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# 1                    **Microplastic leachates disrupt the chemotactic and** 2                    **chemokinetic behaviours of an ecosystem engineer (*Mytilus edulis*)**

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## 18                    **Abstract**

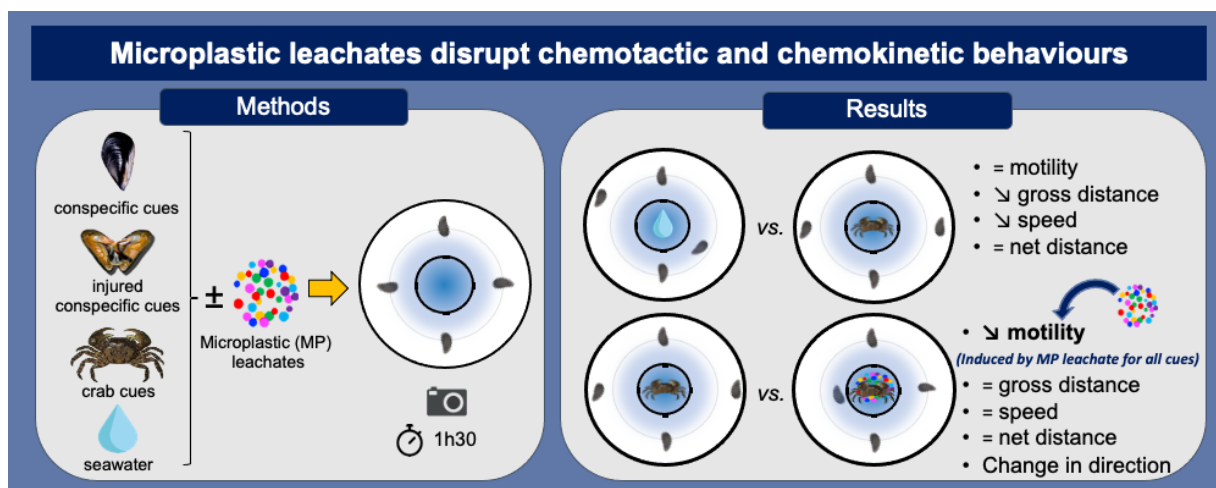
19                    The massive contamination of the environment by plastics is an increasing global  
20                    scientific and societal concern. Knowing whether and how these pollutants affect the behaviour  
21                    of keystone species is essential to identify environmental risks effectively. Here, we focus on  
22                    the effect of plastic leachates on the behavioural response of the common blue mussel *Mytilus*  
23                    *edulis*, an ecosystem engineer responsible for the creation of biogenic structures that modify  
24                    the environment and provide numerous ecosystem functions and services. Specifically, we  
25                    assess the effect of virgin polypropylene beads on mussels' chemotactic (*i.e.* a directional  
26                    movement in response to a chemical stimulus) and chemokinetic (*i.e.* a non-directional change  
27                    in movement properties such as speed, distance travelled or turning frequency in response to a  
28                    chemical stimulus) responses to different chemical cues (*i.e.* conspecifics, injured conspecifics  
29                    and a predator, the crab *Hemigrapsus sanguineus*). In the presence of predator cues, individual  
30                    mussels reduced both their gross distance and speed, changes interpreted here as an avoidance

31 behaviour. When exposed to polypropylene leachates, mussels moved less compared to control  
32 conditions, regardless of the cues tested. Additionally, in presence of crab cues with plastic  
33 leachates, mussels significantly changed the direction of movement suggesting a leachate-  
34 induced loss of their negative chemotaxis response. Taken together, our results indicate that the  
35 behavioural response of *M. edulis* is cue-specific and that its anti-predator behaviour as well as  
36 its mobility are impaired when exposed to microplastic leachates, potentially affecting the  
37 functioning of the ecosystem that the species supports.

### 38 Key-words

39 *Chemical stimulus, Chemodetection, Predators, Blue mussel, Cues, Plastics, Intertidal species*

### 40 Graphical abstract



41

42

## 43 1. Introduction

44 Chemical communication is a key process in ecology that has been extensively studied  
45 over a wide range of organisms, both terrestrial and marine (Zimmer & Butman, 2000;  
46 McClintock & Baker, 2001; Dicke & Takken, 2006; Paul *et al.*, 2006; Ng *et al.*, 2013; Tabata,  
47 2018). Specifically, chemical signals play a key role in intra- and inter-specific interactions  
48 (*e.g.* predation, reproduction, competition, aggregation) and others processes such as habitat  
49 selection, eventually affecting individual fitness, and community and ecosystems dynamics at  
50 larger scales (Murlis *et al.*, 1992; Bradbury & Vahrenkamp, 1998; Dicke & Takken, 2006;  
51 Bornancin *et al.*, 2017; Zimmer & Butman, 2020). Many marine species rely on chemicals to  
52 get information about their environment (Zimmer & Butman, 2000). For instance, the majority  
53 of molluscs possess sensory organs, such as the osphradium that contains chemoreceptors  
54 which allows the detection of *e.g.* food, conspecifics and predators (Lucas, 1931; Morton, 1962;  
55 Lindberg & Sigwart, 2015).

56 Intertidal mussels are considered as key ecosystem engineers on both soft and hard  
57 substrates (Reise, 2002; Borthagaray & Carranza, 2007). Noticeably, these organisms are  
58 motile; they use their foot both to move and attach themselves to their substrate through the  
59 production of byssal threads, extracellular collagenous structure (Schneider *et al.*, 2005; Waite  
60 *et al.*, 2005). Both their motility and byssal production allow them to spatially self-organise into  
61 dense mono- and multi-layered beds, which decrease the vulnerability to predation, desiccation,  
62 heat, and wave dislodgement, while increasing fertilization success and survival (Nicastro *et*  
63 *al.*, 2012; Iwasaki, 2015; Zardi *et al.*, 2021). Through the formation of beds, intertidal mussels  
64 locally dominate rocky shores and operate a biogenic transformation of the habitat (Paine &  
65 Levin, 1981; Menge & Sutherland, 1987; Reise, 2002). These relatively stable and resilient  
66 structures enhance local biodiversity by facilitating the establishment and maintenance of a  
67 range of different species (Reise, 2002; Palomo *et al.*, 2007; Borthagaray & Carranza, 2007;

68 Arribas *et al.*, 2014; Spilmont *et al.*, 2018). Beyond their ecological role, mussels also play an  
69 important economic and heritage value through professional and recreational fishing, *e.g.* global  
70 aquaculture production for *Mytilus edulis* and *M. galloprovincialis* was 287,957 tons in 2016  
71 (FAO Fishstat) with an estimated net worth ranging between US\$2.5 10<sup>6</sup> and US\$100 10<sup>6</sup> in  
72 the United States only (Zippay & Helmuth, 2012).

73 Previous studies have shown that cues from damaged conspecifics and predators of  
74 intertidal mussels can significantly affect their movement and aggregation rate (Côté &  
75 Jelnikar, 1999; Nicastro *et al.*, 2007; Kong *et al.*, 2019), though exceptions exist (Commito *et*  
76 *al.*, 2016; Kong *et al.*, 2019; Manríquez *et al.*, 2021). An exposure to these cues also triggers  
77 morphological and physiological changes such as thicker shell, production and quality of byssal  
78 threads (Leonard *et al.*, 1999; Kong *et al.*, 2019). Intertidal mussels can differentiate chemical  
79 signals from predators and behave accordingly to their specific prey handling techniques, *e.g.*  
80 the mussel *Mytilus edulis* increases byssus production when exposed to crab (*Cancer irroratus*)  
81 cue but not when exposed to starfish cue (*Asterias rubens*; Garner & Litvaitis, 2013). Though  
82 various mussel species share the sensory capacity to perceive, discriminate and adapt to  
83 different environmental chemical signals, the presence and intensity of behavioural responses  
84 to chemical stimuli, in particular in Mytilidae, is fundamentally cue-dependent (Nicastro *et al.*,  
85 2007; Garner & Litvaitis, 2013; Kong *et al.*, 2019; Manríquez *et al.*, 2021).

