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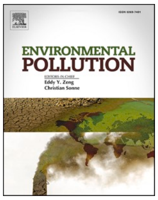

Intraspecific genetic lineages of a marine mussel show behavioural divergence when exposed to microplastic leachates[☆,☆☆]

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ABSTRACT

Worldwide, microplastic pollution has numerous negative implications for marine biota, exacerbating the effects of other forms of global anthropogenic disturbance. Mounting evidence shows that microplastics (MPs) not only cause physical damage through their ingestion, but also act as vectors for hazardous compounds by leaching absorbed and adsorbed chemicals. Research on the effects of plastic pollution has, however, largely assumed that species respond uniformly, while ignoring intraspecific diversity (i.e., variation within a single species). We investigated the effects of plastic leachates derived from factory-fresh (virgin) and beached microplastics on the behavioural responses of two genetic lineages of the Mediterranean mussel *Mytilus galloprovincialis.* Through laboratory behavioural experiments, we found that during exposure to leachates from beached microplastics (beached MPLs), Atlantic specimens moved significantly less than Mediterranean individuals in terms of both (i) proportion of individuals responding through movement and (ii) net and gross distances crawled. In contrast, no significant intraspecific differences were observed in the behaviour of either adults or recruits when exposed to MPLs from virgin microplastics (virgin MPLs). Additionally, the reception of cues from three amino acids (L-cysteine, proline and L-leucine) at increasing concentrations (10^{-5} M to 10^{-3} M in charcoal-filtered seawater) was tested by electrophysiological analysis using mussels exposed to beached MPLs or control seawater. We found significant intraspecific differences in response to 10^{-3} M L-cysteine (regardless of treatment) and 10^{-4} M L-cysteine (in mussels exposed to beached MPLs) and to 10^{-3} M proline (in mussels exposed to beached MPLs) and 10^{-5} M L-leucine. Our study suggests that intraspecific variation in a marine mussel may prompt different responses to plastic pollution, potentially triggered by local adaptation and physiological variability between lineages. Our work highlights the importance of assessing the effects of intraspecific variation, especially in environmental sentinel species as this level of diversity could modulate responses to plastic pollution.

1. Introduction

Ecosystems across the planet are critically and increasingly disrupted by plastic pollution. Along with other global issues such as climate change, this new threat poses multiple risks to the functioning of natural ecosystems and the services they provide to society (Pawar, 2016; Hendry et al., 2017; Heidbreder et al., 2019; Thushari and Senevirathna, 2020; Azevedo-Santos et al., 2021).

Plastic litter is generally defined according to size as micro- (<5 mm) or macroplastics (Barnes et al., 2009; Arthur et al., 2009). Microplastics (MPs) represent the most abundant fraction of plastic in the oceans and constitute a well-known threat to all trophic levels through ingestion (Thushari and Senevirathna, 2020). Acting as carriers for contaminants, MPs also expose organisms to various hazardous compounds (Teuten

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[☆] This paper has been recommended for acceptance by Maria Cristina Fossi.[☆☆] Data are available on figshare online repository at the link https://figshare.com/s/8e6e7aeaa1c4571612f4. Upon acceptance, data on mussel behavioural and physiological responses will be provided via figshare online repository.

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et al., 2009; Lithner et al., 2011; Gunaalan et al., 2020). These can have direct effects by aggravating the physical damage of plastic ingestion (Wright et al., 2013), or indirect effects by releasing both absorbed and adsorbed pollutants that are respectively bounded to the polymer matrix of a plastic item and to its surface (Cormier et al., 2021; see also Delaeter et al., 2022 for a review).

Plastic manufacture involves the use of functional additives (i.e., substances that modify polymer features and impart specific properties to the final product) including flame retardants (e.g., polybrominated diphenyl), plasticizers (e.g., bisphenol A and phthalates; used to impart flexibility), stabilisers (e.g., phenols and arylamines; to prevent UV and heat degradation), metals (e.g., aluminium, chromium, cobalt and nickel), vulcanisers and antimicrobials (Hermabessiere et al., 2017; Hahladakis et al., 2018; Gunaalan et al., 2020; Schiavo et al., 2020; Capolupo et al., 2020). In addition, while exposed to the natural environment, MPs can adsorb other contaminants such as persistent organic pollutants (POPs; e.g., pesticides and dioxins), poly-chlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAH; Fries and Zarfl, 2012; Rodrigues et al., 2019; Yeo et al., 2020; Thushari and Senevirathna, 2020). The release of both absorbed and adsorbed compounds by plastics exposes habitats and organisms to complex toxic mixtures (Thushari and Senevirathna, 2020; Lynch et al., 2022).

The responses of different species to leachates from plastics have been emphasized in several studies. Among the most targeted species are bivalves (Gandara e Silva et al., 2016; Ke et al., 2019; Capolupo et al., 2021; Seuront et al., 2021), which are often regarded as sentinel species because of their filter feeding behaviour, wide distribution and sensitivity to chemical stressors (Phillips, 1990; Fabbri et al., 2014; Amiard and Amiard-Triquet, 2015). Most studies have, however, assumed that species respond to plastic pollution as ecologically and physiologically homogenous entities, potentially underestimating and overgeneralising the effects of plastic pollution. Indeed, it is now well established that genotypic and phenotypic variation within a species can have important ecological implications for processes such as primary productivity (Crutsinger et al., 2006; Zhu et al., 2000), nutrient cycling (Lecerf and Chauvet, 2008), species coexistence (Siefert, 2012; Spasojevic and Suding, 2012) and ecosystem resilience and resistance (Hughes and Stachowicz, 2004; Reusch et al., 2005; Des Roches et al., 2018; Raffard et al., 2018). Importantly, there is compelling evidence of how intraspecific variation can have even greater implications for ecosystem function and services than differences among species; see e.g., Cardinale et al. (2012), Raffard et al. (2018), Nicastro et al. (2020) and Cozzolino et al. (2022).

The Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819) is a key marine ecosystem engineer (Jones et al., 1994; 1997). It is widely distributed in temperate areas of the northern and southern hemispheres (De Moor and Bruton, 1988; Lynch et al., 2020; Ramos-Oliveira et al., 2021) with genetically different entities identified in both hemispheres (Daguin and Borsa, 2000; Zardi et al., 2007). In southern Europe, *M. galloprovincialis* is genetically subdivided into two distinct lineages, one Atlantic (ATL) and one Mediterranean (MED), distributed along either side of the Almeria-Oran Front (Quesada et al., 1995; 1988; Sanjuan et al., 1994), a widely recognised marine barrier to gene flow between populations of several marine species (Galarza et al., 2009; Schunter et al., 2011; Marie et al., 2016; González-Castellano et al., 2020). Despite showing clear genome-wide separation and high heterogeneous genetic differentiation (Gosset and Bierne, 2013; Fraïsse et al., 2016), the Atlantic and the Mediterranean groups can hybridize along the Algerian coast where local introgression of parental backgrounds is observed and populations of mixed ancestry live in sympatry (Gosset and Bierne, 2013; El Ayari et al., 2019).

The distinct clades are adapted to local environmental conditions such as the higher salinity, and temperature (Santinelli et al., 2010) as well as the lower nutrient availability (Krom et al., 1991) of the Mediterranean Sea compared to the Atlantic Ocean. Similarly, the two lineages may have adapted to different levels of anthropogenic pollution, evolving tolerance to local contaminants as suggested by Whitehead et al. (2012). Because of the high residence time of its water (Lacombe et al., 1981), the intensive shipping and industrial activity within its basin and its densely populated coastline, the Mediterranean Sea harbours some of the highest concentrations of floating plastics in the world (Lebreton et al., 2012; Eriksen et al., 2014; Cózar et al., 2015; Sharma et al., 2021; Tsiaras et al., 2021). We hypothesised that the different physical characteristics of water from the Mediterranean Sea and Atlantic Ocean, combined with the resulting differential adaptation of the two lineages, could affect, directly or indirectly, mussel behavioural responses to anthropogenic stressors. Using laboratory behavioural experiments, we assessed whether the two genetic lineages of *Mytilus galloprovincialis* respond differently to exposure to microplastic leachates (MPLs).

