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Size-dependent response of the mussel collective behaviour to plastic leachates and predator cues

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Abstract

Both individual and collective anti-predator behaviours are essential for the survival of many species. This is particularly true for ecosystem engineers like intertidal mussels, which through their collective behaviour create new habitats for a range of organisms and biodiversity hotspots. However, contaminants may disrupt these behaviours and consequently indirectly affect exposure to predation risk at the population level. Among these, plastic litter is a major and ubiquitous contaminant of the marine environment. Here, we assessed the impact of microplastic (MP) leachates of the most produced plastic polymer, polypropylene (PlasticsEurope, 2022), at a high but locally relevant concentration (*i.e. ca.* 12 g L⁻¹) on the collective behaviours and anti-predator responses of small and large *Mytilus edulis* mussels. Indeed, in contrast to large mussels, small ones reacted to MP leachates, showing a taxis towards conspecifics and stronger aggregations. All mussels reacted to the chemical cues of the predatory crab, *Hemigrapsus sanguineus*, but with two different collective anti-predator behaviours. Small mussels only showed a taxis towards conspecifics when exposed to predator cues. This response was also found in large ones with a tendency to form more strongly bound aggregations and an important activity reduction, *i.e.* they significantly delayed their time to start to form aggregations and decreased their gross distance. These anti-

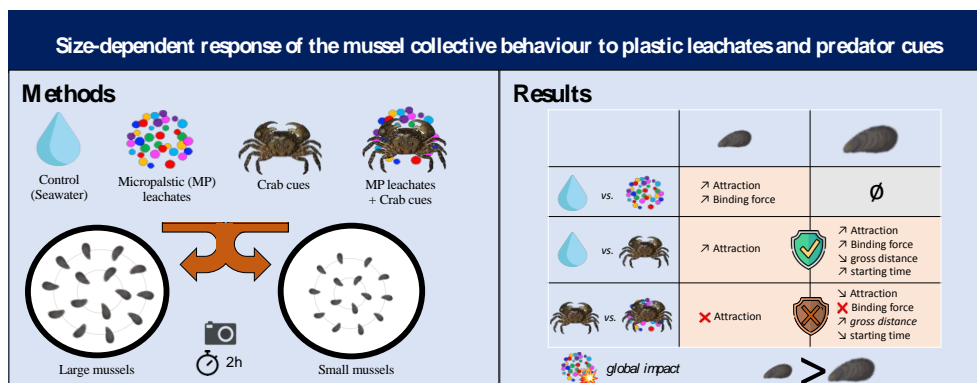
34 predator behaviours were respectively inhibited and impaired in small and large mussels by
 35 MP leachates. The observed collective behavioural changes are likely to reduce individual
 36 fitness and may enhance predation risk, especially for small mussels, that are *H. sanguineus*
 37 favourite preys. Given their key role as ecosystem engineers, our observations suggest that
 38 plastic pollution is likely to have implication on *M. edulis* at the species level, but also
 39 enhancing a cascading effect towards a higher level of organisation such as population,
 40 community and ultimately structure and function of intertidal ecosystem.

41 **Keywords**

42 *Risk of predation, toxicity, clumping behaviour, blue mussel, plastic leachate, pollution*

43

44 **Graphical Abstract**



45

46

47 **1. Introduction**

48 Ecosystems are strongly influenced by prey-predator interactions (Barbosa and
 49 Castellanos, 2005; Belgrad and Griffen, 2016; Lima and Dill, 1990). Predation exerts a strong
 50 selective pressure on prey, leading to the evolution of numerous anti-predator adaptations
 51 (Barbosa and Castellanos, 2005; Dawkins et al., 1979; Lima and Dill, 1990). Prey survival
 52 success is generally linked to the early detection of the predator (Ferrari et al., 2010) which, in
 53 the marine environment, is mainly driven by chemical signals (Zimmer and Butman, 2000).

54 Once a predator is detected, prey survival can be optimised by minimising predator encounter
55 and attack success probabilities through gregarious behaviours and various forms of collective
56 dynamics (Hamilton, 1971; Lehtonen and Jaatinen, 2016; Miller, 1922; Rubenstein, 1978).

57 Mussels are gregarious keystone organisms as their ability to move, aggregate and
58 attach to substrate and to conspecifics using proteogenic filaments (*i.e.*, the byssus; Schneider
59 et al., 2005) lead to the creation of biodiversity hotspots through the formation of biogenic
60 habitats with relatively stable abiotic conditions (Borthagaray and Carranza, 2007;
61 Buschbaum et al., 2009; Reise, 2002; Smaal et al., 2019). Mussels are able to detect,
62 discriminate and adapt to different predator cues either individually (Côté, 1995; Uguen et al.,
63 2022) or collectively, e.g. through an increase in their aggregation behaviour (Côté and
64 Jelnikar, 1999; Kobak et al., 2010; Kong et al., 2019; Nicastro et al., 2007; Reimer and
65 Tedengren, 1997) although some studies do not report changes in maximum aggregation
66 (Commito et al., 2016; Kong et al., 2019; Nicastro et al., 2007). In addition, mussel
67 displacement (Toomey et al., 2002), attachment strength (Garner and Litvaitis, 2013) and
68 predation risk (Brousseau et al., 2001; DeGraaf and Tyrrell, 2004) change over their benthic
69 lifetime potentially driven by the size — hence the age — of individuals.

70 Critically, individual and collective behaviours based on chemical communication can
71 be hindered by environmental pollutants (*e.g.* Atchison et al., 1987; Fleeger et al., 2003;
72 Lüring and Scheffer, 2007), and plastics are no exception (*e.g.* Mannering, 2021; Rondoni et
73 al., 2021; Shi et al., 2021; Uguen et al., 2022). This chemically diverse group of synthetic
74 polymer-based materials is one of the most ubiquitous sources of contamination and pollution
75 of the Anthropocene (Bergmann et al., 2015; Issifu and Sumaila, 2020; Jambeck et al., 2015)
76 and its deleterious effects on wildlife are now widely recognised (Bucci et al., 2020; Cole et
77 al., 2011; Derraik, 2002). While plastic physical and mechanical consequences have been far
78 more studied, their typically far more pernicious chemical effects have received much less

79 attention (Delaeter et al., 2022; Seuront et al., 2022). Indeed, various additives are generally
80 added to plastic polymers to improve their performances, *e.g.* flexibility, hardness, thermo-
81 resistance or flammable retardance (Delaeter et al., 2022; Thevenon et al., 2015). Overall,
82 more than 6,000 different additives are used in plastic production, and the amount of additives
83 is estimated to reach 2,000 Mt produced by 2050 (Aurisano et al., 2021; Geyer et al., 2017).
84 The leaching into the aquatic environment and the toxicity of these additives are influenced
85 by the plastic itself (*e.g.* polymer type, shape, size, weathering), the nature of additives (*e.g.*
86 types, concentration) and the environmental condition (*e.g.* pH, salinity, temperature; Costa et
87 al., 2023; Sridharan et al., 2022).

