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1 Evidence of deleterious effects of microplastics from aquaculture materials on pediveliger  
2 larva settlement and oyster spat growth of Pacific oyster, *Crassostrea gigas*.

3

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10

## 11 **ABSTRACT**

12 Plastic is currently used in aquaculture as a material for settlement and magnification of oyster spats.

13 Plastic weathering and fragmentation under natural conditions can lead to the production of micro and

14 nanoparticles and additive leakage, with potential toxic effects on marine life. This study investigates

15 the effects of the exposure to microplastic (MPs) cocktail derived from aged aquaculture material on

16 oyster pediveliger larvae (*Crassostrea gigas*). The cocktail was made of high-density polyethylene

17 (HDPE), polypropylene (PP) and polyvinyl chloride (PVC). The concentrations tested were 0, 0.1, and

18 10 mg MP. L<sup>-1</sup>. During the 7-day fixation phase, pediveliger larvae (17 days) were exposed to the MP

19 cocktail in laboratory-controlled conditions. After exposure, the success of settlement was

20 significantly lower for larvae exposed to 10 mg MP. L<sup>-1</sup> (49±0.9 %) compared to control ones

21 (61.8±1.6 %). No malformations or metamorphosis abnormalities were observed. Growth of

22 pediveliger and spat stages was monitored up to 11 months. During the first twenty-eight days of

23 development, spat growth was significantly lower for the two MPs exposure conditions (0.1 and 10

24 mg MP. L<sup>-1</sup>; respectively -51.8 % and -44.4 %) compared to control groupe. Subsequently, the

25 previously exposed oysters grew faster than the control condition, resulting in a significantly greater

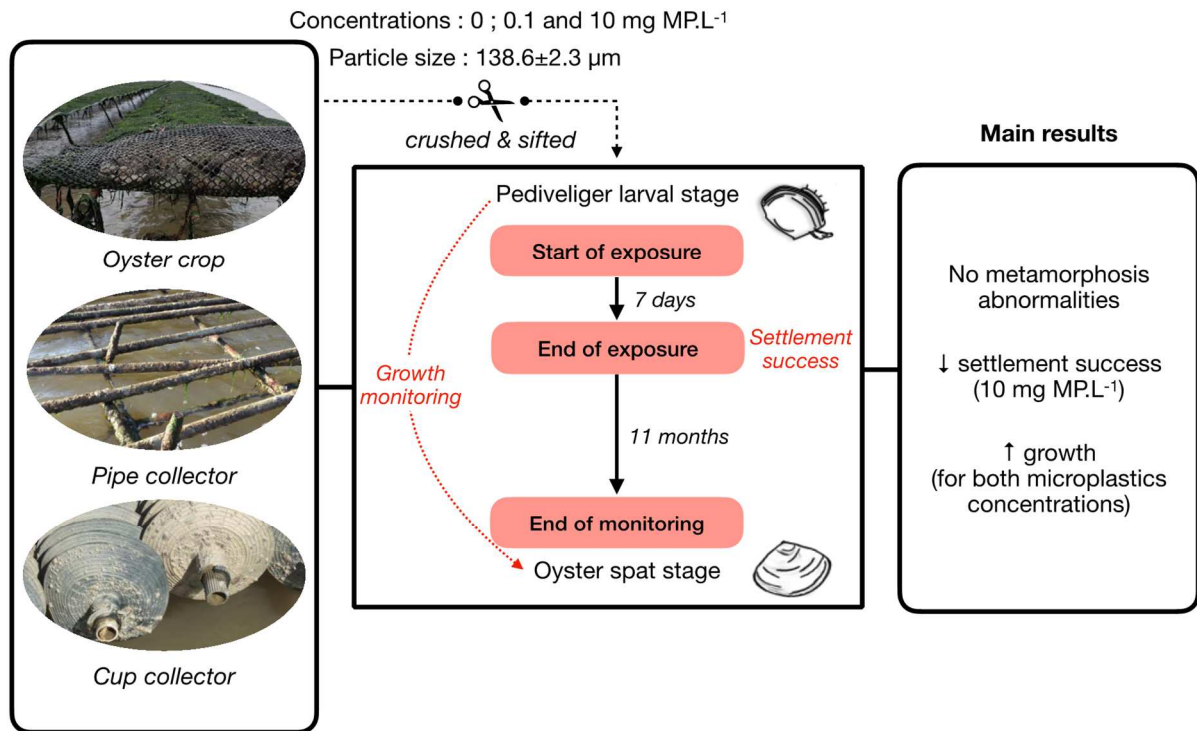
26 growth (0.1 and 10 mg MP. L<sup>-1</sup>: +18.3 % and +19.7 %) than the control group at the end of follow-up.

27 The nearly one-year follow-up highlighted the potential effects of MPs from aquaculture on larvae and

28 spat of *C. gigas*.

29  
30

## GRAPHICAL ANSTRACT



31 **Aquaculture plastic equipment**

32 **KEYWORDS:** Aged microplastics; Oyster; Pediveliger larvae; Spat; Growth; Settlement success.

33

### 34 1. INTRODUCTION

35 In 2019, plastic production reached 370 million tons worldwide, including almost 58 million tons in  
36 Europe (PlasticsEurope, 2020). Seen as revolutionary and versatile, plastic materials are part of one's  
37 daily life. Ideal for the current economic system, they incur low production costs, and are light,  
38 durable and malleable. The first studies revealing MP presence in marine habitats date back to the  
39 1970s (Carpenter *et al.*, 1972). It is estimated that rivers carry between 70 % and 80 % of plastic  
40 waste, most of which ends up in oceans (Horton *et al.*, 2017). Each year, over 9.5 million tons of  
41 plastics are dumped into oceans (Boucher and Friot, 2020). In 2019 (PlasticsEurope, 2020), the  
42 plastics demand focused on Polypropylene (PP, 19.4 %), Low Density Polyethylene (LDPE, 17.4 %),  
43 High Density Polyethylene (HDPE, 12.4 %), and Polyvinyl-Chloride (PVC, 10 %). Plastic gear is  
44 necessary for the viability of many aquaculture and shellfish farming operations (Schoof and DeNike,