Mytilus galloprovincialis is a highly gregarious species that forms extensive beds that perform key ecosystem functions and services (Puri et al., 2021). Mussel beds provide nursery, refuge and feeding areas that enhance local biodiversity (Commito et al., 2005; Borthagaray and Carranza, 2007; Gestoso et al., 2013; Arribas et al., 2014; Lathlean and McQuaid, 2017; Lefcheck et al., 2019) and ensure food provision (Wijsman et al., 2019), water filtration (Newell, 2004; van Leeuwen et al., 2010; Montes et al., 2012) and provide coastal protection (Folkard and Gascoigne, 2009; Borsje et al., 2011; Ysebaert et al., 2019). Despite their crucial role as biogenic habitats, the mechanisms behind the ability of mussels to find each other or aggregate have been largely overlooked. Most likely, these processes are underlain by chemical sensitivity, namely the ability to detect and respond to chemical stimuli.

In bivalves, chemoreception is thought to be mediated by the osphradium (Rato et al., 2023), a patch of nearly unpigmented sensory cells (List, 1902; Clasing, 1923) located on the gill axis or immediately adjacent to it, ventral to the visceral ganglion and innervated by the branchial nerve (Haszprunar and Haszprunar, 1987; 1988). The osphradium of bivalves has been associated with multiple functions including photoreception, regulation of behaviour and reproduction as well as spawning synchronization and the detection of chemical cues (Aiello and Guideri, 1964; Kraemer, 1981; Haszprunar and Haszprunar, 1987; Beninger et al., 1995). Such a chemosensory organ is, however, also directly exposed to chemical pollutants present in seawater. Previous studies have shown pollutant-induced disruption of olfaction in fish and crustaceans (see Olsén (2011) for a review) as well as chemoreception in molluscs (Rózsa and Salánki, 1990; Kamardin et al., 2015), resulting in physiological and behavioural alteration. Similarly, exposure to MPLs may trigger behavioural changes in mussels as a consequence of altered chemoreception as recently illustrated in *Mytilus edulis* (Uguen et al., 2022). We hypothesised that the osphradium might be involved in responses of mussels to microplastic leachates, and that its sensitivity would differ between *Mytilus galloprovincialis* lineages, potentially triggering differential behavioural responses. The negative electrical potential that can be recorded at the surface of the osphradium is thought to be the sum of generator potentials in the olfactory receptor neurons as a response to a given stimulus (Scott and Scott-Johnson 2002), such as an odorant. Many aquatic species including fish (Hara, 2006), amphibians (Heerema et al., 2018), crustaceans (Fuzessery and Childress, 1975) and molluscs (Wedemeyer and Schild, 1995; Rato et al., 2023) for example, have shown strong sensitivity to amino acids. We adapted an electrophysiological technique developed for oysters by Rato et al. (2023) to measure the peak amplitude responses (V) to three L-amino acids (i.e., L-cysteine, L-leucine, and proline) of mussels exposed to beached MPLs and control seawater.

2. Material and methods

2.1. Sampling and treatments

Mussels belonging to Atlantic (ATL) and Mediterranean (MED) lineages of *Mytilus galloprovincialis* were collected at two sites (ca. 25 km apart) for each lineage. At each site, two size classes, adults (5–6 cm in shell length) and recruits (0.4–0.6 mm in shell length), were collected.

Individuals of the MED lineage were collected from a floating instrument buoy in the Gulf of Pozzuoli (Naples; 40°48′29.5″ N, 14°08′35.5″ E) and from a rocky intertidal reef in Castel Volturno (Caserta; 41°02′08.1″ N, 13°55′02.5"E; Fig. 1A). Individuals of the ATL lineage were collected from rocky intertidal shores in Vilamoura (37°04′14.4″ N, −8°06′58.5″ W) and in Farol Island (36°58′39.7″ N, −7°51′58.3″ W; Fig. 1A).

Prior to the laboratory experiments, mussels were cleaned of epifauna and acclimatised for 14 days under controlled laboratory conditions. Populations from each site were housed separately in glass aquaria filled with aerated seawater under standardized conditions of salinity (S = 36 PSU) and temperature (T = 17 °C). For each experiment, three treatments were set up with mussels placed in either control seawater, or seawater treated with either factory-fresh (virgin) or beached microplastics. Control seawater consisted of the seawater used for acclimatisation. Virgin MPLs seawater was prepared from commercially available virgin high density polyethylene pellets (white colour, 3 ± 0.5 mm longest dimension measured on a sample of 50 particles; Materialix, Jaslo, Poland). Beached MPLs seawater was prepared using low density polyethylene pellets (mostly black in colour, 3.8 ± 0.5 mm longest dimension measured for a sample of 50 particles) collected along the shore of Northern France (50°38′27.92"N, 1°34′47.68"E). This site is over 1500 km from the sampling locations for both ATL and MED specimens, minimizing the risk of collecting specimens adapted to the test pollutants. The identification of the additive content of both virgin and beached pellets was carried out using a CDS Pyroprobe 6150 pyrolyzer (CDS Analytical) in conjunction with a GC-HRMS instrument (GC Trace 1310-MS Orbitrap Q Exactive, Thermo Fisher Scientific). Thermal desorption was performed (350 °C) to remove the potential additives from the samples. The samples were then separated using a Restek Rxi-5-MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness) with a cross-linked poly 5% diphenyl-95% dimethyl siloxane stationary phase (slip ratio: 1:5). The acquisition was conducted on full-scan (FS) mode (m/z = 30.00000–600.00000) and the resulting chromatograms were analysed using Xcalibur and TraceFinder software for the identification of organic plastic additives among a selection of 57 additives (i.e., plasticizers, flame retardant, antioxidants and UVs stabilizers; see full list in Table 1). Identification of the respective additives was based on their retention times, m/z values, and specific ions, which were compared with the chromatograms obtained from standard solutions of each additive. Note, however, that because beached pellets were opportunistically collected in situ, their initial additive content is likely to differ from the additive content of virgin pellets, and as such the difference in additive content between beached and virgin pellets cannot be used as a way to identify the additives desorbed from the pellets polymeric matrix during the weathering process. Instead, the difference in their additive content is used hereafter as a proxy of the relative diversity of the chemical compounds likely to be present in the leachate seawater used in the experiments described below. Chemical assessment of heavy metals associated with the same pellets used here has been conducted in a separate study (Zardi et al., in revision) using X-ray Diffraction (XRD) analysis. Heavy metal composition is expressed as a proportion of the overall pellet mass. This approach was chosen as the quantification of the absolute concentration of heavy metals would have necessitated the use of pure chemical standards for each metal, hence *a priori* knowledge of their nature. To prepare the leachate solution, either virgin or beached pellets were mixed with control seawater at a concentration of 20 ml of plastic pellets per litre of water (equivalent, after counting to 405 ± 40 MPs per L) and aerated for 24 h before being use for the behavioural traits experiment (Seuront, 2018; Luo et al., 2019). Despite being relatively high, the MPs concentration (express as number of MPs per mL of water) used here is locally realistic as shown for the same sampling area in Northern France (Seuront, 2018).

2.2. Genetic analysis

To confirm the genetic identity of the populations sampled, total genomic DNA was extracted from a piece of mantle edge from a subsample of 10 individuals per population following a standard proteinase K and ammonium acetate extraction method adapted from Sambrook et al. (1989). A 350 – bp fragment of the gene cytochrome oxidase subunit III was PCR amplified and sequenced using the primers FOR1 (5′-TATGTACCAGGTCCAAGTCCGTG-3′) and REV1 (5′-TGCTCTTCTT GAATATAA GCGTA-3′; Zouros et al., 1994). COIII sequences were aligned and revised manually before being used to construct an unrooted Neighbour-Joining tree with MEGA software v6 (Fig. 1B; Tamura et al., 2013).

