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# The use of stable isotopes to measure the ingestion rate of 1 potentially toxic benthic dinoflagellates by harpacticoid copepods

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1           The use of stable isotopes to measure the ingestion rate of  
2           potentially toxic benthic dinoflagellates by harpacticoid copepods

3  
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15  
16   **Keywords:** *Ostreopsis* cf. *ovata*, *Amphidinium* cf. *carterae*, grazing rate, isotope, meiofauna,  
17   food web

18   **Running title:** Dinoflagellate ingestion by copepods

19  
20   **Abstract**

21       Phycotoxins synthesized by benthic dinoflagellates are known to bioaccumulate in  
22 macrofauna and hence represent a risk for human health. However, the presence of toxins  
23 synthesized by benthic dinoflagellates in smaller marine organisms than macrofauna has not  
24 been considered despite the fact that such small organisms have an important ecological role  
25 in the benthic food web. This present study quantified, for the first time, the trophic  
26 relationship between benthic dinoflagellates and meiofauna by using stable isotope enriched  
27 dinoflagellates during ingestion experiments. Results showed that harpacticoid copepods were  
28 not able to discriminate, during ingestion, between the potentially toxic cells of *Ostreopsis* cf.  
29 *ovata* and the non-toxic cells of *Amphidinium* cf. *carterae*, even when another food resource,  
30 such as diatoms (e.g. *Odontella* sp.), was provided (Kruskal Wallis test,  $p > 0.05$ ).

31

## 32       Introduction

33 Over the last ten years, the frequency and the geographic extent of harmful algal blooms  
34 (HAB) has increased worldwide (Hallegraeff, 1993; Van Dolah, 2000; Cohu et al., 2011).  
35 Among the species which are able to generate HAB, around twenty benthic marine species  
36 have been identified to produce a wide variety of toxins including the most potent toxins  
37 occurring in nature (Accoroni et al., 2016; Chomérat et al., 2019; Hoppenrath et al., 2014;  
38 Litaker et al., 2017; Rodríguez et al., 2018; Tubaro et al., 2011; Verma et al., 2016; Yasumoto  
39 et al., 1987).

40       Benthic dinoflagellates synthesizing these toxins are particularly dangerous due to  
41 their impact on marine life (Shears and Ross, 2009) and human health (Alcala et al., 1988;  
42 Ciminiello et al., 2006; Friedman et al., 2008). Indeed, herbivorous and filter-feeding marine  
43 organisms can ingest and accumulate toxins produced by benthic dinoflagellates throughout  
44 their life (Chungue et al., 1977; Gleibs and Mebs, 1999; Yasumoto et al., 1976, 1971). Toxins  
45 produced by benthic dinoflagellates can transfer between different trophic levels, through

46 predation and bioaccumulation processes thereby reaching high concentrations in top  
47 predators which can be consumed by humans (Lewis and Holmes, 1993; Randall, 1958;  
48 Vernoux, 1988). Ingestion of seafood, previously contaminated by these toxins, can lead to  
49 mass mortalities of marine organisms involving important ecological impacts over large  
50 spatial scales (Shears and Ross, 2009) as well as economic impacts affecting the shellfish  
51 farming sector (Shumway, 1990). Due to the thermostability of these toxins (Kohli et al.,  
52 2015), each benthic toxic genus is responsible for specific human health issues, nevertheless  
53 leading in rare cases to death (Bagnis et al., 1979; Alcala et al., 1988; Onuma et al., 1999).

54 The genera *Gambierdiscus*, *Fukuyoa*, *Ostreopsis*, *Prorocentrum*, *Coolia*, and  
55 *Amphidinium* are frequently involved in benthic dinoflagellate blooms of (Hoppenrath et al.,  
56 2014; Leung et al., 2018; Smith et al., 2017).

57 The most serious human poisoning events are related to the occurrence of *Gambierdiscus*,  
58 *Fukuyoa* (Bagnis et al., 1979; Chinain et al., 2019, 2014; Friedman et al., 2017), and  
59 *Ostreopsis* (Alcala et al., 1988; Randall, 2005) genera which are known to synthesize potent  
60 neurotoxins (Alloisio et al., 2016; Dechraoui et al., 1999). The species of *Gambierdiscus* and  
61 *Fukuyoa* are known to produce ciguatoxins (Yasumoto et al., 1977; Pearn, 2001; Litaker et  
62 al., 2017; Munday et al., 2017) which are potentially lethal for humans, identified as ciguatera  
63 fish poisoning (Chinain et al., 2019, 2014; Yasumoto et al., 1977). Toxic *Ostreopsis* species  
64 synthesize ovatoxins and palytoxins (Ciminiello et al., 2008; Patocka et al., 2018; Rossi et al.,  
65 2010) causing human palytoxicosis and clupeotoxism after the consumption of certain  
66 tropical crustaceans and fish species which are able to bioaccumulate these toxins (Alcala et  
67 al., 1988; Onuma et al., 1999). In temperate regions, toxins synthesized by *Ostreopsis* spp. are  
68 involved in irritations by direct contact (Tichadou et al., 2010) and poisoning by inhalation  
69 (Gallitelli et al., 2005; Durando et al., 2007), however no incident has yet been reported by  
70 ingestion, even though these toxic compounds were detected in seafood.