45 2017). More recently, questions have arisen regarding the potential contributions of aquaculture to  
46 marine debris and the formation of MPs particles. Most varieties of oyster culture around the world  
47 rely on equipment and gear to develop a specific product, respond to environmental conditions, or  
48 provide protection for bivalves (Pacific Coast Shellfish Growers Association, 2011; USACE, 2015).  
49 Plastic is primarily used in gear such as nets, crops, or attachment tubes/cups, designed to contain or  
50 protect farmed molluscs from predators or environmental conditions (Pacific Coast Shellfish Growers  
51 Association, 2011; USACE, 2015). In the seas, macroplastics first undergo physical degradation, by  
52 waves, salt, hydrolysis, temperature, and photodegradation from the sun's ultraviolet rays (Julienne *et*  
53 *al.*, 2019; Andrady, 2017; Alimi *et al.*, 2018; Chubarenko *et al.*, 2019; Hernandez *et al.*, 2019), before  
54 facing biological degradation, by bacterial processes (Dussud and Ghiglione, 2014), creating MPs  
55 (fragments <5 mm, Thevenon *et al.*, 2014). Plastics have invaded all marine habitats around the world  
56 (Kershaw and Rochman, 2015; Li *et al.*, 2016). Scientists have estimated that oceans concentrated  
57 more than 5 trillion pieces of plastic materials, gathering 1 to 102,000 particles/m<sup>3</sup> (Van  
58 Cauwenberghe *et al.*, 2015; Auta *et al.*, 2017). Plastic pollution affects marine ecosystems and human  
59 activities. When ingested by organisms, they cause abrasions, obstructions, and alterations in  
60 physiological structures, directly influencing the survival of individuals (Wright *et al.*, 2013). Recent  
61 studies have shown that commercial MPs can affect the early stages of bivalve development (Tallec *et*  
62 *al.*, 2018; Bringer *et al.*, 2020a). These studies have shown the effects of polystyrene (PS)-  
63 nanoplastics (NPs) (50 nm, 10 µg.mL<sup>-1</sup>) and HDPE-MPs (4-13 µm, 0.1 mg.L<sup>-1</sup>) on development  
64 (malformations and survival) and on swimming behaviour in oyster larvae, *C. gigas*. The early stages  
65 of development are the stages most sensitive to environmental hazards and to potential environmental  
66 pollution and contamination (Gamain *et al.*, 2020). Cole & Galloway (2015) demonstrated the  
67 ingestion of 70 nm to 20 µm PS-MPs/NPs particles in *C. gigas* larvae over the days 3 to 24 after  
68 fertilization. However, the study by Sussarellu *et al.* (2016) showed transgenerational effects of PS-  
69 MPs on larval growth following exposure of parents for two months. Nonetheless, information on  
70 environmentally-aged MPs is limited. Gardon *et al.* (2020) investigated the effects of both new and  
71 aged MPs on the early stages of pearl oyster development (*Pinctada margaritifera*). They concluded  
72 that a 48-hour exposure to MPs significantly increased the larval mortality rate. In addition, MPs with

73 a high surface/volume ratio tended to adsorb persistent organic pollutants while in marine  
74 environments (*e.g.* dichlorodiphenyltrichloroethane [DDT], polycyclic aromatic hydrocarbons and  
75 heavy metals; Beaumont *et al.*, 2019; Rochman *et al.*, 2013; Bakir *et al.*, 2014). During fragmentation  
76 or ageing in an aquatic environment, plastics can release additives, such as phthalates (Koumba,  
77 2018), UV stabilizers, antioxidants (Rani *et al.*, 2017), biocides (Beiras *et al.*, 2021) or dyes. Thus, it  
78 is essential to assess toxicity of environmentally-aged MPs on marine fauna. In 1970, due to the  
79 economic development of modern societies, questions were raised internationally in relation to plastic  
80 waste (Rocher, 2008; Dupré, 2013). Few studies considered plastic materials from aquaculture and  
81 fishing activities. Nevertheless, materials and tools from aquaculture professionals are likely to  
82 interfere with bivalve organisms (production and capture phases). Fishing gear – abandoned, lost, or  
83 discarded – is the main source of plastic waste from the fishing and aquaculture sectors, although their  
84 implication in ocean pollution is overlooked by the public (Lusher *et al.*, 2017). The aquaculture and  
85 shellfish-farming sector has diversified and expanded massively with the importance of exports in  
86 France. Following extreme hydro-climatic events (*e.g.* storms, strong winds or currents, etc.),  
87 professional materials break off and drift to beaches and coasts during high tides. The French shellfish  
88 market is of importance. Each year, nearly 130,000 tons of oysters (*C. gigas*) are produced and  
89 marketed, while the activity generates more than 20,000 jobs (CNC, 2020). The sustainability of  
90 shellfish companies is water-quality dependent. Due to their filtration feeding activities, bivalves such  
91 as oysters absorb and accumulate MPs in marine environments (Li *et al.*, 2018; Phuong *et al.*, 2018;  
92 Teng *et al.*, 2019). In addition, oysters are key ecological and economic species in coastal ecosystems.  
93 They are also used as a model species in laboratory experiments for the assessment of MPs toxic  
94 effects (Teng *et al.*, 2021). Except for the work of Tallec *et al.* (2018) on commercial PS-NPs, no  
95 study has determined the toxicity of MPs or NPs on pediveliger larvae of oyster. For the experiment,  
96 pediveliger larvae were exposed for seven days to MPs from aquaculture (cocktail of HDPE crops, PP  
97 collection cups and PVC collection tubes) at two theoretical concentrations (*i.e.* 0.1 and 10 mg MP. L<sup>-1</sup>).  
98 Study (i) determined larval settlement success after 7 days' exposure to different treatments, and (ii)  
99 monitored growth for nearly a year (11 months), until larvae reached the spat stage.

100

## 101 **2. MATERIALS AND METHODS**

### 102 **2.1 *Pediveliger larvae***

103 *Crassostrea gigas* (Bayne *et al.*, 2019; Bayne *et al.*, 2017) 17-day-old pediveliger larvae were  
104 supplied by a commercial hatchery (France Naissain, France). Upon reception and before starting  
105 experiments, the mobility and good health status of larvae were assessed (Motic 1820, x20 objective).

