THESE DE DOCTORAT

Pour obtenir le grade de docteur de l'Université de Lille

Ecole Doctorale - 104
Sciences de la Matière, du Rayonnement
et de l'Environnement
Université Lille Nord de France

Spécialité : Biologie de l'environnement, des organismes, des populations, écologie

par

Marine UGUEN

New insights into the ecology of a key ecosystem engineer, the blue mussel *Mytilus edulis*, in an era of global change: focus on the effects of plastic leachate pollution

◆

Nouvelles perspectives sur l'écologie d'une espèce clé de l'écosystème, la moule bleue *Mytilus edulis*, à l'ère du changement global : focus sur les effets de la pollution par les lixiviats de plastiques

Soutenue le 25/01/2024

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Christopher MCQUAID, Professeur, Rhodes University, Afrique du Sud	Rapporteur
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Hortense SAINT HILAIRE, France Filière Pêche, France	Invitée
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« The mind is not a vessel to be filled but a fire to be kindled »
Plutarch

SCIENTIFIC CONTRIBUTION

PUBLICATIONS

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- **Uguen, M**., Nicastro, K.R., Zardi, G.I., Gaudron, S.M., Spilmont, N., Akoueson, F., Duflos, G. and Seuront, L., 2022. Microplastic leachates disrupt the chemotactic and chemokinetic behaviours of an ecosystem engineer (*Mytilus edulis*). *Chemosphere*, *306*, p.135425.

- Seuront, L., Zardi, G.I., **Uguen, M.,** Bouchet, V.M., Delaeter, C., Henry, S., Spilmont, N. and Nicastro, K.R., 2022. A whale of a plastic tale: A plea for interdisciplinary studies to tackle micro-and nanoplastic pollution in the marine realm. *Science of the Total Environment*, *846*, p.157187.

- **Uguen, M.,** Gaudron, S.M., Nicastro, K.R., Zardi, G.I., Spilmont, N. and Seuront, L., 2023. Size-dependent response of the mussel collective behaviour to plastic leachates and predator cues. *Science of The Total Environment*, *888*, p.164037.

- **Uguen, M.,** Gaudron, S.M., Nicastro, K.R., Zardi, G.I., Spilmont, N., Henry, S. and Seuront, L., 2024. The tolerance of a keystone ecosystem engineer to extreme heat stress is hampered by microplastic leachates. *Biology Letters, 20*, p.20230457.

Submitted & To be submitted

- **Uguen, M.** and Cozzolino, L. Location-dependent effect of microplastic leachates on the respiration rate of two engineering mussel species. **Submitted to *Environmental Science and Pollution* on 27 December 2023.**

- **Uguen, M.,** Nicastro, K.R., Zardi, G.I., Gaudron, S.M., Spilmont, N. and Seuront, L. Moule, pression de prédation et pollution plastique : la menace des lixiviats. **Submitted as a book chapter to *Presses Universitaires du Septentrion.***

- **Uguen, M.,** Gaudron, S.M. and Seuront, L. Plastic pollution and marine mussels: unravelling disparities in research efforts, biological effects and influences of multiple co-occuring stressors. **To be submitted.**

- **Uguen, M.,** Gaudron, S.M., Rahoui, A., Lefebvre, V. and Seuront, L. Mussel aggregation behaviour: a reproductive strategy impaired by plastic leachates. **To be submitted.**

In preparation

- **Uguen, M.,** Bosc, C., Seuront, L. and Gaudron, S.M. Leachates from plastic pellets and aquaculture materials disrupt the blue mussel’s early life stages.

ORAL COMMUNICATIONS

International Conferences

- **Uguen, M.,** Nicastro, K.R., Zardi, G.I., Gaudron, S.M., Spilmont, N., and Seuront, L. Microplastic leachates induce disruption of chemotactic and chemokinetic responses of an ecosystem engineer to invasive predator. *ICAIS 2022,* 18 – 22 April 2022, Oostende, Belgium — 3rd place in the student oral presentation competition

- **Uguen, M.,** Gaudron, S.M., Nicastro, K.R., Zardi, G.I., Spilmont, N., Henry, S., and Seuront, L. Plastic pollution in a warming world: What are the risks for engineer species? *ASLO Aquatic Science Meeting 2023,* 4 – 9 June 2023, Palma de Mallorca, Spain

National Conferences

- **Uguen, M.,** Nicastro, K.R., Zardi, G.I., Gaudron, S.M., Spilmont, N., and Seuront, L. Moule, pression de predation et pollution plastique : la menace des lixiviats. *MARCO 2021,* 13 – 15 October 2021, Boulogne-sur-mer, France

- **Uguen, M.,** Gaudron, S.M., Nicastro, K.R., Zardi, G.I., Spilmont, N., Henry, S., and Seuront, L. Pollution massive par les larmes de sirène : consequences sur une espèce ingénieure. *GDR Polymères & Océans,* 26 – 28 June 2023, Rennes, France

- **Uguen, M.,** Bosc, C., Gaudron, S.M., Nicastro, K.R., Zardi, G.I., Spilmont, N., and Seuront, L., 2023. Consequences of plastic leachates on common mussel's early life stages. *EGHYMANCHE*, 5 – 7 July 2023, Wimereux, France

POSTER

- **Uguen, M.,** Nicastro, K.R., Zardi, G.I., Gaudron, S.M., Spilmont, N., Akoueson, F., Duflos, G., and Seuront, L. Microplastic leachates impair chemotactic and chemokinetic responses of an ecosystem engineer to an invasive predator. *GDR Polymères & Océans,* 27 – 29 June 2022, Brest, France

TEACHING & CO-SUPERVISION

TEACHING

Biostatistics and Ecosystemic Ecology classes of practical work to second year bachelor students from Lille University 2022 – 2023

Total teaching time: 64 h

CO-SUPERVISION

Co-supervision of a Master 2 student: Alexandre Rahoui, January-June 2022, on the mussel behaviour and reproductive maturity

Co-supervision of a Master 1 student: Cécile Bosc, April-May 2023, on the impact of plastic leachates on fertilisation and mussel early life stages

WORKSHOP ANIMATION

- Animation of a workshop at the Science Sea Festival (08-11 July 2021)

- Animation of a workshop at the Science Festival (13 October 2022)

- Animation of a workshop at the Researchers' Night (29 September 2023)

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GENERAL INTRODUCTION

1. Coastal ecosystems

The ocean covers 70.8% of our planet, yet due to its three-dimensional structure, it alone represents 99% of the biosphere volume (Costanza, 1999). Despite this vastness, the distribution of species richness within marine systems is not uniform, with the highest biodiversity concentrated within coastal ecosystems, which occupy only about 0.5% of the ocean's volume (Hoepffner & Zibordi, 2009; Ray, 1991). At the crossroads between terrestrial, marine and atmospheric systems, coastal systems are transition zones that form a unique and diverse set of ecosystems, essential on a global scale (Ray, 1991). They include estuaries, tidal flats, mangroves, coral reefs, continental shelves, seagrass and algae beds (Costanza et al., 1997; Costanza, 1999). The ecological, economic, and societal benefits these systems provide are numerous and highly valuable, such as nutrient cycling, biological productivity, food production, habitat provision and disturbance regulation (Barbier, 2017; Costanza et al., 1997; Costanza, 1999; Figure 1). Consequently, these systems collectively account for around 43% of the world's ecosystem goods and services (Costanza, 1999).

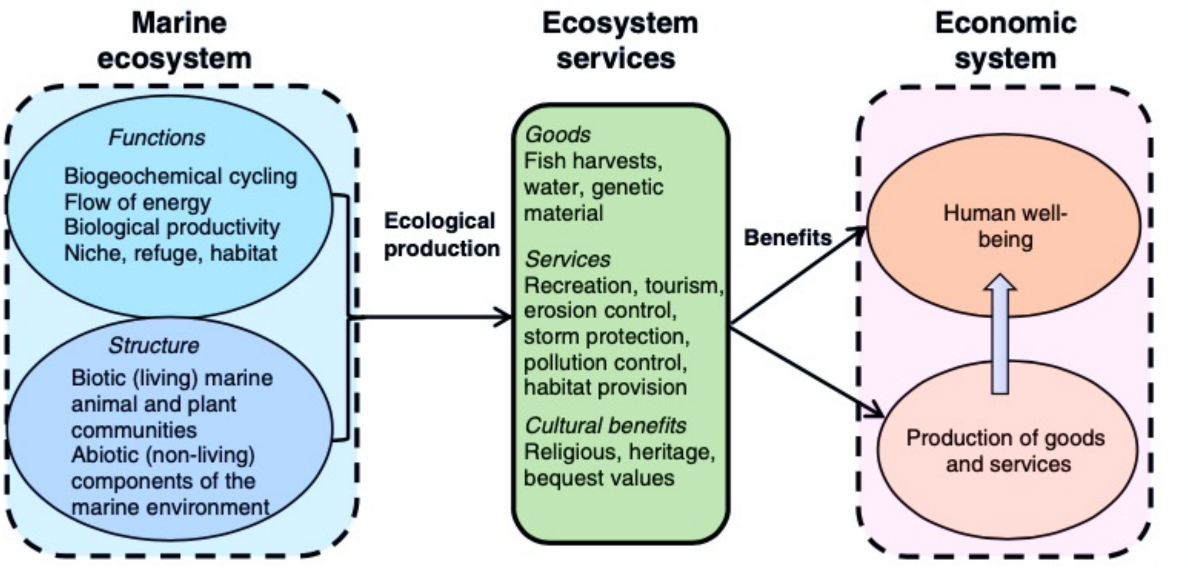


Figure 1: How marine ecosystems generate economic benefits (adapted from Barbier, 2017).

Coastal regions are also the most densely populated areas in the world (Hugo, 2011) and, as such, the most heavily impacted by anthropogenic activities (Williams et al., 2022). Terrestrial and marine anthropogenic pressures, such as urbanisation, industrialisation, agriculture, tourism, maritime transport, and fisheries, may locally act as additional pressure on these complex and vulnerable ecosystems, which are already affected by climate-induced, large-scale disturbances (*e.g.* warming, rising water levels, ocean acidification, deoxygenation, storms, *etc.*; IPCC, 2022; Lu et al., 2018). Indeed, no coastal region is free of human influence, with only 15.5% considered to have a "low" level of disturbance (mainly in Canada, Russia and Greenland; Williams et al., 2022). Since the onset of industrialisation, coastal ecosystems have experienced a rapid decline in biodiversity, with a staggering 91% of coastal species that have declined by more than 50% (Worm et al., 2006). This has led to an alteration of essential ecosystem services such as fisheries (-33%), provision of breeding habitat (-69%), filtering and detoxification services (-63%; Worm et al., 2006). The loss in coastal ecosystem services is estimated to be trillions of dollars each year (Costanza et al., 2014).

Coastal species are thus subjected to multiple stressor situations, as all these natural and anthropogenic drivers interact and mainly exacerbate each other’s effects, leading to major detrimental impacts on these systems (He and Silliman, 2019; IPCC, 2022). Yet, the livelihoods of billions of people directly hinge on the ecosystem services coastal regions provide. Consequently, any degradation of these areas poses a significant challenge to humanity, as we rely on them for various essential services, including climate change mitigation, food supply, and storm protection. It is thus essential to unravel the effects of anthropogenic drivers in order to seek sustainable solutions to safeguard these essential ecosystems for the well-being of both the current generation and the legacy of the next ones.

In this context, the planet boundaries concept identified environmental limits for nine processes within which humanity can safely operate, *i.e.* climate change, rate of biodiversity loss, interference with nitrogen and phosphorus cycles, stratospheric ozone depletion, ocean acidification, global freshwater use, change in land use, atmospheric aerosol loading and chemical pollution (Rockström et al., 2009). One of these nine boundaries, initially referred to as "chemical pollution", updated as "novel entities" planetary boundary by Steffen et al. (2015), includes the "*new substances, new forms of existing substances, and modified life forms that have the potential for unwanted geo- physical and/or biological effects*". Despite its significance, the global risk level of this planetary boundary remains to be quantified, underlining the need to increase research efforts in this area (Persson et al., 2022; Steffen et al., 2015). Within the spectrum of novel entities, plastic pollution has emerged as a high concern prominent environmental issue (Persson et al., 2022), particularly in the coastal system (Díaz-Mendoza et al., 2020; Onink et al., 2021).

2. Plastic pollution

2.1. From a miracle material to a marine scourge

The plastic odyssey: production, consumption, disposition

Amongst environmental pollution, one recent compound stands out: plastic. Its advantageous characteristics, such as its versatility, durability, lightness and affordability, have elevated this material to the third most human-made material in the world (behind steel and cement; Geyer et al., 2017). It is a fundamental part of people's daily lives (*e.g.* through clothing, food packaging, electronic devices, car and house composition) and has thus profoundly shaped the development of modern society (Napper & Thompson, 2020). Undeniably, plastic offers a myriad of benefits, particularly in its contributions to public health (Andrady & Neal, 2009), *e.g.* by ensuring access to clean drinking water and through its pivotal role in the medical sector, enabling the production of surgical equipment, aseptic medical packaging, and the creation of prosthetics. The benefits of plastics extend to energy savings, particularly within the transportation industry, where it enables the production of lighter vehicles, enhancing fuel efficiency. Indeed, every 1 kg of vehicle weight reduction was estimated to result in a reduction of *ca.* 20 kg in CO_2 emissions released into the atmosphere (Szeteiova, 2010). In addition, the plastic industry is a significant source of employment, with the European plastic industry employing about 1.5 million people with a turnover of 405 billion euros (data from 2021; which corresponds to Norway's annual GDP in 2021), ranking 8th in Europe in terms of contribution to industrial value added (data from 2019; PlasticsEurope, 2022). However, despite its advantages and economic importance, plastic represents one of the foremost challenges of the 21st century.

Since its large-scale production in the 1950s, the total amount of plastic produced worldwide is constantly growing and is estimated to be over 9.2 billion tonnes to date (Geyer, 2020) with 400.3 million tonnes in 2022 (excluding fibres; PlasticsEurope, 2023; Figure 2). Projections suggest that if the current trajectory of plastic production persists, the cumulative production could reach 34 billion tonnes by the year 2050 (Geyer, 2020). However, the plastic industry is far from environmentally sustainable. Plastic heavily relies on fossil resources as fuel and feedstock, especially for its production (da Costa et al., 2020). Indeed, nearly half of the petroleum used in the chemical industry is used in plastic production (IPCC, 2022). Thus, plastic global footprint accounts for a noteworthy proportion of global greenhouse gas (GHG) emissions, *i.e.* 4.5% (≈ 2 $GtCO_2$-eq, data from 2015; Cabernard et al., 2022). This figure aligns with the combined GHG emissions from the aviation and shipping sectors (*i.e.* 1.04 and 0.96 $GtCO_2$-eq, respectively; IPCC, 2022).

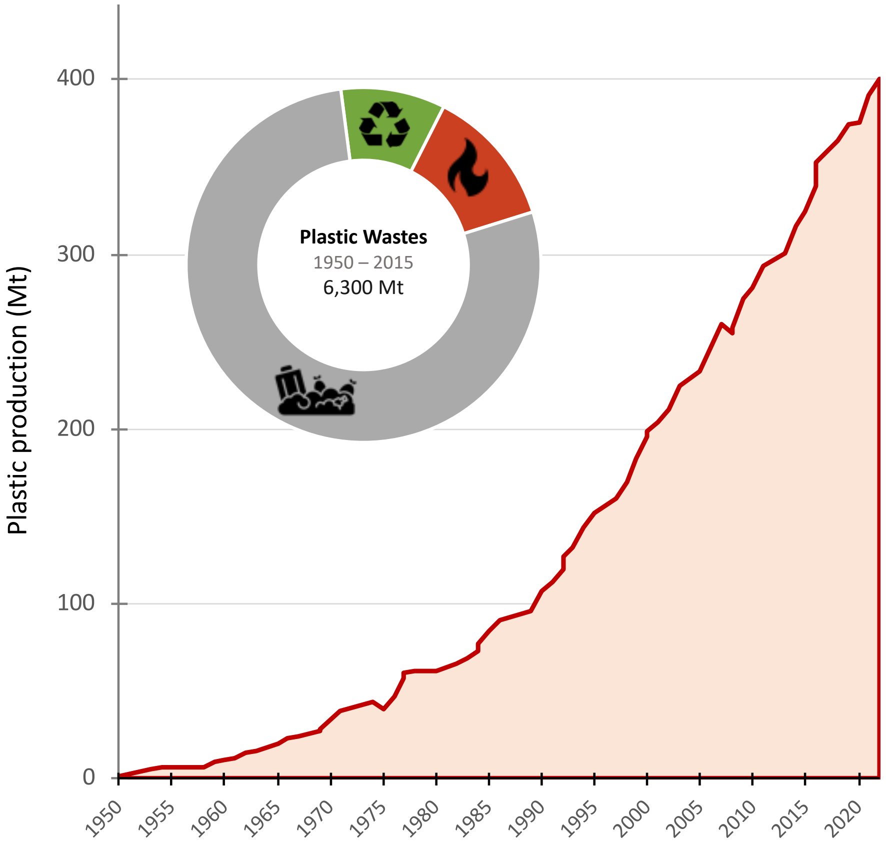


Figure 2: Global plastic production in million tonnes (excluding fibres; density graph), and plastic waste management (from 1950 to 2015; pie chart), between recycling (9% - 600 Mt; green), incineration (12% - 800 Mt; red) and disposal in landfill or in the natural environment (79% - 4,900 Mt; grey).

In addition, most plastics have a short lifespan; only 30% of plastics ever produced worldwide are currently being used (2,500 Mt; Geyer et al., 2017). One of the primary sources of waste is single-use plastics (SUP), which are the most commonly produced plastic despite their very short lifespan, being mostly discarded within a year of production (Chen et al., 2021; Geyer, 2020). However, numerous regulations have noticeably been implemented in recent years to prevent plastic waste pollution, *e.g.* the “Single-Use Plastics Directive” (EU Directive 2019/904) that bans several single-use plastic products; for other regulation examples see da Costa et al. (2020). At the end of their life, a very small proportion of the total unused plastics is recycled (9% - 600 Mt) or incinerated (12% - 800 Mt), and the majority (79% - 4,900 Mt) is disposed of and accumulated in landfills or in the natural environment (data for cumulative plastic waste generated between 1950 and 2015, total = 6,300 Mt; Geyer et al., 2017; Figure 2). Thus, its production and overuse are hampered by rapid disposal and poor management, resulting in a global accumulation and distribution of plastic wastes worldwide, in all major environmental compartments, *i.e.* the atmosphere, hydrosphere and lithosphere (Barnes et al., 2009; Geyer et al., 2017; Hurley et al., 2020; Porta, 2021).

While present in all biosphere compartments, the ocean is the earth's primary plastic waste repository. The major proportion (80%) of marine plastic pollution would be land-based (Andrady, 2011), a global amount estimated between 4.8 and 12.7 million tonnes in 2010 (Jambeck et al., 2015), although potentially underestimated. Indeed, a more recent study, Borrelle et al. (2020) estimated that in 2016, *ca.* 19 to 23 million tonnes of plastic waste entered the oceans or other aquatic systems (here, major lakes and rivers connected to the oceans). Plastic can be transported from land to sea by rivers, municipal wastewater, wind, precipitation and extreme events such as tsunamis, hurricanes, floods and sewage overflows (Napper & Thompson, 2019). Rivers act as a significant transport pathway (Schmidt et al., 2017), being notably polluted by wastewater treatment plants that are estimated to discharge billions of plastic particles each day (Liu et al., 2021). Plastic can also be directly released into the sea; industries related to fishing and aquaculture, offshore platforms, cruises, merchant vessels, and recreational boating are the main sources of ocean-based marine plastic pollution, *e.g.* accidental losses of containers, fishing gear, aquaculture equipment (Deville et al., 2023; Gravier & Haut, 2020; Jayathilaka et al., 2022; Lubchenco & Haugan, 2023; Richardson et al., 2019). Although the ocean-based source has not been globally estimated, it was estimated for Peru to range from 2,715 to 5,584 tonnes in 2018 (Deville et al., 2023).

Box 1. Plastic: A matter of size

Plastic debris can be characterised in various ways, *e.g.* by its shape, polymer, colour, and original purpose. However, size is the most common descriptor. Plastics are primarily classified by their size into four distinct categories: macroplastics (> 20 mm), mesoplastics (5 - 20 mm), microplastics (1 µm - 5 mm; MP), and nanoplastics (< 1 µm; NP; Napper & Thompson, 2020).

MP and NP can be further sub-categorised based on their sources (Figure B1; Napper & Thompson, 2020; Seuront et al., 2022). Primary MP and NP are plastics deliberately produced in these small forms, typically in the shape of tiny beads or pellets. They are commonly employed as raw materials in the manufacturing of plastic items, for padding, or for their exfoliating properties in cleaning, cosmetic, and hygiene products (Figure B1). Secondary MP or NP, conversely, result from the fragmentation or degradation of larger plastic items (Figure B1; Napper & Thompson, 2020; Seuront et al., 2022). They are the most commonly encountered in the marine environment. They may result from the use of products, *e.g.* tyre wear, fibres from washing clothes, or be caused by the degradation of larger items in the environment through physical, chemical and biological processes, *e.g.* mechanical abrasion, UV light, oxidation and biodegradation (Figure B1).

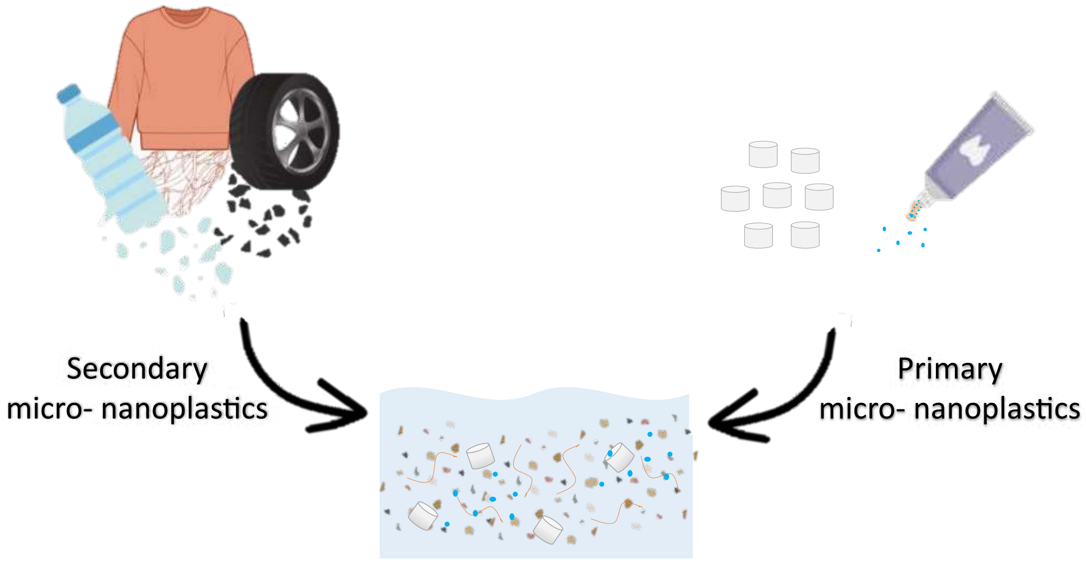


Figure B1: Primary and secondary micro and nanoplastics from various sources entering the marine environment.

As a consequence of the above-mentioned figures, plastic pollution is the most common form of marine litter (*ca.* 80 - 85%; Auta et al., 2017). The smallest sizes of plastic particles are the most abundant, mainly due to the breakdown of larger debris (see Box 1; Eriksen et al., 2014). The global abundance of microplastics (from 1 µm to 5 mm; Box 1) floating on the surface of the world's oceans has been increasing rapidly since 2005, with the latest estimation reaching a new record, *i.e. ca.* 82 - 358 trillion particles in 2019 (≈ 1.1 - 4.9 million tonnes; Eriksen et al., 2023). It should be noted that floating plastics represent only a fraction of the plastic issue (*ca.* 5% of the marine plastics; Andrade et al., 2021). In addition, while an important proportion (> 70%) of plastic waste entering the oceans from land is trapped in coastal regions (Onink et al., 2021), a part will escape and will be scattered throughout the world's oceans. Plastic vertical and horizontal dispersals are subject to a complex interplay of oceanographic and biological processes, enabling plastic to infiltrate and inhabit diverse marine environments (Sebille et al., 2020). Plastic has thus colonised all oceans and accumulated in even the most remote and pristine locations, spanning from the Arctic to the Antarctic, and extending from the ocean's surface to its deepest trenches (Chiba et al., 2018; Sebille et al., 2020).

The unfortunate legacy: plastic adverse effects

Since the first occurrence of plastic in marine organisms was reported in 1962, *i.e.* in the seabird leach’s storm petrel *Oceanodroma leucorhoa* (Rothstein, 1973), the number of studies reporting plastics in marine organisms has been growing. Indeed, because of their ubiquity and their various sizes, plastics are available to a wide range of marine species (Auta et al., 2017). The mechanical impacts of plastic pollution encompass a spectrum of issues, ranging from highly visible problems like the entanglement and strangulation of over 350 marine species (Kühn & van Franeker, 2020) to a less conspicuous but widespread concern: ingestion (Auta et al., 2017; Derraik, 2002; Gall & Thompson, 2015). Plastic ingestion is prevalent due to the abundance of small plastic particles. The smaller the plastic particle is, the greater its likelihood of being ingested by a wider range of organisms (Wright et al., 2013). Recent inventories revealed 1,288 marine species reported ingesting plastic, including a wide variety of organisms: more than 200 seabird species and invertebrates, more than 80 marine mammal species, more than 700 marine fishes (Santos et al., 2021) and various marine reptiles, including all the seven sea turtle species (Kühn & van Franeker, 2020; Santos et al., 2021). Plastic ingestion and entanglement can pose significant dangers to marine life, resulting in drowning, injuries, obstruction of the digestive and/or respiratory tract that can lead to numerous effects, *e.g.* pathological stress, false satiety, reproductive complications, blocked enzyme production, reduced growth rate and oxidative stress, and may sometimes result in the death of the affected organisms (for reviews see: Auta et al., 2017; Derraik, 2002; Gall & Thompson, 2015). It should also be noted that primary producers are not spared from plastic-negative effects, *e.g.* impairment in cell growth, chlorophyll a content and photosynthetic efficiency (Casabianca et al., 2021; Colzi et al., 2022; Sánchez-Fortún et al., 2021; Sjollema et al., 2016; Zhao et al., 2019; Zhu et al., 2019).

Plastic retention time in an organism varies. Particles may either pass through the digestive system and be excreted or accumulate within the organism for varying durations depending on the species and the plastic characteristics (Huang et al., 2022a). Furthermore, recent research showed that the smallest plastic particles can translocate into the circulatory system, cells, and tissues of an organism, a process which, since its initial observation by Browne et al. (2008) in blue mussels, has been documented in a large number of other species (*e.g.* Brennecke et al., 2015; Messinetti et al., 2019; Scanes et al., 2019; Zeytin et al., 2020). This has implications for plastic bioavailability as plastic can enter the trophic chains (i) at the base, through its adsorption or by uptake and translocation in primary producers (Larue et al., 2021), *e.g.* from microalgae to four higher order species in aquatic environment (Chae et al., 2018) or from vascular plants to snails in terrestrial environment (Chae & An, 2020), or (ii) via higher trophic levels through organisms that have ingested plastics directly or indirectly via their prey (*e.g.* from mussel to crab; Farrell & Nelson, 2013; Watts et al., 2014).

Plastic can also lead to less known physical effects, as the substrate itself can act as a vector for invasive species or pathogens (Bowley et al., 2022; Carlton et al., 2017; Gall & Thompson, 2015). Due to its properties, plastic is easily colonised by a wide variety of microorganisms, also referred to as the ‘plastisphere’, communities that may be highly distinctive from the surrounding surface waters (Zettler et al., 2013). Among the microorganisms associated with plastics, some can be pathogenic (*e.g. Escherichia coli, Vibrio parahaemolyticus, Bacillus anthracis* and *Pseudomonas aeruginosa*) and lead to the transmission of disease following plastic particle ingestion (Bowley et al., 2022; Junaid et al., 2023). Additionally, the bacterial communities associated with plastics can exchange genes, such as pathogenic and antibiotic-resistance genes, that can make them more virulent or resistant (Shen et al., 2019). The formation of a biofilm by microorganisms on plastics can create favourable conditions for a succession of communities, including larger species, *e.g.* cnidarians, bivalves and bryozoans (García-Gómez et al., 2021). Due to its dispersal characteristics, plastic can act as rafts, transporting these organisms over long distances to non-native regions where they can become invasive (García-Gómez et al., 2021). Occasionally, extreme weather events may also contribute to the movement of invasive species through the transport of plastic materials, *e.g.* the tsunami in Japan in 2011 led to the transport of 289 living Japanese coastal marine species to American shores, mainly via plastic objects (Carlton et al., 2017). If these invasive species establish themselves successfully in their areas of invasion, they can out-compete native species and disrupt local ecosystems.

Humans are not exempt from the impact of plastics. They can be affected by impacts on their health, associated losses of ecosystem services and economic costs. Humans are exposed to plastic particles in their daily lives through inhalation, skin contact, and ingestion; see Campanale et al. (2020) and Zuri et al. (2023) for reviews. Plastic can be ingested at any level of the food chain, from plants to top predators, and notably seafood, but also through other consumables, *e.g.* sugar, salt, honey, beverages (Campanale et al., 2020; Zuri et al., 2023). Cox et al. (2019) estimated that the annual ingestion of microplastics is between 39,000 and 52,000 particles, with this estimate increasing to 74,000 to 121,000 when the inhalation route is included. However, these values appear to be largely underestimated as, for instance, an apple can contain 223,000 plastic particles per gram, which corresponds to an Estimated Daily Intake (EDI) for children of 1.41 million plastic particles (Conti et al., 2020). A more reliable way to assess plastic exposure will be to quantify quantities in terms of weight rather than particle numbers as analytical techniques evolve, allowing the detection of smaller particles that are present in greater numbers. Thus, plastics have widely contaminated human, being found in, *e.g.* human feces (Zhang et al., 2021a), urine (Pironti et al., 2023), saliva (Abbasi & Turner, 2021), colon (Ibrahim et al., 2021), liver (Horvatits et al., 2022), lungs (Amato-Lourenço et al., 2021), placenta (Ragusa et al., 2021), blood (Leslie et al., 2022) and may cause various health effects (Landrigan et al., 2023). In addition, plastic litter, the degradation of the ecosystems, along with its deleterious impact on charismatic species (*e.g.* turtles, cetaceans), can affect people's emotional state and thus their general well-being (Beaumont et al., 2019).