71           Toxins produced by *Prorocentrum*, *Coolia*, and *Amphidinium* are less dangerous for  
72 humans than those synthesized by the genera *Gambierdiscus*, *Fukuyoa* and *Ostreopsis*. The  
73 genus *Prorocentrum* is distributed worldwide (Rodriguez et al., 2010; Richlen and Lobel,  
74 2011) and is able to produce okadaic acid (Valdiglesias et al., 2013). This phycotoxin  
75 accumulates in shellfish and crustaceans (Kumagai et al., 1986; Vale and Sampayo, 2002) is  
76 responsible for human diarrhetic shellfish poisoning (Tripuraneni et al., 1997). Species of  
77 *Coolia* synthesize lipophilic toxins but these organisms have not yet been related to human  
78 poisoning events (Ben-Gharbia et al., 2016). Some *Amphidinium* species synthesize  
79 amphidinols and amphidinolides (Paul et al., 1997; Kobayashi, 2008). Laboratory scale  
80 experiments have shown that toxins produced by the genus *Amphidinium* can affect marine  
81 organisms (Pagliara and Caroppo, 2012), but there is still no evidence showing that the  
82 bioaccumulation of these toxins throughout trophic levels, including humans, is harmful  
83 (Botana, 2014).

84

85           The effects of toxins on benthic macrofauna consumed by humans have been intensively  
86 studied for several decades, for instance on filter feeders, (Lee et al., 1988; Amzil et al.,  
87 2012), macrophyte grazers (Amzil et al., 2012; Biré et al., 2015, 2013; Chungue et al., 1977;  
88 Yasumoto et al., 1976) and carnivorous fish (Lewis and Endean, 1984), and this due to the  
89 presence of toxic benthic dinoflagellates in coastal areas. However, such effort focusing on  
90 macrofaunal organisms has led to overlook the potential transfer of benthic dinoflagellates  
91 toxins to higher trophic levels through smaller size organisms representing the meiofauna.

92           Meiofauna are a prominent component of the benthos and consist of marine metazoans  
93 with variable sizes ranging from 40  $\mu\text{m}$  to 500  $\mu\text{m}$  (Giere 2009). Meiofauna play two  
94 important roles in the benthic marine food web. They *i*) actively graze microalgae (Blanchard  
95 1991, Montagna et al. 1995) and to a certain extent bacteria (Pascal et al., 2009), and *ii*)

96 constitute the predominant food source for a variety of benthic and pelagic predators (Gee  
97 1989, Coull 1990). In the pelagic environment, zooplankton copepods can ingest  
98 dinoflagellates (Klein Breteler et al. 1990, Klein Breteler et al. 1999) and can consequently  
99 transfer their toxins throughout the pelagic food web (Maneiro et al. 2000, Jansen et al. 2006).  
100 Harpacticoid copepods associated to macrophytes can dominate the meiofauna (Beckley and  
101 McLachlan, 1980; Guidi-Guilvard et al., 2012; Johnson and Scheibling, 1987) and mainly  
102 graze on epiphytic algae (De Troch et al. 2007, Pavaux et al., 2019). Benthic copepods are  
103 ingested by benthic and pelagic predators (Gee 1989, Coull 1990) and could consequently  
104 create an important link between benthic toxic dinoflagellates and higher trophic levels. To  
105 our knowledge, this trophic link has never been measured.

106 The aim of the present study is to quantify ingestion rates of harpacticoid copepods fed  
107 with two species of benthic toxic dinoflagellates (*Ostreopsis* cf. *ovata* and *Amphidinium* cf.  
108 *carterae*), each having a distinct level of toxicity. Feeding experiments were also undertaken  
109 with and without a non-toxic diatom, *Odontella* sp., in order to evaluate the impact of another  
110 food resource on the ingestion rate of dinoflagellates.

111

## 112 **Material and method**

### 113 *Micro-algal culture conditions*

114 The dinoflagellate clonal cultures of *Amphidinium* cf. *carterae* (MCCV092), *Ostreopsis*  
115 cf. *ovata* (MCCV070) and the clonal culture of the diatom *Odontella* sp. (MCCV081) were  
116 initiated using specimens collected in Bois Jolan (16°14'08.2''N — 61°20'59.8W,  
117 Guadeloupe, Caribbean Sea). These strains are maintained in the Mediterranean Culture  
118 Collection of Villefranche, France (MCCV strain number). Non-axenic cultures were grown  
119 in L1 medium (Guillard and Hargraves, 1993) using autoclaved, aged 0.2 µm filtered  
120 seawater with a salinity of 35. The cultures were maintained at 27°C with a 12:12 light: dark

121 cycle provided by cool-white fluorescent tubes in a Memmert incubator. Stock cultures were  
122 grown in 15 mL culture medium in flat culture flasks in order to optimize the surface culture  
123 area for gas exchange and growth of benthic dinoflagellates. During the exponential growth  
124 phase, clonal cultures were successively diluted in order to scale up the culture volume from  
125 15 mL (in tissue culture flask of 25 cm<sup>2</sup> surface area, Corning) to 1 L (in fernbach culture  
126 flasks of 314 cm<sup>2</sup> surface area, Scott/Duran).

127 The biovolume of cells sampled from each cultivated strain was evaluated under an  
128 inverted microscope (Zeiss Axiovert 40 C) using the approximate geometrical shapes of the  
129 dinoflagellates, and the mathematical equation suggested for each genus (Hillebrand et al.,  
130 1999). The ellipsoid shape was chosen for *Amphidinium* cf. *carterae*, the cone and half sphere  
131 shape was chosen for *Ostreopsis* cf. *ovata* and the cylinder shape for *Odontella* sp. Five cells  
132 of each genus were used to determine the biovolume.

### 133 *Dinoflagellates identification*

134 A volume of 5 mL of each clonal culture was centrifuged for 6 min at 2000 rpm. The  
135 supernatant was removed and cell cultures were resuspended with 1 mL of sterile water  
136 (MilliQ, Millipore). After a second centrifugation step (2 min at 2000 rpm), only the cell  
137 pellets were kept and homogenized with 40 µL of sterile water and a fraction of 10 µL of cell  
138 pellet was transferred to 0.2 mL polymerase chain reaction (PCR) tubes. Then, PCR tubes  
139 were stored at —20°C until further analysis.

