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

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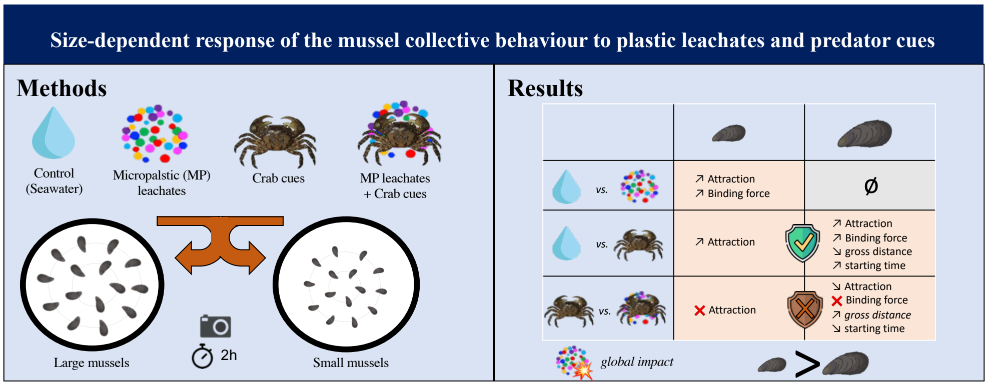
Abstract

Both individual and collective anti-predator behaviours are essential for the survival of many species. This is particularly true for ecosystem engineers such as intertidal mussels, which through their collective behaviour create novel 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 both 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 a considerable reduced activity, *i.e.* they significantly delayed their time to start to form aggregations and decreased their gross distance. These anti-predator behaviours were respectively inhibited and impaired in small and large mussels by MP leachates. The observed collective behavioural changes may reduce individual fitness by enhancing predation risk, particularly in small mussels that are the crab *H. sanguineus's* favourite preys. Given the key role of mussels as ecosystem engineers, our observations suggest that plastic pollution may have implication on *M. edulis* at the species level, but also enhancing a cascading effect towards a higher level of organisation such as population, community and ultimately structure and function of intertidal ecosystem.

Keywords

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

Graphical Abstract



1. Introduction

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

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

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

Critically, individual and collective behaviours based on chemical communication can be hindered by environmental pollutants (*e.g.* Atchison et al., 1987; Fleeger et al., 2003; Lürling and Scheffer, 2007). Plastic pollution is no exception (*e.g.* Mannering, 2021; Rondoni et al., 2021; Shi et al., 2021; Uguen et al., 2022). Formed by a chemically diverse group of synthetic polymer-based materials, plastics is one of the most ubiquitous sources of contamination and pollution of the Anthropocene (Bergmann et al., 2015; Issifu and Sumaila, 2020; Jambeck et al., 2015) and its deleterious effects on wildlife are now widely recognised (Bucci et al., 2020; Cole et al., 2011; Derraik, 2002). While plastic physical and mechanical effects have been far more studied, their typically far more pernicious chemical effects have received much less attention (Delaeter et al., 2022; Seuront et al., 2022). Indeed, various additives are generally added to plastic polymers to improve their performances, *e.g.* flexibility, hardness, thermo-resistance or flammable retardance (Delaeter et al., 2022; Thevenon et al., 2015). Overall, more than 6,000 different additives are used in plastic production, and the amount of additives is estimated to reach 2,000 Mt produced by 2050 (Aurisano et al., 2021; Geyer et al., 2017). The leaching into the aquatic environment and the toxicity of these additives are influenced by the plastic itself (e.g. polymer type, shape, size, weathering), the nature of additives (e.g. types, concentration) and by the environmental conditions (e.g. pH, salinity, temperature; Costa et al., 2023; Sridharan et al., 2022).