88 Leachates of plastic cause adverse effects on mussels at different levels from
89 subcellular and cellular impairments to individual level with notable disturbances on fitness,
90 cell, embryogenic development and behaviour, which could have repercussions at the
91 community level (Capolupo et al., 2021, 2020; Gandara e Silva et al., 2016; Seuront et al.,
92 2021; Trestrail et al., 2020; Uguen et al., 2022). Specifically, the blue mussel (*Mytilus edulis*)
93 behaviour has been shown to be impacted by microplastic (*i.e.* plastic items smaller than 5
94 mm; MP) leachates (Seuront et al., 2021; Uguen et al., 2022). Indeed, *M. edulis* exposed to
95 polypropylene MP leachates (20 mL of MPs L⁻¹, incubation = 24 h) during 8 h showed a
96 change in their collective behaviour with an increase in maximum aggregation, aggregation
97 speed and distance travelled (Seuront et al., 2021). At the individual level, *M. edulis* exposed
98 during 1h30 to polypropylene MP leachates (0.16 – 1.6 mL of MPs L⁻¹; incubation = 24 h)
99 showed a decrease in their motility and anti-predator behaviour (Uguen et al., 2022).
100 However, to the best of our knowledge, the impact of MP leachates on the collective anti-
101 predator behaviour (*e.g.* the ability and celerity to form aggregates) of this keystone engineer
102 species remains unknown.

103 In this context, we tested the hypotheses that (i) the two different sizes of the blue
104 mussel, *Mytilus edulis*, showed a collective response to exposure to cues from their local
105 predator, and (ii) that their potential collective anti-predator behaviours were impaired by MP
106 leachates, as shown at the individual level in previous studies (*i.e.* in *Littorina littorea*,
107 Seuront, 2018, and in small *M. edulis*, Uguen et al., 2022). To test these hypotheses, we
108 quantified their collective behavioural responses following an exposure to cues from the local
109 dominant predatory crab *Hemigrapsus sanguineus*. We subsequently inferred whether
110 leachates from virgin MP polypropylene pellets affected their collective anti-predator
111 behavioural responses.

112

113 2. Material and Methods

114 2.1. Study organism

115 The blue mussel, *M. edulis*, was used as model species due to its ecological and
116 economical importance in coastal intertidal environment. Indeed, as bioengineering species,
117 mussels form dense and dynamic beds that enhance local biodiversity (Borthagaray and
118 Carranza, 2007; Buschbaum et al., 2009). **In addition, mussels also** also hold significant
119 economic value, with their production accounting for one-third of European Union
120 aquaculture production (Avdelas et al., 2021). Thus, *Mytilus edulis* individuals were collected
121 in March 2022 from an intertidal rocky reef, the Pointe aux Oies (Wimereux, France;
122 50°47'08.3"N, 1°36'03.9"E), located along the French coasts of the eastern English Channel.
123 Two different sizes were considered, *i.e.* small mussels (shell length: 1.5 – 2.5 cm, n = 360) as
124 *Hemigrapsus sanguineus*'s preferred prey (Brousseau et al., 2001) and large mussels (shell
125 length: 4.0 – 5.0 cm, n = 360) as commercial mussels (*i.e.* > 4 cm; Seuront et al., 2019).
126 Mussels from both different sizes were acclimated in the laboratory in distinct 85 L tanks

127 filled with aerated running natural seawater representative of in situ conditions ($T = 12^{\circ}\text{C}$, $S =$
128 33‰) for 24 h under a natural 12:12 h light-dark cycle before the behavioural assays took
129 place; an acclimation time typically used in a range of studies on mussel, *e.g.* Auguste et al.,
130 2020; Balbi et al., 2017; Seuront et al., 2021; Uguen et al., 2022). Adult individuals of the
131 Asian shore crab (*Hemigrapsus sanguineus*, $n = 144$, 2.1 ± 0.3 cm in carapace width), one of
132 the dominant mussel predators on rocky shores where this species is now well established
133 (DeGraaf and Tyrrell, 2004; Enderlein et al., 2003; Rolet et al., 2020) were sampled at the
134 same location as the mussels, sorted by sex and acclimated separately in 85 L tanks filled with
135 running aerated natural seawater ($T = 12^{\circ}\text{C}$, $S = 33\text{‰}$) for 2 days. The acclimation period of
136 48 h was chosen following Uguen et al. (2022) to stimulate predator-induced alarm response
137 by feeding them *ad libitum* with mussels and food consumption was enhanced by keeping
138 them in darkness (Spilmont et al., 2015; Uguen et al., 2022). To feed the crabs, mussels were
139 crushed with a natural stone purposely chosen to avoid the use of metal objects and the
140 potentially confounding effect of contamination from metal ions (Commito et al., 2016).

141

142 **2.2. Treatment preparation**

143 The microplastic (MP) leachate treatment was prepared using commercially available
144 virgin polypropylene (PP) pellets. PP is the most produced polymer type worldwide with
145 19.3% of the world plastic production (data from 2021; PlasticsEurope, 2022). The virgin PP
146 non-uniform microbeads used (white colour, 4.04 ± 0.60 mm longest dimension measured on
147 a sample of 50 particles; Pemmiproductions, Aachen, Germany; for a picture see Supplementary
148 Material, S1) were incubated during 24 h in aerated natural unfiltered seawater at a relatively
149 high but locally realistic concentration (Seuront, 2018), *i.e.* 12 g of pellets per litre (20 mL of
150 MPs per L which after counting is equivalent to 404 ± 40 MPs per L). Indeed, plastic pellets

151 pollution is estimated as ca. 17,000 – 165,000 tons per year in Europe (Hann et al., 2018).
152 Plastic pellet transport accidents are frequent. For instance, in 2016 at the study site used in
153 our study a tank-truck lost 8 tons of plastic pellets, leading to high contamination of the
154 coastline even many years after the accident (Gravier and Haut, 2020). Marine organisms may
155 even experience more extreme concentrations, such as seen in Sri Lanka with up to 85 g of
156 pellets per kg of sediment after the spill-over at sea of containers carrying 78 tonnes of plastic
157 pellet in 2021 (Jayathilaka et al., 2022). To study the response of mussels to chemical cues
158 from their predator, a solution of crab cues was used. Crabs were not placed directly in the
159 arena to avoid water movement which could cause mussel rheotactic behaviour (*i.e.*
160 movement towards or away from the water flow). Thus, the crab cue treatment was prepared
161 by adding 3 males and 3 females into 1 L of aerated unfiltered seawater for a 24 h period
162 (Uguen et al., 2022). To prepare the mixed treatment (*i.e.* crab cues + MP leachates), 6 males
163 and 6 females were placed into 1 L of aerated unfiltered seawater for a 24 h period. This
164 doubled crab solution was then mixed at a 1:1 ratio with a double MP leachate solution
165 prepared as above but at a concentration of 24 g of pellets per litre in order to obtain a final
166 concentration comparable to MP leachates and crab cues single stress (Seuront, 2018). The
167 seawater used was pumped directly from the collection site and was not filtered in order to
168 mimic the conditions encountered by our experimental mussels *in situ* and during their
169 acclimation period, and to avoid generated any additional stress to the mussels due to a lack of
170 phytoplankton.