86 Chemotaxis (*i.e.* a directional change in movement in response to a chemical stimulus)  
87 and chemokinesis (*i.e.* a non-directional change in movement properties such as speed, distance  
88 travelled or turning frequency in response to a chemical stimulus) are key behavioural processes  
89 involved in the response to chemical cues (Bell & Tobin, 1982; Wilkinson, 1985). Only a few  
90 studies have addressed chemotaxis in bivalves and interactions with conspecifics, *i.e.*, De  
91 Vooy (2003) with *M. edulis* and Huang *et al.* (2007) with *Tridacna squamosa*, and between a  
92 bivalve (*Montacuta ferruginosa*) and its commensal species (*Echinocardium cordatum*;

93 Morton, 1962). Specifically, the chemical communication on which an important part of  
94 behaviour is based, such as mate and food searching, as well as danger detection, can be altered  
95 by a range of anthropogenic chemicals (Fleeger *et al.*, 2003; Lürling & Scheffer, 2007; Seuront,  
96 2010, 2011, 2018). This disrupting effect of anthropogenic chemicals on intra and inter-species  
97 chemical communication has, to date, received considerably less attention.

98         This issue is, however, particularly relevant given the growing awareness of the ubiquity  
99 and toxicity of plastic compounds and their leachates in marine systems (Gall & Thompson,  
100 2015; Jamieson *et al.*, 2017; Gunaalan *et al.*, 2020). Plastic pollution is one of the main  
101 challenges of the 21<sup>st</sup> century through their deleterious physical and chemical effects on aquatic  
102 life (Derraik, 2002; Gall & Thompson, 2015; Anderson *et al.*, 2016; Rochman *et al.*, 2016;  
103 Sussarellu *et al.*, 2016; Auta *et al.*, 2017), leading to the death of millions of aquatic organisms  
104 annually (Ocean Conference United Nations, 2017). These effects can be due to either  
105 macroplastic (> 5 mm), microplastic (< 5 mm) and nanoplastic (< 100 nm) items either  
106 produced directly in the smaller form industrially or resulting from the breakdown of larger  
107 plastic items (Cole *et al.*, 2011; Paul-Pont *et al.*, 2018). Plastic pollution has also a far more  
108 pernicious effect on marine life through the release of various molecules that are either absorbed  
109 (i.e. intrinsically bounded) to the polymer during the manufacturing process (e.g. light and heat  
110 stabilizers, antioxidants, nucleating and antistatic agents, flame retardants, plasticizers and  
111 colorants) or adsorbed at the surface of a polymer such as persistent organic pollutants, which  
112 include polycyclic aromatic hydrocarbons, polychlorinated biphenyls, polybrominated  
113 diphenyl ethers, pesticides or heavy metals (Delaeter *et al.*, 2022). Recent studies have shown  
114 that direct exposure to either microplastics (Crump *et al.*, 2020) or microplastic leachates  
115 (Seuront, 2018) can interfere with the cognitive system, sensory perception, and thus behaviour  
116 of marine invertebrates. For instance, the intertidal mussel *M. edulis* behaviourally responds to  
117 microplastic leachates through an increase in aggregation rate and frequency (Seuront *et al.*,

118 2021). However, despite the ever-increasing concern about the ecological impact of plastic  
119 leachates (Gunaalan *et al.*, 2020; Delaeter *et al.*, 2022), it is still unknown whether plastic  
120 leachates interfere with conspecific or prey-predator chemical signals in bivalves in general,  
121 and in intertidal mussels in particular.

122 In this context, we aim to determine whether the chemotactic and chemokinetic  
123 responses in an ecologically and economically important intertidal mussel species are affected  
124 by microplastics leachates. To do so, we first assessed the presence of chemotactic and/or  
125 chemokinetic responses of *M. edulis* to cues from intact and injured conspecifics and the  
126 predatory cues from the invasive Asian shore crab *Hemigrapsus sanguineus*. This species is  
127 invasive along European coast and has been reported in other regions, it was first recorded in  
128 France in 1999 (Breton *et al.*, 2002) and became the dominant mussel predator of the rocky  
129 intertidal shores of the eastern English Channel (Rolet *et al.*, 2020) less than two decades after  
130 its first report from our sampling area, *i.e.* 2006 (Dauvin *et al.*, 2009). Noticeably, given their  
131 short common evolutionary history of *M. edulis* and *H. sanguineus*, our behavioural approach  
132 provides a first step towards the assessment of *M. edulis* naivety status (*i.e.* the ability to  
133 recognize a new predator as a threat). We subsequently inferred how leachates from virgin  
134 polypropylene pellets may alter *M. edulis* chemotactic and chemokinetic responses. We further  
135 discuss how the observed behavioural changes are relevant for the ecology of the species in the  
136 context of increasing anthropogenic pressure on marine coastal ecosystems.

## 137 **2. Material and Methods**

### 138 **2.1. Study organisms**

139 Both individual *Mytilus edulis* (1.5–2.0 cm in shell length; Supplementary Materials,  
140 S1) and *H. sanguineus* (1.4–2.4 cm in length; Supplementary Materials, S1) were sampled in  
141 March 2021 at low tide on a rocky intertidal reef of the infra-littoral zone of the Fort de Croy  
142 (Wimereux, France; 50°45'52.3"N, 1°35'55.1"E) along the French coast of the English

143 Channel.

144 Collected organisms were brought back to the laboratory within an hour. Prior to the  
145 experiments, mussels were acclimatised for 1 week under a natural 12:12 light:dark cycle in  
146 85 L tanks filled with running aerated natural seawater ( $T = 10\text{ }^{\circ}\text{C}$ ,  $S = 33\text{‰}$ ,  $\text{pH} = 8.08$ ) directly  
147 pumped from the collection site, and no additional food was provided. *H. sanguineus* were  
148 sorted by sex, and kept in separate 85 L tanks in which crabs were acclimatised for 1 week and  
149 fed *ad libitum* daily with mussels previously crushed with a natural stone (to avoid the use of  
150 metal objects, hence to prevent metal ions from touching mussel tissue; Commito *et al.*, 2016).  
151 Crabs were kept in darkness during the acclimation to enhance food consumption (Spilmont *et*  
152 *al.*, 2015) and thus stimulate predator-induced alarm response in mussels as described here  
153 below.

## 154 **2.2. Chemical cues**

155 The effects of four natural chemical cues were assessed on the chemotactic and  
156 chemokinetic behaviour of *M. edulis*: (1) natural (*i.e.* control) seawater, (2) conspecific cues,  
157 (3) injured conspecific cues, and (4) fed crab cues. To assess the effect of microplastic leachates  
158 on the abovementioned natural cues, these treatments were subsequently mixed with  
159 microplastic (MP) leachate seawater. Note that the seawater used in all our experiments was  
160 consistently pumped directly from the collection site, hence could contain plastic leachates and  
161 cues naturally occurring at this site. As such, in the absence of additional experimentally-  
162 generated cues, natural seawater was considered as cue less.

163 Conspecific cue seawater was prepared through the addition of 20 mussels which were  
164 placed for a 24 h period into 1 L of aerated seawater. Injured conspecific cue seawater was  
165 prepared through the addition of 20 injured mussels previously crushed with a stone, to 1 L of  
166 aerated seawater for a 24 h period. Crab cue seawater was prepared through the addition of 3  
167 males and 3 females *Hemigrapsus sanguineus* (sex ratio of 1:1) into 1L of aerated seawater for



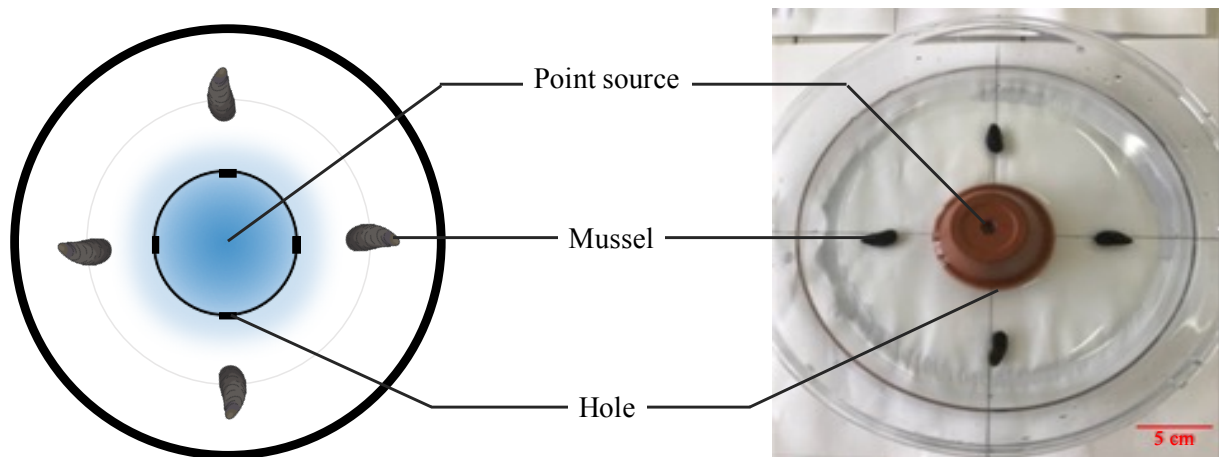
168 a 24h period. For more information about exact sizes, see Supplementary Materials, S1.

