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

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Abstract

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

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

1. Introduction

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

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

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

2. Materials and Methods

2.1. Algal cultures

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

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

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

where V = volume, h = height, D = diameter, d_1 = large diameter, d_2 = small diameter. The biovolume ratio between the two microalgae was 1:6, i.e. *O.* cf. *ovata* (average biovolume = 17 360 µm^3) was 6 times larger than *L. paradoxa* (average biovolume = 2 968 µm^3). 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. LC_{50} and
164 LT_{50} 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

2.2.2. UHPLC-HRMS analyses

The toxicity of the strain was confirmed using chemical analyses. *Ostreopsis* cf. *ovata* cells were grown in 300 mL flasks at previously described conditions, and harvested at day 10. The culture was further centrifuged at 600 g during 10 min at ambient temperature. The cell pellet was flash-freezed using liquid nitrogen and stored at – 80 °C until extraction. The metabolites were extracted using 4 mL of MeOH/H_2O (80:20; v:v) and sonicating during 5 min in a cooled ultra-sonic bath. The extracts were centrifuged at 2500 g during 10 min at ambient temperature and the supernatants were transferred into 20 mL vials. These steps (MeOH, ultra-sonic bath and centrifugation) were repeated 3 times. The resulting organomethanolic extracts were evaporated using a stream of nitrogen avoiding dryness; a volume of 500 µl of DMSO was added in each extract and the remaining H_2O was fully evaporated. All samples were therefore stored in 100% DMSO (500 µl) at -20°C until UHPLC-HRMS analysis.

Ovatoxins quantification by UHPLC-UV-HRMS were performed using an Agilent 1290 system (Agilent Technologies, USA) equipped with a diode array detector and coupled to an Agilent 6540 Qtof mass spectrometer (Agilent Technologies, USA) by the injection of 10µL on T3 column (Acquity UPLC HSS T3 1.8µm, 2.1mm x 100mm, Waters). Separation was achieved using a linear elution gradient of H_2O:MeOH (80:20, v:v)/MeOH with 0.1mM of ammonium formate and 0.1% formic acid from 90:10 (v:v, isocratic from 0 to 2mins) to 0:100 (v:v, isocratic from 12 to 13 mins) with a 0.4 $mL.min^{-1}$ flow rate. UV detection was set at 210, 233 and 263 nm. Ions detections was recorded in positive mode (ESI +) in the range 60 -3000 Da. Collision energies (CE) of 30, 70 and 110 eV were applied to confirm the presence of ovatoxins moieties (Brissard et al., 2014; Ciminiello et al., 2012). The spectrometer analyzer parameters were set as follows: nebulizer sheath gas, N_2 (35 psig);

192 drying gas, N_2 (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*

bioassay. The test involved 72 individuals (12 per concentration and no-food control, 1 per well) and lasted 9 days. The ratio of algal cells/ number of target animals was between 2500:1 to 100 000:1. Mortality (as defined above for *Artemia* bio-assay) was daily recorded in order to calculate LC_{50} and LT_{50}.

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

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

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

Mean fecal pellet production (number of pellets.cop^{-1}) at a given time was calculated only for the copepods that had survived at that given time. Mean egestion rate (number of pellets.cop^{-1}.day^{-1}) for a given treatment and experiment was the slope of the regression line between the corresponding mean fecal pellet production values and time. The R package ecotoxicology was used to calculate median lethal time (LT_{50}) and concentration (LC_{50}). Note that median lethal times were only calculated for the second experiment since a mortality of 100% of the copepods is required to estimate these values.