171 The additive content of the polypropylene pellets was analysed with a pyrolysis
172 analysis coupled to a gas chromatography and a high-resolution spectrometer as described in a
173 previous study (Uguen et al., 2022) before and after their 24h-incubation, for more details on
174 the method used, see Supplementary Material, S2A. The additives identified were: 3
175 brominated flame retardants, 6 phosphorus flame retardants, 5 antioxidants, 13 plasticisers.

176 Specifically, the additives the most released were (i) phthalate plasticisers, as Diisononyl
177 phthalate (DINP) Diisoheptyl phthalate (DIHP), Diisodecyl phthalate (DIDP), Diisononyl
178 hexahydrophthalate (DINCH), Benzylbutyl phthalates (BBP) and in a lesser extent, Diethyl
179 phthalate (DEP) and Dibutyl phthalate (DBP); (ii) the antioxidants: nonylphenol (NPs) and
180 nonylphenol monoethoxylate (NP10E); and (iii) the phosphorus flame retardants: tributyl
181 phosphate (TBP) and Tris(2-Chloroisopropyl)phosphate (TCPP; Supplementary Material,
182 S2B).

183 **2.3. Experimental design**

184 For the two different sizes, mussels ($n = 15$) were placed in 22 cm diameter \times 5 cm
185 high glass arenas with smooth, featureless transparent surfaces under static conditions to
186 avoid passive movements of mussels by water currents (Commito et al., 2016, 2014; Nicastro
187 et al., 2007). Each arena was filled with either 1 L of control or treatment seawater (Crab
188 cues, MP leachates and Crab cues + MP leachates) and the experiment started once the
189 mussels were placed in these arenas, *i.e.* directly after the arena has been filled. Note that
190 before being used to fill the arenas, all treatments were sieved to ensure that no crab or plastic
191 particles were present in the solutions.

192 Following Seuront et al. (2021), mussels were placed, around 1 body size from each
193 other, in concentric circles on their right anterior part, the narrow end facing the centre of the
194 arena (for more details on experimental design see Supplementary Material, S3). Each
195 individual mussel was only used once, prior to the experiment, byssus threads were carefully
196 cut with a pair of scissors to separate the mussels from each other and to allow mussels to
197 establish primary byssus attachment (Kong et al., 2019; Nicastro et al., 2007). A previous
198 study showed that the typical time needed by *M. edulis* to reach a steady-state in their

199 aggregation rate is in the first 2 h of the experiment (Seuront et al., 2021), thus the experiment
200 was run for 2 h.

201 This behavioural experiment was performed on the same day with 6 replicates per
202 treatment for each of the two different sizes at the natural temperature experienced by
203 organisms during this season, *i.e.* 12°C, in a temperature-controlled room under constant
204 conditions of dim cold light illumination and in the absence of any acoustic or mechanical
205 disturbance. Video recording started immediately after all mussels were placed in the arena at
206 a rate of 1 frame per min for 2 h using a GoPro camera (GoPro HERO8 Black, GoPro Inc.,
207 San Mateo, California, USA; see Supplementary Material, S4; S5 for examples) placed 40 cm
208 above the experimental arena.

209

210 **2.4. Behavioural variables**

211 Mussels were visually classified as either solitary or aggregated, *i.e.* two or more
212 mussels with their shells in direct physical contact (Seuront et al., 2021). Aggregation
213 behaviour was quantified at 5 min intervals as the proportion (%) of mussels actively forming
214 aggregates (Seuront et al., 2021). Additionally, three parameters were used to quantitatively
215 assess the aggregation dynamics: the starting time (*i.e.* the time needed to form the first
216 aggregation within an arena), A_{\max} (%; *i.e.* the maximal proportion of aggregation) and k (min;
217 *i.e.*, the time when the proportion of aggregation was half the maximum value; $A(t) = A_{\max}/2$).
218 Indeed, once the first aggregation was formed, the proportion of aggregated mussels as a
219 function of time was fitted with a nonlinear Michaelis-Menten type equation as $A(t) = A_{\max}t/(k$
220 $+ t)$ (Seuront et al., 2021). For each replicate, the non-linear least-squares method was used to
221 estimate A_{\max} and k in order to maximise the coefficient of determination r^2 and to minimise

222 the sum of squared residuals among empirical data. In the rare cases when the model
223 indicated an A_{\max} higher than 100 % ($n = 4$; 8% of the total), the model was manually forced
224 to the maximum aggregation observed. In addition, the arenas were virtually grid-lined on the
225 computer in order to calculate the cluster score at the end of the experiment. This index was
226 calculated, as the maximum number of mussels positioned in a given quadrat divided by the
227 overall number of quadrats they occupied; see *e.g.* Collins et al. (2011), da Costa Araújo and
228 Malafaia (2021), de Faria et al. (2018), Freitas et al. (2023) and Guimarães et al. (2023) for
229 further details and Supplementary Material, S6, for an illustration of the method used.

230 For each treatment and for each arena, the average gross distance (*i.e.* the total
231 distance covered by the mussel between its initial and final position), the average net distance
232 (*i.e.* the distance between its initial and final position) and the average confinement index (*i.e.*
233 the ratio between net distance and gross distance) were measured using the TrackMate plugin
234 of ImageJ (Tinevez et al., 2017). Additionally, for each arena, the total byssus thread
235 production was assessed by counting the number of byssal threads attached to the substratum
236 or to conspecifics at the end of the 2 h experiment.

237 Finally, we assessed the taxis towards conspecifics (*i.e.* attraction) through the
238 presence of a significant positive correlation between the maximum aggregation (A_{\max}) and
239 the distance (gross or net; for a schematic representation, see Supplementary Material, S7).
240 The aggregation strength was subsequently assessed through the presence of a significant
241 positive correlation between byssus thread production and A_{\max} , *i.e.* the more the mussels
242 aggregate, the greater the number of byssal threads produced, which suggests a strong
243 collective binding force (for a schematic representation, see Supplementary Material, S8).

244

245 **2.5. Statistical analyses**

246 As the sample sizes were small ($n = 6$) the use of non-parametric tests is highly
247 recommended (Abdulazeez, 2014; Gibbons and Fielden, 1993; Hoskin, 2012). The normality
248 (Shapiro's test) and heterogeneity of variances (Levene's test) results are given in the
249 Supplementary Material, S9 and S13. Significance levels were set at $p < 0.05$. All statistical
250 analyses were performed using the software R Core Team (2022).

