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

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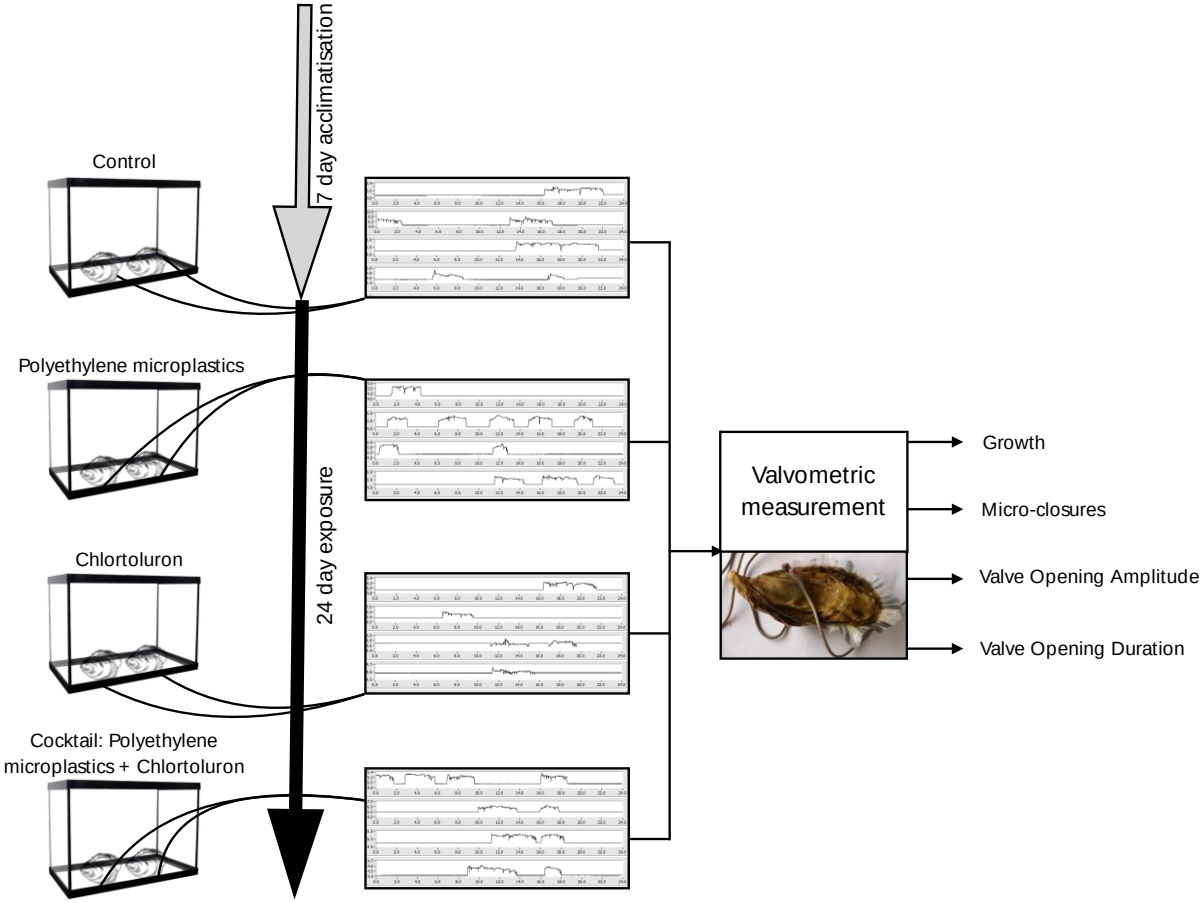
Abstract

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

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

Graphical abstract



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

1. Introduction

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

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

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

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

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

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

2. Material and Methods

2.1. Individuals and acclimatization

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

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

2.2 Experimental procedure

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

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

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

2.5 Statistics

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

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

3. Results

3.1 Pollutant exposure

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

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

and D_{24}) and similar between the exposure conditions (MP alone *vs* cocktail). In addition, an analysis of the particles in the control FSW was performed and no MP particles were detected.

