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1 **Effects of the toxic dinoflagellate *Ostreopsis cf. ovata* on survival, feeding and**
2 **reproduction of a phytal harpacticoid copepod**

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25 Abstract

26 Harmful algal blooms are a source of increasing concern within the health,
27 economic and ecological sectors. In the Mediterranean Sea, severe blooms of the benthic
28 dinoflagellate *Ostreopsis cf. ovata* have been occurring since the beginning of the century,
29 causing human intoxications by inhalation of bio-aerosols or direct contact with cells. The
30 toxicity of this dinoflagellate is attributed to the presence of palytoxin and several of its
31 analogs called ovatoxins, palytoxin being one of the most potent marine toxins. While
32 mass mortalities of marine invertebrates have already been reported in relation with *O.*
33 *cf. ovata* blooms, the toxic effects of this dinoflagellate on benthic organisms is still poorly
34 documented. In the present study, laboratory experiments were performed on a
35 meiobenthic copepod (*Sarsamphiascus cf. propinquus*), which naturally lives on
36 macrophytes in close contact to *O. cf. ovata*, in order to assess its potential toxic effects
37 on mortality, fecal pellet production (as a proxy of feeding), as well as fecundity and
38 fertility ratios. Both, *O. cf. ovata* as well as a non-toxic competitive diatom (*Licmophora*
39 *paradoxa*), were used as food in the experiments. Regarding acute toxicity evaluation,
40 this copepod proved to be the most tolerant organism to *O. cf. ovata* reported to date.
41 Nevertheless, its fecundity and fertility ratios were lower when fed with the toxic
42 dinoflagellate, indicating a possible reprotoxic effect. Moreover, although fecal pellet
43 production decreased significantly when the copepod was fed with a mono-diet of *O. cf.*
44 *ovata*, epifluorescence microscopy observations revealed the presence of the toxic cells
45 inside the digestive track, hence suggesting that these primary grazers could be a vector
46 of toxins through the marine food web.

47

48 Key words: Benthic HABs, *Ostreopsis cf. ovata*, chemical ecology, meiobenthic copepods,
49 reprotoxicity

50

51 **1. Introduction**

52 Over the past decades, the occurrence of Harmful Algal Blooms (HABs) has been
53 increasing in frequency, intensity and geographic distribution (Berdalet et al., 2017;
54 Glibert et al., 2005). These blooms negatively impact human health and wellbeing, by
55 affecting coastal ecosystem services (Berdalet et al., 2015) as well as marine organisms
56 and ecosystems (Dolah et al., 2001). In this regard, the strong geographical expansion of
57 the toxic dinoflagellate *Ostreopsis cf. ovata* in the Mediterranean Sea constitutes an
58 emerging problem (Parsons et al., 2012). This dinoflagellate has long been regarded as a
59 potential vector involved in ciguatera fish poisoning (Glaziou and Legrand, 1994;
60 Hallegraeff, 1993), and it has more recently been shown to produce palytoxin (PLTX) and
61 palytoxin-like compounds (Ciminiello et al., 2006) named ovatoxins (OVTXs) a potential
62 cause of toxic bio-aerosols causing human respiratory illnesses (Ciminiello et al., 2014).
63 *Ostreopsis cf. ovata* is a benthic dinoflagellate usually described as epiphytic on
64 macroalgae and seagrasses growing on rocky shallow seabeds (Mangialajo et al., 2008;
65 Totti et al., 2010) but has also been reported to have a planktonic phase (Vila et al., 2016).

66 Several studies on invertebrate mass mortalities during *Ostreopsis cf. ovata* blooms
67 (Graneli et al., 2002; Shears and Ross, 2009) suggest that this dinoflagellate may be
68 harmful to benthic organisms living nearby even if its effects may be confounded with
69 other stress factors such as pollution or hypo-/anoxic conditions (Shears and Ross, 2010,
70 2009). The toxic effect of *O. cf. ovata* has previously been investigated on several
71 organisms using ecotoxicological bioassays. For instance, the jellyfish *Aurelia sp.*, has

72 been shown to be particularly sensitive at the ephyra (or larval) stage (Giussani et al.,
73 2016) and the mussel *Mytilus galloprovincialis* reacted to *O. cf. ovata* exposures by
74 modifying immunological, histological and oxidative levels (Gorbi et al., 2013). Also,
75 fertilization and early development of the sea urchin *Lytechinus variegatus* were affected
76 by *O. cf. ovata* (Neves et al., 2018). Moreover, a decrease of more than 98.5% of gill cell
77 viability was observed in response to *O. cf. ovata* exposure (Verma et al., 2016). Even
78 though meiofauna living on macrophytes play important roles in benthic biochemical and
79 ecological processes, little is known on the effects of this toxic microalga on the
80 surrounding community. Only one *in situ* study has previously investigated the impact of
81 *O. cf. ovata* on phytal meiofauna, and results showed a severe decrease in the number of
82 nauplii during the toxic blooms, suggesting a reprotoxic effect of the dinoflagellate on
83 harpacticoid copepods (Guidi-Guilvard et al., 2012).