Plastic leachates cause adverse effects on mussels at different levels from subcellular and cellular impairments to individual level with notable disturbances on fitness, cell, embryogenic development and behaviour, which could have repercussions at the community level (Capolupo et al., 2021, 2020; Gandara e Silva et al., 2016; Seuront et al., 2021; Trestrail et al., 2020; Uguen et al., 2022). Specifically, the blue mussel (*Mytilus edulis*) behaviour has been shown to be impacted by microplastic (i.e. plastic items smaller than 5 mm; MP) leachates (Seuront et al., 2021; Uguen et al., 2022). Indeed, *M. edulis* exposed to polypropylene MP leachates (12 g of MPs L^{-1}, incubation = 24 h) during 8 h showed a change in their collective behaviour with an increase in maximum aggregation, aggregation speed and distance travelled (Seuront et al., 2021). At the individual level, *M. edulis* exposed during 1h30 to polypropylene MP leachates (0.12 – 1.2 g of MPs L^{-1}; incubation = 24 h) showed a decrease in their motility and anti-predator behaviour (Uguen et al., 2022). However, to the best of our knowledge, the impact of MP leachates on the collective anti-predator behaviour (*e.g.* the ability and celerity to form aggregates) of this keystone, engineering species remains unknown.

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

2. Material and Methods

2.1. Study organism

The blue mussel, *M. edulis*, was used as a model organism. Marine mussels have been extensively studied as a model system for ecological, physiological, and biomechanical research due to their widespread presence in coastal waters, readily accessibility and key ecological and economic relevance. The blue mussel *Mytilus edulis* was used as model species because it is ecologically and economically important in coastal intertidal environment. It is a bioengineering species and as such it forms dense and dynamic beds that enhance local biodiversity (Borthagaray and Carranza, 2007; Buschbaum et al., 2009). In addition, the species also hold significant economic value, with its production accounting for one-third of European Union aquaculture production (Avdelas et al., 2021).

M. edulis individuals were collected in March 2022 from an intertidal rocky reef (Pointe aux Oies, France; 50°47’08.3"N, 1°36’03.9"E), located along the French coasts of the eastern English Channel. Two different sizes were considered, *i.e.* small mussels (shell length: 1.5 – 2.5 cm, $n = 360$) because *Hemigrapsus sanguineus*’s prefered prey (Brousseau et al., 2001) and large mussels (shell length: 4.0 – 5.0 cm, $n = 360$) because of commercial value (*i.e.* > 4 cm; Seuront et al., 2019). Mussels from both different sizes were acclimated in the laboratory in distinct 85 L tanks filled with aerated running natural seawater representative of *in situ* conditions (T = 12°C, S = 33‰) for 24 h under a natural 12:12 h light-dark cycle before the behavioural assays took place; an acclimation time typically used in a range of studies on mussel, *e.g.* Auguste et al., 2020; Balbi et al., 2017; Seuront et al., 2021; Uguen et al., 2022). Adult individuals of the Asian shore crab (*H. sanguineus*, *n* = 144, 2.1 ± 0.3 cm in carapace width) – one of the dominant mussel predators on rocky shores where this species is now well established (DeGraaf and Tyrrell, 2004; Enderlein et al., 2003; Rolet et al., 2020) – were sampled at the same location as the mussels, and then sorted in the laboratory by sex and acclimated separately in 85 L tanks filled with running aerated natural seawater (T = 12°C, S = 33‰) for 2 days. The acclimation period of 48 h was chosen to stimulate predator-induced alarm response by feeding them *ad libitum* with mussels with enhancing food consumption by keeping them in darkness (Spilmont et al., 2015; Uguen et al., 2022). To feed the crabs, mussels were crushed with a natural stone purposely chosen to avoid the use of metal objects and the potentially confounding effect of contamination from metal ions (Commito et al., 2016).

