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## Subchronic exposure to high-density polyethylene microplastics alone or in combination with chlortoluron significantly affected valve activity and daily growth of the Pacific oyster, *Crassostrea gigas*

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1 Subchronic exposure to high-density polyethylene microplastics alone or in combination  
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3

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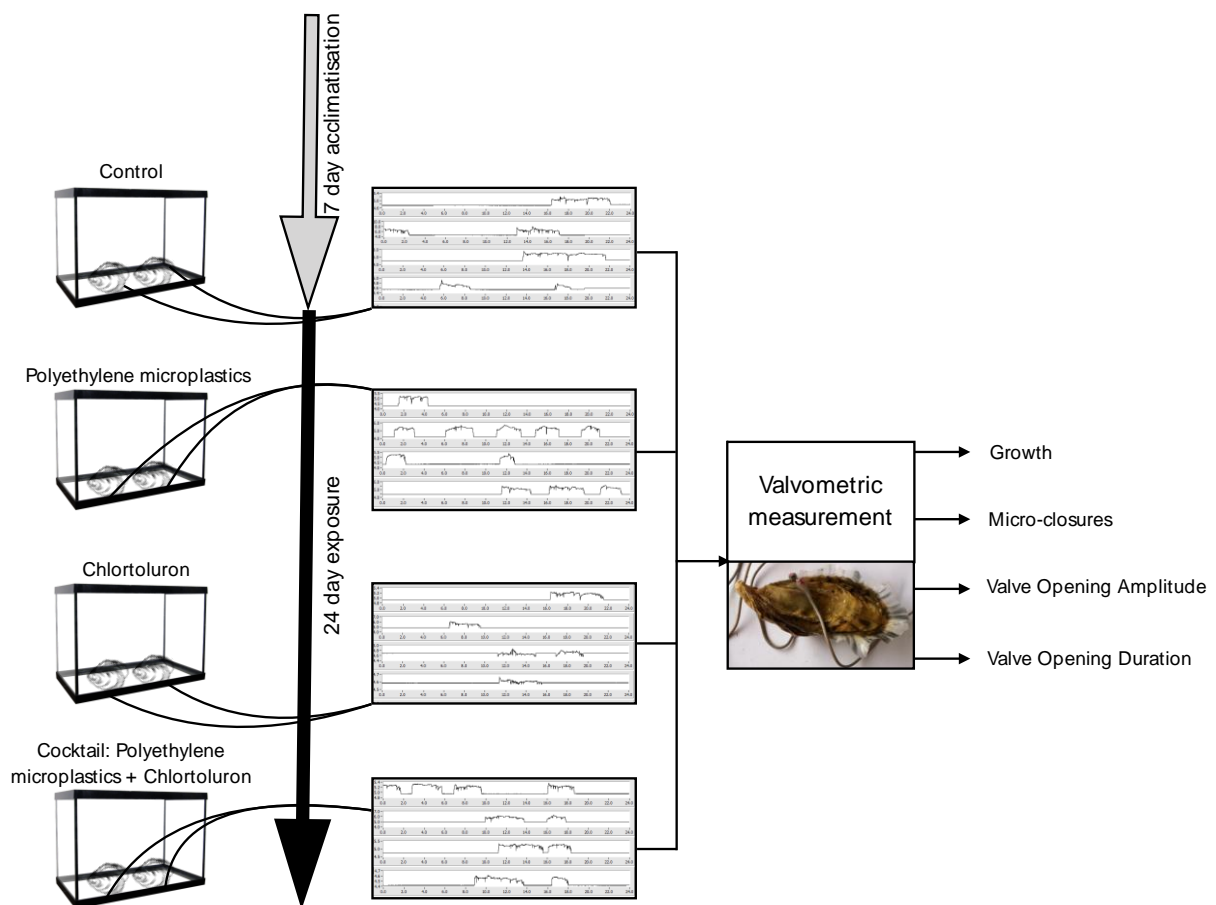
## 14 **Abstract**

15 Nowadays, pesticides and microplastics (MPs) are commonly found in coastal waters worldwide. Due  
16 to their widespread use, their persistence and toxicity, they may induce adverse effects on  
17 physiology and behaviour of marine organisms such as the Pacific oyster (*Crassostrea gigas*). This  
18 study explored the growth and valve activity of juvenile oysters exposed for 24 days to two  
19 frequently detected pollutants in the Pertuis Charentais (South-West France): an herbicide  
20 (chlortoluron, 30  $\mu\text{g.L}^{-1}$ ) and high-density polyethylene microparticles (HDPE 20-25  $\mu\text{m}$ , 10  $\mu\text{g MP.L}^{-1}$ )  
21 alone or in combinaison. The characterisation of the valve activity of juvenile oysters, recorded by  
22 using a High Frequency and Non-Invasive valvometer (HFNI) depended on three parameters: the  
23 number of valve micro-closures (VMC), the Valve Opening Amplitude (VOA) and the Valve Opening  
24 Duration (VOD). Additionally, daily shell growth and the oyster daily rhythm were assessed. The  
25 exposure to MPs of oysters led to a significant, increase of VMC and a decrease of VOD and shell  
26 growth. The exposure to chlortoluron showed a significant, increase of VOA and a decrease of VMC.

27 In mixture with MPs, chlortoluron still increased VOA and decreased VMC but also reduced the shell  
28 growth. Chronobiological analysis did not reveal any effects on the daily rhythm of both  
29 contaminants. High experimental concentrations of MP and chlortoluron were tested. This work has  
30 underlined their selective impacts on the behavioral and physiological parameters studied.

31

## 32 Graphical abstract



33

34

35 **Keywords** Bivalve molluscs, Experimental biomonitoring, Microplastics, Pesticide, Valve behaviour,  
36 Growth.

37

## 38 1. Introduction

39 With a production of 135,000 tons of bivalves in 2017, including 81,000 tons of oysters, France, is the  
40 2<sup>nd</sup> most important producer of bivalves in Europe (France Agrimer, 2020). Shellfish farming turnover  
41 was estimated at 774 million euros in 2019 (CNC, 2020). The Pertuis Charentais area (South-West,  
42 Atlantic coast, France) represents 23 % of the national oyster production (CNC, 2016). Since 2010,  
43 juvenile and adult oysters are facing excess mortality rates, reaching 70 to 80 % of oyster farms  
44 located in the Atlantic coast (Soletchnik *et al.*, 2018 ; Lucasson, 2018 and Girard & Agundez, 2014).  
45 The quality of the coastal waters, where bivalves are grown, is a source of concern today. The  
46 increased use of plastics (Phuong, 2018) in aquaculture generates MPs pollution, which could affect  
47 physiology of bivalves (*e.g.* growth, immune status, reproduction) (Sussarellu *et al.*, 2016; Paul-Pont  
48 *et al.*, 2018; Gardon *et al.*, 2020). Scientific studies are working to offer more and more toxicological  
49 tests on emerging contaminants such as MPs and pesticides, in order to better understand the  
50 modes of action of these pollutants (Wang *et al.*, 2020; Bhagat *et al.*, 2020).