2.3. Behavioural traits experiment

Adult mussels (n = 10) were placed in 26 cm diameter glass arenas with smooth surfaces (n = 4 arenas per site and per treatment) under static conditions to avoid passive movement of mussels by water currents (Commito et al., 2014, 2016; Seuront et al., 2021). Each arena was filled with 2 L of either control, virgin or beached leachate solution. Given the smaller size of recruits, smaller arenas (10.5 cm in diameter), filled with 80 ml of either control, virgin or beached leachate solution were used. Mussels were positioned 3.5 cm (adults) or 1 cm (recruits) equidistant from each other in concentric circles with the anterior

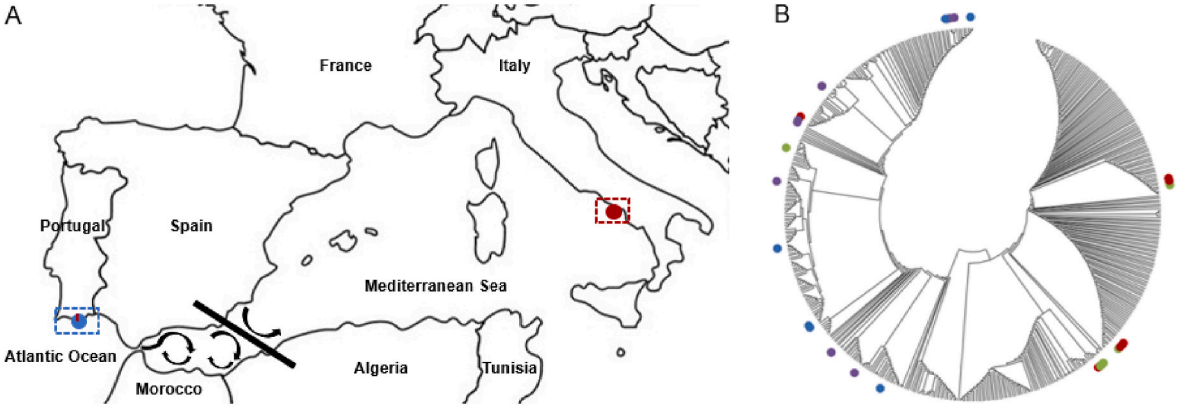

Fig. 1. (A) Sampling locations of *Mytilus galloprovincialis* of the Atlantic (blue) and Mediterranean lineages (red). The black bar indicates the position of the Almeria-Oran Front. (B) Unrooted Neighbour-Joining tree of mtDNA COIII sequences from the two lineages in analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
List of the 57 additives screened by our GC-HRMS analysis. The additives found on virgin (V) and beached (B) polyethylene pellets are indicated by the respective letters.

Function	N°	Molecules	Abbreviation	CAS	Presence
Plasticizers	1	Dimethyl phthalate	**DMP**	**131–11–3**	**V**
	2	Diethyl phthalate	**DEP**	**84–66–2**	**V**
	3	Di-allyl phthalate	**DAlP**	**131–17–9**	**V**
	4	Diisobutyl phthalate	**DIBP**	**84–69–5**	**V–B**
	5	Di-n-butyl phthalate	**DBP**	**84–74–2**	**V**
	6	Tributyl Acetyl Citrate	**ATBC**	**77–90–7**	**V**
	7	Di-n-hexyl phthalate	**DHP**	**84–75–3**	**-**
	8	Benzyl butyl phthalate	**BBP**	**85–68–7**	**V**
	9	Bis-2-Ethylhexyl Adipate	**DEHA**	**103–23–1**	**V–B**
	10	Diisoheptyl phthalate	**DIHP**	**71888–89–6**	**V**
	11	Tri(2-ethylhexyl) phosphate	**TEHPA**	**78–42–2**	**-**
	12	Dicylcohexyl phthalate	**DCHP**	**84–61–7**	**-**
	13	Bis(2-Ethylhexyl) phthalate	**DEHP**	**117–81–7**	**V**
	14	Diisononyl hexahydrophthalate	**DINCH**	**166412–78–8**	**V**
	15	Di-n-octyl phthalate	**DIOP**	**117–84–0**	**-**
	16	Diisononyl phthalate	**DINP**	**68515–48–0**	**V**
	17	Di-nonyl phthalate	**DNP**	**84–76–4**	**-**
	18	Diisodecyl phthalate	**DIDP**	**68515–49–1**	**V**
Flames retardants	19	Triethyl Phosphate	**TEP**	**78–40–0**	**-**
	20	Tripropyl Phosphate	**TPP**	**115–86–6**	**-**
	21	Tributyl Phosphate	**TBP**	**126–73–8**	**V**
	22	2,4,6-Tribromophenol	**2,4,6,TBP**	118-79-6	**-**
	23	Tris(2-Chloroethyl)Phosphate	**TCEP**	**115–96–8**	**V**
	24	Tris(2-Chloroisopropyl)Phosphate	**TCPP**	**13674–84–5**	**V**
	25	2,4,4′-Tribromodiphenyl ether	**BDE-28**	**41318–75–6**	**-**
	26	Tris(1,3-Dichloro-2-Propyl)Phosphate	**TDCPP**	**13674–87–8**	**V**
	27	Triphenyl Phosphate	**TPhP**	**513–08–6**	**-**
	28	2,2′,4,4′-Tetrabromodiphenyl ether	**BDE-47**	**5436–43–1**	**-**
	29	Tricresyl Phosphate	**TCP**	**1330–78–5**	**-**
	30	Tricresyl Phosphate - isomer	**TCrP**	**78–30–8**	**-**
	31	2,2′,4,4′,6-Pentabromodiphenyl ether	**BDE-100**	**60348–60–9**	**-**
	32	Tri-o-tolyl phosphate	**TToP**	**78–30–8**	**-**
	33	2,2′,4,4′,5-Pentabromodiphenyl ether	**BDE-99**	**189084–64–8**	**-**
	34	2,2′,4,4′,5,5′-Hexabromodiphenyl ether	**BDE-153**	**68631–49–2**	**V**
	35	2,2′,4,4′,5,6′-Hexabromodiphenyl ether	**BDE-154**	**207122–15–4**	**V**
	36	2,2′,3,4,4′,5′,6-Heptabromodiphenyl ether	**BDE-183**	**207122–16–5**	**V**
	37	1,2-Bis (2,4,6 Tribromophenoxy) ethane	**BTBPE**	**37853–59–1**	**-**
Antioxydants	38	6,6′-di-tert-butyl-2,2′-thiodi-p-cresol	**Irganox® 1081**	**90–66–4**	**V**
	39	Butylated hydroxytoluene	**BHT**	**128–37–0**	
	40	pentaerythritol tetrakis (3-(3,5-di-t-butyl-4-hydroxyphenyl)propionate	**Irganox® 1010**	**6683–19–8**	**-**
	41	3,5-di-tert-butyl-4-hydroxyhydrocinnamic acid, octadecyl ester	**Irganox® 1076**	**2082–79–3**	**-**
	42	6,6′-ditert-butyl-4,4′-thiodin-m-cresol	**Lowinox® TBM-6**	**96–69–5**	**-**
UV stabiliser	43	2,2-dihydroxy-4,4-dimethoxybenzophenone	**Uvinul® 3049**	**131–54–4**	**-**
	44	2-t-Butyl-6(5-chloro-2H-benzotriazol-2-yl)-4-methylphenol	**UV-326**	**3896–11-5-**	**-**
	45	2-(2H-Benzotriazol-2-yl)-4,6-di-tert-pentylphenol	**UV-328**	**25973–55–1**	**-**
	46	2,4-Di-tert-butyl-6-(5-chloro-2H-benzotriazol-2-yl)phenol	**UV-327**	**3864–99–1**	**-**
	47	2-hydroxy-4-octyloxybenzophenone	**Uvinul 3008**	**1843–05–6**	**-**
Antioxidants – plasticizers – stabilizers	48	4-Tert-Octylphenol	**4-t-OP**	**140–66–9**	**-**
	49	Nonylphenol	**NPs**	**84852–15–3**	**V–B**
	50	4-nonylphenol	**4-NP**	**104–40–5**	**-**
	51	Nonylphenol Monoethoxylate	**NP1EO**	**27986–36–3**	**V**
	52	Bisphenol F	**BPF**	**620–92–8**	**V**
	53	4-Nonylphenol Monoethoxylate	**4-NP1EO**	**104–35–8**	**-**
	54	Bisphenol A	**BPA**	**80–05–7**	**V**
	55	Bisphenol B	**BPB**	**77–40–7**	**-**
	56	Nonylphenol diethoxylate	**NP2EO**	**N/A**	**-**
	57	Bisphenol S	**BPS**	**80–09–1**	**V**