2.2. Treatment preparation

The microplastic (MP) leachate treatment was prepared using commercially available virgin polypropylene (PP) pellets. PP is the most produced polymer type worldwide with 19.3% of the world plastic production (data from 2021; PlasticsEurope, 2022). The virgin PP non-uniform microbeads used (white colour, 4.04 ± 0.60 mm longest dimension measured on a sample of 50 particles; Pemmiproducts, Aachen, Germany; for a picture see Supplementary Material, S1) were incubated during 24 h in aerated natural unfiltered seawater at a relatively high but locally realistic concentration (Seuront, 2018), i.e. 12 g of pellets per litre (20 mL of MPs per L, which, after counting, was equivalent to 404 ± 40 MPs per L). Indeed, plastic pellets pollution is estimated as ca. 17,000 – 165,000 tons per year in Europe (Hann et al., 2018). Plastic pellet transport accidents are frequent. For instance, in 2016 at the studied site, a tank-truck lost 8 tons of plastic pellets, leading to high contamination of the coastline even many years after the accident (Gravier and Haut, 2020). Marine organisms may even experience more extreme concentrations, such as seen in Sri Lanka with up to 85 g of pellets per kg of sediment after the spill-over at sea of containers carrying 78 tonnes of plastic pellet in 2021 (Jayathilaka et al., 2022).

To study the response of mussels to chemical cues from their predator, a solution of crab cues was used. Crabs were not placed directly in the arena to avoid water movement which could cause mussel rheotactic behaviour (*i.e.* movement towards or away from the water flow). Thus, the crab cue treatment was prepared by adding 3 males and 3 females into 1 L of aerated unfiltered seawater for a 24 h period (Uguen et al., 2022). To prepare the mixed treatment (i.e. crab cues + MP leachates), 6 males and 6 females were placed into 1 L of aerated unfiltered seawater for a 24 h period. This doubled crab solution was then mixed at a 1:1 ratio with a double MP leachate solution prepared as above but at a concentration of 24 g of pellets per litre in order to obtain a final concentration comparable to MP leachates and crab cues single stress (Seuront, 2018). The seawater used was pumped directly from the collection site and was not filtered in order to mimic the conditions encountered by our experimental mussels *in situ* and during their acclimation period, and to avoid generated any additional stress to the mussels due to a lack of phytoplankton.

The additive content of the polypropylene pellets was analysed with a pyrolysis analysis coupled to a gas chromatography and a high-resolution spectrometer as described in a previous study (Uguen et al., 2022) before and after their 24h-incubation, for more details on the method used, see Supplementary Material, S2A. The additives identified were: 3 brominated flame retardants, 6 phosphorus flame retardants, 5 antioxidants, 13 plasticisers. Specifically, the additives the most released were (i) phthalate plasticisers, as Diisononyl phthalate (DINP) Diisoheptyl phthalate (DIHP), Diisodecyl phthalate (DIDP), Diisononyl hexahydrophthalate (DINCH), Benzylbutyl phthalates (BBP) and in a lesser extent, Diethyl phthalate (DEP) and Dibutyl phthalate (DBP); (ii) the antioxidants: nonylphenol (NPs) and nonylphenol monoethoxylate (NP10E); and (iii) the phosphorus flame retardants: tributyl phosphate (TBP) and Tris(2-Chloroisopropyl)phosphate (TCPP; Supplementary Material, S2B).

2.3. Experimental design

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

Following Seuront et al. (2021), mussels were placed approximately 1 body size from each other, in concentric circles on their right anterior part, the narrow end facing the centre of the arena (for more details on experimental design see Supplementary Material, S3). Each individual mussel was only used once and, prior to the experiment, byssal threads were carefully cut with a pair of scissors to separate the mussels from each other and to allow mussels to establish primary byssal attachment (Kong et al., 2019; Nicastro et al., 2007). From the stating of the experiment, the typical time needed by *M. edulis* to reach a steady-state in their aggregation rate is 2 h (Seuront et al., 2021), thus here the experiment was consistently run for 2 h.