251 First, to infer the effect of treatments on the behaviour of the mussel, a series of
252 Kruskal-Wallis test (KW test hereafter) were performed for each of the two different sizes
253 with treatment as factor (4 levels: Control, MP leachates, Crab cues, Crab cues + MP
254 leachates) and the different behaviours as dependant variables (Starting time, Gross and Net
255 distances, Confinement index, Byssus, Amax, k, Cluster score). This test was followed by a
256 Conover test with Holm's correction to identify distinct groups of measurements (Conover's
257 test hereafter; Dolgun and Demirhan, 2017). Secondly, to detect the presence of an attraction
258 between conspecifics, and aggregation strength, a series of Pearson correlation tests were
259 performed between behavioural variables for each treatment and each size. Finally, to test the
260 effect of size (small and large) a series Wilcoxon-Mann-Whitney test with continuity
261 correction were used for each behavioural variable (WMW test hereafter; Zar, 1999), except
262 the cluster score to avoid a bias as the same quadrat size was used for both small and large
263 mussels (Supplementary Material, S6).

264

265 **3. Results**

266 Treatment had a significant effect on the starting time (KW test, $H = 17.22$, $df = 3$, $p =$
267 0.0006) and gross distance (KW test, $H = 8.61$, $df = 3$, $p = 0.035$) in large mussels but not in

268 small ones (*i.e.* starting time: KW test, $H = 3.42$, $df = 3$, $p = 0.33$; gross distance: KW test, H
269 $= 3.77$, $df = 3$, $p = 0.29$; Supplementary Material S9). No significant effect between different
270 treatments (control; MP leachates; crab cues; mixture of crab cues and MP leachates) was
271 found in both small and large mussels: net distance (KW test, $H = 4.67$, $df = 3$, $p = 0.20$; $H =$
272 5.17 , $df = 3$, $p = 0.16$, respectively), confinement index (KW test, $H = 1.79$, $df = 3$, $p = 0.62$; $H =$
273 3.04 , $df = 3$, $p = 0.38$, respectively), byssus thread production (KW test, $H = 5.70$, $df = 3$, p
274 $= 0.13$; $H = 5.84$, $df = 3$, $p = 0.12$, respectively; Supplementary Material, S10), maximum
275 aggregation (A_{\max} ; KW test, $H = 1.96$, $df = 3$, $p = 0.58$; $H = 4.85$, $df = 3$, $p = 0.183$,
276 respectively), aggregation speed (k ; KW test, $H = 3.14$, $df = 3$, $p = 0.37$; $H = 0.45$, $df = 3$, $p =$
277 0.93 , respectively) and cluster score (KW test, $H = 3.10$, $df = 3$, $p = 0.38$; $H = 0.74$, $df = 3$, p
278 $= 0.86$, respectively; Supplementary Material S9).

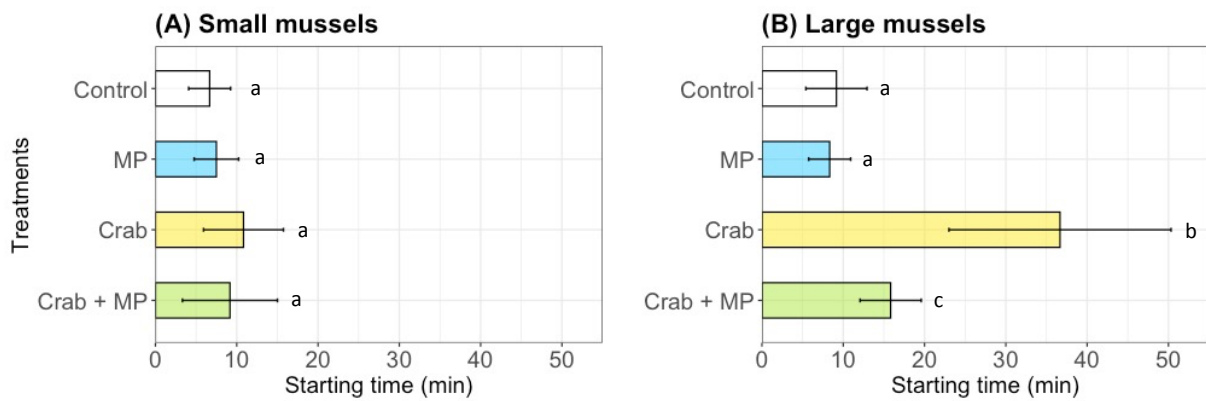
279

280 **3.1. Behavioural effect of microplastic leachates**

281 Treatment had a significant effect on both the starting time (KW test, $H = 17.22$, $df =$
282 3 , $p = 0.0006$) and the gross distance of large mussels (KW test, $H = 8.61$, $df = 3$, $p = 0.035$)
283 (Supplementary Material, S9). In addition, the starting time and gross distance of large
284 mussels bathed with MP leachates were not significantly different from those from control
285 treatment (Conover's test, $p = 0.62$ and $p = 1.00$, respectively; Fig. 1B and 2B; Supplementary
286 Material, S9). In contrast to large ones, small mussels showed both an attraction to
287 conspecifics and a greater aggregation strength in the MP leachate treatment compared to the
288 control treatment (Supplementary Materials, S11, S12). Specifically, when small mussels
289 were exposed to MP leachates, A_{\max} was significantly positively correlated with the number of
290 byssal threads ($p = 0.009$). This result indicates that when small mussels aggregated, they
291 have produced more byssus threads, *i.e.* they attached more to conspecifics in MP leachate

292 treatments. In addition, A_{\max} was also significantly positively correlated with the gross and net
 293 distances ($p = 0.014$ and $p = 0.012$, respectively), indicating that when (net and gross)
 294 distances increased, maximum aggregation increased. Thus, small mussels showed directional
 295 movements to conspecifics in MP leachate treatments.

296

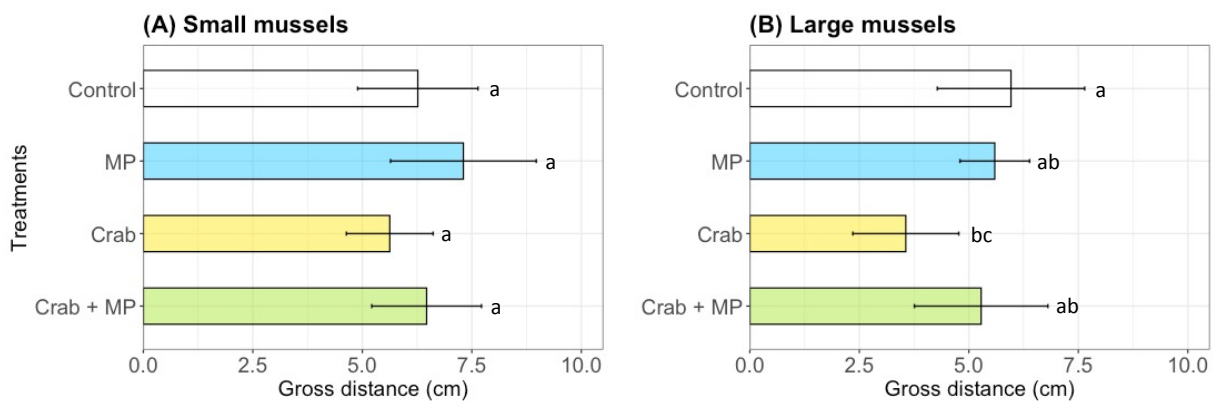


297

298 **Figure 1:** Starting time in minutes (min; mean \pm SD) taken by (A) small and (B) large mussels to form
 299 the first aggregation in control seawater (white; $n = 6$), MP leachates (blue; $n = 6$), crab cues (yellow;
 300 $n = 6$), and 1 : 1 MP leachates and crab cues treatment mixture (green; $n = 6$). Letters depict significant
 301 differences ($p < 0.05$; Conover's test) among treatments.