169 For the mixed cues experiments, commercially available polypropylene pellets  
170 (typically 3.3–4.7 mm in diameter; Pemmiproducs, Aachen, Germany) were added to the  
171 previous treatment (*i.e.* natural seawater, conspecific, injured conspecific and crab cues). They  
172 were left 24 h in aerated tanks at a concentration of 12 g of pellets per litre (ca. 600 MPs per L,  
173 or equivalently 20mL of MPs per L; Seuront, 2018; Seuront *et al.*, 2021), in order to have four  
174 new solutions: seawater with leachates, conspecifics cues with leachates, injured conspecifics  
175 cues with leachates and crab cues with leachates. As a first approximation of the nature of the  
176 additive content of our polypropylene pellets, we ran a pyrolysis analysis coupled to a gas  
177 chromatography and a high-resolution spectrometer (see Supplementary Materials, S2), which  
178 led to the identification of 2 antioxidants (*i.e.* 4-tert-octylphenol, 4tOP, and Butyl  
179 hydroxytoluene, BHT) and 6 plasticizers (*i.e.* Diethyl phthalate, DEP, Diisobutyl phthalate,  
180 DIBP, Dibutyl phthalate, DBP, Bis(2-ethylhexyl)phthalate, DEHP, Di-n-octyl phthalate, DIOP,  
181 and Diisononyl phthalate, DINP).

### 182 **2.3. Chemotactic and chemokinetic assay**

183 Both acclimatation and experiments were consistently carried out under the same  
184 controlled conditions. Temperature was measured at the beginning and at the end of the  
185 experiment. Before the start of each experiment, byssal threads were carefully cut with scissors  
186 to separate the mussels from each other and from the substrate. Behavioural experiments were  
187 conducted in 22 cm diameter glass arenas with smooth, featureless surfaces (Fig. 1). To infer  
188 the presence of a chemotactic and/or chemokinetic response of the individual mussel to the  
189 different cues, their positions were recorded with respect to a point source. This point source  
190 was obtained by four equidistant holes (0.5 cm in diameter) drilled through the side of a clay  
191 pot (6.5 cm in diameter; Fig. 1) which had a top central hole. Arenas were filled with 600 mL  
192 unfiltered seawater saturated in oxygen. Five milliliters (5 mL) of either control or treatment

193 seawater was injected in a clay pot through the top hole at a rate of  $0.7 \text{ mL s}^{-1}$ . Thus, the  
194 concentration of MP leachates, which is the same for all solutions containing plastic leachates,  
195 that flows out of the clay pot, was estimated at  $1.66 \text{ mL L}^{-1}$  (50 MPs per litre). Once fully mixed  
196 in the arena, this concentration reached  $0.166 \text{ mL L}^{-1}$  (5 MPs per litre), a concentration  
197 consistent with those found in coastal waters, *i.e.*  $3.5 \pm 2.0 \text{ MPs L}^{-1}$  with concentrations between  
198 1 and 6.4 MPs per wild *M. edulis* (United Kingdom; Li *et al.*, 2018). Immediately after the  
199 treatment or control injection, each specimen was placed individually at a distance of 4 cm from  
200 each side hole of the pot ( $n = 4$  mussels; Fig. 1). Video recording started immediately after the  
201 injection of water cue in the clay pot, at a rate of 1 frame per minute for 1h30 using a GoPro  
202 camera (GoPro HERO7 Black, GoPro Inc., San Mateo, California, USA) placed 40 cm above  
203 the experimental container. The choice of using four mussels per arena was made to minimize  
204 aggregations between mussels, which decreases with the distance between the mussels (Côté &  
205 Jelnikar, 1999; Nicastro *et al.*, 2007).



207 **Figure 1:** experimental design of one arena of the chemotaxis and chemokinesis experiments. Mussels  
208 were placed at equidistant distances from each other at 4 cm from the holes in a glass arena. To see how  
209 the liquid diffuses from the point source clay pot, see Supplementary Materials, S3.

210

211 Each day, three treatments were run concurrently: seawater (control;  $n = 6$  arenas) and  
212 two different treatments ( $n = 6$  arenas for each treatment; Table 1; Fig. 2). Six additional arenas

213 using seawater as control were also performed. Thus, the sample size for each cue was: seawater  
 214 n = 30 arenas; seawater with leachates n = 12 arenas and n = 6 arenas all other cues tested with  
 215 and without plastic leachates (see the experimental plan; Table 1; Fig. 2). An additional control  
 216 was also performed. Each mussel was only used once and then discarded, no mussels showed  
 217 injury or mortality signs during the experiment.

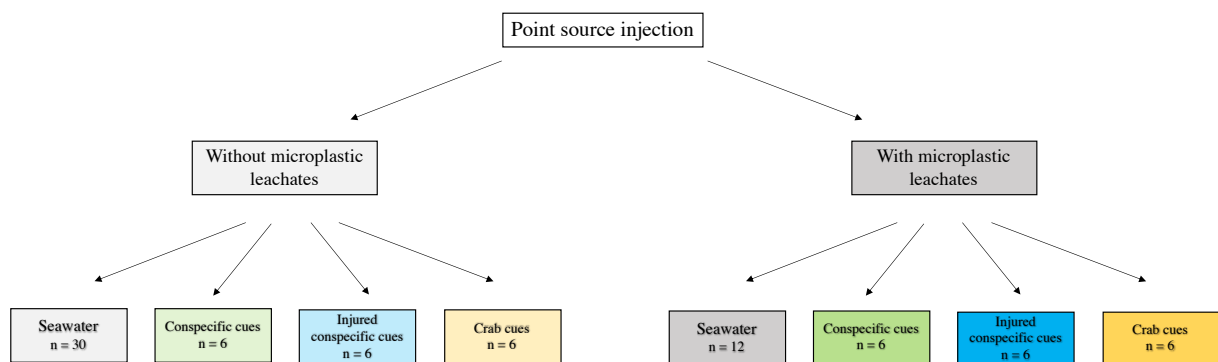
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219 **Table 1:** Experimental timetable of the chemotaxis experiments. Stimuli without plastic leachates (light  
 220 colour): (grey) Control, natural seawater, *i.e.* no stimulus; (green) stimuli from conspecifics *Mytilus*  
 221 *edulis*; (blue) stimuli from injured *M. edulis*; (orange) stimuli from fed *Hemigrapsus sanguineus*; stimuli  
 222 with microplastic (MP) leachates (dark colour). For each treatment, 6 replicates were performed each  
 223 day (two replicates of the same treatment 3 times a day at 2h interval) and an additional control (n = 6)  
 224 was carried out on Day 5.

Day 1	Day 2	Day 3	Day 4
Seawater	Seawater	Seawater	Seawater
Conspecific cues	Seawater + Microplastic Leachates	Seawater + Microplastic Leachates	Crab cues
Injured conspecifics cues	Conspecific cues + Microplastic Leachates	Injured conspecifics cues + Microplastic Leachates	Crab cues + Microplastic Leachates

225

226



227

228 **Figure 2:** Flow diagram of the treatments and their replications. Stimuli without plastic leachates (light  
 229 colour): (grey) Control, natural seawater, *i.e.* no stimulus; (green) stimuli from conspecifics *Mytilus*  
 230 *edulis*; (blue) stimuli from injured *M. edulis*; (orange) stimuli from fed *Hemigrapsus sanguineus*; stimuli  
 231 with microplastic (MP) leachates (dark colour).

232

#### 233 **2.4. Behavioural analysis**

234         Though we specifically chose to use of four mussels per arena to minimize the  
235 probability of occurrence of aggregation behaviour, overall four mussels ended-up as two  
236 aggregates of two mussels (*i.e.* physical contact between their shells) and two as almost-  
237 aggregated (*i.e.* at a distance of less than 2 cm). These 6 mussels represent less than 2% (*i.e.*  
238  $6/312 \times 100$ ) of the total number of mussels used in the experiment, but could may have affected  
239 the behaviour of other mussels in the same arena (Côté & Jelnikar, 1999; Nicastro *et al.*, 2007;  
240 Commito *et al.*, 2014). Consequently, to avoid a potential bias related to the presence of  
241 aggregated conspecifics, the behavioural responses for the different endpoint were not studied  
242 at the level of the individual but at the level of the arena. Thus, for the different behavioural  
243 parameters studied, the data from the four mussels of each arena averaged and used as sample  
244 unit in the statistical analyses. Aggregated and almost-aggregated mussels have been included  
245 in the dataset.