84 The aim of the present study was to provide new insights on the potential effect of
85 the toxic benthic dinoflagellate *Ostreopsis cf. ovata* on meiofauna, and more specifically
86 on benthic copepods. A harpacticoid copepod belonging to the genus *Sarsamphiascus*
87 (formerly *Amphiascus*) was isolated from the natural environment where *O. cf. ovata*
88 summer blooms occur with densities reaching $4 \cdot 10^6$ cells.gFW⁻¹ (Cohu et al., 2013) and
89 was here used as a model organism. Another copepod species belonging to the same
90 genus has already been widely used as a model in toxicity tests (Bejarano and Chandler,
91 2003; Cary et al., 2004; Chandler et al., 2004). This organism was shown to be relevant for
92 ecotoxicological assays due to their small size, short life cycle, simplicity to maintain and
93 manipulate in laboratory conditions and sensitivity to a wide array of toxic substances. In
94 the present laboratory study, the selected copepod was exposed to ecologically relevant
95 concentrations of the dinoflagellate *O. cf. ovata* (Accoroni et al., 2012; Cohu et al., 2013)

96 as well as *Licmophora paradoxa*, a diatom frequently found associated with *O. cf. ovata* in
97 benthic assemblages (Lemée, personal communication), which will here be used as a
98 control. The effects of *O. cf. ovata* were investigated by recording three biological
99 parameters of the copepod: survival, food uptake (assessed through the number of fecal
100 pellets produced) and reproduction (fertility and fecundity ratios). The toxicity of the
101 strain used in these experiments was evaluated using *Artemia franciscana* toxicity bio-
102 assay and confirmed by UHPLC-HRMS quantification of ovatoxins.

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120 2. Materials and Methods

121 2.1. Algal cultures

122 The dinoflagellate *Ostreopsis* cf. *ovata* (MCCV 054) and the diatom *Licmophora*
 123 *paradoxa* (MCCV 033) used as a control microalga were isolated from macroalgal samples
 124 collected at the Rochambeau site (Bay of Villefranche-sur-mer, N-W Mediterranean,
 125 43°41'35.64"N-7°18'31.54"E). The MCCV is the Mediterranean Culture Collection of
 126 Villefranche, where strains are maintained and available for the scientific community.
 127 Both strains were cultured in 150 ml flasks containing L1 medium (Guillard and Hargraves,
 128 1993) prepared with autoclaved aged and 0.2 µm filtered seawater, adjusted to a salinity
 129 of 38. All the cultures were maintained at 24°C, under a 14:10 light/dark cycle (light
 130 intensity 250 µmol.m⁻².s⁻¹). Cells of *O. cf. ovata* used for the experiments were collected
 131 from the cultures during the exponential phase. They were counted in 12 ml aliquots
 132 (triplicates) fixed with acidic lugol (4% v/v) using a liquid particle counter (HIAC/Royco
 133 9703, Pacific Scientific Instruments) following a size range of 2-80 µm (Stramski et al.,
 134 2002).

135 Cell diameter range of *O. cf. ovata* and *L. paradoxa* in culture were 20-40 µm and
 136 15-20 µm, respectively. Biovolumes were calculated assuming *O. cf. ovata* (1) had the
 137 shape of a cone with a half sphere, and *L. paradoxa* (2) was a truncated cone, using the
 138 following equations (Olenina et al., 2006):

$$139 \quad (1) \quad V = \frac{\pi}{12} \times h \times D^2 \quad (2) \quad V = \frac{\pi}{12} \times (d_1^2 + d_1d_2 + d_2^2)$$

140 where V = volume, h = height, D = diameter, d₁ = large diameter, d₂ = small diameter. The
 141 biovolume ratio between the two microalgae was 1:6, i.e. *O. cf. ovata* (average biovolume
 142 = 17 360 µm³) was 6 times larger than *L. paradoxa* (average biovolume = 2 968 µm³).

143 These biovolumes were calculated using 15 cells for each strain.

144

145 2.2. Toxicity of the strain

146 2.2.1. *Artemia franciscana* toxicity bioassay

147 The level of toxicity of the *O. cf. ovata* strain used for this study was checked by
148 performing a standard *Artemia franciscana* test (Faimali et al., 2012; Neves et al., 2017).
149 The high sensitivity of *Artemia* to toxic substances makes it suitable for ecotoxicological
150 assessment (Kalčíková et al., 2012; Nunes et al., 2006). Such bio-assays, described as
151 simple, inexpensive and convenient, can be used to test the toxicity of harmful algal
152 strains (Neves et al., 2017). Cysts of *A. franciscana* (Ocean Nutrition Sep-art) maintained
153 at 4°C in the dark, were incubated in 2 L of filtered seawater (salinity 38), at 20°C with
154 vigorous and continuous aeration until reaching the most sensitive development stages
155 (Stages 2-3, Kerster and Schaeffer, 1983). The larvae were collected using a glass pipette
156 and phototactism and further transferred to 6-well plates. Five larvae were placed in each
157 wells, using one 6-well plate per experimental condition. A total of 4 well-plates were
158 used to investigate the effects of the following cell concentrations of *O. cf. ovata*: 4, 40,
159 400 and 4000 cells.mL⁻¹.

160 The ratio number of algal cells: number of target animals was then between 20:5 to
161 20 000:5. The number of dead larvae was estimated after 2, 4, 24 and 48 h exposure
162 times. Larvae were considered dead when there was no reaction to strong light or to glass
163 pipette aspiration or when a shift to white coloration of the body was observed. LC₅₀ and
164 LT₅₀ values were calculated, corresponding to the concentration which induced the
165 mortality of 50% of the organisms and the lethal time (in hours) to reach 50% mortality,
166 respectively.

167

168 2.2.2. UHPLC-HRMS analyses

169 The toxicity of the strain was confirmed using chemical analyses. *Ostreopsis cf. ovata*
170 cells were grown in 300 mL flasks at previously described conditions, and harvested at
171 day 10. The culture was further centrifuged at 600 g during 10 min at ambient
172 temperature. The cell pellet was flash-freezed using liquid nitrogen and stored at – 80 °C
173 until extraction. The metabolites were extracted using 4 mL of MeOH/H₂O (80:20; v:v)
174 and sonicating during 5 min in a cooled ultra-sonic bath. The extracts were centrifuged at
175 2500 g during 10 min at ambient temperature and the supernatants were transferred into
176 20 mL vials. These steps (MeOH, ultra-sonic bath and centrifugation) were repeated 3
177 times. The resulting organomethanolic extracts were evaporated using a stream of
178 nitrogen avoiding dryness; a volume of 500 µl of DMSO was added in each extract and the
179 remaining H₂O was fully evaporated. All samples were therefore stored in 100% DMSO
180 (500 µl) at -20°C until UHPLC-HRMS analysis.