narrow end facing the centre of the plate. The distance between adult and recruit mussels was calculated over the area of their respective arenas, ensuring equal spacing between the individuals under analysis. Due to variations in the experimental settings (i.e., arena size, distance between individuals and volume of the treatment water) adult mussels and recruits were analysed separately, rather than being compared with one another.

Mussel behaviour was recorded every minute for 2 h using a GoPro camera (GoPro HERO8 Black, GoPro Inc., San Mateo, California, USA) positioned 50 cm above the experimental arenas. The behavioural traits of mussels in each arena were subsequently classified based on: (i) individual motility (total net and gross distances crawled), (ii) proportion of motile individuals in each arena (%) and (iii) aggregation (i.e., number of aggregates (defined as a minimum of 2 individuals in physical contact with each other) and number of individuals in each aggregate; Nicastro et al., 2007, Commito et al., 2016; Seuront et al., 2021). Motile individuals remaining isolated were recorded as “solitary”. No mortality was observed and individuals performing no active movements despite being alive were recorded as “immobilized”.

2.4. *Osphradium chemosensory response experiment*

To test whether the response to increasing concentrations of odorants differed between the two lineages, mussels (n = 3 per population) were kept in control seawater or in beached MPL seawater for 24 h and then their chemosensory activity was recorded. Treatment with virgin

MP leachates was not included in the osphradium chemosensory response experiment because such treatment revealed no significant intraspecific effect on the behavioural responses of mussels. As odorants, the focus was on amino acids as these have been shown to be a powerful tool in triggering differential responses in many aquatic species (Fuzessery and Childress, 1975; Hara, 2006; Rato et al., 2023). Preliminary tests conducted on the osphradium response of adult *Mytilus galloprovincialis* revealed that the amino acids L-cysteine, L-leucine and proline, elicit strong and consistent responses in both Mediterranean and Atlantic individuals. Therefore, they were chosen as stimuli in the follow-up experiments. The chemosensory activity of the osphradium was recorded through Electro-osphradiogram analysis (EosG) during exposure to amino acids diluted in charcoal-filtered sea water at different concentrations (i.e., from 10^{-5} M to 10^{-3} M), with the stimulus administered in variable orders.

2.4.1. *Stimulus preparation*

Odorant solutions were created by directly dissolving each amino acid in charcoal-filtered natural seawater at concentrations ranging between 10^{-5} M and 10^{-3} M. Increasing odorant concentrations were used to assess the threshold of mussel osphradium responses. The stimulus solutions were prepared immediately before Electro-osphradiogram recording. Once prepared, odorant solutions were kept in glassware previously rinsed with charcoal-filtered sea water (Hubbard and Velez, 2020) as this was used as a negative control.

2.4.2. *Sample preparation and electro-osphradiogram analysis (EosG)*

As an extension of the electro-olfactogram (EOG) recording system used in vertebrates (Scott and Scott-Johnson, 2002), the electro-osphradiogram (EOsG) is a D.C. field potential recorded from just above the surface of the osphradium (Rato et al., 2023). It is thought to be a summation of the generator potentials of those sensory neurones responding to a given stimulus; the larger the amplitude, the more cells are responding and/or with larger generator potentials.

Live mussels were carefully opened by cutting the posterior adductor muscle and subsequently moved on one valve without damaging the mantle. Mussels were analysed individually in the EosG experimental chamber where they were continuously irrigated with charcoal-filtered sea water (10 ml min^{-1}) while the osphradium was identified using a stereo microscope (Olympus SZ40) placed over the chamber. The voltage signal was recorded through EosG electrodes made from borosilicate glass micropipettes filled with 3 M NaCl in 4% agar and connected to an amplifier via Ag/AgCl pellets in 3 M KCl. The recording electrode was placed close to the osphradium, under the seawater/odorant stream, at the position that gave the largest response to 10^{-3} M L-cysteine. The reference electrode was placed nearby in contact with the mantle (not the osphradium) of the individual analysed (Haszprunar and Haszprunar, 1987; Rato et al., 2023) and the mussel was connected to earth with an Ag/AgCl pellet. The voltage signal was amplified × 5000 (Neurolog NL 100 head-stage and NL 109 bridge amplifier from Digitimer Ltd., Welwyn Garden City, UK) with the low-pass filter set at 30 Hz (NL 125 filter). The signal was then digitised (Digidata 1440 A, Molecular Devices, Sunnyvale, California, USA) and recorded on a PC running Axoscope TM software (version 12.1, Molecular Devices). The solution containing the stimulus was introduced via a three-way solenoid valve for a period of 6 s. Stimulus order varied among mussels and at least 1 min clearance with charcoal-filtered sea water was allowed between each stimulus. The EosG was set up to allow switching between control sea water and stimulus solution without interrupting the flow over the osphradium. The peak amplitude of each response was measured (Volts) and saved for statistical analysis.

2.5. *Statistical analysis*

Adults and recruits were analysed separately. Differences in mussel aggregate size and number, crawling distances (both net and gross) and percentage of motile individuals were compared between Atlantic and Mediterranean lineages under control seawater and microplastic leachate seawater (beached and virgin) using 3-way ANOVA with “treatment” (Control, Beached, Virgin) and “lineage” (Atlantic and Mediterranean) as fixed factors and “population” (Pozzuoli, Castel Volturno, Vilamoura and Farol) as a random factor nested in lineage. When the data did not conform to the parametric assumptions of normality (Shapiro test) or homoscedasticity (Levene test), they were log-transformed or analysed using non-parametric tests (Wilcoxon, if two groups, or Kruskal-Wallis, when more than two groups).

Prior to statistical analysis, data on the chemosensory activity of the osphradium were blank subtracted and divided by 5000 to correct for the experimental signal amplification to obtain the crude value of the response.

Intraspecific differences in response to each stimulus and concentration were tested between mussels kept in control sea water and beached MPL sea water using two-way ANOVA with “treatment” (Control and Beached) and “lineage” (Atlantic and Mediterranean) as fixed factors and “population” (Pozzuoli, Castel Volturno, Vilamoura and Farol) as a random factor nested into lineage. Tukey’s *post-hoc* test was applied to determine statistical differences between populations of the same lineage when the interaction was significant. When the data did not conform to the parametric assumptions of normality (Shapiro test) or homoscedasticity (Levene test), the analysis was reduced to one-way ANOVA and the intraspecific effect on mussel response was analysed for Treatment (Control vs Beached) separately. When even one-way ANOVA could not apply, the analysis was carried out using non-parametric tests (Wilcoxon, if two groups, or Kruskal-Wallis, when more than two groups). Data analyses and visualization were conducted using RStudio 2022.07.0 (R Core Team, 2021).

3. Results

3.1. *Genetic analysis*

DNA sequences and the genetic tree showed that all the samples from the Mediterranean Sea and the Atlantic Ocean conformed to the allele frequencies previously described for the Mediterranean and Atlantic lineages of *Mytilus galloprovincialis* (El Ayari et al., 2019).

