G.H., Soudant, P., 2003. Flow cytometric analysis of haemocytes
676 from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature
677 elevation II. Haemocyte functions: Aggregation, viability, phagocytosis, and
678 respiratory burst. J Exp Mar Biol Ecol 293, 249–265. [https://doi.org/10.1016/S0022-](https://doi.org/10.1016/S0022-0981(03)00235-1)
679 [0981\(03\)00235-1](https://doi.org/10.1016/S0022-0981(03)00235-1)

- 680 Hine, P.M., 1999. The inter-relationships of bivalve haemocytes. *Fish Shellfish Immunol*
681 9, 367–385. <https://doi.org/10.1006/fsim.1998.0205>
- 682 Hu, T., Curtis, J.M., Walter, J.A., Wright, J.L.C., 1999. Hoffmanniolide: a novel macrolide
683 from *Prorocentrum hoffmannianum*, *Pergamon Tetrahedron Letters* 40(21), 3977 -
684 3980. [https://doi.org/10.1016/S0040-4039\(99\)00513-4](https://doi.org/10.1016/S0040-4039(99)00513-4)
- 685 Jo, P.G., Choi, Y.K., Choi, C.Y., 2008. Cloning and mRNA expression of antioxidant
686 enzymes in the pacific oyster, *Crassostrea gigas* in response to cadmium exposure.
687 *Comp Biochem Physiol C Toxicol Pharmacol* 147, 460–469.
688 <https://doi.org/10.1016/J.CBPC.2008.02.001>
- 689 Lambert, C., Soudant, P., Choquet, G., Paillard, C., 2003. Measurement of *Crassostrea*
690 *gigas* hemocyte oxidative metabolism by flow cytometry and the inhibiting capacity
691 of pathogenic vibrios. *Fish Shellfish Immunol* 15, 225–240.
692 [https://doi.org/10.1016/S1050-4648\(02\)00160-2](https://doi.org/10.1016/S1050-4648(02)00160-2)
- 693 Landsberg, J.H., 2002. The effects of harmful algal blooms on aquatic organisms. *Reviews*
694 *in Fisheries Science* 10, 113–390. <https://doi.org/10.1080/20026491051695>
- 695 Lassudrie, M., Hégaret, H., Wikfors, G.H., da Silva, P.M., 2020. Effects of marine harmful
696 algal blooms on bivalve cellular immunity and infectious diseases: A review. *Dev*
697 *Comp Immunol* 108, 103660. <https://doi.org/10.1016/J.DCI.2020.103660>
- 698 Louzao, M.C., Vilariño, N., Vale, C., Costas, C., Cao, A., Raposo-garcia, S., Vieytes, M.R.,
699 Botana, L.M., 2022. Current trends and new challenges in marine phycotoxins. *Mar*
700 *Drugs*. <https://doi.org/10.3390/MD20030198>
- 701 Mafra, L.L., Sunesen, I., Pires, E., Nascimento, S.M., Álvarez, G., Mancera-Pineda, J.E.,
702 Torres, G., Carnicer, O., Huamaní Galindo, J.A., Sanchez Ramirez, S., Martínez-
703 Goicoechea, A., Morales-Benavides, D., Valerio-González, L., 2023. Benthic harmful
704 microalgae and their impacts in South America. *Harmful Algae* 127, 102478.
705 <https://doi.org/10.1016/j.hal.2023.102478>
- 706 MAPA, 2022. Boletim da aquicultura em águas da união 2021: Relatório. Brasília.
- 707 Matsumoto, H., Ochiai, M., Imai, E., Matsumura, T., Hayakawa, Y., 2021. Stress-derived
708 reactive oxygen species enable hemocytes to release activator of growth blocking
709 peptide (GBP) processing enzyme. *J Insect Physiol* 131.
710 <https://doi.org/10.1016/J.JINSPHYS.2021.104225>
- 711 Mello, D.F., Antonio De Oliveira Proença, L., Barracco, M.A., 2010. Comparative study
712 of various immune parameters in three bivalve species during a natural bloom of
713 *Dinophysis acuminata* in Santa Catarina Island, Brazil. *Toxins (Basel)* 2, 1166–1178.
714 <https://doi.org/10.3390/toxins2051166>