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

3. Results

3.1. *Quantification of the toxins*

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

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

Mortality of *Artemia franciscan*a larvae in the control conditions (without *Ostreopsis* cf. *ovata*) remained below 10% even after 48 hours of experiment (Figure 1), following standard *Artemia* toxicity tests which is less than 10% of mortality in the control. Results showed that *O.* cf. *ovata* induced mortalities of *A. franciscana* larvae and values increased rapidly with exposure time and concentration of the dinoflagellate. Two hours exposure at the lowest concentration (4 cells.mL^{-1}) were sufficient to impact 13% of the larvae and more than 70% were dead at the highest concentration (4 000 cells.mL^{-1}). After 4 hours of exposure, larval mortality almost reached 50% at the lowest concentration of microalgae, and more than 86% at the highest concentration. After 48 hours of exposure, irrespective of the cell concentration tested, all the larvae died while more than 80% survived in the no-food control. Standard measures of toxicity showed a median lethal concentration (LC_{50}) below 4 cells.mL^{-1} after a 48-hour exposure, and a median lethal time (LT_{50}) of 1.3 hours when exposed to the highest cell concentration (4 000 cells.mL^{-1}).

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

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

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

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

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

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

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

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

Results obtained in the 10-day and 14-day experiments showed similar trends (Figure 5). Mortality of *S.* cf. *propinquus* increased with the duration of the experiment, but the magnitude of this increase differed significantly between treatments (log rank test, $p < 0.001$ for the two experiments). The highest mortalities occurred in the absence of food, while when *O.* cf. *ovata* alone was fed to the copepods (mono-diet), mortalities were less

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

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

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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4. Discussion

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

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

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

The *Artemia* larvae were highly sensitive to *O.* cf. *ovata* with a half lethal concentration (LC_{50}) below 4 cells.mL^{-1} and a median lethal time of 2 hours at a 4 000 cells.mL^{-1} cell concentration. These crustacean larvae have previously shown high sensitivity towards other Mediterranean strains of *O.* cf. *ovata* (Faimali et al., 2012; Pezzolesi et al., 2012) with median lethal concentrations varying from 4 to 8 cells.mL^{-1}. The results obtained in the present study were consistent with previous studies undertaken on other toxic dinoflagellates such as *Gambierdiscus excentricus, Prorocentrum lima* (Neves et al., 2017) 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 (LC_{50}), 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

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

In some studies, the toxicity of *O.* cf. *ovata* was also evaluated by using lysed cells or cellular extracts. For exemple, Faimali et al., (2012) compared the effects of different states of *O. ovata* cultures (such as filtered and resuspended *O. ovata* cells, growth medium devoid of algal cells and sonicated cells) on crustacean and fish larvae and found a significant toxic effect only when the whole algal cells were physically present. However, it is difficult to compare such treatments as little is known about released PLTX and OVTXs toxins which are released, their solubility as well as their stability in seawater. Moreover, the lack of harmonization between extraction procedures applied within studies also make comparisons difficult. It is extremely difficult to identify which chemical cues induced these toxic effects.

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

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

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

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

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

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

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

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

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

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

5. Conclusion

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

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

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REFERENCES

Accoroni, S., Colombo, F., Pichierri, S., Romagnoli, T., Marini, M., Battocchi, C., Penna, A., Totti, C., 2012. Ecology of *Ostreopsis* cf. *ovata* Blooms in the Northwestern Adriatic Sea. Cryptogamie, Algologie 33, 191–198. https://doi.org/10.7872/crya.v33.iss2.2011.191

Barone, R., 2007. Behavioural trait of *Ostreopsis ovata* (Dinophyceae) in Mediterranean rock pools: the spider's strategy. Harmful algae news 33, 1–3.

Bejarano, A.C., Chandler, G.T., 2003. Reproductive and developmental effects of atrazine on the estuarine meiobenthic copepod *Amphiascus tenuiremis*. Environmental toxicology and chemistry 22. https://doi.org/10.1897/03-40

Berdalet, E., Fleming, L.E., Gowen, R., Davidson, K., Hess, P., Backer, L.C., Moore, S.K., Hoagland, P., Enevoldsen, H., 2015. Marine harmful algal blooms, human health and wellbeing: challenges and opportunities in the 21st century. Journal of the Marine Biological Association of the United Kingdom. https://doi.org/10.1017/S0025315415001733

