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## Intergenerational effects of environmentally-aged microplastics on the *Crassostrea gigas*

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1 Intergenerational effects of environmentally-aged microplastics on the  
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3  
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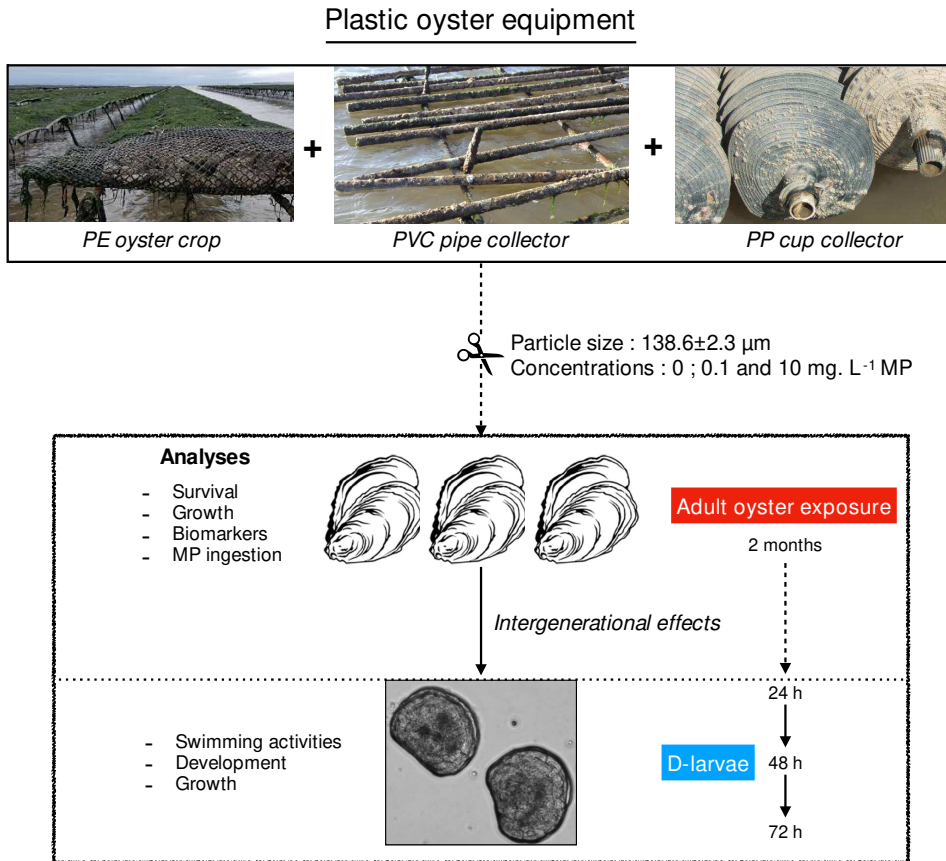
11  
12 **Abstract**

13 This study focused on the impacts of aged aquaculture microplastics (MPs) on oysters (*Crassostrea*  
14 *gigas*). Adult oysters were exposed for two months to a cocktail of MPs representative of the  
15 contamination of the Pertuis Charentais area (Bay of Biscay, France) and issuing from oyster framing  
16 material. The MPs mixture included 28 % of polyethylene, 40 % of polypropylene and 32 % of PVC  
17 (polyvinyl chloride). During the exposure, tissues were sampled for various analyzes (MP  
18 quantification, toxicity biomarkers). Although no effect on the growth of adult oysters was noted, the  
19 mortality rate of bivalves exposed to MPs (0.1 and 10 mg. L<sup>-1</sup> MP) increased significantly (respectively  
20 13.3 and 23.3 % of mortalities cumulative). On the one hand, the responses of biomarkers revealed  
21 impacts on oxidative stress, lipid peroxidation and environmental stress. At 56 days of exposure,  
22 significant increases were noted for Glutathione S-Transferase (GST, 10 mg. L<sup>-1</sup> MP), Malondialdehyde  
23 (MDA, 10 mg. L<sup>-1</sup> MP) and Laccase (LAC, 0.1 and 10 mg. L<sup>-1</sup> MP). No variations were observed for  
24 Superoxyde Dismutase (SOD). Besides, ingestion of MPs in oyster tissues and the presence in  
25 biodeposits was highlighted. In addition, *in vitro* fertilisations were performed to characterize MPs  
26 effects on the offspring. Swimming behavior, development and growth of D-larvae were analyzed at  
27 24-, 48- and 72-hours after fertilisation. D-larvae, from exposed parents, demonstrated reduced  
28 locomotor activity. Developmental abnormalities and arrest as well as growth retardation were also

29 noted. This study highlighted direct and intergenerational effects of MPs from aged plastic materials on  
30 Pacific oysters.

31

## 32 Graphical abstract



33

34 **Keywords** Oyster, microplastics, reproduction, larval behaviour, development and growth,  
35 intergenerational effects, *Crassostrea gigas*

36

## 37 1. Introduction

38 The growing use of plastics dramatically increases their production in Europe (57.9 million tons,  
39 PlasticsEurope, 2020) and in the World (368 million tons). Whether from land, atmosphere or rivers,  
40 anthropogenic pollutants, including plastics, end up in the marine environment (Halpern *et al.*, 2008).

41 In recent years, microplastics (MPs) has drawn the public's attention. MPs are defined as plastic particles  
42 between 1  $\mu\text{m}$  and 5 mm (Cole *et al.*, 2011; Wagner *et al.*, 2014), while nanoplastics (NPs) are smaller

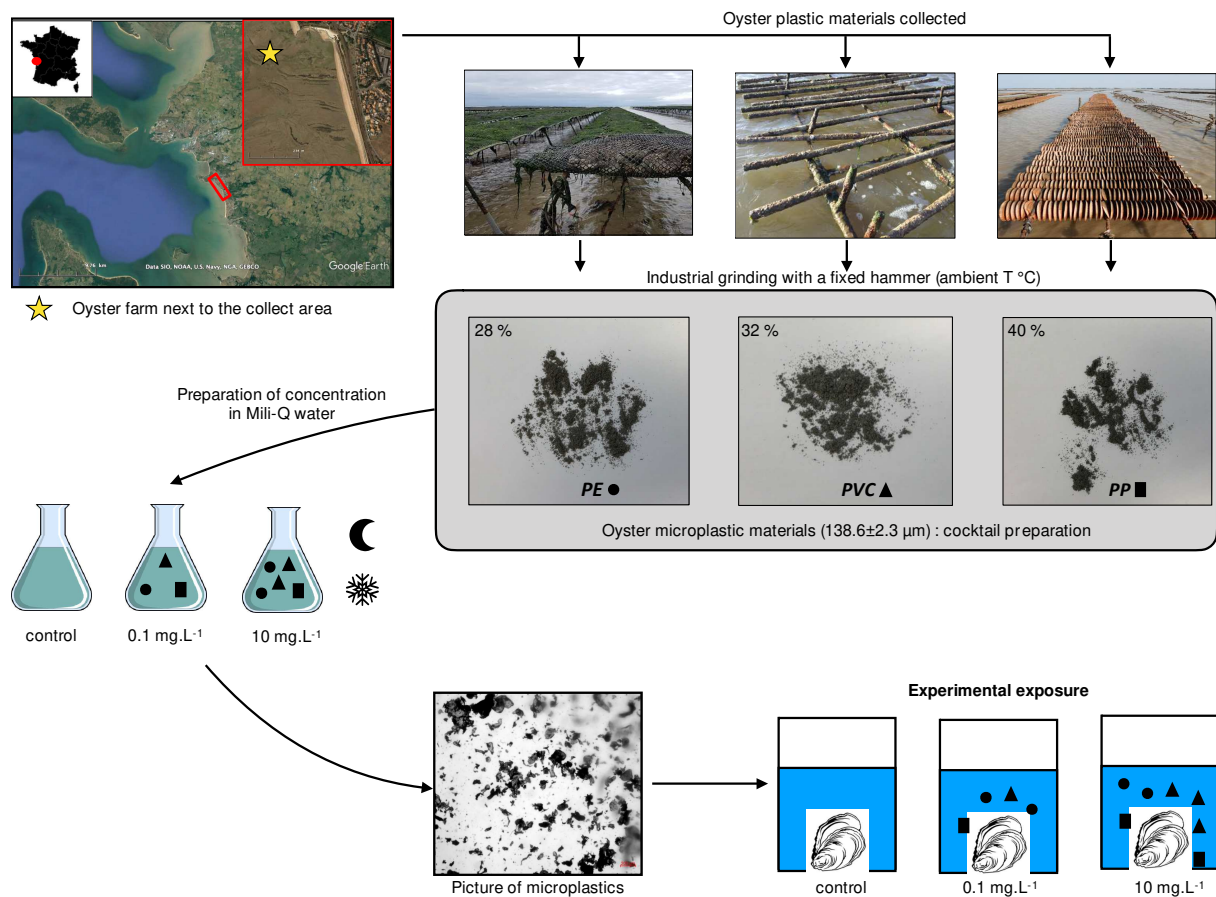
43 than 100 nm (Lambert & Wagner, 2016; Gigault *et al.*, 2016). MPs are spread in all oceans (Barnes *et*  
44 *al.*, 2009), including in the Arctic Ocean (Zarfl & Matthies, 2010). The MP size range distribution in  
45 the environment depends on hydrodynamic conditions, and degradation processes (Enders *et al.*, 2015).  
46 MPs occurrence in ocean is variable over time and space. Oceanic gyres and coastal areas as hot spots  
47 of MP accumulation (Law *et al.*, 2010). In Europe, 73 % of the plastic production resorts to 6 polymers  
48 (PlasticsEurope, 2020): polypropylene (PP), high and low-density polyethylene (HDPE and LDPE),  
49 polyvinyl chloride (PVC), polyurethane (PUR), polyethylene terephthalate (PET) and polystyrene (PS).  
50 Once ingested, MPs cause abrasions, obstructions and alterations of physiological structures, affecting  
51 the survival of individuals (Wright *et al.*, 2013). MPs can also generate chemical damage, as they  
52 contained a large variety of additives (Pérez-Albaladejo *et al.*, 2020; Hermabessiere *et al.*, 2017) and  
53 can sorbed persistent organic pollutants and heavy metals (Rochman *et al.*, 2013; Bakir *et al.*, 2014;  
54 Gorman *et al.*, 2019; Naik *et al.*, 2019). Recent studies have documented the potential effects of MPs  
55 on marine organisms and in particular bivalves (Tallec *et al.*, 2018; Sussarellu *et al.*, 2016; Revel *et al.*,  
56 2020). However, most of the MPs studied were of commercial origin and very few studies focused on  
57 the impacts of environmental MPs (Pannetier *et al.*, 2020; Cormier *et al.*, 2021; Gardon *et al.*, 2020).  
58 Bivalves are commonly used in studies to assess water quality and habitat. These filter feeders, in  
59 particular *Crassostrea gigas* oysters, are sensitive to alterations in water quality, which makes them  
60 good bioindicators and bioaccumulators for the study of pollutants (O'Connor, 2002), and in particular  
61 of MPs (Ward *et al.*, 2019; Bonanno & Orlando-Bonaca, 2018). In fact, oysters (*C. gigas*) are considered  
62 sentinel species of anthropized coastal ecosystems, due to their wide distribution, their sedentary  
63 lifestyle, and their sensitivity to a large range of pollutants (Conger *et al.*, 1978; His *et al.*, 1999; Quiniou  
64 *et al.*, 2007). Found in the bivalve tissues, MPs appear as fibres, fragments or granules (Cho *et al.*, 2019;  
65 Jin-Feng *et al.*, 2018). MP size and shape vary according to the tissue and species (Li *et al.*, 2015).  
66 Phuong *et al.* (2018a) highlighted that MP contamination fluctuated according to seasons, species and  
67 their living conditions (wild or farmed). Several previous studies have shown the presence of MPs in  
68 the tissues of oysters living on the French Atlantic coasts (Van Cauwenbergh & Janssen, 2014; Phuong  
69 *et al.*, 2018b). Sussarellu *et al.* (2016) demonstrated that the PS-MPs ingestion altered energy flows,  
70 metabolism and homeostasis in oysters, resulting in a loss of energy dedicated to their reproductive