51 Plastics are widely used polymers, which came as a revolution in the past decades (Xu *et al.*, 2017). In  
52 2019, nearly 370 million tons of plastics were produced in the world and in Europe, plastics  
53 production almost reached 58 million tons (PlasticsEurope, 2020). In Europe, 80 % of the plastic  
54 production resorts to six main polymers (PlasticsEurope, 2016): polypropylene (PP), high and low-  
55 density polyethylene (HDPE and LDPE), polyvinyl chloride (PVC), polyurethane (PUR), polyethylene  
56 terephthalate (PET) and polystyrene (PS). It is estimated that rivers carry between 70 % and 80 % of  
57 plastic waste, most of which ends up in oceans (Horton *et al.*, 2017). Through wave activities, UV  
58 degradation and physical abrasion, larger plastic pieces in the marine environment are fragmented,  
59 resulting in producing MPs and nanoplastics (NPs) (Born & Schüttrumpf, 2019; Díaz-Mendoza *et al.*,  
60 2020). MPs are plastic particles ranging from 1  $\mu\text{m}$  to 5 mm (Cole *et al.*, 2011; Wagner *et al.*, 2014).  
61 MPs ingestion was observed in numerous aquatic species, notably in filter-feeding bivalves at  
62 different life stages (Rist *et al.*, 2019; Ward *et al.*, 2019; Betsill *et al.*, 2019; Bringer *et al.*, 2020b). It  
63 can cause several harmful effects at the physiological and tissue levels, affecting the growth and  
64 reproduction of bivalves (Lee *et al.*, 2013; Sussarellu *et al.*, 2016; Lo and Chan, 2018), as well as

65 physical injuries (Gall & Thompson, 2015) and reduced eating behavior (Cole *et al.*, 2015). At the  
66 individual level, this can lead to the depletion of energy reserves (Wright *et al.*, 2013 and Xu *et al.*,  
67 2017), larval development leading to delays and malformations (Tallec *et al.*, 2018), and swimming  
68 activity (Bringer *et al.*, 2020a). Toxic effects are mainly observed using high laboratory exposures  
69 (and not *in situ*) compared to environmental concentrations reported in the literature (Paul-Pont *et*  
70 *al.*, 2018). The environmental concentrations mainly consider size classes of MP > 333  $\mu\text{m}$  (Song *et*  
71 *al.*, 2015; Zhao *et al.*, 2014; Baldwin *et al.*, 2016) and therefore contain a significant bias, with respect  
72 to the small sizes used in the laboratory. Environmental concentrations ranging from 0.002 to 0.5 mg  
73 MP. L<sup>-1</sup> have been detected in surface water in different areas of the world (Eriksen *et al.*, 2013;  
74 Enders *et al.*, 2015).

75 Chlortoluron that belongs to substituted urea family is one of the most commonly used herbicides in  
76 wheat and barley crops. Chlortoluron inhibits photosynthesis and affects phytoplankton (Valiente  
77 Moro *et al.*, 2012). In 2017, a maximum value of 0.18  $\mu\text{g.L}^{-1}$  was detected at the mouth of a river  
78 (surface water) flowing into the Pertuis Charentais (Leonard, 2017). A concentration of 0.14  $\mu\text{g.L}^{-1}$  of  
79 chlortoluron was detected in 2018, at the mouth of the Charente (Pertuis Charentais), close to oyster  
80 farms (Action program, 2018). The herbicide family has already been studied on oysters, showing  
81 effects at the early stages of development on survival and development (Mottier *et al.*, 2013; Gamain  
82 *et al.*, 2017; Behrens *et al.*, 2016; Mai *et al.*, 2013), during gametogenesis with genotoxic effects  
83 (Akcha *et al.*, 2020), as well as by modulating molecular and biochemical parameters (Lee *et al.*,  
84 2017).

85 The valve activity on the oyster was measured using an HFNI (High Frequency Non-Invasive)  
86 valvometer biosensor (Andrade *et al.*, 2016). This biosensor has made possible to monitor  
87 behavioural responses of bivalves when exposed to stress events, such as, contaminants. Assessing  
88 the valve activity behaviour through an HFNI valvometer has already been used to study  
89 contaminants, including traces of metals (Tran *et al.*, 2004a, 2007), radionuclides (Tran *et al.*, 2004b,  
90 2005) and harmful algae (Tran *et al.*, 2010, 2015; Haberkorn *et al.*, 2011). To our knowledge, no

91 valvometric behavioral study, under laboratory conditions, has been carried out to assess the  
92 consequences of exposure to pesticides and MPs. However, in recent years, several articles have  
93 underlined the relevance of modelling and analysing the behaviour of marine animals under  
94 experimental conditions mimicking environmental conditions (Mat *et al.*, 2014; Ahmed *et al.*, 2015).  
95 In the present study, under controlled conditions and to characterize the behaviour of the oyster  
96 valve, three parameters were recorded: VMC, VOA and VOD. In addition, a chronobiological analysis  
97 of the daily rhythm of the valve was carried out as well as a measurement of the daily growth. The  
98 aim of this study was to better understand the potentially disrupting effects of emerging  
99 contaminants (MPs and pesticides) alone or in cocktail on the daily growth and the valve activity of  
100 Pacific juveniles oysters (*C. gigas*).

101

## 102 **2. Material and Methods**

### 103 *2.1. Individuals and acclimatization*

104

105 Pacific oysters, *Crassostrea gigas* (Bayne *et al.*, 2019; Bayne *et al.*, 2017), were kindly provided by a  
106 hatchery (™France Naissain, France). This experiment was conducted on 12-month-old juvenile  
107 oysters ( $59.5 \pm 1.1$  mm,  $22.6 \pm 1$  g total mass) during autumn season (after the period of  
108 gametogenesis and fertilization). Four homogenous groups of diploid oysters ( $n = 8/\text{condition}$ ) were  
109 chosen. Each group was distributed in a 37.5 L tank. Each tank was continuously filled with  
110 oxygenated seawater coming from a large buffer tank. For the experiment, oysters were acclimated  
111 for 7 days in UV-treated running ( $78.3 \text{ L}\cdot\text{h}^{-1}$ ) seawater before being exposed to pollutants for 24 days.  
112 Seawater was taken from the Pertuis Breton (South-West, France), which is directly connected to the  
113 experimental area, and filtered using a 50  $\mu\text{m}$  and 10  $\mu\text{m}$  membrane in order to eliminate debris.  
114 Filtered seawater (FSW) was used for experimental exposure tanks. The FSW temperature in tanks  
115 was of  $16.8 \pm 0.4$  °C and the salinity of  $35.7 \pm 0.2$  ‰. The temperature value and salinity were daily  
116 measured. Throughout the experiment, nitrates, nitrites, chlorine and pH ( $7.3 \pm 1.0$ ) were regularly

117 measured to ensure consistency. A continuous bubbling and a brewing system were set up.  
118 Throughout the duration of the experiment, a natural photoperiod was established. In addition, the  
119 oysters were nourished thanks to the natural presence of phytoplankton in the seawater used.  
120 During the acclimatization and experimental periods, no mortality events were recorded.  
121 Nevertheless, failures of the valvometric signal recording, due to an electrical leak at electrodes level  
122 during the study, reduced the number of oysters studied to respectively  $n = 7$  in the control,  $n = 6$  in  
123 the chlortoluron,  $n = 8$  in the MP and  $n = 5$  under cocktail conditions.

124

## 125 *2.2 Experimental procedure*

126

127 MPs were used (<sup>™</sup>HDPE 20-25  $\mu\text{m}$  MPP-1241, density 0.96, Micropowders Inc. USA) and chlortoluron  
128 (<sup>™</sup>Pestanal, analytical standard, 45400-250MG-R reference) was purchased from Sigma-Aldrich  
129 Chemical (St. Quentin Fallavier, France). The solutions containing MPs and chlortoluron were  
130 prepared beforehand in test glass tubes. Stock solutions, prepared in pure Mili-Q-water, showed a  
131 MPs concentration of  $10 \text{ mg MP.L}^{-1}$  and a chlortoluron concentration of  $3 \text{ mg.L}^{-1}$ . They were kept cool  
132 in the dark in order to prevent bacterial growth. The stored solutions were then diluted in FSW to  
133 lower the concentrations to  $10 \mu\text{g MP.L}^{-1}$  and  $30 \mu\text{g.L}^{-1}$  respectively. After the 7-day acclimatization  
134 period, the pollutants are added to the experimental system, in the buffer tanks. Every two days, 30  
135 % of the FSW in the buffer tank was renewed, for the well-health of the oysters. Upstream, FSW  
136 were stored in separate tanks, with the tested pollutants, at a temperature similar to that of tanks  
137 containing oysters. They were used to supply the buffer tanks during the renewal of FSW. Water  
138 stirrers have been installed in each buffer tanks in order to homogenize the plastic microparticles as  
139 much as possible in the seawater. In addition, the cytometric analyzes made it possible to determine  
140 a certain homogeneity in the exposure tanks. Polystyrene foams protected tanks and equipment to  
141 reduce vibrations from electrical equipment. In addition, capacitance chambers were installed to

142 reduce vibrations from the air pipes bringing oxygen into FSW, along with behavioral analyzes (Fig.  
143 S1).