106

### 107 **2.2 *Seawater and preparation of microplastics from aquaculture***

108 Plastic macrodebris were collected on the French Atlantic coast (South West, France), in Angoulins-  
109 sur-mer municipality beach area (Bringer *et al.*, 2021). They were sorted and classified, selecting only  
110 plastics aged in the marine environment, and from the oyster industry. Polypropylene cup collectors  
111 (PP), pipe collector (PVC) and high-density polyethylene oyster culture crops (HDPE) were chosen. A  
112 GC-MS pyrolysis analysis (700 °C) characterised the polymers of the selected materials (manual  
113 cutting to obtain samples of 130-200 µg). After a rough cut (3-4 cm), the plastics were crushed using a  
114 stationary metal hammer, and this was repeated several times. Then, the plastics were sieved using 5  
115 mm, 1 mm and 100 µm sieves. The grinding was carried out at room temperature so as not to  
116 influence the nature of the material. The cocktail consisted of 28 % HDPE, 40 % PP, and 32 % PVC  
117 (Andrady, 2011). The size range of the cocktail of MPs was 138.6±2.3 µm (three analytical replicates  
118 in laser particle size distribution, Malverne). Seawater was sampled in the Atlantic (South West,  
119 France), before being filtered with 50 µm and 10 µm membranes to eliminate debris and  
120 microorganisms (*i.e.* filtered seawater, FSW).

121

### 122 **2.3 *Analysis of exposure solutions***

123 To carry out a quantitative analysis of MPs in experimental seawater, each theoretical cocktail solution  
124 (0, 0.1, and 10 mg MP. L<sup>-1</sup>) was analysed using a flow cytometer (Attune Acoustic Focusing  
125 Cytometer). Two-milliliter samples from each MP exposition solution (n=4/condition) were vortexed  
126 (StarLab Vortex IR, 12,000 rpm for 20 sec) to homogenise the solutions and then 300 µL was taken  
127 for flow cytometry analysis. A calibration was carried out, thanks to previous studies (Bringer *et al.*,  
128 2020a) with commercial MPs, to achieve an analysis rate of 500 µL.min<sup>-1</sup> and a saturation of 10,000

129 particles maximum detected. From the control conditions (10  $\mu\text{m}$  FSW), control analytical samples  
130 (n=12) were created. The blank obtained enabled removal of background particles (naturally present in  
131 seawater). Using seawater filtered at 0.2  $\mu\text{m}$ , a first calibration was conducted to select the < 200-time  
132 detected particles. MPs measurements in seawater were assessed at D<sub>0</sub> (beginning of exposure) and D<sub>7</sub>  
133 (end of exposure) for all exposure conditions.

134

## 135 **2.4 Experimental design**

### 136 **2.4.1 Exposure of pediveliger larvae in experimental microcosms**

137 Pediveliger larvae (D<sub>0</sub>) were acclimatised for a few hours in petri dishes at microcosm temperature  
138 (24 $\pm$ 0.1 °C) for fixation (Figure S1). Microcosms have been defined as experimental systems  
139 simulating real-life conditions (Buffet *et al.*, 2014; Martinez-Sosa, 2019; Enya *et al.*, 2020). Fifteen  
140 thousand (15,000) larvae/condition with three replicates were added to experimental sieves, *i.e.* 45,000  
141 larvae/condition. Then, larvae were exposed in the dark for 7 days (step 1: settlement phase, from D<sub>0</sub>  
142 to D<sub>7</sub>, Figure 1). The exposure conditions were as follows: larvae were put in 195  $\mu\text{m}$  sieves (three  
143 replicates per condition), at the surface of a 300-400  $\mu\text{m}$  microbreak layer (Ovive industry, La  
144 Rochelle, France), and placed in 224 L tanks. Microbreaks correspond to very finely crushed oyster  
145 shells. Microbreaks was cleaned before use in microcosms. The sieves (n=3/conditions) were then  
146 placed on a table inside a tank where the FSW circulated. Sieves were all connected by an air-lift  
147 system creating a bottom-up movement of FSW (2.3 L.h<sup>-1</sup>/sieve), allowing a continuous renewal of  
148 water in the sieves containing the pediveliger larvae. The temperature was 24.0 $\pm$ 0.1 °C, and salinity  
149 28.2 $\pm$ 0.3 (Table 1). Continuous bubbling and brewing systems were set up. Brewers were installed in  
150 the microcosms in order to homogenize the dispersion of the MPs. The temperature value and salinity  
151 were measured daily. Throughout the <sup>[1]</sup><sub>SEP</sub> experiment, nitrates, nitrites, chlorine, and pH (7.1 $\pm$ 0.4) were  
152 regularly measured to ensure consistency. This experimental device was based on Glize's work  
153 (1992). The day before the start of the exposure, the cocktail of MPs was inserted into the tanks (224  
154 L) according to three theoretical concentrations tested: 0 (control without MP); 0.1 and 10 mg MP. L<sup>-1</sup>.  
155 To do this, precise quantities of MPs cocktail were weighed (n=7, *i.e.* 7 days of exposure with one  
156 renewal every day), before being inserted into the microcosms, for the two conditions of exposure to

157 MPs: 22.4 mg for 0.1 mg MP. L<sup>-1</sup> and 2,240 mg for 10 mg MP. L<sup>-1</sup>. The water was changed every day  
 158 and the sieves were transferred to tanks prepared in advance, and the cocktail of MPs was added at  
 159 each renewal according to the conditions. The pediveliger larvae were fed twice a day with algae paste  
 160 (mixed diet of *T. Isochrysis galbana*, *Pavlova*, *Tetraselmis*, *Thalassiosira* and *Nanno*, Shelfish diet).  
 161 At this stage of their development, the larvae are more sensitive to environmental risks (Connor, 1972;  
 162 Martin *et al.*, 1981 and Quiniou *et al.*, 2005), hence the importance of respecting the protocols of  
 163 renewal of water and daily food to avoid significant mortalities.

164

#### 165 **2.4.2 Experimental hatchery for long-term development**

166 After a 7-day exposure period, pediveliger larvae were put in experimental hatchery devices (step 2,  
 167 Figure 1). After D<sub>7</sub>, fixed larvae were placed in a micronursery. In an open circuit, FSW (10 µm)  
 168 circulated at a flow rate of 27 L.h<sup>-1</sup> (132 % renewal every day). The temperature was 21.3±0.5 °C, and  
 169 salinity 25.1±0.3 (Table 1).

170

171 **Table 1.** Essential parameters (temperature, salinity, and light) for development of pediveliger larvae and oyster  
 172 spat in various aquaculture devices.