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

2.4. Behavioural variables

Mussels were visually classified as either solitary or aggregated, *i.e.* two or more mussels with their shells in direct physical contact (Seuront et al., 2021). Aggregation behaviour was quantified at 5 min intervals as the proportion (%) of mussels actively forming aggregates (Seuront et al., 2021). Additionally, three parameters were used to quantitatively assess the aggregation dynamics: the starting time (*i.e.* the time needed to form the first aggregation within an arena), $A_{\max}$ (%; *i.e.* the maximal proportion of aggregation) and k (min; *i.e.,* the time when the proportion of aggregation was half the maximum value; $A(t) = A_{\max}/2$). Indeed, once the first aggregation was formed, the proportion of aggregated mussels as a function of time was fitted with a nonlinear Michaelis-Menten type equation as $A(t) = A_{\max}t/(k + t)$ (Seuront et al., 2021). For each replicate, the non-linear least-squares method was used to estimate $A_{\max}$ and k in order to maximise the coefficient of determination r^2 and to minimise the sum of squared residuals among empirical data. In the rare cases when the model indicated an A_{max} higher than 100 % ($n = 4$; 8% of the total), the model was manually forced to the maximum aggregation observed. In addition, the arenas were virtually grid-lined on the computer in order to calculate the cluster score at the end of the experiment. This index was calculated, as the maximum number of mussels positioned in a given quadrat divided by the overall number of quadrats they occupied; see *e.g.* Collins et al. (2011), da Costa Araújo and Malafaia (2021), de Faria et al. (2018), Freitas et al. (2023) and Guimarães et al. (2023) for further details and Supplementary Material, S6, for an illustration of the method used.

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

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

2.5. Statistical analyses

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

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

3. Results

Treatment had a significant effect on the starting time (KW test, H = 17.22, df = 3, p = 0.0006) and gross distance (KW test, H = 8.61, df = 3, p = 0.035) in large mussels but not in small ones (*i.e.* starting time: KW test, H = 3.42, df = 3, p = 0.33; gross distance: KW test, H = 3.77, df = 3, p = 0.29; Supplementary Material S9). No significant effect between different treatments (control; MP leachates; crab cues; mixture of crab cues and MP leachates) was found in both small and large mussels: net distance (KW test, H = 4.67, df = 3, p = 0.20; H = 5.17, df = 3, p = 0.16, respectively), confinement index (KW test, H =1.79, df = 3, p = 0.62; H = 3.04, df = 3, p = 0.38, respectively), byssus thread production (KW test, H = 5.70, df = 3, p = 0.13; H = 5.84, df = 3, p = 0.12, respectively; Supplementary Material, S10), maximum aggregation ($A_{\max}$; KW test, H = 1.96, df = 3, p = 0.58; H = 4.85, df = 3, p = 0.183, respectively), aggregation speed (*k;* KW test, H = 3.14, df = 3, p = 0.37; H = 0.45, df = 3, p = 0.93, respectively) and cluster score (KW test, H = 3.10, df = 3, p = 0.38; H = 0.74, df = 3, p = 0.86, respectively; Supplementary Material S9).

3.1. Behavioural effect of microplastic leachates

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

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

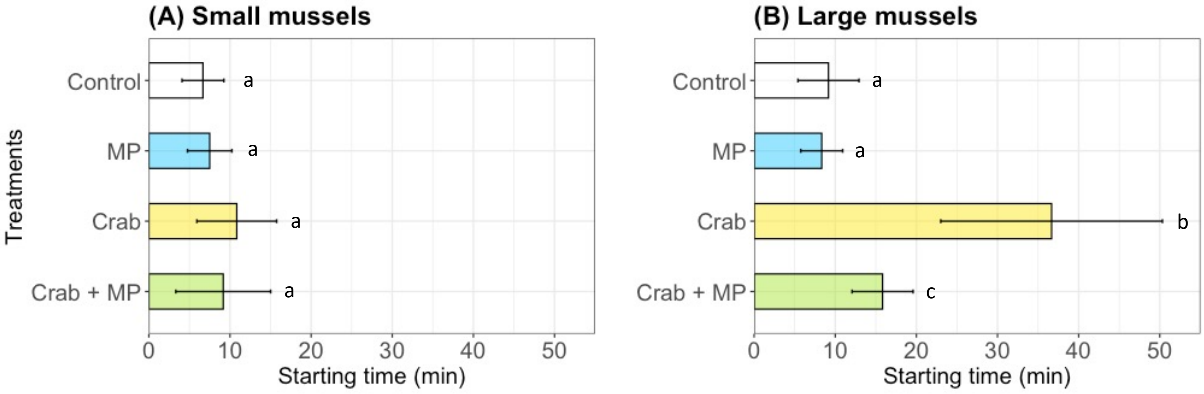


Figure 1: Starting time in minutes (min; mean ± SD) taken by (A) small and (B) large mussels to form the first aggregation in control seawater (white; $n = 6$), MP leachates (blue; $n = 6$), crab cues (yellow; $n = 6$), and 1 : 1 MP leachates and crab cues treatment mixture (green; $n = 6$). Letters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