181 Ovatoxins quantification by UHPLC-UV-HRMS were performed using an Agilent 1290
182 system (Agilent Technologies, USA) equipped with a diode array detector and coupled to
183 an Agilent 6540 Qtof mass spectrometer (Agilent Technologies, USA) by the injection of
184 10µL on T3 column (Acquity UPLC HSS T3 1.8µm, 2.1mm x 100mm, Waters). Separation
185 was achieved using a linear elution gradient of H₂O:MeOH (80:20, v:v)/MeOH with 0.1mM
186 of ammonium formate and 0.1% formic acid from 90:10 (v:v, isocratic from 0 to 2mins) to
187 0:100 (v:v, isocratic from 12 to 13 mins) with a 0.4 mL.min⁻¹ flow rate. UV detection was
188 set at 210, 233 and 263 nm. Ions detections was recorded in positive mode (ESI +) in the
189 range 60 -3000 Da. Collision energies (CE) of 30, 70 and 110 eV were applied to confirm
190 the presence of ovatoxins moieties (Brissard et al., 2014; Ciminiello et al., 2012). The
191 spectrometer analyzer parameters were set as follows: nebulizer sheath gas, N₂ (35 psig);

192 drying gas, N₂ (11 L.min⁻¹); Gas Temperature, 300°C; capillary, 4.129 μA; Vaporizer/Sheat
193 Gas Temp, 350°C.

194 Ovatoxins quantification was performed by extracting and integrating all tri-charged ions
195 (from 858 to 910 m/z) from the base peak chromatogram. The concentration of ovatoxins
196 is given in palytoxin equivalent as commercially available standard of palytoxin (Wako
197 Chemicals GmbH, Neuss, Germany) was used to perform a calibration curve, assuming
198 their ionization pattern is similar.

199

200 2.3. The model copepod

201 The harpacticoid copepod used in the experiments thrives in the shallow rocky
202 shore of the Marinières site (Bay of Villefranche-sur-Mer, N-W Mediterranean,
203 43°42'21.51"N – 7°19'07.44"E) where it was collected in 2012 using a WP2 net towed
204 over the macroalgal cover. In the laboratory, samples were maintained in 10 L tanks
205 together with a planktonic copepod (*Acartia clausi*) in 0.2-μm filtered aged seawater
206 (salinity 38) at 22°C, in the dark to avoid phototactism, and fed three times a week with a
207 mixture of the microalgae *Dunaliella salina* (MCCV 20) and *Tisochrysis lutea* (CCAP
208 927/14). The copepod belongs to the family Miraciidae Dana, 1946 (formerly
209 Diosaccidae), and is part of the *varians*-Group. Within this group, the genus *Amphiascus*,
210 renamed *Sarsamphiascus* by Huys (2009), is well known for taxonomic discrepancies and
211 extreme difficulty in species identification (Hicks, 1971; Wells, 2007). In the present study,
212 the species name remains uncertain due to inter-individual variation in the parapodial
213 setation. However, among the possible *varians*-Group species, only *Amphiascus*
214 *propinquus* had been previously reported in areas close to the Bay of Villefranche-sur-mer

215 (i.e. the Italian Ligurian shore, Ceccherelli, personal communication). For all these
216 reasons, this species was named *Sarsamphiascus cf. propinquus* (Sars, 1906) in our study.

217

218 2.4. Experimental setup

219 All experiments were run in 6-well plates, each well containing 4 mL of autoclaved
220 aged and 0.2- μm filtered seawater (salinity 38), at 24°C, in the dark. One milliliter of
221 microalgae at the appropriate cell concentration was added to the wells as food, except in
222 the no-food controls which received instead 1mL of clean culture medium. The content of
223 each well was renewed every 48 hours and wells were checked daily under a binocular
224 microscope (ZEISS, SteREO Discovery V12). Experiments involving the copepod *S. cf.*
225 *propinquus* started with adults and late copepodites. Prior to the experiments, individuals
226 were collected from the culture tanks, sorted with an elongated Pasteur pipette, flushed
227 twice in seawater and transferred to the 6-well plates. They were left for 2 days without
228 food to allow gut clearance, except in the reproduction experiments where the food was
229 added from the start. At the end of some of the experiments, copepods were fixed with
230 formalin (4% v/v) to determine sex and overall size (from the tip of the cephalosome,
231 excluding the rostrum, to the end of the last body somite, excluding the caudal rami)
232 using a NIKON AZ100 microscope coupled to a Digital Imaging System equipped with the
233 NIS-Elements software (Nikon Instruments Inc., New-York , U.S.A.).

234

235 2.5. Acute toxicity of *O. cf. ovata* on Copepods

236 To estimate and compare the level of toxicity of *O. cf. ovata*, the copepods were
237 exposed to five increasing cell concentrations (500, 1 000, 2 000, 4 000 and 20 000
238 cells.mL⁻¹) and one no-food control in six-well plates, as previously described for *Artemia*

239 bioassay. The test involved 72 individuals (12 per concentration and no-food control, 1
240 per well) and lasted 9 days. The ratio of algal cells/ number of target animals was
241 between 2500:1 to 100 000:1. Mortality (as defined above for *Artemia* bio-assay) was
242 daily recorded in order to calculate LC₅₀ and LT₅₀.