644 Berdalet, E., Tester, P.A., Chinain, M., Fraga, S., Lemée, R., Litaker, W., Penna, A., Usup, G.,
645 Vila, M., Zingone, A., 2017. Harmful Algal Blooms in Benthic Systems: Recent
646 Progress and Future Research. Oceanography. https://doi.org/10.2307/24897840
647 Brissard, C., Herrenknecht, C., Séchet, V., Hervé, F., Pisapia, F., Harcouet, J., Lemée, R.,
648 Chomérat, N., Hess, P., Amzil, Z., 2014. Complex toxin profile of French
649 Mediterranean *Ostreopsis* cf. *ovata* strains, seafood accumulation and ovatoxins
650 prepurification. Marine Drugs 12, 2851–2876.
651 Cary, T.L., Chandler, G.T., Volz, D.C., Walse, S.S., Ferry, J.L., 2004. Phenylpyrazole
652 Insecticide Fipronil Induces Male Infertility in the Estuarine Meiobenthic
653 Crustacean *Amphiascus tenuiremis*. Environmental Science and Technology 38,
654 522–528. https://doi.org/10.1021/es034494m
655 Chandler, G.T., Cary, T.L., Volz, D.C., Walse, S.S., Ferry, J.L., Klosterhaus, S.L., 2004. Fipronil
656 effects on estuarine copepod (*Amphiascus tenuiremis*) development, fertility, and
657 reproduction: a rapid life-cycle assay in 96-well microplate format. Environmental
658 toxicology and chemistry 23, 117–124. https://doi.org/10.1897/03-124
659 Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Forino, M., Grauso, L.,
660 Tartaglione, L., Guerrini, F., Pezzolesi, L., Pistocchi, R., Vanucci, S., 2012. Isolation
661 and structure elucidation of ovatoxin-a, the major toxin produced by *Ostreopsis*
662 *ovata*. Journal of the American Chemical Society 134, 1869–1875.
663 https://doi.org/10.1021/ja210784u
664 Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L.,
665 Grillo, C., Melchiorre, N., 2006. The Genoa 2005 Outbreak. Determination of
666 putative palytoxin in mediterranean *Ostreopsis ovata* by a new liquid

667 chromatography tandem mass spectrometry method. Analytical Chemistry 78,
668 6153–6159. https://doi.org/10.1021/ac060250j
669 Ciminiello, P., Dell'Aversano, C., Iacovo, E.D., Fattorusso, E., Forino, M., Tartaglione, L.,
670 Benedettini, G., Onorari, M., Serena, F., Battocchi, C., Casabianca, S., Penna, A.,
671 2014. First Finding of *Ostreopsis* cf. *ovata* Toxins in Marine Aerosols.
672 Environmental Science & Technology 48, 3532–3540.
673 https://doi.org/10.1021/es405617d
674 Cohu, S., Mangialajo, L., Thibaut, T., Blanfuné, A., Marro, S., Lemée, R., 2013. Proliferation
675 of the toxic dinoflagellate *Ostreopsis* cf. *ovata* in relation to depth, biotic substrate
676 and environmental factors in the North West Mediterranean Sea. Harmful Algae
677 24, 32–44. https://doi.org/10.1016/j.hal.2013.01.002
678 Colin, S.P., Dam, H.G., 2005. Testing for resistance of pelagic marine copepods to a toxic
679 dinoflagellate. Evolutionary Ecology 18, 355–377. https://doi.org/10.1007/s10682-
680 004-2369-3
681 De Troch, M., Chepurnov, V., Gheerardyn, H., Vanreusel, A., Ólafsson, E., 2006. Is diatom
682 size selection by harpacticoid copepods related to grazer body size? Journal of
683 Experimental Marine Biology and Ecology 332, 1–11.
684 https://doi.org/10.1016/j.jembe.2005.10.017
685 Dolah, F.M. Van, Roelke, D., Greene, R.M., 2001. Health and Ecological Impacts of Harmful
686 Algal Blooms: Risk Assessment Needs. Human and Ecological Risk Assessment 7,
687 1329–1345. https://doi.org/10.1080/20018091095032
688 Faimali, M., Giussani, V., Piazza, V., Garaventa, F., Corrà, C., Asnaghi, V., Privitera, D.,
689 Gallus, L., Cattaneo-Vietti, R., Mangialajo, L., Chiantore, M., 2012. Toxic effects of
690 harmful benthic dinoflagellate *Ostreopsis ovata* on invertebrate and vertebrate