144

### 145 *2.3 HFNI valvometry analysis*

146

147 To assess the pollutant impact (MPs and chlortoluron) on the oyster's valve behaviour, the valve  
148 activity was recorded using a High Frequency Non-Invasive (HFNI) valvometer. The oysters were  
149 equipped with light electromagnetic electrodes coated in resin ( $\approx 1$  g), which were glued at the same  
150 spot on both shells. The electrodes were attached to free covered stranded copper wires (diameter:  
151 0.98 mm, length: 120 cm), sending data to a device. The induced voltage, measured by biosensors,  
152 varied according to the distance between the electromagnetic electrodes. For each oyster, a  
153 measure was taken every 4.8 sec., *i.e.* 18 000 data/oyster/day. For more details, see Tran *et al.*  
154 (2015) and Andrade *et al.* (2016). Bivalve behaviour was monitored 24/24 hours and 7/7 days (Tran  
155 *et al.*, 2004a and Haberkorn *et al.*, 2011). The biosensor used consists of very sensitive electrodes,  
156 resulting in minimal experimental constraints. The valve activity was recorded non-stop, both for the  
157 7-day acclimatisation and the 24-day experimental periods. Then, the data were analysed using the  
158 LabView 8.6 software (<sup>TM</sup>National Instruments, Austin, TX, USA).

159

160 Three behavioural parameters were assessed: daily and hourly VMC, VOA and VOD. Micro-closures  
161 (VMC) designate partial and rapid closures followed by a re-opening of the valves. A VOA equal to  
162 100 % and 0 % meant that the valves were opened at maximal and minimal amplitudes, respectively,  
163 during the studied duration. Finally, A VOD equal to 100 % and 0 % meant that the valves were  
164 opened or closed, respectively, during the studied duration. Mean daily VMC, VOA and VOD were the  
165 average results of the hourly values recorded for each oyster. The HFNI biosensor enabled to  
166 individually measure the daily growth of shells by calculating the minimal distance between the  
167 electrodes when the oyster valves were closed. For instance, in bivalve molluscs, calcification



168 appears at the shell internal surface. Thus, the cumulative daily shell length growth was calculated as  
169 indicated: minimal distance between electrodes (day (n) – day (1)) x 100 % (n/24), n representing the  
170 number of the day. To obtain the actual shell length growth, manual measurements (digital caliper, ±  
171 0.1 mm) were performed at the start ( $D_0$ ) and at the end ( $D_{24}$ ) of the experiment. The difference in  
172 mm equals 100 % growth. Growth rates have been measured using the HFNI valvometer based on  
173 the fact that calcification in bivalves occurs in the mantle cavity, all over the shell's internal structure  
174 (Figure 4). When daily growth layers are deposited, the minimum distance between the electrodes  
175 glued to the shells increases providing a good proxy of growth (Schwartzmann *et al.*, 2011; Berge *et*  
176 *al.*, 2015).

177 To study the daily biological rhythm ( $\tau = 24$  h) of the valve activity, a statistical chronobiological  
178 analysis was conducted using the software Time Series Analysis Seriel Cosinor 6.3  
179 (<http://www.euroestech.com>). Several steps were required to validate a significant biological rhythm  
180 (Gouthiere *et al.*, 2005). The first step included verifying the data quality of biological and physical  
181 phenomena. The absence of random distribution in data was assessed using an autocorrelation  
182 diagram, whereas the absence of a stationary phenomenon was calculated with a partial  
183 autocorrelation function (Box *et al.*, 2015). The second step involved searching significant periodicity  
184 ( $p = 0.95$ ), using the Lomb and Scargle periodogram (Scargle, 1982). The third step added a modelling  
185 and statistical validation using the Cosinor model, which is a cosine function included in a regression  
186 model (Nelson *et al.*, 1979). Two tests validated the model and, thus, the existence of a biological  
187 rhythm. The elliptic test (Bingham *et al.*, 1982) had to be rejected, leading to a probability of null  
188 amplitude hypothesis of  $< 0.05$ . When conditions were met, a chronobiometric parameter computed  
189 the Percent Rhythm (PR, %), which is the percentage of cyclic behaviour determined by the Cosinor  
190 model.

191

## 192 *2.4 Contaminant analysis*

193

194 Chlortoluron and MPs concentrations were measured twice: on day 5 ( $D_5$ , 5 days after the start of  
195 the experiment) and day 24 ( $D_{24}$ , the last day of the experiment). Water samples ( $V = 100$   
196 mL/replicate with  $n = 3$  replicates) and oyster tissues ( $n = 7$ /condition) exposed to both chlortoluron  
197 and the cocktail solution were assayed using the QuEChERS method (Quick Easy Cheap Efficient  
198 Rugged and Safe, Anastassiades *et al.*, 2003) and the SBSE analysis (Stir Bar Sorptive Extraction,  
199 Baltussen *et al.*, 1999) (Pfannkoch *et al.*, 2010 and from the AOAC 2007.01 method of Lehotay,  
200 2007).

201 To analyse the oyster tissue and determine chlortoluron concentrations, 3 g of oyster tissue was  
202 spiked with an internal standard solution. Milli-Q water was added to the solution to reach 15 g.  
203 After being vortexed once, samples were added with 15 ml of acetonitrile (Pestnorm, VWR  
204 Chemicals), before being vortexed a second time (1 min, 160 rpm). Then, 6 g of magnesium sulphate  
205 (anhydrous, BioReagent, Sigma-Aldrich) and 1.5 g of sodium acetate (anhydrous, >99 %, Sigma-  
206 Aldrich) were added to the samples, which were then vortexed again (10 min, 250 rpm) and  
207 centrifuged (5 min, 4700 rpm, 5 °C). Two phases were formed and supernatant containing the  
208 organic phase with acetonitrile was sampled. Then, 10 mL of the supernatant were collected in the  
209 previous step and were taken and placed in a 125 mL flask, where 40 ml of sodium carbonate (0.1 M,  
210  $\geq 99.7$  %, Sigma-Aldrich) was added. A Twister (magnetic stir bar coated with polydiméthylsiloxane)  
211 was inserted into the bottle. SBSE extraction was performed (16 h, 700 rpm), in the dark). After the  
212 completion of the extraction, the bars were rinsed off with Milli-Q water before being dried on a  
213 fabric.

214 To analyse the chlortoluron seawater exposure samples, 10 mL of methanol (Pestnorm, VWR  
215 Chemicals) was put in a bottle containing the internal standard solution. 100 mL of seawater and a  
216 Twister were then added. Protected from light, the solution was stirred for 16 hours at 700 rpm.  
217 Chlortoluron was quantified by gas chromatography (HP 7890N equipped with a Combipal MPS2  
218 multifunction injection system, 300 °C, Gerstel, Switzerland) and mass spectrometry (GC/MS),  
219 coupled with a TDU (Thermal Desorption Unit, Gerstel: 50 °C (0.5 min) to 280 °C (6 min) at 15 °C/min)

220 and a temperature-programmed injector (Cooled Injection System, Gerstel: -10 °C (0.05 min) to 300  
221 °C (10 min) at 12 °C/s). The temperature was programmed from 70 °C (5 min) to 150 °C increasing by  
222 20 °C/min, then to 320 °C (5 min) increasing by 7 °C/min. The carrier gas, helium, was at constant  
223 flow rate (1 mL/min). The capillary column was a 5-ms RXi (Restek, Bellefonte, USA): 30m x 0.25mm  
224 ID x 0.25µm (film thickness) column. Through tandem mass spectrometry (Agilent 7000 Triple Quad),  
225 a chromatograph and a detector were coupled together. The quantitative analysis of chlortoluron  
226 was performed in MRM mode by internal calibration. Two transitions for each compound were set  
227 up: the first to quantify the molecules and the second to confirm their nature. The second step  
228 defined the nature by calculating the qualifier/quantifier ratio and comparing them to the reference  
229 values of pure compounds. The acquisition frequency of each fragment was of 2 cycles/s.