140 Approximately 400 base pairs of the internal transcribed spacer region (ITS1-5.8s-  
141 ITS2) ribosomal DNA (rDNA) were amplified by PCR using the primer 329F (5'-  
142 GTGAACCTGCRGAAGGATCA-3') which is the inverse complementary sequence of the  
143 universal eukaryote primer 329-R (Moon-van der Staay et al., 2001) and the designed primer  
144 DIR-R (5'-TATGCTTAAAATTCAGCAGGT-3') which is the inverse complementary  
145 sequence of the primer DIR-F (Scholin et al., 1994).

146 Each PCR tube containing the cell pellets was resuspended in 1  $\mu\text{L}$  of each primer at  
147 10  $\mu\text{M}$ , 1  $\mu\text{L}$  of dNTP at 10 mM, 1  $\mu\text{L}$  of the 50X *Taq* Advantage 2 DNA polymerase  
148 (Clontech), 5  $\mu\text{L}$  of 10X Advantage 2 PCR Buffer and 31  $\mu\text{L}$  of sterile water in order to  
149 perform PCR reactions in a final volume of 50  $\mu\text{L}$ . The PCR was performed using a  
150 MasterGradient thermocycler (Eppendorf) with the following conditions: one initial  
151 denaturation at 94°C for 10 min followed by 35 cycles each consisting of 1 min at 94°C, 1  
152 min at 53°C, 1 min at 68°C and a final elongation for 10 min at 68°C. The PCR products were  
153 purified using QIAGEN MinElute PCR Purification kit according to the recommendations.  
154 After sequencing by Genewiz, sequences were treated using BioEdit software and were  
155 compared to the National Center for Biotechnology Information database using BLASTn tool.  
156 The rDNA sequences obtained have been deposited on GenBank with the GenBank accession  
157 nos. **MK543271** and **MK543258** for the strains MCCV092 and MCCV070 respectively.

#### 158 *Labeling of micro-algal cultures*

159 The experiments were run using a dinoflagellate culture (*Amphidinium* cf. *carterae* or  
160 *Ostreopsis* cf. *ovata*) and a diatom culture (*Odontella* sp.) in the exponential growth phase  
161 characterized by a cell density above 1000 cells  $\text{mL}^{-1}$ . Cultures of *Amphidinium* cf. *carterae*  
162 and *Ostreopsis* cf. *ovata* were labeled with  $^{15}\text{N}$  a week before the beginning of feeding  
163 experiments.

164 Before deploying the stable isotope enrichment experiment, cultures of dinoflagellates ( $>$   
165 1000 cells  $\text{mL}^{-1}$ ) were filtered through a 10  $\mu\text{m}$  mesh and rinsed with autoclaved 0.2  $\mu\text{m}$   
166 filtered aged seawater at a salinity of 35. Dinoflagellates remaining on the mesh were  
167 collected with a pipette. The collection, rinsing, and suspension steps were performed on  
168 successive small aliquots of culture (35-40 mL) in order to avoid net clogging due to mucus  
169 accumulation. Rinsed dinoflagellates (1500 cells  $\text{mL}^{-1}$ ) were placed in 1 L of L1 culture  
170 medium with enriched sodium nitrate ( $\text{Na}^{15}\text{NO}_3$ , 99%, MERCK) for one week. During this



171 period, the majority of N available for dinoflagellate intake was in the form of  $^{15}\text{N}$ . The  
172 suspension and rinsing steps were used to start cultures in low nitrogen medium (Jauzein et  
173 al., 2017) and to remove the majority of bacteria present in the growth medium (Rausch de  
174 Traubenberg and Soyer-Gobillard, 1990). The same method was used to rinse dinoflagellates  
175 and diatoms from their culture medium before the beginning of the feeding assays. In order to  
176 evaluate the cell abundances for each culture, suspensions at adequate concentrations were  
177 done by using a 1 mL Sedgewick Rafter<sup>®</sup> counting cell under a standard light microscope.

#### 178 *Harpacticoid copepods culture conditions*

179 Copepods were collected from stands of *Penicillus* sp. (Ulvophyceae) growing in the  
180 region of Bois Jolan (Guadeloupe, Caribbean Sea). They were identified as *Canthocamptus*  
181 sp. based on morphological characteristics. To obtain a monospecific culture of copepods, a  
182 unique female carrying eggs was isolated in 5 mL of GF/F filtered seawater. The volume of  
183 the culture was increased gradually until reaching a final volume of 1 L and the cultures were  
184 kept at 25-27 °C with a natural day/night cycle. Copepods were fed weekly with a mixture of  
185 2/3 canned spinach and 1/3 dried fish food. Once a month, 2/3 of the culture volume water  
186 was removed and replaced by the same volume of 0.22  $\mu\text{m}$  filtered seawater.

#### 187 *Feeding assay*

188 Three different controls were designed with: a) 150 live copepods in 0.2  $\mu\text{m}$  filtered  
189 seawater, b) 150 dead copepods (frozen and then thawed) in contact with dinoflagellates and  
190 c) 150 live copepods trapped in a tube closed by a GF/F filter and immersed in the microcosm  
191 containing dinoflagellates. Feeding experiments were performed with d) 150 live copepods in  
192 contact with  $^{15}\text{N}$  enriched dinoflagellates only (*Amphidinium* sp. or *Ostreopsis* sp.), and e)  
193 150 live copepods in contact with a mixture of enriched  $^{13}\text{C}$  diatoms and  $^{15}\text{N}$  dinoflagellates  
194 (*Amphidinium* sp. or *Ostreopsis* sp.), see Figure 1. Each treatment was performed in triplicate

195 conditions (n=3) where 150 adult harpacticoid copepods were placed in a 100 mL microcosm  
196 (64 cm<sup>2</sup>).