71 function. They also noted impacts on the gametogenesis, with a decrease in quantity and quality of the  
72 gametes produced. Several studies underlined MPs effects on adult bivalves – including on their  
73 gametogenesis mechanism, and early larval stages. Cole & Galloway (2015) demonstrated that oyster  
74 larvae ingested PS-MPs, although their study differed in terms of larval stages and particle sizes. Tallec  
75 *et al.* (2018) reported a significant increase in malformation and developmental arrest (early mortality)  
76 frequencies in oyster larvae exposed to PS-NPs. Bringer *et al.* (2020b) studied the ingestion and effects  
77 of fluorescent MPs (mixed polymers) on oyster larvae. They documented that 72h-old D-larvae ingested  
78 MPs, which were detected in their digestive tract. This study also highlighted that MPs are able to stick  
79 to the locomotor cilia of D-larvae, influencing their swimming behaviour at 24-h of development. In  
80 addition, Bringer *et al.* (2020a) demonstrated that an exposure to 4-13  $\mu\text{m}$  HDPE-MPs, affected the  
81 oyster maximum swimming speed and trajectories of oyster larvae. A recent study focused on effects  
82 environmental MPs on the early stages of the pearl oyster, *Pinctada margaritifera* (Gardon *et al.*, 2020).  
83 This work documented the impacts of aged plastics on the embryo-larval and larval stages, leading to  
84 malformations and mortalities depending on the tested concentrations (Gardon *et al.*, 2020).  
85 Aquaculture professionals regularly use plastic tools (Lusher *et al.*, 2017). Following extreme  
86 hydroclimatic events (storms, winds or strong currents, etc.), professional materials become detached  
87 and drift towards beaches and coasts during high tides. Plastics, used in French oyster farming, could  
88 deteriorate, fragment and become toxic when broken down into particles of MPs/NPs. In addition, the  
89 additives released could potentially be more harmful to the marine environment than the polymer itself  
90 (phthalates, PAHs, metals ; Bringer *et al.*, 2021b). The French shellfish market is important. The  
91 sustainability of shellfish companies depends on the quality of the water.  
92 This study aimed at assessing effects of environmentally-aged MPs (from shellfish farming) on adult  
93 oysters (*C. gigas*) and their offspring. The MPs used ( $138.6 \pm 2.3 \mu\text{m}$ ) were a cocktail of three different  
94 aged-polymers: oyster crops (HDPE), collector cups (PP) and collector tubes (PVC). The data obtained  
95 included: (i) MP exposure of two-month-old adult oysters, with a follow-up on survival, biometrics,  
96 potential ingestion/excretion (tissues/biodeposits) and biomarker responses; (ii) larvae from exposed  
97 parents, with a 72-hour follow-up on swimming behaviour, development (malformations and  
98 developmental arrest) and length growth.

## 100 2. Materials and Method

### 101 2.1 Preparation and analysis of environmentally-aged cocktail of MPs

102 Plastic macrodebris were recovered on the French Atlantic beach (South-West, France), more precisely  
103 in the Angoulins-sur-Mer municipality (Bringer *et al.*, 2021b). These were sorted to identify plastics  
104 from the oyster farming industry. HDPE oyster culture crops, PP cup collectors and PVC tube collectors,  
105 were selected. A GC-MS pyrolysis analysis (700 °C) of the three plastic materials selected (manual  
106 cutting of 130-200 µg samples) allowed the identification of the main polymer. After a rough cut (3-4  
107 cm), the plastics were <sup>[1]</sup><sub>SEP</sub>crushed using a stationary metal hammer, crushing the plastics several times.  
108 Then, the plastics were <sup>[1]</sup><sub>SEP</sub>sieved using 5 mm, 1 mm and 100 µm sieves. The grinding was carried out at  
109 room temperature so as <sup>[1]</sup><sub>SEP</sub>not to influence the nature of the polymers materials. MPs cocktail of 138.6  
110 ± 2.3 µm were recovered (three analytical replicates in laser granulometry, Malverne ®). MPs cocktail  
111 was composed of 28 % HDPE, 40 % PP and 32 % PVC (Andrady, 2011). Each solution was prepared  
112 in pure Milli-Q-water (Millipore ®) and kept in the dark at 4 °C (Figure 1). To carry out a quantitative  
113 analysis of MPs in experimental seawater, each cocktail solution (0.1, and 10 mg. L<sup>-1</sup> MP) was tested  
114 through a flow cytometer (Attune Acoustic Focusing Cytometer ®) according to the protocole described  
115 previously (Bringer *et al.*, 2020a ; Bringer *et al.*, 2020b). Two-millilitre samples from each MP  
116 exposition solution were vortexed (StarLab Vortex IR ®, 12,000 rpm for 20 sec) to homogenise the  
117 solutions and then 300 µL were taken for flow cytometry analysis. A calibration was carried out, thanks  
118 to previous studies (Bringer *et al.*, 2020a) with commercial MPs, to achieve an analysis rate of 500  
119 µL.min<sup>-1</sup> and a saturation of 10,000 particles maximum detected. From the control conditions (10 µm  
120 filtered seawater), control analytical <sup>[1]</sup><sub>SEP</sub>samples (n=12) were created. The blank obtained enabled to  
121 remove background particles (natural present in seawater). Using seawater filtered at 0.2 µm, a first  
122 calibration was conducted to select the < 200-time detected particles. MPs measurements in seawater  
123 were assessed at T<sub>1</sub> (D<sub>17</sub>), T<sub>2</sub> (D<sub>35</sub>) and T<sub>3</sub> (D<sub>56</sub>) for all exposure conditions.



125

126 **Figure 1.** Experimental design of adult oysters exposed to MPs cocktail obtained from different aged-plastic  
 127 materials used in French oyster farms.