- 715 Morton, S.L., Bomber, J.W., Tindall, P.M., 1994. Environmental effects on the production
716 of okadaic acid from *Prorocentrum hoffmannianum* Faust I. temperature, light, and
717 salinity. *J Exp Mar Biol Ecol* 178, 67–77. [https://doi.org/10.1016/0022-](https://doi.org/10.1016/0022-0981(94)90225-9)
718 0981(94)90225-9
- 719 Napolitano, J.G., Norte, M., Padrón, J.M., Fernández, J.J., Hernández Daranas, A., 2009.
720 Belizeanolide, a cytotoxic macrolide from the dinoflagellate *Prorocentrum*
721 *belizeanum*. *Angewandte Chemie - International Edition* 48, 796–799.
722 <https://doi.org/10.1002/anie.200804023>
- 723 Nascimento, S.M., Neves, R.A.F., De'carli, G.A.L., Borsato, G.T., da Silva, R.A.F., Melo,
724 G.A., de Moraes, A.M., Cockell, T.C., Fraga, S., Menezes-Salgueiro, A.D., Mafra,
725 L.L., Hess, P., Salgueiro, F., 2020. *Ostreopsis* cf. *ovata* (Dinophyceae) molecular
726 phylogeny, morphology, and detection of ovatoxins in strains and field samples from
727 Brazil. *Toxins* 2020, Vol. 12, Page 70 12, 70.
728 <https://doi.org/10.3390/TOXINS12020070>
- 729 Neves, R. A. F., Fernandes, T., Dos Santos, L. N., & Nascimento, S. M. (2017). Toxicity
730 of benthic dinoflagellates on grazing, behavior and survival of the brine shrimp
731 *Artemia salina*. *PLoS ONE*, 12(4), 1–17.<https://doi.org/10.1371/journal.pone.017516>
- 732 Neves, R.A.F., Contins, M., Nascimento, S.M., 2018. Effects of the toxic benthic
733 dinoflagellate *Ostreopsis* cf. *ovata* on fertilization and early development of the sea
734 urchin *Lytechinus variegatus*. *Mar Environ Res* 135, 11–17.
735 <https://doi.org/10.1016/J.MARENRES.2018.01.014>
- 736 Neves, R.A.F., Nascimento, S.M., Santos, L.N., 2021. Harmful algal blooms and shellfish
737 in the marine environment: an overview of the main molluscan responses, toxin
738 dynamics, and risks for human health. *Environ Sci Pollut Res* 28, 55846–55868.
739 <https://doi.org/10.1007/s11356-021-16256-5>
- 740 Neves, R.A.F., Pardal, M.A., Nascimento, S.M., Oliveira, P.J., Rodrigues, E.T., 2020.
741 Screening-level evaluation of marine benthic dinoflagellates toxicity using
742 mammalian cell lines. *Ecotoxicol Environ Saf* 195, 110465.
743 <https://doi.org/10.1016/J.ECOENV.2020.110465>
- 744 Patocka, J., Nepovimova, E., Wu, Q., Kuca, K., 2018. Palytoxin congeners. *Arch Toxicol*
745 92, 143–156. <https://doi.org/10.1007/s00204-017-2105-8>
- 746 Pelin, M., Ponti, C., Sosa, S., Gibellini, D., Florio, C., Tubaro, A., 2013. Oxidative stress
747 induced by palytoxin in human keratinocytes is mediated by a H⁺-dependent
748 mitochondrial pathway. *Toxicol Appl Pharmacol* 266, 1–8.
749 <https://doi.org/10.1016/J.TAAP.2012.10.023>
- 750 Pistocchi, R., Pezzolesi, L., Guerrini, F., Vanucci, S., Dell'Aversano, C., Fattorusso, E.,
751 2011. A review on the effects of environmental conditions on growth and toxin

- 752 production of *Ostreopsis ovata*. *Toxicon* 57, 421–428.
753 <https://doi.org/10.1016/J.TOXICON.2010.09.013>
- 754 Prego-Faraldo, M., Valdiglesias, V., Laffon, B., Mendez, J., Eirin-Lopez, J., 2016. Early
755 genotoxic and cytotoxic effects of the toxic dinoflagellate *Prorocentrum lima* in the
756 mussel *Mytilus galloprovincialis*. *Toxins* (Basel) 8, 159.
757 <https://doi.org/10.3390/toxins8060159>
- 758 Randall, J.E., 2005. Review of clupeotoxism, an often fatal illness from the consumption
759 of clupeoid fishes. *Pac Sci* 59, 73–77.
- 760 Rodríguez, F., Riobó, P., Crespín, G.D., Daranas, A.H., de Vera, C.R., Norte, M.,
761 Fernández, J.J., Fraga, S., 2018. The toxic benthic dinoflagellate *Prorocentrum*
762 *maculosum* Faust is a synonym of *Prorocentrum hoffmannianum* Faust. *Harmful*
763 *Algae* 78, 1–8. <https://doi.org/10.1016/J.HAL.2018.06.009>
- 764 Schmitt, P., Duperthuy, M., Montagnani, C., Bachère, E., Destoumieux-Garzón, D., 2011.
765 Immune responses in the pacific oyster *Crassostrea gigas*: An overview with focus on
766 summer mortalities, in: Qin, J.G. (Ed.), *Oyster Physiology, Ecological-Distribution and*
767 *Mortality*. Nova Science Publishers, New York, pp. 1–47.
- 768 Soliño, L., García-Altare, M., Godinho, L., Costa, P.R., 2020. Toxin profile of *Ostreopsis*
769 *cf. ovata* from Portuguese continental coast and Selvagens Islands (Madeira, Portugal).
770 *Toxicon* 181, 91–101. <https://doi.org/10.1016/J.TOXICON.2020.04.102>
- 771 Song, L., Wang, L., Qiu, L., Zhang, H., 2011. Bivalve immunity, in: Söderhäll, K. (Ed.),
772 *Invertebrate Immunity*. New York: Spring.
773 https://link.springer.com/chapter/10.1007/978-1-4419-8059-5_3
- 774 Soudant, P., E. Chu, F.L., Volety, A., 2013. Host–parasite interactions: Marine bivalve
775 molluscs and protozoan parasites, *Perkinsus* species. *J Invertebr Pathol* 114, 196–216.
776 <https://doi.org/10.1016/J.JIP.2013.06.001>
- 777 Souza, R.V., Silva, B.C., Novaes, A.L.T., 2022. A aquicultura em Santa Catarina em
778 números. Epagri, Florianópolis. 39 p.
- 779 Suplicy, F.M., 2022a. O negócio da ostreicultura, in: Felipe Matarazzo Suplicy (Ed.),
780 *Manual Do Cultivo de Ostras*. Epagri, Florianópolis, pp. 19–25.
- 781 Suplicy, F.M., 2022b. Controle sanitário de ostras, in: Suplicy, F.M. (Ed.), *Manual Do*
782 *Cultivo de Ostras*. Epagri, Florianópolis, pp. 157–169.
- 783 Suzuki, T., Yoshinaka, R., Mizuta, S., Funakoshi, S., Wada, K., 1991. Extracellular matrix
784 formation by amoebocytes during epithelial regeneration in the pearl oyster *Pinctada*
785 *fucata*. *Cell Tissue Research* 266, 75–82.