197 Concentrations of diatoms and dinoflagellates were determined in order to reach an  
198 equal total biovolume of diatoms and dinoflagellates in each experiment (Table 1). Feeding  
199 experiments lasted 4h with a salinity of 35 and a temperature of 27 °C (Memmert incubator).  
200 Experiments were stopped by removing all copepods through a sieve (50 µm mesh).  
201 Copepods were preserved in a —80 °C freezer for subsequent chemical analysis.

202

### 203 *Isotope analysis and calculations*

204 The  $\delta^{15}\text{N}$  of prey (*Amphidinium* cf. *carterae* and *Ostreopsis* cf. *ovata*) and predators  
205 (copepods) were measured by EA-IRMS (Elemental Analysis – Isotope Ratio Mass  
206 Spectrometry). Nitrogen isotope composition is expressed in the delta notation ( $\delta^{15}\text{N}$ ) relative  
207 to air N<sub>2</sub>:  $\delta^{15}\text{N} = [((^{15}\text{N}/^{14}\text{N})_{\text{sample}} / (^{15}\text{N}/^{14}\text{N})_{\text{reference}}) - 1] \times 1000$ . For each experimental condition,  
208 copepod sample was composed by 150 specimens representing a total of  $109 \pm 7 \mu\text{g}$  ( $\pm$   
209 standard error).

210 Incorporation of  $^{15}\text{N}$  is defined as excess levels which are above the background  $^{15}\text{N}$  level  
211 (i.e. copepods in control treatment incubated without  $^{15}\text{N}$  enriched dinoflagellates) and is  
212 expressed in terms of specific uptake ( $I$ ).  $I$  was calculated as the product of excess  $^{15}\text{N}$  ( $E$ ) and  
213 biomass of N per grazer.  $I$  was converted in dinoflagellate carbon grazed using C/N ratio of  
214 each dinoflagellate species measured with EA-IRMS. This C/N ratio was obtained from stable  
215 isotope measurements (30 samples of 150 specimens).  $E$  is the difference between the  
216 background ( $F_{\text{background}}$ ) and the sample ( $F_{\text{sample}}$ )  $^{15}\text{N}$  fraction:  $E = F_{\text{sample}} - F_{\text{background}}$ , with  $F =$   
217  $^{15}\text{N}/(^{15}\text{N}+^{14}\text{N}) = R / (R + 2)$  and  $R =$  the nitrogen isotope ratio. For the  $F_{\text{background}}$ , we used  
218 control values measured with control grazers.  $R$  was derived from the measured  $\delta^{15}\text{N}$  values:  
219  $R = [(\delta^{15}\text{N}/ 1000) + 1] \times R_{\text{airN}_2}$ . The ingestion of dinoflagellates was calculated as  $[I \times (\text{C/N}$

220 ratio of enriched dinoflagellate)/ ( $F_{\text{enriched dinoflagellate}} \times \text{incubation time}$ )] (Pascal et al., 2008)

221 Individual weights were derived from stable isotope samples and used in ingestion rate  
222 calculations.

### 223 *Data analysis*

224 The  $\delta^{15}\text{N}$  of copepods after all incubations was not normally distributed which led to the  
225 use of non-parametric tests. Kruskal-Wallis tests were used to assess  $\delta^{15}\text{N}$  and average  
226 ingestion rate of copepods. Dunn's test is a multiple pairwise comparison method allowing  
227 comparisons of the mean of the rank of each treatment after a Kruskal-Wallis test. Values are  
228 presented as means  $\pm$  standard deviations (SD), except when specified otherwise.

229

### 230 **Results**

231 The ITS sequence of 411 bp from the strain MCCV092 collected in Guadeloupe  
232 matched with a sequence of *Amphidinium* cf. *carterae* (KY697961.1) through GenBank. No  
233 data is available on the exact location where the strain was sampled. This match was made  
234 with a E-value of 0, an identity percentage of 98.56% and on 100% of query coverage.

235 The ITS sequence of 370 bp for the strain MCCV070 collected in Guadeloupe matched in  
236 GenBank with a sequence of *Ostreopsis* cf. *ovata* (MH844087.1) collected in Ecuador. This  
237 match was made with a E-value of 0, a percentage of identity of 98,64% and on 100% of  
238 query coverage.

239

240 The  $^{15}\text{N}$  enriched dinoflagellates *Ostreopsis* cf. *ovata* and *Amphidinium* cf. *carterae* used  
241 during ingestion experiments presented a  $\delta^{15}\text{N}$  of 96,569 ‰ and 555,151 ‰ respectively.  
242 Their individual ratios of C/N were 18.4 and 11.7, respectively. *Ostreopsis* cf. *ovata* and  
243 *Amphidinium* cf. *carterae* cell biovolumes reached  $17,933 \pm 7081 \mu\text{m}^3$  and  $677 \pm 64 \mu\text{m}^3$

244 respectively. The clonal culture of *Odontella* sp. used during ingestion experiments presented  
245 a biovolume of  $3874 \pm 508 \mu\text{m}^3$ . A total of 4500 harpacticoid copepod specimens were  
246 isotopically determined from which weight, C and N contents were derived. The individual  
247 weight of an adult copepod was estimated at  $724 \pm 99 \text{ ng copepod}^{-1}$  with 30.9 % of C and  
248 14.5 % of N.

249 Copepods were incubated in three control conditions a) copepods which were not fed  
250 with  $^{15}\text{N}$  enriched dinoflagellates and presented a  $\delta^{15}\text{N}$  of  $7.0 \pm 0.7 \text{ ‰}$  ( $n = 3$ ), b) copepods  
251 which were euthanized (frozen and then thawed) before incubation and c) copepods trapped  
252 in GF/F filter without having access to  $^{15}\text{N}$  enriched dinoflagellates. The  $\delta^{15}\text{N}$  of copepods  
253 from both b) and c) control conditions were not significantly different from control a),  
254 Kruskal-Wallis,  $p > 0.05$ , and this conclusion was validated with clonal cultures of both  
255 *Ostreopsis* cf. *ovata* and *Amphidinium* cf. *carterae* (Figure 1 and letters associated).