230

231 Due to the small sizes of particles (20-25 µm), the MPs analysis in oyster tissues was not performed.  
232 According to the protocol described in Bringer *et al.* (2020a & 2020b), flow cytometer (Attune  
233 Acoustic Focusing Cytometer) enabled a quantitative analysis of MPs in the 10 µg MP.L<sup>-1</sup> condition. 2  
234 mL samples were prepared with the MPs solution (n=8/condition). The samples were vortexed  
235 (StarLab Vortex IR, 12,000 rpm for 20 s) before being transferred into the cytometer to homogenise  
236 the solution. 300 mL were taken to carry out a flow cytometry analysis. Calibration was conducted to  
237 reach an analysis rate of 500 mL.min<sup>-1</sup> and a saturation of maximum 10,000 particles detected. The  
238 blank obtained enabled to remove background particles (naturally present in seawater). Using  
239 seawater filtered at 0.2 µm, a first calibration step was conducted to select the < 200-time detected  
240 particles.

241

## 242 2.5 Statistics

243

244 All data were expressed as means ± standard error of the mean (SEM). Treatment differences were  
245 determined using a one-way analysis of variance (ANOVA), when both criteria of homogeneity of

246 variance (Levene's test) and normality of distribution (Shapiro-Wilk) were met. When these  
247 requirements were not met, a statistical analysis was performed using the non-parametric Kruskal-  
248 Wallis test, followed by the Nemenyi Post-hoc test for pair comparisons. Statistical comparisons were  
249 made on the means of each parameter according to the exposure conditions. Significant difference  
250 was accepted when  $p$ -value  $< 0.05$ . The statistical analysis was conducted using R.

251

### 252 **3. Results**

#### 253 *3.1 Pollutant exposure*

254

255 It is noted, that no significant difference was observed concerning the assays carried out for  
256 chlortoluron (FSW and total tissues), nor for MP (FSW). Chlortoluron concentrations were  
257 determined in exposure waters (Table 1.a). Under control, water contamination increased over time.  
258 On  $D_{24}$  (condition chlortoluron vs cocktail), an increase in the concentration of chlortoluron was  
259 noted. The concentration of chlortoluron in the cocktail condition was slightly higher than for the  
260 condition of chlortoluron alone (at  $D_5$  and  $D_{24}$ ). In both cases, the measured concentrations were well  
261 above the theoretical concentration of  $30 \mu\text{g.L}^{-1}$ . The concentrations of chlortoluron measured on the  
262 soft body of the oyster are shown in Table 1.a. No trace of herbicide was detected in control  
263 individuals. While in exposed individuals, tissue concentrations of chlortoluron were measured at  
264 both sampling times  $D_5$  and  $D_{24}$ .

265

266 The concentrations of MP in FSW (Table 1.b) were above the desired theoretical concentration of 10  
267  $\mu\text{g of MP.L}^{-1}$ . The approximate actual concentrations calculated in this study were determined using  
268 additional data from a previous study (Bringer *et al.*, 2020a), which used and tested the same  
269 commercial MP (HDPE, 20-25  $\mu\text{m}$ ), at different concentrations. The additional data allowed for  
270 calibration and validation of the current results. The concentrations of MP were stable over time ( $D_5$

271 and D<sub>24</sub>) and similar between the exposure conditions (MP alone vs cocktail). In addition, an analysis  
 272 of the particles in the control FSW was performed and no MP particles were detected.

273

274 **Table 1. (a)** Theoretical and measured chlortoluron (herbicide) concentrations in oyster tissues (dry weight)  
 275 and seawater for three conditions: control, chlortoluron and cocktail (MPs + chlortoluron). Values are mean ±  
 276 SEM for n=7/condition for oyster tissues and n=3/condition for water samples. **(b)** Theoretical, measured MPs  
 277 and approximate concentrations concentrations in three conditions: control (n=12), MPs (n=8) and cocktail  
 278 (MPs + chlortoluron, n=8). Values are mean ± SEM.

279 **a**

Conditions	Samples time	Theoretical ( $\mu\text{g.L}^{-1}$ )	Oyster tissues ( $\text{ng.g}^{-1}$ )	Water exposure ( $\mu\text{g.L}^{-1}$ )
control	Day 5	0	0	0.06 ± 0.0
	Day 24	0	0	1.5 ± 0.9
chlortoluron	Day 5	30	226.9 ± 69.5	58.3 ± 25.1
	Day 24	30	229.5 ± 92.0	85.5 ± 17.0
cocktail	Day 5	30	366.0 ± 132.7	74.6 ± 4.9
	Day 24	30	242.3 ± 199.6	96.6 ± 25.8

280 **b**

Conditions	Samples time	Theoretical ( $\mu\text{g MP.L}^{-1}$ )	Measured (MP.mL <sup>-1</sup> ) 20-25 $\mu\text{m}$ size	Approximate concentrations ( $\mu\text{g MP.L}^{-1}$ )
control	Day 5	0	0	0
	Day 24	0	0	0
MPs	Day 5	10	93.9 ± 31.2	47
	Day 24	10	111.7 ± 11.5	56
cocktail	Day 5	10	124.4 ± 30.3	60
	Day 24	10	107.6 ± 49.3	54

281

## 282 3.2 Valve activity of oysters

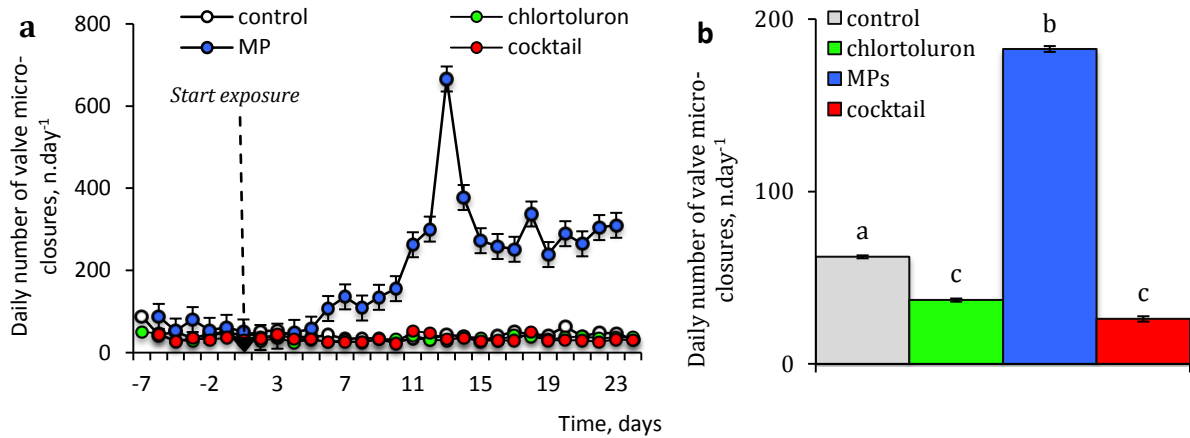
### 283 3.2.1 Valve Micro-Closures (VMC)

284

285 The number of VMC per day and per individual is shown on Fig. 1a. Compared to the other  
 286 conditions, the daily VMC in MPs over the 24-day experiment highlighted a significant increase ( $p <$   
 287 0.001). The mean VMC for MPs was 2.9 times higher than for the control condition (Fig. 1b). Fig. 1b  
 288 clearly showed a significant decrease in the chlortoluron and cocktail VMC, compared to the control

289 condition VMC. The mean VMC for the conditions of exposure to chlortoluron and cocktail were  
290 respectively 40.3 % and 58 % lower compared to the control condition.

291



292

293 **Fig. 1. (a)** Daily VMC showing the 7-day acclimatisation and the 24-day exposure periods. **(b)** Histograms of  
294 mean VMC  $\pm$  SEM for the 24-day exposure period. The different conditions are: control (white,  $n = 7$ ),  
295 chlortoluron alone (green,  $n = 6$ ), MPs alone (blue,  $n = 8$ ) and cocktail (red,  $n = 5$ ). Different letters indicate  
296 significant differences between concentrations ( $p < 0.05$ ).