243

244 2.6. Impact of *Ostreopsis cf. ovata* on copepod survival and food uptake

245 To investigate potential toxic effects of *O. cf. ovata* cells, mono- and mixed diets
246 were fed to the copepod. Five different treatments were applied: (i) *O. cf. ovata* at a
247 concentration of 100 cells.mL⁻¹ reflecting the realistic bloom alert threshold measured in
248 the natural environment (Lemée et al., 2012; Tichadou et al., 2010) ; (ii) *L. paradoxa* at a
249 concentration of 600 cells.mL⁻¹ corresponding to the microalgae supplied in treatment (i)
250 in terms of bio-volumes ; (iii) a mixed diet of *O. cf. ovata* and *L. paradoxa* (i.e. treatments
251 (i) and (ii) combined) to investigate if the presence of the two microalgae could change
252 the response of the copepods; (iv) the mixed diet (iii) at half concentrations (i.e. *O. cf.*
253 *ovata* at 50 cells.mL⁻¹ + *L. paradoxa* at 300 cells.mL⁻¹) ; (v) a no-food treatment. To study
254 both mortality and ingestion of *S. cf. propinquus*, two experiments were performed
255 during 10 days and 14 days, each involving 150 individuals (30 per treatment, 1 per well).
256 Mortality (as previously defined) was assessed daily, while feeding (assessed through the
257 number of fecal pellets produced by alive copepods ; Souza-santos et al., 1995) every 48
258 h, at the time of medium renewal. Copepods that had been in contact with *O. cf. ovata*
259 alone were observed under an epifluorescence inverted microscope (Axio Scope.A1, UV
260 excitation, red fluorescence) to check for the presence of *O. cf. ovata* cells in the digestive
261 track.

262

263 2.7. Impact of *Ostreopsis cf. ovata* on reproductive performances of Copepods

264 The potential effects of *O. cf. ovata* on the reproduction capacity of *S. cf. propinquus* were
265 assessed through 3 identical experiments, which ran for 7 days with only mono-diets. The
266 treatments were the same as (i), (ii) and (v) in the previous experiments, i.e. *O. cf. ovata*
267 at 100 cells/mL⁻¹, *L. paradoxa* at 600 cells/mL⁻¹ and a no-food control. For each
268 experiment, 120 males and females were first equally distributed in 3 glass crystallizing
269 dishes and acclimatized to the corresponding treatment for 72h. Then, copepods were
270 randomly transferred to 6-well plates (10 individuals per well to facilitate fecundation).
271 The formation of egg sacs was checked every day to estimate fecundity ratios, i.e. number
272 of ovigerous females to total number of females. Ovigerous females were isolated
273 immediately after egg sacs were observed, but were still exposed to algal treatments
274 described above. Hatching was monitored daily by counting the number of nauplii.
275 Fertility ratios, i.e. number of nauplii to number of ovigerous females, were subsequently
276 calculated, assuming that egg mortality and cannibalism were negligible. Indeed, we
277 assumed that no egg mortality occurred only in control condition because the effect of
278 the toxins on egg mortality was not tested. It is noteworthy that no treatment was used
279 to induce reproduction in this experiment. At the end of the experiments, sex and size of
280 all adult individuals were determined. Body length (Mean ± Standard Deviation) of adult
281 *S. cf. propinquus* reared in the laboratory and used in the experiments was 479 ± 65 µm
282 (n=68) for females and 407 ± 43 µm (n = 79) for males. No mortality was observed in this
283 experiment since it only lasted 7 days and with very low concentrations of *O. cf. ovata*.

284

285 2.8. Statistical analyses

286 Mean fecal pellet production (number of pellets.cop⁻¹) at a given time was
287 calculated only for the copepods that had survived at that given time. Mean egestion rate
288 (number of pellets.cop⁻¹.day⁻¹) for a given treatment and experiment was the slope of the
289 regression line between the corresponding mean fecal pellet production values and time.
290 The R package ecotoxicology was used to calculate median lethal time (LT₅₀) and
291 concentration (LC₅₀). Note that median lethal times were only calculated for the second
292 experiment since a mortality of 100% of the copepods is required to estimate these
293 values.

294 Kruskal-Wallis tests were used to assess the influence of *O. cf. ovata* on egestion; a
295 Dunn's post hoc tests was used *a posteriori* to identify which experimental treatment
296 differed from the others when the Kruskal-Wallis test showed significant differences
297 ($p < 0.05$). Kaplan Meier curves and a log-rank test were applied to evaluate the influence
298 of *O. cf. ovata* on mortality of *S. cf. propinquus*. The Fisher Exact Test was used to assess
299 the potential toxicity of *O. cf. ovata* on the reproduction performances of the copepod.
300 All tests were performed using the PAST software (Hammer et al., 2001).

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310 3. Results

311 3.1. Quantification of the toxins

312 The *Ostreopsis cf. ovata* strain used in the present study (MCCV 054) was shown to
313 produce 44 ± 17 pg PLTX eq/cell at the end of its exponential phase (day 10).

314

315 3.2. Acute toxicity of the *Ostreopsis cf. ovata* on *Artemia franciscana*

316 Mortality of *Artemia franciscana* larvae in the control conditions (without *Ostreopsis cf.*
317 *ovata*) remained below 10% even after 48 hours of experiment (Figure 1), following
318 standard *Artemia* toxicity tests which is less than 10% of mortality in the control.

319 Results showed that *O. cf. ovata* induced mortalities of *A. franciscana* larvae and values
320 increased rapidly with exposure time and concentration of the dinoflagellate. Two hours
321 exposure at the lowest concentration (4 cells.mL^{-1}) were sufficient to impact 13% of the
322 larvae and more than 70% were dead at the highest concentration ($4\ 000 \text{ cells.mL}^{-1}$). After
323 4 hours of exposure, larval mortality almost reached 50% at the lowest concentration of
324 microalgae, and more than 86% at the highest concentration. After 48 hours of exposure,
325 irrespective of the cell concentration tested, all the larvae died while more than 80%
326 survived in the no-food control. Standard measures of toxicity showed a median lethal
327 concentration (LC_{50}) below 4 cells.mL^{-1} after a 48-hour exposure, and a median lethal time
328 (LT_{50}) of 1.3 hours when exposed to the highest cell concentration ($4\ 000 \text{ cells.mL}^{-1}$).