256 In contrast, copepods incubated with  $^{15}\text{N}$  enriched dinoflagellates presented a  
257 significantly higher  $\delta^{15}\text{N}$  compared to the control a). This difference was observed for both  
258 dinoflagellate species (Kruskal-Wallis,  $p < 0.05$ ). The  $\delta^{15}\text{N}$  of copepods incubated with  
259 enriched *Ostreopsis* cf. *ovata* and *Amphidinium* cf. *carterae* was not affected by the addition  
260 of diatoms (Kruskal Wallis,  $p > 0.05$ ).

261 The average ingestion rate of copepods was not significantly different between  
262 *Ostreopsis* cf. *ovata* ( $372 \pm 412 \text{ pg C ind}^{-1} \text{ h}^{-1}$ ) and *Amphidinium* cf. *carterae* ( $49 \pm 82 \text{ pg C}$   
263  $\text{ind}^{-1} \text{ h}^{-1}$ ), Kruskal Wallis test,  $p > 0.05$ .

## 264 **Discussion**

### 265 *Potential toxicity of benthic dinoflagellates in the Caribbean Sea*

266 Investigating the toxicity of benthic dinoflagellates in the Caribbean Sea is recent and  
267 studies generally focus only on several genera such as *Gambierdiscus* (Díaz-Asencio et al.,

268 2019; Litaker et al., 2017), *Prorocentrum* (Moreira González, 2013) and *Amphidinium*  
269 (Moreira González, 2013).

270 Despite a great diversity of *Ostreopsis* species observed in the Caribbean Sea  
271 (Ballantine et al., 1988; Besada et al., 1982; Bomber et al., 1988; Faust, 2009, 1995) no  
272 studies have yet focused on the actual production of toxins by *Ostreopsis* cf. *ovata* in this  
273 area. In the Mediterranean Sea, environmental conditions have been shown to modify the  
274 toxicity of *Ostreopsis* cf. *ovata* (Pezzolesi et al., 2012; Scalco et al., 2012). Indeed, high  
275 toxicity levels were measured when water temperatures increased above 25° (Pezzolesi et al.,  
276 2012; Scalco et al., 2012) and with a salinity of 32 (Pezzolesi et al., 2012). These  
277 environmental conditions, which seem to optimize the toxin production of *Ostreopsis* cf.  
278 *ovata* in the Mediterranean Sea, were similar to those applied in this present study which  
279 suggests that Caribbean strains could be toxic (Boisnoir et al., 2019, 2018). In addition, toxin  
280 production by *Ostreopsis* cf. *ovata* strains collected in the Mediterranean Sea increased during  
281 cell growth (Guerrini et al., 2010; Pistocchi et al., 2011). This trend was also observed for the  
282 Brazilian strains (Nascimento et al., 2012). However, the increase of toxin production during  
283 growth was not measured for all the Mediterranean strains of *Ostreopsis* cf. *ovata* (Scalco et  
284 al., 2012). The risk of using aged cells, in post-exponential growth phase, is that the toxins  
285 produced by *Ostreopsis* cf. *ovata* are released in the medium (Guerrini et al., 2010) which can  
286 hence reduce the feeding efficiency of harpacticoid copepods (Pavaux et al., 2019).

287 The toxicity of the genus *Amphidinium* from the Caribbean and inherent toxin content,  
288 was studied on one single species *A. massartii* (Moreira González, 2013). To our knowledge,  
289 no study has yet investigated the relationship between the environmental conditions and the  
290 toxicity of *Amphidinium* cf. *carterae*. However, strains of *Amphidinium* cf. *carterae* collected  
291 off the coasts of Bahamas, in the Mediterranean Sea and in China had hemolytic and  
292 antilarval activities (Kong et al., 2016; Meng et al., 2010; Pagliara and Caroppo, 2012).

293           Moreover, cells of *Amphidinium* cf. *carterae* from Egypt collected during the  
294 exponential growth phase were found to be toxic on *Artemia salina*, whilst cells collected in  
295 the post-exponential phase had no effect (Ismael et al., 1999). On the other hand strains from  
296 the Northern Arabian Sea did not show any significant toxicity on *Artemia salina* (Baig et al.,  
297 2006).

#### 298           *Methodological considerations*

299 Identifying and quantifying copepod ingestion rates of specific microalgae species are  
300 relevant practices when considering the context of harmful algal blooms (Haley et al., 2011).  
301 Similarly to planktonic organisms, several methods can be used to quantify the ingestion rate  
302 of benthic dinoflagellates by epiphytic copepods. However, it remains difficult to undertake *in*  
303 *situ* copepod feeding experiments without depending on laborious, intrusive and potentially  
304 biased incubation approaches (Nejstgaard et al., 2008). Methods used to quantify ingestion  
305 rates of microalgae all present both strengths and weaknesses. Furthermore, methods which  
306 are initially adapted for planktonic organisms can be difficult to implement on benthic  
307 organisms.