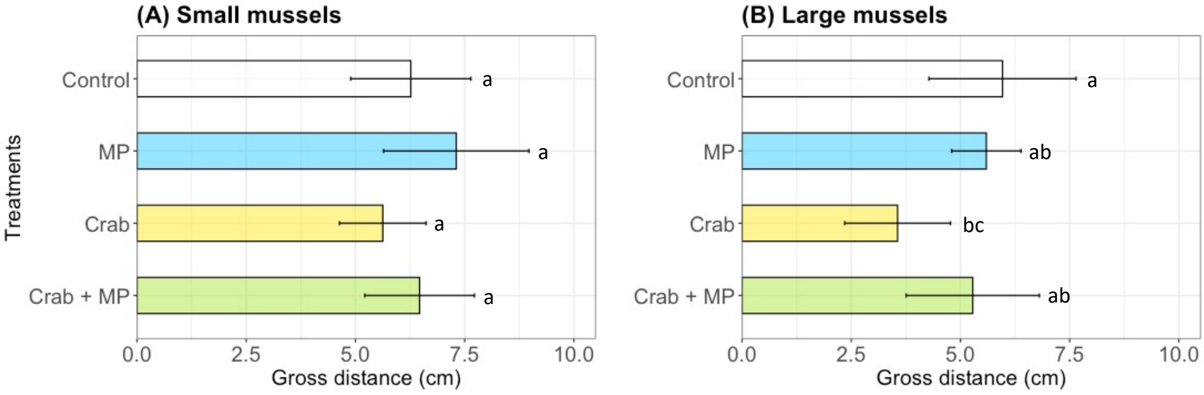


Figure 2: Gross distance in centimetres (cm; mean ± SD) travelled by (A) small and (B) large mussels in control seawater (white; $n = 6$), MP leachates (blue; $n = 6$), crab cues (yellow; $n = 6$), and 1 : 1 MP leachates and crab cues treatment mixture (green; $n = 6$). Letters depict significant differences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

3.2. Behavioural effect of crab cues

Hemigrapsus sanguineus cues had a significant size-specific effect on the collective behaviour of the blue mussel *Mytilus edulis* (Figs. 1 & 2; Supplementary Material S9). For large mussels, crab cue treatment had a significant effect on starting time (KW test, H = 17.22, df = 3, $p = 0.0006$) and gross distance (KW test, H = 8.61, df = 3, $p = 0.035$). In particular, when exposed to crab cues, the time needed by large mussels to form the first aggregation was highly significantly longer (*i.e.* 4-fold) than in the control treatment (Conover's test, $p = 2.4\times10^{-5}$; Fig. 1B). Similarly, the average mussel gross distance was significantly lower (*ca.* 2-fold) than in the control treatment (Conover's test, $p = 0.035$; Fig. 2B). In contrast to what observed in control seawater, large mussels exposed to crab cues exhibited a taxis towards conspecifics, as shown by the significant positive linear correlations between both gross and net distances with $A_{\max}$ ($p = 0.020$ and $p = 0.023$; Supplementary Material S12). Finally, the observed significant positive linear correlation between $A_{\max}$ and byssus thread production ($p = 0.013$; Supplementary Material S12) is indicative of a greater aggregation strength in the presence of crab cues compared to control seawater. In line with what observed in large mussels, small mussels exhibited a significant ($p = 0.028$; Supplementary Material S11) positive linear correlation between the gross distance and $A_{\max}$, thus a taxis behaviour towards conspecifics when exposed to crab cues compared to control treatment.