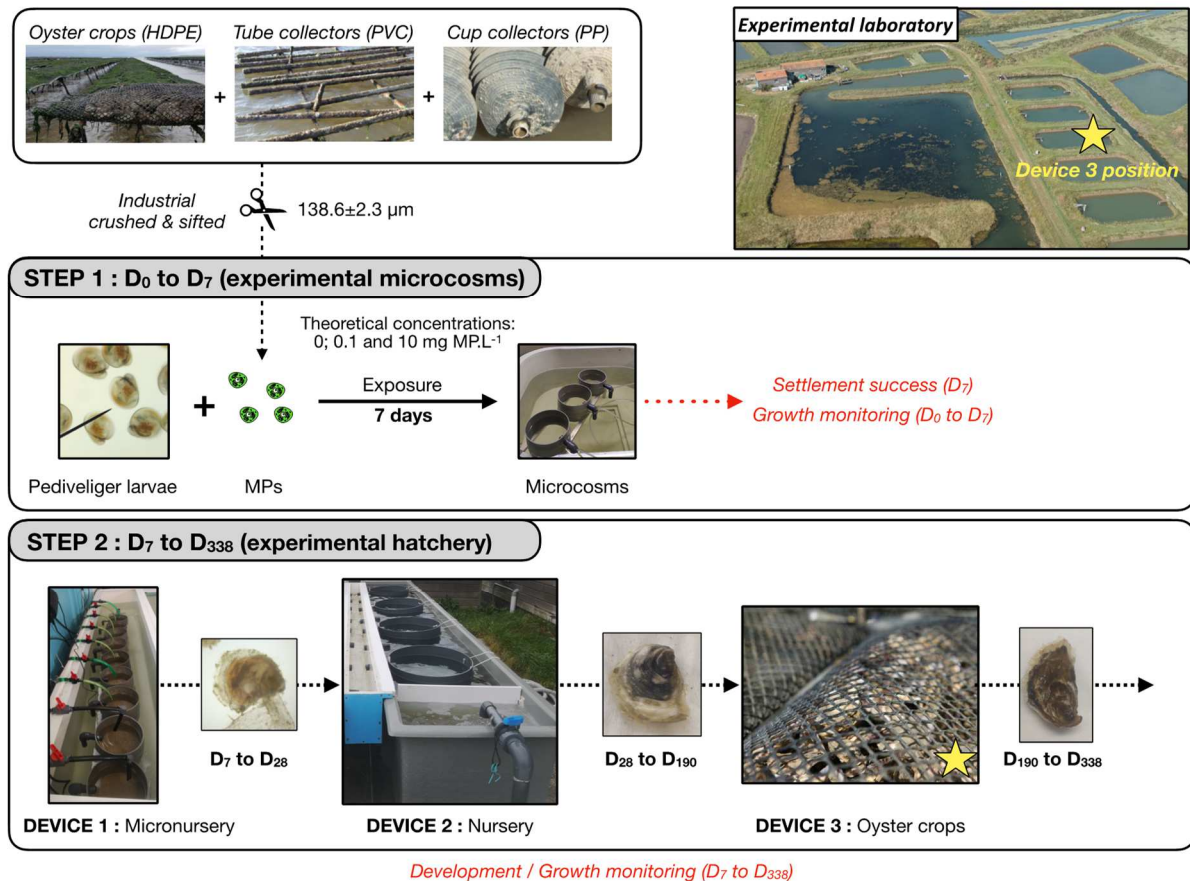
<b>Water parameters</b>	<b>Microcosms</b>	<b>Micronursery</b>	<b>Nursery</b>	<b>Oyster farm</b>
<b>Temperature (°C)</b>	24±0.1	21.3±0.5	19.4±0.6	15.9±2.5
<b>Salinity</b>	28.2±0.3	25.1±0.3	32.0±0.7	32.6±1.5
<b>Light</b>	dark	natural	natural	natural

173

174 At D<sub>28</sub>, oyster spat were transferred to an outdoor nursery (seawater filtered at 30 µm). The flow rate  
 175 was 2,117.6 L.h<sup>-1</sup> (7,260 % renewal every day), temperature 16.3±1.0 °C, and salinity 27.4±0.7 (Table  
 176 1). Once in the nursery, spat were exclusively fed with naturally-present phytoplankton. Larger mesh  
 177 sieves were used (500, 1000 and 3000 µm) to allow larval magnification. Then, oyster spat were  
 178 placed in 4000 µm mesh crops and placed in experimental beds (D<sub>190</sub>, CNRS platform, La Rochelle,  
 179 France). Figure 1 summarises the development cycle from pediveliger larvae to oyster spat. It includes  
 180 the aquaculture devices used for oyster development, and indicates the beginning of the exposure



181 period (D<sub>0</sub>), the settlement success (end of exposure; D<sub>7</sub>), and the growth monitoring analysis (D<sub>0</sub> to  
 182 D<sub>338</sub>),  
 183



184  
 185 **Figure 1.** Experimental devices to develop pediveliger larvae into oyster spat *C. gigas*. Step 1: 7-day exposure  
 186 (D<sub>0</sub> to D<sub>7</sub>, settlement success) in experimental microcosms. Step 2: development (D<sub>7</sub> to D<sub>338</sub>) in experimental  
 187 hatchery. Length growth was monitored for the entire experiment (from D<sub>0</sub> to D<sub>338</sub>, *i.e.* 11 months).  
 188

189 **2.5 Settlement success and growth monitoring of pediveliger larvae**

190 After a 7-day exposure period to MPs cocktail, at different concentrations, an analysis of the larval  
 191 settlement ratio was performed, quantifying larvae that were fixed in each condition and replicate  
 192 (n=3/condition). A ratio was calculated from the weight of samples taken (n=3/replicates, *i.e.*  
 193 n=9/conditions) and the total weight corresponding to the total quantity of pediveliger larvae at the  
 194 start of the experiment. After being weighed, the samples taken were analysed under a microscope  
 195 (Motic 1820, 200x) and the number of larvae fixed was quantified. The oyster growth in length was

196 monitored at pediveliger and early spat stages, taking microphotographies under the Motic 1820  
197 Binocular microscope (200x). Length of larvae was measured on photographs with Image J.  
198 Pediveliger larvae were oriented lengthwise under the microscope (Motic 1820, x20 objective) and a  
199 line was drawn with Image J (Talmage and Gobler, 2009; Helm *et al.*, 2004; Bringer *et al.*, 2020b). To  
200 carry out the growth analysis, pediveliger larvae (n=30) in each replicate and condition  
201 (n=90/conditions) were measured. Results are expressed in mean  $\pm$  SEM. Then, oyster spat  
202 (n=30/replicate, *i.e.* n=90/condition) were measured (length, width, and thickness) and weighed (total  
203 weight) using a digital caliper (0.1 mm) and precision balance (0.1 g, Denver Instrument). Results are  
204 expressed in mean  $\pm$  SEM. During the exposure period (D<sub>0</sub> to D<sub>7</sub>, microcosms, Figure 1), growth was  
205 quantified every two days. During the experimental hatchery period (D<sub>7</sub> to D<sub>190</sub>, device 1 and 2, Figure  
206 1), growth was quantified every 4 days. Finally, during the period in oyster beds, growth was  
207 quantified once a month (D<sub>190</sub> to D<sub>338</sub>, device 3, Figure 1).