691 marine organisms. Marine Environmental Research 76, 97–107.
692 https://doi.org/10.1016/j.marenvres.2011.09.010
693 Gasparini, S., Daro, M.H., Antajan, E., Tackx, M., Rousseau, V., Parent, J.-Y., Lancelot, C.,
694 2000. Mesozooplankton grazing during the *Phaeocystis globosa* bloom in the
695 southern bight of the North Sea. Journal of Sea Research 43, 345–356.
696 https://doi.org/10.1016/S1385-1101(00)00016-2
697 Giussani, V., Costa, E., Pecorino, D., Berdalet, E., De Giampaulis, G., Gentile, M., Fuentes,
698 V., Vila, M., Penna, A., Chiantore, M., Garaventa, F., Lavorano, S., Faimali, M.,
699 2016. Effects of the harmful dinoflagellate *Ostreopsis* cf. *ovata* on different life
700 cycle stages of the common moon jellyfish *Aurelia* sp. Harmful Algae 57, 49–58.
701 https://doi.org/10.1016/j.hal.2016.05.005
702 Giussani, V., Sbrana, F., Asnaghi, V., Vassalli, M., Faimali, M., Casabianca, S., Penna, A.,
703 Ciminiello, P., Dell'Aversano, C., Tartaglione, L., Mazzeo, A., Chiantore, M., 2015.
704 Active role of the mucilage in the toxicity mechanism of the harmful benthic
705 dinoflagellate *Ostreopsis* cf. *ovata*. Harmful Algae 44, 46–53.
706 https://doi.org/10.1016/j.hal.2015.02.006
707 Glaziou, P., Legrand, A.-M., 1994. The epidemiology of ciguatera fish poisoning. Toxicon
708 32, 863–873. https://doi.org/10.1016/0041-0101(94)90365-4
709 Glibert, P.M., Anderson, D.M., Gentien, P., Granéli, E., Sellner, K.G., 2005. The Global,
710 Complex Phenomena of Harmful Algal Blooms. Oceanography 1818.
711 Gorbi, S., Avio, G.C., Benedetti, M., Totti, C., Accoroni, S., Pichierri, S., Bacchiocchi, S.,
712 Orletti, R., Graziosi, T., Regoli, F., 2013. Effects of harmful dinoflagellate *Ostreopsis*
713 cf. *ovata* exposure on immunological, histological and oxidative responses of

714 mussels *Mytilus galloprovincialis*. Fish & Shellfish Immunology 35, 941–950.
715 https://doi.org/10.1016/j.fsi.2013.07.003
716 Graneli, E., Ferreira, C., Yasumoto, T., Rodrigues, E., Neves, M., Granéli, E., 2002. Sea
717 urchins poisoning by the benthic dinoflagellate *Ostreopsis ovata* on the Brazilian
718 Coast.
719 Guidi-Guilvard, L.D., Gasparini, S., Lemée, R., 2012. The negative impact of *Ostreopsis* cf.
720 *ovata* on phytal meiofauna from the coastal NW Mediterranean. Cryptogamie,
721 Algologie 33, 121–128.
722 Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a
723 chrysophyte. Phycologia 32, 234–236. https://doi.org/10.2216/i0031-8884-32-3-
724 234.1
725 Hallegraeff, G.M., 1993. A Review of Harmful Algal Blooms and Their Apparent Global
726 Increase. Phycologia 32, 79–99. https://doi.org/10.2216/i0031-8884-32-2-79.1
727 Hammer, O., Harper, D.A.T., Ryan, P.D., 2001. PAST: Paleontological Statistics Software
728 Package for Education and Data Analysis. Electronia 9.
729 Hicks, G.R.F., 1971. Some littoral harpacticoid copepods, including five new species, from
730 Wellington, New Zealand. New Zealand Journal of Marine and Freshwater
731 Research 5, 86–119. https://doi.org/10.1080/00288330.1971.9515371
732 Huys, R., 2009. Unresolved cases of type fixation, synonymy and homonymy in
733 harpacticoid copepod nomenclature (Crustacea: Copepoda). Zootaxa 2183, 1–99.
734 Ianora, A., Miralto, A., Buttino, I., Romano, G., Poulet, S.A., 1999. First evidence of some
735 dinoflagellates reducing male copepod fertilization capacity. Limnology and
736 Oceanography 44, 147–153. https://doi.org/10.4319/lo.1999.44.1.0147