All the concerns raised by plastic pollution above can generate economic consequences. For instance, plastic may lead to health-related economic costs (Landrigan et al., 2023), in addition to the unappealing presence of plastics on the shoreline that may pose a significant detriment to the tourism industry, discouraging visitors and resulting in financial losses, parallel with the costs of cleaning and maintaining beaches (Beaumont et al., 2019). Furthermore, due to the damage they can cause to organisms, plastic pollution has the potential to reduce the efficiency and productivity of commercial fisheries and aquaculture and lead to important economic loss in these industries, although difficult to quantify (Conti et al., 2020). The disruptions caused by plastics to ecosystem services could amount to several billion USD annually (for a 1–5% decline in marine ecosystem services; Beaumont et al., 2019).

2.2. Plastic-associated chemicals: the pernicious cocktail

While the vast majority of research on the impacts of plastic on organisms typically considers plastics as inert and pure items, the actual situation is far more complex. Plastics are intimately associated with a diverse array of chemical compounds that can have profound and wide-ranging effects. These chemicals may have been added during the manufacturing process of plastics or adsorbed during their life cycle, and particularly at the end of their life for those that end up in the environment.

The plastic recipe: chemicals used in its manufacture

All plastic items are composed of one or more polymers and chemicals, *i.e.* some residual solvents, unreacted monomers, processing aids (*e.g.* catalysts) and non-intentionally added substances (*e.g.* impurities), as well as a wide variety of plastic additives (Aurisano et al., 2021). Although there is a general lack of transparency regarding the chemicals present in plastic, a recent inventory identified more than 13,000 plastic associated chemicals that are intentionally or unintentionally added during the plastic manufacture (UNEP, 2023; based on data from Aurisano et al. (2021) and Wiesinger et al. (2021).

Among these chemicals, additives are added to improve plastic functionalities, performances and/or appearances according to the expected characteristics of the manufactured item, such as plasticisers (*e.g.* phthalates), antioxidants (*e.g.* bisphenols) and flame retardants (*e.g.* organophosphorus and organobromine compounds) that respectively increase plastic flexibility, ageing properties and fire resistance (Wiesinger et al., 2021). In general, a plastic item is associated with a large diversity of additives, *e.g.* biodegradable bioplastics household items from local stores contained an average of 123 additives (Savva et al., 2023). Plastic additives also vary in quantity, from trace amounts (< 1% by weight) to major components, sometimes exceeding 50% by weight, *e.g.* PVC typically can contain 10 to 60% phthalates by weight (Net et al., 2015).

The demand for additives is continuously growing, following the increasing demand for plastic. Additives have, therefore, a high market value, *i.e.* around 45.6 billion USD in 2021 (Markets & Markets, 2023a). Plasticisers, which are one of the most extensively produced and consumed categories of plastic additives, notably through phthalates constituting over half of the plasticiser category, reached a market value of 17 billion USD in 2022 (Markets & Markets, 2023b). Assuming an average mass fraction of 7% additives in plastics (Geyer et al., 2017), the total amount of additives incorporated in plastics in 2021 could be estimated at *ca.* 27 million tonnes. Projections from Geyer et al. (2017) suggest that the cumulative production of additives will approximately reach a staggering 2,000 million tonnes by 2050.

Plastic is thus associated with an important cocktail of chemical compounds from the moment it leaves the factory, whether in the form of pellets or as the final manufactured product (Box 2). However, many chemicals associated with plastics in their manufacture are recognised as toxic. Specifically, out of more than 7,000 plastic manufacture associated chemicals screened, over 3,200, including plastic monomers, additives, processing aids, and non-intentionally added substances, were identified as having hazardous properties, *e.g.* carcinogenicity, mutagenicity, reproductive and organ toxicity, and their potential to act as endocrine disruptors (Aurisano et al., 2021; UNEP, 2023; Wiesinger et al., 2021).

Box 2. Plastic pellets: an invasive fundamental intermediate of plastic production

Pre-production raw resin plastic pellets, also referred to as nurdles or granules, are primary microplastics used as the building block material in the manufacture of plastic items, making them fundamental intermediaries in plastics production. It should be noted that these raw pellets are not free of chemicals, although the majority of chemicals are added when these pellets are melted and moulded into the desired plastic product (Hunter *et al*., 2022; Figure B2).

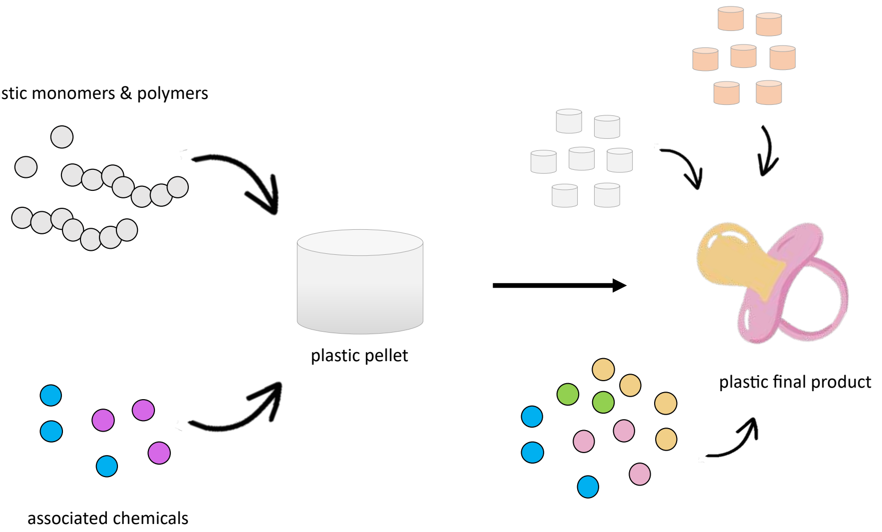


Figure B2: From plastic pellet production to final plastic item.

However, plastic pellets represent high levels of marine pollution, being often accidentally leaked into the environment throughout their production chain or during their transportation (Gravier & Haut, 2020; OSPAR Commission, 2018). In the EU alone, it is estimated that the loss of plastic pellets ranges from approximately 17,000 to 165,000 tonnes annually, with over 40,000 tonnes ultimately reaching the oceans (Hann et al., 2018). In addition, localised spill incidents can lead to extreme pollution events, *e.g.* the shore contamination by the 8-tonne spill from a tanker truck on the Opal Coast in northern France in 2016 (Gravier & Haut, 2020), or the largest marine plastic spill in history in Sri Lanka in 2021, where 70 billion plastic pellets were discharged due to a shipwreck (James et al., 2022; Jayathilaka et al., 2022). These make them accessible to a wide range of organisms. In addition, these pellets can accumulate several chemicals, and they are notably used in the international pellet watch survey, which aims to monitor POPs worldwide using beached plastic pellets (http://pelletwatch.org).

Plastic: a magnet for the surrounding chemicals

In addition, as the majority of plastics are hydrophobic, they tend to accumulate a wide variety of pollutants from their surrounding environment on their surface. As such, persistent organic pollutants (POPs; *e.g.* polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and pesticides) as well as inorganic contaminants (*e.g.* trace elements, halogens, and heavy metals) have been identified on plastic particles (Verla et al., 2019). Interestingly, additives dissolved in water can also be sorbed by a different plastic particle that did not initially contain these molecules (Lynch et al., 2022). The diversity and quantity of contaminants sorbed are influenced by local activities and can exhibit significant variations from one location to another (Fisner et al., 2013; Ogata et al., 2009; Taniguchi et al., 2016; Vedolin et al., 2018). Chemical equilibrium kinetics drive the chemical sorption and desorption processes that occur continuously in the environment (Lynch et al., 2022). Thus, plastics act as the mixer of cocktails of chemicals that would otherwise not necessarily be present concurrently. All the accumulated chemical compounds, sometimes in concentrations far higher than in the surrounding, *e.g.* until concentration up to 10^6 times higher than the environment in which it was collected (Mato et al., 2001), can be transported to remote areas (Yamashita et al., 2019). Plastics will thus act as a sink, vector and source for a wide variety of chemical compounds, processes that depend on particle size, degradation state, and various abiotic factors such as salinity, temperature, and UV exposure (Dhavamani et al., 2022; Liu et al., 2020).

Plastic leachates and their adverse effects

The chemicals associated with plastics can be desorbed from the particles, which we refer to as ‘plastic leachates’. There are two types of plastic leachates according to their origin: primary and secondary leachates (Delaeter et al., 2022). Primary leachates are leachates from chemicals associated during plastic manufacture (*e.g.* monomers, plastic additives). Indeed, with the exception of a limited number of additives (*e.g.* certain organic flame retardants), plastic-associated chemicals found in plastics are typically not chemically bound to the polymer matrix (Delaeter et al., 2022; Hahladakis et al., 2018). Although the quantity of plastic-associated chemicals in the oceans is difficult to estimate, Andrade et al. (2021) estimated that plastics in the oceans were involved in the introduction of between *ca.* 578,100 and 1,838,000 tonnes of additives worldwide. In turn, secondary leachates are those from chemicals associated to plastic during the rest of their life cycle (*e.g.* POPs, metals; Delaeter et al., 2022).

Although plastic-associated chemicals and their implications have gained attention only recently, nearly half a century ago, Carpenter et al. (1972) described for the first time the presence of plastic in coastal waters and, at the same time, their ability to accumulate toxic substances, stressing their potential ingestion by marine organisms. Today, uptake of these chemicals is acknowledged, with a transfer of chemicals from plastic to organisms taking place through the water column (*e.g.* Gandara e Silva et al., 2016; Nobre et al., 2015; Seuront, 2018; Seuront et al., 2021) or following the ingestion of plastic particles or contaminated preys (*e.g.* Chua et al., 2014; Hasegawa et al., 2022; Rochman et al., 2013a; Tanaka et al., 2015, 2020). Aquatic organisms, such as seafood, can concentrate chemicals even if their plastic potential origin is not always known; for instance, phthalate concentration in crabs ranged from 84 to 258 $\mu g\ g^{-1}$ (Tiwari et al., 2019); see also Hermabessiere et al. (2017) and Liu et al. (2020) for reviews. Chemicals can be transferred through the food chain, and in contrast to plastics (Miller et al., 2020), numerous are acknowledged to biomagnify, *i.e.* increase concentration with increasing trophic level (*e.g.* Liu et al., 2019; Madgett et al., 2022; Peng et al., 2017; Figure 3).

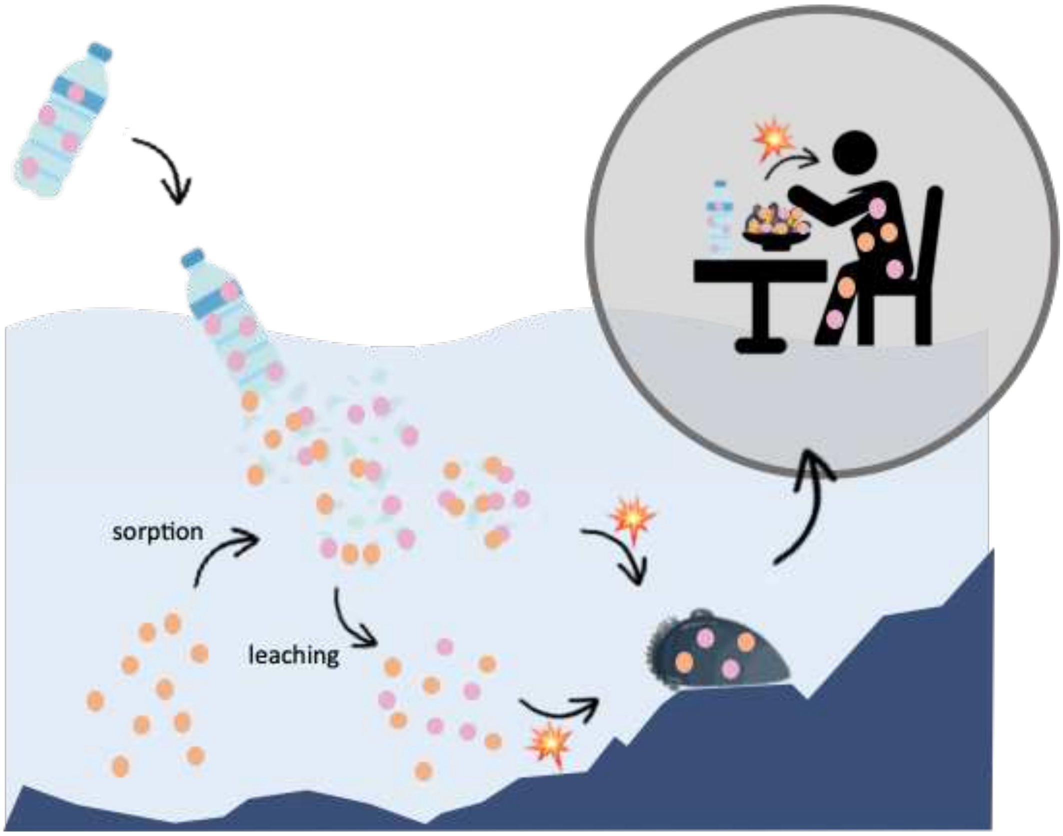


Figure 3: Schematic behaviour of plastic-associated chemicals used in their manufacture (pink dot) and chemicals from their surrounding (orange dot) and their transfer and impact to marine organisms and human.

Although far less studied than plastic's physical effects (Figure 4), plastic's chemical effects on organisms are numerous and pernicious. For instance, Rodrigues et al. (2019) point out that although most studies use virgin polymers, real-life scenarios could considerably increase the toxicity of particles due to chemical migration. Indeed, both primary and secondary plastic leachates may be highly toxic, with several hazards to human life, *e.g.* recognised carcinogenic, mutagenic, or endocrine-disrupting properties (Burgos-Aceves et al., 2021; Landrigan et al., 2023; Lithner et al., 2011; Menéndez-Pedriza & Jaumot, 2020; Oehlmann et al., 2009; Rochman, 2015; UNEP, 2023). A recent review on the impact of plastic leachates on marine organisms showed that their effect was only assessed in 26 studies, showing adverse effects at molecular, cellular, reproductive, behavioural and survival levels across 29 species (Delaeter et al., 2022).

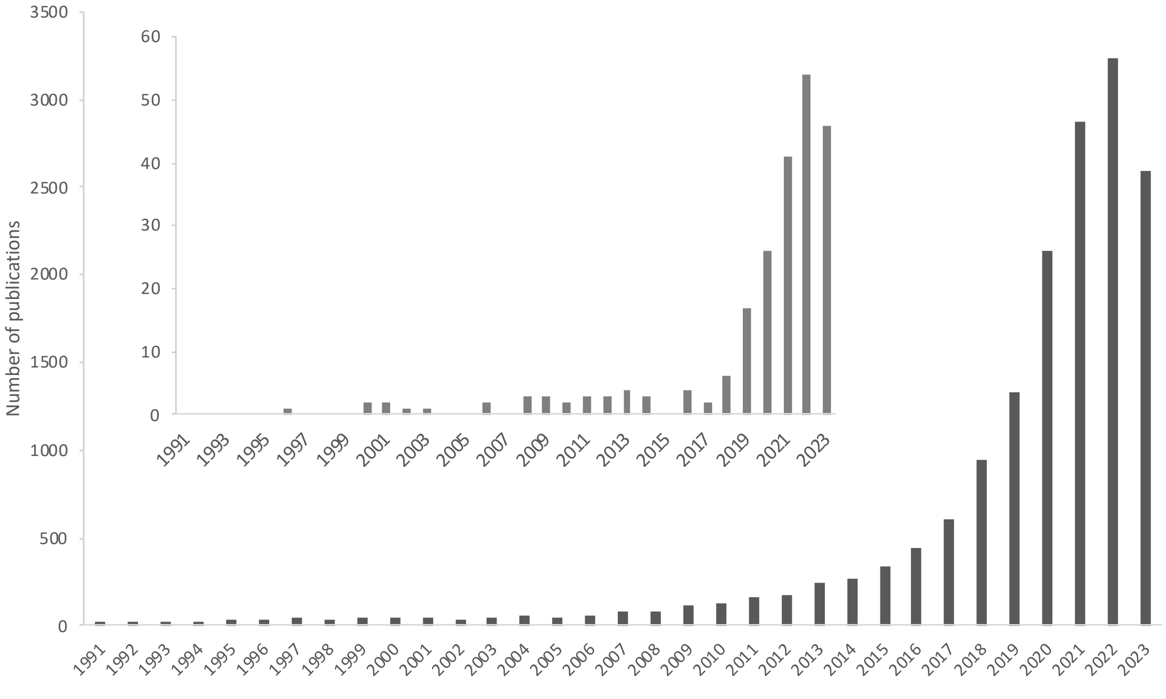


Figure 4: Number of publications on plastic (black; keywords used: "*plastic**" and "*pollution**") and plastic leachates (grey; keywords used: "*plastic**" and "*pollution**" and "*leachate**") from 1991 to 2023 on Web of Science (assessed November 10, 2023).

As for plastic particles, humans are also contaminated by plastic leachates, *e.g.* additives were detected in their blood (Cobellis et al., 2009), eyes (Jolanta et al., 2022), and urine (Dong et al., 2020). As the plastic-associated chemicals can be easily leached out at all stages of the life cycle of plastics, human exposures are multiple, *e.g.* migration from food containers or seafood (*e.g.* Hermabessiere et al., 2017; Landrigan et al., 2023; Ong et al., 2022; Sewwandi et al., 2023; Figure 3). The impact of chemicals on human health is also recognised, being associated with diseases prevalent in developed nations, such as cancer, diabetes, infertility, obesity, premature death and neurodevelopmental disorders (Landrigan et al., 2023; Rodrigues et al., 2019a; Sendra et al., 2021). Thus, it was estimated that the health costs of disease, disability, and premature death caused by just three plastic additives, *i.e.* the Polybrominated diphenyl ethers (PBDE; flame retardant), the Bisphenol A (BPA; antioxidant) and the Bis(2-

ethylhexyl) phthalate (DEHP; plasticisers), exceeded 920 billion USD in 2015 in the US alone (Landrigan et al., 2023). Currently, there is a growing trend in implementing additional regulations on these plastic-associated chemicals, which may include restrictions on their use in certain products, authorised exposure limits or bans in specific applications. For instance, in the European Union, BPA has been banned in plastic containers for babies and young children under three years since September 2018 but remains permitted in food contact materials under Regulation 10/2011/EU. Additionally, a resolution (UNEA resolution 5/14) initiated the formation of an Intergovernmental Negotiating Committee (INC) under the UN Environment Program to develop a comprehensive, legally binding instrument addressing plastic pollution across its lifecycle, with negotiations that began in 2022 and should be completed by end 2024 (https://www.unep.org/). One goal of the Global Plastics Treaty is to address the lack of transparency in the plastic industry, compelling manufacturers to give information about chemical constituents in plastics that are often undisclosed (https://www.unep.org/).

The economic costs on marine ecosystem services are largely unquantified, indeed, research on the impact of plastic leachates is still in its infancy. As suggested by Delaeter et al. (2022), to gain a more comprehensive understanding, it is imperative to investigate these effects on key ecosystem engineers. Investigating these organisms is crucial as they play a fundamental role in maintaining ecological balance and stability within the ecosystem (Lawton & Jones, 1995). Encompassing these pivotal organisms in future research becomes central to accurately assessing the impact of plastic leachate on the overall functioning of ecosystems.

3. Intertidal mussels

Intertidal mussels are filter-feeder bivalves thriving in the intertidal zone where they face constant exposure to both air and seawater as the tides ebb and flow. These organisms are found on the shorelines across continents (Seed & Suchanek, 1992), where they hold essential roles in coastal ecosystems. However, the balance they maintain could be threatened by anthropogenic pressure, that could disrupt their essential functions.

3.1. Mussels: invaluable species

High ecologic value

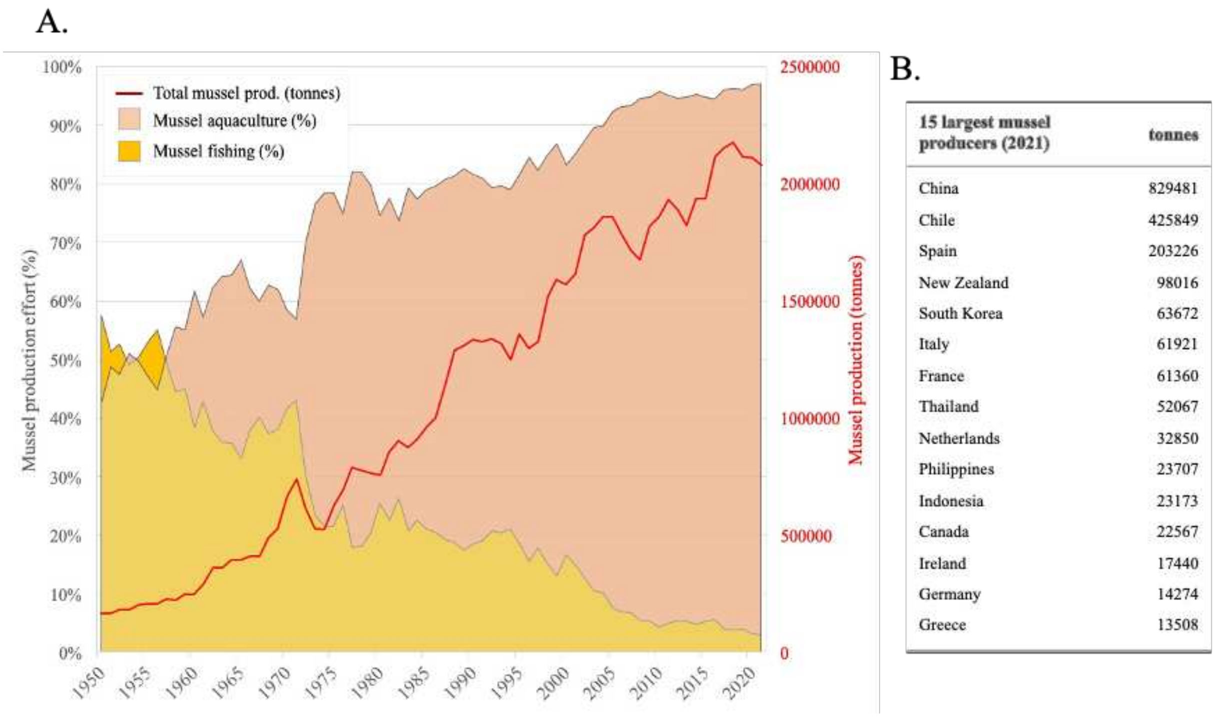
Intertidal mussels play a pivotal role in coastal ecosystems, notably through their role as a bioengineer species (Borthagaray & Carranza, 2007; Buschbaum et al., 2009). They locally dominate rocky shores through the formation of reefs (Buschbaum et al., 2009; Rossi et al., 2017; Seed & Suchanek, 1992). These reefs, also referred to as mussel beds, are highly dynamic three-dimensional structures through the mussel's ability to move, aggregate, attach (through the production of extracellular collagenous structure: the byssus) and detach themselves (Commito et al., 2014; Seuront et al., 2021; Zardi et al., 2021). The self-organisation into dense mono- and multi-layered beds operates a biogenic transformation of the habitat, modifying the physical nature of their environment (Nicastro et al., 2012; Reise, 2002; Seed & Suchanek, 1992; Zardi et al., 2021). By enhancing the complexity, they buffer several intertidal stressors (*e.g.* hydrodynamics, water retention, heat during emersion) and also increase the surface area and diversity of colonisable habitats and the availability of refugia from predators (Buschbaum et al., 2009; Commito & Dankers, 2001; Nicastro et al., 2012; Reise, 2002; Zardi et al., 2021). Consequently, these unique habitats contribute to the establishment and maintenance of a range of different species, thereby enhancing local biodiversity (Borthagaray & Carranza, 2007; Buschbaum et al., 2009; Commito & Dankers, 2001; Romero et al., 2015; Figure 5). Therefore, mussel beds are considered as biodiversity hot-spots (Borthagaray & Carranza, 2007; Buschbaum et al., 2009).

Figure 5: Overview of the services provided by mussel beds.

In addition, mussel beds contribute to the natural protection and stability of the coastlines by hampering erosion and wave action (Borsje et al., 2011; Figure 5). Indeed, the raised and rough surfaces formed by mussel beds modify the local hydrodynamic and sedimentary environment; they reduce current velocities and increase turbulence, effectively damping waves and facilitating the trapping of sediments, leading to a soil elevation, that through a positive feedback loop consequently further enhances wave attenuation and sediment trapping (Borsje et al., 2011; Reise, 2002; Figure 5). Additionally, through their high filtration ability—their filtration rate can exceed 10 litres per hour per individual—mussels act actively on water quality and nutrient cycling (Lindahl et al., 2005; Newell, 2004; Suplicy, 2020; Timmermann et al., 2019; Figure 5). They can control phytoplankton biomass and act on organic and inorganic matter in the water column, which can reduce turbidity, improve water transparency and light penetration (Newell, 2004; Timmermann et al., 2019; Figure 5). Mussels can also mitigate excessive nutrient (typically nitrogen and phosphorus) loading. For instance, the establishment of a mussel farm at a Swedish fjord mouth has been shown to reduce the nitrogen flux by 20% (Lindahl et al., 2005; Figure 5). Their high filtration capacity also allows mussels to efficiently accumulate pollutants from seawater, making them sentinel species that reflect the overall health of the ecosystems, and, as such, they have been widely used in the biomonitoring of coastal water pollution, *e.g.* metals, PAHs, PCBs, dioxins, pharmaceuticals, pesticides, microplastics, PCBs and PAHs; see Beyer et al. (2017) for a review. Finally, bivalve shells can be used in paleoenvironmental reconstruction as they provide high-resolution environmental archives of environmental variations, *e.g.* temperature, pH, salinity and pollution events (Schöne and Krause, 2016).

High economic value

Beyond their key ecological role, mussels also have important economic and heritage values through professional and recreational fishing (Figure 5). The earliest evidence of mussel fishing dates back to the Middle Pleistocene, *ca.* 164,000 (±12,000) years BP, from an archaeological site in South Africa (Marean, 2010). Notably, it has been suggested that the consumption of shellfish played a pivotal role in human history, the nutrient provided by their consumption may have allowed the development of the human brain (Broadhurst et al., 2002; Crawford et al., 1999; Parkington, 2003). Despite the fact that mussel consumption remains prevalent today, mussel fishing (distinct from mussel aquaculture) has been greatly reduced in recent years (FAO, 2023). In the 1970s, global mussel fishing exceeded 300,000 tonnes, falling to 61,898 tonnes in 2021, a decline that contrasts with the mussel aquaculture production trend (FAO, 2023). Indeed, in 1950, mussel fishing accounted for 58% of the overall mussel production, whereas in 2021, it constitutes less than 3%, aquaculture contributing to 97% of the mussel production (FAO, 2023; Figure 6A).



15 largest mussel producers (2021)	tonnes
China	829481
Chile	425849
Spain	203226
New Zealand	98016
South Korea	63672
Italy	61921
France	61360
Thailand	52067
Netherlands	32850
Philippines	23707
Indonesia	23173
Canada	22567
Ireland	17440
Germany	14274
Greece	13508

Figure 6: (A) Global mussel production (tonnes; red line) and the relative proportion of mussel aquaculture (%; orange) and mussel fishing per year (%; yellow). (B) The world's 15 largest mussel producers in 2021 and respective quantities (tonnes). Data collected on FAO website (FAO, 2023).

Mussel aquaculture's global production grew from *ca.* 70,000 tonnes in 1950 to more than 2 million tonnes in 2021, with an estimated value of 4.5 billion USD in 2019 (FAO, 2023). Among the 42 mussel-producing countries, three countries account for more than 70% of global mussel production. China is the largest leading producer with 829,481 tonnes (57%), followed by Chile (19%; 425,849 tonnes) and Spain (14%; 203,226 tonnes; data from 2021; FAO, 2023; Figure 6B). The most farmed mussel species are the blue mussel (*Mytilus edulis*; 159,466 tonnes), the Mediterranean mussel (*M. galloprovincialis*; 103,011 tonnes), the Chilean mussel (*Mytilus chilensis*; 365,595 tonnes), the Asian green mussel (*Perna viridis*; 124,184 tonnes) and the New Zealand green mussel (*P. canaliculus*; 86,176 tonnes; data from 2018; FAO, 2023).

Although mussels account for one-third of the European Union (EU) aquaculture production, EU mussel production has declined since the late 1990s (Avdelas et al., 2021). Nevertheless, mussel farming can contribute to global food security and socio-economic development in a more environmentally sustainable way than current sources of animal protein (Parodi et al., 2018; Suplicy, 2020; Willer & Aldridge, 2020; Figure 7). Mussels offer significant nutritional benefits, being a cost-effective source of high-quality protein with essential amino acids (*e.g.* Leucine, Lysine, Threonine) and providing a rich source of vitamins (*e.g.* Vitamin C, B12, Thiamin, Riboflavin), minerals (*e.g.* magnesium, zinc, iron, selenium, manganese and phosphorus) and fatty acid (*e.g.* omega-3 polyunsaturated fatty acids; see Suplicy (2020), and the references therein). For instance, the protein content of mussels is *ca.* 61.3 g per 100g of dry matter (DM), which is higher than many meats and plant crops (Parodi et al., 2018; Figure 7), *e.g.* beef (51.5 g $100gDM^{-1}$), chicken (51.3 g $100gDM^{-1}$), pork (40.7 g $100gDM^{-1}$) or maise (12.6 g $100gDM^{-1}$).