208

## 209 **2.6 Statistics**

210 A statistical analysis was conducted using R Studio and graphs on Microsoft Excel. Homogeneity of  
211 variance (Levene's test) and normality of distribution (Shapiro-Wilk) were assessed. To compare the  
212 settlement success in each treatment, an analysis of variance (ANOVA) was performed followed by a  
213 Tukey's post-hoc test. A statistical analysis of growth biomonitoring data was carried out through the  
214 Kruskal-Wallis test. Then, differences in mean concentrations were assessed using the Kruskal  
215 Nemenyi Post-hoc test, coupled with the PMCMR package (equivalent to the Tukey's test for non-  
216 parametric data). A significance was accepted for  $p < 0.05$ . Data are expressed in mean  $\pm$  SEM.

217

## 218 **3. RESULTS AND DISCUSSION**

### 219 **3.1 MP exposure concentrations**

220 The concentrations of MPs for the two exposure conditions (Table 2) varied between D<sub>0</sub> (start of  
221 exposure) and D<sub>7</sub> (end of exposure). The results of this present study are comparable to the  
222 concentrations assayed by flow cytometry in a previous study on commercial HDPE-MPs of 20-25  $\mu\text{m}$

223 (Bringer *et al.*, 2020a). In accordance with theoretical factors, the dilution factor of the two  
 224 concentrations tested should equate to 100. However, factors at D<sub>0</sub> and D<sub>7</sub> were of 8.88±0.62 and  
 225 9.17±0.37. The theoretical 10 mg MP. L<sup>-1</sup> condition suggested that the real dilution factor was lower in  
 226 display tanks. The differences observed between the theoretical and measured concentrations of MPs  
 227 could come from aggregation of MPs together (Alimi *et al.*, 2018; Michels *et al.*, 2018) and binding on  
 228 the walls of exposure microcosms. Despite the use of brewers, MPs might be heterogeneously  
 229 dispersed in the exposure medium. By taking into account the assays carried out at D<sub>0</sub> and D<sub>7</sub>, we can  
 230 calculate the mean concentrations corresponding to the theoretical concentrations: 0.9±0.08 (0.1 mg  
 231 MP. L<sup>-1</sup>) and 8.4±0.5 (10 mg MP. L<sup>-1</sup>). No microparticles were found in the waters of the control  
 232 condition..

233

234 **Table. 2** Theoretical and measured MP concentrations (mean ± SEM) at the beginning (D<sub>0</sub>) and at the end of the  
 235 pediveliger exposure (D<sub>7</sub>).

236

Sampling times	Theoretical (mg MP. L <sup>-1</sup> )	Measured (MP. µL <sup>-1</sup> )	Concentration factor
D <sub>0</sub>	0.1	0.88±0.07	-
D <sub>7</sub>	0.1	0.97±0.09	-
D <sub>0</sub>	10	7.8±0.65	8.88±0.12
D <sub>7</sub>	10	8.9±0.27	9.17±0.54

237

238 Through flow cytometry, MP concentrations in exposure conditions were determined. Long *et al.*  
 239 (2017) assessed PS-MPs concentrations using flow cytometry. Recent studies have measured the  
 240 concentrations of nano and microparticles through flow cytometry (Caputo *et al.*, 2021; Kaile *et al.*,  
 241 2020). In this work, concentrations were higher than measured in the marine environment (Van  
 242 Cauwenberghe *et al.*, 2015; Auta *et al.*, 2017). A previous study measured 13.4±0.9 MP. L<sup>-1</sup> in the  
 243 coastal Atlantic sector (Green *et al.*, 2018). In addition, the study published by Frere *et al.* (2017),  
 244 showed concentrations of 0.24±0.35 MP.m<sup>-3</sup> in the Bay of Brest (France). These different results prove  
 245 to us that our experimental concentrations are stronger than the natural environment. However, the use  
 246 of aged aquaculture MPs in the marine environment has increased knowledge on the toxicity of plastic

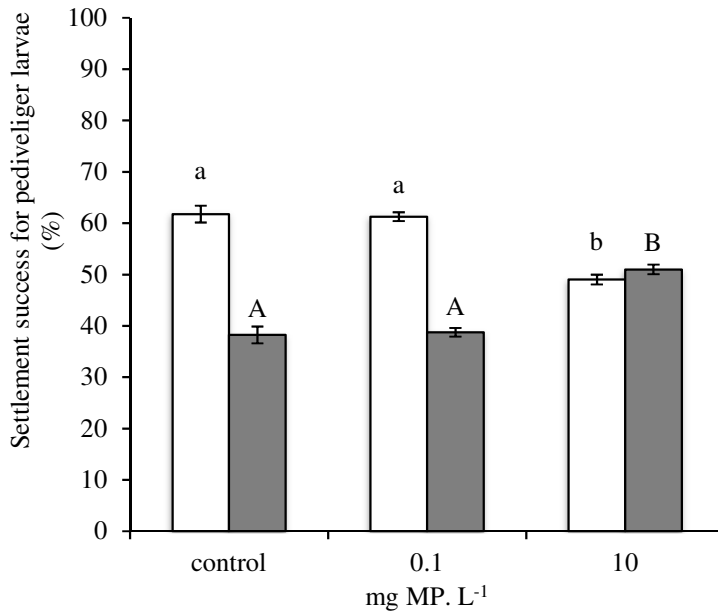
247 particles at early stages of development in *Crassostrea gigas*. Apart from this research, few studies  
248 focused on aquaculture plastics and their impacts.