302

303



304

305 **Figure 2:** Gross distance in centimetres (cm; mean \pm SD) travelled by (A) small and (B) large mussels
 306 in control seawater (white; $n = 6$), MP leachates (blue; $n = 6$), crab cues (yellow; $n = 6$), and 1 : 1 MP
 307 leachates and crab cues treatment mixture (green; $n = 6$). Letters depict significant differences ($p <$
 308 0.05 ; Conover's test) among treatments.

309

310 **3.2. Behavioural effect of crab cues**

311 *Hemigrapsus sanguineus* cues had a significant size-specific effect on the collective
312 behaviour of the blue mussel *Mytilus edulis* (Figs. 1 & 2; Supplementary Material S9). For
313 large mussels, crab cue treatment had a significant effect on starting time (KW test, $H =$
314 17.22 , $df = 3$, $p = 0.0006$) and gross distance (KW test, $H = 8.61$, $df = 3$, $p = 0.035$). In
315 particular, when exposed to crab cues, the time needed by large mussels to form the first
316 aggregation was highly significantly longer (*i.e.* 4-fold) than in the control treatment
317 (Conover's test, $p = 2.4 \times 10^{-5}$; Fig. 1B). Similarly, the average mussel gross distance was
318 significantly lower (*ca.* 2-fold) than in the control treatment (Conover's test, $p = 0.035$; Fig.
319 2B). In contrast to observations conducted in control seawater, large mussels exposed to crab
320 cues exhibited a taxis towards conspecifics, as shown by the significant positive linear
321 correlations between both gross and net distances with A_{\max} ($p = 0.020$ and $p = 0.023$;
322 Supplementary Material S12). Finally, the observed significant positive linear correlation
323 between A_{\max} and byssus thread production ($p = 0.013$; Supplementary Material S12), is
324 indicative of a greater aggregation strength in the presence of crab cues compared to control
325 seawater. Small mussels, in agreement to observations conducted on large mussels, exhibited
326 a significant ($p = 0.028$; Supplementary Material S11) positive linear correlation between the
327 gross distance and A_{\max} , thus a taxis behaviour towards conspecifics when exposed to crab
328 cues compared to control treatment.

329

330 **3.3. Behavioural effect of a microplastic leachate - crab cue mixture**

331 When exposed to a mixture of crab cues and MP leachates, large mussels showed
332 weaker behavioural responses than after an exposure to crab cues alone (Figs. 1B & 2B).

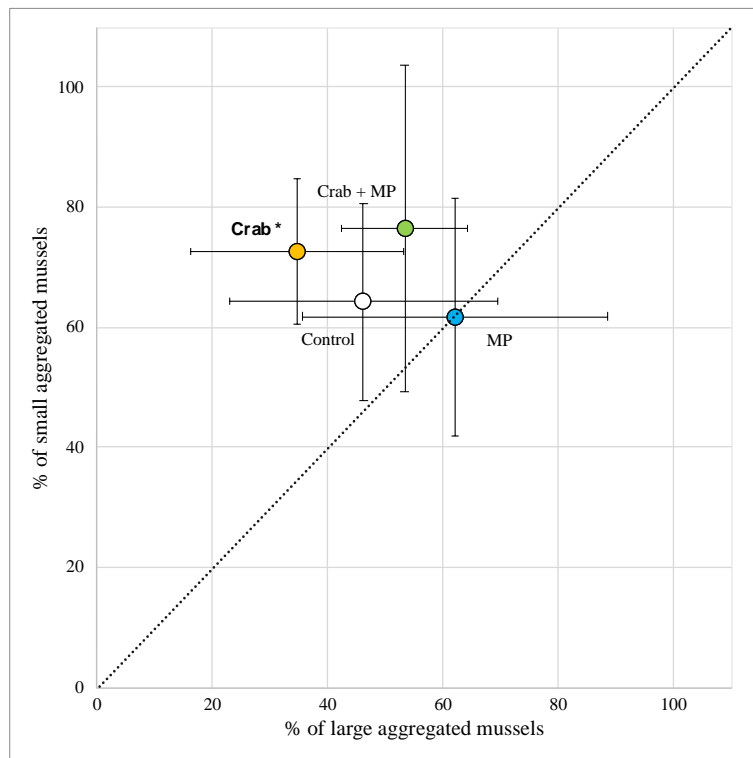
333 Specifically, though large mussels showed a highly significantly longer starting time than in
334 control seawater and MP leachates (Conover's test, $p = 0.0066$ and $p = 0.0027$ respectively;
335 Fig. 1B), they were highly significantly slower (*c.a.* 2.3-fold) to form the first aggregate than
336 when exposed to crab cues alone (Conover's test, $p = 0.028$; Fig. 1B). In contrast, although
337 the gross distance increased 1.5-fold when exposed to a mixture of crab cues and MP
338 leachates compared to crab cues alone, this treatment did not significantly differ from all other
339 treatments, *i.e.* control, MP leachate, crab cue treatments (Conover's test, $p = 1.00$, $p = 1.00$
340 and $p = 0.115$ respectively; Fig. 2B). Finally, when exposed to a mixture of crab cues and MP
341 leachates, large mussels showed a weaker taxis behaviour towards conspecifics (only one
342 significant correlation was found: between net distance and A_{\max} ; $p = 0.023$; Supplementary
343 Material S12) and a loss in aggregation strength compared to mussels exposed to crab cues.
344 For small mussels, an exposition to a mixture of crab cues and MP leachates leads to a loss in
345 their taxis behaviour towards conspecifics that was observed when exposed to crab cue alone.