297

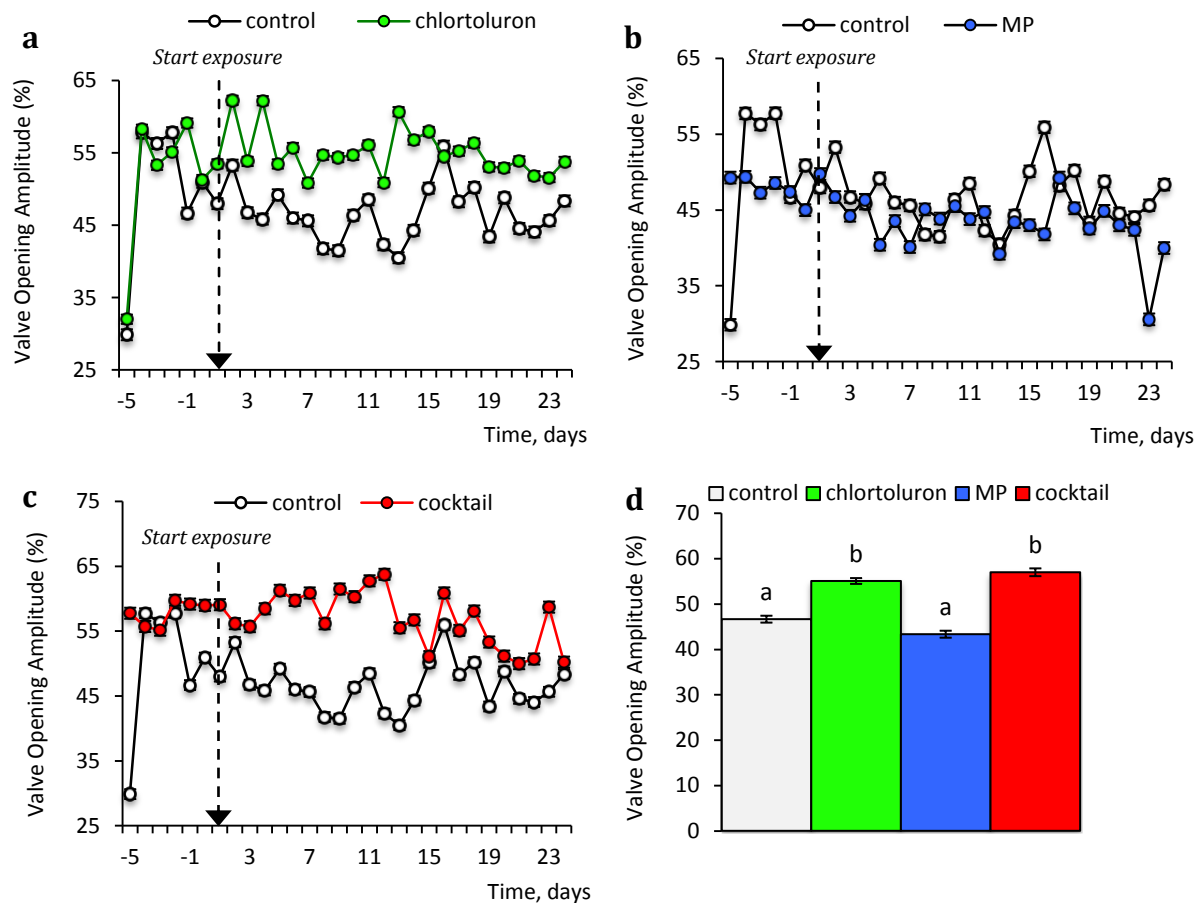
### 298 3.2.2 Valve Opening Amplitude (VOA)

299

300 In terms of VOA, the 24-day experiment underlined contrasting results between the MPs condition  
301 and the two other conditions containing herbicides (Fig. 2a, b, c). Although the VOA was similar for  
302 the MPs and control conditions (with no significant differences), it was significantly higher in  
303 chlortoluron and cocktail conditions compared to control (both  $p < 0.001$ )

304 Over the entire experiment, the mean VOA displayed major differences between the four conditions  
305 ( $p < 0.001$ ). Compared to the control condition, a significant increase in mean VOA of 18.2 % and 22.3  
306 % were respectively noted for the chlortoluron alone and cocktail conditions (Fig. 2d).

307



308

309

310 **Fig. 2.** Valve Opening Amplitude (VOA) in percentage for each condition: **(a)** chlortoluron alone (green), **(b)** MPs  
 311 alone (blue) and **(c)** cocktail (red), in comparison with the control condition (white). **(d)** Histograms of VOA (%)  
 312 for the entire exposure and all conditions. Values are mean  $\pm$  SEM for control ( $n = 7$ ), chlortoluron ( $n = 6$ ), MPs  
 313 ( $n = 8$ ) and cocktail ( $n = 5$ ) conditions. Different letters indicate significant differences between concentrations  
 314 ( $p < 0.05$ ).

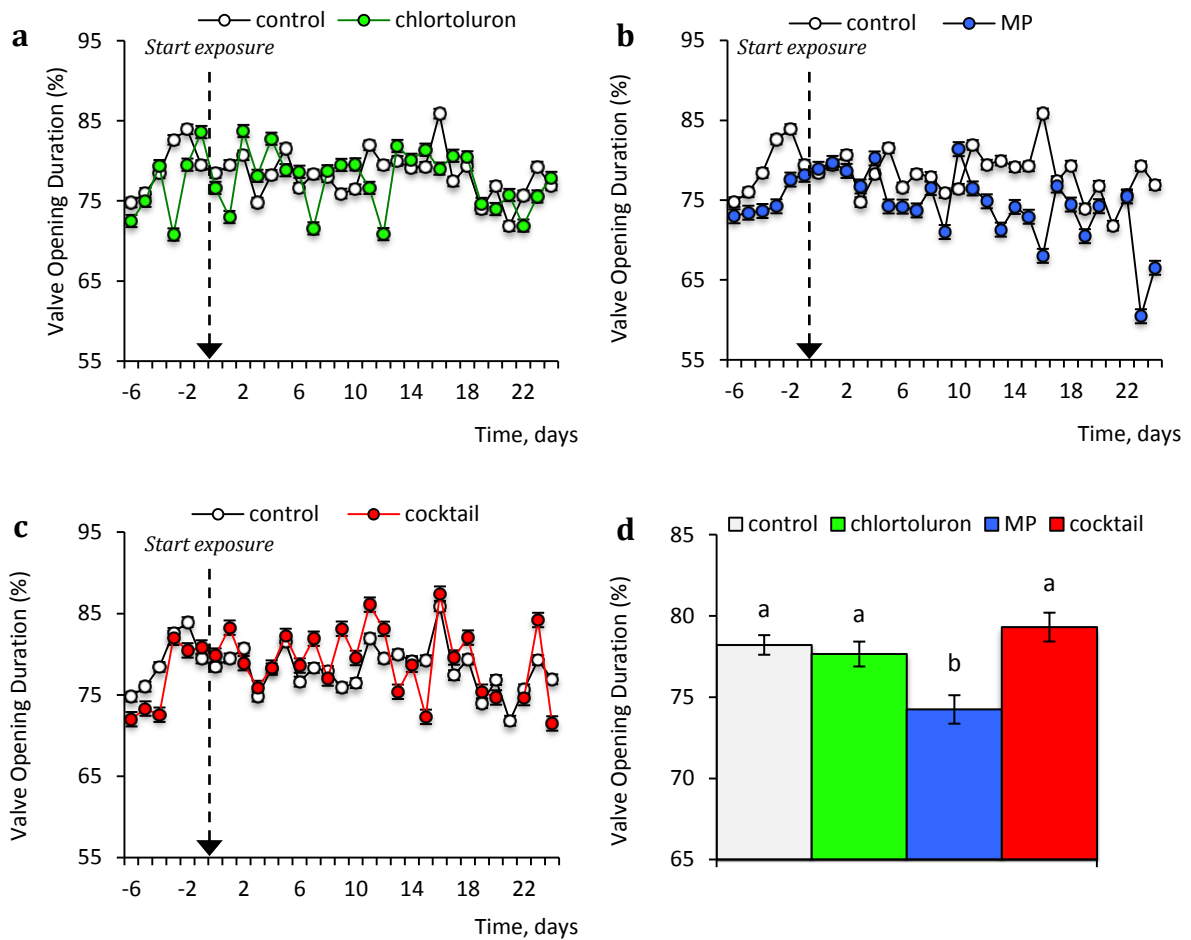
315

### 316 3.2.3 Valve Opening Duration (VOD)

317

318 Over the 24-day experiment (Fig. 3 a, b, c), the VOD for both the chlortoluron and cocktail conditions  
 319 remained unchanged. In contrast, the VOD for the MPs condition significantly decreased compared  
 320 to the control condition. Indeed, over the entire experiment, the mean VOD highlighted a significant  
 321 decrease ( $p < 0.001$ ) of 5.1 % for the MPs condition, compared to the control one (Fig. 3d).