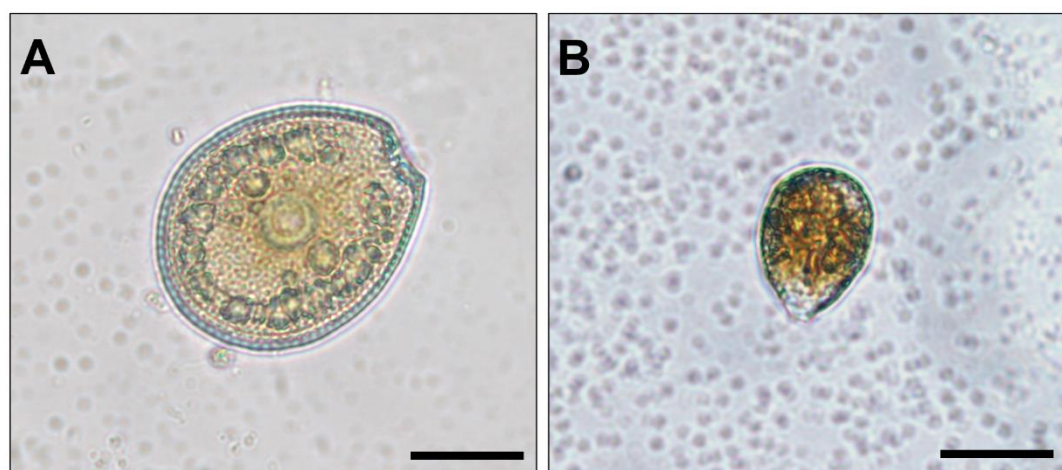
- 786 Tan, K., Sun, Y., Zhang, H., Zheng, H., 2023. Effects of harmful algal blooms on the
787 physiological, immunity and resistance to environmental stress of bivalves: Special
788 focus on paralytic shellfish poisoning and diarrhetic shellfish poisoning. *Aquac.* 563,
789 739000. <https://doi.org/10.1016/j.aquaculture.2022.739000>
- 790 Varela, A.T., Neves, R.A.F., Nascimento, S.M., Oliveira, P.J., Pardal, M.A., Rodrigues,
791 E.T., Moreno, A.J., 2021. Exposure to marine benthic dinoflagellate toxins may lead
792 to mitochondrial dysfunction. *Comp Biochem Physiol C Toxicol Pharmacol* 240,
793 108937. <https://doi.org/10.1016/J.CBPC.2020.108937>
- 794 Wang, L., Song, X., Song, L., 2018. The oyster immunity. *Dev Comp Immunol* 80, 99–
795 118. <https://doi.org/10.1016/J.DCI.2017.05.025>
- 796 Zhang, D., Dong, M., Song, X., Qiao, X., Yang, Y., Yu, S., Sun, W., Wang, L., Song, L.,
797 2022. ROS function as an inducer of autophagy to promote granulocyte proliferation
798 in Pacific oyster *Crassostrea gigas*. *Dev Comp Immunol* 135, 104479.
799 <https://doi.org/10.1016/j.dci.2022.104479>
- 800 Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., Dong, W., 2016. ROS and ROS-
801 mediated cellular signaling. *Oxid Med Cell Longev* 2016, 1–18.
802 <https://doi.org/10.1155/2016/4350965>

803

804

805 8. SUPPLEMENTARY MATERIAL

806



807

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