329

330 3.3. Acute toxicity of *Ostreopsis cf. ovata* on Copepods

331 *Ostreopsis cf. ovata* exposures induced mortality of *Sarsamphiascus cf. propinquus* and
332 the impact increased with exposure time and cell concentration (Figure 2). However,
333 results were highly variable and remained relatively low irrespective of the microalgal

334 concentration tested. Even after 9 days of exposure at the highest cell concentration,
335 mortality did not exceed 50% (on average), while it reached more than 58% in the no-
336 food control. However mortality was higher at low concentration (i.e. below 1 000
337 cells.mL⁻¹) than at medium concentration (i.e. between 1 000 cells.mL⁻¹ to 4 000 cells.mL⁻¹).
338 Indeed after 9 days of experiment, 40% of the copepods died at 500 cells.mL⁻¹ although
339 only 20% died at 4 000 cells.mL⁻¹. Median lethal concentration (LC₅₀) calculated after an
340 exposure time of 48 hours was more than 20 000 cells.mL⁻¹, and half lethal time (LT₅₀) for
341 the highest cell concentration (20 000 cells.mL⁻¹) was 120 hours. This latter value was
342 lower compared to that found in the no-food control, i.e. LT₅₀ (no-food) = 192 h.

343

344 3.4. Impact of *Ostreopsis cf. ovata* on food intake of Copepods

345 Fecal pellet production (or egestion) was used as a proxy to estimate food intake. In both
346 the 10-day and 14-day experiments, the number of fecal pellet increased with time in the
347 four feeding treatments, except in the no food treatment (Figure 3). The Kruskal-Wallis
348 analysis of variance showed that mean values differed significantly between treatments
349 ($p < 0.001$, for the two experiments). In the absence of food, egestion was extremely low,
350 and ceased shortly after the start of the experiments. Only 3 and 6 fecal pellets were
351 collected in total from the 10-day and 14-day controls, respectively. When the copepods
352 were fed with *O. cf. ovata* alone, fecal pellet production first increased sharply, and
353 slowed down after a few days. A total of 189 and 45 fecal pellets were collected from the
354 10-day and 14-day experiments with mean egestion rates reaching 0.62 and 0.19
355 pellets.copepod⁻¹.day⁻¹, respectively. The Dunn's post hoc test showed that the mean
356 pellet production measured in the *O. cf. ovata* 14-day experiment was not significantly
357 different from the corresponding no-food control, but was significantly different from

358 measurements in the 10-day experiment. Mean fecal pellet production was significantly
359 different between all treatments (including controls), except for the above stated
360 treatment. It was also significantly different between the *O. cf. ovata* fed as mono-diet
361 and the half concentration of both microalgae mixed-diet as well as between both mixed
362 diets. The highest fecal pellet production values were measured with the diatom mono-
363 diet. Egestion increased linearly with time ($\alpha < 0.005$) with a mean production rate of 5.1
364 and 4.0 pellets.copepod⁻¹.day⁻¹ in the 10-day and 14-day experiments, respectively. When
365 the diatom was supplemented together with *O. cf. ovata* in the food (mixed diets),
366 copepod egestion followed the same trend, but mean egestion rates were lower. With
367 the full concentration of both microalgae in the mixed diet, copepods produced on
368 average 2.5 and 3.8 pellets.copepod⁻¹.day⁻¹, while with the half-mixed diets the mean
369 pellet production decreased to 1.6 and 1.4 pellets.copepod⁻¹.day⁻¹ in the 10-day and 14-
370 day experiments, respectively.

371 Copepods fed with *O. cf. ovata* alone (mono-diet) were observed under an
372 epifluorescence microscope and images revealed an intense fluorescence of their
373 digestive tracks due to the autofluorescence of chlorophyll indicating the presence of
374 dinoflagellate chloroplast (Figure 4).

375

376 3.5. Impact of *Ostreopsis cf. ovata* on survival of Copepods

377 Results obtained in the 10-day and 14-day experiments showed similar trends (Figure 5).

378 Mortality of *S. cf. propinquus* increased with the duration of the experiment, but the
379 magnitude of this increase differed significantly between treatments (log rank test, $p <$
380 0.001 for the two experiments). The highest mortalities occurred in the absence of food,
381 while when *O. cf. ovata* alone was fed to the copepods (mono-diet), mortalities were less

382 pronounced. Feeding a mixed diet by introducing the diatom *L. paradoxa* with *O. cf. ovata*
383 in the food, reduced mortalities. Finally, when the diatom alone was used as food,
384 mortality along time was almost null and only slightly increased towards the end of the
385 experiments. The log-rank test showed that mortality associated to the diatom mono-diet
386 did not significantly differ from mortality measured when copepods were fed with the
387 mixed diet (iii) (i.e. *O. cf. ovata* at 100 cells.mL⁻¹ + *L. paradoxa* at 600 cells.mL⁻¹). On the
388 other hand, mortality values with *O. cf. ovata* differed significantly from mortality values
389 observed in all other treatments. Moreover, mortality due to the absence of food differed
390 significantly from mortality observed in all the other treatments. In other words, *O. cf.*
391 *ovata* alone offered to the copepods and the absence of food, both, strongly affected
392 their survival.