249

### 250 **3.2 MP effects on the settlement success of pediveliger larvae**

251 After a 7-day exposure, pediveliger larvae were removed from the microcosm devices and were put in  
252 the micronursery (device 1 of experimental hatchery, Figure 1). Fixed larvae were counted using a  
253 microscope. In the control condition (without MPs), 9,266±246 (n=3 replicates) out of the 15,000  
254 larvae at D<sub>0</sub> were fixed. In the 0.1 and 10 mg MP. L<sup>-1</sup> conditions, 9,188±129 and 7,352±142 larvae  
255 were fixed (Figure 2).

256



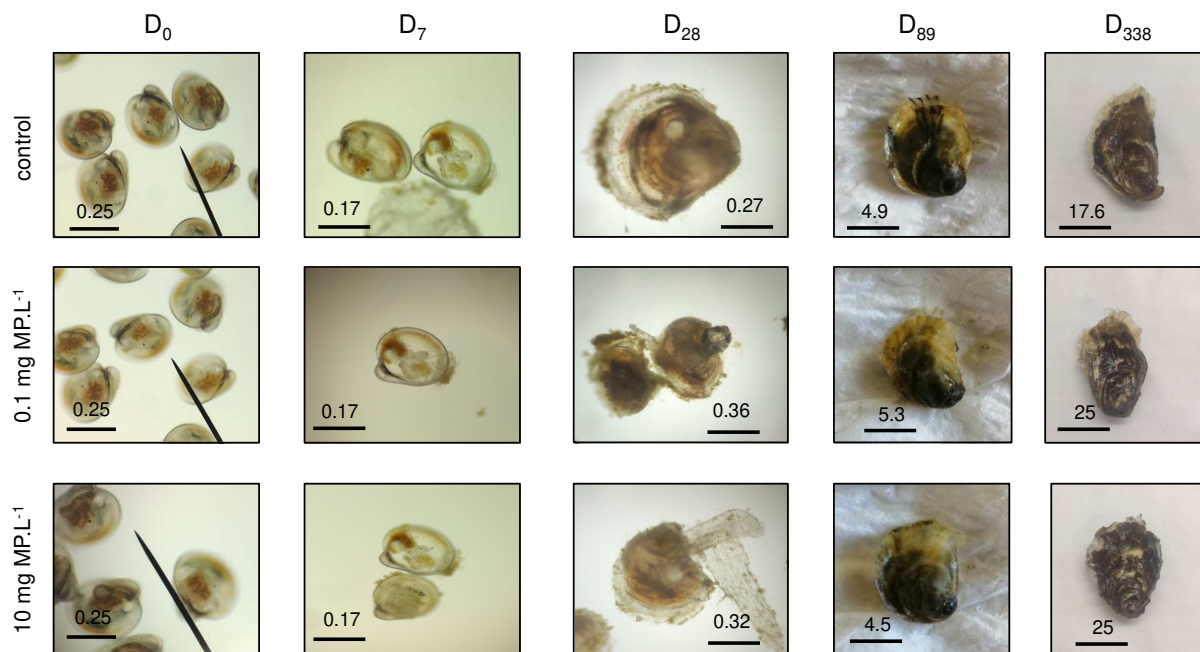
257

258 **Figure 2.** Settlement success of pediveliger larvae of *C. gigas* after a 7-day exposure: control (0 mg MP. L<sup>-1</sup>),  
259 0.1 and 10 mg MP. L<sup>-1</sup>. Values are expressed as mean ± SEM (n=3 replicates/conditions, *i.e.* n=9/conditions).  
260 Different letters at the top of the bars indicate significant differences between concentrations ( $p < 0.05$ , Anova  
261 and Tukey test).

262

263 In the control condition, 61.8±1.6 % larvae were successfully fixed. In the 0.1 mg MP. L<sup>-1</sup> condition,  
264 the ratio indicated 61.3±0.9 %, which did not statistically differ from the control condition ( $p=0.95$ ).  
265 However, in the 10 mg MP. L<sup>-1</sup> condition, the ratio was 49.0±0.9 %, differing significantly from the

266 other two conditions ( $p < 0.001$ ). The average rate of *C. gigas* larval settlement in remote microbreaks  
 267 currently reaches 65 % (Bodoy, 1990). In this work, the control condition displayed a binding rate of  
 268 approximately 62 %. The experimental system was thus considered valid. After a few days of  
 269 development in the micronursery, fixed pediveliger larvae began to grow shells (Figure 3). In all  
 270 conditions, no delay in shell development nor developmental abnormalities (malformations) were  
 271 noted.  
 272



273  
 274 **Figure 3.** Microphotographs and pictures of pediveliger larvae from the beginning (D<sub>0</sub>) to the end of the  
 275 experiment (D<sub>338</sub>) – including MP exposure (D<sub>7</sub> – step 1), spat development (D<sub>28</sub> and D<sub>89</sub> – step 2), and  
 276 monitoring (D<sub>338</sub>). Photos were taken in each condition: control (0 mg MP. L<sup>-1</sup>); 0.1 and 10 mg MP. L<sup>-1</sup>. Scales  
 277 are expressed in mm. Observations and photographs were performed at 5X or 20X using a binocular microscope  
 278 (Motic). “D<sub>x</sub>” indicates the sampling times in days.