322



323

324

325 **Fig. 3.** Valve Opening Duration (VOD) in percentage for each condition: **(a)** chlortoluron alone (green), **(b)** MPs  
 326 alone (blue) and **(c)** cocktail (red), in comparison with the control condition (white). **(d)** Histograms of VOA (%)  
 327 for the entire exposure and all conditions. Values are mean  $\pm$  SEM for control ( $n = 7$ ), chlortoluron ( $n = 6$ ), MPs  
 328 ( $n = 8$ ) and cocktail ( $n = 5$ ) conditions. Different letters indicate significant differences between concentrations  
 329 ( $p < 0.05$ ).

330

### 331 3.2.4 Chronobiological analysis

332

333 Through a statistical chronobiological approach, a daily rhythm analysis was performed using the  
 334 Cosinor method. When comparing the three conditions to the control one, no significant disruption  
 335 of VOA rhythm was observed. Figure S2 displays for each condition, the spectral analysis of the valve  
 336 activity (Lomb and Scargle periodogram). It highlighted a significant periodicity in the valve  
 337 behaviour. The daily mean VOA for all conditions were:  $\tau = 24.0 \pm 0.1$  (control);  $23.8 \pm 0.1$



338 (chlortoluron);  $23.9 \pm 0.1$  (MPs);  $23.9 \pm 0.1$  h (cocktail). Periodicities were observed using the Cosinor  
 339 model, which validated the presence of a daily rhythm for all conditions ( $p < 0.0001$  for all  
 340 conditions). The percent rhythms (PR) between conditions were quite close, except for chlortoluron,  
 341 which had a lower PR. PR were 46.4 % (control), 32.3 % (chlortoluron), 42.8 % (MPs) and 45 %  
 342 (cocktail).

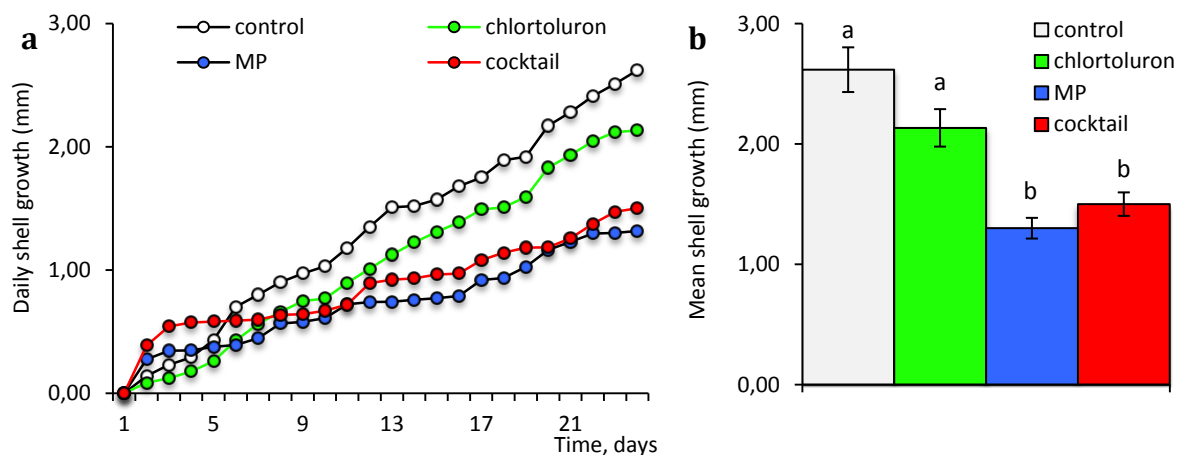
343

### 344 3.2.5 Shell Growth

345

346 The Fig. 4a displays the cumulative daily shell growth, monitored for 24 days. No significant  
 347 difference was calculated between the control and chlortoluron conditions ( $p = 0.07$ ) but this p-value  
 348 is close to the statistical significance and, therefore express a trend. In contrast, over 24 days, oysters  
 349 exposed to the MPs and cocktail conditions underlined a lower shell growth. Indeed, after the 24-day  
 350 period, the mean shell length growth for the MPs and cocktail conditions respectively was reduced of  
 351 50.4 % ( $p < 0.0001$ ) and 42.7 % ( $p = 0.021$ ), in comparison with the control one (Fig. 4b). The oyster  
 352 shell growth observed for the MPs and cocktail conditions were also lower than observed for the  
 353 exposure to chlortoluron alone.

354



355

356 **Fig. 4. (a)** Daily shell length growth monitored for all conditions (mm). **(b)** After the completion of the 24-day

357 experiment, histograms displaying the mean shell growth (mm) of the oysters equipped with electrodes. The

358 different conditions are: control (no pollutants, white), chlortoluron alone (green), MPs alone (blue) and  
359 cocktail (red). Values are mean  $\pm$  SEM for control (n = 7), chlortoluron (n = 6), MPs (n = 8) and cocktail (n = 5)  
360 conditions. Different letters indicate significant differences between concentrations ( $p < 0.05$ ).

361

### 362 *3.2.6 Summary of the main results*

363

364 The actual concentrations measured on D<sub>24</sub>, in the FSW exposure (for MP and chlortoluron alone and  
365 cocktail), are presented in Table 2, as well as in the remainder of this present study. In our work, the  
366 analysis validated the existence of a daily rhythm for all tested conditions. No significant differences  
367 in chronobiological rhythms were noted between the four conditions tested. Regarding the  
368 behavioural parameters, our results suggest that, when exposed to chlortoluron, the oyster valve  
369 activity was significantly disrupted (Table 2). Indeed, in comparison with the control condition, a  
370 decrease in the number of VMC and an increase in VOA were noted. In terms of MPs exposure, a  
371 significant increase in VMC, and a decrease in VOD and growth were observed. Concerning the  
372 cocktail condition, a decrease in VMC and growth, and an increase in VOA were highlighted.  
373 Depending on the exposure conditions, the valve behaviour in oysters responded differently.

374

375 **Table 2.** Summary of the effects on the Pacific oyster valve activity induced after a 24-day exposure to  
376 chlortoluron alone (85  $\mu\text{g}\cdot\text{L}^{-1}$ ), 20-25  $\mu\text{m}$  HDPE MPs alone (112 MP.  $\text{mL}^{-1}$ ) and a combination of both (cocktail,  
377 97  $\mu\text{g}\cdot\text{L}^{-1}$  of chlortoluron + 108 MP.  $\text{mL}^{-1}$ ). Measured parameters are VMC (Valve Micro-Closures), VOA (Valve  
378 Opening Amplitude), VOD (Valve Opening Duration), daily rhythm (chronobiological analysis) and shell growth (  
379  $\uparrow$  : significant increase compared to the control condition,  $\downarrow$  : significant decrease compared to the control  
380 condition and - : absence of significant effects compared to the control condition).

381

Conditions	VMC	VOA	VOD	Daily rhythm	Shell growth
Chlortoluron alone	↓	↑	-	-	-
MPs alone	↑	-	↓	-	↓
Cocktail	↓	↑	-	-	↓