393 However, mortality associated to *O. cf. ovata* in the food always remained lower
394 compared to starvation, excepted during the first 7 days. This difference is reflected in
395 the median lethal time values, i.e. LT₅₀ under starvation was 9 days, while LT₅₀ with *O. cf.*
396 *ovata* (at 100 cells.mL⁻¹) was 11 days. When the copepods were fed with *O. cf. ovata*
397 alone at 100 cells.mL⁻¹, mortality was significantly different from results obtained with
398 mixed diets. For example, after 14 days, ca. 67% of the copepods died with the *O. cf.*
399 *ovata* mono-diet, while they were only ca. 13% when fed with the same concentration of
400 *O. cf. ovata* supplemented with the diatom at 600 cells.mL⁻¹. However, when reducing the
401 cell concentrations of both micro-algae by two fold in the mixed diet (i.e. *O. cf. ovata* at
402 50 cells.mL⁻¹ + *L. paradoxa* at 300 cells .mL⁻¹), copepod mortality increased to ca. 37%.
403 Finally, when the diatom was fed alone to the copepods, mortality values remained below
404 10%.

405

406 3.6. *Impact of Ostreopsis cf. ovata* on reproductive performances of Copepods

407 Depending on the experiment, when the copepods were fed with the diatom *L.*
408 *paradoxa*, 55 to 88% of the females present were gravid (Table 1). On the other hand,
409 when *O. cf. ovata* was used as food, only 13 to 18% of the females were gravid, and in the
410 absence of food (starvation) gravid females did not exceed 10%. The Fisher test showed
411 that fecundity ratios obtained with the *O. cf. ovata* mono-diet were actually not
412 significantly different from values in the no-food controls, but significantly lower than
413 with the diatom mono-diet (Table 2).

414 Depending on the experiment, between 88 and 210 nauplii hatched in the
415 presence of the diatom *L. paradoxa* (Table 1). Only 2 to 11 nauplii hatched in the
416 presence of the dinoflagellate *O. cf. ovata*, and between 0 and 5 nauplii hatched in the
417 absence of food. More specifically, each gravid female produced on average 9.3 nauplii
418 with the diatom mono-diet, 2.7 nauplii with the *O. cf. ovata* mono-diet, and 1.7 nauplii
419 when not fed. Here again, the Fisher test showed no significant difference in the fertility
420 ratios derived from the two latter treatments (Table 2). Moreover, the number of nauplii
421 per ovigerous female was significantly lower when the copepods were exposed to *O. cf.*
422 *ovata* compared to copepods fed *L. paradoxa*. Irrespective of the experimental
423 treatment, all the nauplii that hatched were viable, at least until the end of the
424 experimental period.

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430 4. Discussion

431 The physiological state of the copepod *Sarsamphiascus cf. propinquus* was altered
432 when exposed to the dinoflagellate *Ostreopsis cf. ovata*. Although this copepod appeared
433 as very resistant to the toxic dinoflagellate during acute toxicity evaluation tests, changes
434 in fecal pellet production as well as reduced fecundity and fertility indexes were
435 observed.

436

437 4.1. Acute toxicity of *Ostreopsis cf. ovata*

438 The toxicity of the dinoflagellate strain used in this study was estimated by a
439 simple ecotoxicological assay using *Artemia franciscana* larvae and the content in
440 ovatoxins was further assessed by UHPLC-UV-HRMS. Using a palytoxin standard for
441 calibration, we estimated that the ovatoxin content of the dinoflagellate strain used in
442 this study was comparable to other Mediterranean strains (Brissard et al., 2014), although
443 more enriched than previously assessed for this same strain (18 pg PLTX eq/cell, Ternon
444 et al., 2018). This enrichment can be easily explained by the protocol for cell extraction
445 and data analysis that differed from the previous study.

446 The *Artemia* larvae were highly sensitive to *O. cf. ovata* with a half lethal concentration
447 (LC_{50}) below 4 cells.mL⁻¹ and a median lethal time of 2 hours at a 4 000 cells.mL⁻¹ cell
448 concentration. These crustacean larvae have previously shown high sensitivity towards
449 other Mediterranean strains of *O. cf. ovata* (Faimali et al., 2012; Pezolesi et al., 2012)
450 with median lethal concentrations varying from 4 to 8 cells.mL⁻¹. The results obtained in
451 the present study were consistent with previous studies undertaken on other toxic
452 dinoflagellates such as *Gambierdiscus excentricus*, *Prorocentrum lima* (Neves et al., 2017)
453 and *Ostreopsis siamensis* (Rhodes et al., 2002). The extreme sensitivity exhibited by the

454 *Artemia* larvae in the present study confirms that *Artemia* is a suitable model for
455 assessing the toxicity of *O. cf. ovata*.

456 The toxicity of *O. cf. ovata* has previously been investigated on benthic and pelagic
457 model organisms. Regarding crustaceans, previous studies have shown clear differences
458 between species. For instance, the isopod *Sphaeroma serratum* exhibited lower acute
459 toxicity compared to the amphipod *Corophium insidiosum* (Prato et al., 2011). Our study
460 highlighted (1) a higher mortality of the copepod *S. cf. propinquus* at low concentration
461 due to a starvation. At medium concentration, the mortality was the lowest suggesting an
462 optimal balance between food needs and toxicity effects due to *O. cf. ovata*; (2) a strong
463 resistance of *S. cf. propinquus*, with a median lethal concentration (LC₅₀), reaching more
464 than 20 000 cells.mL⁻¹ after 48 hours of exposure. Indeed, *S. cf. propinquus* was at least 80
465 times more tolerant compared to *Tigriopus fulvus*, for which the LC₅₀ after a 48 hour
466 exposure was 250 cells.mL⁻¹ (Faimali et al., 2012). Such differences could be explained by
467 variable environmental conditions experienced by each species (Prato et al., 2011) or
468 strains used for toxicity assays. We suggest that *S. cf. propinquus* might have adapted to
469 the toxicity of *O. cf. ovata* due to the natural co-occurrence of both organisms. Resistance
470 of grazers facing toxic dinoflagellates has already been reported for calanoid copepod
471 populations that have frequently experienced blooms of the highly toxic *Alexandrium spp.*
472 These copepods were reported to be more resistant to toxic blooms compared to naive
473 populations and had a relatively better fitness in the presence of the toxic dinoflagellates
474 (Colin and Dam, 2005). The difference of sensitivity to *O. cf. ovata* exposure observed
475 between the two benthic copepods could also be explained by the strains used for
476 toxicity assays. Indeed, very few studies have tested the variability among strains (see

477 Faimali et al., 2012; Giussani et al., 2016) and it was impossible to realise this test for
478 technical constraints.