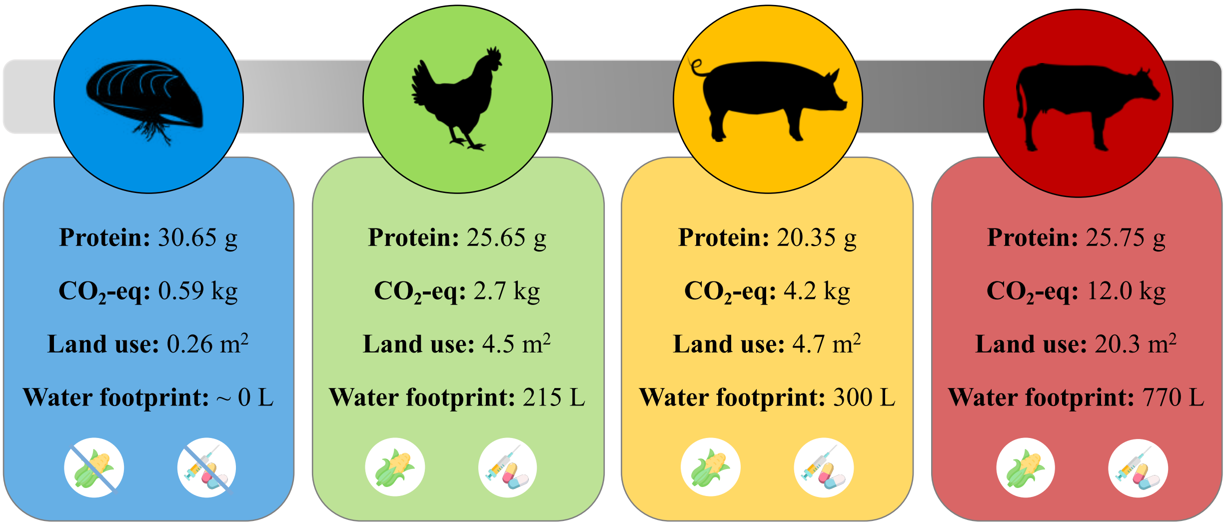


Figure 7: Comparison between four different types of animal-source foods, *i.e.* mussel, chicken, pork and beef, in terms of (i) nutritional profiles, *i.e.* protein content (g per 50g of dry matter), (ii) greenhouse gas emissions ($kgCO_2$-eq per 50g of protein), (iii) land use (m^2 per 50g of protein), (iv) water footprints (L per 50g) and (v) feed, antibiotics and vaccines. Data from Parodi et al., 2018 (i-iii); Ibidhi and Ben Salem, 2020 (iv); and Suplicy, 2020 (iv-v).

While the protein content per 100g is relatively similar between these different animal food sources, their environmental impacts vary greatly. Indeed, modern terrestrial agriculture, despite having enabled a considerable increase in food production, generates a considerable environmental footprint—food and agriculture account for 26% of greenhouse gas emissions (13.7 GtCO_2-eq), uses 50% of the habitable land (covering 51 million km^2), and 70% of the world's freshwater withdrawal—with a significant portion attributed to livestock rearing and meat production (Ritchie et al., 2022). For 50g of proteins, the production of mussels would emit 0.59 kgCO_2-eq, which is less than the production of maize (0.76 kgCO_2-eq), chicken (2.7 kgCO_2-eq), pork (4.2 kgCO_2-eq) or beef (12.0 kgCO_2-eq; Parodi et al., 2018; Figure 7). Mussel farming does not consume any habitable surface, and the land used is minimal*, i.e.* 0.26 m^2 to produce 50 g of proteins; for an equivalent value, maize requires 2.4 m^2, chicken 4.5 m^2, pork 4.7 m^2, and beef 20.3 m^2 (Parodi et al., 2018; Figure 7). Furthermore, mussel farming does not use freshwater and does not require exogenous food supply, or the use of vaccines, antibiotics, or probiotics in contrast to traditional animal farming (Ibidhi and Ben Salem, 2020; Suplicy, 2020; Figure 7). These numerous advantages make them a favourable substitute for conventional meats (Parodi et al., 2018; Suplicy, 2020; Willer & Aldridge, 2020; Figure 7). In addition, mussel wastes and by-products, *e.g.* mussel shell, byssus and residual meat, hold significant potential for valorisations, including, for example, soil amendment, fertilisers, concrete aggregate, handicraft, jewellery, bio-adsorbant for pollutants, collagen, bio-adhesives, fish-meal; see Medina Uzcátegui et al. (2022) for a review.

3.2. The intertidal zone: a highly stressful environment

Mussels thrive across global intertidal substrates, from subtidal to high intertidal areas, from estuaries to tropical and polar seas (Seed & Suchanek, 1992). They have evolved to withstand an array of natural drivers, most of which are orchestrated by a key process in these systems: the tide (Raffaelli & Hawkins, 1996). Tides compel mussels to face a myriad of biotic and abiotic environmental gradients, *e.g.* desiccation, extreme temperature, varying salinity conditions, wave impact, competition for space, and predation (Kunze et al., 2021; Raffaelli & Hawkins, 1996). Of these numerous natural stressors, three are designated as major in the delimitation of the mussel vertical distribution (Seed & Suchanek, 1992). Thus, their upper limit is dictated by abiotic factors, notably temperature and desiccation challenges, while their lower limit is intricately tied to a biotic factor, the pressure of predation (Seed & Suchanek, 1992). These natural factors collectively have shaped the mussel distribution and, thus, the boundaries of the ecosystems they support.

The equilibrium established at the evolutionary scale between these mussels and their surrounding stressors is, however, now facing new disturbances linked to human activities. They are, therefore, subjected to large-scale anthropogenic stressors, such as global warming, with, for instance, the rise in both seawater and aerial temperatures (Galil et al., 2022; IPCC, 2022; Seuront et al., 2019). In addition, intertidal systems experience more pronounced impacts from anthropogenic stressors compared to other coastal systems (Branch et al., 2008; Thompson et al., 2002) because of their unique position at the interface of land and sea, exposing them to both terrestrial (land-based) and marine (sea-based) sources of human-caused influences. Indeed, local anthropogenic stressors, *e.g.* habitat degradation, alien species introduction and pollution, are highly challenging to these intertidal communities. As intertidal species have evolved along varying natural environmental conditions, they already live near their upper tolerance limits (Somero, 2002), making them vulnerable to more extreme conditions, which could lead to mass mortality.

In recent years, mussels' mass mortality episodes have been numerous (*e.g.* Charles, 2019; Galil et al., 2022; Harley, 2008; Polsenaere et al., 2017; Raymond et al., 2022; Seuront et al., 2019). In some parts of the world, the decline in mussel populations has reached a concerning extent, so the European Red List of Habitats, which provides an overview of the risk of collapse of various habitats in the European Union and adjacent regions, classifies the mussel beds in the Atlantic littoral zone in the category Endangered of the list of the threatened North-East Atlantic habitats (Gubbay et al., 2016). In addition, the Convention for the

Protection of the Marine Environment of the North-East Atlantic (the “OSPAR Convention”) includes intertidal *Mytilus edulis* beds on mixed and sandy sediments of the Greater North Sea and Celtic Sea (Region II and III) in the OSPAR List of Threatened and/or Declining Species and Habitats (OSPAR Agreement 2008-6). These mass mortalities underline the urgent need to take a closer look at the stresses that mussels undergo. Moreover, as the stressors can act synergistically, it is also essential to understand how these factors may interact and act on organisms, especially in bioengineering species, such as mussels, to avoid (negative) ‘ecological surprises’ (Paine et al., 1998).

4. The case of the Eastern English Channel

The English Channel is a temperate sea, extending over 77,000 km^2, bordered by the United Kingdom to the north and France to the south (Dauvin, 2012, 2019; Figure 8). It is an exceptionally productive region from a biological point of view, but it is also one of the most heavily impacted seas in the world due to anthropogenic pressures (Dauvin, 2019). Note that the hydrological, oceanographic and biogeographic characteristics of the English Channel are very distinct between the western and the eastern basins (Dauvin, 2012; Figure 8). Here, we will focus on the Eastern English Channel (EEC), and specifically, on the French part.

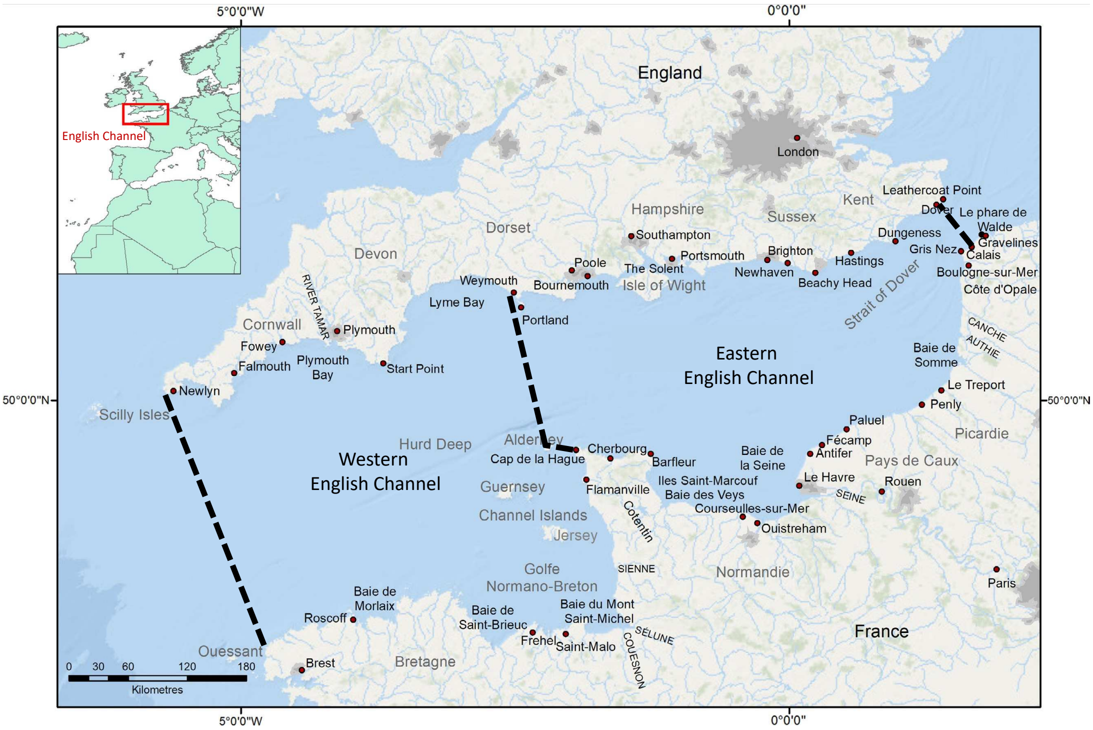


Figure 8: Map of the English Channel (adapted from Dauvin, 2019), with the two main basins, *i.e.* the Western and Eastern part, according to Dauvin, 2012.

4.1. The local mussel: Mytilus edulis

The farming, harvesting and consumption of mussels are an integral part of French cultural heritage. In France, mussels are the most consumed shellfish, with an average of 2.4 kg/inhabitant/year (data from 2017; FranceAgriMer, 2020). France ranked 7th with 61,360 mussel tonnes produced in 2021 (4% of the global mussel production; FAO, 2023). However, French mussel production is not sufficient to meet national consumption demand. Based on FAO data, the apparent mussel consumption in 2021 was estimated at 107,526 tonnes (*i.e.* Production (61,360 tonnes) + Imports (52,425 tonnes) – Exports (6,258 tonnes)), which highlights the importance of import that accounts for *ca.* 50% of the mussels consumed in France (FAO, 2023). Spain (22,282 tonnes), Chile (10,132 tonnes), and the Netherlands (9,233 tonnes) are the primary countries exporting mussels to France, collectively accounting for approximately 80% of these imports (FAO, 2023).

Moreover, there are inter-regional disparities in the quantity of fresh mussels consumed by French households, with a very strong dominance by the populations of the North of France (FranceAgriMer, 2021). In the Hauts-de-France state, *Mytilus edulis*, the local mussel species, is farmed by different techniques either subtidally on 'longlines' or in intertidal areas, either 'flat' or on 'piles'. The latter, known as 'bouchots', represents the most important part of mussel production in France (Lebon et al., 2023; Figure 9A). Mussel farming production in the Hauts-de-France state is around 3,000 tons, to which are added 300 to 500 tonnes collected by professional fishermen on natural beds (Lebon et al., 2023; Verin & Devreker, 2020; Figure 9B). In addition, mussel recreational fishing is highly cherished by the local population (Seuront et al., 2017; Figure 9C). Indeed, mussels hold significant social, cultural and patrimonial values in this region, where mussels even have their own festival annually in the coastal town of Wimereux (https://www.ville-wimereux.fr/events/fete-de-la-moule-2/) and the "moules-frites" (literally mussels with french fries) is the traditional dish served during the 'braderie de Lille' (the largest flea market in Europe held during the first week-end of September) where the 2 to 3 millions visitors consumed over 500 tonnes of mussels (https://www.lille.fr/Braderie-de-Lille/Actualites/L-histoire-des-moules-frites). At the end of the 'braderie de Lille', the restaurants make the 'tas de moules', *i.e.* piles of mussels, to compete and determine the highest sales (Figure 9D).

In addition, the mussel beds formed by *M. edulis* in this region significantly enhance the biodiversity and are recognised as a biodiversity hotspot (Seuront et al., 2017; Spilmont et al., 2018; Figure 9F). The species richness associated with these mussel beds has been measured in the region to range between 50 and 100 species (Hennion, *pers. comm*), some of them being exclusive to this specific habitat (Seuront et al., 2017). In addition, mussels are also used for the biomonitoring of various contaminants in the region (*e.g.* Johansson et al., 2006; Olenycz et al., 2015; Przytarska et al., 2010), including microplastics (Kazour and Amara, 2020).

Figure 9: Pictures taken in the Hauts-de-France region of (A) a bouchot mussel farming (© Marine Uguen), (B) a professional fisherman technique of mussel collection on a natural bed (© Aurélie Lutrand), (C) a local recreational fisherman collecting mussels on a natural bed (© Marine Uguen), (D) the ‘tas de moules’ from the ‘braderie de Lille’ (© Benoît Bugnicourt – France Télévision) (E) a *Mytilus edulis* individual and its byssal threads (© Solène Henry) and (F) a *Mytilus edulis* mussel bed (© Julie Anquetin).

4.2. Threats to mussels in the Eastern English Channel

A highly polluted area

The English Channel is one of the world's most anthropologically impacted seas, especially in its eastern part (Eastern English Channel, EEC hereafter; Dauvin, 2012). Given its strategic location, it is one of the busiest marine traffic thoroughfares in the world, with *ca.* 500 vessels crossing each day its eastern limit with the North Sea, *i.e.* the Dover Strait (Dauvin, 2019). Additionally, each day, several hundred vessels connect France to England, *e.g.* there are 837 ferries that shuttle passengers from France to England every week (https://www.directferries.com/england.htm). The predominant vessels in this region are commercial, and numerous carry hazardous cargo, *e.g.* oil tankers constitute *ca.* 75% of the traffic (Dauvin, 2019). The heavy maritime traffic in this region has historically led to significant pollution incidents, such as the sinking of tankers as the *Ievoli Sun* in 2000, resulting in the release of 4,000 tonnes of styrene and 1,000 tonnes each of methyl ethyl ketone and isopropyl alcohol (Law et al., 2003), as well as the *Ece* in 2006, which caused the release of 14,000 tonnes of phosphoric acid, alongside multiple instances of hydrocarbon pollution (Dauvin, 2019).

The French coasts of the EEC are also heavily urbanised and noticeably host two of the five main commercial French harbours, *i.e.* Rouen and Le Havre (Lacoste & Bouchet, 2012), the largest industrial French fishing harbour, *i.e.* Boulogne-sur-mer (Jouanneau et al., 2013) and the largest harbour in Europe for passenger traffic, *i.e.* Calais (Dauvin, 2019; Figure 10). This area is also home to various heavy industries (Bourennane et al., 2010; Figure 10); the EEC has been widely known for the contamination of its sediment and coastal waters related to its history of metallurgical plants (Boughriet et al., 1994; Cuvillier-Hot et al., 2018; Kerambrun et al., 2012; Proix, 1989; Wartel et al., 1990). This is also a key area for electricity production in France, *i.e.* five nuclear power plants are located along the EEC coasts or close to it, including the third largest in the world (Gravelines); and numerous offshore wind farms are rising in this zone (Dauvin, 2019; Figure 10). In addition to these urban and industrial pressures, this area is also prone to tourist and agricultural pressures (Dauvin, 2019; Figure 10). For instance, the *Baie de Somme* and the *Caps Blanc Nez* and *Gris Nez*, classified as “Grands Sites de France”—a French label for remarkable landscapes—attract respectively 2 and 1.2 million tourists every year (MEEM, 2017). The characteristic agricultural practice in this area is intensive open-field farming (Perrier, 1937; Preux, 2021), which can lead to the runoff of fertilisers and pesticides into rivers, estuaries, and coastal seas (Dauvin, 2019). Notably, four main rivers flow into the EEC: the Canche, the Authie, the Somme and the major one, the Seine (Brylinski et al., 1991; Figure 10). These river inputs generate a coastal water mass along the French coasts of the EEC, characterised by its freshness, turbidity, phytoplankton richness and pollution content (Brylinski et al., 1991). The Seine is notably acknowledged to be highly polluted by various contaminants, *e.g.* metals, POPs, PAHs, and plasticisers, that spill into the EEC; see Tappin and Millward (2015) for a review. Indeed, its watershed encompasses 30% of the French population, 40% of the French industry and 25% of the French agriculture (Thibault et al., 2019). Thus, the sources of pollution in the EEC are multiple.

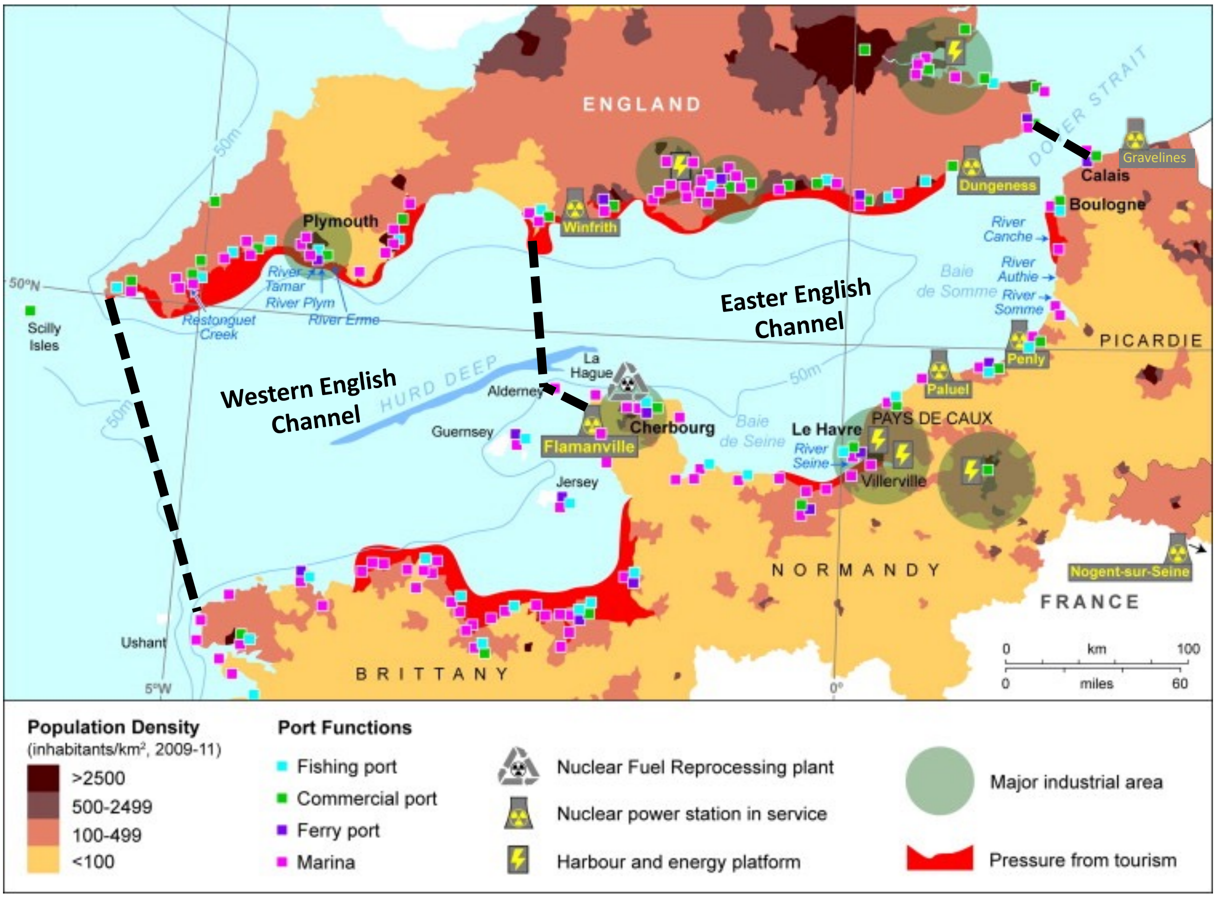


Figure 10: Geography and bathymetry of the English Channel. The population density, coastal industries, and harbours are also shown (adapted from: Dauvin, 2019, 2012; Tappin & Millward, 2015; Uncles & Stephens, 2007; University of Caen, 2014).

What about plastic?

The EEC is not spared from plastic pollution. Plastics are present along the EEC coastline, in water, sediment, and biota samples (Doyen et al., 2019; Green & Johnson, 2020; Hermabessiere et al., 2019; Steer et al., 2017). Mussels are among the species contaminated by microplastics in the EEC, and they also showed other contaminants in their tissue, *e.g.* phthalates, PAHs and PCBs (Hermabessiere et al., 2019). The Seine has noticeably the highest phthalate levels in Europe (DEHP emission: > 5 kg km^{-1} a^{-1}), implying a potential impact in the eastern English Channel (Tappin & Millward, 2015). In addition, certain events can also generate localised plastic pollution, *e.g.* in 2016, an incident occurred, and *ca.* 8 tonnes of plastic pellets were accidentally spilt from a tanker truck on a highway situated between Boulogne-sur-mer and Marquise, approximately 2.5 km away from the shoreline (Gravier & Haut, 2020). These pellets dispersed into the surrounding waterways, causing substantial contamination of the shoreline that endured for several years following the event (Gravier & Haut, 2020). These virgin plastic pellets—as discussed above—can sorbed and leached out various contaminants that can be found in the EEC, as pollution sources are numerous (see previous paragraph). The International Pellet Watch (http://pelletwatch.org/) use these plastic pellets to monitor local POP pollution. They notably reveal an alarmingly high level of Polychlorinated Biphenyls (PCBs) in these pellets in the EEC with concentrations exceeding 500 ng g^{-1}. Thus, mussels in the EEC have to deal with this plastic pollution, but despite *M. edulis* economic, social and patrimonial importance in this area, the biological and ecological effects of this pollution on them are unknown.

Additional stressors in the EEC

Pollution is not the only anthropogenic disturbance that mussels have to face in the EEC. Indeed, in this area, the heavy marine traffic—notably through ballast water and biofouling—contributes to the introduction of invasive species; in France, *ca.* 50 new invasive species per decade have been reported since 1970 (Massé et al., 2023). Among them, the Japanese crab *Hemigrapsus sanguineus* represents a problem for native species, especially mussels and their local predator, the European green crab *Carcinus maenas*. This species was first recorded in Le

Havre harbour in 1999 (Breton et al., 2002) and now extends from the Mont-Saint-Michel Bay to Dunkirk (Dauvin et al., 2019). Sharing the same ecological niche as the native crab *C. maenas*, it has now supplanted it in many locations along the EEC coastline (Rolet et al., 2020). For instance, initially recorded in Boulogne-sur-Mer in 2006 (Dauvin et al., 2009), *H. sanguineus* and *C. maenas* accounted for 6% and 94%, respectively, in 2008 (Rolet et al., 2020). Today, the scenario has reversed, with *H. sanguineus* representing 95% and *C. maenas* comprising only 5% (Rolet et al., 2020). The mussel beds in the EEC are ideal habitats for *H. sanguineus*, the density record being 528 ind m^2 (Pezy & Dauvin, 2015). As *C. maenas, H. sanguineus* feeds on *M. edulis*, yet it has the ability to feed on larger size classes of mussels (DeGraaf & Tyrrell, 2004). Brousseau et al. (2014) showed that *H. sanguineus* can contribute to *ca.* 25% of *M. edulis* mortality. The mussel are able to adapt their behaviour in response to their native predator, *e.g.* change in aggregation, motility, or byssus production (Côté, 1995; Côté & Jelnikar, 1999; Nicastro et al., 2007; Reimer & Tedengren, 1997). However, prey can be unable to recognise a new predator as a threat and develop effective anti-predator behaviour due to the absence of coevolution between them, a process referred to as prey naivety (Anton et al., 2020). Thus, given the relatively recent introduction of *H. sanguineus*, the potential naivety of the blue mussel to this new predator remains, to the best of my knowledge, not yet investigated.

The EEC is also prone to climate change, notably climate warming. Air and sea temperature anomalies have shown a significant increase in the EEC since the mid-1990s (Cornes et al., 2023; Tinker et al., 2020). Projections indicate an expected rise in the annual mean near-bottom temperature in the English Channel of +3.15°C (± 0.85°C) in 2079-2098 compared to the 2000-2019 baseline (Cornes et al., 2023). In addition to this general increase in temperature, there is also an increase in the frequency, intensity and duration of these extreme events, such as aerial and marine heatwaves (IPCC, 2022). For instance, the number of marine heatwaves by days doubled their frequency between 1982 and 2016 and are projected to increase by a factor of 16 following a +1.5°C climate warming compared to pre-industrial levels (Frölicher et al., 2018). These temperature changes expose species, especially ectothermic poikilothermic organisms such as mussels, to conditions of intense stress that regularly lead to mass mortality. Thus, in 2018, the EEC shores experienced unprecedented waves of mussel mortality in August and October, which resulted in a mussel loss of 90 to 95% of the volume of some natural sites (Seuront et al., 2019). Experiments revealed that these mortalities were due to the aerial temperature experienced by the mussels, *M. edulis* body temperatures exceeding 30°C for 5 to 6 consecutive days and occasionally reaching more than 40°C (Seuront et al., 2019).

5. Objectives

This thesis aims to unravel the effects of plastic leachate pollution on intertidal mussels using the model species *Mytilus edulis* within the Eastern English Channel ecosystem. The research is structured into six chapters, each addressing specific issues related to mussel responses to plastic leachate pollution.

Chapter 1: Plastic pollution and marine mussels: unravelling disparities in research efforts, main biological effects and implications of multiple, co-occurring stressors

The first chapter reviews the existing research on the effects of plastics on mussels. It includes an analysis of the research efforts and their disparities and reviews the effects of plastics at each organisational level. It concluded by highlighting the limited research on multi-stress studies involving plastics, illustrated through the example of temperature stress.

Chapter 2: **The tolerance of a keystone ecosystem engineer to extreme heat stress is hampered by microplastic leachates**

Building on the gaps identified in Chapter 1, the second chapter explores how mussels respond to the combined stress of plastic leachates and temperature. Given the numerous pressures faced by mussels in the Eastern English Channel, such as extreme heatwave events (Seuront et al., 2019) and high plastic pollution (Gravier & Haut, 2020), this chapter investigates (i) the mussels' response to endure varying levels of aerial heat stress (from 20 to 35°C) and (ii) assesses whether a combined exposure to a mixture of plastic leachates from beached or virgin plastic pellets shapes their tolerance.

Chapter 3: Microplastic leachates disrupt the chemotactic and chemokinetic behaviours of an ecosystem engineer (*Mytilus edulis*)

Given the complex response resulting from the interaction of two stressors found in Chapter 2, the third chapter delves into another combination of stressors faced by mussels in the English Channel: plastic pollution (Gravier & Haut, 2020) and predation pressure by the invasive crab *Hemigrapsus sanguineus* (Rolet et al., 2020). Specifically, this chapter explores (i) the individual response of small mussels to different chemical cues, including conspecifics, injured conspecifics, and the predator *Hemigrapsus sanguineus* cues, and assesses (ii) whether leachates from virgin plastic pellets influence their chemical communication.

Chapter 4: Size-dependent response of the mussel collective behaviour to plastic leachates and predator cues

The response of a prey to its predator can greatly vary based on the organism size (DeGraaf & Tyrrell, 2004) and the presence of conspecifics (Schaerf et al., 2017). Thus, this chapter expands on the findings from Chapter 3 by investigating (i) the collective behaviour of small and large mussels to their *Hemigrapsus sanguineus* predator cues and (ii) whether leachates from virgin plastic pellets influence their responses.

Chapter 5: Mussel aggregation behaviour: a reproductive strategy impaired by plastic leachates

After a first step into the mussel collective dynamics in the previous chapter, this chapter will delve deeper into it through the study of the temporal aspect of mussel aggregation with a particular emphasis on its potential role as a reproductive strategy. Indeed, aggregation before spawning was observed in numerous broadcast benthic spawners (*e.g.* Himmelman et al., 2008; Keesing et al., 2011; Marquet et al., 2018; Mendo et al., 2014; Shepherd, 1986; Simon &

Levitan, 2011), but has not yet been studied in mussels. Thus, this chapter investigates (i) whether mussel aggregation behaviour can be considered a reproductive strategy and (ii) whether leachates from virgin plastic pellets may impair their response. To do so, mussel aggregation behaviour and histological analyses were conducted over an eight-month study period.

Chapter 6: Location-dependent effect of microplastic leachates on the respiration rate of two engineering mussel species

Building upon our results on the impacts of plastic leachates on *Mytilus edulis* in the preceding chapters, we extended our investigations by examining plastics sourced from various locations and studying mussels of different species. Thus, this chapter assesses whether the effects of plastic leachates are (i) contingent on their source and (ii) specific to a mussel species. To do so, we used *Mytilus edulis* and *Mytilus galloprovincialis* as models and focused on a key physiological parameter, *i.e.* the respiration rate, a response that has not yet been explored in mussels in regard to plastic leachates.

Finally, this thesis will conclude with a general discussion of the combined results obtained, with a set of perspectives highlighting new questions arising from this work.