3.2. *Additive and heavy metal assessment*

Virgin polyethylene pellets were composed of 26 additives including 13 plasticizers, 6 antioxidants, 4 phosphorylated and 3 brominated flame retardants (Table 1; Fig. 2A–B). In contrast, beached pellets contained only 3 additives, including two plasticizers (DIBP, and DEHA) and one antioxidant (Nonylphenol).

X-ray Diffraction (XRD) assessment of heavy metals associated with pellets revealed high levels of heavy metals (including Pb, Cd, As, Br and Sn) in beached MP pellets (Zardi et al., in revision).

3.3. *Behavioural experiment*

3.3.1. *Percentage of motile individuals*

In beached MPLs, adults and recruits belonging to the Mediterranean lineage were significantly more active than Atlantic individuals (Table 1-2SM; $P_{\text{recruits}} = 0.002$ Wilcoxon Rank Sum Test; Fig. 3A and D). No significant differences in percentage of motile individuals were observed between lineages in control sea water or virgin MPLs for either adults or recruits (Table 1-2SM and 3-4SM).

Additionally, within each lineage, significant differences among treatments were detected for adults (Table 2SM). Specifically, between the Beached and Control treatments and between Beached and Virgin. Significant differences among treatments were also observed for Atlantic recruits (Table 5-6SM) between the Beached and Control treatments and between Beached and Virgin.

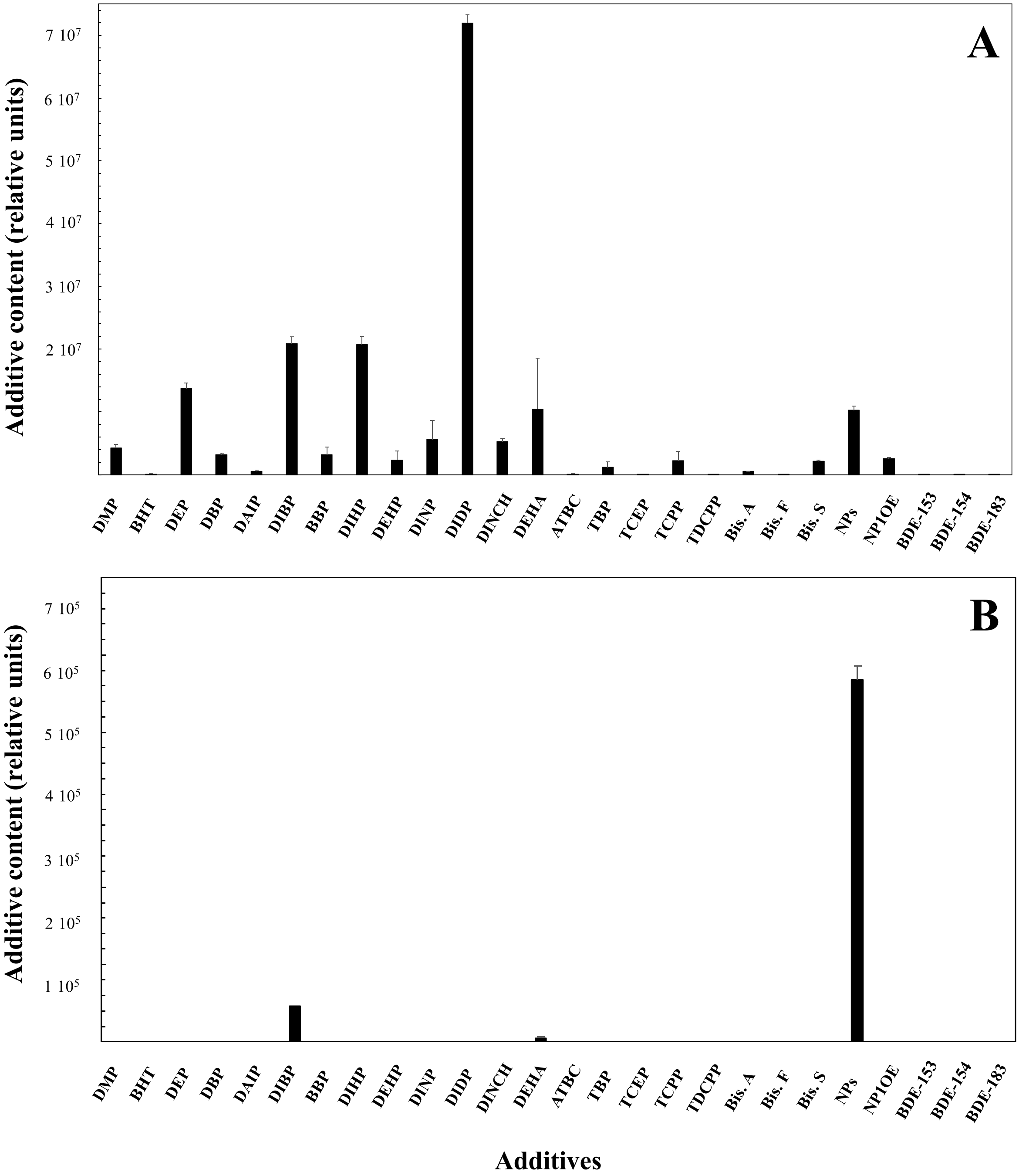

Fig. 2. (A) Additive content of virgin polyethylene pellets (mean ± standard deviation; n = 3) and (B) beached polyethylene pellets. Acronyms as in Table 1. Note that the two-orders of magnitude differ in the additive contents of virgin and beached pellets.

3.3.2. *Crawling distance*

In beached MPLs, MED adults crawled significantly greater net and gross distances than ATL individuals (Table 8SM; Fig. 3B–C). Similarly, MED recruits crawled significantly greater net and gross distances than ATL individuals (Tables 11–13SM; Fig. 3E–F). No significant intraspecific differences in crawled distance were found in control sea water or virgin MPLs for either adults (Tables 9–10SM) or recruits (Tables 12–14SM).

Additionally, within each lineage, significant differences among treatments were detected for recruits for both the net (Table 12SM) and the gross distance (Table 14SM), specifically, for the Atlantic, between Beached and Control treatment and between Beached and Virgin.

3.3.3. *Aggregation behaviour*

Regardless of treatment, the number of aggregates of adult mussels did not differ significantly between lineages (Table 27SM; Fig. 4A). Yet, during exposure to beached MPLs, the average number of adults per aggregate was significantly higher in MED clumps (Tables 29–30SM, Fig. 4B). For recruits, however, the number of clumps in beached MPLs was significantly higher for MED than ATL (Tables 31–32SM; Fig. 4C), despite there being no significant difference in the average number of individuals comprising clumps (Tables 33–34SM; Fig. 4D).

Additionally, within each lineage, significant differences among treatments were detected for the number of aggregates formed by recruits (Table 32SM). Specifically, for the Atlantic, between Beached and Control treatment and between Beached and Virgin.