346

347 **3.4. Large vs. small mussel behaviour**

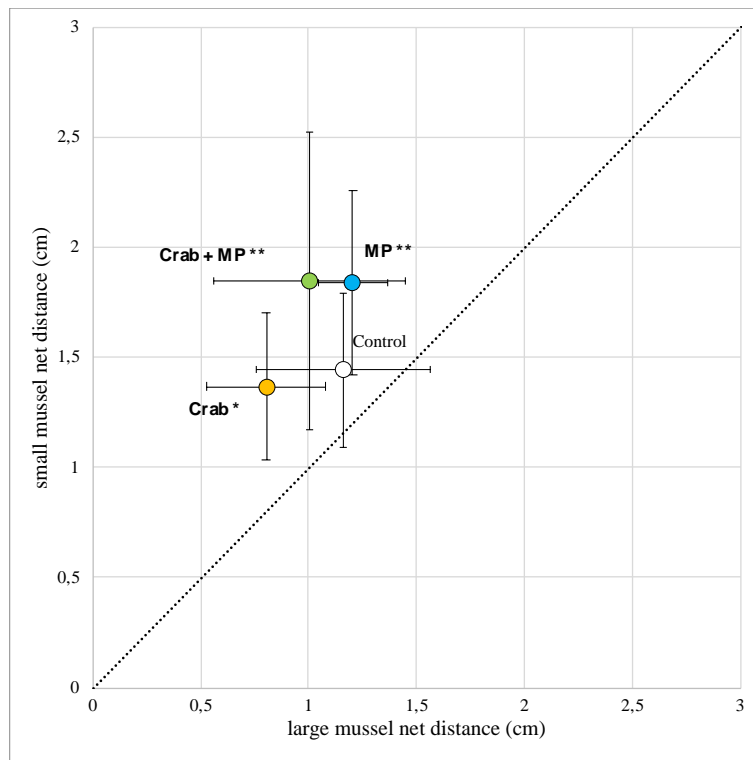
348 Although in the control, none of the behavioural variables considered significantly
349 differed between large and small mussels (WMW test, Supplementary Material, S13), this
350 was not the case for the other treatments. When exposed to crab cues, large mussels covered
351 significantly lower gross and net distances than small ones (*i.e.* 3.56 ± 1.21 vs. 5.63 ± 0.99 cm
352 for gross distance and 0.80 ± 0.28 cm vs. 1.37 ± 0.34 cm for net distance; WMW test, $p =$
353 0.013 and $p = 0.020$, respectively; Figs. 2 & 4; Supplementary Material S13). For the same
354 treatment, large mussels compared to small ones also significantly delay their starting time
355 (36.67 ± 13.66 vs. 10.83 ± 4.92 min; WMW test, $p = 0.007$; Fig. 1) to aggregate and, reached
356 a significantly lower maximum aggregation (A_{\max} : $34.74 \pm 12.08\%$ vs. $72.63 \pm 18.54\%$;

357 WMW test: $p = 0.02$; Fig. 3; Supplementary Material S13). Moreover, small mussels covered
 358 significantly more net distances than large ones when exposed to MP leachates (1.84 ± 0.42
 359 cm vs. 1.21 ± 0.16 cm; WMW test: $p = 0.008$) and to the mixture of MP leachates and crab
 360 cues (1.85 ± 0.67 cm vs. 1.01 ± 0.45 cm; WMW test: $p = 0.008$; Fig. 4; Supplementary
 361 Material S13).
 362



363
 364 **Figure 3:** Maximal proportion of aggregation (A_{max} ; %) at the end of the 2 h experiment for small and
 365 large *Mytilus edulis* in control seawater (white), MP leachates (blue), crab cues (yellow) and 1 : 1 MP
 366 leachates and crab cues mixture (green). Treatment name in bold indicates the presence of significant
 367 differences between small and large mussels (*: $p < 0.05$; Wilcoxon-Mann-Whitney test). The dotted
 368 line is the first bissectrix, *i.e.*, % of small aggregated mussels = % of large aggregated mussels, and the
 369 error bars are the standard deviations. Thus, the further the points (treatments) are from the first
 370 bissectrix (dotted line), the more the behavioural response differs between large and small mussels.
 371

372



373

374 **Figure 4:** Net distance (cm) travelled by small and large *Mytilus edulis* in control seawater (white),
 375 MP leachates (blue), crab cues (yellow) and 1 : 1 MP leachate and crab cue treatment mixture (green).
 376 Treatment names in bold indicate the presence of significant differences between small and large
 377 mussels (*: $p < 0.05$; **: $p < 0.01$; Wilcoxon-Mann-Whitney test). The dotted line is the first
 378 bissectrix, *i.e.*, small mussel net distance = large mussel net distance, and the error bars are the
 379 standard deviations. Thus, the further the points (treatments) are from the first bissectrix (dotted line),
 380 the more the behavioural response differs between large and small mussels.

381

382

383

384 4. Discussion

385 4.1. Microplastic leachates affect only the collective behaviour of small mussels

386 Exposure to leachates of virgin polypropylene MP pellets did not affect the collective
 387 behaviour of large mussels. In contrast, small mussels were sensitive to these MP leachates. In
 388 particular, their movements were more directional, they showed signs of taxis towards
 389 conspecifics and also a greater aggregation strength than under control conditions.
 390 Additionally, small mussels travelled a greater net distance than large ones when exposed to
 391 MP leachates. Noticeably, the absence of taxis behaviour towards conspecifics found in the
 392 control experiment suggests that even if mussels can detect each other (de Vooy, 2003), they

393 do not have particular benefits (*e.g.* reproduction, anti-predator behaviour, protection against
394 storm) to move towards their conspecifics and increased their aggregation strength in the
395 control at this time of the year (March – relatively calm weather, no reproduction). However,
396 the presence of stress, such as here MP leachates, may increase mussel cooperation
397 (aggregation and attachment to neighbours; de Jager et al., 2017). The aggregation strength, a
398 cooperative behaviour which provides a mutual protection by increasing the number of byssus
399 threads produced according to the number of neighbours, found here in small mussels when
400 exposed to MP leachates is consistent with the finding of de Jager et al. (2017) which showed
401 an increase of this parameter in stressful conditions. In contrast, no byssal alteration
402 production nor aggregation strength in large mussels were altered, Seuront et al., (2021) also
403 found no effect of MP leachates on byssal production in large (3 – 4 cm) *M. edulis*. Although
404 MP leachates do not appear to affect large *M. edulis* byssal thread production, mechanical
405 byssal thread characteristics may be altered, *e.g.* adhesion strength, as shown in another
406 mussel species (*Mytilus coruscus*) when exposed to MP particles (Shang et al., 2021).

407 The overall lack of response from large mussels when exposed to MP leachates
408 compared to small ones can be interpreted as a difference in chemical sensitivity which can be
409 due to a dilution by growth (Jager, 2013). Indeed, an exposure to the same concentration of
410 contaminant leads to a higher internal concentration of contaminant in smaller organisms than
411 in larger ones, the latter having a larger volume, the contaminant will be more strongly diluted
412 in its body than in smaller organisms (Jager, 2013). Thus, the threshold concentration of MP
413 leachates to induce a behavioural response in mussel could be reached here in small *M. edulis*
414 but not in larger ones with the concentration used in this study, *i.e.* 12 g of virgin
415 polypropylene pellets per litre. Size is well documented to affect the response to pollutants,
416 with evidence for a greater sensitivity to pollutants in smaller individuals in various taxa (*e.g.*,
417 Alonso et al., 2010; Coeurdassier et al., 2004; Kiffney and Clements, 1996; Luan et al., 2019;

418 Orr et al., 2022). This highlights the importance of the selected life stage to assess ecological
419 risk of a contaminant.