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

a

Conditions	**Samples time**	**Theoretical** ($\mu g.L^{-1}$)	**Oyster tissues** ($ng.g^{-1}$)	**Water exposure** ($\mu g.L^{-1}$)
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

b

Conditions	**Samples time**	**Theoritical** ($\mu g\ MP.L^{-1}$)	**Measured** ($MP.mL^{-1}$) ***20-25 µm size***	**Approximate concentrations** ($\mu g\ MP.L^{-1}$)
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

3.2 Valve activity of oysters

3.2.1 Valve Micro-Closures (VMC)

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

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

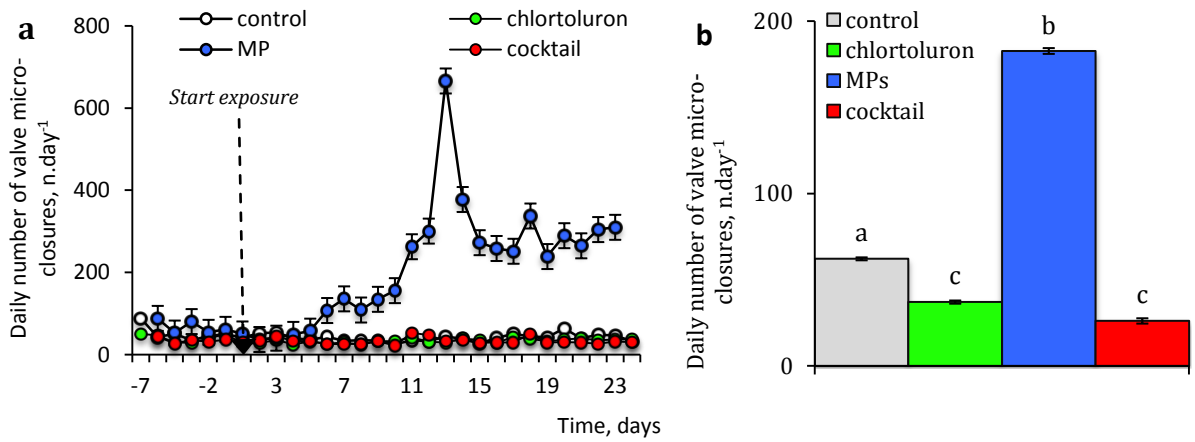


Fig. 1. **(a)** Daily VMC showing the 7-day acclimatisation and the 24-day exposure periods. **(b)** Histograms of mean VCM ± SEM for the 24-day exposure period. The different conditions are: control (white, n = 7), chlortoluron alone (green, n = 6), MPs alone (blue, n = 8) and cocktail (red, n = 5).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