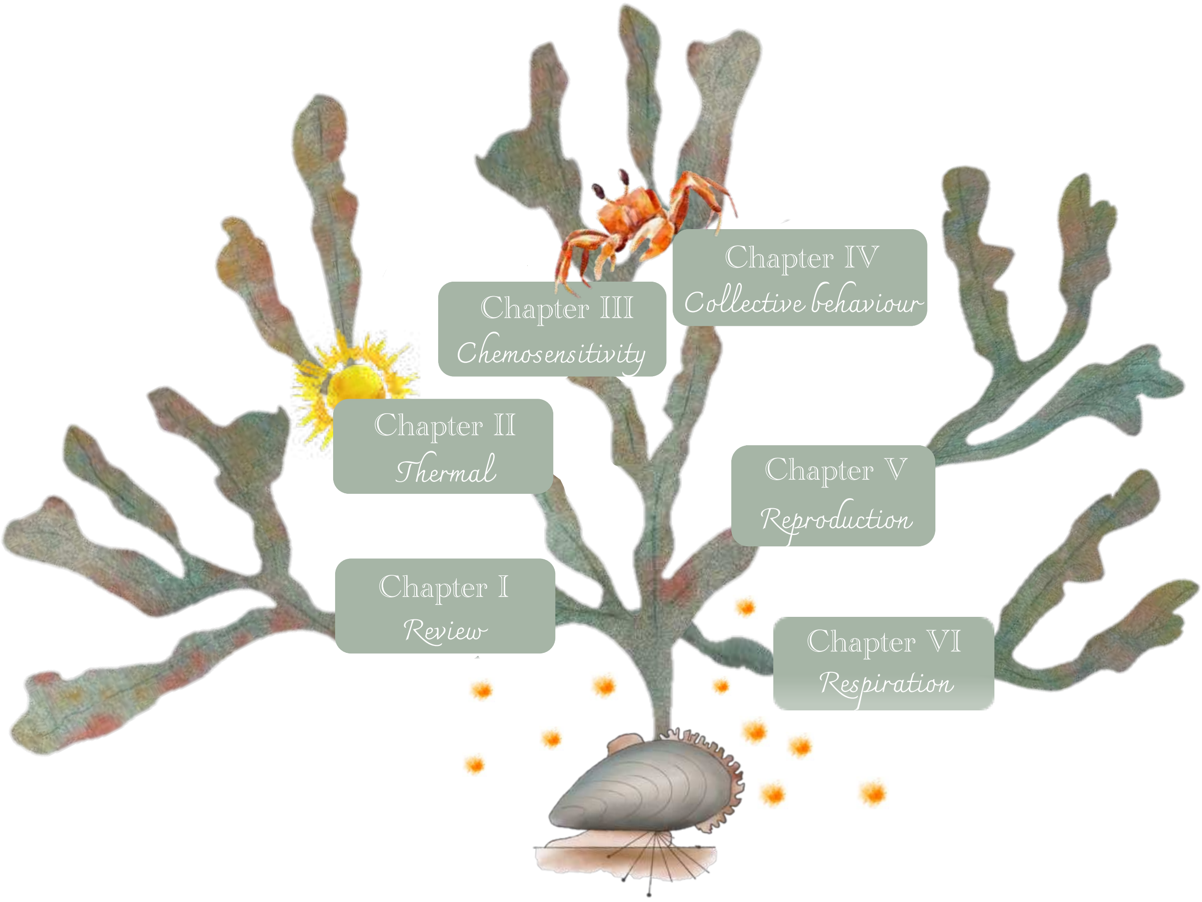


Figure 11: Thesis objectives. © Solène Henry

CHAPTER I

PLASTIC POLLUTION AND MARINE MUSSELS: UNRAVELLING DISPARITIES IN RESEARCH EFFORTS, BIOLOGICAL EFFECTS AND INFLUENCES OF MULTIPLE, CO-OCCURING STRESSORS

Plastic pollution and marine mussels: unravelling disparities in research efforts, biological effects of plastics and influences of multiple, co-occurring stressors

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1. Introduction

The numerous advantageous characteristics of plastic, *e.g.* its versatility, resistance, lightness and affordability, have elevated this material to the third most man-made material worldwide (behind cement and steel; Geyer et al., 2017). Since the onset of its large-scale production in the 1950s, the total amount of plastic produced worldwide has been exponentially increasing, reaching 400.3 million tons in 2022 (PlasticsEurope, 2023). However, this production has profound disadvantages. The ever-growing production, overuse and subsequent rapid disposal and poor management of plastics have resulted in the global accumulation and distribution of plastic wastes (Geyer et al., 2017; Geyer, 2020; Hurley et al., 2020; Porta, 2021). Plastic is subsequently carried by several forces, such as the wind, wastewaters and rivers, to the ocean, which is the ultimate receptacle of these wastes. Although the quantity of plastic in the oceans each year is difficult to accurately quantify, it is estimated that *ca.* four to 12 million tonnes of plastic enter the marine environment yearly; this figure is expected to increase by an order of magnitude by 2050 (Jambeck et al., 2015), making plastics the most abundant and widespread marine waste globally (Auta et al., 2017).

Plastics typically vary in size, shape, colour, polymers and additive content and are mainly categorised by size. The largest ones (> 5 mm) are referred to as macroplastics, but the most abundant by far are those of smaller sizes (Eriksen et al., 2014): microplastics (1 µm – 5 mm; MPs) and nanoplastics (< 1 µm; NPs). Beyond their physical characteristics, plastic acts as a vector of numerous chemical compounds, which include (i) the large variety of additives incorporated into a plastic polymer during its manufacture to improve its performance (*e.g.* plasticisers, antioxidants, flame retardants) and (ii) the wide range of environmental contaminants sorbed from the surrounding environment (*e.g.* persistent organic pollutants and inorganic contaminants; Delaeter et al., 2022; Rochman, 2015). Through desorption, both of these types of chemical compounds associated with the plastic polymer may cause chemical damage to marine organisms, which adds to the physical damage caused by plastic particles (*e.g.* entanglement and obstruction of the digestive and/or respiratory tract; Auta et al., 2017; Delaeter et al., 2022; Seuront et al., 2022).

As filter feeders, mussels can filter several litres per hour (Riisgård, 2001) and are thus highly exposed to plastic pollution. Mussels ingest large amounts of plastic particles, and for the blue mussel *Mytilus edulis* on European shores, contaminants levels are as high as 4.44 MPs g^{-1} of tissue wet weight (Courtene-Jones et al., 2017); a review on the quantity of plastic in bivalves was also provided by Hermabessiere et al. (2019). After ingestion, both MPs and NPs can migrate to mussel cells, tissues or the circulatory system, where MPs have been shown to persist for more than 48 days (Browne et al., 2008; Sendra et al., 2020a). Plastic particles can also be transferred through the food web, *e.g.* from mussels (*M. edulis*) to crabs (*Carcinus maenas*; Farrell & Nelson, 2013; Watts et al., 2014). Beyond the abovementioned absorption, translocation, accumulation and transfer phenomena, plastic particles and their leachates may cause numerous deleterious effects on mussels from subcellular to population levels (*e.g.* Avio et al., 2015; Capolupo et al., 2021a; Choi et al., 2022; Delaeter et al., 2022; Seuront et al., 2021).

Intertidal species, including mussels, are subject to a wide range of anthropogenically induced stressors, such as thermal stress and acidification (Kunze et al., 2021), that introduce an additional level of complexity to research fields devoted to assessing the impacts of plastic. Temperature is a particularly important stressor for intertidal mussels, as these organisms live close to their tolerance limits due to the emersion process (Somero, 2002). In this context, the combined impact of temperature and plastic pollution may amplify the adverse effects experienced by intertidal mussels, with synergistic effects representing the prevalent form of multistress interaction observed in marine species (Gunderson et al., 2016). In an era of global change, temperatures rise and heatwaves are expected to become more frequent and intense (IPCC, 2022). As such, there is a critical need to determine the combined effects of these stressors, which have predominantly been studied independently (Ford et al., 2022).

Adverse effects on intertidal mussels are likely to act on larger scales, given the valuable ecological and economic roles of these species. As key ecosystem engineers, mussels form unique habitats that enhance local biodiversity as biodiversity hotspots (Borthagaray & Carranza, 2007; Buschbaum et al., 2009). Mussels also have important economic and heritage value due to professional and recreational fishing, *e.g.* mussels account for one-third of European Union aquaculture production, *i.e.* around USD 465 10^6 (Avdelas et al., 2021). In this context, any direct and/or indirect effects that plastics have on mussels are likely to cascade through this ecosystem, as well as mussel goods and services, and may be further affected by higher temperatures.

In this context, this review provides a state-of-the-art analysis of the impacts of plastic pollution on marine mussels. By synthesising the available literature, we aim first to shed light on the disparities present in research efforts, encompassing aspects such as plastic characteristics and exposure conditions used. Second, we aim to relate the disparities to their environmental relevance and highlight potential similarities between exposure conditions in the laboratory and those in nature. We subsequently examined the plastic-induced effects on mussels across diverse organisational levels, ranging from cellular to organism levels. We finally discuss the gaps in our knowledge on the effects of exposure to multiple stressors, including plastics, with a specific emphasis on the role of temperature as a central factor, which is often underestimated, and its potential to shape the impacts of plastics. Thus, we offer a comprehensive review of plastic pollution in marine mussels, emphasising our current knowledge on the complexity of the factors faced by these key organisms. We then conclude by highlighting areas in which research efforts need to be intensified to better develop and implement effective conservation and management strategies for intertidal ecosystems.

2. Methodology

The Web of Science database (assessed on May 25, 2023) was used with a search in Topics of the following terms in 3 distinctive rows: (i) "*microplastic**" OR "*nanoplastics**" (ii) AND "*effect**" OR "*impact**" OR "*affect**" (iii) AND "*mussel**". The document type restriction "*Article*" was added. A total of 368 articles were returned, among which a preliminary screening was performed based on a close examination of their titles and abstracts. The articles excluded were those that (i) belonged to another research domain (*e.g.* physics, chemistry), (ii) focused on species other than marine mussels (other taxa or freshwater mussels), (iii) were not related to plastic pollution and (iv) were review papers. A secondary screening excluded the literature focusing on mussel plastic ingestion (53 papers), modelling (2 papers) and other aspects that lie well beyond the scope of the present work, *i.e.* how mussels enhance the sinking of plastic particles (5 papers). This approach resulted in the selection of 106 studies that are described in detail in the present work (Figure 12).

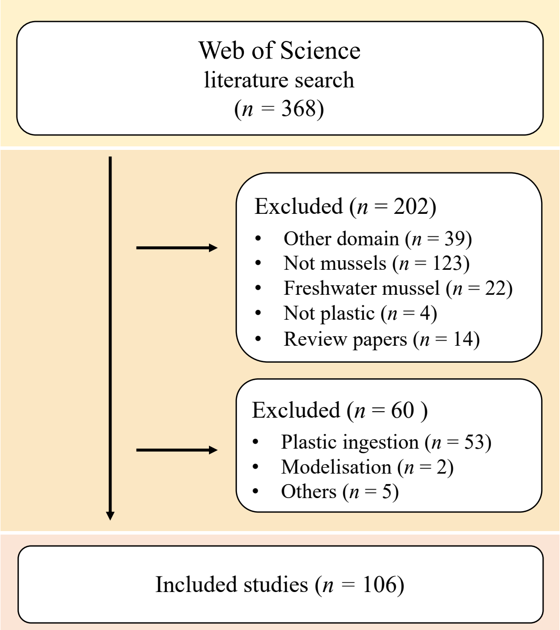


Figure 12: Flow chart of literature selection. Values (*n*) are the number of papers excluded at each stage of our screening process.

The main information of each article, *i.e.* reference, publication year, mussel species, life stage, plastic polymer, shape and size, qualitative and quantitative assessment of the chemical compounds attached or bound to the particle and/or in the leachate solution, minimum concentration, maximum exposure duration, location, presence or absence of plastic particles during exposure, and consideration of the temperature factor as well as their main effects are provided in Supplementary Material S1.

3. Disparities in research effort and their environmental relevance

While plastic research is among the most prolific fields of the last 50 years (see Seuront et al. (2022) for a review), the effects of plastics on marine mussels have only recently been addressed, starting with an article published in 2008 (Browne et al., 2008). Nevertheless, this research area has been growing rapidly, as more than 90% of the 106 papers selected have been published since 2017, with 60% within the last three years only (2021–2023; Figure 13).

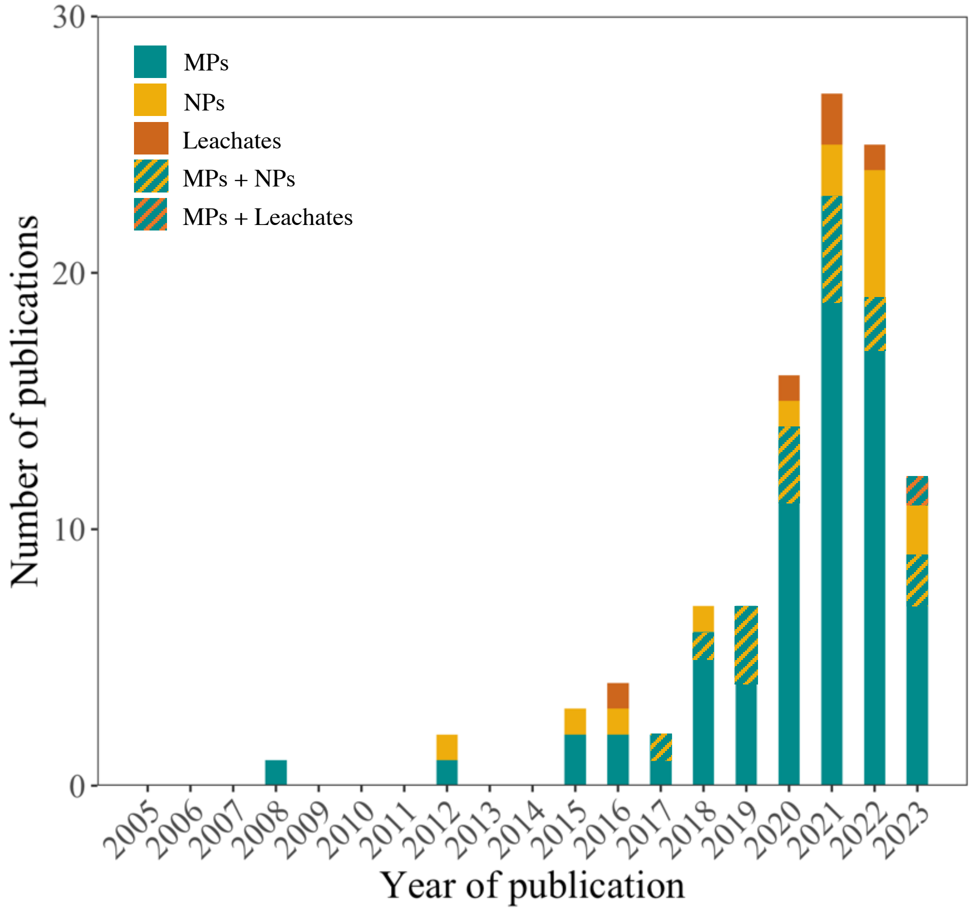


Figure 13: Temporal trends in the total number of publications included in this review (*n* = 106). Studies exploring the effects of microplastics and nanoplastics on marine mussels are indicated in blue and yellow, respectively, while those focusing on the effects of leachates (*i.e.* specifically in the absence of MPs and NPs) are in orange. Studies examining multiple categories are denoted by cross-hatched patterns corresponding to the respective colours.

Despite the growing interest in this issue, research efforts have not been evenly distributed between the categories described above, leading to clear disparities. Many studies have concentrated on specific parameters, *e.g.* one plastic polymer, shape, and concentration, that may not be consistent with *in situ* observations. In this section, we have highlighted these disparities and provided recommendations to steer future research in a more comprehensive and effective direction.

3.1. Spatial and dominant species disparities

Among the 106 studies that directly addressed the effects of plastics on marine mussels, experiments were conducted in 32 different countries. However, the research effort is far from being evenly distributed (Figure 14). Among the 32 countries represented, four collectively accounted for 50% of research efforts, with China displaying the highest publication record (18%), followed by Italy (16%), Spain (9%), and France (7%). On a global scale, Europe (54%) and Asia (28%) dominated the research effort by far. Although the African continent is noticeably recognised for its high levels of plastic pollution (Lebreton & Andrady, 2019), this topic accounted for less than 5% of the global research effort, as only 5 papers were spread among three countries, Tunisia (3 papers), Cape Verde (1 paper), and South Africa (1 paper). In addition, we did not find any study relevant to the issues discussed in the present work in Central America or most South American countries.

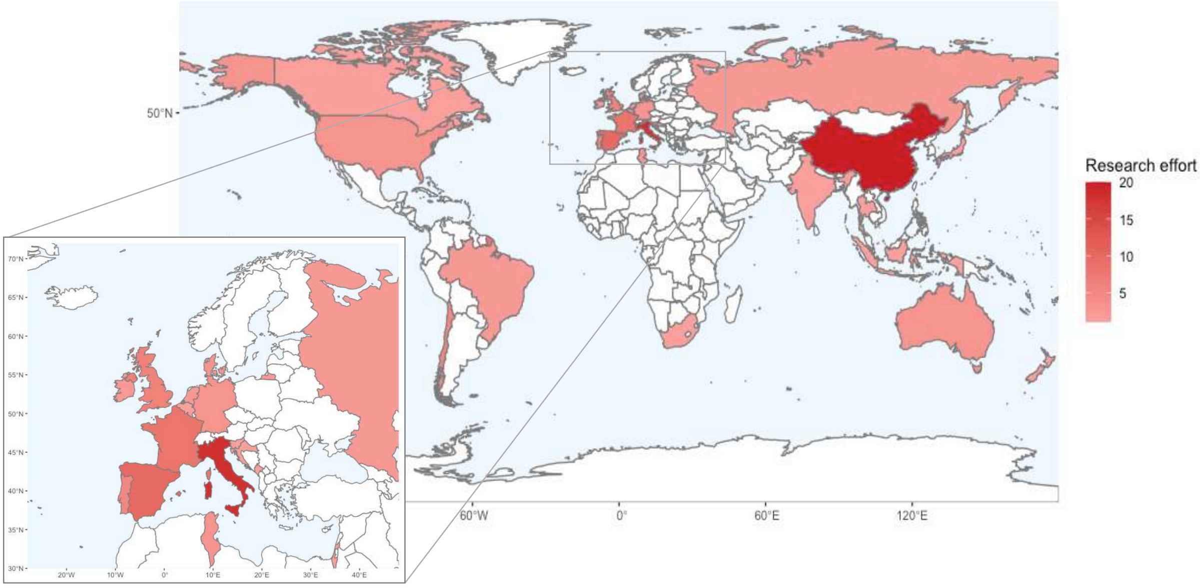


Figure 14: Research effort based on the number of publications per country that discuss the impacts of plastic pollution on marine mussels from the start of the research (2008) to 25 May 2023.

The spatial disparities observed in research efforts (Figure 14) mirror the diversity of mussel genera and species that have been scrutinised (Figure 15A, B). Fourteen mussel species belonging to five different genera were studied, including *Brachidontes, Choromytilus, Mytilus, Perna* and *Semimytilus.* The genus *Mytilus* accounted for a substantial 88% of the total research effort (Figure 15A). This dominance is predominantly due to the use of *Mytilus galloprovincialis* as a model species, which accounts for almost 50% of the total research effort (Figure 15A), followed by *M. edulis* (18%) and *M. coruscus* (12%). *Mytilus* and *Perna* spp. dominate the majority of the world's coastlines, with *Perna* spp. prevalent in the tropical zone and *Mytilus* spp. in temperate zones (Figure 15B). However, even though *Perna* was the second most studied genus, it only accounts for 8% of the research effort. The limited research effort on *Perna* spp. (8%) in comparison to *Mytilus* spp. (47%) likely results from disparities in research focus between tropical and temperate zones (Figures 14 and 15).

The concentration of research efforts towards specific countries and specific mussel species may lead to skewed knowledge on plastic pollution in other coastal regions and different mussel species. Despite being underrepresented, tropical regions are not exempt from plastic pollution. Due to the lack of proper regulations, adequate waste management practices, and the import of plastic waste from developed countries, tropical countries are significant contributors to plastic waste in the ocean (Lebreton & Andrady, 2019; Meijer et al., 2021). In addition, the consumer demand for plastic is projected to increase, especially in developing countries, *e.g.* by 375% from the current demand in Africa by 2060 (Lebreton & Andrady, 2019).

Research in tropical zones is relatively scarce despite the global presence of *Perna* spp. (Figure 15C), possibly because research groups in many parts of Asia (excluding China) and Africa may not have the necessary infrastructures and financial resources to purchase the needed tools, such as Fourier transform infrared spectrometer, Raman spectrometer or pyrolysis gas chromatography mass spectrometry packages (see Seuront et al. (2022) for a discussion on

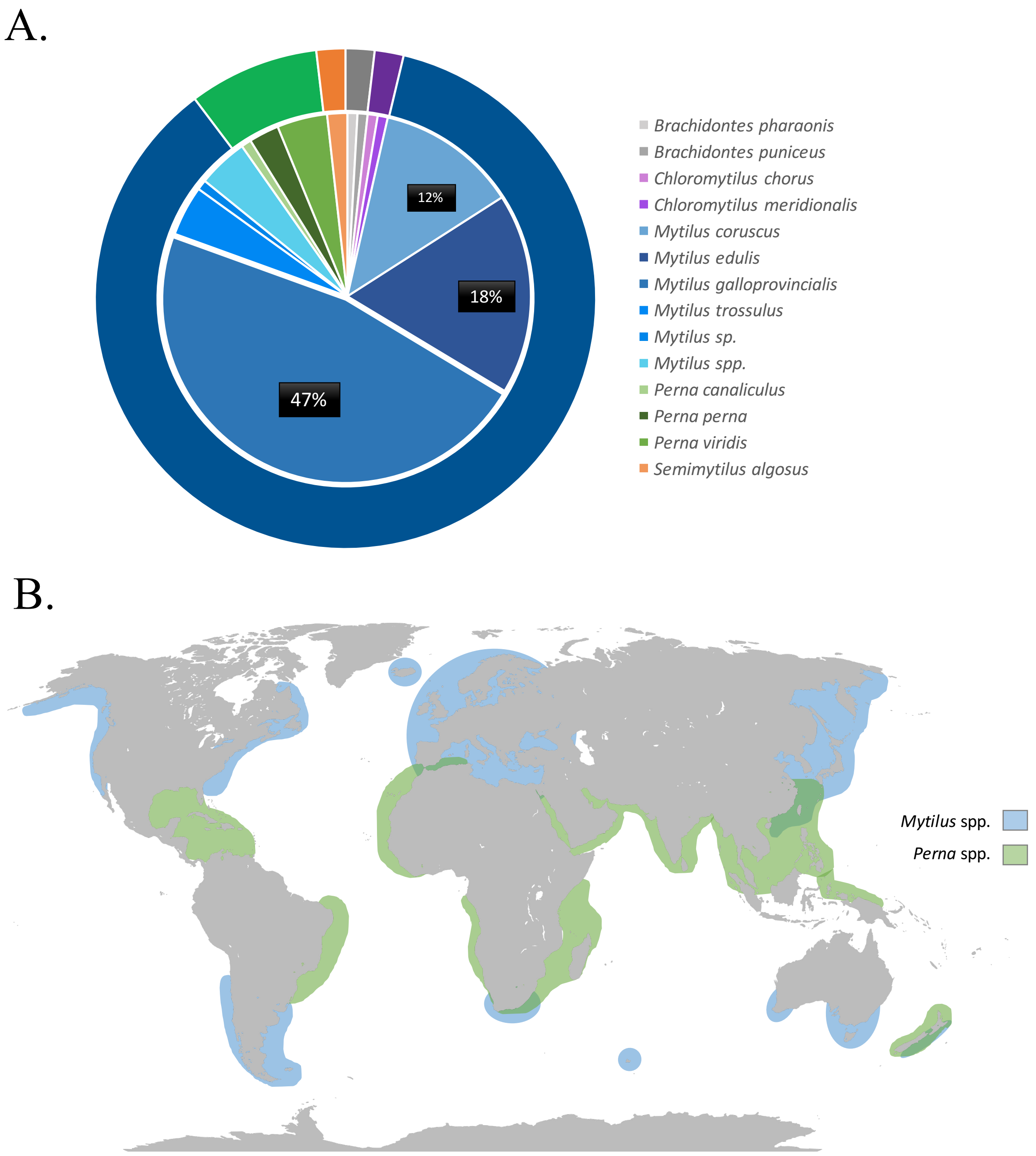


Figure 15: (A) Relative distribution of (i) the mussel genera (outer pie chart), *i.e. Mytilus* spp. (blue), *Perna* spp. (green), *Semimytilus* spp. (orange), *Brachidontes* spp. (grey), *Choromytilus* spp. (purple) and (ii) species (inner pie chart), with the value for the three most studied species, *i.e. M. galloprovincialis* (47%), *M. edulis* (18%), and *M. coruscus* (12%). (B) Global distribution of two mussel genera, *Mytilus* spp. (blue) and *Perna* spp. (green); adapted from Gardner et al., 2016; Hilbish et al., 2012.

this topic), despite the growing concern of the issues related to plastic pollution in these areas (*e.g.* Akindele & Alimba, 2021; Kapinga & Chung, 2020; Marnn et al., 2021; Matsuguma et al., 2017). The situation may be similar in Central America and South American countries, in which plastic pollution is a growing and pressing issue (*e.g.* Ardusso et al., 2021; Blettler et al., 2020; Denuncio et al., 2017; Ita-Nagy et al., 2022; Ivar do Sul & Costa, 2007). To address these challenges and achieve a more balanced representation of plastic pollution's impact on marine mussels worldwide, it is crucial to promote international collaborations, increase research capacity in underrepresented regions, and foster awareness of the importance of studying different mussel species in different geographical areas as well as their inter- and intraspecific variability in plastic pollution (Nicastro et al., 2023a). Notably, only a limited number of cross-border studies investigated the same parameter across multiple mussel species (Hamm et al., 2022; Seuront et al., 2021) or in the same species across different locations (Kolarević et al., 2023; Provenza et al., 2023).

3.2. Plastic characteristics: size, polymer, shapes and leachates

3.2.1. Size

Most studies that investigated the impact of plastic on marine mussels primarily focused on microplastics (MPs; 1 µm – 5 mm), accounting for 71% of the research effort (Figure 16A). Nanoplastic (NPs; < 1 µm) studies represented 24% of the research effort. The remaining 5% of the research effort was specifically dedicated to studying the impact of plastic leachates, a topic that is further explored in detail hereafter (Section 3.2.4); notably, leachate solutions were consistently prepared from MP but not NP particles. Most studies focused on the individual impacts of MPs and NPs on mussels, and only six articles examined the effects of a mixture of both particle sizes. The use of a NP and MP mixture provides a more realistic scenario, given the ubiquity of these two particle size classes in the marine environment (Eriksen et al., 2014;

Ter Halle et al., 2017). Although the presence of MPs in wild mussels has often been reported (Li et al., 2019), the presence of NPs remains more elusive, even though their retention and accumulation have been demonstrated in the laboratory; see Zaki & Aris (2022) for a review. However, given that the number of particles in the environment is known to increase as the size decreases (Eriksen et al., 2014), NPs are expected to be more widely abundant than MPs. NPs are emerging contaminants and constitute a relatively new research field, indeed the impact of NPs on mussel was first studied relatively recently (Wegner et al., 2012) in contrast to studies on MPs (Browne et al., 2008; Figure 13). In addition, the methods and technologies currently available to detect and quantify NPs in environmental matrices remain in their infancy (Zaki & Aris, 2022).

To thoroughly assess the overall impact on marine ecosystems, researchers must understand the influence of particle size on the toxicity and ecological consequences of plastic pollution. Particle size may induce different toxicity, as it influences the interaction mode between the particle and the organism, *e.g.* ingestion, retention time, translocation, and biomagnification (Zaki & Aris, 2022). Notably, compared to larger particles, smaller MPs have a higher potential to be translocated across the epithelial membrane of the gut to the mussel haemolymph and haemocytes, where they can persist for more than a month (Browne et al., 2008). In addition, these small MPs can be transferred through the food web, *e.g.* from mussels to crabs (Farrell & Nelson, 2013). The size of particles also affects their surface-to-volume ratio; thus, compared to larger particles, smaller particles exhibit a greater adsorption capacity, which can result in higher toxicity due to the adsorption of toxic compounds on their larger surface (Rodrigues et al., 2019b).

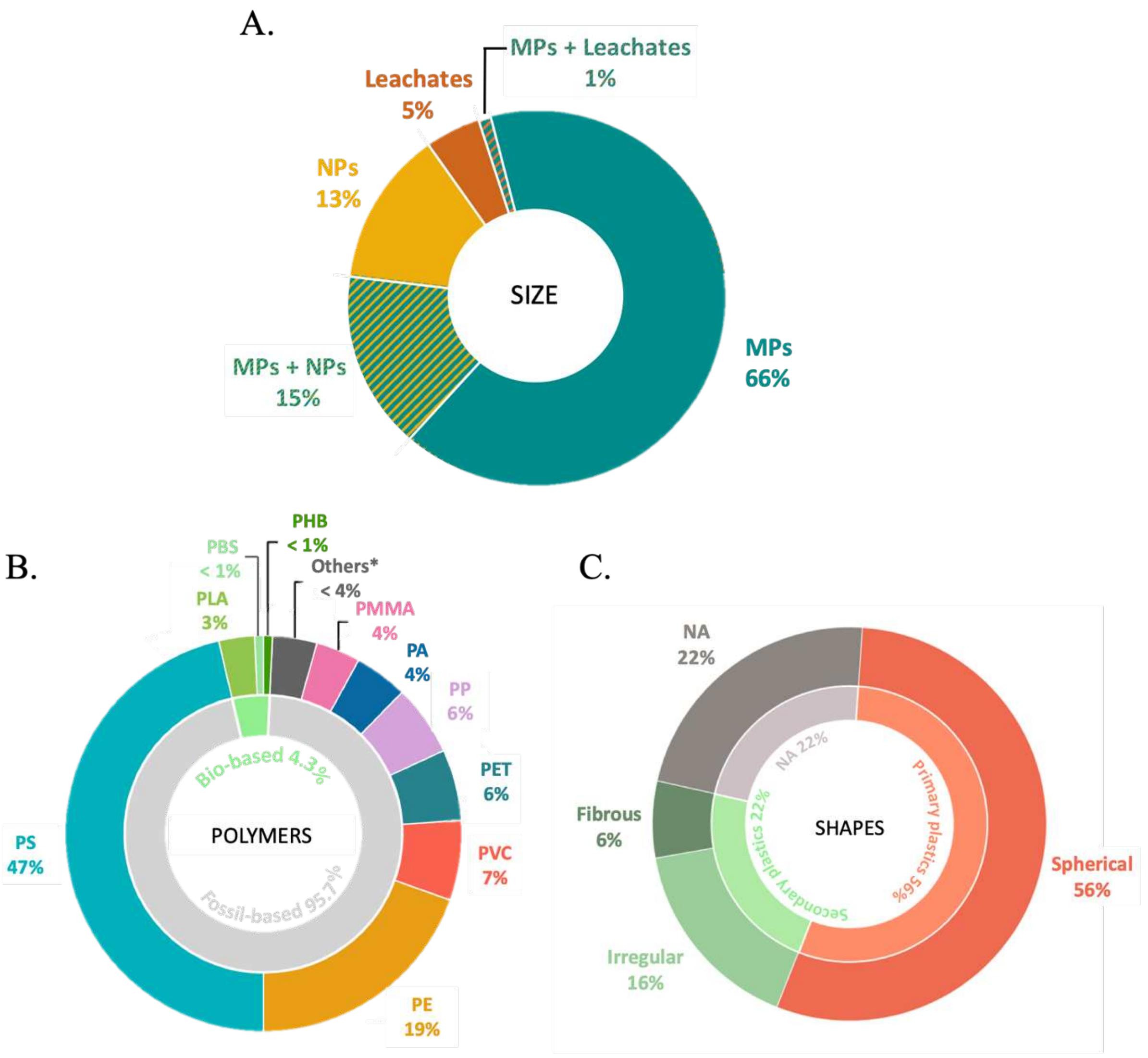


Figure 16: Relative distribution of the plastic size (A) polymers (B) and shapes (C) used in plastic impact studies on marine mussels. MPs: Microplastics, NPs: Nanoplastics. PS: polystyrene, PE: polyethylene, PVC: polyvinyl chloride, PP: polypropylene, PET: polyethylene terephthalate, PA: polyamide, PMMA: poly(methyl methacrylate), PEST: polyester fossil-based, PLA: polylactic acid, PBS: polybutylene succinate, PHB: poly-β-hydroxybutyrate, PEVA: ethylene-vinyl acetate, paraffin and rubber. Asterisk indicates other polymers used, *i.e.* PEST: polyester fossil-based, PEVA: ethylene-vinyl acetate, paraffin and rubber. NA indicates no available data.