479 In some studies, the toxicity of *O. cf. ovata* was also evaluated by using lysed cells
480 or cellular extracts. For exemple, Faimali et al., (2012) compared the effects of different
481 states of *O. ovata* cultures (such as filtered and resuspended *O. ovata* cells, growth
482 medium devoid of algal cells and sonicated cells) on crustacean and fish larvae and found
483 a significant toxic effect only when the whole algal cells were physically present.

484 However, it is difficult to compare such treatments as little is known about released PLTX
485 and OVTXs toxins which are released, their solubility as well as their stability in seawater.

486 Moreover, the lack of harmonization between extraction procedures applied within
487 studies also make comparisons difficult. It is extremely difficult to identify which chemical
488 cues induced these toxic effects.

489

490 4.2. Was *Ostreopsis cf. ovata* ingested by *Sarsamphiascus cf. propinquus*?

491 Although fecal pellet production is rarely used to evaluate the food uptake in
492 copepods (Souza-santos et al., 1995), the method is simple, fast and quite satisfying when
493 only estimates are needed. In our study, mean egestion rates was between 0.19 and 0.62
494 pellets.copepod⁻¹.day⁻¹ when copepods were fed with *O. cf. ovata*. This suggests a true
495 ingestion of the toxic dinoflagellate by *S. cf. propinquus* even if the egestion rates were
496 significantly lower than those obtained with all other food treatments.

497 This ingestion was confirmed by epifluorescence microscopy observations showing
498 the presence of algal chlorophyll in the gut content. The copepods even seemed to have
499 obtained an energetic advantage since mortality rates were lower when they were fed
500 with *O. cf. ovata* compared to the no-food control. Nevertheless, *O. cf. ovata* alone was

501 not enough to sustain the energy requirements of the copepods as mortality rates were
502 higher in the mono-diet experiment compared to the mixed diet.

503

504 4.3. Negative impacts of *Ostreopsis cf. ovata* on the copepod physiology

505 The food uptake results show a clear decrease of fecal pellet production when the
506 copepods fed on *O. cf. ovata*. Significant differences of fecal pellet production observed
507 when the diatom was offered in mono-diet and in mixed-diet clearly, show that the
508 presence of *O. cf. ovata* is deleterious. This difference could be due to the higher bio-
509 volume of *O. cf. ovata* (compared to the control diatom *L. paradoxa*) or to its toxicity.
510 Copepods are known to actively select the size of their prey (Mullin, 1963). However,
511 some studies suggest that prey size might not be the main factor controlling ingestion.
512 For example, De Troch et al. (2006) showed that the harpacticoid copepod *Harpacticus*
513 *obscurus* had no clear preference for small diatoms. In the present study, the difference
514 in fecal pellet production by *S. cf. propinquus* between the feeding treatments could then
515 rather be explained by the presence of toxic secondary metabolites produced by *O. cf.*
516 *ovata* since mortality was also higher in the presence of *O. cf. ovata*. The addition of the
517 toxic dinoflagellate to the diet clearly contributed to increase copepods mortality. It might
518 hence be suggested that the copepod could avoid toxic cells by chemosensory detection
519 of toxic cues. Some studies have previously shown the ability of pelagic copepods to
520 select a non-toxic prey in a mixed algal culture including the toxic dinoflagellate
521 *Alexandrium excavatum* (Turriff et al., 1995). Such a selection capacity, as suggested for *S.*
522 *cf. propinquus*, is highlighted by a difference in fecal pellet production between mixed-
523 and mono-diets of the diatom *L. paradoxa*. Even if the concentration of the diatom was
524 the same in both diets, the decrease of egestion might be due to an effort to select non-

525 toxic preys, leading to a lower ingestion rate of the algal particles. In addition to the
526 decrease in fecal pellet production of copepods fed *O. cf. ovata*, hatching success was
527 also significantly lower. In fact, compared to the no-food treatment, the presence of the
528 toxic dinoflagellate reduced the fecundity and fertility ratios by 4.7-fold and 3.5-fold,
529 respectively. These results agree with *in situ* observations of Guidi-Guilvard et al (2012)
530 who showed changes in the composition of the meiobenthic community when *O. cf.*
531 *ovata* blooms occurred in summer 2008, with a 72% decrease in the number of nauplii,
532 hence suggesting that the dinoflagellate affected benthic harpacticoid copepods
533 reproduction. These impacts on reproduction are comparable to results obtained in
534 previous studies for planktonic copepod species, such as *Temora stylifera* fed
535 *Prorocentrum micans*, *Gymnodinium sanguinium*, and *Gonyaulax polyedra*, (Ianora et al.,
536 1999; Laabir et al., 2001), as well as *Calanus finmarchicus* (Roncalli et al., 2016) and
537 *Calanus sinicus* (Liu and Wang, 2002) fed *Alexandrium fundyense* and *Alexandrium*
538 *tamarense* respectively. The reprotoxicity of microalgae on copepods could have various
539 origins. For *Temora stylifera* fed on *Prorocentrum micans*, the reduced fertilization was
540 due to an effect on the sperm. Maternal effects or male age are excluded since hatching
541 rates returned to normal when new males were introduced in the experiment (Ianora et
542 al., 1999).