420

421 No differences were found for the two mussel sizes in the maximum aggregation
422 between the control and the MP leachate treatments, despite the observed attraction behaviour
423 of small mussels towards conspecifics. This is in contradiction with previous observations
424 conducted on *M. edulis* in the 3 – 4 cm size range, which significantly increased their
425 maximum aggregation when exposed to polypropylene leachates (Seuront et al., 2021). The
426 significant differences observed between the seasons when the experiments took place (*i.e.*
427 October 2018 *vs.* March 2021), in our study and previous work may, however, partially
428 explain this discrepancy. Indeed, the existing differences in experimental thermal conditions,
429 *i.e.* 12°C for our experiment in March 2021 and 18°C for Seuront et al. (2021) experiment
430 conducted in October 2018, and also thermal history, the French coasts of the eastern English
431 Channel suffered a heat wave in August 2018 that led to *M. edulis* mass mortality (Seuront et
432 al., 2019) may have contributed to weaken the mussels, making them more sensitive in their
433 study compared to ours. In addition to these thermal differences, the discrepancies between
434 our studies may also be related to the mussel reproductive stages. Indeed, *M. edulis* spawning
435 events at the study site typically take place continuously from May to September (Uguen et
436 al., unpublished data), our study takes place before the onset of spawning events (March) and
437 Seuront et al. (2021) study after it (October). *M. edulis* reproduction is energetically costly
438 (Bayne et al., 1983) and after spawning *M. edulis* energetic reserves are low which make them
439 less resistant to stress (Tremblay et al., 1998). Thus, post-spawning weakness may also be
440 responsible for a higher sensitivity to MP leachates contaminants found in the study by
441 Seuront et al. (2021) compared to ours. The sensitivity of *M. edulis* to microplastic-borne
442 pollutants might be much more complex than previously thought, highlighting the need for

443 further work to understand the impacts of reproductive stages and heat stress on the mussel
444 sensitivity to MP leachates.

445 Another hypothesis to this lack of difference in the maximum aggregation, is that the
446 aggregation benefit – cost balance was already reached under the control conditions used in
447 our study. Indeed, aggregations are not uniquely beneficial, if the aggregation maximum is
448 already high enough, there will be no additional individual benefit from aggregating more but
449 rather disadvantages, *e.g.* increased competition for food and space, decreased in condition
450 index and growth rate, and thus despite an additional stress (Bertness and Grosholz, 1985;
451 Commito et al., 2016; Cubillo et al., 2012; van de Koppel et al., 2008; Kong et al., 2019). The
452 maximum aggregations observed under our control condition were high (*i.e.* 64.34 ± 23.14 %
453 and 46.18 ± 16.41 % for small and large mussels, respectively) and were similar to the
454 maximum aggregation found in *M. edulis* mussels when exposed to stress, *i.e.* around 60% for
455 small mussels exposed to predator cues (Reimer and Tendengren, 1997; Côté and Jelnikar,
456 1999) and around 40% for large mussels when exposed to MP leachates (Seuront et al., 2021).
457 Thus, *ca.* 60% and 40% may be the thresholds in the aggregation benefit – cost balance for
458 small and large mussels respectively and an additional source of stress such as MP leachates
459 is unlikely to trigger any additional aggregation.

460

461 **4.2. Small and large mussels show different collective anti-predator behaviours in** 462 **response to crab cues**

463 Noticeably, both large and small mussels adapt their collective behaviour to chemical
464 cues from their predator, the Asian shore crab, but not in the same way. For both mussel sizes,
465 predator cues seem to be perceived by the mussels as a stress as they stimulate a taxis
466 behaviour towards conspecifics. In contrast to small individuals, large mussels decreased their
467 gross distance, delayed their starting time to form aggregation and increased the strength of

468 their aggregation compared to control seawater. Large mussels also travelled over
469 significantly smaller gross and net distances, started to aggregate much latter and aggregated
470 less than small ones.

471 The observed reduction in activity of large mussels is consistent with an anti-predator
472 strategy (Clements et al., 2020; Garner and Litvaitis, 2013; Ishida and Iwasaki, 2003; Uguen
473 et al., 2022). It allows the preys to reduce their probability of being detected and thus captured
474 by a predator. A decrease in their movements (Reimer and Tedengren, 1997) and gross
475 distance (Uguen et al., 2022) were observed in *Mytilus edulis* when exposed to predator cues.
476 In addition, despite smaller distances travelled and longer times to start aggregating, a
477 maximum aggregation was found similar to our control treatments. This is consistent with a
478 taxis behaviour towards their conspecifics, hence can be seen as part of an anti-predatory
479 strategy. This behaviour allows, despite a reduction of their activity, to maintain a maximum
480 aggregation similar to that observed under control conditions. The absence of an increase in
481 large mussel maximum aggregation when exposed to predator cues is in accordance with
482 previous studies conducted on large *Mytilus* sp. (Kong et al., 2019, on *M. edulis*; Nicastro et
483 al., 2007, on *M. galloprovincialis*). Indeed, large mussels, because of their size, may not
484 necessarily need to form larger aggregations to protect themselves from predators as *H.*
485 *sanguineus* feeds mainly on small individuals. However, large mussels increase their byssus
486 attachment when exposed to predators (Côté, 1995; Kong et al., 2019; Leonard et al., 1999).
487 These observations are consistent with our study as we found a collective solidification of
488 aggregations in large mussels exposed to crab cues. Thus, the most effective protection
489 strategy for large mussels would be to reduce their activity through limited but directed
490 displacement towards their conspecifics and form more tightly bound aggregations.

491 Small mussels do not show a reduction in their activity when exposed to crab cues,
492 although small mussels have been shown to decrease their gross distance when exposed to

493 crab cues (Uguen et al., 2022). In this present work, collective behaviour study, the presence
494 of conspecifics combined with stress (here predator cue pressure) may stimulate mussel
495 displacement, which is supported by the observed taxis behaviour toward conspecifics. This
496 behaviour, however, did not lead to an increase in the maximum aggregation in small *M.*
497 *edulis* exposed to predator cues, which contrasts with two other studies (Côté and Jelnikar,
498 1999; Reimer and Tedengren, 1997). Increasing the maximum of aggregation is an effective
499 protection method against predators, but when this maximum is high enough, increasing it
500 further will not provide any additional benefit but rather high costs (Bertness and Grosholz,
501 1985; Commito et al., 2016; Cubillo et al., 2012; van de Koppel et al., 2008). The maximum
502 aggregation when exposed to crab cues in our study is 72.63 ± 18.64 %, which is consistent
503 with the maximum aggregation found for other small *Mytilus edulis* exposed to predator cues,
504 *i.e.* around 60% (Côté and Jelnikar, 1999; Reimer and Tedengren, 1997). Thus, the
505 aggregation benefit – cost balance threshold was potentially already reached in our study.