246         First, motility was assessed by comparing the proportion of motile and non-motile  
247 mussels, to have an average percentage of mussel motility per arena. Chemokinesis was  
248 subsequently assessed through mean speed, gross distance (*i.e.* the total distance covered by the  
249 mussel between its initial and final position), net distance and confinement index (*i.e.* the ratio  
250 between net distance and gross distance) per arena. These parameters were measured only for  
251 mussels moving a net distance above 1 cm (*i.e.* approximately half mussel's shell length) using  
252 the TrackMate plugin of ImageJ (Tinevez *et al.*, 2017). Note that the choice of a net distance  
253 greater than 1cm was specifically made because distances less than 1 cm were assigned by  
254 TrackMate to mussels oscillating around their center of mass without actually actively moving  
255 as their foot was never observed outside their valves (see Supplementary Material, S4).  
256 Chemotaxis was estimated by recording the final motile mussel position with reference to the

257 source point, in order to have an average percentage of mussels with a negative chemotaxis  
258 (repulsion from the source point) per arena.

## 259 **2.5. Statistical analyses**

260 To compare differences among all treatments and to take into account the potential  
261 confounding effect of running treatments on different days, the motility, speed, gross distance,  
262 net distance, confinement index and chemotaxis of mussels, observed in the control seawater,  
263 were compared between days with an ANOVA (for more details, see Supplementary Material,  
264 S5). As no significant differences ( $p > 0.05$ ; see Supplementary Material, S5) were found  
265 among control replicates run in different days, behavioural data for each treatment from controls  
266 and treatments were pooled across days for further analyses.

267 Motility data were not normally distributed (Shapiro's test,  $p = 0.004$ ), but showed a  
268 homogeneity of variance (Levene's test,  $p = 0.931$ ). Parametric analysis was used based on the  
269 assumption that ANOVA is relatively robust to the effects of non-normality (Zar, 1999);  
270 therefore, data were analysed using a 2-way ANOVA with treatment (control or microplastic  
271 leachates) and cues (seawater, conspecifics, injured conspecifics or crabs) as fixed factors.  
272 Significant effects were examined using Tukey-HSD post-hoc test.

273 In order to measure the speed, gross distance, net distance, confinement index and  
274 chemotaxis, only mussels with a net distance greater than 1 cm (about half a body length) were  
275 considered, hence the sample size per treatment was: seawater  $n = 27$ , seawater with leachates  
276  $n = 12$  and  $n = 6$  for the other treatments. The distribution of speed, gross distance, net distance  
277 and confinement index measurements were tested for normality (Shapiro's test,  $p = 0.00005$ ,  
278  $p = 0.00004$ ,  $p = 0.070$ ,  $p = 0.406$ , respectively) and homogeneity of variance (Levene's test,  
279  $p = 0.141$ ,  $p = 0.616$ ,  $p = 0.582$ ,  $p = 0.060$ , respectively). As all parameters showed a  
280 homogeneity of variance, but were not always normally distributed, parametric analyses were  
281 consistently used as described above. A 2-way ANOVA with treatment (control or microplastic

282 leachates) and cues (seawater, conspecifics, injured conspecifics or crabs) as fixed factors was  
283 finally performed and significant effect were further examined using a Tukey-HSD post-hoc  
284 test.

285 To assess the chemotaxis, *i.e.* to compare the direction of motile mussels (with a net  
286 distance greater than 1cm), attraction (positive chemotaxis) or repulsion (negative chemotaxis)  
287 to the source point, a 2-way ANOVA with treatment (control or microplastic leachates) and  
288 cues (seawater, conspecifics, injured conspecifics or crabs) as fixed factors was used, followed  
289 by a Tukey-HSD post-hoc test when significant differences were identified. Prior to this test,  
290 normality ( $p = 0.013$ ) and homogeneity of variance ( $p = 0.109$ ) were measured, as homogeneity  
291 of variance was confirmed, this test could be performed (Zar, 1999). All statistical analyses  
292 were performed using the software R Core Team (2021).

### 293 **3. Results**

#### 294 **3.1. Chemokinesis**

##### 295 **3.1.1. Motility**

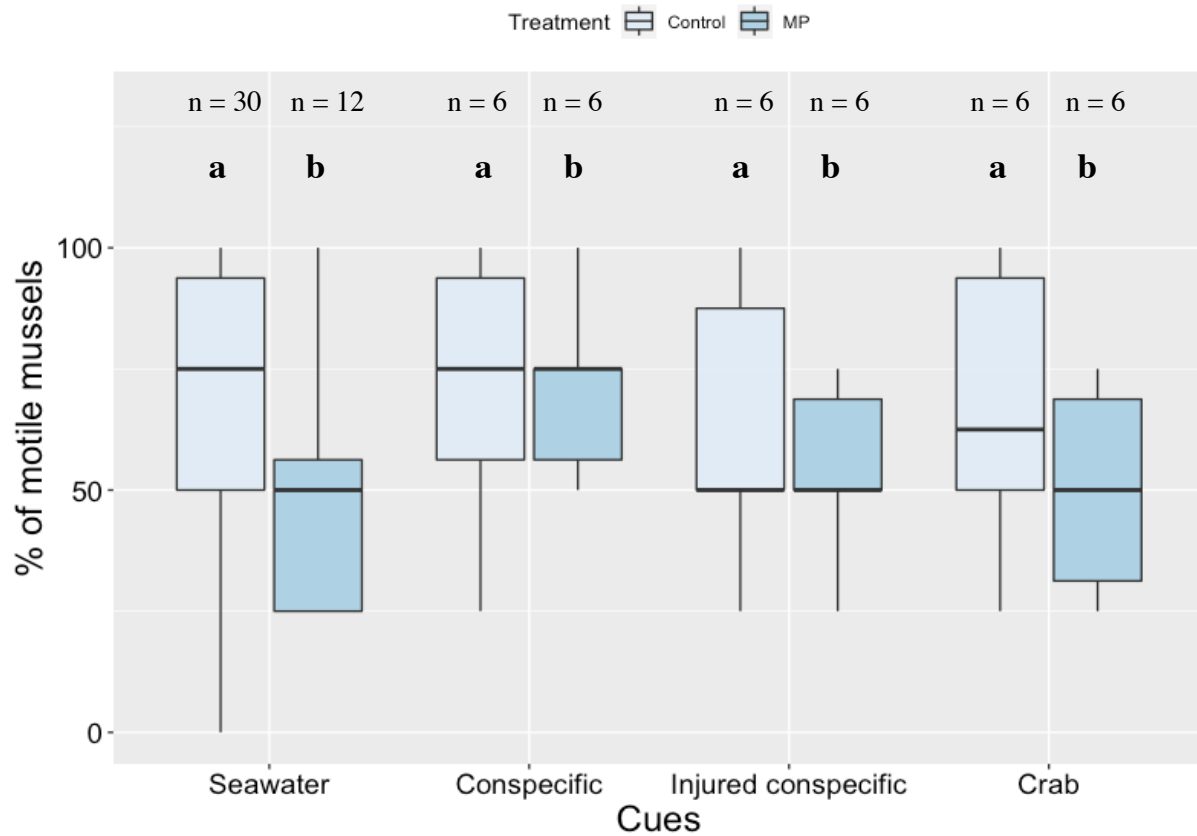
296 Polypropylene leachates inhibited significantly mussel motility for all cues tested  
297 (Treatment;  $p = 0.043$ ; Fig. 3, Table 2). Specifically, a significantly higher percentage of  
298 mussels moved in the control treatment (no leachate) than when they were exposed to  
299 microplastic leachate (Tukey's test:  $p = 0.049$ ).

300 **Table 2:** results of 2-way ANOVA applied to the percentage of motile mussels with Treatment (control  
301 or microplastic leachates) and Cue (seawater, conspecifics, injured conspecifics or crabs) as fixed  
302 factors.

Source of variation	df	MS	<i>F</i>	p
Treatment	1	3066.9	4.230	0.0434
Cue	3	419.0	0.578	0.6315
Treatment × Cue	3	285.2	0.393	0.7581
Residuals	70	725.0		

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**Figure 3:** percentage of motile *Mytilus edulis* by replicate in control seawater and natural cue treatments

307

(light blue) and microplastic leachates treatments (dark blue). Significant differences ( $p < 0.05$ ; Tukey-

308

HSD) between treatments were identified by different letters and the number, n, of arenas used was also

309

indicated.