3.2.2. Polymers

With the exception of one paper (Alnajar et al., 2021), polymer types were consistently indicated in the papers considered here. Specifically, a range of 14 different polymer types were used to assess the effect of plastic on marine mussels (Figure 16B), with polystyrene (PS) being the most widely studied, accounting for *ca.* 50% of the papers. The remaining polymer types ranked as follows: polyethylene (PE; 19%, including low and high density), polyvinyl chloride (PVC; 7%), polypropylene (PP; 6%), polyethylene terephthalate (PET; 6%), polyamide (PA; 4%) and poly(methyl methacrylate) (PMMA; 4%). These types were followed by polyester fossil-based (PEST; <1%) and biobased, *i.e.* polylactic acid (PLA; 3%), polybutylene succinate (PBS; <1%), poly-β-hydroxybutyrate (PHB; <1%). Finally, less than 1% of the research effort involved ethylene-vinyl acetate (PEVA), paraffin and rubber.

Conducting experiments with representative polymer compositions in a given region is necessary to obtain valuable insights into the real ecological impact of plastic pollution on marine mussels. The dominant use of PS in marine mussel impact studies (Figure 16B) conflicts with the typical polymer composition commonly observed in the natural environment. Specifically, PE is the most common polymer found in marine mussels, followed by PP; see Bajt (2021) for a review. PE and PP are the most produced polymers worldwide, with 26.9% and 19.3% of the global production in 2021, respectively (PlasticsEurope, 2022), and are the most frequently observed polymers in the sea-surface environment (Erni-Cassola et al., 2019). As mussels are filter feeders, the polymer composition inside mussels is representative of the polymer composition found in their environment (Li et al., 2019). Mussels typically filter not only one type of polymer but also a variety of polymers depending on their region and the related level of contamination (Bajt, 2021; Li et al., 2019). The ecological consequences of this diverse polymer variety have, however, only recently attracted attention, and a mere fraction of the research (1%) investigated the effects of mixed polymer exposures (Bandini et al., 2021; Romdhani et al., 2022).

In addition, there is growing interest in the so-called ‘biodegradable plastics’ (*i.e.* PLA, PBS and PHB), which were first used in experiments with mussels in 2017 and only accounted for 4% of the research effort; Figure 16B). While these biodegradable plastics accounted for *ca.* 0.5% of the global plastic production in 2022 (PlasticsEurope, 2023), they are often considered a potential solution to plastic pollution. However, their biodegradable properties are subject to considerable debate due to their restricted decomposition in natural environments; see Paul-Pont et al. (2023) for a recent discussion on this topic. These plastics are also not risk-free, as they induce a range of alterations at various levels in mussels (Green et al., 2019, 2017; Khalid et al., 2021; Magara et al., 2019; Phothakwanpracha et al., 2021), although exceptions exist (*e.g.* Joyce & Falkenberg, 2022).

3.2.3. Shape

Various particle shapes were used to investigate the effect of plastics on marine mussels, in which the following main shapes can be defined: spherical (*e.g.* beads and pellets), irregular (*e.g.* fragments and films) and fibrous (*e.g.* fibres and filaments; Figure 16C). Among these, spherical shapes constitute the highest proportion of the research effort, *i.e.* 56%. Irregular and fibrous shapes represent 16% and 6% of the research effort, respectively. Notably, 22% of the studies did not specify the shape of the plastics used.

While spherical particles are the most commonly used in laboratory experiments, fibrous shapes are highly predominant, followed by irregular shapes in field investigations on plastic inside mussels; see Bajt (2021) and Li et al. (2019) for reviews. These plastics are defined as secondary plastics, *i.e.* they result from the degradation of larger plastic debris, and are the most prevalent plastic in marine environments (Paul-Pont et al., 2018). Secondary plastics are different from primary plastics, which are mainly spherical and directly produced in this form. However, plastic toxicity is intrinsically shape-dependent, as shape leads to different levels of potential tissue irritability (Ziajahromi et al., 2017) and to different mussel plastic uptake, retention and elimination (Li et al., 2021). These observations highlight the significance of providing accurate information on the shape of the plastic used, particularly in experiments focusing on the impact of plastic ingestion.

As fibres and filaments are the predominant plastic shapes found in marine mussels but the least studied, it is critical to intensify efforts to clarify their impact. Additionally, investigating a combination of shapes that mirrors the proportions found in the environment is essential to ensure the ecological coherence of this research area. In addition, although most studies showed a dominance of fibrous shapes in mussels followed by irregular and spherical shapes, some studies found opposite results (Ibryamova et al., 2022). Noticeably, local waste sources and accidental events, *e.g.* the X-press Pearl shipwreck in Sri Lanka, which released 70 billion pellets into the environment (James et al., 2022), can influence the proportion of the different plastic shapes. As such, it is highly recommended to systematically conduct *in situ* measurements before laboratory experiments to obtain environmentally relevant and meaningful results.

3.2.4. Plastic-associated chemicals

A neglected aspect: absence of chemical composition analyses

Plastics are fundamentally loaded with chemical compounds. Numerous additives (*e.g.* plasticisers, antioxidants and flame retardants) are incorporated into plastic during the manufacturing process. In addition, during their residence in the aquatic environment, plastics typically sorbe and accumulate a wide range of chemicals from the environment, *e.g.* heavy metals and persistent organic pollutants (POPs; Fries & Zarfl, 2012; Rodrigues et al., 2019b; Yeo et al., 2020). However, among the 106 papers analysed, only 8% performed—at least partially—a chemical content analysis (Figure 17). Therefore, most of the literature addressing the effect of plastic pollution on mussels (92%) did not provide any information about the potential chemical composition of the microplastic particles that were employed. Notably, among them, 15% considered implementing measures to avoid chemical compounds by using 'additive-free' plastics (7%) or by 'washing' the particles to allegedly release their additives (8%). However, none of the studies conducted a chemical analysis to validate the absence of additives or the efficacy of the washing process. In the 8% of articles that conducted chemical content analyses, a specific list of compounds (*e.g.* bisphenol A, bis(2-ethylhexyl) phthalate) from particular chemical classes (*e.g.* additives, mineral contaminants) was targeted. For instance, the lists of screened additives are often very limited compared to the ever-growing number of additives used in the plastic industry (*i.e.* 481 so far as listed under the mapping exercise of the European Chemical Agency plastic additives initiative; www.echa.europa.eu, accessed June 2023), which *de facto* potentially overlooks important (in terms of, *e.g.* toxicity) chemical components of the particles. In addition, these studies essentially carried out qualitative rather than quantitative analyses, potentially overlooking the role of additive doses.

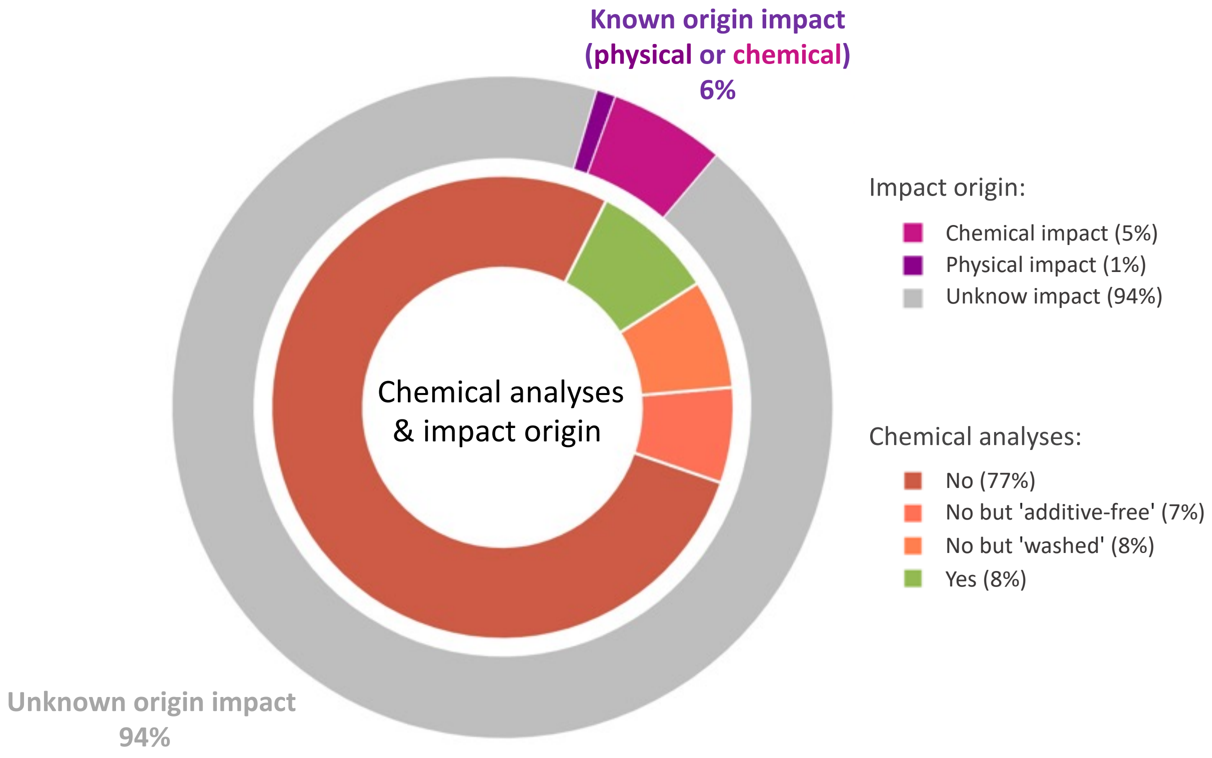


Figure 17: Relative distribution of chemical composition analyses (inner pie chart) and origin of the effect found (outer pie chart) in the 106 studies reviewed.

Chemical compounds can constitute a substantial part of the plastic, with plasticisers up to 60% of the polymer's weight (Net et al., 2015). These chemical compounds can be released (i) inside the organisms after particle filtration or/and (ii) in seawater and interact directly or/and indirectly with the organisms. High quantities of chemical contaminants, including plastic additives, such as phthalates and bisphenols, have been recorded in mussels in nature (Castro et al., 2022; Hermabessiere et al., 2019; Rios-Fuster et al., 2022) and are known to have negative effects on organisms (Delaeter et al., 2022). Thus, the dearth of chemical screening analyses represents a significant knowledge gap that can hinder progress in research areas involving the assessment and clarification of the biological effects of plastic pollution. To address this gap, interdisciplinary collaborations are crucial, as a wide range of analytical methods are available to qualify and quantify these additives; see Seuront et al. (2022) for a statement on this specific aspect of plastic research.

Physical or chemical impact?

Among the 106 studies that examined the impacts of plastic on mussels, only 6% knew the origin of the impact, *i.e.* physical or/and chemical impact, exerted on organisms (Figure 17). Thus, five papers specifically assessed the impact of plastic leachates by removing plastic particles from the solution to which the organisms were exposed (Capolupo et al., 2020, 2021a; Gandara e Silva et al., 2016; Seuront et al., 2021; Uguen et al., 2022), and one paper assessed the exposure to particles and leachates individually, showing that the effect resulted from the physical impact of plastic particles (Wang et al., 2023a). In contrast, in 94% of the screened literature, the experimental design did not reveal whether the observed effects resulted from the physical presence of plastic particles, the chemical impact of the particles' leachates, or a combination of both factors. To address this concern, future studies should focus on isolating the impacts of plastic particles from those of leachates to clarify the relative contribution of each factor to the overall harm caused by plastics on marine organisms. Interestingly, 22 papers investigated the effect of plastic particles alone or in combination with an added pollutant (*e.g.* benzo(a)pyrene, fluoranthene, cadmium) on marine mussels. However, only two of these 22 papers screened the chemical composition of their plastic. Rather than adding a specific type of pollutant, leachates should be used from plastic particles collected from (or incubated in) the field to clarify the environmental chemical cocktail that plastic might accumulate.

One study compared the effects of leachates from raw industrial plastic pellets (virgin pellets) with those from plastic pellets found on beaches (beached pellets) in marine mussels (Gandara e Silva et al., 2016), showing that leachates from beached pellets exert a higher impact than that of virgin pellets. However, the lack of leachate chemical screening prevents direct comparison with other studies with an opposite pattern, *e.g.* on oysters at early life stages (Gardon et al., 2020; Tallec et al., 2022a). Gandara e Silva et al. (2016) were nevertheless the first authors to investigate the impact of leachates on marine mussels, and most (80%) subsequent papers conducted chemical screenings on plastic leachates. However, the methodological approaches to prepare leachate solutions are quite different. For instance, the particle incubation time varied from 24 h (3 papers) to 14 days (2 papers), while the leaching temperatures varied from 10 °C (1 paper) to 25 °C (2 papers; the remaining papers provided no information about these parameters) and were not always aligned with the incubation temperature of the model organism. Although they do not jeopardise the central point of these papers, these discrepancies nevertheless prevent the results from being unambiguously compared. Notably, a protocol has been recently proposed to standardise the methodology for obtaining microplastic leachates (Almeda et al., 2023).

Compared to the abundant literature on micro- and nanoplastics physical effects, the impact of leachates is generally neglected (Delaeter et al., 2022; Seuront et al., 2022); however, leachates are a major contaminant transfer route. A study that compared two contaminant bioaccumulation pathways in mussels found that uptake through the aqueous phase (leachates) was a more significant pathway than plastic particle ingestion (Jang et al., 2021). By (i) isolating the effects of plastic particles from leachates, (ii) considering the environmental chemical composition, (iii) using a standardised protocol and, finally, (iv) conducting chemical screenings, future studies can provide more comprehensive and accurate insights into the complex interactions between plastics and marine ecosystems.

In addition to plastic chemical and physical impacts, the colonisation of plastic by organisms, referred to as the "plastisphere", may also induce biological impacts; see Bowley et al. (2022) and Sooriyakumar et al. (2022) for reviews. The surfaces of plastics are colonised by a succession of organisms, including bacteria that form a biofilm and then more complex eukaryotic organisms; these plastic-associated communities generally differ from those in the surrounding environment (Bowley et al., 2022). Plastic facilitates the transport of their associated communities over long distances, thereby accelerating the dispersal of organisms and potentially leading to biological invasions (Bowley et al., 2022; Carlton et al., 2017; Shen et al., 2019). The transport-associated organisms may also include pathogenic species, *e.g.* *Vibrio* spp. and *Escherichia* spp., which may induce disease upon ingestion of plastic particles; see Bowley et al. (2022) for a review. In marine mussels, exposure to plastic with bacterial biofilm induced a significant immune response and affected the microbial composition of their gills in comparison to sterile mussels (Bandini et al., 2021). In addition, plastic particles enhance gene exchange among attached biofilm communities, fostering the transfer of pathogenic and antibiotic resistance genes (Shen et al., 2019). Given the multifaceted potential biological impacts of plastic particles outlined above, future studies must delve deeper into their exact role as biological vectors (Sooriyakumar et al., 2022).

3.3. Exposure Conditions

3.3.1. Plastic concentration

All studies provided information about the concentrations of plastics they used. However, these concentrations were expressed in a range of units, with the most common being mass/volume (ng mL^{-1}, µg L^{-1} or mg L^{-1}) and items/volume (MP mL^{-1}, MP L^{-1}, MP mm^{-3}), while the least frequently used unit was volume/volume (mL L^{-1}). According to the plastic characteristics (polymer, size and shape), these three units of measurement may vary considerably. Due to their complementarity, reporting the concentration in mass/volume, item/volume and volume/volume would provide a more straightforward overview of the concentration used and thus improve the reproducibility of research.

For a representative overview of the concentration used in the plastic impact on marine mussel studies, we focus on studies that used mass/volume units (66% of the studies; 70 papers). Among them, 52% (36 papers) used a range of different concentrations. To study the environmental relevance of the concentration used, we only examined the minimum concentration employed in each study. All concentrations were converted to µg L^{-1} to ensure comparability.

The minimum concentrations used varied from 10^{-4} to 10^{8} µg L^{-1}, with almost 50% (49%, 34 papers; Figure 18) of the research effort in the 1 – 1000 µg L^{-1} range (median = 200 µg L^{-1}). Based on the highest plastic concentration reported (*i.e.* 1890 µg L^{-1}; see Isobe et al., 2015 in Beiras & Schönemann, 2020), 71% (Figure 18) of the research papers reviewed used environmentally relevant concentrations. Notably, environmental plastic mean concentrations were generally far below 1890 µg L^{-1}, ranging from 0.01 to 34.09 µg L^{-1}, depending on the geographical region (Beiras & Schönemann, 2020). However, these measurements were taken at the sea surface, with a defined mesh size and far from the coast, which may bias the estimation of the plastic concentration faced by mussels, especially those living in coastal waters. Coastal systems are among the most heavily plastic-polluted ecosystems (Serra-Gonçalves et al., 2019; Wu et al., 2022), and the plastic concentration faced by benthic species at the water-sediment interface can be even higher (Besseling et al., 2014). Additionally, mussel beds, with their spatial structure and important filtration rate, alter the water flow, increase turbulence, and act as local hotspots of benthic microplastic pollution (*e.g.* Lim et al., 2020; Van Colen et al., 2021); this effect can be significantly enhanced by mussel shell endolithic infestation, which further increases the rugosity of beds (Nicastro et al., 2022). As a consequence, mussels may be exposed to concentrations greater than expected, and the plastic concentration used in the experiment may not be as unreasonable as previously thought (*e.g.* Delaeter et al., 2022; Paul-Pont et al., 2018; though these figures were obtained outside the mussel literature).

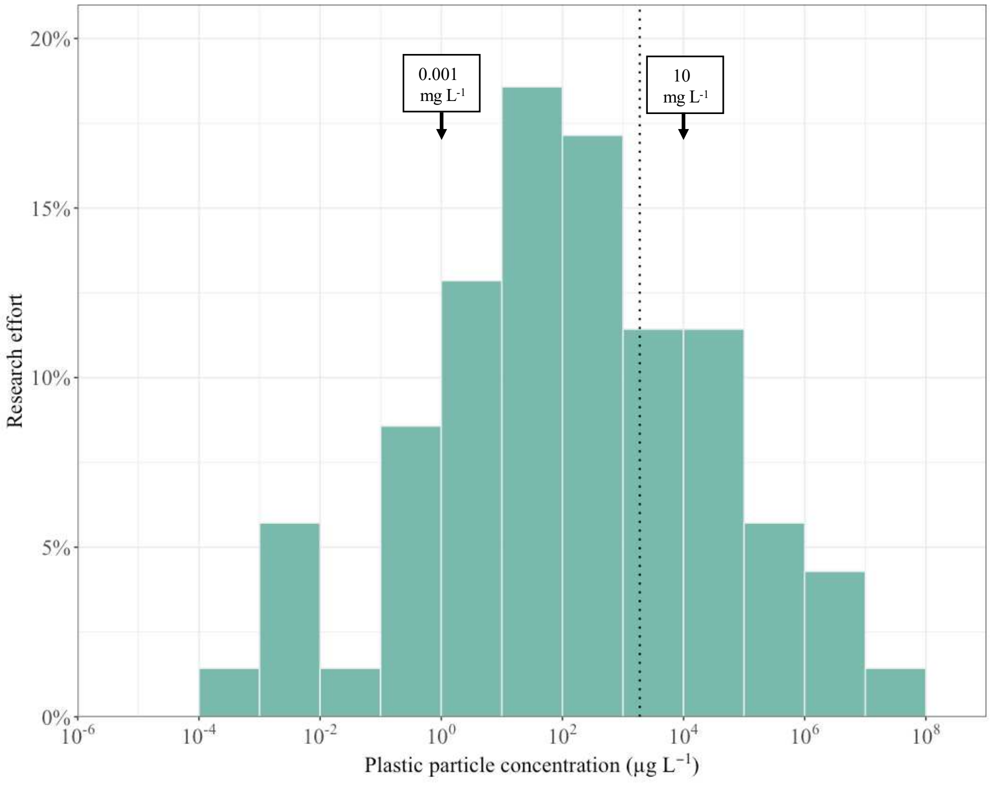


Figure 18. Distribution of the minimum concentration used in plastic impact studies on marine mussels. The dashed line represents the highest concentration recorded in the environment, *i.e.* 1890 µg L^{-1} (= 1.89 mg L^{-1}; see Isobe et al., 2015 in Beiras & Schönemann, 2020). The data presented here are based only on concentrations in µg L^{-1}, available in 70 studies.

The highest minimum concentration found in this review, *i.e.* a concentration of 1.2 × 10^7 µg L^{-1} (= 1.2 × 10^4 mg L^{-1}), was used to study the impact of plastic leachate on marine mussels (Seuront et al., 2022). In this experiment, particles were incubated in seawater for 24 hours before being removed. As (i) the particles are not intended to be ingested by mussels and (ii) the desorption of additives may be a lengthy process that can take several weeks (Paluselli et al., 2019), the short incubation time used potentially counterbalances the high plastic concentration employed. Although the resolution of this specific issue lies well beyond the scope of the present review, these observations warrant the need for further work to decipher the relative effect of particle concentrations, the chemical components of particles, the leaching time and the duration of exposure to the resulting leachate solutions.

3.3.2. Exposure duration

When an experiment used several exposure durations (11%), only the maximum duration was considered here for clarity. Each experiment covered a wide range of exposure times, *i.e.* from a few hours to a maximum of 91 days, with a median exposure duration of 7 days. Most studies (*ca.* 50%) used standard exposure durations, *i.e.* 7 days (15%), 4 days (12%), 21 days (12%) and less than 24 hours (10%). Long-term exposures are typically chosen to reflect environmental conditions, involving lower plastic concentrations than in shorter experiments. However, no significant correlation was found between the maximum duration of the experiment and the minimum concentration used (p = 0.6618). Additionally, only a limited number of studies investigated how mussels recover after plastic exposure. Further experiments are needed to address these gaps.

Finally, despite the growing interest in (i) full life cycle tests (*i.e.* stressor exposure from early-life stages to mature individuals), and (ii) multigenerational tests (*i.e.* stressor exposure across multiple generations; Guimarães et al. (2023)), neither approach has been explored to examine the impact of plastics on marine mussels. Plastics and their associated chemicals induce several adverse intergenerational effects in terrestrial and aquatic species (see Junaid et al. (2023) for a review), including the marine bivalve *Magallana gigas* (Bringer et al., 2022; Sussarellu et al., 2016). Parental exposure to a different type of plastic was found to result in motility and development impairment of their (nonexposed) offspring (Bringer et al., 2022; Sussarellu et al., 2016). Utilising these approaches in mussel studies would provide an opportunity to clarify the impacts of plastics on these organisms.

3.3.3. Mussel life stage and other internal factors

The sensitivity to a contaminant has been shown to vary with the life stage of an organism (Mohammed, 2013). Mussel life stages were defined here as (i) pelagic early-life stages (gametes to larvae) or (ii) benthic life stages (juvenile to adult). Among the 106 papers screened, nine did not contain specific information on mussel size or life stages. However, through contextual information in these nine papers, we could classify the individuals in the benthic mussel life stage category. In the plastic impact on marine mussel fields, benthic life stages received much more attention than pelagic life stages, accounting for 94% and 6% of the research effort, respectively (Figure 19A). Among them, only a few studies (4 papers) examined the plastic impact on both stages (Auguste et al., 2021; Capolupo et al., 2020, 2021b; Franzellitti et al., 2019; Figure 8A).

Morphological and physiological differences between pelagic and benthic life stages can lead to variations in their exposure and vulnerability to contaminants, with early life stages generally being more sensitive to toxicants (Mohammed, 2013; Figure 19B). This phenomenon was also recently described for microplastics in a global pattern analysis of benthic species (Mason et al., 2022), highlighting the importance of considering the full life cycle of mussels when assessing the effects of pollutants.

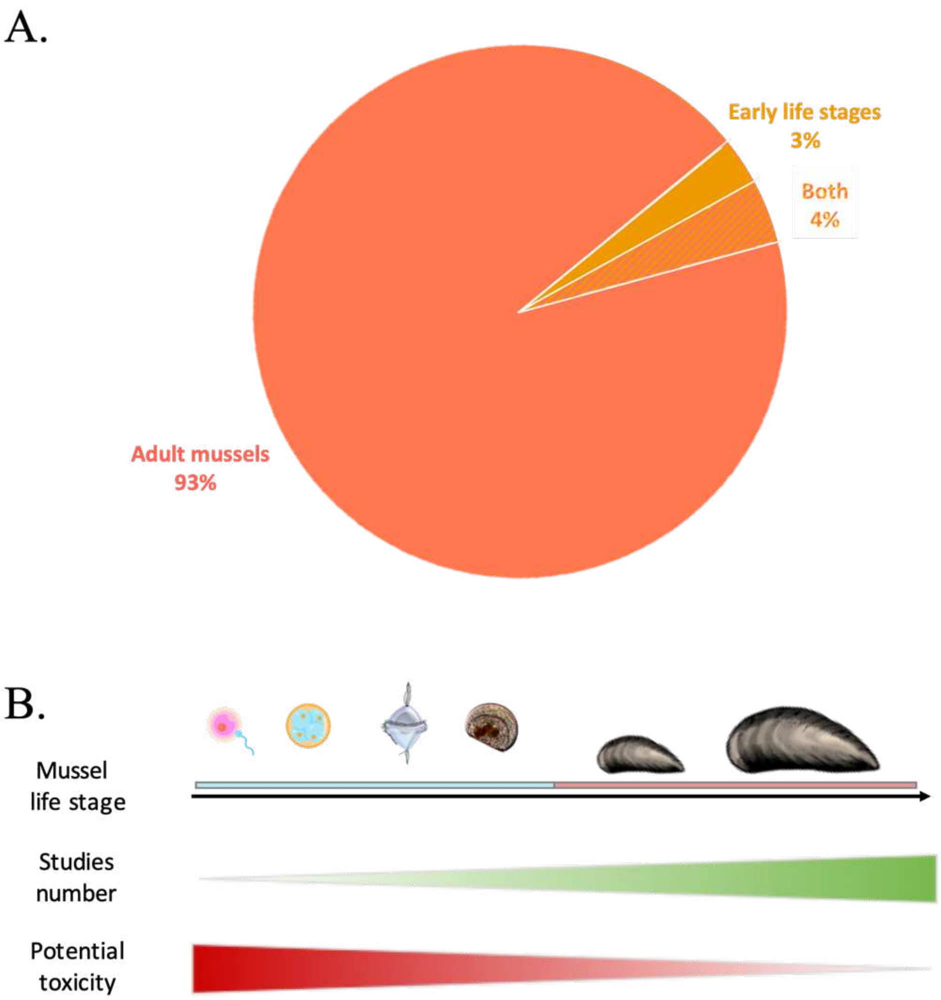


Figure 19. Relative distribution of the mussel life stages used to assess the impact of plastics on marine mussels (A) and estimation of the number of studies and plastic toxicity by mussel life stage (B) pelagic (gametes, embryos, larvae; blue) and benthic (juveniles and mature; red).

Note that other factors internal to an organism, such as its sex, sexual maturity, size, and genotype, can also influence the bioaccumulation of a pollutant (Boening, 1999) and its potential effects. The effects of contaminants can be size-dependent inside the same life stage category, *e.g.* small adult mussels were found to be more sensitive to plastic leachates than larger ones (Uguen et al., 2023). Sex is also a critical parameter in pollutant impact studies, although this parameter has received very little attention, especially in 'invertebrate' studies (Burger, 2007). Among the 106 papers, only one has shown that plastics impact females (*i.e.*

through a significant decrease in catalase (CAT) activity), which was not observed in males (Abidli et al., 2021). Sex-specific effects may be caused by plastic additives, as some can mimic hormones and act as endocrine disruptors; a typical example is polybrominated diphenyl ethers (PBDEs), flame retardants that induce mussel sex-specific effects (Ji et al., 2013). Sexual maturity can also modulate the plastic effects, *e.g.* MP impact on clearance rate was more pronounced in mussels that had recently spawned (Laubscher et al., 2023). This highlights the importance of sex and sexual maturity when evaluating the effects of pollutants on organisms and underlines the need for further research in this area to clarify the potential sex-related differences in pollutant sensitivity.