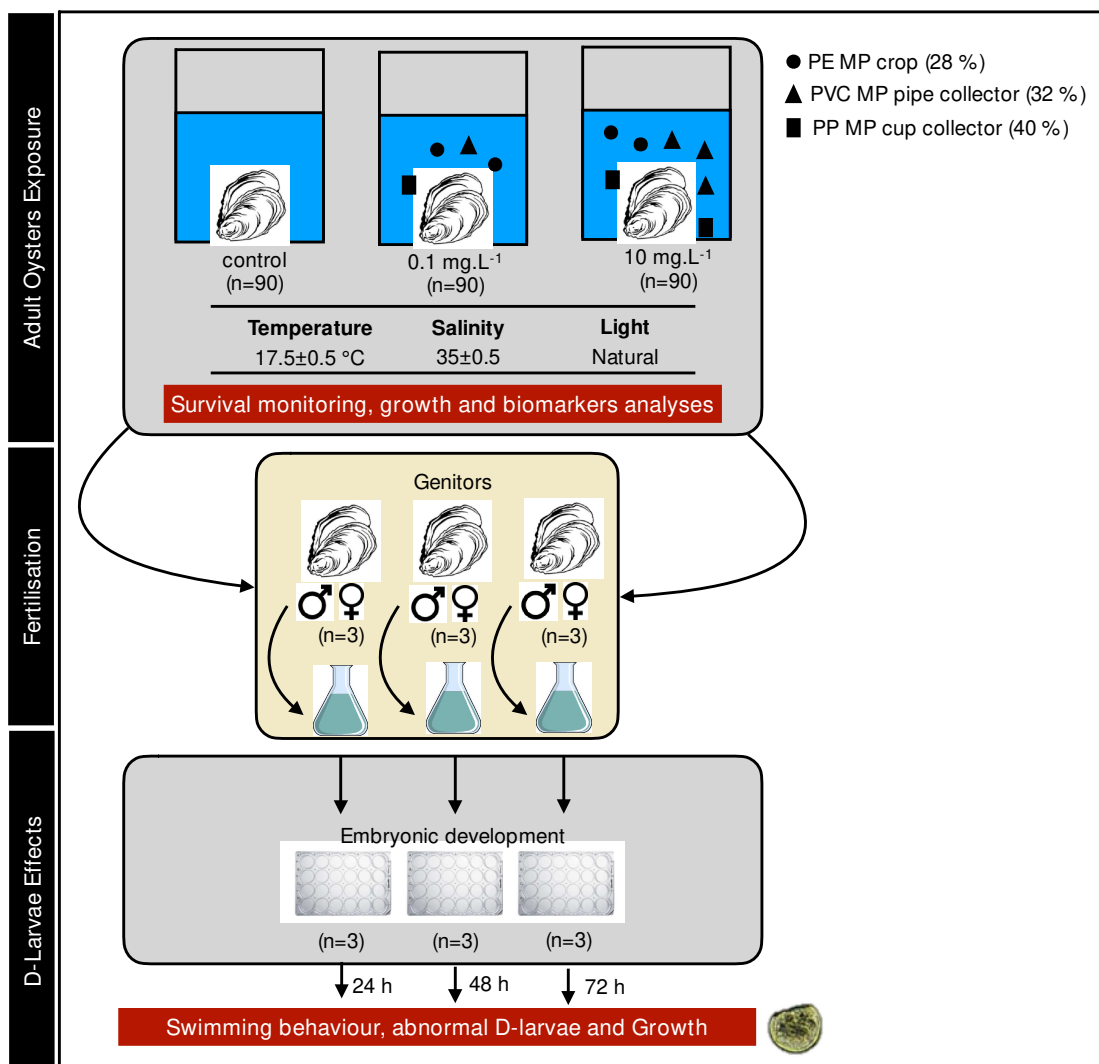
128

## 129 2.2 Experimental exposure of adult oysters and larvae

130 In June 2019, adult oysters *Crassostrea gigas* (Bayne *et al.*, 2019; Bayne *et al.*, 2017) purchased from  
 131 a commercial hatchery (24 mo, 15.75 ± 1.5 g) were transferred to our laboratory. After a 7-day  
 132 acclimatisation period, the oysters were placed in 9 experimental tanks of 37.5 L (n=30 oysters per tank,  
 133 with three replicates tanks per condition) with 3 large buffer tanks (one buffer tank per condition). Tanks  
 134 were supplied with filtered (10 μm) UV-treated (78.3 L.h<sup>-1</sup>) running seawater (filtered seawater, FSW)  
 135 at a temperature of 18.5 ± 0.1 °C, salinity of 35.3 ± 0.06 and natural photoperiod. During the experiment,  
 136 the tank temperature was gradually increased to faithfully recreate the environmental conditions of  
 137 spring on the French Atlantic coast (Zapata-Restrepo *et al.*, 2019). Seawater came from the Atlantic  
 138 Ocean (South-West, France) in the Pertuis Breton and more precisely it was pumped in the Baie de  
 139 l'Aiguillon (La Rochelle, France). Once a day, oysters were fed with a mixed diet (*T.Isochrysis galbana*,

140 *Pavlova*, *Tetraselmis*, *Thalassiosira* and *Nanno*, Shelfish diet, VariconAqua). Tanks (n=3/condition)  
 141 containing the exposed oysters, were connected to a buffer tank, equipped with an air bubbling system  
 142 in order to aerate FSW, and carry out a continuous water renewal in a closed system. The buffer tanks  
 143 had the same physicochemical parameters as the exposure tanks. Every two days, water was renewed to  
 144 prevent bacterial growth and provide good conditions for oysters. Three conditions were set up: control  
 145 condition (without MPs), 0.1 mg. L<sup>-1</sup> MP, and 10 mg. L<sup>-1</sup> MP (Figure 2). In each buffer tank, a pumping  
 146 system was set up to homogenise MPs in water. After a two-month exposure, oysters were mating to  
 147 obtain embryos. Then, oyster larvae were grown in filtered seawater (0.2 µm) at 24 °C (optimal  
 148 temperature for development, Robinson, 1992) and potential effects of MPs on their swimming  
 149 behaviour and development were recorded at 24-, 48- and 72-hours (Figure 2).

150



151



152 **Figure 2.** Experimental design of adult oysters exposed to environmentally-aged MPs cocktail for two months.  
153 After fertilisation, behaviour, development, and growth analyses were carried out on D-larvae.

154

### 155 *2.3 Biometric analysis and survival*

156 A length growth monitoring was performed on adult oysters exposed to MPs for two months. A follow-  
157 up was carried out at D<sub>0</sub> (T<sub>0</sub>), D<sub>17</sub> (T<sub>1</sub>), D<sub>35</sub> (T<sub>2</sub>) and D<sub>56</sub> (T<sub>3</sub>). Oysters (n=30/condition) were measured  
158 (length, width and thickness) with a digital calliper and weighted (whole weight, wet flesh and empty  
159 shell). A condition index was calculated in order to compare the growth of exposed oysters to control  
160 ones. Bodoy *et al.* (1986) defined the index condition by the ratio of wet flesh to whole weight minus  
161 empty shell. The delta condition index was the difference between the beginning and end of the exposure  
162 period. A survival monitoring was carried out, removing dead oysters every two days and counting the  
163 total dead oysters for each condition over the two-month period.

164

### 165 *2.4 Microplastic analysis in adult oysters tissues and faeces*

166 Methodological details have been placed in the section Supplementary data (S1).

167

### 168 *2.5 Analyses of biochemical biomarkers in adult oysters*

#### 169 *Individual sample processing*

170 Adult oysters were removed from their experimental tank, dissected to extract shell tissue, and  
171 submerged in liquid nitrogen and stored at -80 °C for analysis. For each condition and each sampling  
172 date (T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub>), digestive glands (n=5/conditions) were homogenised in ice-cold phosphate buffer  
173 (100 mM, pH 7.2, 1100 mOsm). The homogenates were then centrifuged for 15 min at 12,500 g at 4 °C  
174 and the Final Fractions (FF), corresponding to the supernatant, were used for biochemical assays. FF  
175 was stored at -80 °C for analyzes. Analytical protocols for each biomarker have been placed in the  
176 Supplementary data section (S2).

177

178

179 *2.6 Reproduction of adult oysters after a two-month exposure*

180 Mature oysters were cleaned to avoid external contaminations and remove stuck microorganisms. Male  
181 and female oysters were induced to spawn by thermal stimulation (alternating immersion in FSW at 18  
182 °C and 28 °C for 30 min) or by stripping the gonad (when thermal stimulation was ineffective) (Mai *et*  
183 *al.*, 2013; Gamain *et al.*, 2017; Bringer *et al.*, 2020a). Males and females were individually isolated in  
184 beakers (500 mL of FSW) at their spawning temperatures (Parker *et al.*, 2009) for 10 minutes, before  
185 being removed. Eggs and sperm from two individuals were selected to create a single pairing. To  
186 eliminate debris and faeces, both were sieved separately using 50 µm (sperm) and 100 µm (eggs) meshes  
187 (Sefar Nitex ®). Sperm mobility was assessed and eggs counted under a microscope (Motic ®) at 100x  
188 magnification. Eggs were fertilised with sperm at a ratio of 1:10 (egg:sperm). Fertilisation success was  
189 studied under a microscope at 100x magnification, 1 hour after the start of the fertilisation. Embryos  
190 were then counted and transferred to a 24-well microplate (Greiner Bio-One, Cellstar without detectable  
191 DNase, RNase, human DNA and pyrogens ®) used in embryotoxicity according to previously published  
192 protocols (His *et al.*, 1999; Quiniou *et al.*, 2005) and the AFNOR standard procedure (NF ISO 17244,  
193 2015). The fertilized eggs (approximately 300-350 eggs) were placed in wells containing 2 mL of  
194 filtered seawater (0.2 µm) and incubated in a dark climatic chamber at 24 °C for 24-, 48- and 72-hour.