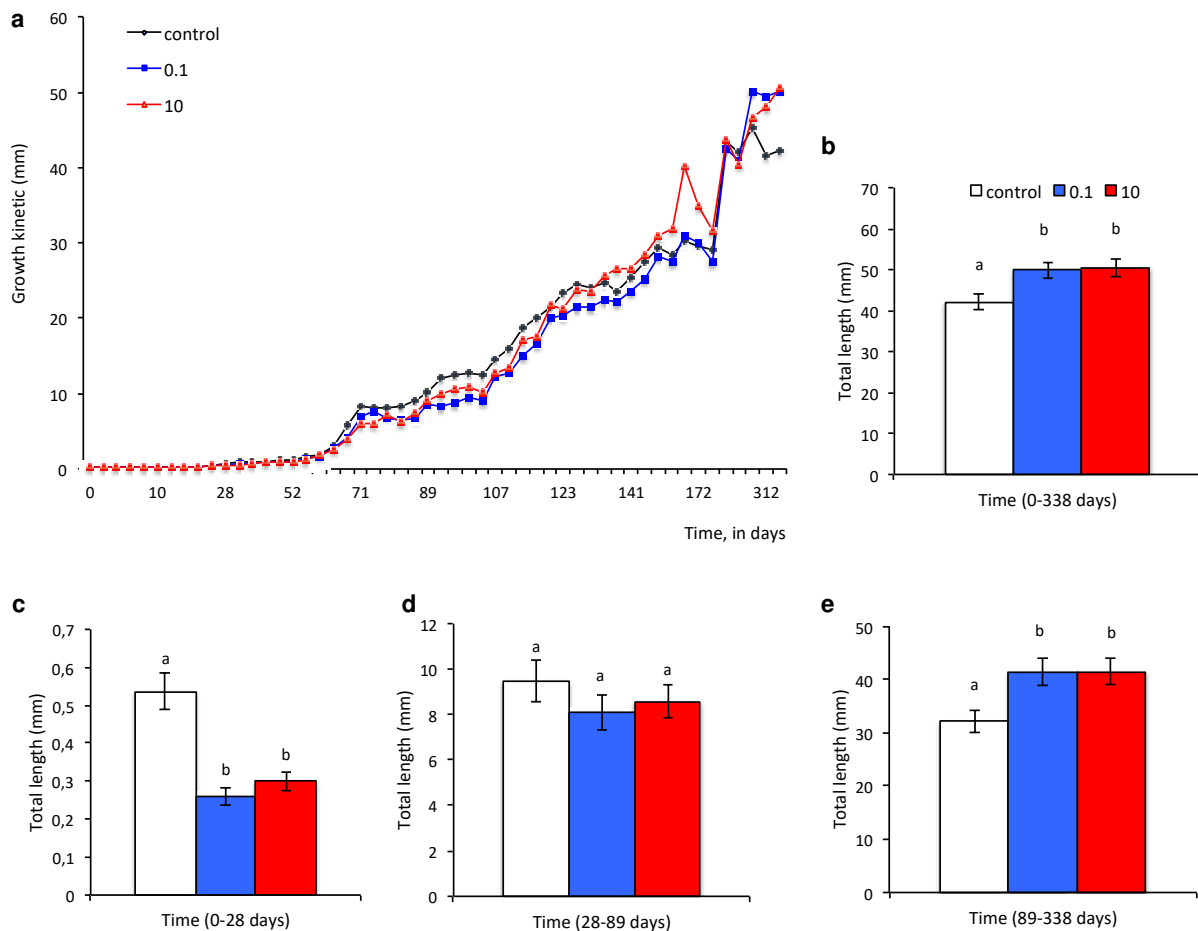
279  
 280 In an earlier study, pediveliger larvae (*C. gigas*) were exposed for a period of 24 hours, showing  
 281 decreased metamorphosis rates with increasing drug concentrations tested, greater than what is found  
 282 in the natural environment (Di Poi *et al.*, 2014). In the study conducted by Vignier *et al.* (2016),  
 283 exposure of oyster pediveliger larvae (*C. virginica*) for 72 h to increasing doses of CEWAF (dispersed  
 284 oil) induced settlement inhibition in a dose-dependent manner. The authors concluded that the  
 285 settlement success was sensitive to environmental parameter variations and pollutant exposure

286 (Vignier *et al.*, 2016). Wang *et al.* demonstrated that the pediveliger larvae of *Pinctada maxima* are  
287 more prone to attach to natural collectors (palm rope collector) than to plastic collectors (Wang *et al.*,  
288 2017). Larvae were considered metamorphosed if they displayed clear loss of velum and foot, shell  
289 growth and well-developed gills. Mottier *et al.* (2014) exposed pediveliger larvae for 24 h to  
290 glyphosate in commercial formulations and observed a reduction of the larval metamorphosis rate.  
291 Tallec *et al.* (2018) did not observe impacts on the metamorphosis of exposed *C. gigas* following 24 h  
292 of exposure. A variation in particle size (MPs or NPs) or concentration had no influence. In addition,  
293 after PS-MPs/NPs treatments, there were no developmental abnormalities in oysters (Tallec *et al.*,  
294 2018). These results echoed our own observations, showing no impacts on larval metamorphosis and  
295 no developmental abnormalities during fixation. MPs had no impacts on the development of  
296 pediveliger larvae, most likely due to their protective shell (Hickman, 1999; Liebig and Vanderploeg,  
297 1995; Schiaparelli *et al.*, 2004). Nonetheless, no studies reported MP impacts on fixation, *i.e.*, with  
298 lower fixation rate in larvae exposed to high concentrations. MPs could affect the energy dynamics of  
299 pediveliger larvae (Sussarellu *et al.*, 2016; Rico-Villa *et al.*, 2010). Larvae would lose their fixation  
300 capacities as they lack energy reserves, constantly fighting against MPs in their environment. Effects  
301 were noted on the energy reserves of sediment-dwelling bivalves. Indeed, the lipid content was lower  
302 in bivalves exposed to MPs (Auclair *et al.*, 2020; Bour *et al.*, 2018). The drifting and structure of  
303 marine organisms could be influenced by chronic MP exposure (Sendra *et al.*, 2021). In addition,  
304 recent studies have highlighted that MPs may affect immune enzyme activities and immune-related  
305 gene expression (Gardon *et al.*, 2020; Huang *et al.*, 2020). Authors hypothesised that the defence  
306 mechanisms at larval stages could be weakened and therefore individuals could be more sensitive to  
307 environmental factors such as pathogens or potential MPs (Thomas-Guyon *et al.*, 2009). Larvae of the  
308 Pacific oyster *C. gigas* appeared to acquire their own defence mechanisms during development.  
309 Potentially weakened by MPs, pediveliger larvae would have recourse to an adapted defense system to  
310 fight against plastic contamination, which can generate significant energy consumption, detrimental to  
311 growth for example.