4. Main effects of plastics on mussels: an overview

This section aims to examine the state-of-the-art knowledge and understanding of the influence of plastic on marine mussels. The impact of plastics on mussels has been studied in depth at different organisational levels, from small-scale impacts encompassing molecular, cellular, tissue and organ levels to organism-level effects encompassing functional, physiological, and behavioural traits, and, more generally, in terms of their broader effects, *e.g.* on associated communities. Most research effort has focused on small-scale impacts (57.2%), followed by effects at the organism level (41.4%), with limited exploration of larger-scale impacts (1.4%; Figure 20). For more details about the presence/absence of effects by organisational levels for each study, see Table 1.

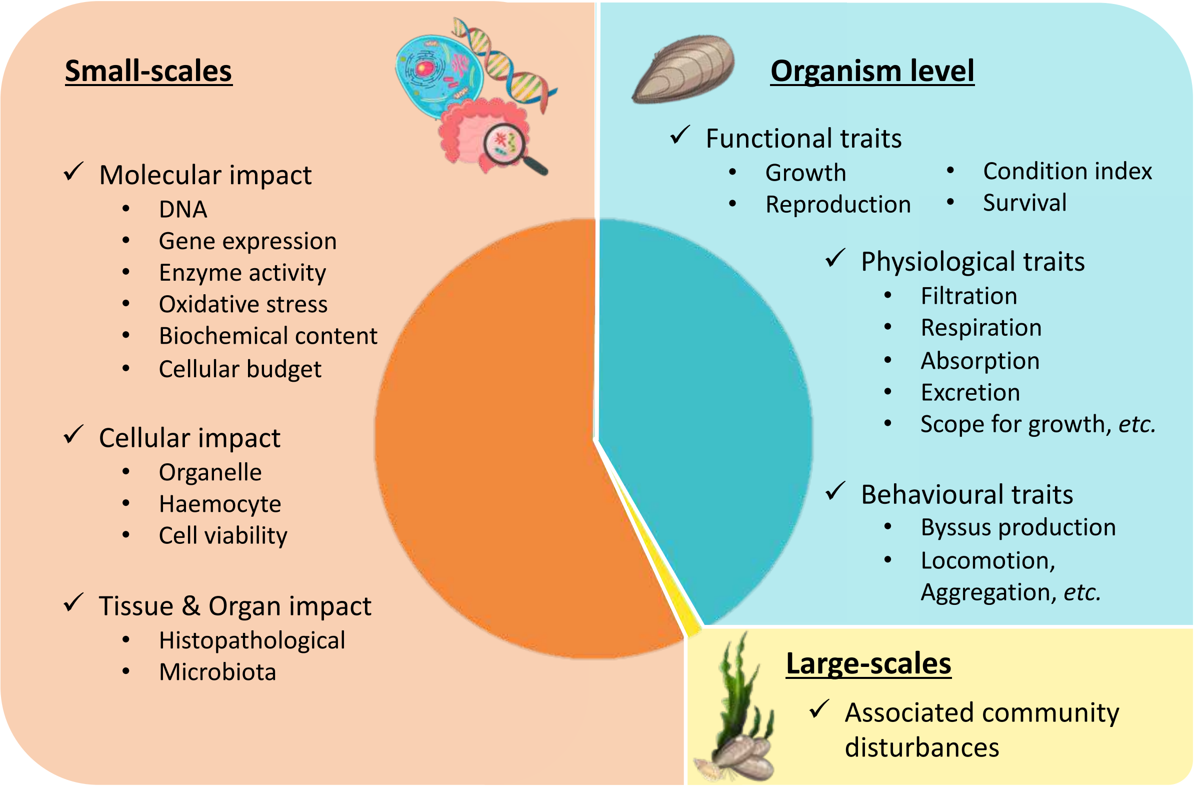
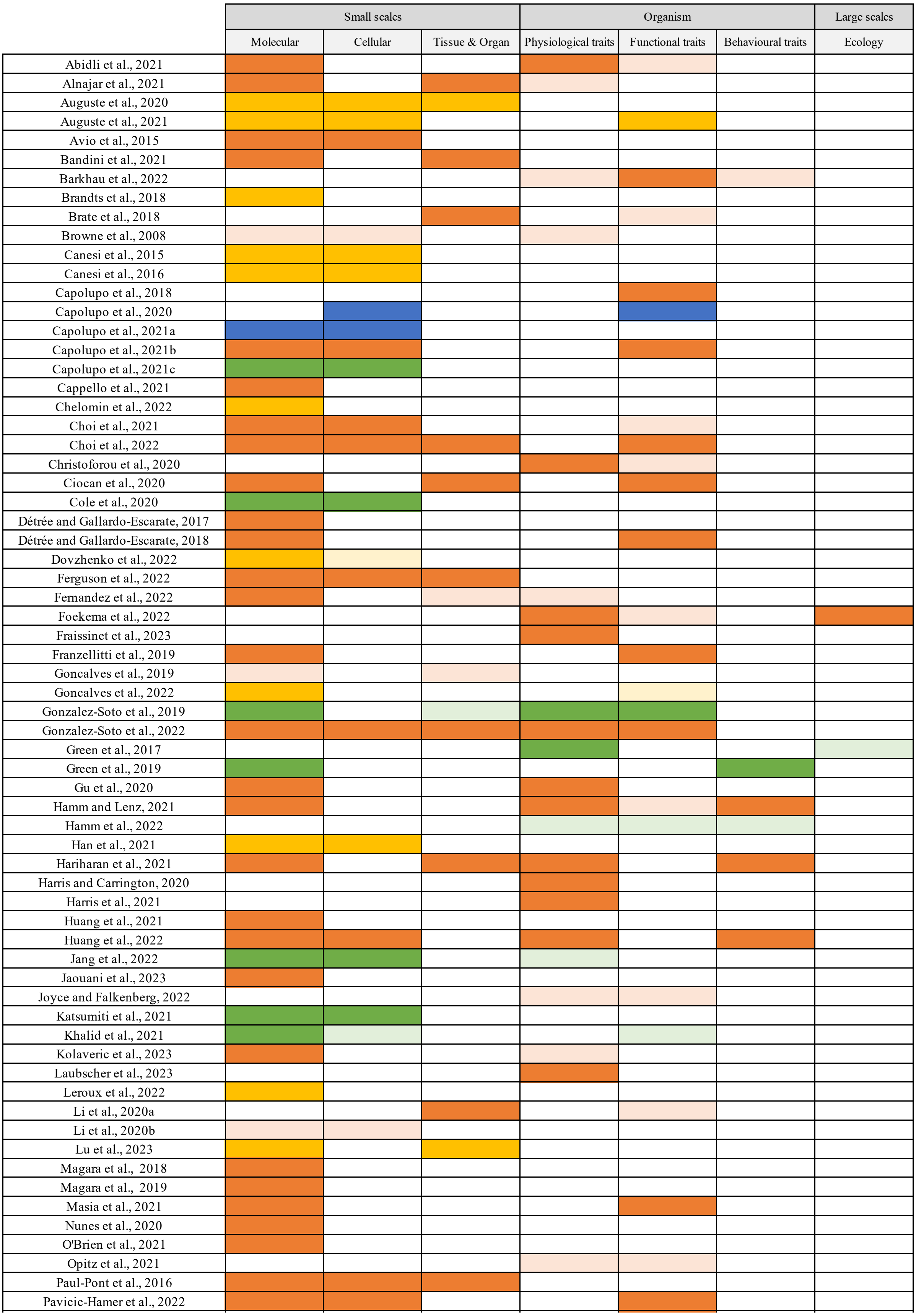


Figure 20: Relative distribution of the research effort across impact levels, *i.e.* small scales (57.2%), organism level (41.4%) and large scales (1.4%) and the main effects found.

Table 1. Presence (dark colour) or absence (light colour) of impact of microplastics (orange), nanoplastics (yellow), plastic leachates (blue), or a mix (green; *e.g.* microplastics + nanoplastics) on mussels across various organisational levels for each of the 106 articles.



	Small scales			Organism			Large scales
	Molecular	Cellular	Tissue & Organ	Physiological traits	Functional traits	Behavioural traits	Ecology
Abidli et al., 2021	🟧			🟧	🟠		
Alnajar et al., 2021	🟧		🟧	🟠			
Auguste et al., 2020	🟨	🟨	🟨				
Auguste et al., 2021	🟨	🟨			🟨		
Avio et al., 2015	🟧	🟧					
Bandini et al., 2021	🟧		🟧				
Barkhau et al., 2022				🟠	🟧	🟠	
Brandts et al., 2018	🟨						
Brate et al., 2018			🟧		🟠		
Browne et al., 2008	🟠	🟠		🟠			
Canesi et al., 2015	🟨	🟨					
Canesi et al., 2016	🟨	🟨					
Capolupo et al., 2018					🟧		
Capolupo et al., 2020		🟦			🟦		
Capolupo et al., 2021a	🟦	🟦					
Capolupo et al., 2021b	🟧	🟧			🟧		
Capolupo et al., 2021c	🟩	🟩					
Cappello et al., 2021	🟧						
Chelomin et al., 2022	🟨						
Choi et al., 2021	🟧	🟧			🟠		
Choi et al., 2022	🟧	🟧	🟧		🟧		
Christoforou et al., 2020				🟧	🟠		
Ciocan et al., 2020	🟧		🟧		🟧		
Cole et al., 2020	🟩	🟩					
Détrée and Gallardo-Escarate, 2017	🟧						
Détrée and Gallardo-Escarate, 2018	🟧				🟧		
Dovzhenko et al., 2022	🟨	🟡					
Ferguson et al., 2022	🟧	🟧	🟧				
Fernandez et al., 2022	🟧		🟠	🟠			
Foekema et al., 2022				🟧	🟠		🟧
Fraissinet et al., 2023				🟧			
Franzellitti et al., 2019	🟧				🟧		
Goncalves et al., 2019	🟠		🟠				
Goncalves et al., 2022	🟨				🟡		
Gonzalez-Soto et al., 2019	🟩		🟢	🟩	🟩		
Gonzalez-Soto et al., 2022	🟧	🟧	🟧	🟧	🟧		
Green et al., 2017				🟩			🟢
Green et al., 2019	🟩					🟩	
Gu et al., 2020	🟧			🟧			
Hamm and Lenz, 2021	🟧			🟧	🟠	🟧	
Hamm et al., 2022				🟢	🟢	🟢	
Han et al., 2021	🟨	🟨					
Hariharan et al., 2021	🟧		🟧	🟧		🟧	
Harris and Carrington, 2020				🟧			
Harris et al., 2021				🟧			
Huang et al., 2021	🟧						
Huang et al., 2022	🟧	🟧		🟧		🟧	
Jang et al., 2022	🟩	🟩		🟢			
Jaouani et al., 2023	🟧						
Joyce and Falkenberg, 2022				🟠	🟠		
Katsumiti et al., 2021	🟩	🟩					
Khalid et al., 2021	🟩	🟢			🟢		
Kolaveric et al., 2023	🟧			🟠			
Laubscher et al., 2023				🟧			
Leroux et al., 2022	🟨						
Li et al., 2020a			🟧		🟠		
Li et al., 2020b	🟠	🟠					
Lu et al., 2023	🟨		🟨				
Magara et al., 2018	🟧						
Magara et al., 2019	🟧						
Masia et al., 2021	🟧				🟧		
Nunes et al., 2020	🟧						
O'Brien et al., 2021	🟧						
Opitz et al., 2021				🟠	🟠		
Paul-Pont et al., 2016	🟧	🟧	🟧				
Pavicic-Hamer et al., 2022	🟧	🟧			🟧		

Phothakwanpracha et al., 2021					🟧		
Pittura et al., 2018	🟧	🟧					
Pittura et al., 2022	🟧	🟧					
Provenza et al., 2020	🟠						
Provenza et al., 2022	🟧						
Provenza et al., 2023	🟧				🟧		
Qi et al., 2023	🟩		🟩				
Revel et al., 2019	🟧		🟠	🟠	🟠		
Rist et al., 2016				🟧	🟧	🟧	
Rist et al., 2019					🟩		
Rodrigues et al., 2022	🟧					🟧	
Roman et al., 2023	🟩	🟩					
Romdhani et al., 2022	🟧	🟧					
Santana et al., 2018	🟢	🟢		🟩	🟢		
Sendra et al., 2020a	🟩	🟩					
Sendra et al., 2020b	🟩	🟩					
Seuront et al., 2021						🟦	
Shang et al., 2021a	🟧				🟧	🟧	
Shang et al., 2021b	🟧						
Silva et al., 2016					🟦		
Sui et al., 2022				🟧			
Sui et al., 2023				🟧			
Sun et al., 2023	🟧	🟧					
Tang et al., 2022	🟧	🟧		🟧			
Trestrail et al., 2021	🟧						
Uguen et al., 2022						🟦	
Van Cauwenberghe et al., 2015	🟧						
von Hellfeld et al., 2022	🟠	🟠	🟠				
von Moos et al., 2012	🟠	🟧	🟧		🟠		
Wang et al., 2020	🟧						
Wang et al., 2021	🟩			🟩			
Wang et al., 2022	🟨	🟨		🟨			
Wang et al., 2023a	🟨						
Wang et al., 2023b	🟩		🟩				
Webb et al., 2020				🟠		🟧	
Wegner et al., 2012				🟨		🟨	
Wei et al., 2021	🟧						
Yang et al., 2021			🟠				
Yap et al., 2020				🟠	🟠	🟠	
Yu et al., 2022	🟧			🟧			

Among the 106 studies we examined in the present work, the vast majority (91%) found plastic-induced effects on at least one mussel organisational level. Notably, microplastics have been the most extensively studied in relation to mussels, although nanoplastics and leachates have shown more pronounced effects on these organisms (Figure 21). The effects found following the addition of pollutants to plastics (see Section 3.2.4; *e.g.* benzo(a)pyrene, fluoranthene, cadmium), their combined effects with plastic particles and effects alone are not considered in this review.

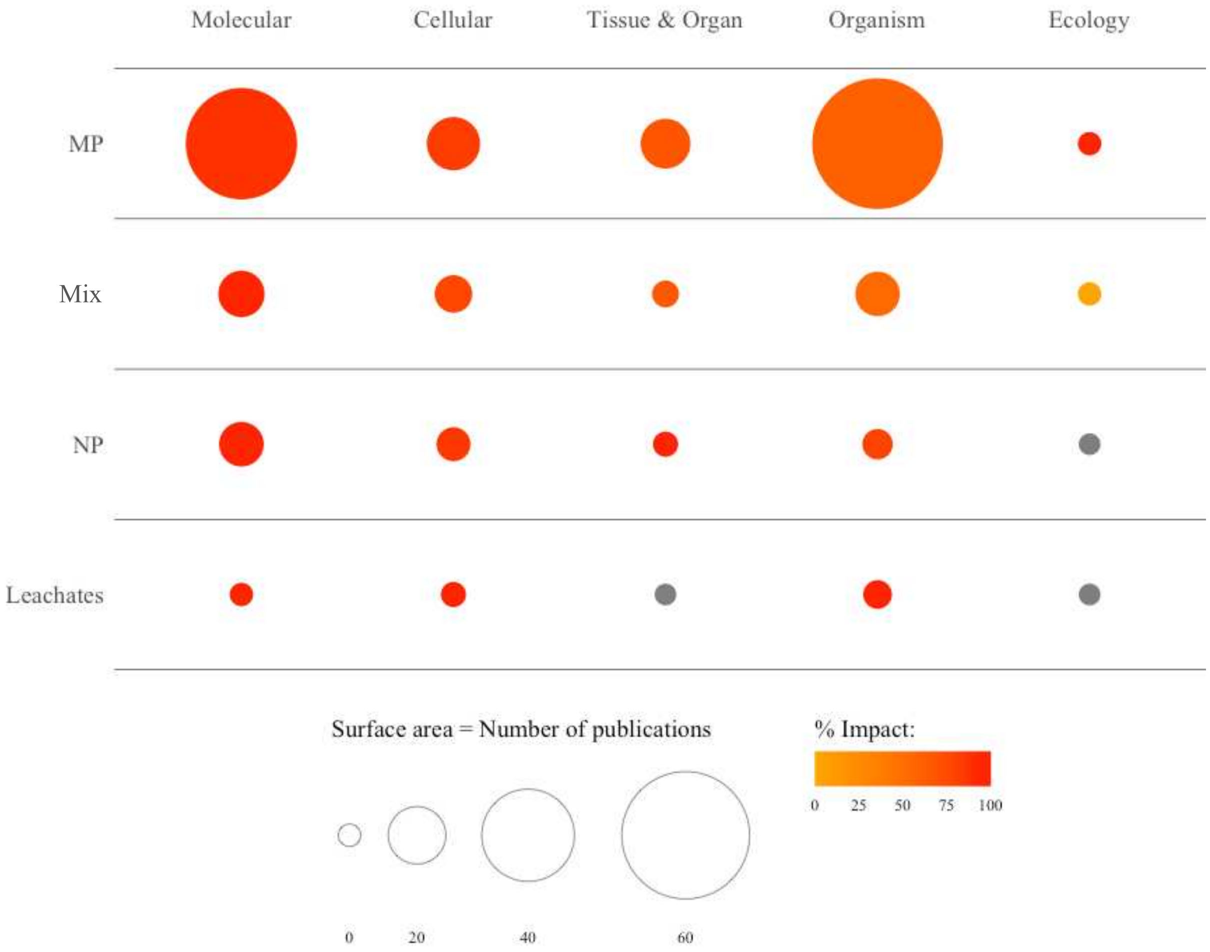


Figure 21: Research has been conducted across various organisational levels, and the proportion (%) of studies as well as the number of publications varies according to the plastic type studied, *i.e.* microplastic (MP), nanoplastic (NP), a combination of multiple treatments (Mix; *e.g.* MP + NP) and plastic leachates.

4.1. Small-scale impacts

The small-scale effects of plastics range from molecular alterations to tissue and organ impairments. Molecular impact was the most studied of the small-scale effects (58.1%), followed by cellular impacts (26.5%) and tissue and organ impacts (15.4%). Among the actual number of papers that focused on small-scale effects, 92% found an effect. These effects were predominantly observed at the molecular level (91%; 72 out of 79 papers), followed by the cellular level (83%; 30 out of 36 papers) and finally in tissues and organs (71%; 15 out of 21 papers). The molecular level is often the first level affected by toxicants, leading to disruptions in DNA, gene expression, enzymatic activities, oxidative stress and biochemical content (see Section 4.1.1 for more details). The cellular level may be affected through changes in organelles, haemocytes, and directly in cell viability (see Section 4.1.2 for more details). Finally, these alterations may subsequently extend to the tissue and organ levels, with histopathological effects on different tissues and organs, and disrupt mussel microbiota (see Section 4.1.3 for more details). The effects and studies are detailed in the sections below.

4.1.1. Molecular impact

DNA damage and changes in gene expression

Genotoxicity was investigated in 21 articles, with 17 of them reporting significant effects. DNA damage, *i.e.* DNA fragmentation and the presence of micronuclei increase following exposure to MPs (Alnajar et al., 2021; Avio et al., 2015; Choi et al., 2021, 2022; González-Soto et al., 2019, 2022a; Katsumiti et al., 2021; Kolarević et al., 2023; Masia et al., 2021; Revel et al., 2019; Roman et al., 2023; Romdhani et al., 2022) and NPs (Brandts et al., 2018; Chelomin et al., 2022; Dovzhenko et al., 2022; Gonçalves et al., 2022; González-Soto et al., 2019; Jang et al., 2021; Katsumiti et al., 2021; Roman et al., 2023), although exceptions exist (Cole et al., 2020; Pittura et al., 2022, 2018; Santana et al., 2018). MPs and NPs also induce changes in DNA content (Sendra et al., 2020b) and several changes in gene expression in mussels. Thus, among the 22 studies that determined gene expression, 18 found alterations following exposure to MPs (Bandini et al., 2021; Détrée & Gallardo-Escárate, 2017, 2018; Franzellitti et al., 2019; Huang et al., 2022b; Jaouani et al., 2023; Paul-Pont et al., 2016; Pittura et al., 2018; Qi et al., 2023; Romdhani et al., 2022; Shang et al., 2021a; Sun et al., 2023; Tang et al., 2022; Wang et al., 2023a; Yu et al., 2022) and NPs (Brandts et al., 2018; Han et al., 2021; Lu et al., 2023; Qi et al., 2023). This encompassed a wide variety of genes, such as those linked to oxidative stress responses, immune system function, apoptosis regulation, byssus protein production, shell matrix formation, and various metabolic pathways. However, some studies did not find that

MPs (Li et al., 2020a; O'Brien et al., 2021; Revel et al., 2019; Sendra et al., 2020a), NPs (Sendra et al., 2020a) or plastic leachates (Wang et al., 2023a) affect mussel gene expression.

Change in enzymatic activity

A wide range of enzymes involved in various physiological processes, such as digestion, neurotransmission, metabolism, detoxification, and antioxidant defense, were significantly influenced by plastic exposure. The activities of digestive enzymes (*i.e.* amylase, cellulase, lipase, protease, trypsin and pepsin) were altered by MPs (O'Brien et al., 2021; Trestrail et al., 2021; Wang et al., 2020) and NPs (Wang et al., 2023b), with two exceptions (Fernández et al., 2022; Wang et al., 2021). Following exposure to MPs (Avio et al., 2015; Choi et al., 2022, 2021; Pittura et al., 2022), NPs (Brandts et al., 2018; Capolupo et al., 2021c) and plastic leachates (Capolupo et al., 2021a), neurotoxic effects were also induced, such as changes in the activity of the neurotransmitter enzyme cholinesterase. However, seven studies reported no plastic-induced effects on this enzyme (Capolupo et al., 2021b; Khalid et al., 2021; Kolarević et al., 2023; Pavičić-Hamer et al., 2022; Pittura et al., 2018; Provenza et al., 2020; Rodrigues et al., 2022). Metabolism-associated enzymes, *i.e.* lactate and isocitrate dehydrogenase, acid and alkaline phosphatase, esterase, aspartate aminotransferase, alanine transaminase and acyl-CoA oxidase were also affected by MPs (González-Soto et al., 2022b; Gu et al., 2020; Huang et al., 2021; Katsumiti et al., 2021; Pittura et al., 2022; Revel et al., 2019; Roman et al., 2023) and NPs (Brandts et al., 2018; Katsumiti et al., 2021; Roman et al., 2023), with the exception of six out of the fourteen studies (Avio et al., 2015; Huang et al., 2022b; Khalid et al., 2021; Pittura et al., 2018; Rodrigues et al., 2022; von Hellfeld et al., 2022). Moreover, plastic exposure led to changes in cellular defence mechanisms. Activity alterations were observed in antimicrobial enzymes, mainly lysosomes, following exposure to MP (Capolupo et al., 2021c; Fernández et al., 2022; Wang et al., 2020) and NP (Auguste et al., 2020, 2021; Canesi et al., 2015; Capolupo et al., 2021c). Plastic exposure also affects xenobiotic detoxification enzymes (Franzellitti et al., 2019) and antioxidant enzyme production (see the next part on oxidative stress).

In addition to enzyme-related effects, plastic exposure led to an increase in heat shock proteins (HSP70; Huang et al., 2021). Plastics also disrupted the regulation of signaling proteins, including p38 MAPK and PKC (Canesi et al., 2016), and resulted in a decrease in the production of male and female hormones, namely, testosterone and oestradiol, respectively (Choi et al., 2022).

Oxidative stress

Oxidative stress, the most studied parameter, was examined in 56 studies, with 43 showing an impact of plastics. Reactive oxygen species (ROS) generation was quantified in 17 studies, in 15 of them the production of ROS was altered (mainly through an increase) when exposed to MPs (Choi et al., 2021; Gu et al., 2020; Huang et al., 2022b; Paul-Pont et al., 2016; Qi et al., 2023; Sendra et al., 2020b; Sun et al., 2023; Y. Wang et al., 2023a) or NPs (Auguste et al., 2020, 2021; Canesi et al., 2015; Han et al., 2021; Qi et al., 2023; Sendra et al., 2020a, 2020b; Wang et al., 2022). Nonetheless, four studies did not detect any significant alterations in ROS content after exposure to MPs (Katsumiti et al., 2021; Revel et al., 2019; Sendra et al., 2020a), NPs (Katsumiti et al., 2021), or plastic leachates (Wang et al., 2023a).

To counteract the potential damage caused by ROS, the cells produce antioxidants. The content of antioxidant enzymes, *i.e.* catalase, superoxide dismutase, peroxidase, glutathione S-transferase, glutathione peroxidase and/or glutathione reductase, was affected by MPs (Abidli et al., 2021; Avio et al., 2015; Capolupo et al., 2021b, 2021c; Choi et al., 2022; Cole et al., 2020; González-Soto et al., 2022a; Hamm & Lenz, 2021; Hariharan et al., 2021; Magara et al., 2018, 2019; Nunes et al., 2020; Paul-Pont et al., 2016; Pavičić-Hamer et al., 2022; Provenza et al., 2022, 2023; Revel et al., 2019; Sun et al., 2023; Wang et al., 2020, 2021; Wei et al., 2021), NPs (Capolupo et al., 2021c; Cole et al., 2020; Gonçalves et al., 2022; Han et al., 2021; Wang et al., 2022; Wang et al., 2023b) and plastic leachates (Capolupo et al., 2021a). Nonenzymatic antioxidant molecule content, such as glutathione, was also modified by exposure to MPs (Abidli et al., 2021; Avio et al., 2015; Capolupo et al., 2021b, 2021c; Choi et al., 2022; Cole et al., 2020; González-Soto et al., 2022; Hamm & Lenz, 2021; Hariharan et al., 2021; Magara et al., 2018, 2019; Nunes et al., 2020; Paul-Pont et al., 2016; Pavičić-Hamer et al., 2022; Provenza et al., 2022, 2023; Revel et al., 2019; Sun et al., 2023; Wang et al., 2020, 2021; Wei et al., 2021) and NPs (Qi et al., 2023). Out of the 40 articles that examined the antioxidant system, only 12 reported no observed effects caused by plastic (Fernández et al., 2022; Gonçalves et al., 2019; González-Soto et al., 2019; Jang et al., 2021; Khalid et al., 2021; Kolarević et al., 2023; Pittura et al., 2018, 2022; Provenza et al., 2020; Rodrigues et al., 2022; von Hellfeld et al., 2022; Wang et al., 2023b). In addition, the total antioxidant capacity was impaired after exposure to MPs (Pittura et al., 2022; Qi et al., 2023) and NPs (Brandts et al., 2018; Dovzhenko et al., 2022; Qi et al., 2023), although there are exceptions (Avio et al., 2015; Browne et al., 2013; Jang et al., 2021; Pittura et al., 2018). However, several methods were used (*e.g.* ferric reducing antioxidant potential, total oxyradical scavenging capacity towards peroxyl and hydroxyl) and a universal protocol for estimating total antioxidant capacity is lacking (Flieger et al., 2021).

If the antioxidant defense system cannot adequately counterbalance ROS production, oxidative stress may occur. Oxidative stress induces cellular damage, such as oxidative modifications of lipids, proteins, and DNA. Notably, lipid peroxidation (LPO) was altered by MPs (Abidli et al., 2021; Capolupo et al., 2021c; Gu et al., 2020; Hariharan et al., 2021; Huang et al., 2021; Paul-Pont et al., 2016; Provenza et al., 2022, 2023; Sun et al., 2023; Tang et al., 2022; Wang et al., 2021), NPs (Brandts et al., 2018; Capolupo et al., 2021c; Cole et al., 2020; Gonçalves et al., 2022; Han et al., 2021; Wang et al., 2021, 2022, 2023), and plastic leachates (Capolupo et al., 2021a). Of the 28 studies examining lipid peroxidation, ten reported no discernable plastic-induced effects (Avio et al., 2015; Dovzhenko et al., 2022; Ferguson et al., 2022; Gonçalves et al., 2019; Hamm & Lenz, 2021; Pittura et al., 2018; Provenza et al., 2020; Qi et al., 2023; Rodrigues et al., 2022; Santana et al., 2018). Although lipofuscin and protein carbonylation are less extensively studied than LPO, the accumulation of these compounds was associated with oxidative stress. Increased levels of lipofuscin content in the cells were measured after exposure to MPs (Avio et al., 2015; Capolupo et al., 2021b, 2021c; Ferguson et al., 2022; Pittura et al., 2022), NPs (Capolupo et al., 2021c) and plastic leachates (Capolupo et al., 2020), although von Moos et al. (2012) did not find any effect on this parameter. Changes in protein carbonyl concentrations were also observed following NP and MP exposure (Dovzhenko et al., 2022; Rodrigues et al., 2022, respectively).

Other changes in biochemical content and cellular budgets

Biomolecule content is affected by plastics, which may lead to cellular energy budget impairment. All metabolomics studies revealed the effects of plastics. Numerous changes in metabolites, *e.g.* amino acids, carbohydrates, and nucleotides, were observed following exposure to MPs (Cappello et al., 2021; Fernández et al., 2022; Huang et al., 2021; Khalid et al., 2021; Shang et al., 2021b; Wei et al., 2021) and NPs (Khalid et al., 2021; Leroux et al., 2022). Neutral lipid content was assessed in 13 studies, among which six recorded an alteration (mainly through an increase), *i.e.* by MPs (Capolupo et al., 2021b, c; Ciocan et al., 2020; Pittura et al., 2022; Shang et al., 2021b), NPs (Capolupo et al., 2021c) and plastic leachates (Capolupo et al., 2021a). MPs did not impair neutral lipid content in the remaining studies (Avio et al., 2015; Fernández et al., 2022; Nunes et al., 2020; Pittura et al., 2018; Rodrigues et al., 2022; Van Cauwenberghe et al., 2015; von Moos et al., 2012). Other changes, such as protein abundance and sugar content, were also identified (Green et al., 2019; Shang et al., 2021b). A decrease in cellular energy allocation by MPs was found in two studies (Shang et al., 2021b; Yu et al., 2022), although no effect was found in two others (Rodrigues et al., 2022; Van

Cauwenberghe et al., 2015). These changes in the cellular energy budget may compromise vital cellular processes and overall organism health and performance.