506 Among the differences in anti-predator behaviour observed between our two mussel
507 sizes, no strong reduction in the small mussel activity was observed compared to large ones.
508 In contrast, small mussels travelled greater distances, and form aggregations earlier and much
509 larger than large mussels. As a consequence, and in contrast to small mussels, the anti-
510 predator behaviour of large mussels is relatively similar to the anti-predator behaviour
511 displayed by solitary mussels, *i.e.* a reduction in activity (Uguen et al., 2022). The dilution
512 effect and the protection by conspecifics in aggregations is much more advantageous in small
513 mussels, which have a lower survival probability than large ones by remaining solitary as *H.*
514 *sanguineus* feeds mainly on smaller individuals (Brousseau et al., 2001; DeGraaf and Tyrrell,
515 2004). In addition, the cost of movement is much lower in small mussels than in large ones,
516 thus, the collective anti-predator response of small mussels may be more related to the
517 formation of aggregates based on displacement.

518

519 **4.3. Small and large mussel collective anti-predator behaviours are differently altered by**
520 **MP leachates**

521 Finally, the MP leachate and crab cue mixture led to a mitigation of the collective anti-
522 predator response of large mussels, while small mussels showed no response at all.
523 Specifically, for large mussels, the lower gross distance travelled, the longer time to start to
524 aggregate and the taxis behaviour towards conspecifics were attenuated while the aggregation
525 strength was inhibited by the addition of plastic leachates. For small mussels especially, their
526 unique anti-predator response (*i.e.* a taxis behaviour towards conspecifics) was totally
527 inhibited by the addition of plastic leachates. Similar results showing an impairment in anti-
528 predator behaviour have been reported (i) at the collective level in various organisms exposed
529 to plastic particles, e.g. mice (da Costa Araújo and Malafaia, 2021; Guimarães et al., 2023),
530 tadpoles (da Costa Araújo and Malafaia, 2020) and freshwater fish (da Costa Araújo et al.,
531 2020), and (ii) at the individual level when exposed to MP leachates in the gastropod *Littorina*
532 *littorea* (Seuront, 2018) and in small mussel, *M. edulis* (Uguen et al., 2022). Indeed, these two
533 last studies demonstrated that when exposed to crab cues with polypropylene leachates, *L.*
534 *littorea* showed an alteration of their righting time, skioptic withdrawal, time to explore, and
535 avoidance response compared to an exposition to predator cues only (Seuront, 2018) and
536 small *M. edulis* presented an alteration in their motility and in their negative chemotactic
537 behaviour toward crab cues (Uguen et al., 2022). Partial and total loss of prey anti-predator
538 behaviours can be related to a disruption of the chemical communication chain (*i.e.* cue
539 release, transmission and/or reception) by this anthropogenic compound (Olsén, 2011). Thus,
540 these anti-predator behavioural alterations may be due to the prey inability to recognise the
541 chemical cues of its predator which is linked to an interaction of MP leachates (i) with crab
542 cue molecules directly in the water leading to a modified cue, or (ii) with the prey sensory

543 system, hindering the cue reception and/or perception abilities of predator cues (Olsén, 2011).
544 The latter hypothesis is supported by a recent study that showed the disruption of the hermit
545 crab (*Pagurus bernhardus*) cognition, *i.e.* assessment and/or decision-making, in a shell
546 selection test when exposed to MP leachates (Crump et al., 2023). They hypothesised, as in
547 Uguen et al. (2022), that the MP leachates caused a neurosensory disruption, an hypothesis
548 that is also supported here by the phthalates contained in our leachates which are known to be
549 neurotoxic in a wide variety of species (*e.g.* DINP, BBP, DEP, DBP; for reviews, see: Shincy
550 and Chitra, 2020; Zhang et al., 2021). Additional experiments are nevertheless required to
551 further investigate these assumptions and to understand the detailed mechanisms underlying
552 these processes.

553 The partial and total loss of the anti-predator behaviour of large and small
554 mussels respectively, suggest that smaller mussels are the most sensitive to the contaminant,
555 in addition to being the most at risk of being predated. In a broader context, the lack and
556 mitigation of an anti-predator response for both mussel sizes when in presence of plastic
557 pollution, could fundamentally represent a crucial evolutionary and ecological change at the
558 species level. Indeed, by modifying predator-prey interactions through the predator detection,
559 the avoidance effectiveness and the ability to escape of the prey, pollutants may cause
560 changes at population and community levels (Weis et al., 2001). Vulnerability to MP
561 leachates and predation by crabs, which both act in a size-selective manner to the detriment of
562 small mussels, can affect the abundance and size structure of the mussel population. Small
563 mussels, the favourite crab preys, are thus more impacted by MP leachates pollution than
564 large mussels, leading to an inhibition of their anti-predator collective response. This could
565 lead to an excessive mortality of small mussels, and lead in the short term to a shift of the
566 mussel size-frequency distribution to the right in favour of large mussels, but in the long term
567 to a depletion of the mussel stock as the number of large mussels could be reduced, limiting

568 the reproductive effort (i.e., the largest individuals contributing the most to the egg output;
569 Sprung, 1983). Note that large mussels are not spared by the effect of leachates towards their
570 behavioural anti-predator response, but their mortality can be expected to be lower than for
571 small mussels. Thus, the sensitivity of individuals to pollutants may impact the sensitivity at
572 the population level (Gergs et al., 2013), which could lead to a risk of local extinction of
573 populations of such a commercially and ecologically important species. However, it is
574 important to note that the assumptions made are therefore applicable in very specific cases,
575 these results having been obtained on defined mussel sizes under controlled laboratory
576 conditions following a short-time exposure to a particular type and concentration of polymer
577 without evaluating the potential impact of MP leachates on the predator's abilities.

578

579 **5. Conclusion**

580 Our hypothesis that both small and large mussels showed collective behavioural
581 response to predator cues, a response impaired by MP leachates, was supported. However,
582 mussel collective anti-predator behaviour and sensitivity to MP leachates differed according
583 to their size, with a greater effect of MP leachates on small mussels. The change in collective
584 anti-predator behaviour of both large and small *M. edulis* when exposed to MP leachates may
585 hamper mussel survival. Such effects are likely to have cascading effects at larger scales
586 because of the central role of this species in intertidal ecosystems. Indeed, as ecosystem
587 engineers, mussels create microhabitats that allow a wide range of organisms to settle and live
588 in the intertidal environment. Thus, by disrupting the mussel collective anti-predator
589 behaviour, MP leachates may enhance predation risks that will have consequences at the
590 individual that extend to the community and ecosystem. Further experiments are needed to (i)
591 explore the mechanisms of action of these pollutants, (ii) set up long-term monitoring

592 experiment and (iii) test their impact on predators and establish the overall ecological risk of
593 these pollutants.

594

595 **Declaration of competing interest**

596 The authors declare that they have no known competing financial interests or personal
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598

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