310

### 3.1.2. Speed

311

There was a significant effect of Cue (Cue;  $p = 0.005$ ; Fig. 4, Table 3). Specifically,

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regardless of the treatment, mussels always moved significantly slower when exposed to crab

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cues than when exposed to conspecifics cues or to control seawater (Tukey's test:  $p = 0.043$

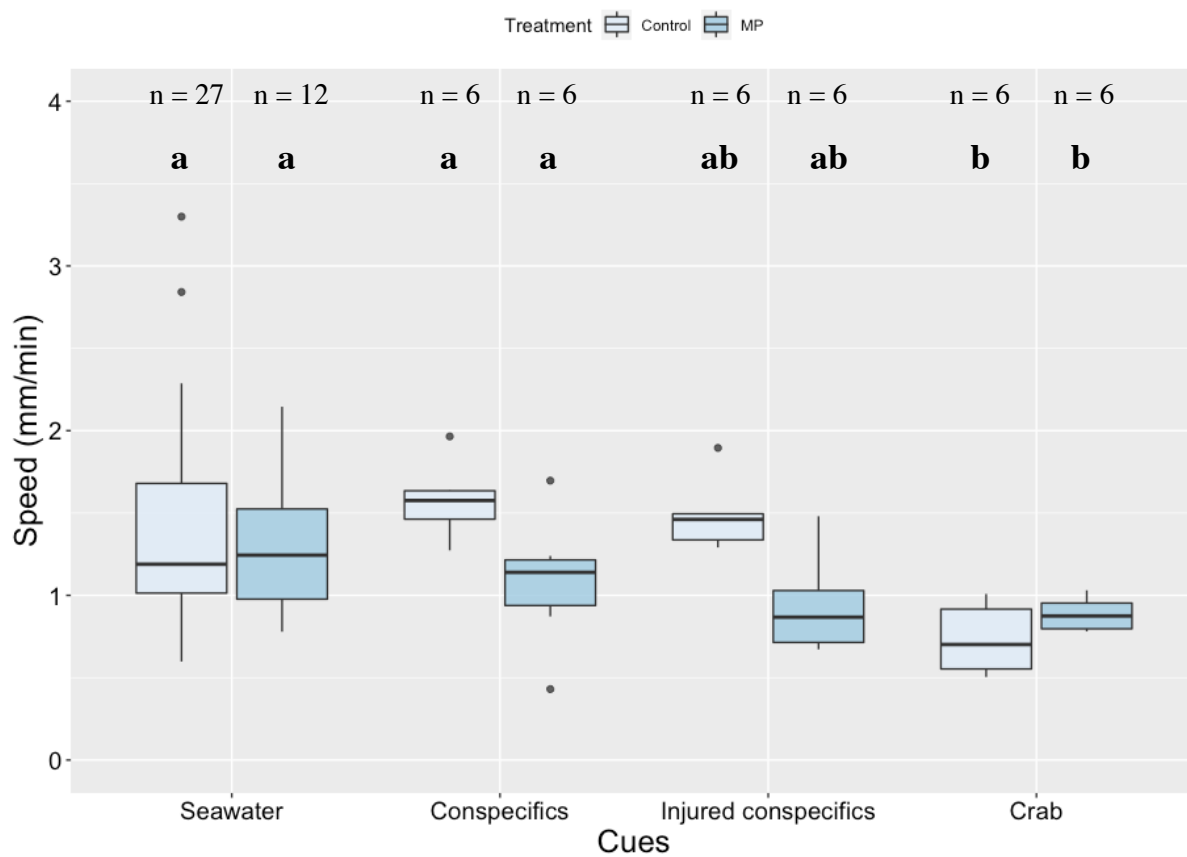
314 and  $p = 0.002$  respectively). However, the speed of mussels exposed to conspecific cues, injured  
 315 conspecific cues and control seawater did not significantly differ (Seawater; Tukey's test:  $p >$   
 316  $0.05$ ).

317 **Table 3:** results of 2-way ANOVA applied to the mean speed by replicate with Treatment (control or  
 318 microplastic leachates) and Cue (seawater, conspecifics, injured conspecifics or crabs) as fixed factors.

Source of variation	df	MS	<i>F</i>	<i>p</i>
Treatment	1	0.7950	3.536	0.06439
Cue	3	1.0567	4.700	0.00488
Treatment × Cue	3	0.3418	1.520	0.21723
Residuals	67	0.2248		

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321

322 **Figure 4:** mean speed of *Mytilus edulis* by replicate in control seawater and natural cue treatments (light  
 323 blue) and microplastic leachates treatments (dark blue). Significant differences ( $p < 0.05$ ; Tukey-HSD)



324 between treatments were identified by different letters and the number of arenas used was also indicated  
325 (n =).

### 326 3.1.3. Gross Distance

327 The gross distance travelled by mussels varied significantly depending on the Cue (Cue;  
328  $p = 0.0498$ ; Fig. 5, Table 4). Specifically, regardless of the presence of leachates, the gross  
329 distance was always lower in mussels exposed to crab cues than when exposed to sea water  
330 (Tukey's test:  $p = 0.032$  respectively). There were no significant differences for the mussel  
331 speed when exposed to conspecific cues, injured conspecific cues and the control (Seawater;  
332 Tukey's test:  $p > 0.05$ ).

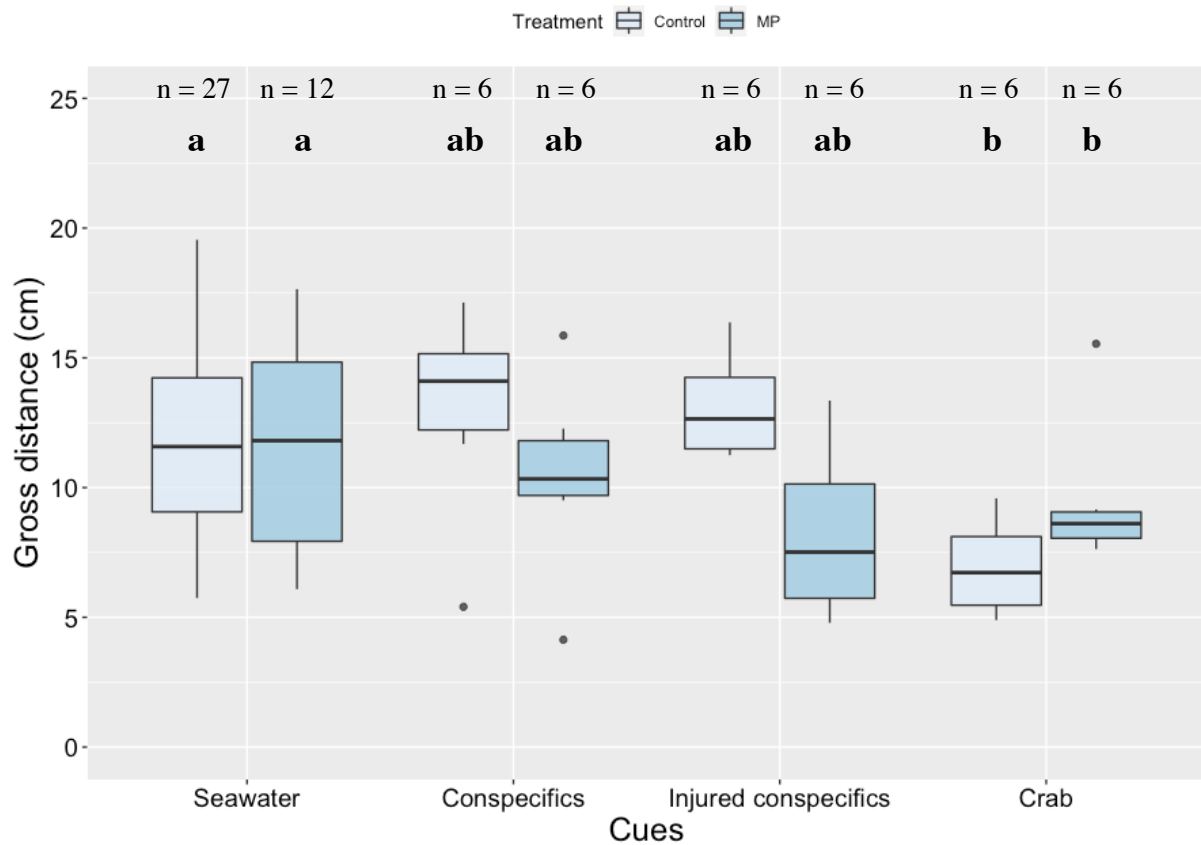
333

334 **Table 4:** results of 2-way ANOVA applied to the mean gross distance by replicate with Treatment  
335 (control or microplastic leachates) and Cue (seawater, conspecifics, injured conspecifics or crabs) as  
336 fixed factors.