382

## 383 4. Discussion

### 384 4.1 Experimental concentrations versus natural environment

385

386 In our FSW exposures, concentrations between 58 and 96  $\mu\text{g.L}^{-1}$  were measured The study by  
387 Valiente Moro *et al.* (2012) reported an  $\text{IC}_{50}$  (half-maximum inhibitory concentration) of 50  $\mu\text{g.L}^{-1}$  on  
388 the freshwater phytoplankton *Ankistrodesmus fusiformis*. Although this concentration is higher than  
389 those commonly found in the Pertuis Charentais, it is quite close to the maximum concentrations  
390 detected in some European rivers. Indeed, concentrations of herbicide (chlortoluron in particular) of  
391 20  $\mu\text{g.L}^{-1}$  and more (Beitz *et al.*, 1994) were determined. For herbicide exposure, control FSW  
392 highlighted a slight contamination to chlortoluron. Being volatile, it could easily evaporate in the  
393 atmosphere and contaminate the other tanks (Ineris, 2013). Nevertheless, the concentrations of  
394 chlortoluron measured in the control FSW samples were satisfactory, to validate the study. An  
395 increase of chlortoluron concentrations was observed between the start ( $D_5$ ) and the end of the  
396 experiment ( $D_{24}$ ). This increase could be explained by the initial presence of chlortoluron in natural  
397 seawater (0.14  $\mu\text{g.L}^{-1}$  in 2018, Action program, 2018) and regular water changes during the  
398 experiment. In this present study, the theoretical concentration of HDPE MPs (20-25  $\mu\text{m}$ ) was set at  
399 10  $\mu\text{g MP.L}^{-1}$ , where other studies have tested these same concentrations (Beiras *et al.*, 2018;  
400 Constant, 2018; Lei *et al.*, 2018 and Dris *et al.*, 2016). To assess MPs/NPs in water, flow cytometry  
401 was performed, as used in some studies (Long *et al.*, 2017; Caputo *et al.*, 2021; Kaile *et al.*, 2020).  
402 Instead of being 10  $\mu\text{g MP.L}^{-1}$ , the average concentration for MPs-contained solutions was  
403 approximately of 50  $\mu\text{g MP.L}^{-1}$  at the two sampling periods. However, the assays by cytometry, we

404 confirm contents between 93.9 and 107.6 MP.mL<sup>-1</sup>. The highest environmental concentration of MP  
405 detected in Artic sea ice cores is around 13.7 MP.mL<sup>-1</sup> (Peeken *et al.*, 2018). However, the size classes  
406 of MP detected in the natural environment are larger than MP tested in the laboratory (Paul-Pont *et*  
407 *al.*, 2018). Our present study could be defined as realistic in terms of size tested but not in terms of  
408 concentration. According to a study on the characterization of MP on the Atlantic coast, the majority  
409 of MP (64 %) were < 40 µm. In addition, about half (42 %) of them were PE (Enders *et al.*, 2015).  
410 These results justify the use of HDPE-MP (20-25 µm) in this study. In addition, some equipment used  
411 in aquaculture for bivalves consists mainly of PE (Lusher *et al.*, 2017 and Bringer *et al.*, 2021). The  
412 differences observed between the theoretical and measured MP concentrations could come from  
413 the aggregation of MP (Alimi *et al.*, 2018; Michels *et al.*, 2018) on the walls of the exposure tanks.  
414 Long *et al.* (2017) clearly showed that micro-PS may attach to glassware, form homo-aggregates and  
415 hetero-aggregates. In addition, regular water changes, for the welfare of oysters, could also have  
416 played a role in increasing theoretical concentrations.

417

#### 418 *4.2 Behavioural responses of juveniles' oysters (C. gigas) to chlortoluron exposure*

419

420 Chlortoluron is a photosynthesis-disrupting herbicide, and more specifically as an inhibitor of  
421 photosystem II (Faggiano *et al.*, 2010). Phytoplankton communities have been shown to be very  
422 sensitive to herbicides (Solomon *et al.*, 1996). Indeed, effects on phytoplankton growth and  
423 development have been observed (El-Sheekh *et al.*, 1994), and could indirectly affect filter feeders  
424 such as oysters when they feed. Chlortoluron showed a significant decrease in VMC in number  
425 compared to condition control. An equivalent result has already been demonstrated for *Mytilus*  
426 *galloprovincialis* exposed to cypermethrin (insecticide) with a reduced the valve closure in a time-  
427 dependent manner (Ayad *et al.*, 2011). Higher VOA, at condition control, was also detected following  
428 exposure to chlortoluron. An apparent increase in the rate of opening of the shell under  
429 contamination with an herbicide (lenacil) was reported in an earlier study (Chmist *et al.*, 2019).

430 However, this parameter has been little studied in order to obtain relevant interpretations (Bae &  
431 Park, 2014). Chlortoluron may have a specific reducing effect on some distinctive phytoplankton  
432 species important for the physiology, development and behaviour of oysters (Stachowski-Haberkorn  
433 *et al.* 2008). Additional studies should be carried out in order to better understand the effect of this  
434 herbicide on phytoplankton populations and on oyster nutrition. **Moreover**, combined effect of VMC  
435 decrease and VOA increased may suggest that the herbicide lead to an apparent relaxed behaviour  
436 of **valve activity**. Next investigations should focus on **putative** neurotoxic effects of chlortoluron on  
437 oyster valve activity. Indeed, it would be of interest to study if the chlortoluron may block conduction  
438 of action potential at the voltage-gated sodium channels level, which plays a crucial role in  
439 membrane excitability in nerve cell, and lead to a decrease of neuro-muscular response. **This**  
440 **neurotoxic effect has already been shown** for many neurotoxins produced by animals or plants, such  
441 as saxitoxin and tetrodotoxin (Boulot *et al.*, 2017).

442

#### 443 *4.3 Behavioural responses of juveniles' oysters (C. gigas) to microplastics exposure*

444

445 Oysters exposed to MP showed a higher number of VMC compared to the others conditions. VMC is  
446 defined as a marker of stress in oysters (Tran *et al.*, 2010). The presence of MP in the seawater,  
447 which could be detected by oysters, can induce mechanical stress (Sow *et al.*, 2011). The presence of  
448 harmful substances could activate chemoreceptors on the edge of the oyster mantle or directly  
449 affect the gills when the pollutants are in direct contact with the tissue of the pallial cavity,  
450 responsible for the rapid increase in the frequency of VMC. According to the work of Castrec *et al.*  
451 (2018), a similar hypothesis could be made, implying that the increase in the frequency of VMC is a  
452 protective behavioral response to accelerate interval water turnover and minimize potential contacts  
453 with MP, irritating the gills (Zhu *et al.*, 2020). A significant decrease in VOD was also measured.  
454 Likewise, a freshwater bivalve (*Corbicula fluminea*) had a behavioral closure reaction - as a protective  
455 strategy - when exposed to a metallic contaminant (Tran *et al.*, 2004a). The branchial cavities and the

456 labial palps (Ward *et al.*, 1998) determine the different modes of selection and sorting of particles,  
457 which could slow down the filtration of nutrients of exposed oysters, affect their feeding activity,  
458 their behaviour, and reduce their metabolism and their growth. Indeed, several studies have noted  
459 that toxins and foreign bodies can modify the nutritional activity of oysters through behavioral  
460 modifications (Tran *et al.*, 2010; Haberkorn *et al.*, 2010). Oysters exposed to MP grow more slowly  
461 than control oysters. Some previous work has shown non-effects of MP on oysters during subchronic  
462 exposure (Green, 2016; Revel *et al.*, 2020). However larger sizes of MP (103  $\mu\text{m}$  and 300  $\mu\text{m}$ ) were  
463 tested contrary to this present study (20-25  $\mu\text{m}$ ). The study by Gardon *et al.* (2018) showed a  
464 decrease in the scope for growth from 25  $\mu\text{g MP.L}^{-1}$  of PS-MP in *P. margaritifera*. These results  
465 indicate that the exposed oysters must have drawn their energy from the reserves. In addition, the  
466 assimilation of microalgae was disturbed by the presence of micro-PS, and the energy provided by  
467 food intake was lower for exposed oysters (Gardon *et al.*, 2018). In the study conducted by Thomas  
468 *et al.* (2020), the condition index at the highest concentration (106  $\text{MP.L}^{-1}$ ) decreased significantly  
469 over time. The concentration tested is similar to our concentrations measured in MP exposure  
470 waters.

471

#### 472 *4.4 Effects of co-contamination by microplastics and chlortoluron on juveniles' oysters (C.* 473 *gigas)*

474

475 For oysters exposed to the cocktail, results similar to the chlortoluron condition were noted, on VMC  
476 and VOA. No effect on VOD was noted in contrast to its decrease for exposure to MP. Chlortoluron  
477 and MP may have antagonistic effects. A previous study demonstrated antagonistic effects between  
478 NP and glyphosate on the microalgae *Microcystis aeruginosa* (Zhang *et al.*, 2018). Combined  
479 exposure to diclofop-methyl and silver nanoparticles has been shown to antagonize the growth  
480 inhibition of *A. Thaliana* (Li *et al.*, 2018). In our study, growth decreased under the cocktail condition  
481 as for the MP condition. Studies have pointed out that MP can make hydrophobic organic chemicals

482 bioavailable by acting as an absorption vector (Horton *et al.*, 2018). However, our results do not  
483 show significantly different effects for the cocktail, compared to MP and therefore we cannot  
484 conclude on a potential vectorization of chlortoluron on MP. The results showed that the synergistic  
485 effect of chlortoluron and MPs is not significant, compared to the contamination conditions alone.  
486 Their common ecotoxic effects are affected by physical and chemical factors such as particle size,  
487 color, composition, function (Yu *et al.*, 2021). Due to their high filtration rate, marine bivalves, like  
488 oysters, are particularly sensitive to chemical contamination, which can be a major source of stress  
489 and behavioral alteration (Sokolova and Lannig, 2008; Islam and Tanaka, 2004).