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

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

3.4. Large *vs.* small mussel behaviour

Although in the control none of the behavioural variables considered significantly differed between large and small mussels (WMW test, Supplementary Material, S13), this was not the case for the other treatments. When exposed to crab cues, large mussels covered significantly lower gross and net distances than small ones (*i.e.* 3.56 ± 1.21 *vs.* 5.63 ± 0.99 cm for gross distance and 0.80 ± 0.28 cm *vs.* 1.37 ± 0.34 cm for net distance; WMW test, p = 0.013 and p = 0.020, respectively; Figs. 2 & 4; Supplementary Material S13). For the same treatment, large mussels compared to small ones also significantly delay their starting time (36.67 ± 13.66 *vs.* 10.83 ± 4.92 min; WMW test, p = 0.007; Fig. 1) to aggregate and, reached a significantly lower maximum aggregation ($A_{\max}$: 34.74 ± 12.08% *vs.* 72.63 ± 18.54%; WMW test: p = 0.02; Fig. 3; Supplementary Material S13). Moreover, small mussels covered significantly more net distances than large ones when exposed to MP leachates (1.84 ± 0.42 cm *vs.* 1.21 ± 0.16 cm; WMW test: $p = 0.008$) and to the mixture of MP leachates and crab cues (1.85 ± 0.67 cm *vs.* 1.01 ± 0.45 cm; WMW test: $p = 0.008$; Fig. 4; Supplementary Material S13).

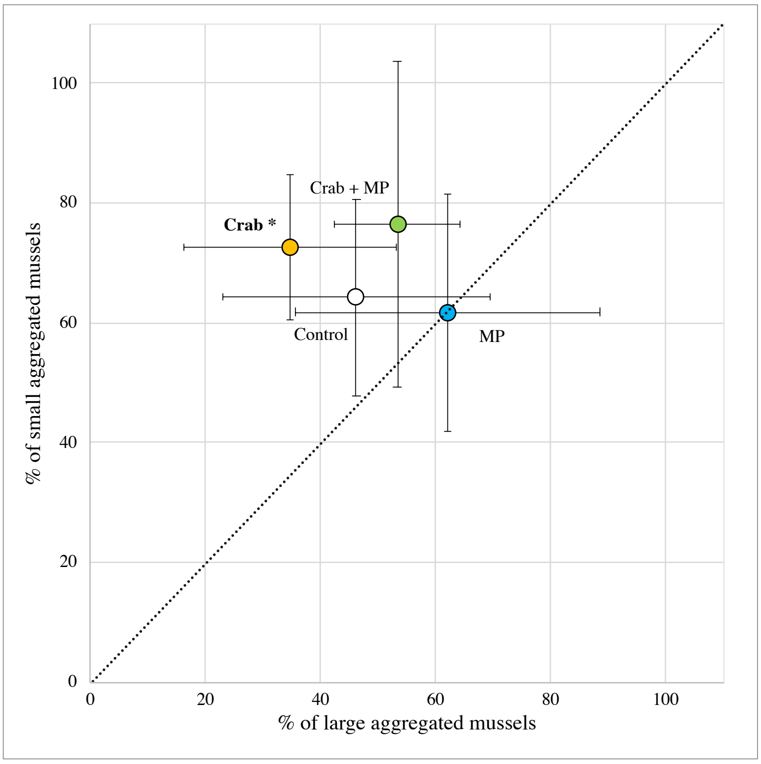


Figure 3: Maximal proportion of aggregation ($A_{\max}$; %) at the end of the 2 h experiment for small and large *Mytilus edulis* in control seawater (white), MP leachates (blue), crab cues (yellow) and 1 : 1 MP leachates and crab cues mixture (green). Treatment name in bold indicates the presence of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