737 Kalčíková, G., Zagorc-Končan, J., Žgajnar Gotvajn, A., 2012. *Artemia salina* acute
738 immobilization test: a possible tool for aquatic ecotoxicity assessment. Water
739 Science & Technology 66. https://doi.org/10.2166/wst.2012.271
740 Kerster, H.W., Schaeffer, D.J., 1983. Brine shrimp (*Artemia salina*) nauplii as a teratogen
741 test system. Ecotoxicology and Environmental Safety 7, 342–349.
742 https://doi.org/10.1016/0147-6513(83)90079-9
743 Kyoungsoon, S., Min-Chul, J., Pung-Kuk, J., Se-Jong, J., Tea-Kyun, L., Man, C., 2003.
744 Influence of food quality on egg production and viability of the marine planktonic
745 copepod *Acartia omorii*. Progress in Oceanography 57, 265–277.
746 https://doi.org/10.1016/S0079-6611(03)00101-0
747 Laabir, M., Buttino, I., Ianora, A., Kattner, G., Poulet, S.A., Romano, G., Carotenuto, Y.,
748 Miralto, A., 2001. Effect of specific dinoflagellate and diatom diets on gamete
749 ultrastructure and fatty acid profiles of the copepod *Temora stylifera*. Marine
750 Biology 138, 1241–1250. https://doi.org/10.1007/s002270100547
751 Lemée, R., Mangialajo, L., Cohu, S., Amzil, Z., Blanfune, A., Chomerat, N., Ganzin, N.,
752 Gasparini, S., Grossel, H., Guidi-Guivard, L., Hoareau, L., Duff, F. le, Marro, S.,
753 Simon, N., Nezan, E., Pedrotti, M.-L., Sechet, V., Soliveres, O., Thibaut, T., 2012.
754 Interactions between Scientists, Managers and Policy Makers in the Framework of
755 the French MediOs Project on *Ostreopsis* (2008–2010). Cryptogamie, Algologie 33,
756 137–142. https://doi.org/10.7872/crya.v33.iss2.2011.137
757 Liu, S., Wang, A.W.-X., 2002. Feeding and reproductive responses of marine copepods in
758 South China Sea to toxic and nontoxic phytoplankton. Marine Biology 140, 595–
759 603. https://doi.org/10.1007/s00227-001-0714-4

760 Lundholm, N., Krock, B., John, U., Skov, J., Cheng, J., Pančić, M., Wohlrab, S., Rigby, K.,
761 Nielsen, T.G., Selander, E., Harðardóttir, S., 2018. Induction of domoic acid
762 production in diatoms—Types of grazers and diatoms are important. Harmful
763 Algae 79, 64–73. https://doi.org/10.1016/j.hal.2018.06.005
764 Mangialajo, L., Bertolotto, R., Cattaneo-Vietti, R., Chiantore, M., Grillo, C., Lemee, R.,
765 Melchiorre, N., Moretto, P., Povero, P., Ruggieri, N., 2008. The toxic benthic
766 dinoflagellate *Ostreopsis ovata*: Quantification of proliferation along the coastline
767 of Genoa, Italy. Marine Pollution Bulletin 56, 1209–1214.
768 https://doi.org/10.1016/j.marpolbul.2008.02.028
769 Migliaccio, O., Castellano, I., Di Cioccio, D., Tedeschi, G., Negri, A., Cirino, P., Romano, G.,
770 Zingone, A., Palumbo, A., 2016. Subtle reproductive impairment through nitric
771 oxide-mediated mechanisms in sea urchins from an area affected by harmful algal
772 blooms. Scientific Reports 6, 1–14. https://doi.org/10.1038/srep26086
773 Morton, B., Fraser, C., Thenawidjaja, M., Albagli, L., Rayner, M., 1982. Potent inhibition of
774 sperm motility by palytoxin. Experimental Cell Research 140, 261–265.
775 https://doi.org/10.1016/0014-4827(82)90113-6
776 Mullin, M.M., 1963. Some factors affecting the feeding of marine copepods of the genus
777 *Calanus*. Limnology and Oceanography 8, 239–250.
778 https://doi.org/10.4319/lo.1963.8.2.0239
779 Neves, R.A.F., Contins, M., Nascimento, S.M., 2018. Effects of the toxic benthic
780 dinoflagellate *Ostreopsis* cf. *ovata* on fertilization and early development of the
781 sea urchin *Lytechinus variegatus*. Marine Environmental Research.
782 https://doi.org/10.1016/J.MARENVRES.2018.01.014