490

## 491 **5. Conclusions**

492 On a larger scale, valvometric technology is a tool for monitoring water quality, using oysters as  
493 biosensors (Ahmed *et al.*, 2015). This study provided valuable data on the behavior of bivalves  
494 exposed to pollutants potentially present in the marine environment. Exposure to chlortoluron had  
495 little impact on their behavior and none on their growth. This study provided insight into the  
496 evidence that MP could potentially affect the physiology and behavior of *C. gigas*. MPs tended to act  
497 as stressors in bivalves, triggering urgent behavioral responses. In addition, MP could affect the  
498 filtration capacity of bivalves (nutrient system) and, indirectly, their growth. The combination of the  
499 two pollutants showed antagonistic responses to the conditions of contamination alone. Rarely used  
500 in studies, valvometric technology is an innovative, integrated and comprehensive marker that helps  
501 to understand the effects of environmental pollution. Future studies on the effects of MP on bivalves  
502 could use HFNI valvometry, mimicking environmental conditions (*e.g.* size range, concentrations,  
503 pollutant cocktail).

504

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516

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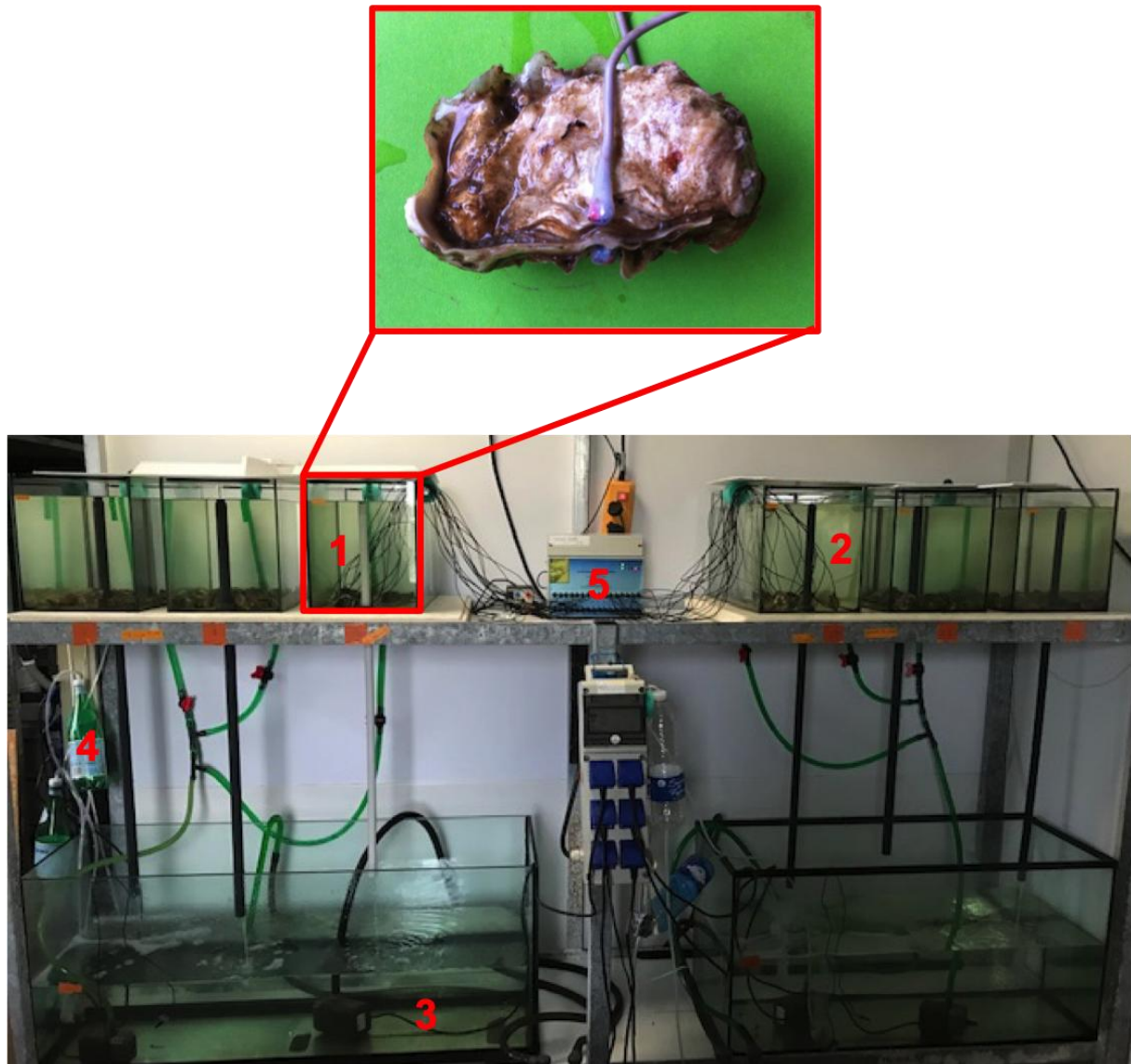
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911 **Supplementary data**

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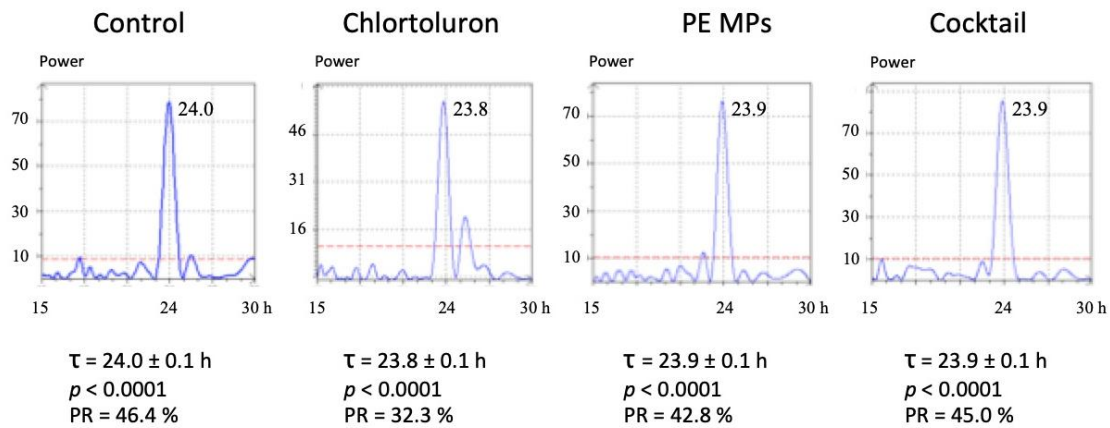
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915 **Fig. S1.** Experimental design located in an experimental laboratory (LIENSs La Rochelle – CNRS). The numbers  
916 correspond to: **(1)** experimental batch of exposed oysters equipped with valvometric electrodes (zoom  
917 picture), **(2)** second experimental batch of exposed oysters, **(3)** buffer tank, **(4)** reduced vibrations thanks to the  
918 capacitance chamber and **(5)** HFNI valvometer (High Frequency Non-Invasive) connected to the electrodes  
919 stuck on oysters.

920



## Chronobiological analysis



921

922 **Fig. S2.** Rhythmic valve behaviour of *C. gigas*. For each condition, the daily periodicity ( $\tau$ ) was determined by

923 spectral analysis (Lomb and Scargle periodogram). The X axis indicates the test duration in order to determine a

924 significant period. The Y axis displays the power (arbitrary units) of the determined period. The red dotted line

925 highlights a significant period for  $p$ -value = 0.95.