Source of variation	df	MS	<i>F</i>	p
Treatment	1	31.94	1.617	0.2080
Cue	3	54.21	2.744	0.0498
Treatment × Cue	3	30.38	1.538	0.228
Residuals	67	19.76		

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340 **Figure 5:** mean gross distance travelled by *Mytilus edulis* by replicate in control seawater and natural  
 341 cue treatments (light blue) and microplastic leachates treatments (dark blue). Significant differences  
 342 ( $p < 0.05$ ; Tukey-HSD) between treatments were identified by different letters and the number of arenas  
 343 used was also indicated (n =).

#### 344 3.1.4. Net Distance and Confinement index

345 There was a significant effect of the Treatment  $\times$  Cue interaction (Treatment  $\times$  Cue;  
 346  $p = 0.036$ ; Fig. 6, Table 5). However, the Tukey's test revealed no significant differences  
 347 ( $p > 0.05$ ) for the net distance traveled by mussels between every cues and treatments, even if  
 348 a marginal difference was found between conspecifics with and without microplastic leachates  
 349 ( $p = 0.094$ ).

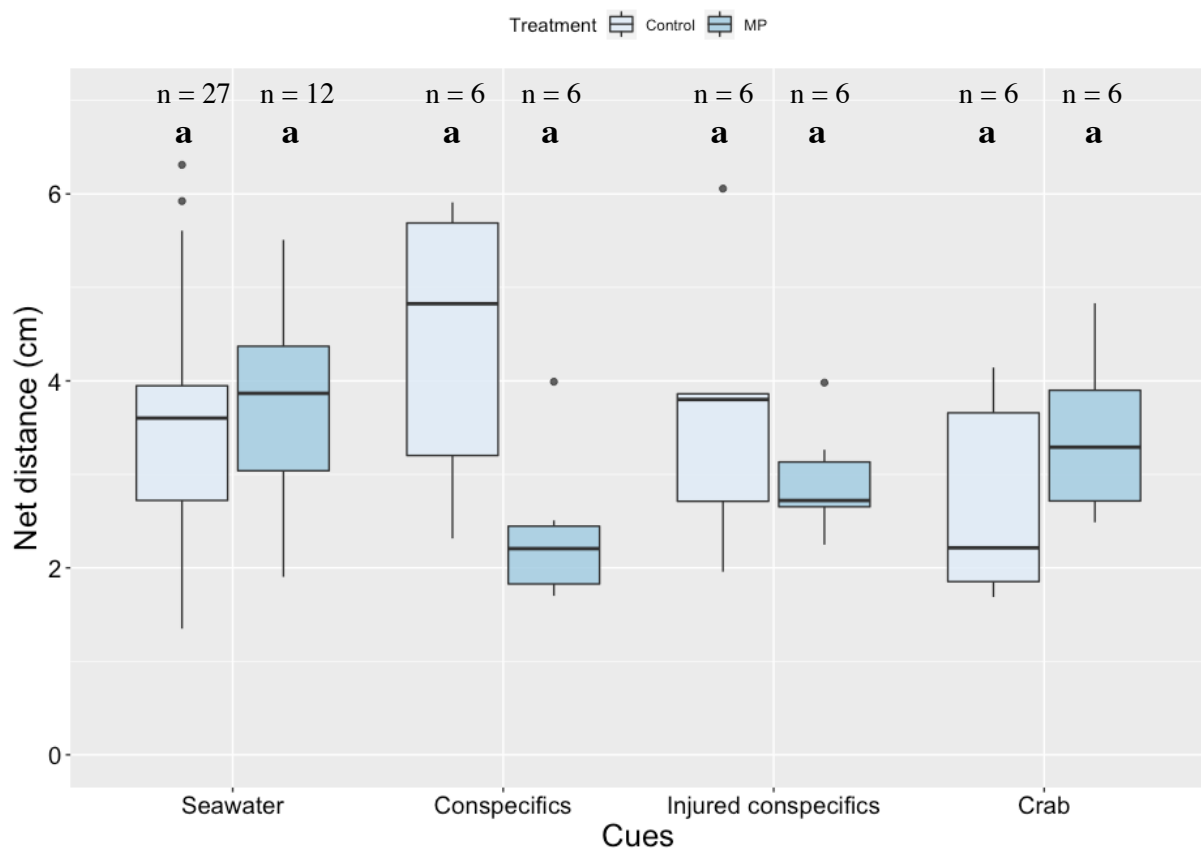
350 The confinement index did not differ among factors nor treatments (Table 6).

351 **Table 5:** results of 2-way ANOVA applied to the mean net distance by replicate Treatment (control or  
 352 microplastic leachates) and Cue (seawater, conspecifics, injured conspecifics or crabs) as fixed factors.

Source of variation	df	MS	F	p
Treatment	1	2.031	1.360	0.2477
Cue	3	2.042	1.367	0.2604
Treatment × Cue	3	4.491	3.006	0.0363
Residuals	67	1.494		

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354



355

356 **Figure 6:** mean net distance travelled by *Mytilus edulis* by replicate in control seawater and natural cue  
 357 treatments (light blue) and microplastic leachates treatments (dark blue). Significant differences  
 358 ( $p < 0.05$ ; Tukey-HSD) between treatments were identified by different letters and the number of arenas  
 359 used was also indicated (n =).

360

361 **Table 6:** Results of 2-way ANOVA applied to the mean confinement index by replicate with Treatment  
 362 (control or microplastic leachates) and Cue (seawater, conspecifics, injured conspecifics or crabs) as  
 363 fixed factors.

Source of variation	df	MS	<i>F</i>	p
Treatment	1	0.003253	0.257	0.614
Cue	3	0.007719	0.609	0.611
Treatment × Cue	3	0.024363	1.923	0.134
Residuals	67	0.012667		

364

365

### 366 **3.2. Chemotaxis**

367 There was a significant effect of the Treatment × Cue interaction on the mussel  
 368 chemotaxis (Treatment × Cue;  $p = 0.038$ ; Fig. 7, Table 7), as significantly more mussels showed  
 369 a negative chemotaxis to crab cue without microplastic leachates than with microplastic  
 370 leachates (Tukey's test:  $p = 0.047$ ).

371

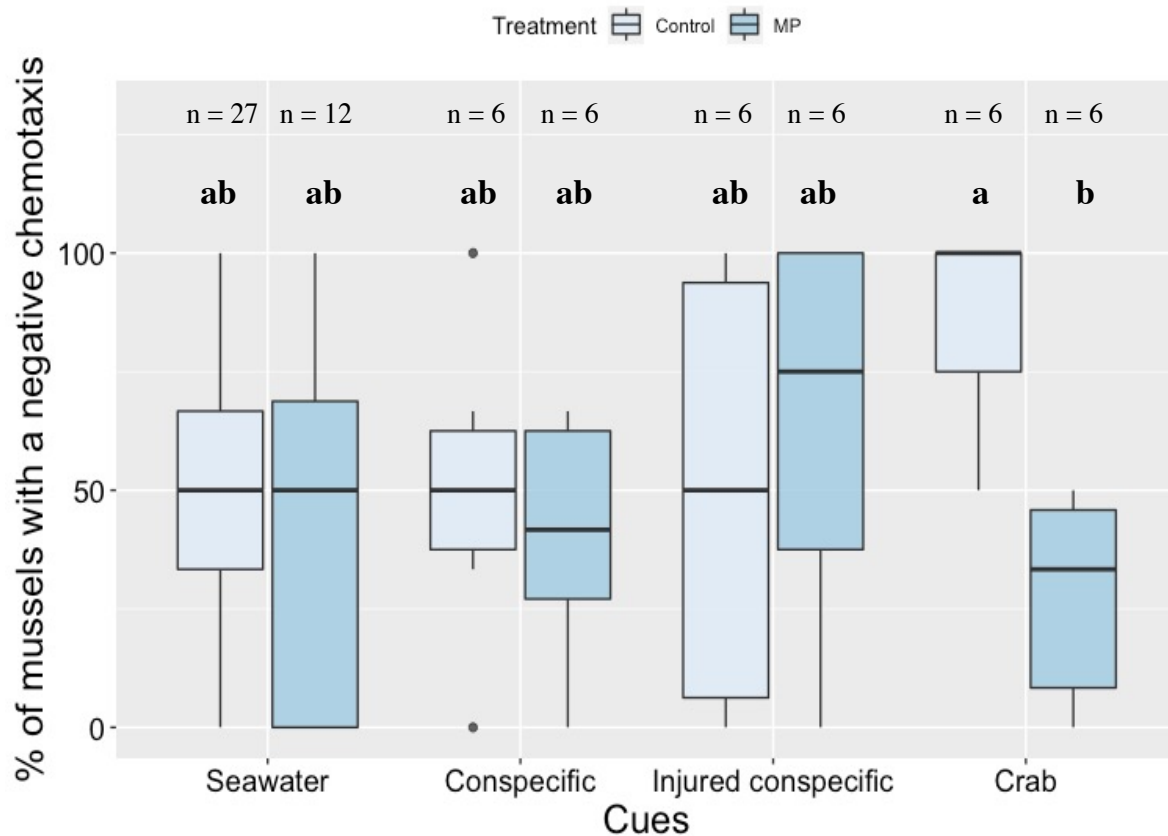
372 **Table 7:** results of 2-way ANOVA applied to the percentage of mussels moving away from the source  
 373 point with Treatment (control or microplastic leachates) and Cue (seawater, conspecifics, injured  
 374 conspecifics or crabs) as fixed factors.