195

196 *2.7 Larval behaviour, development and growth*

197 After a 24-, 48- and 72-hour incubation, the swimming behaviour was recorded under a microscope  
198 (ZEISS Axio Observer Z1 ®) at 100x magnification in a naturally-lit air-conditioned room, set at 24 °C.  
199 Two-minute videos were recorded. For the three replicates (corresponds to three different couples with  
200 fertilisation success), results are expressed in mean ± SEM, with 6 videos by couple, *i.e.*, n=18  
201 videos/condition. A freeware application (VirtualDub, Windows ®) was used to subsample videos to 4  
202 fps (frame per second) and convert them to AVI format (Gamain *et al.*, 2019 and Bringer *et al.*, 2020a,b).  
203 ImageJ (1,52a software) was used to analyse the videos. These (AVI format) were first converted to  
204 grayscale image, before being transformed into a stack of binary images. Swimming parameters of  
205 numbered D-larvae, including their maximum speed (pixel/s) and trajectories, were calculated using the

206 wrMTrack plugin. Consequently, each larva was assigned an identification number reported in the result  
207 file. Using the protocol published by Gamain *et al.* (2019) and improved by Bringer *et al.*, 2020a, three  
208 different types of larval path were noted: (1) rectilinear; (2) circular and (3) motionless. A graphic tablet  
209 (Wacom Bamboo Pen&Touch ®) and image processing software (Photos, Windows 10) enabled to  
210 quantify and characterise the swimming trajectories. Once processed with a plugin, the videos were  
211 analysed to ensure each larva was only detected once. Then, 25 µL of 1 % buffered formaldehyde  
212 (Sigma- Aldrich Chemical ®, St. Quentin Fallavier, France) were added to each well. The percentage  
213 of abnormal D-larvae (developmental abnormalities) and developmental arrests were recorded (His *et*  
214 *al.*, 1999; Quiniou *et al.*, 2005) according to the standardised AFNOR procedure (NF ISO 17244, 2015).  
215 To validate the bioassays, the control condition should have contained less than 20 % of abnormal larvae  
216 (NF ISO 17244, 2015). For the three replicates (three couples of oyster genitors), results are expressed  
217 in mean ± SEM. In each condition, twelve wells of approximately 200 D-larvae were analysed, *i.e.*,  
218 n=2400 D-larvae counted/condition.

219 A length growth analysis using the ImageJ software was carried out, studying larvae exposed for 24-,  
220 48- and 72-hours (n=20/condition with three replicates, *i.e.*, a total of n=60/condition). Each larva were  
221 measured lengthwise (Talmage *et al.*, 2009; Helm *et al.*, 2006; Bringer *et al.*, 2021a). Exposed larvae  
222 were then visualised (ZEISS Axio Observer Z1, x20 objective) and photographed. Body length was  
223 determined from photographs. Results are expressed in mean ± SEM.

224

## 225 2.8 Statistics

226 Data are expressed in mean ± SEM. A logrank test, using the Kaplan-Meier method, was carried out to  
227 analyse the survival of adult oysters. In case of abnormal distribution, data were transformed using the  
228 following formula:  $P' = \arcsin \sqrt{r}$ ; P corresponds to raw data (frequency of larvae abnormalities) specified  
229 in P values from 0 to 1 (Legendre & Legendre, 1998). Homogeneity of variance (Levene's test) and  
230 normality of distribution (Shapiro-Wilk) were assessed. In order to compare the various treatments, an  
231 analysis of variance (ANOVA) using Tukey's post-hoc test was performed. For data not entering in the  
232 scope of a parametric test, a statistical analysis was carried out through the Kruskal-Wallis Test.

233 Differences in tested concentration means were then assessed using the Kruskal Nemenyi Post-hoc test  
234 with the PMCMR package (the Tukey test equivalent for non-parametric data).

235 Significance difference was accepted when  $p$ -value  $< 0.05$ . Statistical analysis was completed using R  
236 and graphs from Microsoft Excel ®.

237

### 238 3. Results

#### 239 3.1 Analysis of MP concentrations

240 **Table 1.** Theoretical and measured MP concentrations in FSW at different sampling times.

Conditions	Sampling time	Theoretical (mg. L <sup>-1</sup> MP)	Measured (MP. μL <sup>-1</sup> )	Measured (MP. mL <sup>-1</sup> )
MPs cocktail	T1	0.1	0.68 ± 0.067	677 ± 67.6
	T2	0.1	0.66 ± 0.05	655 ± 53.0
	T3	0.1	0.94 ± 0.06	943 ± 60.7
MPs cocktail	T1	10	3.30 ± 0.32	3,297 ± 319.0
	T2	10	3.47 ± 0.14	3,466 ± 142.7
	T3	10	3.21 ± 0.22	3,205 ± 221.3

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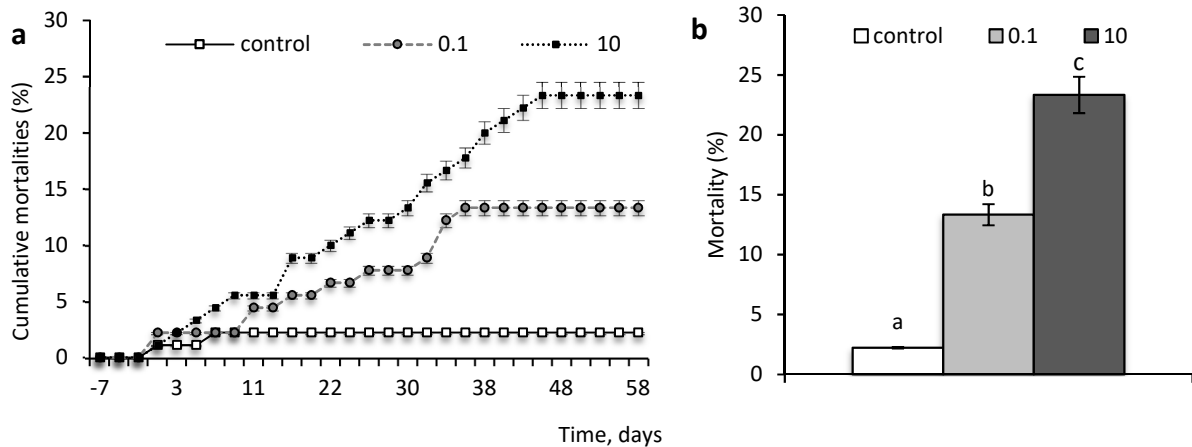
242 Through flow cytometry, the different concentrations of the MPs cocktail were measured in the  
243 experimental waters used for the exposure of adult oysters (Table 1) at different sampling times (T<sub>1</sub>, T<sub>2</sub>  
244 and T<sub>3</sub>). At 0.1 mg. L<sup>-1</sup> MP, very little variation was noted between the first two sampling times (T<sub>1</sub> and  
245 T<sub>2</sub>), ranging from 655 ± 53.0 to 677 ± 67.6 MP.mL<sup>-1</sup>. At T<sub>3</sub>, the concentrations of MPs increased slightly,  
246 with 943 ± 60.7 MP.mL<sup>-1</sup>. At 10 mg. L<sup>-1</sup> MP, values at the three sampling times (T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub>) were  
247 stable. However, the difference in concentration factor between the two conditions of exposure to MPs  
248 seems to be lower than theory.

249

#### 250 3.2 State of health and reproductive effort in adult oysters

251 To monitor growth and shape, a condition index was built from biometric measurements on adult  
252 oysters. However, there was no significant difference in the condition index between the control and  
253 exposed oysters (0.1 and 10 mg. L<sup>-1</sup> MP) with respectively 0.36 ± 0.03; 0.30 ± 0.02 and 0.31 ± 0.04.  
254 Following a two-month exposure, oyster mortality rate differed significantly between the three exposure  
255 conditions (0, 0.1 and 10 mg. L<sup>-1</sup> MP). The control condition exhibited the lowest mortality rate of 2.2

256 % (n=90/condition). At 0.1 mg. L<sup>-1</sup> MP, a cumulative mortality of 13.3 % was quantified. At 10 mg. L<sup>-1</sup>  
 257 MP, the mortality rate reached 23.3 % (Figure 3.a). Over the exposure period, the cumulative mortality  
 258 rate differed significantly between the conditions tested (Figure 3.b).  
 259



260  
 261 **Figure 3. (a)** Survival monitoring of adult oysters exposed to MPs for two months: control (without pollutants);  
 262 0.1 mg. L<sup>-1</sup> MP and 10 mg. L<sup>-1</sup> MP. **(b)** Histogram of cumulative mortalities for each exposure condition. Expressed  
 263 in mean ± SEM. Different letters indicate significant differences between concentrations ( $p < 0.05$ , Kaplan-Meier  
 264 Test, n=90/condition).  
 265

266 After two months exposure under controlled laboratory conditions, some adult oysters reached sexual  
 267 maturity and developed mature gonads, allowing reproduction (n=10/conditions). Six oysters from the  
 268 control condition emitted gametes. Seven oysters from the 0.1 mg MP. L<sup>-1</sup> condition and 8 oysters from  
 269 the 10 mg. L<sup>-1</sup> MP condition were mature and ejected gametes through thermal shock (Table S3). In  
 270 terms of sex determination, oysters in the control condition were predominantly maturing females (4:2,  
 271 female: male) while oysters exposed to MPs were mostly males with mature gametes (2:5 for 0.1 mg.  
 272 L<sup>-1</sup> MP and 3:5 for 10 mg. L<sup>-1</sup> MP, Table S1). The quality of gametes (well-formed oocytes and motile  
 273 spermatozoa) and was observed under a microscope (Motic). In the control condition, 100 % of the  
 274 gametes ejected being of good quality (Figure S4). 57 % and 50 % of the gametes from oysters exposed  
 275 to 0.1 and 10 mg. L<sup>-1</sup> MP were of good quality (Table S3). When bivalves expelled poor-quality gametes,  
 276 they were not selected to proceed to *in vitro* fertilisation. Three couples for each condition (*i.e.* three  
 277 replicates) were formed to obtain embryos, then becoming D-larvae.

278

### 279 3.3 MP analysis in adult oyster tissues and biodeposits

280 MPs were quantified in digested tissues and biodeposits from exposed adult oysters (Table 2).

281

282 **Table 2.** Concentrations of environmental MP cocktail (PE, PP and PVC) in oyster tissues (MPs.g<sup>-1</sup>) and  
283 biodeposits (cumulate faeces and pseudo-faeces, MPs.mg<sup>-1</sup>) at three sampling times (T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub>). Data are  
284 expressed in mean ± SEM. Different letters indicate significant differences between concentrations ( $p < 0.05$ ,  
285 ANOVA and Tukey post-hoc test).