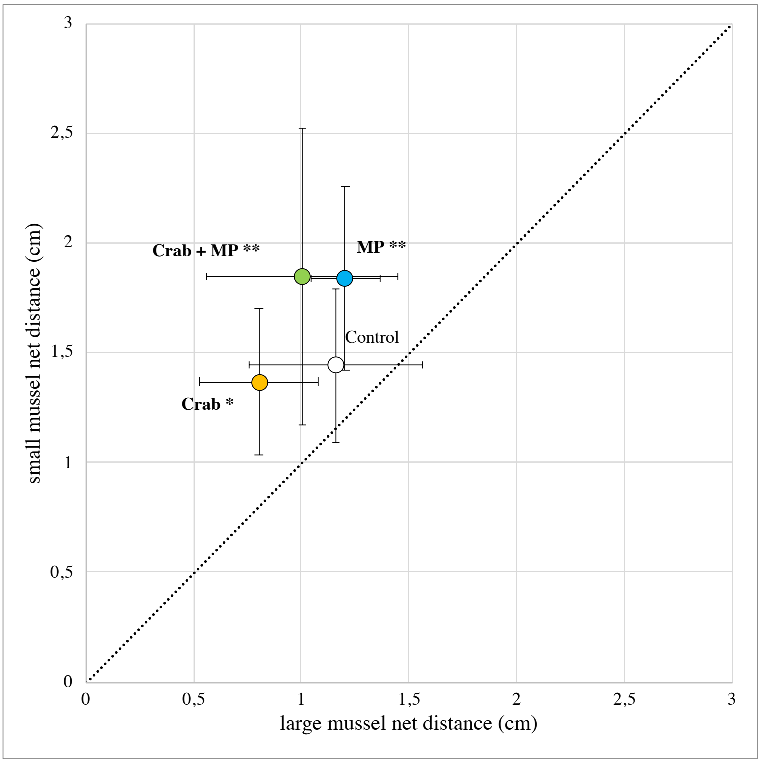


Figure 4: Net distance (cm) travelled by small and large *Mytilus edulis* in control seawater (white), MP leachates (blue), crab cues (yellow) and 1 : 1 MP leachate and crab cue treatment mixture (green). Treatment names in bold indicate the presence of significant differences between small and large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

4. **Discussion**

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

Exposure to leachates of virgin polypropylene MP pellets did not affect the collective behaviour of large mussels. In contrast, small mussels were sensitive to these MP leachates. In particular, their movements were more directional, they showed signs of taxis towards conspecifics and also a greater aggregation strength than under control conditions. Additionally, when exposed to MP leachates, small mussels travelled a greater net distance than large ones. Noticeably, the absence of taxis behaviour towards conspecifics found in the control experiment suggests that even if mussels can detect each other (de Vooys, 2003), they do not have particular benefits (*e.g.* reproduction, anti-predator behaviour, protection against storm) to move towards their conspecifics and increased their aggregation strength in the control at this time of the year (March – relatively calm weather, no reproduction). However, the presence of stress, such as here MP leachates, may increase mussel cooperation (aggregation and attachment to neighbours; de Jager et al., 2017). The aggregation strength, a cooperative behaviour which provides a mutual protection by increasing the number of byssal threads produced according to the number of neighbours, found here in small mussels when exposed to MP leachates is consistent with the finding of de Jager et al. (2017), who showed an increase of this parameter in stressful conditions. In contrast, no byssal alteration production nor aggregation strength in large mussels were altered, Seuront et al. (2021) also found no effect of MP leachates on byssal production in large (3 – 4 cm) *M. edulis*. Although MP leachates do not appear to affect large *M. edulis* byssal thread production, mechanical byssal thread characteristics may be altered, *e.g.* adhesion strength, as shown in another mussel species (*M. coruscus*) when exposed to MP particles (Shang et al., 2021).

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

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

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

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

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

Noticeably, both large and small mussels adapt their collective behaviour to chemical cues from their predator, the Asian shore crab, but not in the same way. For both mussel sizes, predator cues seem to be perceived by the mussels as a stress as they stimulate a taxis behavioural response towards conspecifics. In contrast to small individuals, large mussels decreased their gross distance, delayed their starting time to form aggregation and increased the strength of their aggregation compared to control seawater. Large mussels also travelled over significantly smaller gross and net distances, started to aggregate much later and aggregated less than small ones.