3.2.2 Valve Opening Amplitude (VOA)

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

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

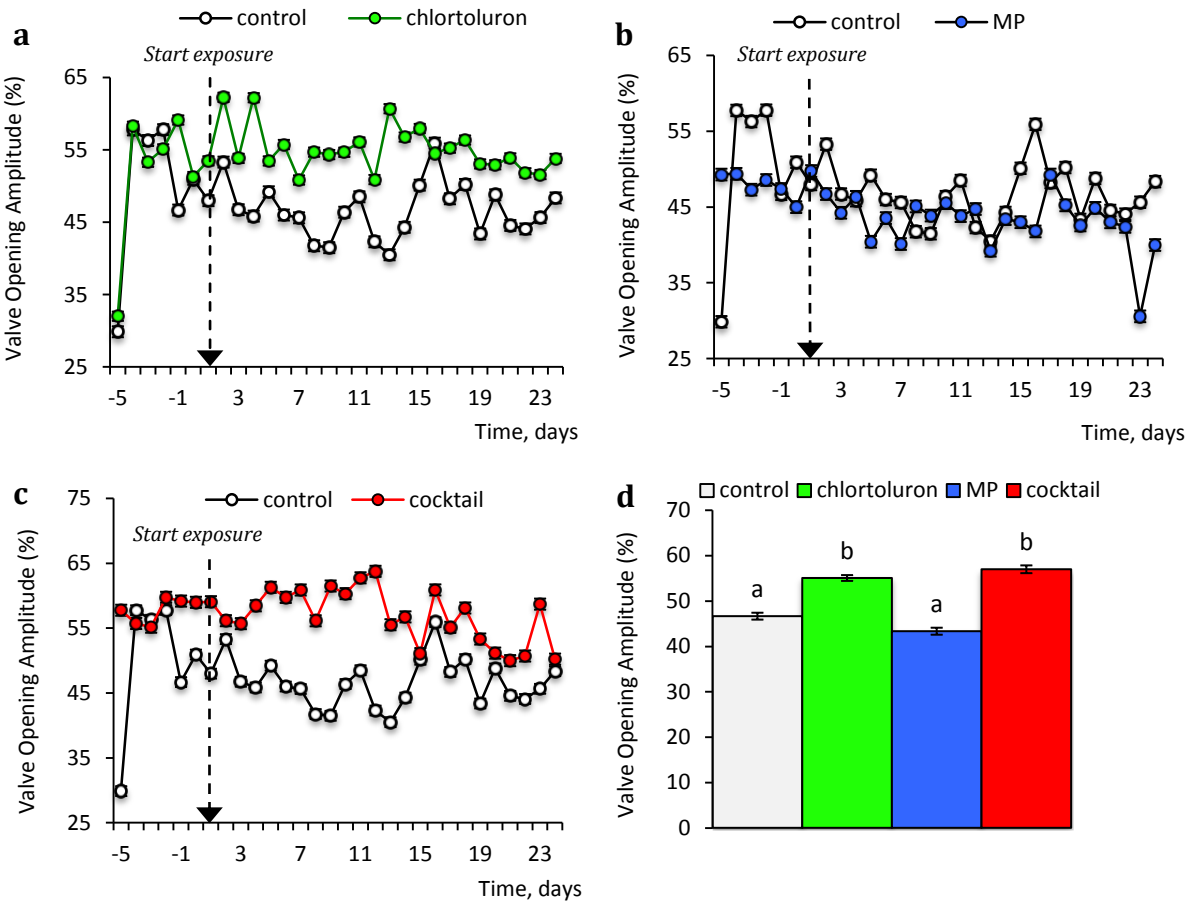


Fig. 2. Valve Opening Amplitude (VOA) in percentage for each condition: **(a)** chlortoluron alone (green), **(b)** MPs alone (blue) and **(c)** cocktail (red), in comparison with the control condition (white). **(d)** Histograms of VOA (%) for the entire exposure and all conditions. Values are mean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