312

### 313 **3.3 MP effects on pediveliger larvae development and oyster spat growth**

314 Length growth (in mm, Figure 4.a) was monitored at pediveliger larval stage from the beginning to the  
 315 end of exposure (D<sub>0</sub> to D<sub>7</sub>, Figure 3) and then until the spat stage, 11 months later (D<sub>338</sub>, Figure 3). A  
 316 7-day exposure to MPs led to growth retardation of larvae up to 28 days (D<sub>0</sub> to D<sub>28</sub>, Figure 4.c).  
 317 Indeed, there was a significant difference of growth rate between the control condition (0.54±0.05 mm  
 318 of growth for 28 days) and the 0.1 and 10 mg MP. L<sup>-1</sup> conditions (0.26±0.02 and 0.30±0.02 mm of  
 319 growth for 28 days). Oysters had similar growth rate between D<sub>28</sub> and D<sub>89</sub> (Figure 4.d), with no  
 320 significant variation between the conditions tested. The larvae from the control had a growth of  
 321 9.5±0.9 mm, while the larvae from the 0.1 and 10 mg MP. L<sup>-1</sup> conditions grew at 8.1±0.7 and 8.6±0.7  
 322 mm for 61 days (from 28 to 89 days). From D<sub>89</sub> to D<sub>338</sub> (end of growth monitoring), pre-exposed  
 323 oysters exhibited high growth during their development (Figure 4.e). Control spat grew 32.1±2.0 mm  
 324 while the spat from the 0.1 and 10 mg MP. L<sup>-1</sup> conditions had similar growth of 41.5±2.5 mm and  
 325 41.3±2.1 mm, for 249 days (from 89 to 338 days, Figure 4.e).  
 326



327

328 **Figure 4. (a)** Growth kinetic (D<sub>0</sub> to D<sub>338</sub>) **(b)** and total length of *C. gigas* pediveliger larvae and oyster spat. **(c)**  
329 Graphical displays on total length from D<sub>0</sub> to D<sub>28</sub>; **(d)** D<sub>28</sub> to D<sub>89</sub> and **(e)** D<sub>89</sub> to D<sub>338</sub> in various exposure  
330 conditions: control (0 mg MP. L<sup>-1</sup>), 0.1 and 10 mg MP. L<sup>-1</sup>. Values are expressed in mean ± SEM  
331 (n=30/replicates, *i.e.* n=90/conditions). Different letters at the top of the bars indicate significant differences  
332 between exposure conditions ( $p < 0.05$ , Kruskal-Wallis and Nemenyi test).

333

334 The total growth (D<sub>0</sub> to D<sub>338</sub>) was significantly higher in MPs-exposed oysters in comparison to  
335 control individuals (Figure 4.b). Indeed, the control condition observed a total growth of 42.1±1.9 mm  
336 while the 0.1 and 10 mg MP. L<sup>-1</sup> conditions displayed a total growth of 49.8±2.0 and 50.4±2.1 mm  
337 (Figure 4.b). Our results indicate that the oyster growth during the fixation phase was delayed by MPs  
338 exposure. Then, the growth at the end of experiment turned out to be higher than for oysters from the  
339 control condition. Few studies monitored the growth of exposed invertebrates on a long-term basis. In  
340 some cases, growth, respiration and filtration rates of oysters were not impacted by MPs, compared to  
341 other benthic study models that were weakened by exposures to MPs (Green, 2016). Revel *et al.*  
342 (2020) showed that PE and PP-MPs (<400 µm) had no significant toxicity on adult oysters after 10  
343 days of exposure to 0-100 µg MP. L<sup>-1</sup>. Cole and Galloway (2015) reported that PS-MPs of 1 and 10  
344 µm restricted algal feeding of oyster larvae, but there were no consequences on the growth of oyster  
345 larvae exposed up to 100 MP. mL<sup>-1</sup>. After a two-month exposure to low and high doses of PLA  
346 (polyactic acid) or HDPE-MPs, filtration and growth rates of adult *Ostrea edulis* were unaltered  
347 (Green, 2016). Because of the relatively low toxicity and long-term effects of MPs and additives, toxic  
348 effects analysis over a long periode of time is particulary relevant.. Analysis of metabolic rates and  
349 assimilation efficiency indicated a decrease in mean energy balance, when comparing adults from the  
350 control condition to those exposed for two months to 0.25 and 2.5 µg. L<sup>-1</sup> micro-PS (Gardon *et al.*,  
351 2018). The impacts on growth and energy dynamics are likely interconnected. Widdows and Johnson  
352 (1988) suggested that the energy available for growth should be a balance between the energy from  
353 food consumption and that used for respiration. Oyster larvae facing chronic MPs exposure could  
354 consume the energy dedicated to the fixation and development. Sussarellu *et al.* (2016) exposed adult  
355 oysters during gametogenesis for two-months to 23 µg. L<sup>-1</sup> PS-microbeads (2-6 µm) and reported



356 delayed larval growth. Watts *et al.* (2015) fed crabs (*Carcinus maenas*) over a period of 4 weeks (fed  
357 2-3 times per week) with different amounts of PP rope microfibers (0; 0.3; 0.6 and 1 % added to food).  
358 Those fed with plastics had a significant decrease in growth, mainly driven by a reduced food  
359 consumption over time. Larvae (*Crepidula onyx*) fed with higher concentrations (30 and 70 % of the  
360 algal concentration with final concentration of  $6 \times 10^4$  and  $1.4 \times 10^5$  particles.mL<sup>-1</sup> respectively) of  
361 micro-PS (2-5  $\mu$ m) than those found in the environment were smaller due to their reduced growth rate,  
362 in comparison to the control species (Lo and Chan, 2018).

363

## 364 **5. CONCLUSIONS**

365 This study observed the effects of a cocktail of MPs ( $138.6 \pm 2.3 \mu$ m) containing particles of HDPE, PP  
366 and PVC from aquaculture plastics aged in the marine environment. After an exposure period of 7  
367 days, the settlement success of the pediveliger larvae was evaluated. Then, an 11-month follow-up was  
368 carried out to monitor the growth in length of the pediveliger larvae up to the spat stage. Larvae  
369 exposed to 10 mg MP. L<sup>-1</sup> showed a lower binding rate to substrate. In addition, the larvae exposed to  
370 the two concentrations of MPs (0.1 and 10 mg MP. L<sup>-1</sup>) exhibited growth retardation over 28 days of  
371 follow-up. At the end of the experiment (at 338 days), the spat produced from the larvae exposed to  
372 the MPs recovered their growth retardation, and over 11 months had a total growth greater than the  
373 control group. The energy used by larvae facing MPs exposure could affect the oysters' ability to  
374 grow. It may be relevant to conduct further studies on the potential effects of MPs on energy reserves  
375 and the immune system of oysters at early stages of development. This study provides new evidences  
376 on the environmental risks of realistic high concentrations of MPs on aquatic invertebrates.

377

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