308           Relationships between different trophic levels can be evaluated by identifying ingested  
309 organisms in the feces or in the gut content and thus by undergoing microscope observations.  
310 This method is nevertheless laborious even for a trained observer (Nejstgaard et al., 2008) and  
311 a high fraction of food items remains impossible to identify (Gowing and Wishner, 1992). In  
312 order to bypass direct microscope identification, another indirect approach based on the  
313 measurement of specific pigments of dinoflagellates in the gut content of copepods can be  
314 applied (Kleppel et al., 1988; Oechsler-Christensen et al., 2012). Actually, most studies  
315 involving copepod feeding rely on this approach although several limitations have been  
316 highlighted (Bustillos-Guzmán et al., 2002; Pandolfini et al., 2000) such as *i*) pigment  
317 degradation during the digestion process (Pandolfini et al., 2000) and *ii*) limited specificity of

318 pigments for a given microalgal group (Antajan et al., 2004; Irigoien et al., 2004). However,  
319 this method remains largely used as it is quick and inexpensive (Nejstgaard et al., 2008).  
320 Biomarkers could be an alternative, however there is a lack of specificity for dinoflagellates,  
321 particularly regarding amino acid (Guisande et al., 2002) and fatty acid (Desvillettes et al.,  
322 1994; Graeve et al., 1994) compositions. A new promising strategy is the use of prey-specific  
323 DNA barcodes (Sheppard and Harwood, 2005). This approach was used to quantify specific  
324 phytoplanktonic species present inside gut contents and fecal pellets of zooplankton  
325 (Nejstgaard et al., 2008, 2003). The DNA-based approach may provide a way to rapidly  
326 measure ingestion rates, even when the targeted microalgae are present in low abundances  
327 within a mixed community (Haley et al., 2011). With this approach, cells of interest which are  
328 used as prey must be previously sequenced in order to develop specific primer sets in order to  
329 amplify their DNA (Sheppard and Harwood, 2005). Another inconvenience of this method is  
330 the possible interference of a large amount of non-target DNA with the primer or the presence  
331 of co-purifying material from the host copepod during quantification (Nejstgaard et al., 2008).  
332 However, the use of high throughput DNA sequencing combined with better-designed  
333 primers and improved databases will undoubtedly generate more studies employing DNA-  
334 based approaches (Ho et al., 2017).

335         Alternatively, grazing experiments offer a straightforward method to estimate prey  
336 consumption and do not present drawbacks associated with label specificities. The most  
337 current grazing experiment method is based on the disappearance of prey cells over time  
338 (Frost, 1972). This method is reliable for planktonic organisms (Campbell et al., 2005; Haley  
339 et al., 2011; Turner, 2014) but presents limitations for benthic organisms. When microalgae  
340 are present in low concentrations, the accuracy of cell counting decreases (Campbell et al.,  
341 2005; Haley et al., 2011) which leads to an overestimation of ingestion rates. Moreover,  
342 benthic dinoflagellates can form agglomerates, which may sink and attach to the surface of

343 the cultivation flasks, thereby increasing this bias. Methods used to detach benthic cells can  
344 damage cell integrity and introduce a bias in interpretations. Indeed, an empty theca can be  
345 considered as a consumed cell with an ingested cytoplasmic content or as a cell which is not  
346 grazed.

347 Grazing experiments can also be performed using pre-labeled prey. In this case,  
348 dinoflagellates can be labeled using radioactive isotopes (Lampert and Taylor, 1985; Napp  
349 and Long, 1989; White and Roman, 1991) but with the inconvenience to present legal  
350 restrictions. Compared to radioactive isotopes, using stable isotopes to enrich dinoflagellates  
351 is more appropriate especially for investigators who are limited by radioactive material  
352 regulations. The method using pre-labeled dinoflagellates enriched with stable isotopes was  
353 consequently chosen in this present study and controls were conducted to assess its efficiency.  
354 For instance, passive adhesion of labeled dinoflagellates on the cuticle of copepods (Figure  
355 1.b) could overestimate the ingestion of dinoflagellates. However, controls using dead  
356 copepods revealed a limited bias due to such events. Caged copepods were not able to ingest  
357 benthic dinoflagellates but could consume their soluble secretions through cage meshes.  
358 Results showed a limited labeling for caged copepods which highlighted that the transfer of  
359 dinoflagellate compounds through the dissolved form was limited (Figure 1.c). Even if 150  
360 specimens were pooled for each sample, measured ingestion rates presented a high variability  
361 potentially due to different feeding behaviors between specimens.

### 362 *Ingestion rate*

363 Copepod ingestion of toxic planktonic dinoflagellates have been previously measured  
364 for genera such as *Alexandrium* (Lasley-Rasher et al., 2016; Sopenan et al., 2011; Teegarden  
365 and Cembella, 1996), *Karenia* (Prince et al., 2006; Schultz and Kiørboe, 2009; Walsh and  
366 O'Neil, 2014), and *Gymnodinium* (da Costa et al., 2012; Koski et al., 1998; Paffenhöfer,  
367 1971). These studies revealed that different factors can lead to difficulties in interpretation



368 when describing interactions between grazers and cell prey. Furthermore, different predators  
369 and various clonal cultures of dinoflagellates which are often used during the feeding assay  
370 experiments make the comparison between studies problematic (Teegarden and Cembella,  
371 1996). The interaction between grazers and cell prey can be highly specific (Teegarden, 1999;  
372 Teegarden and Cembella, 1996) and even site-specific when a single grazer is considered  
373 (Teegarden and Cembella, 1996; Uye and Takamatsu, 1990). Indeed, within a species, trophic  
374 interactions can be population-specific and linked with population history (Colin and Dam,  
375 2002). For instance, a same species of grazer, present at two different geographic sites, and  
376 fed with the same toxic dinoflagellate species, can present different ingestion rates, which  
377 suggests that historical exposure of grazers can impact the ingestion (Colin and Dam, 2003,  
378 2002). There are also ontogenetic considerations, such as different developmental stages (e.g.  
379 nauplii vs. copepodides vs adult copepods) which certainly display diet differences in the type  
380 and amount of preyed items.