Source of variation	df	MS	<i>F</i>	p
Treatment	1	1976.1	1.920	0.1704
Cue	3	596.3	0.579	0.6305
Treatment x Cue	3	3051.7	2.965	0.0382
Residuals	67	1029.2		

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379 **Figure 7:** percentage of *M. edulis* moving away from the point source by replicate in control seawater  
 380 and natural cue treatments (light blue) and microplastic leachates treatments (dark blue). Significant  
 381 differences ( $p < 0.05$ ; Tukey-HSD) between treatments were identified by different letters and the  
 382 number of arenas used was also indicated ( $n =$ ).

## 383 4. Discussion

### 384 4.1. Cue-specific chemotactic and chemokinetic responses in *Mytilus edulis*

385 Taken together, our results show that *M. edulis* exhibit both chemotactic and  
 386 chemokinetic response to chemical cues, and that these responses are cue-dependent.

#### 387 4.1.1. Lack of behavioural response towards conspecific cues

388 *Mytilus edulis* did not show any behavioural change in response to conspecific cues, in  
 389 accordance with previous work on the effect of conspecific cues on *M. edulis* aggregation  
 390 patterns (Commito *et al.*, 2016). These observations are, however, in contrast with previous  
 391 studies which showed that positive chemotaxis was the main driver of conspecific aggregation

392 in bivalves (Huang *et al.*, 2007) and in particular in *M. edulis* (De Vooy, 2003). These  
393 discrepancies may, however, be related to (i) potential behavioural divergence in *M. edulis*  
394 between those living on the bottom of a concrete ditch with running seawater or on beach  
395 groynes as seen in Netherlands (De Vooy, 2003) with those living on natural rocky shores  
396 habitats as seen in France (present work) and/or (ii) the presence of a seasonal component in  
397 the behavioural response of *M. edulis* to conspecific cues (De Vooy, 2003). Though the  
398 identification of the mechanisms at the origin of the above-mentioned behavioural differences  
399 lies well beyond the scope of the present work, it warrants the need for further work.

400

#### 401 4.1.2. *Lack of behavioural response toward injured conspecifics cues*

402 No behavioural changes were observed either in terms of chemotaxis or chemokinesis  
403 in response to injured conspecifics cues. These results are consistent with previous observations  
404 conducted on *Mytilus edulis* (Comitato *et al.*, 2016). They contrast, however, with the results  
405 of studies conducted on other mussel species, which may be indicative of a species-dependent  
406 behaviour. For instance, the motility of the zebra mussel (*Dreissena polymorpha*) decreases in  
407 response to crushed conspecifics cues (Toomey *et al.*, 2002; Czarnołęski *et al.*, 2010).  
408 *Brachidontes variabilis* has been shown to adapt its behaviour when exposed to injured  
409 conspecifics by seeking and selecting the most appropriate refuges (Shin *et al.*, 2008). Other  
410 studies showed an increase in aggregation rates, which is implicitly driven by a change in  
411 motility in both the Mediterranean mussel *Mytilus galloprovincialis* and the brown mussel  
412 *Perna perna* in the presence of injured conspecifics (Nicastro *et al.*, 2007).

413

#### 414 4.1.3. *Crab cues induce a chemokinetic and potentially a chemotactic responses in Mytilus* 415 *edulis*

416 *M. edulis* behaviour was significantly altered by the presence of  
417 *Hemigrapsus sanguineus* cues through a chemokinetic response. Specifically, although motility  
418 is not affected, a decrease in both gross distance and speed compared to seawater control  
419 treatment was recorded. These results are consistent with previous experiments highlighting a  
420 decrease in movement of *M. edulis* and of another intertidal mussel (*Hormomya mutabilis*) in  
421 response to predator cues (Reimer & Tedengren, 1997; Ishida & Iwasaki, 2003). These  
422 observations are consistent with the activity reduction used by many aquatic animals as an anti-  
423 predator strategy (Clements *et al.*, 2020). Indeed, low movement in mussels decreases water  
424 motion, visibility and odour dispersal and thus the probability of being spotted by predators  
425 relying on hydrodynamical, visual and chemical cues (Ishida & Iwasaki, 2003; Garner &  
426 Litvaitis, 2013; Clement *et al.*, 2020). In addition, our results suggest a negative chemotactic  
427 response of *M. edulis* to crab cues. This observation would be consistent with early evidence of  
428 chemodetection and chemotaxis in bivalves (Morton, 1962; De Vooy, 2003, Huang *et al.*,  
429 2007). However, to our knowledge, a chemotactic response of a bivalve to its predator has yet  
430 to be documented. Here, we move a step forward by showing that *M. edulis* was able to have  
431 an adapted behaviour response to predator cues by limiting its movements and by appearing to  
432 move in the opposite direction to the source. The combination of these two complementary  
433 adaptive strategies to predator cues may provide an important defence strategy against predators  
434 and may add a short-term component to the acknowledged long-term anti-predator traits of *M.*  
435 *edulis*, such as its phenotypic plasticity (*i.e.* increase in its byssal attachment strength and the  
436 thickness of its shell following the presence of crab cues; Leonard *et al.*, 1999; Cheung *et al.*,  
437 2004).

438 We finally stress that the chemokinetic and possible chemotactic behavioural changes  
439 observed in the native *M. edulis* in response to the invasive crab predator *H. sanguineus* indicate  
440 that *M. edulis* is not naive to this predator. Prey naiveté is a lack of its ability to recognize a

441 new predator as a threat and subsequently develop an effective anti-predator behaviour, due to  
442 the lack of coevolution between the predator and the prey (Anton *et al.*, 2020). The invasion of  
443 *H. sanguineus* in the eastern English Channel is relatively recent, *i.e.* about 16 years at our study  
444 site (Dauvin *et al.*, 2009). The invader has now supplanted the native populations of the  
445 European green crab *Carcinus maenas* (Rolet *et al.*, 2020). The lack of naiveté of *M. edulis*  
446 reported in the present work noticeably contrasts with observations conducted in South Africa  
447 where, 28 years after its introduction, the invasive mussel (*M. galloprovincialis*) still had not  
448 developed any anti-predator responses to cues from a native predator (the spiny lobster *Jasus*  
449 *lalandii*), which instead had an effect on the native mussel (*P. perna*; Nicastro *et al.*, 2007).  
450 These observations suggest different predator-prey co-evolution history between *M. edulis* and  
451 *M. galloprovincialis*.

452         The overall *M. edulis* behavioural response to the different chemical cues inferred in the  
453 present work highlights a cue-specific response. Specifically, *M. edulis* responds to predatory  
454 crab cue previously fed on conspecifics (diet cue) but not to damaged released conspecific cues  
455 (alarm cue). This result is consistent with previous observation conducted on *Macoma baltica*,  
456 a marine bivalve which showed a strong response to fed crab effluents but a lack of response to  
457 injured conspecifics cues (Griffiths & Richardson, 2006). However, this behavioural response  
458 is likely to be species-dependent, as the same pattern was not observed for  
459 *Mercenaria mercenaria* (Smee & Weissburg, 2006). This observation further suggests the  
460 existence of a hierarchy in the behavioural responses. Predator cues may be a more  
461 unambiguous threat compared to crushed conspecific cues which could instead be caused by a  
462 variety of risks, such as wave action, sand, storms, rockslides, and trampling (Zardi *et al.*, 2006;  
463 Nicastro *et al.*, 2019).

464



465 **4.2. Alteration of *Mytilus edulis* chemotactic and chemokinetic responses by**  
466 **microplastic leachates**

467 The addition of plastic leachates disrupted the chemotactic and/or chemokinetic  
468 behaviour of *M. edulis* from all treatments, indicating that polypropylene leachates can alter the  
469 neurosensory abilities of this species, hence its way of acquiring and processing information  
470 and therefore their decision-making.