3.2.3 Valve Opening Duration (VOD)

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

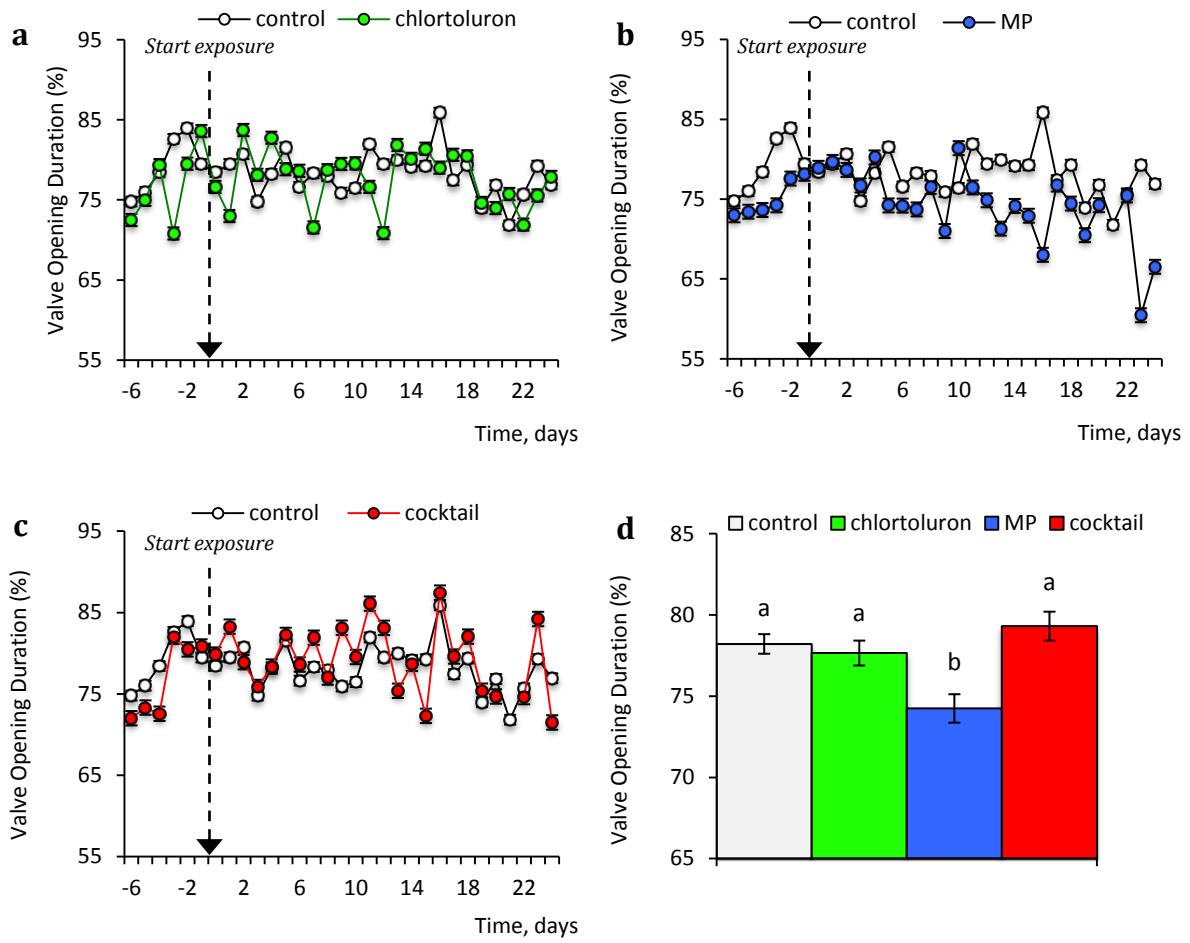


Fig. 3. Valve Opening Duration (VOD) in percentage for each condition: **(a)** chlortoluron alone (green), **(b)** MPs alone (blue) and **(c)** cocktail (red), in comparison with the control condition (white). **(d)** Histograms of VOA (%) for the entire exposure and all conditions. Values are mean ±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

3.2.4 Chronobiological analysis

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

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

3.2.5 Shell Growth

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

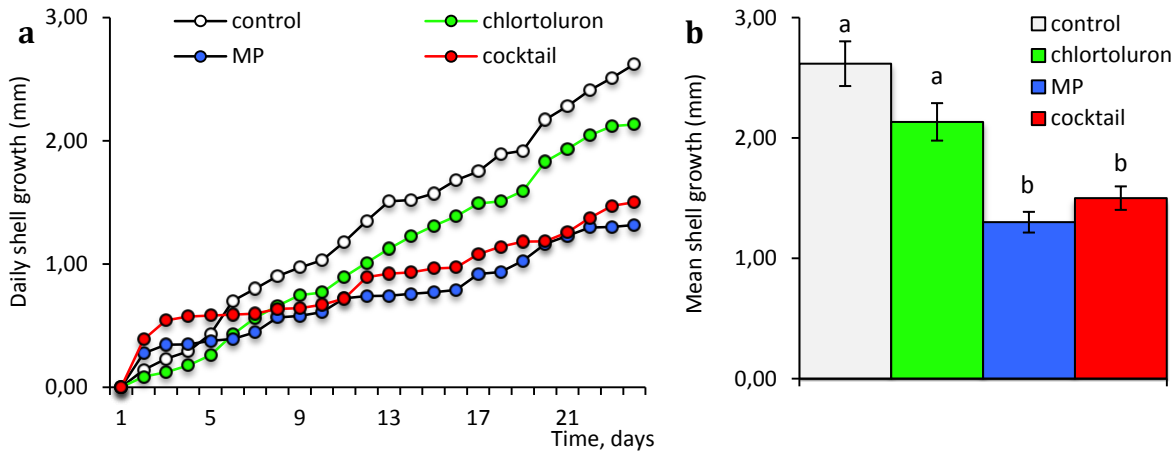


Fig. 4. (a) Daily shell length growth monitored for all conditions (mm). **(b)** After the completion of the 24-day experiment, histograms displaying the mean shell growth (mm) of the oysters equipped with electrodes. The