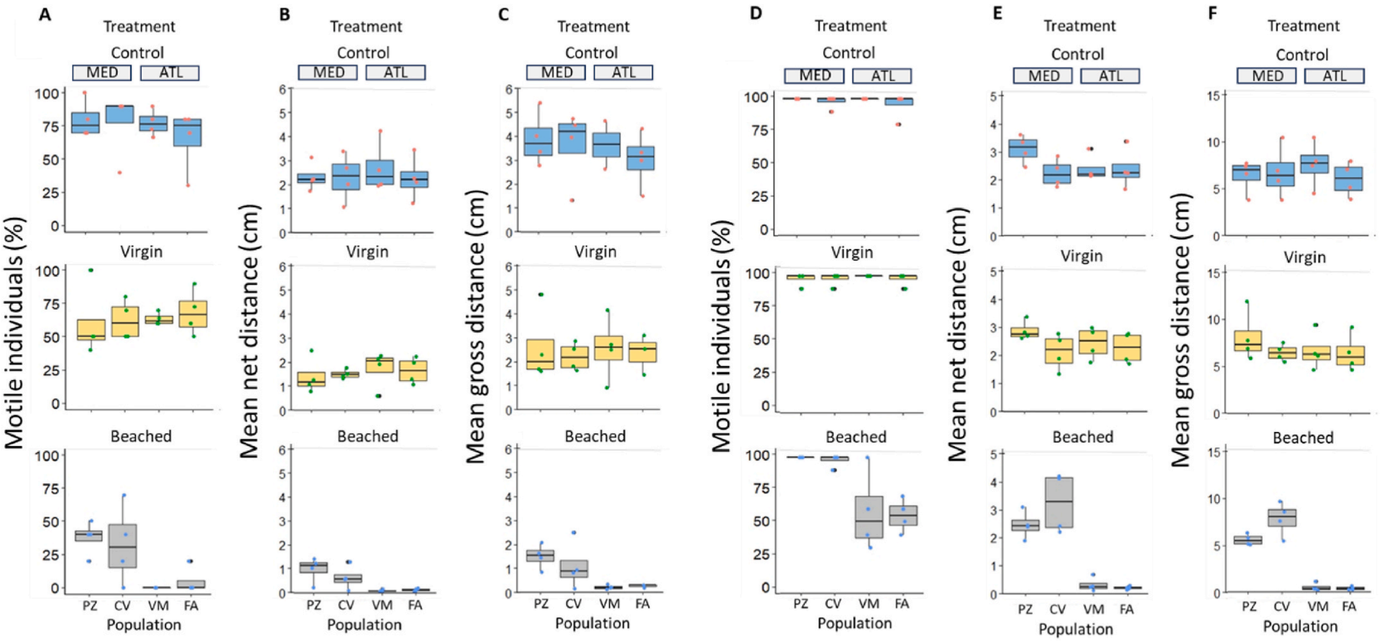

Fig. 3. Behavioural analysis of adults (A, B, C) and recruits (D, E, F) of *M. galloprovincialis* from Mediterranean (Pozzuoli, PZ – Castel Volturno, CV) and Atlantic (Faro, FA – Vilamoura, VM) lineages under 3 treatments (Control, C – Virgin MPLs, V - Beached MPLs, B). (A–D) Percentage of motile individuals, (B–E) Mean net (C–F) and mean gross distance moved (cm, n = 10 individuals, n = 4 replicates; t = 120 min).

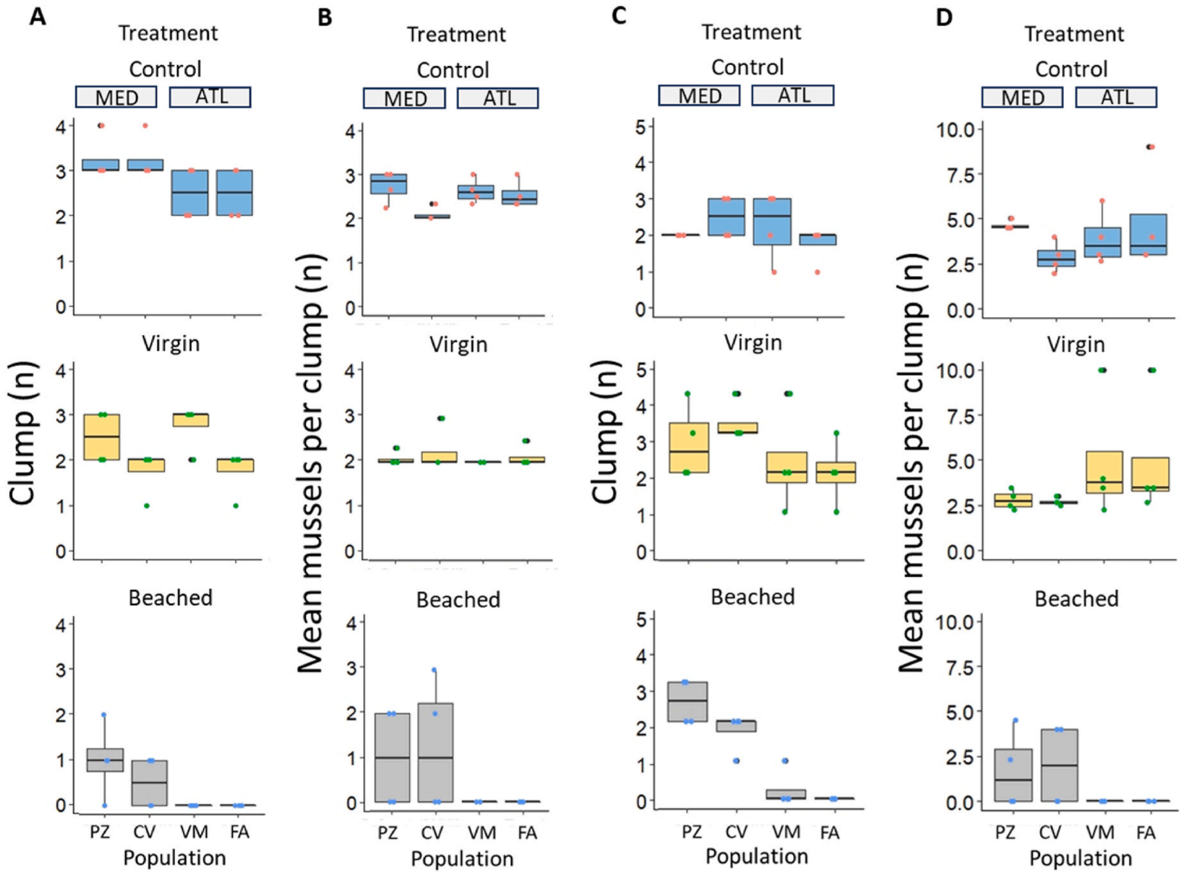

Fig. 4. Mean number of aggregates (A and C) and aggregate size (B and D) of *Mytilus galloprovincialis* adults (A and B) and recruits (C and D). Treatment and lineage abbreviations as in Fig. 3.

3.4. Osphradium experiment

The amplitude of the Electro-Osphradiogram (EOsG) responses varied fundamentally according to the type of stimulus and its concentration (Fig. 5).

At a concentration of 10^{-3} M, responses to L-cysteine differed significantly between lineages and between Mediterranean populations (Tables 35–36SM, Fig. 6A), regardless of treatment (Control seawater or Beached MPLs). Specifically, MED mussels harvested from Pozzuoli showed the highest response. Responses to L-leucine did not differ (Tables 41–42-43SM; Fig. 6B) between lineages or populations, while significant intraspecific differences were observed in response to proline (Tables 48–49SM; Fig. 6C) but only in the beached MPLs treatment.

At a concentration of 10^{-4} M, intraspecific differences in response to L-cysteine were only observed in mussels exposed to beached MPLs (Tables 37–38-39SM). No significant differences in lineage responses to L-leucine or proline were found, regardless of the treatment. However, an effect of the nested factor “population” was found as the two Mediterranean populations responded differently (Tables 44–45SM for Leucine and Tables 50–51SM for Proline respectively).

At the lowest concentration of 10^{-5} M, significant differences between lineages and between Mediterranean populations were found in response to L-leucine alone (Tables 46–47SM). Specifically, Mediterranean mussels from Pozzuoli showed the highest mean amplitude response.

4. Discussion

We show that microplastic leachates (MPLs) affect the behaviour of *Mytilus galloprovincialis* and that mussel responses may vary within the different genetic lineages of the species. Our findings consistently demonstrated a significant decrease in the proportion of motile individuals (both adults and recruits), the distances they crawled (both net and gross), and the number of aggregates they formed in beached MPLs compared to virgin MPLs or control seawater. Importantly, mussel responses to beached MPLs consistently differed between Atlantic and Mediterranean lineages of *Mytilus galloprovincialis*. We found adults and recruits of the Mediterranean lineage to be significantly more active and crawl significantly longer net and gross distances than those of the Atlantic lineage. Remarkably, only 2% of Atlantic lineage mussels actively moved during exposure to beached MPLs, the remaining 98% remained immobile despite being alive. In contrast, 35% of MED adults responded to beached MPLs.