286

Sampling time	T <sub>1</sub>		T <sub>2</sub>		T <sub>3</sub>	
	Tissues	Biodeposits	Tissues	Biodeposits	Tissues	Biodeposits
Control	0	0	0	0	0	0
MP 0.1 mg. L <sup>-1</sup>	2.1 ± 0.8 <sup>a</sup>	0.084 ± 0.02 <sup>a</sup>	6.3 ± 2.8 <sup>a</sup>	0.78 ± 0.2 <sup>a</sup>	5.4 ± 2.5 <sup>a</sup>	0.93 ± 0.2 <sup>a</sup>
MP 10 mg. L <sup>-1</sup>	11.3 ± 3.6 <sup>b</sup>	6.4 ± 2.2 <sup>b</sup>	23.6 ± 9.2 <sup>b</sup>	7.6 ± 2.7 <sup>b</sup>	21.4 ± 7.3 <sup>b</sup>	7.3 ± 3.1 <sup>b</sup>

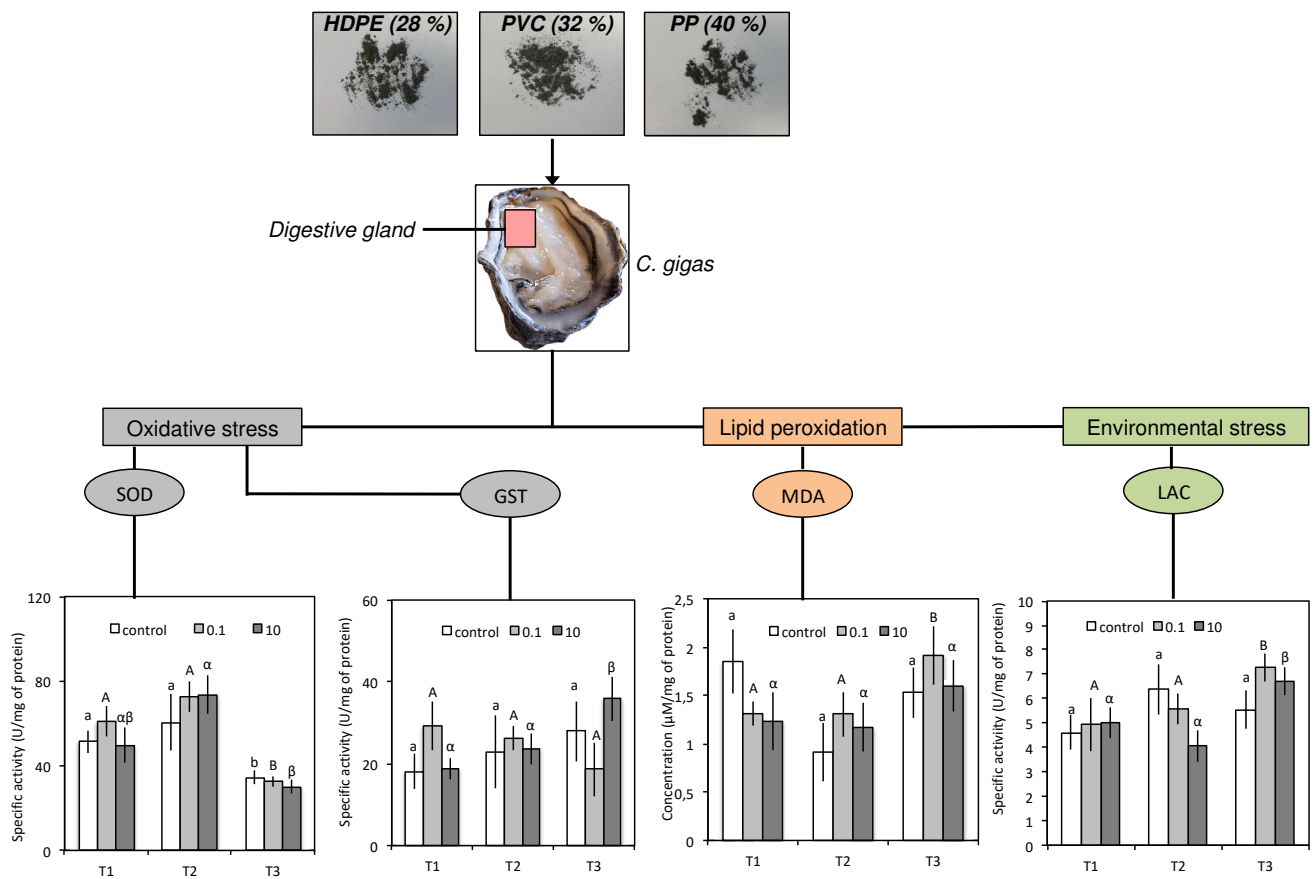
287

288 In the control oysters, there were no microplastics in the tissues for the three sampling times. In exposed  
289 oysters, MPs were detected in the tissues at all sampling times (T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub>). At T<sub>1</sub>, a greater quantity  
290 (increase factor of 5.4) of MPs is noted in the tissues of oysters exposed to 10 mg. L<sup>-1</sup> MP compared to  
291 the 0.1 mg. L<sup>-1</sup> MP condition. Same observation at T<sub>2</sub> and T<sub>3</sub>, with increase factors of 3.7 and 4  
292 respectively. The two exposure conditions noted a decrease in the MPs present in the tissues at T<sub>3</sub>,  
293 compared to T<sub>2</sub>. Under the control conditions, there were no MPs in the biodeposits. In exposed oysters,  
294 MPs at T<sub>1</sub> were quantified as follows: 0.084 ± 0.02 at 0.1 mg of MP .L<sup>-1</sup> and 6.4 ± 2.2 MPs.mg<sup>-1</sup> at 10  
295 mg. L<sup>-1</sup> MP. At T<sub>2</sub> and T<sub>3</sub>, the MPs in the biodeposits were markedly increased compared to T<sub>1</sub>.

296

### 297 3.4 Analysis of biochemical biomarkers in the adult oyster digestive gland

298 Figure 4 presents biomarker results on adult oysters exposed to environmental MPs (cocktail of HDPE,  
299 PVC and PP from oyster farming). SOD and GST activities are both induce under oxidative stress. The  
300 MDA activity is defined as lipid peroxidation and the LAC activity as environmental stress.



302

303 **Figure 4.** Effects of environmental MPs on oxidative stress (Superoxide Dismutase (SOD) and Glutathione S-  
 304 transferase (GST)), lipid peroxidation (Malondialdehyde (MDA) and environmental stress Laccase (LAC) in *C.*  
 305 *gigas* adult digestive gland. The different sampling times correspond to: T<sub>1</sub> (D<sub>17</sub>), T<sub>2</sub> (D<sub>35</sub>) and T<sub>3</sub> (D<sub>56</sub>). Three MP  
 306 conditions were tested: 0 (control), 0.1 and 10 mg. L<sup>-1</sup> MP. The histogram data are expressed in mean ± SEM.  
 307 Within series, the letters (a/b, A/B and α/β) above each bar indicate significant differences ( $p < 0.05$ , ANOVA and  
 308 Tukey test, n=5/condition) between different sampling times for each condition.

309

#### 310 Superoxide Dismutase (SOD) and Glutathione S-transferase (GST) assays: oxidative stress

311 The SOD activity in oysters from the three conditions tested (control, 0.1 and 10 mg. L<sup>-1</sup> MP) decreased  
 312 significantly at T<sub>3</sub> (respectively 34.3 ± 3.3, 32.6 ± 2.5 and 30.0 ± 3.3 U/mg of protein), in comparison  
 313 with T<sub>1</sub> (respectively 51.4 ± 5.4, 60.9 ± 7.0 and 49.7 ± 8.3 U/mg of protein) and T<sub>2</sub> (respectively 60.5 ±  
 314 13.4, 72.8 ± 7.2 and 73.5 ± 20.5 U/mg of protein; ANOVA,  $p < 0.01$ ). Both the control and exposed  
 315 oysters followed similar trends. It cannot be concluded that there is a significant difference in SOD

316 activity at T<sub>3</sub> for the exposure conditions because the control condition follows the same trend. The  
317 GST activity in control condition had no significant change at the three sampling times (ANOVA,  
318  $p=0.9$ ). The trend was similar to oysters exposed to 0.1 mg MP. L<sup>-1</sup>, observing no significant difference  
319 between the three sampling times (ANOVA,  $p=0.5$ ). In the 10 mg. L<sup>-1</sup> MP condition, the GST activity  
320 increased at T<sub>3</sub> ( $35.9 \pm 5.4$  U/mg of protein; ANOVA,  $p<0.05$ , Figure 4).

321

#### 322 Malondialdehyde (MDA) assay: lipid peroxidation

323 In the control and 10 mg. L<sup>-1</sup> MP conditions, the MDA activity in oysters had no significant difference  
324 at the three sampling times (respectively, ANOVA,  $p=0.2$  and  $p=0.9$ ). In the 0.1 mg. L<sup>-1</sup> MP condition,  
325 the MDA activity increased between T<sub>2</sub> ( $1.3 \pm 0.2$  μM/mg of protein) and T<sub>3</sub> ( $1.9 \pm 0.3$  μM/mg of  
326 protein; ANOVA,  $p < 0.05$ ) (Figure 4).