381 Trophic interactions between dinoflagellates and the meiobenthos are less investigated  
382 than with the macrobenthos and zooplankton. Harpacticoid copepods can graze on different  
383 food items due to the large diversity of microalgal species present in the microphytobenthos  
384 (Azovsky et al., 2013) however, they are usually selective in their ingestion (Azovsky et al.,  
385 2005; Buffan-Dubau et al., 1996). Indeed, in temperate regions, copepods were found to feed  
386 on a broad diversity of diatoms (Cnudde et al., 2011; Decho, 1986; Rzeznik-Orignac and  
387 Fichet, 2012; Wyckmans et al., 2007) and bacteria (Cnudde et al., 2013, 2011; Pascal et al.,  
388 2013, 2009).

389 In tropical areas, potentially toxic dinoflagellates can be a major component of the  
390 microphytobenthic communities (MacIntyre et al., 1996). To our knowledge, the ingestion  
391 rate of potentially toxic dinoflagellates by meiofauna has never been measured in benthic  
392 environments.

393 The present study indicates that harpacticoid copepods can feed on potentially toxic  
394 benthic dinoflagellates even when another food source is available. Indeed, ingestion rates of  
395 dinoflagellates were not affected when adding diatoms as a food resource, which suggests that  
396 dinoflagellates are *i*) part of the regular copepod diet and *ii*) are not neglected when another  
397 food resource is available. Similar ingestion rates were found when copepods were fed with  
398 *Ostreopsis* sp. and with *Amphidinium* sp. even though *Ostreopsis* cells have a cellulosic theca  
399 (Schmidt, 1901) whilst *Amphidinium* cells have none (Fensome et al., 1993). This result  
400 suggests that rigid cellulosic walls can be easily broken down by copepods in order to ingest  
401 the cytoplasmic content. Moreover, the high  $\delta^{15}\text{N}$  variability found in the present study when  
402 applying feeding conditions d) and e) could be due to fluctuations of the body size explained  
403 by *i*) sexual dimorphism in harpacticoid copepods where the females are larger than the males  
404 and/or *ii*) different shapes of the mouthpart which determine food ingestion (Giere, 2009).  
405 Observations showed that the body size of copepods could influence the ingestion rate of  
406 planktonic copepods (Turner and Tester, 1989). Indeed, the body size is an important factor  
407 for many physiological processes (Peters, 1985) since in general terms, maximal ingestion  
408 rates are inversely correlated to the body size (Moloney and Field, 1989).

#### 409 *Ingestion role in regulation of blooms*

410 Predation of dinoflagellates by meiofauna have received only little attention and this  
411 mainly due to technical constraints associated to the quantification procedure (Danovaro et  
412 al., 2007). Blooms of benthic toxic dinoflagellates are currently increasing worldwide  
413 (Hallegraeff, 1993; Van Dolah, 2000; Cohu et al., 2011) and the position of the meiofauna  
414 within these events needs further clarification mainly regarding the regulation of toxic  
415 dinoflagellate abundances through ingestion and transfer of toxins in the food web. In the  
416 global context of climate change now experienced by the oceans, quantification of predation

417 rates might be useful to understand the ecological role of benthic toxic dinoflagellates and to  
418 forecast how these relationships will evolve.

419 Algal blooms are possible only if algal growth exceeds the loss by predation and  
420 senescence (Buskey et al., 1997). The role of predation must hence be further assessed in  
421 order to better describe the regulation of benthic toxic dinoflagellate blooms. Population  
422 blooms of benthic dinoflagellates may be controlled by meiobenthic organisms in a  
423 comparable way to those occurring in planktonic environments. Model simulations have  
424 already shown that during the first stages of pelagic dinoflagellate blooms, growth can be  
425 retarded or inhibited with a low pressure of predation by copepods since each grazed cell is,  
426 in proportion, an important loss when considering such a small local population (Haley et al.,  
427 2011). However, the impact of low predation was minimal when simulations were carried out  
428 with higher micro-algae abundances, above 100 cells L<sup>-1</sup> (Haley et al., 2011), which  
429 highlights the fact that predation pressure has an important role in bloom dynamics, especially  
430 in the early development stages. Some dinoflagellates are able produce cysts (Anderson,  
431 1998; Faust, 1992; Tian et al., 2017) which make them sink down to the seabed, thereby being  
432 less available for planktonic copepods (Butman et al., 2014; Dale et al., 1978; Mohamed and  
433 Al-Shehri, 2011). It has been suggested that a decrease in the abundance of dinoflagellates  
434 due to grazing is relatively minor compared to the increase of abundance resulting from a  
435 gradual release of germinated cells from the benthic cyst beds (Anderson et al., 2005).

436 Planktonic dinoflagellates are ingested by copepods even at low concentrations (Haley  
437 et al., 2011). Therefore, benthic toxic dinoflagellates, which are found all year round in  
438 Guadeloupe and Martinique at viable densities (Boisnoir et al., 2019), could consequently be  
439 grazed and assimilated by copepods on a permanent basis. Meiofauna represent a key  
440 component of coastal benthos, since over 75 % of the total meiofauna production is  
441 transferred to higher trophic levels through predation (Danovaro et al., 2007), especially due

442 to macrofaunal and other epibenthic predators (Chardy and Dauvin, 1992). Harpacticoid  
443 copepods ingesting toxic benthic cells could bioaccumulate the toxins synthesized by the  
444 benthic dinoflagellates and contaminate secondary consumers when they are consumed.  
445 Furthermore, the toxicity of dinoflagellate cysts has been shown to be higher in benthic cells  
446 than in planktonic cells (Dale et al., 1978) which can expose meiobenthic grazers to a more  
447 important toxic risk, able to induce mass mortalities and promote the development of  
448 dinoflagellate blooms. Natural marine toxins are a considerable increasing threat when  
449 bioaccumulation takes place within the food chain (Ramos and Vasconcelos, 2010). Indeed,  
450 the ingestion of benthic toxic dinoflagellates is a way of introducing phycotoxins in the food  
451 web, and to our knowledge, this transfer has been rarely considered. The role of the  
452 meiofauna in this transfer of toxins has been considered as minor, however, more interest  
453 should be given to estimate the bio-magnification potential through specific models since the  
454 amplification effect of such toxins is liable to contribute to the emergence of diseases related  
455 to toxic benthic dinoflagellates.