The implications of our findings extend from the immediate effects of MPLs on mussels to the broader consequences at the ecosystem level for the numerous functions and services that mussels provide. In coastal habitats, mussels have a major role as ecosystem engineers (e.g., Suchanek, 1985). They modify their environment primarily through their aggregations (mussel beds), acting as autogenic and allogenic bioengineers that increase habitat complexity and modify the surrounding physical and chemical environment (e.g., Bertness, 1984; Borthagaray and Carranza, 2007; Nicastro et al., 2012). We showed that MPLs reduce the ability of Atlantic mussels to move, a prerequisite for the formation of mussel aggregations, the maintenance of their spatial organization, their capacity to withstand disturbances over shorter temporal scales, and their ability to reorganise aggregations following mortality events that increase the availability of space (Nicastro et al., 2008; de Paoli et al., 2017). By associating closely with others to form a bed, mussels benefit from a number of advantages including reduced risk of predation and amelioration of environmental conditions, which in turn increases mussel resistance to abiotic stressors. Notably, mussel aggregations benefit not only the mussels themselves, but are key to ecosystem functioning. Apart from being a primary food commodity, mussel beds provide habitat for many fish and invertebrates, act as nurseries for higher-trophic level taxa and ensure water clearance and nutrient turnover (Costanza et al., 1997; Raffaelli and Hawkins, 1999; Borthagaray and Carranza, 2007; Puri et al., 2021), as well as playing a key role in shoreline stabilization (Ysebaert et al., 2019) and the capture of ‘blue carbon’ (Macreadie et al., 2019).

Previous studies have reported effects of MP leachates on mussel behavioural responses similar to those observed in our study, but, in those studies, only virgin polypropylene pellets were used (Seuront et al., 2021; Uguen et al., 2022). Here, virgin MPLs showed a lower effect on mussel behaviour than beached MPLs. Specifically, they only affected

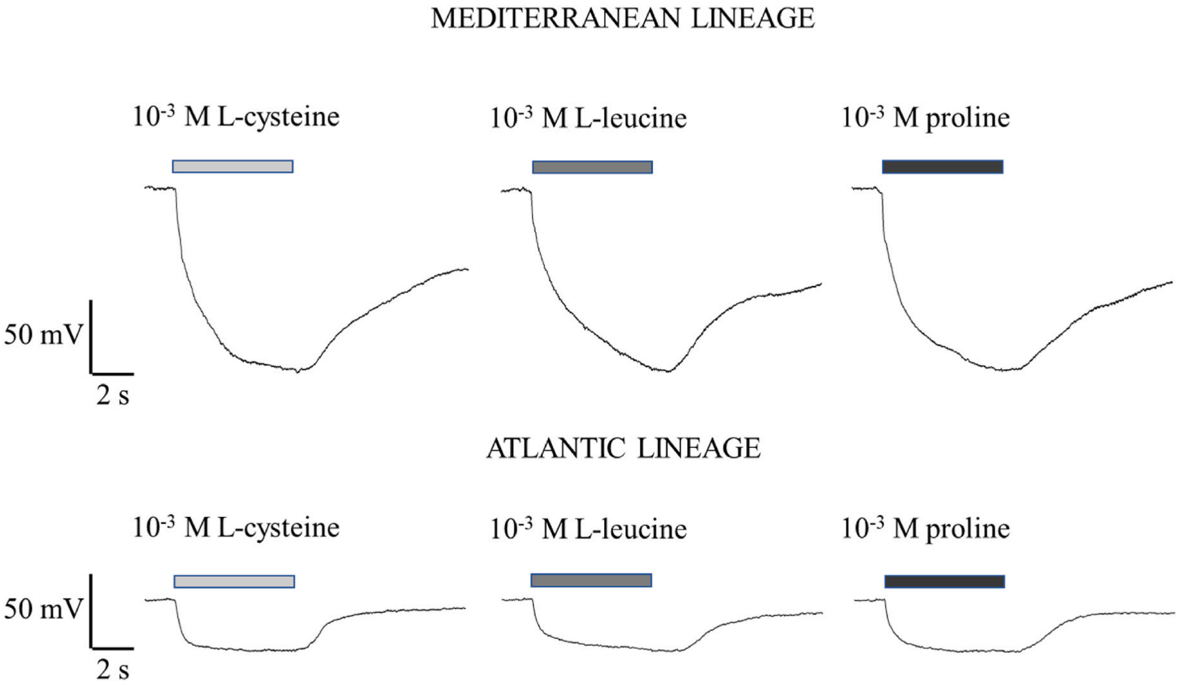

Fig. 5. Typical “EOsG” responses recorded from a mussel of the Mediterranean lineage (upper three traces) and Atlantic lineage (lower three traces) in response to 1 μM L-cysteine, 1 μM L-leucine and 1 μM proline. Mediterranean mussels typically gave larger amplitude responses than Atlantic mussels.

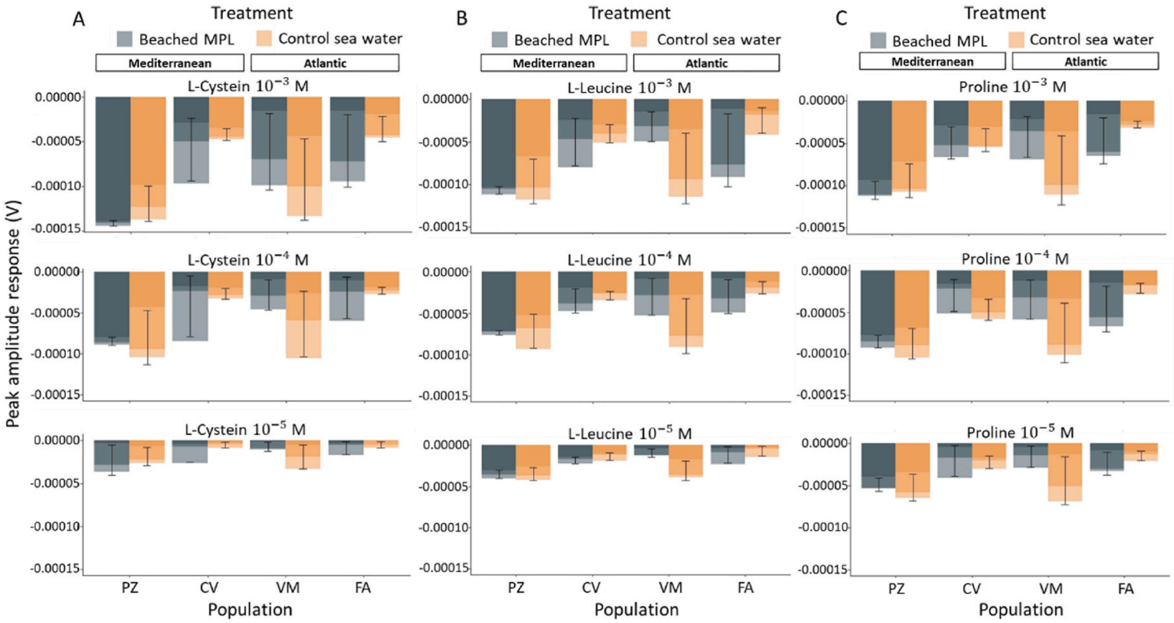

Fig. 6. Peak amplitude response of Mediterranean and Atlantic adults of *M. galloprovincialis* (n = 3; SD is provided) kept in control sea water and beached MPL sea water for 24 h prior analysis. Different shades of the same colour represent the different replicates. The mussels were tested using three stimuli (A) L-cysteine, (B) L-leucine and (C) proline at three increasing concentrations (10^{-5} M, 10^{-4} M and 10^{-3} M). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the responses of adult individuals (in terms of distances crawled and number of aggregates formed) and did not trigger intraspecific responses in mussel behaviour. Indeed, intraspecific differences in behaviour were only observed when using beached MPLs. Virgin and beached microplastic pellets often carry and release different chemicals (Fries and Zarfl, 2012; Capolupo et al., 2021; Gunaalan et al., 2020). Virgin MPs are a well-known source of primary leachates (e.g., plastic additives), whereas beached MPs can act as a source of both primary and secondary leachates (e.g., POPs, PCBs, DDT, heavy metals; Holmes et al., 2012; Delaeter et al., 2022). Several studies comparing the effects of virgin and beached MPLs have emphasized the higher toxicity of the latter (see e Silva et al., 2016; Seuront, 2018; Pannetier et al., 2019; Wang et al., 2020), though exceptions exist (Nobre et al., 2015). Our findings support the stronger effects of beached MPLs on key behavioural traits of *M. galloprovincialis* compared to virgin pellets. Other studies have emphasized the effects of leachates obtained from plastic/rubber polymers in reducing larval motility and survival and affect cellular and developmental parameters in *M. galloprovincialis* to varying degrees, depending on polymer type (Capolupo et al., 2020; 2021).