327

#### 328 Laccase (LAC) assay: environmental stress

329 In control oysters, there were no significant differences of laccase activity between the three sampling  
330 times. The activity in oysters exposed to MPs (0.1 and 10 mg. L<sup>-1</sup> MP) increased significantly between  
331 T<sub>2</sub> (respectively  $5.6 \pm 0.6$  and  $4.1 \pm 0.6$  U/mg of protein) and T<sub>3</sub> (respectively  $7.3 \pm 0.6$  and  $6.7 \pm 0.6$   
332 U/mg of protein; ANOVA,  $p < 0.05$ , Figure 4).

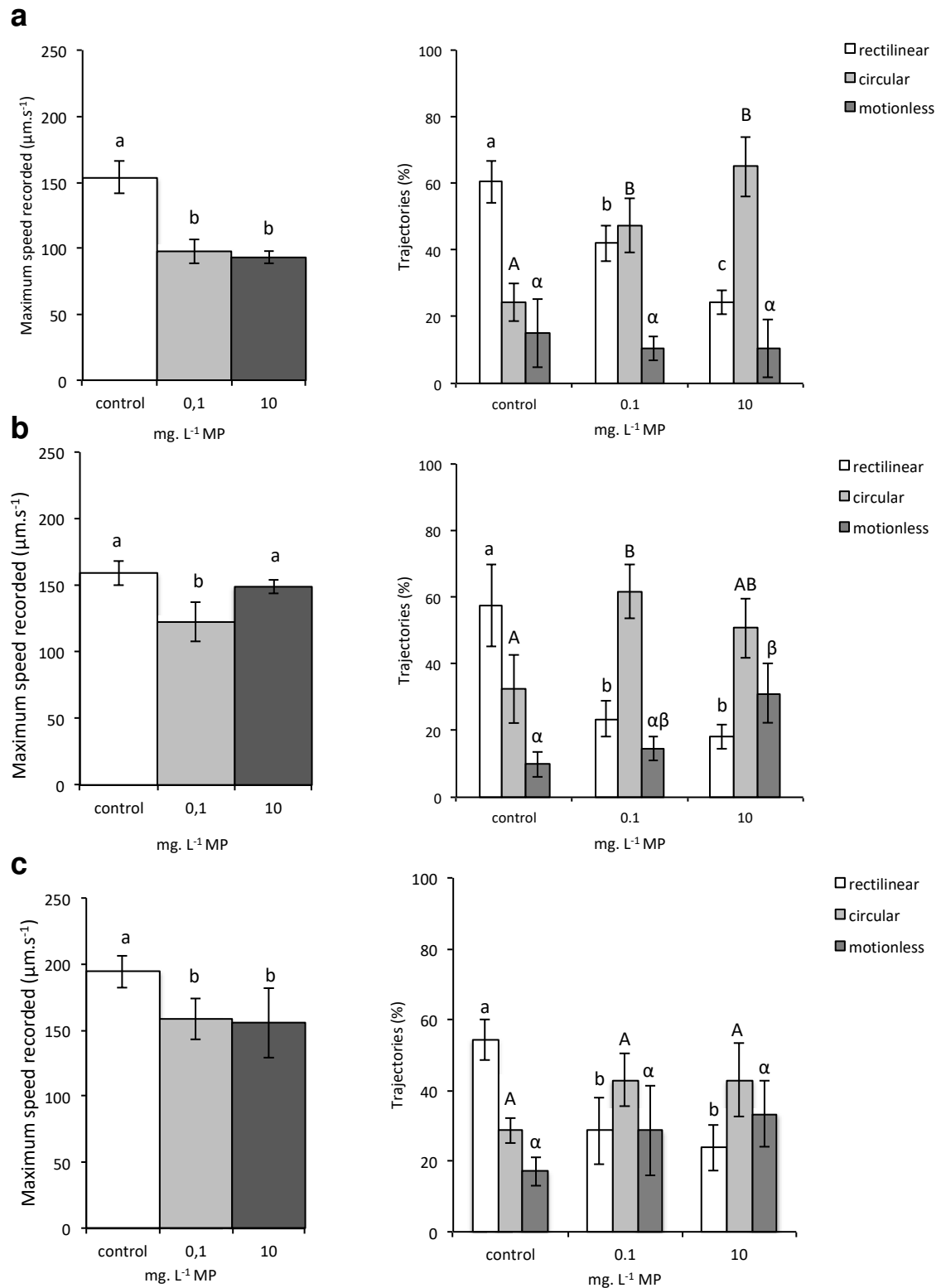
333

#### 334 *3.5 Larval behaviour, development and growth*

335 The recorded videos (ZEISS Axio Observer Z1) highlighted a swimming activity (maximum swimming  
336 speed) increasing with the larvae age. The swimming speed was faster in 72-hour than for 24-hour larvae  
337 (Figure 5). Compared to control larvae ( $153.3 \pm 12.2$  μm. s<sup>-1</sup>), 24-hour D-larvae (Figure 5.a) from  
338 exposed adult oysters swam significantly slower (respectively  $97.6 \pm 9.0$  and  $93.1 \pm 5.0$  μm. s<sup>-1</sup> at 0.1  
339 and 10 mg. L<sup>-1</sup> MP). 48-hour larvae (Figure 5.b) from parents exposed to 0.1 mg. L<sup>-1</sup> MP had a lower  
340 swimming activity. 72-hour larvae (Figure 5.c) from parents exposed to the highest MP concentration  
341 (10 mg. L<sup>-1</sup> MP,  $155.6 \pm 26.4$  μm. s<sup>-1</sup>) swam slower than oyster larvae from other conditions (respectively  
342  $194.5 \pm 12.2$  and  $158.5 \pm 16.0$  μm. s<sup>-1</sup> in the control and 0.1 mg. L<sup>-1</sup> MP conditions). In addition, over a



343 two-month period, the swimming trajectories of larvae from exposed parents were characterised (Figure  
344 5). 24-hour D-larvae from unexposed parents (control condition) displayed rectilinear swimming  
345 trajectories ( $60.6 \pm 6.4 \%$ , Figure 5.a). 48- and 72-hour larvae had predominant rectilinear swimming  
346 paths ( $57.5 \pm 12.4 \%$  and  $54.3 \pm 5.8 \%$  respectively, Figure 5.b & c). 24-hour D-larvae from parents  
347 exposed to 0.1 and 10 mg. L<sup>-1</sup> MP mainly swam in circular trajectories, for  $47.4 \pm 8.1$  and  $65.0 \pm 8.9 \%$   
348 of the larvae, respectively (Figure 5.a). Forty-eight hour D-larvae from the 0.1 mg. L<sup>-1</sup> MP condition  
349 showed predominant circular trajectories ( $61.8 \pm 7.4 \%$ ), in higher proportion than the control condition  
350 ( $32.5 \pm 10.4 \%$ ). D-larvae from the 10 mg. L<sup>-1</sup> MP condition had motionless trajectories ( $31.1 \pm 6.0\%$ ),  
351 in higher proportion than the control condition ( $10.0 \pm 3.8\%$ , Figure 5.b). 72-hour larvae from parents  
352 exposed to 0.1 and 10 mg. L<sup>-1</sup> MP displayed fewer rectilinear trajectories (respectively,  $28.6 \pm 9.4$  and  
353  $23.8 \pm 6.5 \%$ ), in comparison with larvae from the control condition ( $54.3 \pm 5.8 \%$ , Figure 5.c).  
354



355

356 **Figure 5.** Maximum speed and trajectories (rectilinear, circular or motionless) of 24- (a), 48- (b) and 72-hour (c)

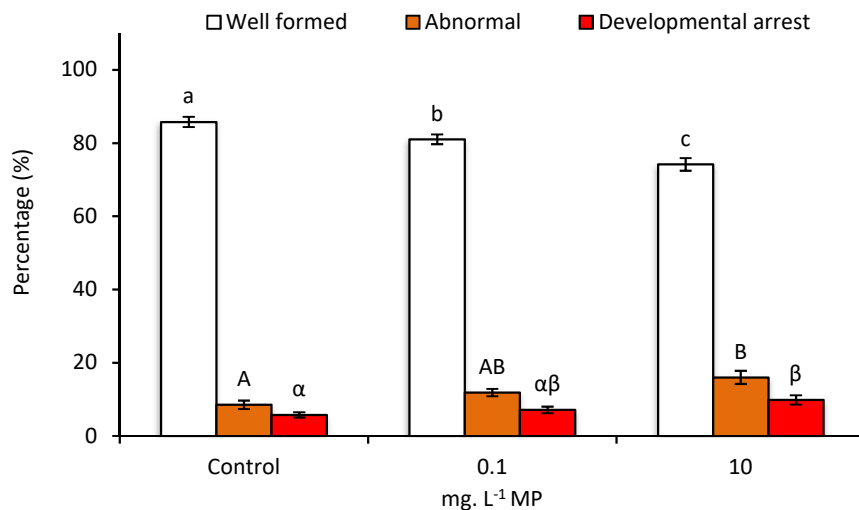
357 D-larvae from adult oysters exposed for two months to 0 (control), 0.1 and 10 mg. L<sup>-1</sup> MP. For the three replicates

358 (*i.e.* three couples), values are expressed in mean ± SEM. Different letters indicate significant differences between

359 concentrations ( $p < 0.05$ , Kruskal-Wallis and Nemenyi post-hoc test for maximum recorded speed and ANOVA  
360 and Tukey post-hoc test for the trajectory statistical analysis,  $n=18$  videos/condition).