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

3.2.6 Summary of the main results

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

Table 2. Summary of the effects on the Pacific oyster valve activity induced after a 24-day exposure to chlortoluron alone (85 µg.L^{-1}), 20-25 µm HDPE MPs alone (112 MP. mL^{-1}) and a combination of both (cocktail, 97 µg.L^{-1} of chlortoluron + 108 MP. mL^{-1}). Measured parameters are VMC (Valve Micro-Closures), VOA (Valve Opening Amplitude), VOD (Valve Opening Duration), daily rhythm (chronobiological analysis) and shell growth (↑ : significant increase compared to the control condition, ↓ : significant decrease compared to the control condition and - : absence of significant effects compared to the control condition).

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 µg.L^{-1} were measured The study by
387 Valiente Moro *et al.* (2012) reported an IC_{50} (half-maximum inhibitory concentration) of 50 µg.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 µg.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 µg.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 µm) was set at
399 10 µg 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 µg MP.L^{-1}, the average concentration for MPs-contained solutions was
403 approximately of 50 µg 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^{-1}. The highest environmental concentration of MP
405 detected in Artic sea ice cores is around 13.7 MP.mL^{-1} (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).

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

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

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

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

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

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

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

5. Conclusions

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

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Supplementary data

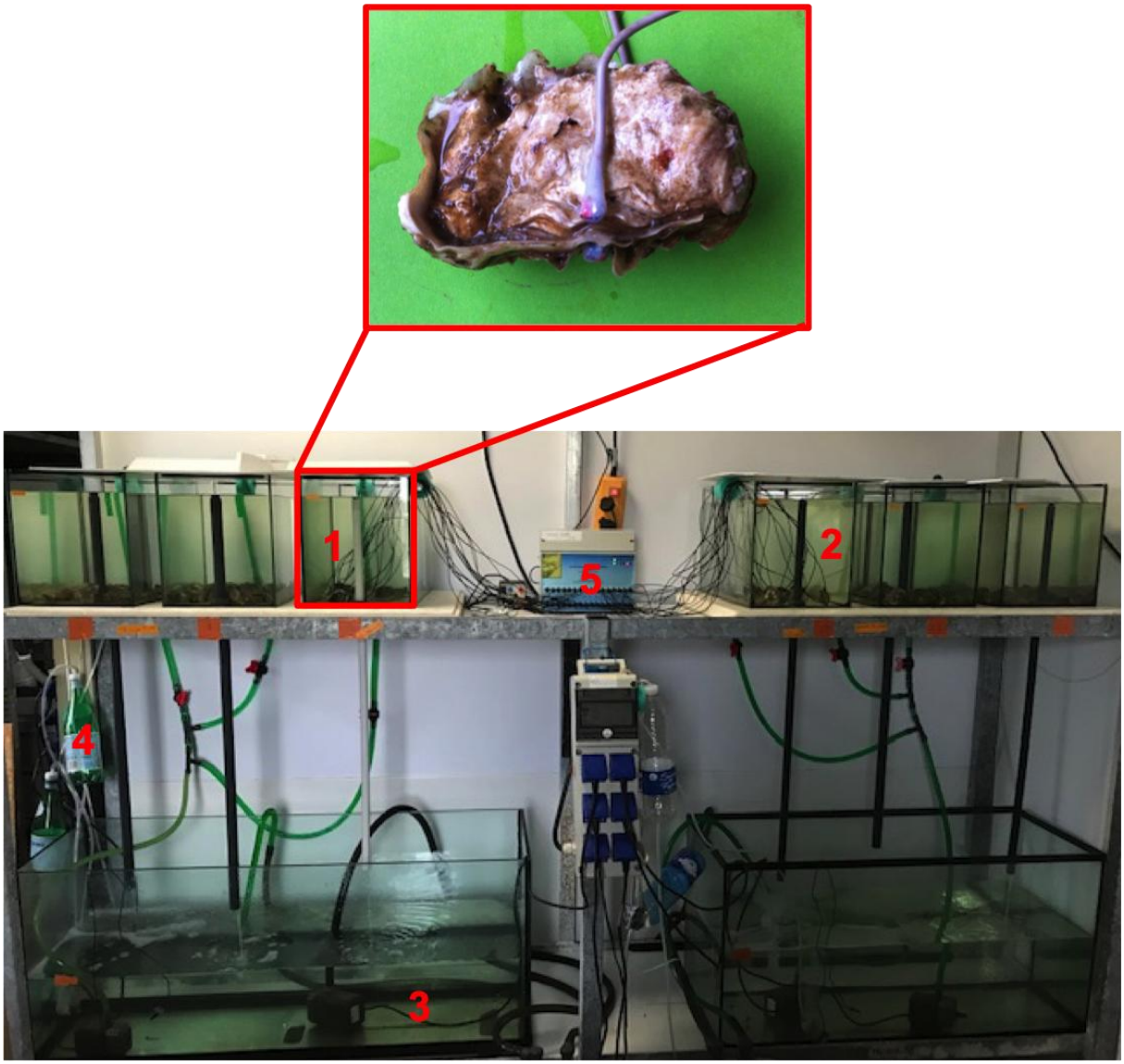

Fig. S1. Experimental design located in an experimental laboratory (LIENSs La Rochelle – CNRS). The numbers correspond to: **(1)** experimental batch of exposed oysters equipped with valvometric electrodes (zoom picture), **(2)** second experimental batch of exposed oysters, **(3)** buffer tank, **(4)**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

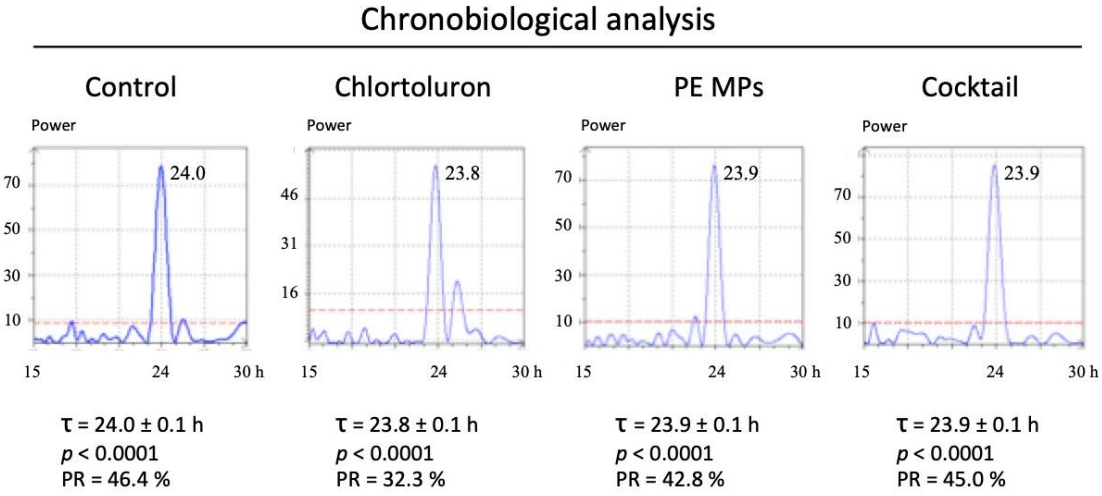

Fig. S2. Rhythmic valve behaviour of *C. gigas*. For each condition, the daily periodicity (τ) was determined by spectral analysis (Lomb and Scargle periodogram). The X axis indicates the test duration in order to determine a significant period. The Y axis displays the power (arbitral units) of the determined period. The red dotted line highlights a significant period for *p*-value = 0.95.