4.1.2. Cellular impact

Organelle impact

Among the effects reported at the cellular level, lysosomal membrane stability (LMS) was the most extensively studied effect (22 studies; 13 found an effect). While 9 studies did not observe any impact (Auguste et al., 2020; Cole et al., 2020; Dovzhenko et al., 2022; Jang et al., 2021; Li et al., 2020a; Pavičić-Hamer et al., 2022; Pittura et al., 2018; Santana et al., 2018; von Hellfeld et al., 2022), a decrease in LMS was reported following exposure to MPs (Avio et al., 2015; Capolupo et al., 2021b, 2021c; Pittura et al., 2022; Romdhani et al., 2022; Sun et al., 2023), NPs (Auguste et al., 2021; Canesi et al., 2016, 2015; Capolupo et al., 2021c), both MPs and NPs (Khalid et al., 2021) and plastic leachates (Capolupo et al., 2020, 2021a). Other impacts on lysosomes were observed, *e.g.* changes in lysosome abundance (Sendra et al., 2020b) and increases in the lysosome–cytoplasm volume ratio (Capolupo et al., 2021a, 2021c), as well as impacts on other organelles, *e.g.* damage to the rough endoplasmic reticulum and mitochondria (Sendra et al., 2020b; Tang et al., 2022).

Haemocyte impairment

Alteration of cell phagocytic activity was examined in 19 studies, and 15 studies reported an effect. Most studies found a decrease in mussel cell phagocytic activity upon exposure to MPs (Capolupo et al., 2021c; Huang et al., 2022b; Jang et al., 2021; Pittura et al., 2022; Sendra et al., 2020a,b; Sun et al., 2023) and NPs (Auguste et al., 2020, 2021; Canesi et al., 2015, 2016; Han et al., 2021; Jang et al., 2021; Sendra et al., 2020a; Wang et al., 2022). The remaining studies found a more nuanced response, with some indicating an increase (Katsumiti et al., 2021; Pittura et al., 2018) or no significant alteration in the phagocytosis response (Avio et al., 2015; Browne et al., 2008; González-Soto et al., 2022a; Paul-Pont et al., 2016). Plastic also influences haemocyte motility (Sendra et al., 2020a). The decrease in haemocyte cytoskeleton actin filaments (F-actin) due to NP exposure (Han et al., 2021) may have contributed to these changes in motility and phagocytosis. Moreover, the granulocyte-hyalinocyte ratio (two distinct types of haemocytes) was found to decrease (Avio et al., 2015; Pittura et al., 2018), although there are exceptions (Cole et al., 2020; Pittura et al., 2022), which affected the number of cells involved in the phagocytic process.

Global cell viability

Cell morphology was altered by MPs, NPs and plastic leachates, leading to changes such as reduced membrane integrity, cell vacuolisation or loss of filopodia (Canesi et al., 2016; Katsumiti et al., 2021; Pavičić-Hamer et al., 2022; Sendra et al., 2020b). All these alterations might have implications for cell survival. MPs and NPs were found to increase the number of apoptotic and necrotic cells (Han et al., 2021; Huang et al., 2022b; Katsumiti et al., 2021; Paul-Pont et al., 2016; Pavičić-Hamer et al., 2022; Roman et al., 2023; Sendra et al., 2020b), decrease cell viability (Canesi et al., 2015; Choi et al., 2021, 2022; Sendra et al., 2020b) and cause a shift in haemocyte number (Ferguson et al., 2022; Han et al., 2021; Huang et al., 2022b; Pavičić-Hamer et al., 2022).

Notably, all these cellular effects were modulated by various parameters, such as the polymer type (Avio et al., 2015; Capolupo et al., 2020), concentration (Auguste et al., 2021; Canesi et al., 2015, 2016; Huang et al., 2022b; Katsumiti et al., 2021; Pavičić-Hamer et al., 2022), particle size (Auguste et al., 2021; Capolupo et al., 2021c; Choi et al., 2021; Cole et al., 2020; Jang et al., 2021), exposure duration (Auguste et al., 2021; Pavičić-Hamer et al., 2022; von Moos et al., 2012), or the type of cell studied (Sendra et al., 2020b).

4.1.3. Tissue and organ impact

Histopathological changes

Mussel histopathology was assessed in 17 studies, among which 12 found significant changes. The gills, the digestive gland and other tissues from the digestive system (*e.g.* stomach and gut) were the most extensively studied tissues. Significant abnormalities in these tissues, *e.g.* hypoplasia, hypertrophy, atrophy, inflammation, haemocyte infiltration, ceroids and necrosis, were found after exposure to MP (Alnajar et al., 2021; Bråte et al., 2018; Choi et al., 2022; Ciocan et al., 2020; Ferguson et al., 2022; González-Soto et al., 2022b; Hariharan et al., 2021; Paul-Pont et al., 2016; Qi et al., 2023; von Moos et al., 2012; Wang et al., 2023a) and NP (Lu et al., 2023; Qi et al., 2023). Abnormalities generally increase with plastic concentration (Alnajar et al., 2021) and were more severe in the presence of NPs than MPs (Qi et al., 2023). Interestingly, no tissue recovery was observed after one week of depuration (Hariharan et al., 2021). Although less studied, exposure to MPs also led to histological alterations in other tissues, such as the foot and adductor muscles (Hariharan et al., 2021). Gonadal inflammation of both sexes and oocyte degeneration were also found after plastic particle exposure (Choi et al., 2022; Ciocan et al., 2020; González-Soto et al., 2022a; Paul-Pont et al., 2016). No histopathological anomalies were found in the remaining studies following exposure to MPs, NPs, a mix of both or plastic leachates (Fernández et al., 2022; Gonçalves et al., 2019; González-Soto et al., 2019; Revel et al., 2019; von Hellfeld et al., 2022; Wang et al., 2023a).

Changes in microbiota composition

There has been a recent and growing interest in studying microbiota due to the essential role played by these complex microbial communities in maintaining tissue integrity and contributing to the overall health of their host. As a relatively new research field, the impact of plastic on mussel microbiota has only been investigated by five studies. Of these, four studies found significant effects of plastics. The gill (Bandini et al., 2021), digestive gland (Ferguson et al., 2022) and gut (Li et al., 2020a) microbiota compositions were altered by MP exposure. NPs also affected microbiota composition in mussel haemolymph (Auguste et al., 2020), predominantly towards a reduction in microbial abundance and diversity. Notably, the severity of the impact was enhanced by higher plastic concentrations, longer experiment durations, and the presence of biofilms on plastic particles (Bandini et al., 2021; Ferguson, 2022; Li et al., 2020a). Interestingly, an 8-day depuration period was insufficient to restore the mussel microbiota after plastic exposure (Li et al., 2020a). Although one study (Yang et al., 2021) found that MPs did not significantly affect the mussel gut microbiota composition, it is noteworthy that plastic pollution may impact the overall microbiome; the observed alterations in microbial communities indicate the potential for broader ecological consequences.

4.2. Organism-level impacts

At the organism level, the impacts of plastic were categorised through their effects on (i) functional traits (41.7%), (ii) physiological traits (41.7%), and (iii) behavioural traits (16.6%). Functional traits encompassed parameters related to growth, survival, and reproduction. Within the reproduction category, we included the effects of different organisational levels when they were exerted on mussels at early-life stages. Physiological endpoints comprised filtration, respiration, adsorption, excretion, scope for growth, heart rate, and shell regeneration. Finally, the behavioural traits category included byssus production, aggregation, displacement, valve movement and anti-predator behaviours. Plastics were found to affect mussels at the organism level in 73% of the studies. Interestingly, the less frequently studied category, *i.e.* mussel behavioural traits, occurred the most in papers identifying a significant impact of plastics (77%), followed by physiological traits (66%) and functional traits (54%). The effects and studies are detailed in the sections below.

4.2.1. Functional traits

Growth and condition index

The effect of plastics on the mussel growth rate, which is typically estimated from length, width, or weight, was assessed in seven studies. With the exception of one study, in which a decrease in mussel growth rate was observed following exposure to MPs (Détrée & Gallardo-Escárate, 2018), no effect was ever reported after exposure to MPs (Choi et al., 2022; Christoforou et al., 2020; Foekema et al., 2022; Hamm & Lenz, 2021; Opitz et al., 2021) or to a combination of MPs and NPs (Santana et al., 2018). To the best of our knowledge, there are no studies on the impact of NPs or plastic leachates on mussel growth. Larval growth was also altered by plastic; see Section '*Reproduction and larval development*' for further details.

Based on mussel morphological parameters, the mussel condition index was assessed in 18 studies, and five studies identified a significant impact of plastics. Specifically, exposure to MP led to a decrease in the condition index (Barkhau et al., 2022; Masiá et al., 2021; Pavičić-Hamer et al., 2022; Shang et al., 2021a), except for one study, in which an increase was observed (González-Soto et al., 2019). The response to plastic exposure is complex and influenced by several factors, such as the mussel feeding regime (Shang et al., 2021a), plastic size (González-Soto et al., 2019; Pavičić-Hamer et al., 2022), its concentration (Pavičić-Hamer et al., 2022) and its polymer composition (Barkhau et al., 2022), *e.g.* the condition index was impacted by PVC but not PMMA particles (Barkhau et al., 2022). In addition, differences in research methodologies (*e.g.* used of dry weight, ash-free dry weight, mussel shell length) may contribute to the diverse findings reported in the literature. No significant effects on the condition index were detected in the remaining studies, regardless of whether the exposure involved MP (Bråte et al., 2018; Choi et al., 2021; González-Soto et al., 2022a; Hamm et al., 2022; Hamm & Lenz, 2021; Joyce & Falkenberg, 2022; Li et al., 2020b; Revel et al., 2019; von Moos et al., 2012; Yap et al., 2020), NP (Gonçalves et al., 2022), or a combination of both (Hamm et al., 2022; Khalid et al., 2021; Santana et al., 2018).

Survival rate

The survival rate following plastic exposure was investigated in 12 papers, among which four studies found an effect. MPs were found to decrease the mussel survival rate in two species, the green mussel *Perna viridis* (Phothakwanpracha et al., 2021; Rist et al., 2016) and the Mediterranean mussel *Mytilus galloprovincialis* (Pavičić-Hamer et al., 2022; Provenza et al., 2023). Mussel mortality was influenced by several factors, including polymer, colour and size as well as plastic concentration, duration of the experiment, presence or absence of food and the site of mussel collection (Pavičić-Hamer et al., 2022; Phothakwanpracha et al., 2021; Provenza et al., 2023; Rist et al., 2016). Compared to a lack of mortality in control experiments, *M. galloprovincialis* reached a mortality maximum of 66.7% and 63.3% in unfed and fed conditions, respectively, when exposed to 5.0 10^4 µg L^{-1} PE for 7 days (Provenza et al., 2023). Exposure to a concentration of 2.16 10^6 µg L^{-1} of PVC led to total mortality in *Perna perna* in the first *ca.* 50 days (Rist et al., 2016). Compared to control conditions in which no mortality occurred, small, medium and large MPs induced *ca.* 100% mortality in a 4-day experiment on *Perna viridis* at concentrations ranging from 66 to 1,333 MP L^{-1} for PP and at concentrations of 333 to 1,333 MP L^{-1} for PBS and PS (Phothakwanpracha et al., 2021). Finally, Pavičić-Hamer et al. (2022) assessed mussel mortality through a stress-on-stress experiment, showing that mortality occurred more quickly in mussels contaminated by PMMA (5 to 10 days earlier than in the control) depending on the concentration used (0.0001 to 0.01 µg L^{-1}) and size of the particles used. For the remaining 8 papers, no effect of MPs (Abidli et al., 2021; Barkhau et al., 2022; Choi et al., 2022; Foekema et al., 2022; Joyce & Falkenberg, 2022; Santana et al., 2018; Yap et al., 2020) or NP (Auguste et al., 2020) on mussel survival was recorded. No studies investigated the impact of plastic leachates on mussel survival. Mussel early-life stage survival was also affected; see Section '*Reproductive success and larval development*' for further details.

Reproductive success and larval development

Mussels are broadcast spawners and gonochoric species, in which fertilisation and larval development occur in the water column (pelagic stage) until the veliger larvae settle to the benthic hard substrates at the pediveliger stage. Metamorphosis is subsequently completed and the larvae reach the juvenile stage, starting their benthic life (Bayne, 1976). Ten scientific articles investigated parameters related to reproductive success, *e.g.* fertilisation rate, embryo and larval survival rates, or reproductive system impairment of adult mussels. All these papers showed that plastics affect at least one of these parameters.

The mussel fertilisation rate was investigated in MP and leachate exposures but not under NP exposure. While there was no observed effect of polystyrene (PS) MP particles on the *Mytilus galloprovincialis* fertilisation rate (Capolupo et al., 2021b), leachates (no particles) from all the tested polymers (PS, PP, PET, PVC, rubber) were found to induce a decrease in this parameter (Capolupo et al., 2020). In contrast, mussel embryos and larvae were consistently impacted by MPs, NPs and leachates. Larval motility was only analysed under plastic leachate exposure, revealing a polymer-dependent impact. A decrease in larval motility was found with rubber and PVC leachates but not PS, PP, or PET leachates (Capolupo et al., 2020). Larval development was impaired (*i.e.* developmental delay, shell formation, malformation and/or mortality) by both plastic leachates (Capolupo et al., 2020; Gandara e Silva et al., 2016) and NPs (Auguste et al., 2021; Rist et al., 2019). Except in one study (Rist et al., 2019), MPs did not impact mussel larval development (Capolupo et al., 2018, 2021b) or larval food consumption (Capolupo et al., 2018). While MPs did not greatly impact the mussel early life stages at the individual level, they exhibited several effects at lower-scale levels. MPs may impact mussel cellular defense mechanisms at early life stages, as they induce a change in the multixenobiotic resistance system (Franzellitti et al., 2019). Changes in the expression of genes involved in shell biogenesis, immunomodulation, neuroendocrine signalling, and antioxidant and lysosomal responses were shown after exposure to MPs; see Capolupo et al. (2018) for more details. Other lower-scale effects, as a decrease in lysosomal membrane stability was found when exposed to plastic leachates (Capolupo et al., 2020). These sublethal processes may weaken the early life stages of mussels and potentially compromise their survival and ability to reach the juvenile stage, which coincides with the settlement stage.

As discussed previously, in the context of exposure duration (Section 3.3.2), knowledge of the impact of plastics on mussel reproductive output following parental exposure remains limited. MPs can affect gonads, gametes and sex hormones, *i.e.* oestradiol and testosterone, of adult mussels (Choi et al., 2022; Ciocan et al., 2020; González-Soto et al., 2022b). Thus, mussel reproductive success is affected by plastic, which directly affects mussel offspring from gametes to larvae and the adult reproductive system, potentially impairing mussel recruitment on a broader scale.

4.2.2. Physiological traits

The impact of plastic on physiological traits was assessed in 35 studies through several parameters, such as filtration, respiration, absorption, excretion, scope for growth, heart rate and shell regeneration. Among these studies, 66% found an effect in at least one parameter, with the magnitude of the effects being dependent on several factors, such as MP properties (*e.g.* polymer, size) and exposure mode (*e.g.* concentration and duration). However, the research effort is uneven, as most studies focused on MPs, only a few addressed the potential impact of NPs (2 papers), and none investigated the impact of plastic leachates on these parameters. As such, increased research efforts in these plastic categories would be advisable, as demonstrated in another bivalve, the oyster *Magallana gigas*, with the impact of plastic leachates on different physiological parameters (Tallec et al., 2022b).

Filtration rate

The impact of plastic on the mussel filtration rate has been extensively studied (29 papers), and almost 50% of the studies reported an effect (14 papers). Specifically, with the exception of one paper (Fraissinet et al., 2023) that reported an increase in mussel filtration rate with increasing MP concentrations, MPs consistently decreased filtration rates in 13 papers (Albidi et al., 2021; Christoforou, 2020; Gu et al., 2020; Hamm & Lenz, 2021; Hariharan et al., 2021; Harris & Carrignton, 2020; Harris et al., 2021; Huang et al., 2022b; Laubscher et al., 2023; Rist et al., 2016; Sui et al., 2022; 2023). The combined exposure of NPs and MPs led to a decrease in the mussel filtration rate in one study (Green et al., 2019) and no effect in two others (Hamm et al., 2022; Santana et al., 2018). The remaining papers (*n* = 16) reported no significant effect on the filtration rate of MPs (Alnajar et al., 2021; Barkhau et al., 2022; Browne et al., 2008; Fernandez et al., 2022; Gonzalez-Soto et al., 2019, 2022; Jang et al., 2022; Joyce & Falkenberg, 2022; Optiz et al., 2021; Revel et al., 2019; Tang et al., 2022; Wang et al., 2021; Webb et al., 2020) and NPs (Gonzalez-Soto et al., 2019; Jang et al., 2022; Wang et al., 2021). The disparities in the mussel filtration rates are epitomised by differences in experimental protocols, such as the polymer used and their concentrations. For instance, the mussel filtration rate was impacted by PVC microplastics but not by PS microplastics (Hamm & Lenz, 2021). In turn, the effect of PE microplastics on the mussel filtration rate was only significant at high concentrations (Harris et al., 2021). These observations further stress the need to diversify the range of polymers, concentrations, and experimental parameters examined; in addition, the impact of plastic leachates on this parameter should be emphasised, as this aspect has not been studied in marine mussels.

Respiration rate

The respiration rate was studied in 17 papers, and only 7 papers found that plastic affected this parameter. Oxygen consumption decreased in four studies after exposure to MPs (Rist et al., 2016; Huang et al., 2022b; Tang et al., 2022) or NPs (Wang et al., 2022). In contrast, mussel respiration was found to be enhanced in three other studies after exposure to MPs (Sui et al., 2022; 2023; Wang et al., 2021) and NPs (Wang et al., 2021). Respiration impairment is noticeably dependent on several factors, such as plastic concentration and experiment duration. For instance, the significant increase in respiration observed by Sui et al. (2022, 2023) between their control conditions and the highest plastic concentration (1,000 MP L^{-1}) only appeared during the first week of the experiment and remained stable afterwards. The remaining studies did not observe any significant impact on the respiration rate due to MP exposure (Barkhau et al., 2022; Fernandez et al., 2022; Gonzalez-Soto et al., 2019, 2022; Gu et al., 2020; Joyce & Falkenberg, 2022; Opitz et al., 2021; Webb et al., 2020; Yap et al., 2020), NPs (Gonzalez-Soto et al., 2019) or a combination of both (Hamm et al., 2022).

Absorption efficiency

The absorption efficiency of food organic content (based on the proportions of organic dry weights in food and in feces; Conover, 1966) was studied in 10 papers; among these, 6 papers found that plastic impacted this parameter. Specifically, absorption efficiency decreased in three studies following exposure to MPs (Gonzalez-Soto et al., 2022; Huang et al., 2022b) and NPs (Wang et al., 2021) but increased in three other studies following exposure to MPs (Sui et al., 2022, 2023) and NPs (Gonzalez-Soto et al., 2019). Among them, two studies compared the effects of MPs and NPs, and both showed that exposure to NPs led to a disruption in absorption efficiency (Gonzalez-Soto et al., 2019; Wang et al., 2021), highlighting the significant role of particle size as a crucial determining factor. The remaining studies did not observe any impact of plastic on the absorption efficiency, either after exposure to MPs (Fernandez et al., 2022; Gonzalez-Soto et al., 2019; Optiz et al., 2021; Tang et al., 2022; Wang et al., 2021) or a mixture of NPs and MPs (Santana et al., 2018).

Excretion

The excretion rate, measured through ammonia concentration in seawater, was assessed in five studies. Although one study did not observe any plastic-induced impact on excretion (Optiz, 2021), the others showed conflicting effects. Some studies found an increase in ammonia concentration in the presence of MP (Sui et al., 2022, 2023; Wang et al., 2021) or NP (Wang et al., 2021), while one study reported a decrease in ammonia concentration following MP exposure (Huang et al., 2022b). The effect of plastic on the excretion rate was not constant throughout the experiment and depended on the timing and concentration (*e.g.* Sui et al., 2022, 2023). An increase in the amount of pseudofaeces was also observed following exposure to MPs (Foekema et al., 2022), NPs (Wegner et al., 2012), and a mixture of both (Santana et al., 2018).

Scope for growth

The scope for growth (SFG)—the difference between the acquisition of energy by filtration and/or assimilation and the loss of energy by respiration and/or excretion to produce somatic tissue and gametes (Widdows & Jonson, 1988)—is estimated from the physiological rate data mentioned above and provides a proxy of the mussel overall condition and energy budget. However, SFG calculation methods vary among studies, potentially leading to discrepancies in reported values; for instance, SFG was estimated as SFG = Absorption - (Respiration + Excretion) in Wang et al. (2022) and as SFG = (Filtration × Absoption) – Respiration in Fernandez et al. (2022). Among the eight papers that estimated SFG, four found that plastic affects this parameter. Thus, a decrease in SFG was found following MP (Huang et al., 2022b; Wang et al., 2021) and NP exposures (Wang et al., 2021). In contrast, an increase in SFG was found following NP (González-Soto et al., 2019) and MP (Sui et al., 2023) exposures, although this effect was not consistently observed throughout the duration of the experiment. Size, concentration, and exposure duration were the factors that mainly impacted this parameter (González-Soto et al., 2019; Sui et al., 2023). The remaining studies did not observe any impact of MPs on the ability to grow (Fernández et al., 2022; González-Soto et al., 2019, 2022a; Opitz et al., 2021; Sui et al., 2022). More recently, the Dynamic Energy Budget model (DEB; Kooijman, 2010) has emerged as a powerful tool that surpasses SFG, providing holistic insights into an organism's energy dynamics and life cycle; promising results have been obtained, as shown for *M. edulis* (*e.g.* Larsen et al., 2014). Additionally, DEBtox, an extension of DEB, was designed to simulate the effects of toxic substances on an organism's physiology, energy allocation, and life cycle (Jager & Ashauer, 2018; Jager, 2019), making it a valuable tool for studying the environmental impact of plastics and other pollutants on organisms, such as mussels.

Others

Two other studies focused on other physiological parameters, *i.e.* mussel heart rate and mussel shell regeneration rate under MP exposure (Kolarević et al., 2022; Yu et al., 2022). When used in combination with fluoranthene, MPs act on the mussel heart rate, but MPs alone do not induce an effect (Kolarević et al., 2023). A shell study showed that the regeneration rate of mussel shell was significantly inhibited after an exposure to MPs (Yu et al., 2022). Notably, plastic also affects shell formation (the prodissoconch) of the veliger larvae during the larval stage; see Section '*Reproductive success and larval development*' above.

4.2.3. Behavioural traits

The effect of plastics on mussel behaviour was assessed in 14 studies, and 79% of studies found at least one effect among the following studied parameters: byssus production (12 papers), displacement (4 papers), aggregation (one paper), valve movement (one paper), and antipredator behaviour (one paper).

Byssus

The majority (75%) of studies that assessed the impact of plastic on byssal thread properties found a significant effect. Byssus (*i.e.* the proteinic filaments produced by the byssal gland at the base of the foot) production was consistently reduced after exposed to MPs (Hamm & Lenz, 2021; Hariharan et al., 2021; Huang et al., 2022b; Rist et al., 2016; Rodrigues et al., 2022; Webb et al., 2020) or a combination of NPs and MPs (Green et al., 2019). Other byssal properties, such as the adhesion strength, decreased after exposure to plastic particles (Green et al., 2019; Shang et al., 2021a). Only one study showed an increase in byssus production, and interestingly, this was the only study that examined the impact of plastic leachates (Seuront et al., 2021). Byssus properties were not affected by MPs (Barkhau et al., 2022; Yap et al., 2020) or a combination of MPs and NPs (Hamm et al., 2022) in the remaining studies. Notably, the plastic effects were dependent on the mussel species studied (Seuront et al., 2021), the plastic concentration (Hamm & Lenz, 2021) and the polymer used (Green et al., 2019; Hamm & Lenz, 2021), *e.g.* PE induced an effect in *Mytilus edulis* byssus properties, but not PLA (Green et al., 2019).

Other behavioural changes

A single study focused on mussel valve movements, revealing that valve opening decreases after exposure to NPs (*M.* edulis; Wegner et al., 2012). Movement behaviour has been studied more extensively, as four studies showed that mussel movement decreased after exposure to MP (Hariharan et al., 2021) and MP leachates (Seuront et al., 2021; Uguen et al., 2022). Specifically, Seuront et al. (2021) showed that MPs impact mussel locomotion and aggregation in a species-specific way (Seuront et al., 2021). *M. edulis* and *Choromytilus meridionalis* shared the same response, *i.e.* a decrease in locomotion and aggregation in MP leachate seawater, while this parameter remained unaffected in *M. galloprovincialis* and *Perna perna*. Finally, the mussel anti-predator response was also impaired by MP leachates, as they induced a loss in their negative chemotaxis to crab cues (Uguen et al., 2022).

4.3. Large-scale impacts and implications for mussel populations, communities and ecosystems

Mussels play a crucial role as ecosystem engineers, and the effect of plastic pollution may extend from the species to the biodiversity and functioning of the ecosystem they support.

Especially given the ability of mussel beds to trap microplastic particles, indeed, through their high filtration ability, their spatial arrangements and shell structure mussels enhance turbulence and plastic retention (Lim et al., 2020; Nicastro et al., 2022; Van Colen et al., 2021). However, only two studies have assessed the influence of plastic on mussel bed-associated communities (Foekema et al., 2022; Green et al., 2017). Interestingly, exposure to HDPE and PLA NPs and MPs did not result in significant effects on ecosystem functioning or the community assemblages of associated invertebrates (Green et al., 2017). This greatly conflicts with the same experiment conducted on oysters (*Ostrea edulis*), in which plastic exposure caused effects on their associated community assemblage (*e.g.* benthic cyanobacteria, polychaetes, oligochaetes; Green et al., 2017). Species diversity was also unaffected by exposure to PS microplastics, although an increase in the abundance of the benthic community was observed (Foekema et al., 2022).

These investigations were conducted in mesocosms, with study periods ranging from 50 to 56 days. Importantly, mesocosm studies, which offer a more realistic environment than laboratory settings, still oversimplify natural conditions; for example, the effect of wave actions is removed. While MPs alter byssus production (see Section 4.2.3), the potential influence of MPs on mussel dislodgment and the subsequent cascading effects at larger ecological scales may be underestimated when the wave action in this mesocosm is absent. Additionally, no top-down effects were measured in the mesocosm set-ups, oversimplifying a system without predators and potentially causing the effects of plastics on the mussel bed ecosystem to be underestimated. Finally, the impairment of mussel reproduction and early life stages may act on mussel recruitment and underscore the potential far-reaching consequences of plastic pollution on the population dynamics and resilience of these marine organisms and their ecosystems. Mesocosm studies are effective tools for incorporating multiple stressors and increasing the similarity of the results to those obtained under environmental conditions.

Considering the changing climate that is increasingly disrupting ecosystem structures and functions, multistress studies are very important in understanding the toxicity of plastic pollution on these keystone species in the environment.

5. Multistress experiment: the case of global warming

Environmental factors, such as temperature, may influence the toxicity of a pollutant by directly affecting the nature of the pollutant, its bioavailability, and/or the interplay of an *a priori* toxicity of a chemical compound and its actual effect, which depends on the target organisms and the thermal conditions; thus, its toxic effects either increase or decrease (Mohammed, 2013). Although plastic pollution and climate change are fundamentally linked (Ford et al., 2022), their combined effects have received limited attention; see also, *e.g.* Nicastro et al. (2023) for an illustration of the gaps between literature that has focused on climate change and plastic pollution. However, the potential interaction between these stressors fundamentally introduces an additional level of complexity and irregularity in how organisms and ecosystems respond. In this context, it is necessary to consider the effect of temperature in plastics research to improve our knowledge of the complex interactions that may arise between these two major global change stressors.

5.1. Temperature: a research gap in plastic studies

5.1.1. Heat stress in intertidal mussel research

Plastic pollution does not occur alone but fundamentally interacts with various other environmental stressors. Among them, global warming stands out as a critical factor that affects various biological systems. The rapid increase in greenhouse gases, which resulted from human activities since the Industrial Revolution, has led to a dramatic rise in CO_2 levels, triggering an increase in global temperature. This rapid change in climate also led to an increase in the frequency, severity and duration of extreme weather events, such as terrestrial and marine heatwaves (IPCC, 2022). For example, the frequency of marine heatwaves doubled between 1982 and 2016 and is expected to increase by a factor of 16 following a climate warming of 1.5 °C compared to preindustrial levels (Frölicher et al., 2018).

Mussels from intertidal coastal systems inhabit one of the most challenging and stressful habitats on the planet (Harley, 2008), as mussels must address challenging fluctuating conditions caused by immersion and emersion cycles (*e.g.* temperature, salinity, oxygen and food availability). During emersion, mussels experience temperatures close to those of substrates, which exhibit strong spatial and temporal variability (Seuront and Ng, 2016). For instance, mussels situated on rocky intertidal shores may face substratum temperatures of over 55 °C during low tide (Seuront and Ng, 2016). While the temperature experienced by the mussel at emersion can strongly fluctuate within a single tidal cycle, changes at immersion occur over longer time scales. However, both temperatures may occasionally exceed the tolerance range of mussels and cause mass mortality of mussels.

5.1.2. Emersion: an overlooked factor in temperature studies

The tidal cycle plays a role in climate change studies that focus on intertidal species and ecosystems. However, as recently stressed for the fraction of plastic research focusing on plastic leachates (Delaeter et al., 2022; Seuront et al., 2022), emersion represents only a tiny fraction of climate change research. To illustrate this, a Web of Science search (accessed on October 28, 2023) returned 2,745 articles using the keywords "*effect**", "*impact**", "*affect**", "*mussel**", "*temperature**" and "*heat**". However, when the keywords "*aerial**", "*emersion**", and "*air**" were added, only 235 articles emerged (Figure 22). Emersion thus represents less than 10% of the research effort on the impacts of climate warming on intertidal mussels. Despite the ever-growing research efforts devoted to clarifying the global impacts of temperature on mussels, research into the specific effects of emersion shows a more erratic trend (Figure 22).