361  
362 After being fixed with formalin (1 %), 72-h D-larvae were classified according to their developmental  
363 states (well-formed, malformed or in developmental arrest, Figure 6). D-larvae from unexposed adult  
364 oysters were well-formed ( $85.8 \pm 1.4$  %) and statistically different ( $p < 0.05$ ) from oysters exposed to 0.1  
365 ( $p < 0.01$ ) ( $81.1 \pm 1.3$  %) and 10 mg.  $L^{-1}$  MP ( $p < 0.01$ ) ( $74.2 \pm 1.7$  %). Malformed D-larvae from the  
366 control and 0.1 mg.  $L^{-1}$  MP conditions were not statistically different, with respectively  $8.5 \pm 1.2$  and  
367  $11.8 \pm 1.0$  % ( $p=0.08$ ). On the contrary, malformed larvae in the 10 mg.  $L^{-1}$  MP condition were  
368 significantly higher than in the control condition ( $16.0 \pm 1.8$  % ( $p < 0.01$ )). In addition, D-larvae with  
369 developmental arrest had no significant difference between the first two conditions (control and 0.1 mg.  
370  $L^{-1}$  MP, with respectively  $5.7 \pm 0.7$  and  $7.1 \pm 0.9$  %,  $p=0.6$ ). In comparison with the control condition  
371 ( $p=0.03 < 0.05$ ), the 10 mg.  $L^{-1}$  MP condition differed significantly, with  $9.8 \pm 1.2$  %. Representative  
372 photographs of malformed and developmental arrest larvae are presented in Figure S5.

373

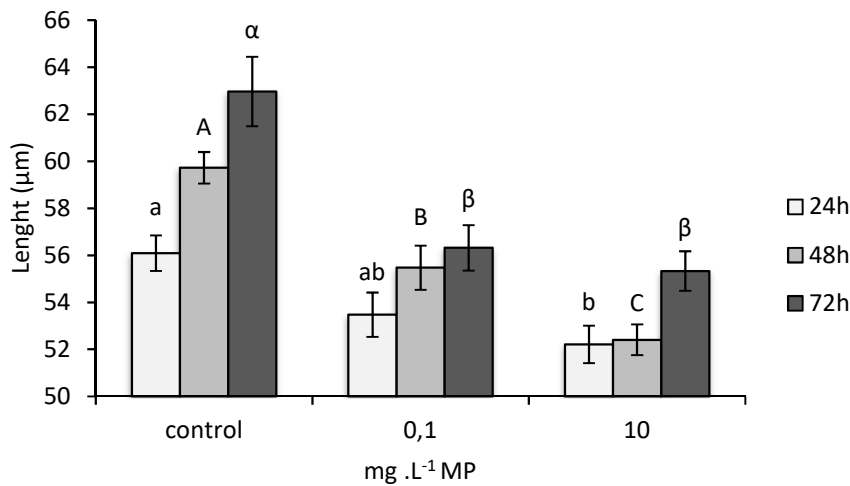


374

375 **Figure 6.** Percentages of well-formed, abnormal and developmental arrest in 72-hour *C. gigas* larvae. Over a two-  
376 month period, genitor oysters were exposed to three conditions: 0 (control), 0.1 and 10 mg.  $L^{-1}$  MP. For the three  
377 replicates, values are expressed in mean  $\pm$  SEM. Different letters indicate significant differences between  
378 concentrations ( $p < 0.05$ , ANOVA and Tukey test,  $n=2400$  larvae/condition).

379

380 Larval growth was slower in exposed than in control larvae (Figure 7). Significant differences of size  
381 reductions of 10.6 % and 12.3 %, were observed in 72-hour larvae from parents exposed to 0.1 and 10  
382 mg. L<sup>-1</sup> MP. The total length of control D larvae was 63.0 ± 1.5 µm, and significantly greater than larvae  
383 of parents exposed to 0.1 mg. L<sup>-1</sup> MP and up to 10 mg. L<sup>-1</sup> MP measured 56.3 ± 1.0 µm and 55.3 ± 0.8  
384 µm.



385  
386 **Figure 7.** Larva total length (µm) of *C. gigas* after 24-, 48- and 72-h of development from parents exposed for 2  
387 months to different concentrations of MPs cocktail : 0 (control), 0.1 and 10 mg. L<sup>-1</sup> MP. Values are means ± SEM.  
388 Different letters indicated significant differences between different concentrations and development time ( $p < 0.05$ ,  
389 ANOVA and Tukey test, n=60/condition).

390

#### 391 4. Discussion

392 The study aimed at evaluating the effects of environmentally-aged MPs (plastic materials from oyster  
393 farms) on *C. gigas* oysters. Over a two-month period, adult oysters were exposed to aged MPs  
394 obtained from a mixture of HDPE, PP and PVC. Their growth, survival, MP ingestion/excretion and  
395 the response of biomarkers were monitored. Adult oysters were fertilised in laboratory conditions to  
396 produce embryos. Then, the development and swimming activity of 72-hour oyster D-larvae (early  
397 stages of development) were observed.

398

399 4.1 Microplastics concentrations in experimental seawater

400 MP concentrations in the exposure medium were assessed through flow cytometry. Except for the 0.1  
401 mg. L<sup>-1</sup> MP condition at T<sub>3</sub>, concentrations measured were constant. In the latter condition at T<sub>3</sub>, the  
402 increase in MP concentration over time might be due to particles binding on the tank walls while water  
403 was renewed (Summers *et al.*, 2018). Our present study focused on ecotoxicological test concentrations,  
404 stronger than what is found in the marine environment (Lenz *et al.*, 2016; Paul-Pont *et al.*, 2018). The  
405 previous study by Dubaish & Liebezeit (2013) determined concentrations in the North Sea of 1.7  
406 MP.mL<sup>-1</sup> (MP > 80 μm). In our work, the ranges of concentrations assayed reported means of 758 ± 60  
407 and 3,322 ± 228 MP.mL<sup>-1</sup> for the two theoretical mass concentrations (0.1 and 10 mg. L<sup>-1</sup> MP). It is  
408 therefore estimated that our study would have used concentrations 445 and 1,954 higher than the natural  
409 environment. However, our concentrations are close to certain ecotoxicological studies that took into  
410 account doses of 1,000 MP.mL<sup>-1</sup> (Sussarellu *et al.*, 2016 and Paul-Pont *et al.*, 2016). According to  
411 literature, MP loads in seawater are within the μg.L<sup>-1</sup> range, and the highest MP loads reported in the  
412 sea area round 0.08 to 0.3 mg.L<sup>-1</sup> (Lusher *et al.*, 2014; Frias *et al.*, 2014). Thereby, the theoretical 0.1  
413 mg. L<sup>-1</sup> MP concentration correspond to the contamination peak of MPs in the marine environment  
414 (Beiras *et al.*, 2018). A recent study in the Bay of Biscay (South-West, Atlantic, France) near the study  
415 area where our MPs were collected indicated MP concentrations between 0.03 and 0.06 MP.L<sup>-1</sup> in  
416 water, with a size distribution between 4-2,000 μm, at the surface of the studied marine environment  
417 (Lerebours *et al.*, 2021). Wang *et al.* (2021), reported higher concentrations of 15-20 MP.L<sup>-1</sup> in  
418 seawater from the coastal areas in Zhuhai (China), with a size range of 101-500 μm for 45 % of the  
419 detected MPs.

420

#### 421 *4.2 Microplastic in adult oysters tissues and faeces*

422 MPs were not detected in tissues of control oysters. Like most animals, oysters lack enzymes and  
423 enzymatic pathways enabling to break down plastics (Aladaileh *et al.*, 2007; Wright *et al.*, 2013). After  
424 filtration-ingestion, MPs cannot be absorbed or used as energy, leading to their accumulation in the gills  
425 and digestive gland (Teng *et al.*, 2020). This work echoed previous results highlighting that bivalve  
426 accumulated MPs in both experimental and natural conditions (Browne *et al.*, 2008; Von Moos *et al.*,  
427 2012; Magni *et al.*, 2018; Teng *et al.*, 2019; Birnstiel *et al.*, 2019 and Ward *et al.*, 2019). The presence

428 of MPs accumulated in the tissues at the end of exposure ( $T_3$ ) could potentially affect reproductive  
429 efforts and subsequently the quality of the gonads and embryo-larval development (Sussarellu *et al.*,  
430 2016). One must recognize that the concentrations tested and MP in tissues were much higher than those  
431 in the environment (Keisling *et al.*, 2020). Regarding the quantities of MPs contained in the biodeposits,  
432 these present results are comparable to the results published by Revel *et al.* (2020). Oysters tended to  
433 quickly excrete MPs (Graham *et al.*, 2019). In a recent study (Thomas *et al.*, 2020), MPs were detected  
434 in tissues and biodeposits. Oysters could excrete MPs in faeces through cleaning processes, preventing  
435 the gills from being clogged by particles (Jorgensen, 1981) and rejecting indigestible particles (Wegner  
436 *et al.*, 2012). Our results indicate low amounts of MPs in faeces. Although this suggests that oysters  
437 might excrete MPs through feces, a small fraction of these microparticles might persist in the tissues or  
438 be re-ingested by filtration, potentially leading to longer-term effects (Farrell & Nelson, 2013; Ribeiro  
439 *et al.*, 2017).