In general, to ensure a comprehensive understanding of the effects of MPLs on organisms, it is important to identify the plastic polymers and leachates screened. In the present work, virgin and beached pellets drastically differed in their additive load, the former and the latter containing respectively 26 and 3 additives. The leaching of contaminants from both the matrix and the surface of plastics depends on environmental factors and weathering forces, as well as intrinsic factors such as polymer features including texture (Brydson, 1999) and molecular binding (Paluselli et al., 2018; Tang, 2021; see also Delaeter et al., 2022 for a review). Such effects are exacerbated by the fact that MPs exhibit a high surface area to volume ratio that enhances sorption-desorption dynamics on their surface (Agboola and Benson, 2021). Hence, it is likely that beached microplastics have fewer additives than virgin ones as a result of their long-term residence in the natural environment. Virgin pellets were characterized by a range of phthalate plasticizers—in particular Diisodecyl phthalate, DIDP—that are widely acknowledged as endocrine disruptors even at low concentration, and induce numerous effects on aquatic organisms, including their behaviour; see Oehlmann et al. (2009) for a review of the effects of phthalates on aquatic organisms. Despite leaching considerably fewer additives than virgin pellets, the beached microplastics also carried heavy metals. X-ray diffraction (XRD) analysis performed in an earlier study (Zardi et al., in review) on the same batch of pellets used here, showed high contamination by Pb, Cd, As, Ti, Cu, Fe and Si. Many of these have previously been classified as priority pollutants by the U.S. Environmental Protection Agency because of their high toxicity and their capacity for persistence, bioaccumulation and biomagnification within natural ecosystems (Bryan and Langston, 1992; Luoma and Rainbow, 2008; Li et al., 2015a,b). Iron, copper and lead, for example, have been shown to have severe effects on fish olfaction at structural, biochemical and physiological levels even at concentrations far below lethal values (Baatrup, 1991; Olsén, 2011). In aquatic molluscs, heavy metals have been found to change the electrical processes in the osphradial receptor cells (Rózsa and Salánki, 1990). These findings strongly support the role of the osphradium in the response to heavy metal exposure and in turn, corroborate our initial hypothesis involving the osphradium in the mechanisms behind responses to MPLs. Importantly, the concentrations of metals on the surface of plastic particles become higher than concentrations in the water column and these metals are highly bioavailable, posing even greater ecological threats (Holmes, 2013).

To our knowledge this is the first investigation into the effects of MPLs on the chemosensory activity of mussels. As expected, we found the peak amplitude response of mussels to be dependent upon stimulus concentration. Hence, in response to stimuli of fixed duration (i.e., 6 s) and increasing concentration (10^{-5} to 10^{-3} M), the peak amplitude response increased until reaching plateau values and subsequently exhibited a decay slope. Our analysis highlighted high inter-individual variability in the amplitude of response, potentially linked to inter-individual sensitivity, as suggested for the oyster *Magallena gigas* (Boullot et al., 2021). We found significant between-lineage differences in response to L-cysteine (10^{-4} M) and proline (10^{-3} M) in mussels pre-exposed to beached MPLs. This differential sensitivity in the osphradium of the two lineages may be at least partially responsible for the intraspecific differences observed in the behavioural responses.

Intraspecific differences were also found in response to L-cysteine (10^{-3} M) and L-leucine (10^{-5} M) regardless of mussel treatment (control sea water or beached MPLs). The different control responses of the two lineages suggest potential differences in their physiology that could be related to genotypic or phenotypic factors as well as individual sensitivity or specific adaptation to local contaminants. Such differential adaptation could also explain the different behaviours observed between lineages. Specifically, Mediterranean mussels appeared to be more adapted to leached contaminants to the extent that they did not exhibit such strong behavioural effects as shown by Atlantic mussels. Reinforcing this interpretation are numerous studies carried out near our study sites in the Gulf of Pozzuoli and in Castel Volturno that highlight the high levels of contamination by metals (e.g., Cu, Fe, Hg, As, Cd, Pb), PAHs and PCBs in the areas surrounding the sampling sites (see Arienzo et al., 2017; Trifuoggi et al., 2017; Romano et al., 2018; De Rosa et al., 2022). Whether the history of mussel exposure to chemicals in the water affects their response to experimental treatments is only a hypothesis. The mussels sampled in Pozzuoli, for example, differed from the others in their exposure to chemicals due to differences in attachment substratum and immersion time, as well as differences in geographical location. Despite the behavioural analysis failing to show significant inter-population differences, the analysis of the osphradium often showed stronger responses in mussels from Pozzuoli. These individuals may have had life-long exposure to heavy metals due to their attachment to a metallic buoy possibly coated with antifouling compounds. In order to overcome the potential effects of such environmental factors, future research should be carried out using specimens cultivated under laboratory conditions.

5. Conclusions

While current belief generally suggests a low global risk posed by microplastic pollution to aquatic ecosystems, growing data on hazards and exposure indicate that risk is possible in specific locations. This is particularly true where sensitive organisms inhabit regions where plastics, particularly microplastics, accumulate. Considering that the abundance (mass and count) of microplastics is likely to keep increasing in the coming years due to the breakdown of existing and new debris, this risk is expected to rise. It is clear that we have a significant journey ahead before we can confidently claim to comprehend the long-term consequences of ongoing and future plastic contamination in the environment.

We are of the opinion that our results are novel and contribute essential insights to the continuous and worldwide research efforts that strive to furnish the information necessary for mitigating or reducing any potential harm in the future. Here we demonstrate for the first time the effect of intraspecific variation on the behavioural and chemosensory responses of a marine mussel during exposure to a complex mixture of contaminants leached from microplastic pellets. Our research revealed distinct intraspecific behavioural and chemosensory responses to leachates from beached microplastics. Such differences can be potentially attributed to local adaptation and physiological variability between lineages. Taken together, our results suggest that not all populations of the same species respond equally to a given stimulus and underline the mechanisms by which intraspecific variation can modulate the responses to microplastic leachates. We further emphasise the ecological relevance of intraspecific diversity and indicate the need for a critical re-evaluation of strategies for the management of plastic contamination in natural ecosystems.

CRediT authorship contribution statement

Lorenzo Cozzolino: Conceptualization, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Katy R. Nicastro:** Conceptualization, Formal analysis, Resources, Writing – review & editing, Funding acquisition, Project administration, Supervision. **Peter C. Hubbard:** Investigation, Formal analysis, Resources, Writing – review & editing, Funding acquisition. **Laurent Seuront:** Formal analysis, Writing – review & editing. **Christopher D. McQuaid:** Writing – review & editing, Supervision. **Gerardo I. Zardi:** Conceptualization, Investigation, Formal analysis, Resources, Writing – review & editing, Funding acquisition, Supervision.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122779.

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