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

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

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

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

Finally, the MP leachate and crab cue mixture led to a mitigation of the collective anti-predator response of large mussels, while small mussels showed no response at all. Specifically, for large mussels, the lower gross distance travelled, the longer time to start to aggregate and the taxis behaviour towards conspecifics were attenuated while the aggregation strength was inhibited by the addition of plastic leachates. For small mussels especially, their unique anti-predator response (*i.e.* a taxis behaviour towards conspecifics) was totally inhibited by the addition of plastic leachates. Similar results showing an impairment in anti-predator behaviour have been reported (i) at the collective level in various organisms exposed to plastic particles, e.g. mice (da Costa Araújo and Malafaia, 2021; Guimarães et al., 2023), tadpoles (da Costa Araújo and Malafaia, 2020) and freshwater fish (da Costa Araújo et al., 2020), and (ii) at the individual level when exposed to MP leachates in the gastropod *Littorina littorea* (Seuront, 2018) and in small mussel, *M. edulis* (Uguen et al., 2022). Indeed, these two last studies demonstrated that when exposed to crab cues with polypropylene leachates, *L. littorea* showed an alteration of their righting time, skioptic withdrawal, time to explore, and avoidance response compared to an exposition to predator cues only (Seuront, 2018) and small *M. edulis* presented an alteration in their motility and in their negative chemotactic behaviour toward crab cues (Uguen et al., 2022). Partial and total loss of prey anti-predator behaviours can be related to a disruption of the chemical communication chain (*i.e.* cue release, transmission and/or reception) by this anthropogenic compound (Olsén, 2011). Thus, these anti-predator behavioural alterations may be due to the prey inability to recognise the chemical cues of its predator which is linked to an interaction of MP leachates (i) with crab cue molecules directly in the water leading to a modified cue, or (ii) with the prey sensory system, hindering the cue reception and/or perception abilities of predator cues (Olsén, 2011). The latter hypothesis is supported by a recent study that showed the disruption of the hermit crab (*Pagurus bernhardus*) cognition, *i.e.* assessment and/or decision-making, in a shell selection test when exposed to MP leachates (Crump et al., 2023). As also in Uguen et al. (2022), they hypothesised that the MP leachates caused a neurosensory disruption, an hypothesis supported here by the phthalates contained in our leachates which are known to be neurotoxic in a wide variety of species (*e.g.* DINP, BBP, DEP, DBP; for reviews, see: Shincy and Chitra, 2020; Zhang et al., 2021). Additional experiments are nevertheless required to further investigate these assumptions and to understand the detailed mechanisms underlying these processes.

The partial and total loss of the anti-predator behaviour of large and small mussels respectively, suggest that smaller mussels are the most sensitive to the contaminant, in addition to being the most at risk of being predated. In a broader context, the lack and mitigation of an anti-predator response for both mussel sizes when in presence of plastic pollution, could fundamentally represent a crucial evolutionary and ecological change at the species level. Indeed, by modifying predator-prey interactions through the predator detection, the avoidance effectiveness and the ability to escape of the prey, pollutants may cause changes at population and community levels (Weis et al., 2001). Vulnerability to MP leachates and predation by crabs, which both act in a size-selective manner to the detriment of small mussels, can affect the abundance and size structure of the mussel population. Small mussels are the preferred crab preys and thus more affected by MP leachates pollution than large mussels as showed by an inhibition of their anti-predator collective response. This could eventually cause to an excessive mortality of small mussels and lead, in the short term, to a shift of the mussel size-frequency distribution to the right in favour of large mussels while, in the long term, to a depletion of the mussel stock as the number of large mussels could be reduced, limiting the reproductive effort (i.e., the largest individuals contributing the most to the egg output; Sprung, 1983). Note that large mussels are not spared by the effect of leachates in terms of their behavioural anti-predator response, but their mortality can be expected to be lower than for small mussels. Thus, the sensitivity of individuals to pollutants may impact the sensitivity at the population level (Gergs et al., 2013), which could lead to a risk of local extinction of populations of such a commercially and ecologically important species. However, it is important to note that the assumptions made are applicable in very specific cases, given that these results have been obtained on specific mussel sizes, under controlled laboratory conditions and following a short-time exposure to a particular type and concentration of polymer without evaluating the potential impact of MP leachates on the predator's abilities.

5. Conclusion

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

Declaration of competing interest

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

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