471

472 **4.2.1. Microplastic leachates alter *M. edulis* chemokinetic behaviour**

473 The percentage of non-motile mussels was significantly greater in the presence of  
474 microplastic leachates, regardless of the cues. This may be the result of an inhibition of the  
475 neuromuscular performance of *M. edulis* by polypropylene leachates. Previous studies showed  
476 a deleterious effect of plastic additives on motility of mussel D-shape larvae (*M. edulis*;  
477 Capolupo *et al.*, 2020), fish larvae (*Danio rerio*; Kim *et al.*, 2020), cladoceran (*Daphnia magna*;  
478 Lithner *et al.*, 2009), drosophila (*Drosophila melanogaster*; Kaur *et al.*, 2015), nematodes  
479 (*Caenorhabditis elegans*; Tseng *et al.*, 2013) and rats (Vermeer *et al.*, 2014). Thus, there is a  
480 significant effect of polypropylene leachates on mussel motility which could lead to larger scale  
481 disturbances. Indeed, any changes related to the chemosensory, referential, and behavioural  
482 abilities of *M. edulis* may have implications on their aggregation rate and consequently for the  
483 survival and reproduction of the species but also for intra- and interspecific competition for  
484 space with potentially cascading effects for coastal ecosystems (Borthagaray & Carranza, 2007;  
485 van de Koppel *et al.*, 2008; Nicastro *et al.*, 2012; Iwasaki, 2015, Zardi *et al.*, 2021).

486 Thus, in seawater contaminated with leachates, mussels were significantly less motile  
487 than in the control seawater treatment. This contrasts with the behaviour of the mollusc  
488 *Littorina littorea*, which was not affected by the presence of leachates from virgin  
489 polypropylene microbeads, although leachates concentration was higher (*ca.* 10- to 100-fold

490 higher) than in our study (Seuront, 2018). This observation highlights distinct species  
491 chemosensitivity to plastic leachates.

492

#### 493 **4.2.2. *Microplastic leachates alter M. edulis response to conspecific cues***

494 In the present study, *M. edulis* behaviour towards conspecific cues was impacted by  
495 plastic leachates, via a decrease in their motility. There were less motile mussels, which in  
496 comparison to mussels exposed to conspecific cues without leachates, seems to cover a smaller  
497 net distance, however this last response was not significant. This observed decrease in motility  
498 and possible smaller net distance in response to conspecific cues with leachates may impact the  
499 aggregation rate of mussels which may have larger scale spatial and ecosystem implications.  
500 However, *M. edulis* motility and aggregation rate have been shown to increase in seawater  
501 contaminated by plastic leachate (Seuront *et al.*, 2021). Higher leachates concentration (*ca.* 10-  
502 to 100-fold more concentrated than in our study; Seuront *et al.*, 2021) may, however, account  
503 for this discrepancy.

504

#### 505 **4.2.3. *Microplastic leachates alter M. edulis response to injured conspecific cues***

506 The combined signal of the injured mussels and leachates impact their chemokinetic  
507 response through a large percentage of non-motile mussels. Since no behavioural changes have  
508 been demonstrated in response to chemical cues from injured conspecifics, polypropylene  
509 leachates appear to affect the mussel itself and not its behaviour in response to injured  
510 conspecifics. Thus, the addition of leachates could lead to the reduction of motility found in the  
511 presence of injured conspecific cues.

512

#### 513 **4.2.4. *Microplastic leachates alter M. edulis response to crab cues***

514           When exposed to a mixture of crab cue and microplastic leachates, *M. edulis* showed a  
515 significantly lower proportion of motile mussels and a significant change in direction compared  
516 to the crab cue treatment without leachates, which may result in an inhibition of its negative  
517 chemotaxis. In addition, although the gross distance and speed did not significantly differ for  
518 this treatment than for the crab cues without leachates treatment, they were significantly  
519 reduced compared to the control and control with leachates. This change in speed and distance  
520 is related to the behavioural response of the mussel to the crab and does not appear to be affected  
521 by polypropylene leachates at the concentration used in this study. However, these leachates  
522 reduce mussel motility and affect chemotaxis, the latter suggesting a neurosensory impairment  
523 rather than a neuromuscular one. This could indicate that plastic leachates may be masking the  
524 signal and/or lead to sensory deficiencies. Plastics leachates have also been reported to have  
525 neurological effect and act on the cognition of organisms in their way of acquiring and  
526 processing information and thus their decision-making (Seuront, 2018; Crump *et al.*, 2020). For  
527 instance, the cognitive response of a prey to its predator is affected by plastic leachates in  
528 another mollusc, the gastropod *Littorina littorea* (Seuront, 2018). Indeed, the vigilance and  
529 anti-predation behaviours of gastropods (*i.e.* righting time, skioptic withdrawal, time to explore,  
530 avoidance response) have been shown to be altered and/or inhibited in the presence of plastic  
531 leachates (Seuront, 2018).

532           The impairments described above are in line with previous work showing that phthalates  
533 (DEHP, DBP, DIBP) can alter the neurological behaviour in nematodes (Tseng *et al.*, 2013).  
534 Indeed, these are the dominant additives found in our polypropylene beads (Supplementary  
535 Materials, S2). Phthalates disrupt the nematode's antioxidant defence system, the morphology  
536 of some thermosensory neurons and the genes involved in this thermotaxis function (Tseng *et*  
537 *al.*, 2013), altering their locomotion, *i.e.*, body bends, head thrashes, and reversal frequencies  
538 (Tseng *et al.*, 2013). In similar fashion, these additives could induce a neurotoxicity in mussels,

539 which would act on the neurosensory system and lead to an alteration of their movements and  
540 chemotactic capacities. A disturbance in *M. edulis* motility and neuro-sensory response could  
541 have consequences in aggregation rate and would impact the mussel growth, reproduction and  
542 anti-predator responses (Harger, 1968; Schneider *et al.*, 2005; van de Koppel *et al.*, 2008; Zardi  
543 *et al.*, 2021). These impairments could lead to changes in mussel bed formations, to a decrease  
544 in mussel population and ultimately impact the ecosystem structure and functioning in *M. edulis*  
545 (Borthagaray & Carranza, 2007).

## 546 **5. Conclusion**

547 Taken together, our results show that *M. edulis* behavioural responses are cue-specific.  
548 Critically, exposure to plastic leachates altered mussel behavioural responses by altering their  
549 motility. The fitness of mussels is directly related to their motility through their ability to  
550 aggregate. The impact of microplastic leachates also altered the response of mussels to  
551 conspecific cues, which is a direct link to aggregation, and to their crab predator cues. This  
552 observation indicates that chemical communication among mussels may be disrupted by plastic  
553 leachates, suggesting that aggregation rate and bed formation and also prey-predator interaction  
554 may be impacted on the short-term, at least by polypropylene leachates and at the concentration  
555 tested here which is consistent to the contamination occurring in the marine environment (*i.e.*  
556 5 MPs L<sup>-1</sup>). Prey-predator interactions are essential traits of the ecology of marine systems and  
557 variations in the ability to detect predation risk can significantly influence population and  
558 community structures (Ferrari *et al.*, 2010). The changes caused by plastic leachates may have  
559 important effects on the bioengineering role of *M. edulis* and have cascading effects on the  
560 associated communities. Given the predicted increase in plastic pollution and the relative  
561 limited information on plastic leachates compared to plastic ingestion (Fauvelle *et al.*, 2021),  
562 our work highlights effects of plastic leachates that may have fundamental knock-on effects on  
563 the functioning of intertidal ecosystems and the services provided to society. However, before

564 extrapolating our results any further, it is crucial to look at the impact of plastic leachates on  
565 the predator behaviour, to see if its predatory performance is also affected or not in a plastic-  
566 contaminated environment.

## 567 **Credit author statement**

568 **Marine Uguen:** Conceptualization, Formal Analysis, Methodology, Investigation, Writing –  
569 Original Draft, Editing. **Katy R Nicastro:** Investigation, Methodology, Resources, Writing –  
570 Review & Editing. **Gerardo I Zardi:** Investigation, Methodology, Resources, Writing –  
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572 **Nicolas Spilmont:** Investigation, Methodology, Resources, Writing – Review & Editing.  
573 **Fleurine Akoueson:** Methodology, Resources. **Guillaume Duflos:** Methodology, Resources.  
574 **Laurent Seuront:** Supervision, Conceptualization, Methodology, Resources, Writing –  
575 Review & Editing, Funding acquisition.

## 576 **Declaration of competing interest**

577 The authors declare that they have no known competing financial interests or personal  
578 relationships that could have appeared to influence the work reported in this paper.

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