543 It is worth noting that, due to technical constraints, our results were obtained for only a
544 single *O. cf. ovata* strain. These effects on survival and reproduction should be
545 ascertained using other strains from different sites even if these effects of *O. cf. ovata* on
546 reproduction have already been demonstrated on sea urchin (Migliaccio et al., 2016).

547

548 4.4. Were these negative effects due to toxins produced by *O. cf. ovata*?

549 After ingestion of *O. cf. ovata* cells, the release of toxins could have caused a gradual
550 physical incapacitation of the copepod leading to a reduced feeding efficiency. Indeed,
551 during the first 7 days mortality rates were higher with *O. cf. ovata* than in the no-food
552 control. Moreover, during the first 4 days of the “food uptake” experiments, there was no
553 significant difference between ingestion when the diatom was offered alone or when it
554 was mixed with *O. cf. ovata*, suggesting again that the ingestion decrease observed after
555 the first 5 days could be due to an alteration of the copepod’s physiology due to chemical
556 cues produced by the dinoflagellate. This is supported by a previous study by Sykes and
557 Huntley (1987), reporting that ingestion of *Gonyaulax grindleyi* and *Karenia brevis* (ex.
558 *Ptychodiscus brevis*) cells can cause physiological reactions such as regurgitation or
559 elevated heart rate. Other studies have moreover demonstrated an induction of domoic
560 acid production by *Pseudo-nitzschia sp.* exposed to herbivorous copepods (Lundholm et
561 al., 2018) which could act as zooplankton grazing deterrents. The question arises about
562 the role of toxins produced by *O. cf. ovata* in the bloom formation and maintenance by
563 deterring copepods. The ability of *Alexandrium minutum* to increase its toxin production
564 in response to the exposition to copepods has already been demonstrated as a way to
565 facilitate its bloom formation by being more resistant to grazers (Selander et al., 2006).
566 However, the question concerning the digestibility of *O. cf. ovata* arises. *Acartia clausi* fed
567 with the dinoflagellate *Prorocentrum micans*, showed a longer gut transit time suggesting
568 that the digestibility of these cells was difficult, probably because of the presence of
569 cellulose-rich compounds in the theca hence requiring additional enzymes to break down
570 these compounds (Tirelli and Mayzaud, 2005).

571 Very little information is available regarding the effects of PLTX and OVTXs on sperm
572 viability or as antimetabolic compounds. Nonetheless, this impairment on reproductive

573 ability caused by *O. cf. ovata* has previously been described in two species of sea urchins.
574 In *Paracentrotus lividus*, fertilization success and progeny development processes were
575 both compromised (Migliaccio et al., 2016) and in *Lytechinus variegatus*, fertilization and
576 early stage development were also affected (Neves et al., 2018). The mode of action of
577 toxins (i.e. OVTXs) produced by *O. cf. ovata* is poorly documented. PLTX, for instance, is
578 known as a potent inhibitor of sperm motility in several species including the sea urchin
579 *Triploneustes gratilla* (Morton et al., 1982). However, PLTX represents only 8 % of the
580 known toxins contained in Villefranche *O. cf. ovata* (Brissard et al., 2014) and OVTXs
581 toxicity still needs to be evaluated.

582 Reprotoxicity could also be related to the inadequate nutritive values of the
583 dinoflagellate. A previous study has shown that the number of copepod nauplii depends
584 on the quality of the ingested food, suggesting that the biochemical composition of the
585 toxic dinoflagellate *Cochlodinium polykrikoides* may significantly affect *Acartia omorii*
586 reproduction (Kyoungsoon et al., 2003). Such a hypothesis needs to be explored in detail
587 by performing an assessment of the reproductive impacts of *O. cf. ovata* on other
588 copepods or meiobenthic organisms, to confirm if the reproductive effect was due to an
589 inadequate or poor nutritive value or to the toxicity of *O. cf. ovata* secondary
590 metabolites.

591 Interaction between *O. cf. ovata* and its environment could also be mediated by the
592 presence of an extracellular mucilage (Giussani et al., 2015). Indeed, this dinoflagellate
593 produces an abundant mucilaginous matrix, which helps the cells to adhere to substrates.
594 This mucus, which plays diverse roles such as to improve competition with other micro-
595 organisms (for space and nutrients) or as a physical barrier against predators, is actually
596 increasingly reported for dinoflagellates (Barone, 2007; Giussani et al., 2015). During the

597 present study, we often observed an intense production of mucus when *O. cf. ovata*
598 concentrations were high. This mucilage helped the algal cells to adhere to the body of
599 the copepods, mostly on their furca. This could have compromised the copepod's
600 movements, and hence their ability to find food, which furthermore could explain the
601 clear decrease in fecal pellet production when the toxic dinoflagellate was used as food. It
602 could also have altered fertilization (Gasparini et al., 2000).

603

604 **5. Conclusion**

605 This study highlights the high resistance of *S. cf. propinquus* to acute toxicity from
606 *O. cf. ovata*, compared to other tested animals. The observed resistance can result from
607 an acclimatization process developed by this copepod species after frequent exposures to
608 the toxic algae.

609 The present study, together with the previous *in situ* analyses undertaken by
610 Guidi-Guilvard et al (2012), clearly shows the impact of *O. cf. ovata* on the reproduction
611 of benthic copepods, and more precisely on the fecundity and fertility ratios. These
612 deleterious effects on reproduction and consequently on population growth, will modify
613 the composition of benthic communities and can also suggest the existence of chemical
614 defence mechanisms exposed by *O. cf. ovata* as a survival strategy against grazers.

615

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