456

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464

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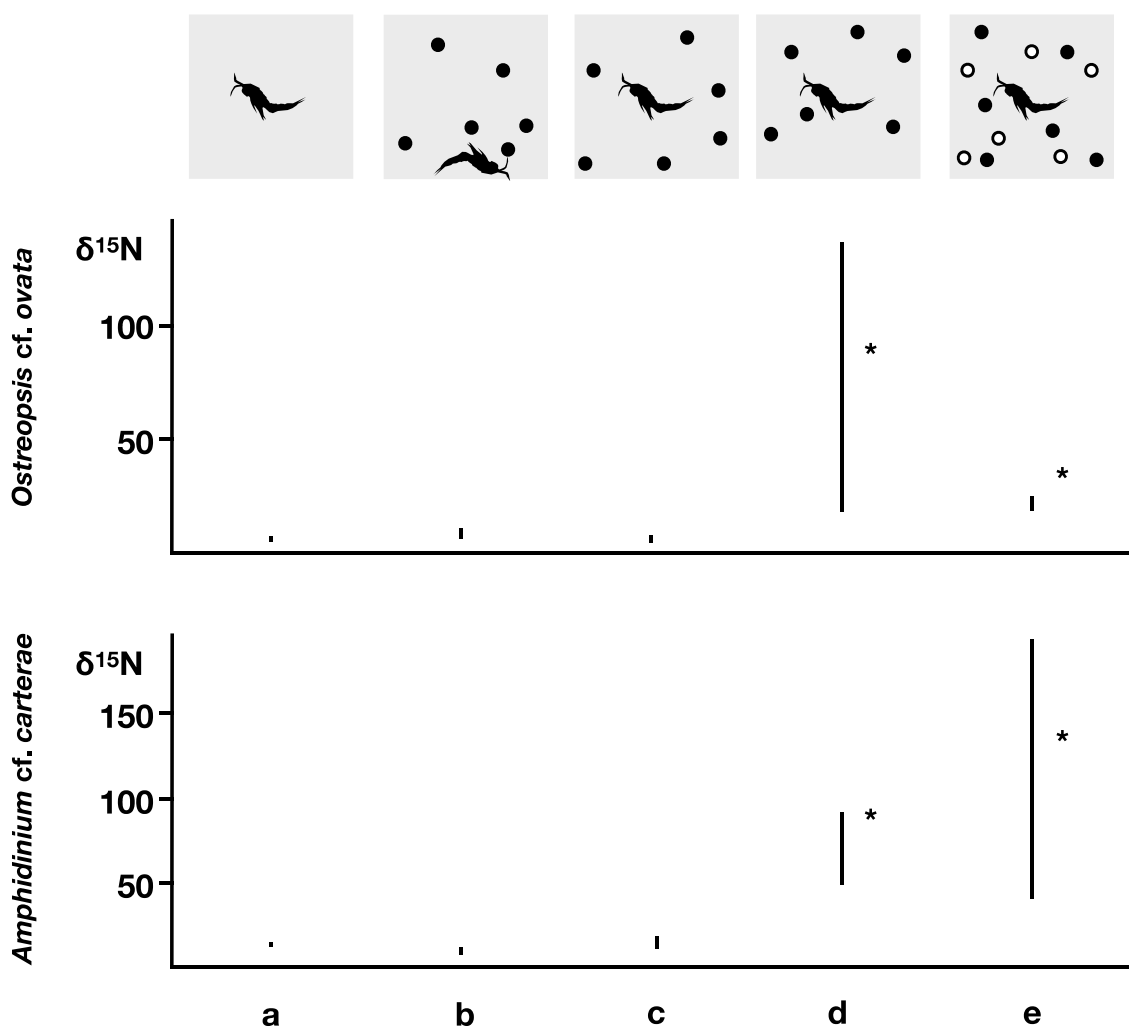
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900 **Figures and tables**



901  
 902 Figure 1.  $\delta^{15}\text{N}$  (‰) of copepods ( $\pm$  SE ; n = 3, 150 specimens per sample) after  
 903 incubation (4 h):

- 904 a) without dinoflagellate
- 905 b) with copepods previously killed before incubation with  $^{15}\text{N}$  enriched dinoflagellates ●
- 906 c) caged copepods (GF/F filter) without access to  $^{15}\text{N}$  enriched dinoflagellates
- 907 d) with  $^{15}\text{N}$  enriched dinoflagellates ●
- 908 e) with diatoms ○ and  $^{15}\text{N}$  enriched dinoflagellates ●

909 \* significant difference between a) and other incubation conditions (Kruskal Wallis tests,  
910 p<0.05)

911

912 Table 1: Cell concentrations of labeled diatoms and dinoflagellates during feeding assays

	Concentration (cells.mL <sup>-1</sup> )	Individual biovolume ( $\mu\text{m}^3$ )	Total biovolume ( $\mu\text{m}^3$ )
<hr/>			
Experiment 1			
Diatom ( <i>Odontella</i> )	100	3874	387,400
Dinoflagellate ( <i>Amphidinium</i> )	600	677	406,200
<hr/>			
Experiment 2			
Diatom ( <i>Odontella</i> )	2400	3874	9,297,600
Dinoflagellate ( <i>Ostreopsis</i> )	500	17,933	8,966,500

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