440

#### 441 *4.3 Oysters state of health*

442 In the present study, MP exposure of adult oysters did not affect their growth, but their survival. The  
443 highest dose of exposure (10 mg. L<sup>-1</sup> MP) led to a significant increase of mortality rate. Sussarellu *et al.*  
444 (2016) obtained similar results, showing that oysters exposed to PS-MPs had disrupted energy flows,  
445 metabolism and homeostasis, increasing the mortality rate. Browne *et al.* (2013) reported in *Arenicola*  
446 *marina*, accumulation of nonylphenol and triclosan from PVC that led to altered physiological stress,  
447 immune system responses and increasing mortality. Stabilisation of mortality were noted at 35 and 44  
448 days for oysters exposed to respectively 0.1 and 10 mg. L<sup>-1</sup> MP. The evolution in cumulative mortality  
449 of exposed individuals may suggest an adaptation to MP exposure (Guo *et al.*, 2015). However, it  
450 remains complicated to conclude on the lethal effects of microplastics without setting up exclusion  
451 analyzes for other factors (*i.e.* bacteria, nutrition, toxic algae, etc.). In addition, an impact on fertilisation  
452 success was noted. The oysters exposed to MPs, having reached the stage of sexual maturation, had  
453 more males than females, unlike the control oysters. In addition, the quality of female gametes was  
454 poorer in adults exposed to MPs than in control. A previous study revealed that marine medaka (*Oryzias*  
455 *melastigma*) exposed to PS-MPs faced sex-specific endocrine disruption. Wang *et al.* (2019) suggested

456 that PS-MPs had sex-specific reproductive disruption and decreased sex hormone levels in female  
457 plasma by inhibiting their synthesis. Sussarellu *et al.* (2016) indicated that the quality of gametes  
458 decreased following parental exposure to PS-MPs. This work reached similar conclusions on the quality  
459 of gametes from exposed parents.

460

#### 461 *4.4 Biomarkers activities*

462 The activities of biomarkers (SOD, GST, MDA and LAC) that can be modulated by environmental  
463 pollutants were used as defense markers, providing information on the level of exposure and the  
464 response capacities of oysters exposed to cocktail of MPs. Oxidative stress biomarkers were chosen for  
465 their early stress responses in bivalves (Valavanidis *et al.* 2006). Milinkovitch *et al.* (2015) confirmed  
466 that SOD was an early indicator of oxidative stress. In their study, Revel *et al.* (2019) indicated that the  
467 SOD activity in the digestive gland of blue mussels (*Mytilus edulis*) exposed for 10 days to a mixture of  
468 MPs (PE, PP) was not affected. Our results show a decrease in SOD over time and with increasing  
469 gonadal maturity in all conditions tested, including condition control. Similar effects have been  
470 demonstrated in *C. gigas* with a decrease in SOD due to seasonal variations (Luna-Acosta *et al.*, 2010b).  
471 In contrast to this study, Ribeiro *et al.* (2017) revealed that the SOD activity in the digestive gland of  
472 *Scrobicularia plana* exposed to PS-MPs increased at the end of the exposure period.

473 GST is involved both in the biotransformation of xenobiotics (Dos Santos *et al.*, 2021) but also in the  
474 recycling of reduced glutathione and therefore in the fight against oxidants (Fabioux *et al.*, 2015). This  
475 phenomenon could be a reaction to an increased xenobiotic biotransformation, as underlined by  
476 Breitwieser *et al.* (2016). Excessive oxidative stress can lead to a significant increase in oxidative  
477 damage (Araújo *et al.*, 2016), potentially due to the depletion of organisms due to the activation of  
478 defense mechanisms to fight against the polluted environment. Concerning the GST enzyme, also linked  
479 to oxidative stress, an increase in this enzymatic activity was noted in freshwater clams *Corbicula*  
480 *fluminea* exposed to PS-NP (Li *et al.*, 2020). GSTs are key players in the removal of reactive oxygen  
481 species (ROS), intervening indirectly by controlling the level of reduced glutathione.

482 If the balance is upset in favor of ROS, oxidative stress is generated due to environmental stress and  
483 xenobiotics could influence this balance by catalyzing the production of ROS, and altering the metabolic

484 balance (Donaghy *et al.*, 2015; Manduzio *et al.*, 2015). In addition, Wang *et al.* (2019) noted an  
485 increased GST activity in the intestine and gills of the marine medaka (*Oryzias melastigma*) exposed to  
486 20 and 200 µg PS-MP. L<sup>-1</sup>, concentrations similar to this study.

487 The MDA content reflects the rate and intensity of lipid peroxidation (Qiao *et al.*, 2019a, b). In the 0.1  
488 mg. L<sup>-1</sup> MP condition at T<sub>3</sub>, the MDA content increased. The increase in MDA content, decreased the  
489 antioxidant protection and immune modulation (Pan *et al.*, 2009; Breitwieser *et al.*, 2016). Indeed,  
490 Sussarellu *et al.* (2016) and Palais *et al.* (2012) suggested that oysters exposed to particles can modify  
491 their energy flow and induced antioxidant and detoxification processes.

492 Laccase (LAC) is a marker of environmental stress (Luna-Acosta *et al.*, 2010a). In the two exposure  
493 conditions (0.1 and 10 mg. L<sup>-1</sup> MP) at T<sub>3</sub>, the LAC activity increased over time. The environmental study  
494 conducted by Breitwieser *et al.* (2020) correlated an increase of the LAC activity in *Mimachlamys varia*  
495 over time in presence of metals (Cu, Cr, Mn, Sn, and As). Bivalves are able to detect pollutants in their  
496 environment and respond to stress by increasing their LAC activity (Luna-Acosta *et al.*, 2015). At T<sub>3</sub> –  
497 the last sampling time – LAC and MDA biomarkers were significantly increased in comparison to  
498 control group. Exposure to MPs should be investigated on a long-term basis to understand adaptive  
499 responses or toxic effects on bivalves. Understanding mechanisms triggered by organisms to fight  
500 against chronic exposure to MPs is key to conduct risk assessments. It is crucial as coastal areas are  
501 constantly impacted by plastics (Santana *et al.*, 2018). The main limitations of biomarker measurements  
502 for assessing the quality of ecosystems are thus the difficulty in discriminating between “natural”  
503 adaptive responses and responses to chemical stress, in extrapolating responses from one scale of  
504 biological organization to another (cell-individual-population-community) and from one species to  
505 another (Forbes *et al.*, 2006). In addition, the use of biomarkers such as SOD and GST lead to increased  
506 sensitivity in the results which could create bias in the observed effects.

507

#### 508 *4.5 Intergenerational effects on D-larvae*

509 The present study also focused on the offspring obtained from adult oysters exposed to MPs over a two-  
510 month period during gametogenesis (Enríquez-Díaz *et al.*, 2009). The larval growth was slower in the  
511 MPs-exposed groups in comparison to the control group. This result is consistent with those obtained



512 by Sussarellu *et al.* (2016) and Bringer *et al.* (2020b), suggesting that MPs exposure can delay *C. gigas*  
513 larval growth. In this work, the exposure conditions revealed a significant increase in developmental  
514 abnormalities, including malformations and developmental arrest. Thus, when parents were exposed to  
515 high MP concentrations, developmental abnormalities were more frequent in their offspring. A lot  
516 studies highlighted that micro-pollutants such as heavy metals and pesticides, caused developmental  
517 defects in oysters (Mai *et al.*, 2012; Gamain *et al.*, 2016, 2017; Bringer *et al.*, 2021b). Tallec *et al.* (2018)  
518 reported greater toxicity of smaller plastic particles. In comparison with PS-MPs (2  $\mu\text{m}$ ), PS-NPs (500  
519 and 50 nm) had greater impacts on the fertilisation success and embryo-larval development of oyster *C.*  
520 *gigas*. Our results revealed a shift in the swimming behaviour of larvae from parents exposed to MPs.  
521 Indeed, circular trajectories increased, in comparison to rectilinear trajectory. Gamain *et al.* (2019)  
522 demonstrated that, under optimal developmental conditions, *C. gigas* D-larvae tended to have a  
523 rectilinear swimming behaviour (2-D analysis). In a previous study, Bringer *et al.* (2020a) noted that  
524 circular trajectories were significantly increased in larvae exposed to HDPE-MPs (4-13  $\mu\text{m}$ ). For both  
525 MP concentrations, a decrease in maximum speed recorded was noted in 24- and 72-hour D-larvae.  
526 These results are consistent with previous studies (Bringer *et al.*, 2020a & b) in which D-larvae exposed  
527 to MPs (HDPE and polymer cocktail) showed a decrease in maximum swimming speed. Yin *et al.*  
528 (2019) indicated that black rockfish *Sebastes schlegelii* exposed to MPs (0.19 mg. L<sup>-1</sup> PS-MP of 15  $\mu\text{m}$ )  
529 had a restricted swimming speed and range of motion, affecting their hunting behaviour and exploration  
530 skill.

531 Altogether our data reinforced the hypothesis that MPs impacted both the physiology and behaviours of  
532 oysters *C. gigas*. This study relied on the use of secondary MPs – aged in the marine environment – as  
533 well as on plastic materials from oyster farming.

534

## 535 **5. Conclusions and perspectives**

536 To conclude, this experiment showed that the cocktail of MPs obtained from oyster material and aged  
537 in the marine environment can be toxic for adult oysters and the larval generation. The results indicated  
538 that exposure to MPs could affect survival, responses to defense biomarkers, and fertilization success,  
539 in adults (*C. gigas*) exposed for 2-months. D-larvae obtained from exposed oysters, having reached a

540 mature stage, exhibited alterations in swimming behavior, development and growth. Bad weather and  
541 degradation of plastics from mariculture could therefore represent a possible threat to oyster farming  
542 and surrounding marine life. In the future, it would be useful to study the life cycle of aquaculture  
543 plastics and the potential risk of additives present on plastics on the *Crassostrea gigas* study model and  
544 the health of marine ecosystems in general.

545

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