783 Neves, R.A.F., Fernandes, T., Dos Santos, L.N., Nascimento, S.M., 2017. Toxicity of benthic
784 dinoflagellates on grazing, behavior and survival of the brine shrimp *Artemia*
785 *salina*. PLoS ONE 12, 1–17. https://doi.org/10.1371/journal.pone.0175168
786 Nunes, B.S., Carvalho, F.D., Guilhermino, L.M., Stappen, G. Van, 2006. Use of the genus
787 *Artemia* in ecotoxicity testing. Environmental Pollution 144, 453–462.
788 https://doi.org/10.1016/j.envpol.2005.12.037
789 Olenina, I., Andersson, A., Wasmund, N., Busch, S., 2006. Biovolumes and size-classes of
790 phytoplankton in the Baltic Sea Helsinki Commission Baltic Marine Environment
791 Protection Commission.
792 Parsons, M.L., Aligizaki, K., Bottein, M.-Y.D., Fraga, S., Morton, S.L., Penna, A., Rhodes, L.,
793 2012. *Gambierdiscus* and *Ostreopsis*: Reassessment of the state of knowledge of
794 their taxonomy, geography, ecophysiology, and toxicology. Harmful Algae 14,
795 107–129. https://doi.org/10.1016/j.hal.2011.10.017
796 Pezzolesi, L., Guerrini, F., Ciminiello, P., Dell'Aversano, C., Iacovo, E.D., Fattorusso, E.,
797 Forino, M., Tartaglione, L., Pistocchi, R., 2012. Influence of temperature and
798 salinity on *Ostreopsis* cf. *ovata* growth and evaluation of toxin content through HR
799 LC-MS and biological assays. Water Research 46, 82–92.
800 https://doi.org/10.1016/j.watres.2011.10.029
801 Prato, E., Biandolino, F., Bisci, A.P., Caroppo, C., 2011. Preliminary assessment of
802 *Ostreopsis* cfr. *ovata* acute toxicity by using a battery bioassay. Chemistry and
803 Ecology 27, 117–125. https://doi.org/10.1080/02757540.2011.625930
804 Rhodes, L., Towers, N., Briggs, L., Munday, R., Adamson, J., 2002. Uptake of palytoxin-like
805 compounds by shellfish fed *Ostreopsis siamensis* (Dinophyceae). New Zealand

Journal of Marine and Freshwater Research 36, 631–636. https://doi.org/10.1080/00288330.2002.9517118

Roncalli, V., Turner, J.T., Kulis, D., Anderson, D.M., Lenz, P.H., 2016. The effect of the toxic dinoflagellate *Alexandrium fundyense* on the fitness of the calanoid copepod *Calanus finmarchicus*. Harmful Algae 51, 56–66. https://doi.org/10.1016/j.hal.2015.11.003

Sars, G.O., 1906. An Account of the Crustacea of Norway, Vol. V: Copepoda (Harpacticoida): Georg Ossian Sars | NHBS Shop.

Selander, E., Thor, P., Toth, G., Pavia, H., 2006. Copepods induce paralytic shellfish toxin production in marine dinoflagellates. Proceedings of the Royal Society B: Biological Sciences 273, 1673–1680. https://doi.org/10.1098/rspb.2006.3502

Shears, N.T., Ross, P.M., 2010. Toxic cascades: multiple anthropogenic stressors have complex and unanticipated interactive effects on temperate reefs. Ecology Letters 13, 1149–1159. https://doi.org/10.1111/j.1461-0248.2010.01512.x

Shears, N.T., Ross, P.M., 2009. Blooms of benthic dinoflagellates of the genus *Ostreopsis*; an increasing and ecologically important phenomenon on temperate reefs in New Zealand and worldwide. Harmful Algae 8, 916–926. https://doi.org/10.1016/j.hal.2009.05.003

Souza-santos, L.P., Castel, J., dos Santos, P.J.P., 1995. Feeding rate cycle of the epibenthic harpacticoid copepod *Harpacticus flexus*: Laboratory experiments using fecal pellets counts. Vie milieu 45, 75–83.

Stramski, D., Sciandra, A., Claustre, H., 2002. Effects of temperature, nitrogen, and light limitation on the optical properties of the marine diatom *Thalassiosira*

pseudonana. Limnology and Oceanography 47, 392–403. https://doi.org/10.4319/lo.2002.47.2.0392

Sykes, P.F., Huntley, M.E., 1987. Acute physiological reactions of *Calanus pacificus* to selected dinoflagellates: direct observations. Mar. Biol. 94, 19–24. https://doi.org/10.1007/BF00392895

Ternon, E., Pavaux, A.-S., Marro, S., Thomas, O.P., Lemée, R., 2018. Allelopathic interactions between the benthic toxic dinoflagellate *Ostreopsis* cf. *ovata* and a co-occurring diatom. Harmful Algae 75, 35–44. https://doi.org/10.1016/j.hal.2018.04.003

Tichadou, L., Glaizal, M., Armengaud, A., Grossel, H., Lemée, R., Kantin, R., Lasalle, J.-L., Drouet, G., Rambaud, L., Malfait, P., de Haro, L., 2010. Health impact of unicellular algae of the *Ostreopsis* genus blooms in the Mediterranean Sea: experience of the French Mediterranean coast surveillance network from 2006 to 2009. Clinical Toxicology 48, 839–844. https://doi.org/10.3109/15563650.2010.513687

Tirelli, V., Mayzaud, P., 2005. Relationship between functional response and gut transit time in the calanoid copepod *Acartia clausi*: role of food quantity and quality. Journal of Plankton Research 27, 557–568. https://doi.org/10.1093/plankt/fbi031

Totti, C., Accoroni, S., Cerino, F., Cucchiari, E., Romagnoli, T., 2010. *Ostreopsis ovata* bloom along the Conero Riviera (northern Adriatic Sea): Relationships with environmental conditions and substrata. Harmful Algae 9, 233–239. https://doi.org/10.1016/j.hal.2009.10.006

Turriff, N., Runge, J.A., Cembella, A.D., 1995. Toxin accumulation and feeding behaviour of the planktonic copepod *Calanus finmarchicus* exposed to the red-tide

852 dinoflagellate *Alexandrium excavatum*. Marine Biology 123, 55–64.
853 https://doi.org/10.1007/BF00350323
854 Verma, A., Hoppenrath, M., Dorantes-Aranda, J.J., Harwood, D.T., Murray, S.A., 2016.
855 Molecular and phylogenetic characterization of *Ostreopsis* (Dinophyceae) and the
856 description of a new species, *Ostreopsis rhodesae* sp. nov., from a subtropical
857 Australian lagoon. Harmful Algae 60, 116–130.
858 https://doi.org/10.1016/j.hal.2016.11.004
859 Vila, M., Abós-Herràndiz, R., Isern-Fontanet, J., Àlvarez, J., Berdalet, E., 2016. Establishing
860 the link between *Ostreopsis* cf. *ovata* blooms and human health impacts using
861 ecology and epidemiology. Scientia Marina 80, 107–115.
862 https://doi.org/10.3989/scimar.04395.08A
863 Wells, J.B.J., 2007. Zootaxa,An annotated checklist and keys to the species of Copepoda
864 Harpacticoida 4.