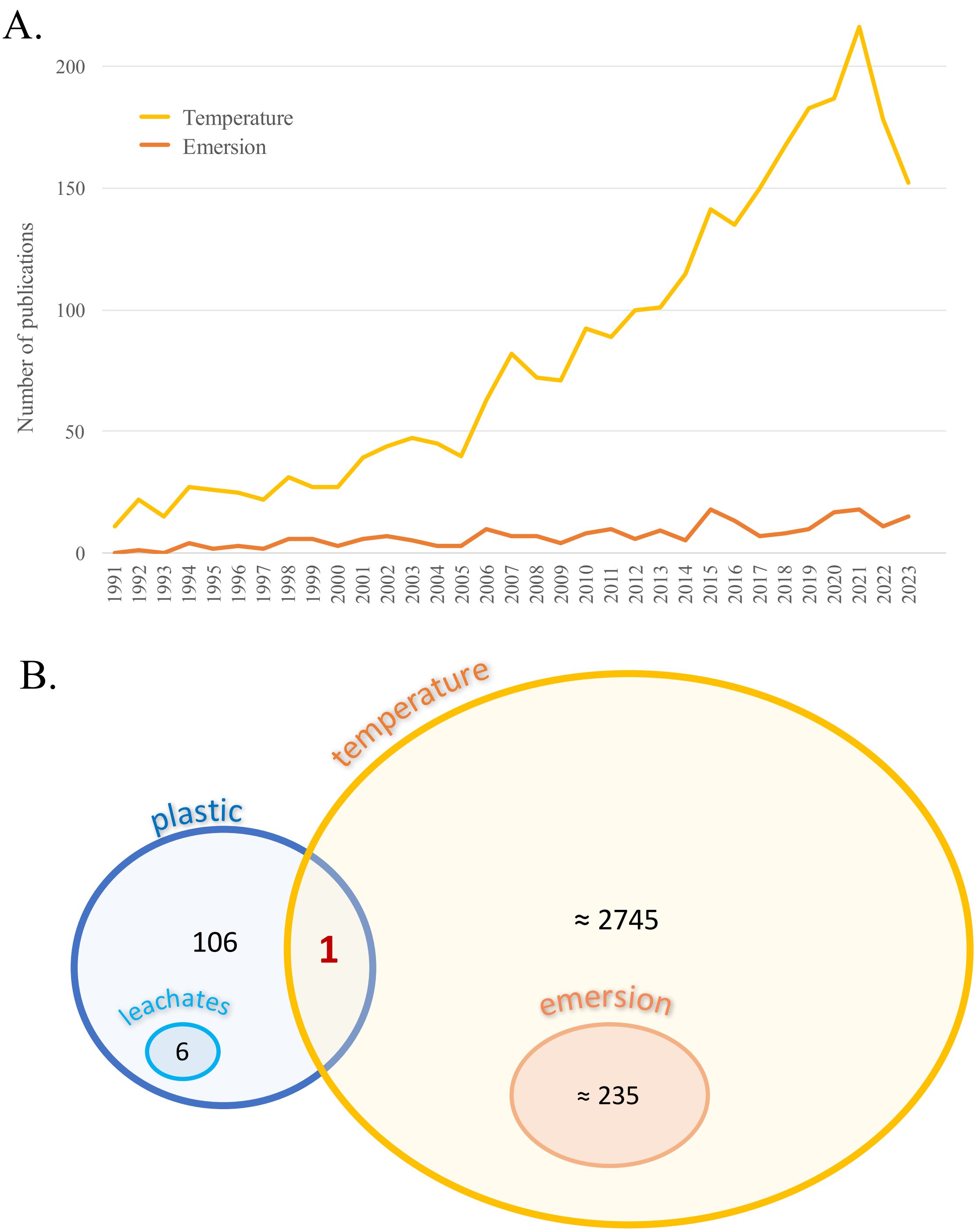


Figure 22: (A) Temporal trends in the total number of publications on the impact of temperature on mussels (light orange; keywords on Web of Science were "*effect**", "*impact**", "*affect**", "*mussel**", "*temperature**" and "*heat**") and specifically on the impact of emersion temperature on mussels (dark orange; keywords on Web of Sciences were "*effect**", "*impact**", "*affect**", "*mussel**", "*temperature**", "*heat**", "*aerial**", "*emersion**", and "*air**") between 1991 (first study) and October 28, 2023. (B) Distribution of the research effort, represented by the number of studies, in two marine mussel research fields, *i.e.* plastic impacts (blue) and temperature impacts (orange). The intersection area shows the studies that examined the combined effect of both factors, while the distinct sections highlight the lack of combined research between these research fields.

5.1.3. Temperature as an overlooked factor in plastic studies

Despite the significance and growing interest in plastic pollution and climate change, a notable research gap persists regarding their combined impact. Thus, among the marine mussel studies that examined the effects of plastic and temperature, only one paper addressed their combined effect (Pittura et al., 2022; Figure 22B). This study revealed the complexity of plastic and temperature interactions. It estimated a higher molecular and cellular impact with plastic following marine heat stress for one polymer (PES) and the opposite for another (PLA; Pittura et al., 2022). Thus, plastic and temperature interactions could yield unpredictable outcomes for organisms and ecosystems. As these two stressors potentially amplify each other's impact, expanding the research is crucial for obtaining comprehensive knowledge on the multifaceted interplay between these stressors and their potential implications. For instance, there is a gap in studies that explore the combined effects of NP or plastic leachates with temperature, as well as investigations into the effects of thermal stress during emersion. Moreover, an encompassing approach to research involving a wider variety of polymers, mussel species, endpoints, and temperatures is essential to fathom the effects of plastic in our changing world.

5.2. Plastic pollution in a warming world: a roadmap for future research

5.2.1. Temperature: a significant stressor

Mussels are ectothermic poikilothermic organisms, *i.e.* their temperature varies with the environmental temperature; thus, temperature is among the most important stress factors (Guschina & Harwood, 2006). Although mussel body temperature is close to that of the surrounding water, their body temperature can be significantly warmer than the surrounding air and substrate temperatures at low tide (10 to 17 °C and 5 to 14 °C warmer, respectively), as recorded by *M. edulis* biomimetic loggers (maximum biomimetic mussel body temperature = 41.7 °C; Seuront et al., 2019). In addition, mussels have a limited capacity of movement and, depending on the season, live close to the upper limit of their thermal tolerance window;

therefore, mussels are particularly susceptible to extreme weather conditions (Mislan & Wethey, 2015; Somero, 2002). Thermal stress can be buffered by behavioural responses, such as gaping behaviour or interaction with other individuals through aggregation (Lathlean et al., 2016; Nicastro et al., 2012). As the temperature is directly linked to metabolism, if the behavioural responses do not sufficiently limit the thermal stress, exposure to extreme heat events may induce subcellular damage and alterations in functional traits, such as growth, reproduction and survival; see Zippay & Helmuth (2012) for a review. In addition to this direct effect, temperature affects mussels indirectly through altering the feeding performances of their predators, *e.g.* sea stars and dogwhelks (Contolini et al., 2020; Pincebourde et al., 2008). Similar to plastic pollution, temperature affects mussels across all levels of organisation; as mussels are ecosystem engineers, temperature may affect the thermal, humid and protective microhabitats formed that other species depend upon (Jurgens et al., 2022).

Mussel sensitivity to thermal stress is dependent on several factors. First, sensitivity to thermal stress is species-dependent, *e.g. M. galloprovincialis* exhibits a higher tolerance temperature than that of *M. edulis* (Thomas & Bacher, 2018). This adaptation to thermal stress is transferred from female mussels to their offspring. For instance, *M. galloprovincialis-M. edulis* hybrid larvae from *M. galloprovincialis* females exhibit lower sensitivity to heat stress than those from *M. edulis* females (Mlouka et al., 2020). In addition to interspecific variability, sensitivity to thermal stress is influenced by intraspecific diversity and by the type of temperature exposure (*i.e.* temperature immersion or emersion), as shown recently with *Perna perna* mussels (Nicastro et al., 2023b). Heat stress exposure conditions (*e.g.* duration, acclimation) are also an important parameter to consider. For instance, exposure to marine heatwaves may affect mussel survival (*e.g.* Venter et al., 2023; although Ducker et al., 2023, did not find any effect), a response modulated by the temperature to which they have been acclimated (*e.g.* Brownlee, 2022). Although studies have demonstrated that mussels can adapt to aerial thermal stress (Moyen et al., 2020; Schneider, 2008), others found that multiple exposures to high aerial temperature greatly decreased the thermal tolerance of mussels (Jones et al., 2009; Seuront et al., 2019; Sorte et al., 2018). The mussel response to aerial heat stress is also typically modulated by emersion time (Seuront et al., 2019), exposure month (Jones et al., 2009; Sorte et al., 2018), relative humidity (Delorme et al., 2021), aggregation and gaping behaviour (Nicastro et al., 2012).

5.2.2. Temperature may increase the impact of plastics on mussels

Stressor interactions can fundamentally be additive, antagonistic or synergistic (Crain et al., 2008). Warming and contaminant stressors—mainly metals and pesticides, which are much more studied than plastics—showed synergistic effects in most studies; see Dinh et al. (2022) for a review. Specifically, in marine mussels, many studies have observed synergistic effects between temperature and contaminants (Andrade et al., 2022; Bordalo et al., 2023; Leite et al., 2020; Lopes et al., 2022; Moleiro et al., 2022), including plastic pollution (Pittura et al., 2022), although our literature review shows that this area of research remains in its infancy. Thus, each stressor could alter an organism's resistance to the other stressor (Sokolova & Lannig, 2008); for instance, compared to mussels from 'pristine' environments, mussels from polluted environments showed a significantly lower thermal tolerance (Hellou and Law, 2003; Smaal et al., 1991; Thomas et al., 1999). The combined exposure to climate-related stressors and toxic substances may affect individuals as well as populations and communities, which can lead to large-scale shifts (Moe et al., 2013). Although these two stressors can directly act on mussels and the ecosystem they support, the stressors can also impact mussel food sources, predators or competitors and indirectly affect mussel populations and their ecosystem. Thus, these stressor interactions emphasise the complexity of potential consequences for organisms and ecosystems. Understanding these implications remains essential for effectively evaluating chemical risks and improving our ability to protect ecosystems.

5.2.3. Temperature may impact plastic physical and chemical properties

Temperature may also directly influence the physical, chemical, and mechanical properties of any plastic item. Elevated temperatures can accelerate the degradation of plastics through abiotic (*e.g.* thermal oxidation; Chowdhury et al., 2022; Zhang et al., 2021b) or biotic processes (*e.g.* increasing enzymatic activity; Ge et al., 2023). The breakdown of polymer chains induced by these processes may impact the structural integrity of plastics and result in the formation of smaller and coarser particles (Villamizar and Morillas, 2018). For instance, increasing temperatures strongly promote the release of microplastics from disposable face masks (Jiang et al., 2022).

Higher temperatures can also influence the interactions between plastic particles and other environmental components, potentially enhancing the adsorption and leaching of chemicals; as a result, the transport and fate of plastic-associated chemicals in ecosystems are altered. The adsorption of chemical compounds (*e.g.* perfluorinated compounds, pharmaceutical drugs, plasticisers, flame retardants, oils and heavy metals) by plastic is enhanced by temperature up to a certain optimal temperature (Guan et al., 2022; Khumalo et al., 2022; Liu et al., 2018, 2022; Oz, 2019; Tang et al., 2021; Xu et al., 2019; Yap & Tan, 2021). In turn, an increase in temperature enhanced the leaching of plastic additives (*e.g.* phthalates ester plasticisers and flame retardants; Dhavamani et al., 2022; Kida et al., 2022; Kida & Koszelnik, 2021; Li & Tang, 2023; Ye et al., 2020; Zhang et al., 2022) and other environmental chemicals adsorbed on plastic particles (*e.g.* polycyclic aromatic hydrocarbons (PAHs) and antibiotics; see Godoy et al., 2020; Kida & Koszelnik, 2021). Notably, the adsorption and desorption optimal temperatures are dependent on the chemical compound (Khumalo et al., 2022; Kida et al., 2022) and the plastic polymer (Du et al., 2022; Zhang et al., 2022).

Note that while certain chemical compounds might migrate faster at elevated temperatures, high temperatures may also accelerate the degradation of the compound, potentially impacting its bioavailability (Kang & Kondo, 2005; Manzano et al., 1999; Saeed et al., 2011; Yang et al., 2018). However, elevated temperatures might also modify the transformation of contaminants into more toxic metabolites; for example, although the half-life of polychlorinated biphenyls (PCBs) in rainbow trout decreases with higher water temperature, this compound was biotransformed into toxicologically more active hydroxylated PCBs with increasing temperature (Buckman et al., 2007). Overall, in efforts to understand and mitigate the environmental consequences of plastic pollution, the impact of temperature on plastic materials, a complex and multifaceted issue, should be carefully considered. Thus, future knowledge on the role that plastics play as direct or indirect carriers of molecules with different levels of toxicity, and their influence on organisms and ecosystems, partially depends on our efforts to investigate how temperature affects the adsorption, desorption, and degradation dynamics of various contaminants in or on plastic particles and within the surrounding environment.

6. Conclusion

This review highlights the profound and widespread impact of plastic pollution on marine mussels, as keystone engineer species. In addition, the urgency of more holistic research that considers the challenges posed by multiple stressors, particularly in an era of global warming, is emphasised. While plastic pollution affects mussels at multiple levels, strong disparities in research efforts persist; thus, our efforts to clarify the role of plastic in the biology and ecology of mussels, as well as marine invertebrates in general, remain in their infancy. The asymmetric concentration of research effort in specific countries, mussel species, life stages and specific plastic types (*e.g.* MP size, polystyrene polymer and sphere shape) raises concerns about the possibility of generalising the findings. To address these challenges, we advocate for more coordinated interdisciplinary and international collaborations. In addition, awareness of environmental consistency in the choice of experimental protocols as well as their standardisation should be increased (*e.g.* in plastic chemical compound analyses). Furthermore, it is essential to recognise that plastic pollution interacts with numerous stressors in the Anthropocene era, resulting in intricate and often unpredictable effects. To address this issue, we must assess plastic pollution in conjunction with temperature, as discussed here, and potentially extend the framework to other stressors, such as acidification, hypoxia, food availability, and salinity. Implementing the above recommendations is imperative for fostering comprehensive knowledge of the ecological consequences of plastic pollution and promoting effective conservation strategies to ensure the health and sustainability of our oceans in our changing world.

Supplementary Material

S1: Main information of each of the 106 articles used in the Chapter I, *i.e.,* reference, mussel life stage, plastic polymer and potential contaminant or biofilm presence, shape and size, qualitative and quantitative assessment of the chemical compounds attached or bound to the particle and/or in the leachate solution, plastic main effect for endpoints of each organisational level, minimum concentration (mc; $\mu g\ L^{-1}$), maximum exposure duration (med; days), mussel species, location, presence or absence of plastic particles during exposure (MP filtration).

articles	stages	polymer	shape	size	leachate analyses	endpoint organisational level (effect / no effect)	mc (µg/L)	med (d)	species	location	filtration
Abidli et al., 2021	adults	PE	NA	MP	NA	**molecular; organism**	1	14	*Mytilus galloprovincialis*	Tunisia	NO
Alnajar et al., 2021	adults	NA	fibers	MP	YES	**molecular; tissues & organs;** organism	56000	7	*Mytilus galloprovincialis*	UK	NO
Auguste et al., 2020	adults	PS	NA	NP	NA	**molecular; cellular; tissues & organs**	10	4	*Mytilus galloprovincialis*	Italy	NO
Auguste et al., 2021	adults	PS	NA	NP	NA	**molecular; cellular; organism**	0,001	2	*Mytilus galloprovincialis*	Italy	NO
	larvaes										
Avio et al., 2015	adults	PE	spheres	MP	YES	**molecular; cellular**	1500000	7	*Mytilus galloprovincialis*	Italy	NO
		PS									
		PE + **Pyrene**									
		PS + **Pyrene**									
Bandini et al., 2021	adults	PE; PET; PP; PA; PS ± **Biofilm**	fragments	MP	NA	**molecular; tissues & organs**	50	3	*Mytilus galloprovincialis*	Tunisia	NO
Barkhau et al., 2022	NA	PVC	NA	MP	NA	**organism**	1500	63	*Semimytilus algosus*	Chile	NO
		PMMA									
Brandts et al., 2018	adults	PS	spheres	NP	NA	**molecular**	50	91	*Mytilus galloprovincialis*	Portugal	NO
		PS + **Carbamazepine**									
		Carbamazepine	Ø	Ø							
Brate et al., 2018	adults	PE	irregular	MP	NA	**tissues & organs;** organism	10	21	*Mytilus galloprovincialis*	Spain	NO
Browne et al., 2008	adults	PS	spheres	MP	NA	molecular; cellular; organism	NA	< 1	*Mytilus edulis*	UK	NO
Canesi et al., 2015	adults	PS	spheres	NP	NA	**molecular; cellular**	1	< 1	*Mytilus galloprovincialis*	Italy	NO
Canesi et al., 2016	adults	PS	NA	NP	NA	**molecular; cellular**	1	< 1	*Mytilus galloprovincialis*	Italy	NO
Capolupo et al., 2018	larvaes	PS	spheres	MP	NA	**organism**	NA	2	*Mytilus galloprovincialis*	Italy	NO
Capolupo et al., 2020	larvaes	PET	NA	MP	YES	**cellular; organism**	480000	6	*Mytilus galloprovincialis*	Italy	YES
		PP									
		PS									
		PVC									
		Rubber									
Capolupo et al., 2021a	adults	PET	NA	NA	YES	**molecular; cellular**	80	7	*Mytilus galloprovincialis*	Italy	YES
		PP									
		PS									
		PVC									
		Rubber									
Capolupo et al., 2021b	larvaes	PS	spheres	MP	NA	**molecular; cellular; organism**	NA	2	*Mytilus galloprovincialis*	Italy	NO
		PS + **Chrysene (PAH)**									
		Chrysene (PAH)	ø	Ø							
	adults	PS	spheres	MP							
		PS + **Chrysene (PAH)**									
		Chrysene (PAH)	ø	Ø							
Capolupo et al., 2021c	adults	PS	spheres	MP	NA	**molecular; cellular**	0,0015	21	*Mytilus galloprovincialis*	Italy	NO
				NP							
Cappello et al., 2021	adults	PS	spheres	MP	NA ("additives free")	**molecular**	NA	3	*Mytilus galloprovincialis*	Italy	NO
Chelomin et al., 2022	adults	PS	spheres	NP	NA	**molecular**	NA	3	*Mytilus trossulus*	Russia	NO
Choi et al., 2021	adults	PET	fibers	MP	NA	**molecular; cellular;** organism	0,5	4	*Mytilus galloprovincialis*	Korea	NO
Choi et al., 2022	adults	PET	fibers	MP	YES	**molecular; cellular; tissues & organs; organism**	0,5	32	*Mytilus galloprovincialis*	Korea	NO
Christoforou et al., 2020	adults	PA	fibers	MP	NA	**organism**	NA	52	*Mytilus edulis*	UK	NO
Ciocan et al., 2020	adults	PE + glass	fibers	MP	NA	**molecular; cellular**	60000	7	*Mytilus edulis*	UK	NO
Cole et al., 2020	adults	PA	spheres	MP	NA (washed)	**molecular; cellular; organism**	500	7	*Mytilus spp.*	UK	NO
		PS	spheres	NP							
				MP							

articles	stages	polymer	shape	size	leachate analyses	endpoint organisational level (effect / no effect)	mc (µg/L)	med (d)	species	location	filtration
Détrée and Gallardo-Escarate, 2017	adults	HDPE	spheres	MP	NA ("additives free")	**molecular**	NA	1	*Mytilus galloprovincialis*	Chile	NO
Détrée and Gallardo-Escarate, 2018	adults	HDPE	NA	MP	NA ("additives free")	**molecular; organism**	30	18	*Mytilus galloprovincialis*	Chile	NO
Dovzhenko et al., 2022	adults	PS	NA	NP	NA	**molecular;** cellular	NA	5	*Mytilus trossulus*	Russia	NO
		PS + **Copper (II) oxide nanoparticles (CuO–NPs)**									
		Copper (II) oxide nanoparticles (CuO–NPs)	ø	Ø							
Ferguson et al., 2022	NA	PS	spheres	MP	NA	**molecular; cellular; tissues & organs**	NA	42	*Mytilus edulis*	Canada	NO
Fernandez et al., 2022	adults	HDPE	irregular	MP	NA	**molecular;** tissues & organs; organism	1500	21	*Mytilus galloprovincialis*	Spain	NO
		HDPE + **Chlorpyrifos (CPF)**									
		Chlorpyrifos (CPF)	ø	Ø							
Foekema et al.,2022	adults	PS	spheres	MP	YES	**organism; ecology**	47000	56	*Mytilus edulis*	Netherlands	NO
Fraissinet et al., 2023	adults	PS	spheres	MP	NA	**organism**	NA	< 1	*Mytilus galloprovincialis*	Italy	NO
Franzellitti et al., 2019	larvaes	PS	spheres	MP	NA	**molecular; organism**	NA	4	*Mytilus galloprovincialis*	Italy	NO
	adults										
Goncalves et al., 2019	adults	PS	spheres	MP	NA	molecular; tissues & organs	NA	21	*Mytilus galloprovincialis*	Portugal	NO
Goncalves et al., 2022	adults	PS	NA	NP	NA	**molecular;** organism	10	21	*Mytilus galloprovincialis*	Portugal	NO
Gonzalez-Soto et al., 2019	adults	PS	spheres	NP ; MP	NA	**molecular;** tissues & organs; **organism**	58	21	*Mytilus galloprovincialis*	Spain	NO
		PS + **B[a]P**									
Gonzalez-Soto et al., 2022	adults	PS	spheres	MP	NA	**molecular; cellular; tissues & organs; organism**	58	21	*Mytilus galloprovincialis*	Spain	NO
		PS + **Water accomodated fraction (WAF)**									
		Water accomodated fraction (WAF)	Ø	Ø							
Green et al., 2017	adults	PLA	NA	NP + MP	NA	**organism;** ecology	2,5	50	*Mytilus edulis*	Ireland	NO
		HDPE									
Green et al., 2019	adults	PLA	fragments	NP + MP	NA	**molecular; organism**	25	52	*Mytilus edulis*	Ireland	NO
		HDPE									
Gu et al., 2020	NA	PS	spheres	MP	NA	**molecular; organism**	2,5	21	*Mytilus coruscus*	China	NO
		PS + **Polybrominated diphenyl ethers (BDE-47)**									
		Polybrominated diphenyl ethers (BDE-47)	ø	Ø							
Hamm and Lenz, 2021	juveniles	PVC	irregular	MP	NA	**molecular; cellular**	NA	42	*Mytilus spp.*	Germany	NO
		PS	spheres								
Hamm et al., 2022	adults	PMMA	fragments	NP ; MP	NA ("additives free")	organism	1500	42	*Brachidontes puniceus*	Cabo Verde	NO
									Semimytilus algosus	Chile	
		PVC	pellets						*Brachidontes pharaonis*	Israel	
									Mytilus trossulus	Japan	
									Mytilus galloprovincialis	Australia	
Han et al., 2021	NA	PS	spheres	NP	NA	**molecular; cellular**	260	28	*Mytilus coruscus*	China	NO
		PS + **Oxytetracycline**									
		PS + **Florfenicol**									
		PS + **Sulfamethoxazole**									
		Oxytetracycline	Ø	Ø							
		Florfenicol									
		Sulfamethoxazole									
Hariharan et al., 2021	adults	PE	fibers	MP	NA (washed)	**molecular; tissues & organs; organism**	1	30	*Perna viridis*	India	NO
Harris and Carrington, 2020	adults	PE	spheres	MP	NA	**organism**	NA	< 1	*Mytilus trossulus*	USA	NO
Harris et al., 2021	adults	PE	spheres	MP	NA	**organism**	NA	< 1	*Mytilus trossulus*	USA	NO
Huang et al., 2021	adults	PS	spheres	MP	NA ("additives free")	**molecular**	NA	14	*Mytilus coruscus*	China	NO
Huang et al., 2022	adults	PS	spheres	MP	NA	**molecular; cellular; organism**	2,5	21	*Mytilus coruscus*	China	NO
Jaouani et al., 2023	NA	LDPE	pellets	MP	NA (new and aged)	**molecular; cellular;** organism	600	7	*Mytilus edulis*	France	NO
Jang et al., 2022	adults	PS	spheres	NP ; MP	NA	**molecular**	0,008	4	*Perna viridis*	Singapore	NO
Joyce and Falkenberg, 2022	adults	PET	NA	MP	NA	organism	100	28	*Perna viridis*	Hong Kong	NO
		PLA									
Katsumiti et al., 2021	adults	PS	NA	NP	NA	**molecular; cellular**	NA	1	*Mytilus galloprovincialis*	Spain	NO
				MP							
				MP							
		PS + **Benzo(a)Pyrene**		NP							
				MP							
		Benzo(a)Pyrene		Ø							
Khalid et al., 2021	adults	PLA	NA	NP + MP	NA	**molecular;** cellular; organism	10	8	*Mytilus edulis*	France	NO

articles	stages	polymer	shape	size	leachate analyses	endpoint organisational level (**effect** / no effect)	mc (µg/L)	med (d)	species	location	filtration
Khalid et al., 2021	adults	PLA	NA	NP + MP	NA	**molecular;** cellular; organism	10	8	*Mytilus edulis*	France	NO
Kolaveric et al., 2023	adults	PS	NA	MP	NA	**molecular**; organism	10000	4	*Mytilus galloprovincialis*	Slovenia	NO
		PS + **Fluoranthene (PAH)**									
		Fluoranthene (PAH)	ø	Ø							
	NA	PS	NA	MP						Montenegro	
		PS + **Fluoranthene (PAH)**									
		Fluoranthene (PAH)	ø	Ø							
Laubscher et al., 2023	adults	PS	spheres	MP	NA	**organism**	NA	< 1	*Mytilus spp.*	Germany	NO
Leroux et al., 2022	adults	PS	NA	NP	NA	**molecular**	NA	1	*Mytilus galloprovincialis*	Spain	NO
Li et al., 2020a	adults	PVC	NA	MP	NA (virgin)	molecular; cellular	NA	7	*Mytilus edulis*	UK	NO
		PVC + **Cadmium (CdCl2)**									
		Cadmium (CdCl2)	ø	Ø							
Li et al., 2020b	adults	HDPE	NA	MP	NA (new and aged)	**tissues & organs;** organism	200	42	*Mytilus edulis*	France	NO
Lu et al., 2023	adults	PS	spheres	NP	NA	**molecular; tissues & organs**	20	7	*Mytilus galloprovincialis*	China	NO
Magara et al., 2018	NA	PE	spheres	MP	NA	**molecular**	NA	4	*Mytilus edulis*	Denmark	NO
		PE + **Fluoranthene**									
		Fluoranthene	ø	Ø							
Magara et al., 2019	NA	PE	spheres	MP	NA	**molecular**	NA	4	*Mytilus edulis*	Denmark	NO
		PHB									
		PE + **Fluoranthene**									
		PHB + **Fluoranthene**									
		Fluoranthene	Ø	Ø							
Masia et al., 2021	adults	PS	spheres	MP	NA	**molecular; organism**	20	21	*Mytilus galloprovincialis*	Spain	NO
Nunes et al., 2020	adults	Paraffin	irregular	MP	NA	**molecular**	5000	4	*Mytilus* sp.	Portugal	NO
O'Brien et al., 2021	adults	PS	spheres	MP	NA	**molecular**	NA	7	*Mytilus galloprovincialis*	USA	NO
Opitz et al., 2021	juveniles	PS	spheres	MP	NA	organism	250	40	*Chloromytilus chorus*	Chile	NO
Paul-Pont et al., 2016	adults	PS	spheres	MP	YES	**molecular; cellular; tissues & organs**	32	7	*Mytilus spp.*	France	NO
		PS + **Fluoranthene**									
		Fluoranthene	Ø	Ø							
Pavicic-Hamer et al., 2022	adults	PMMA	spheres	MP	NA	**molecular; cellular; organism**	100	3	*Mytilus galloprovincialis*	Croatia	NO
Phothakwanpracha et al., 2021	adults	PS	films	MP	NA	**organism**	NA	4	*Perna viridis*	Thailand	NO
		PP									
		PBS									
Pittura et al., 2018	adults	LDPE	NA	MP	NA	**molecular; cellular**	10000	28	*Mytilus galloprovincialis*	Italy	NO
		LDPE + **B[a]P**									
		B[a]P		Ø							
Pittura et al., 2022	adults	PEST	fibers	MP	NA	**molecular; cellular**	NA	14	*Mytilus galloprovincialis*	Italy	NO
		PA									
Provenza et al., 2020	adults	PET	irregular	MP	NA (washed)	molecular	100000	7	*Mytilus galloprovincialis*	Italy	NO
Provenza et al., 2022	adults	PA	glitters	MP	NA	**molecular**	NA	7	*Mytilus galloprovincialis*	Italy	NO
		PMMA									
		PE									
Provenza et al., 2023	adults	HDPE	fragments	MP	NA (washed)	**molecular; organism**	50000	7	*Mytilus galloprovincialis*	Italy	NO
Qi et al., 2023	adults	PS	NA	NP ; MP	NA	**molecular; tissues & organs**	20000	2	*Mytilus coruscus*	China	NO
Revel et al., 2019	adults	PE + PP	fragments	MP	NA	**molecular;** tissues & organs; organism	0,008	10	*Mytilus spp.*	France	NO
Rist et al., 2016	adults	PVC	fragments	MP	NA	**organism**	2160000	91	*Perna viridis*	Indonesia	NO
		PVC + **Fluoranthene**									
Rist et al., 2019	larvaes	PS	spheres	MP	NA	**organism**	42	15	*Mytilus edulis*	Denmark	NO
				NP							
Rodrigues et al., 2022	adults	PA	irregular	MP	NA	**molecular; organism**	1000	4	*Mytilus galloprovincialis*	Portugal	NO
		PA + ***Asparagopsis armata* algae**									
		***Asparagopsis armata* algae**	ø	Ø							
Roman et al., 2023	NA	PE	fragments	MP	NA	**molecular; cellular**	0,008	1	*Mytilus edulis*	France	NO
		PE		NP							
		PS	spheres								
Romdhani et al., 2022	adults	PE + PET + PP + PEVA + HDPE	fragments	MP	NA	**molecular; cellular**	1000	3	*Mytilus galloprovincialis*	Tunisia	NO
		PE + PET + PP + PEVA + HDPE **+ B[a]P**									
		B[a]P		Ø							
Santana et al., 2018	adults	PVC	NA	NP + MP	NA	molecular; cellular; **organism**	125000	90	*Perna perna*	Brazil	NO
Sendra et al., 2020a	adults	PS	spheres	NP ; MP	NA	**molecular; cellular**	10000	< 1	*Mytilus galloprovincialis*	Spain	NO
Sendra et al., 2020b	adults	PS	spheres	NP ; MP	NA	**molecular; cellular**	1000	1	*Mytilus galloprovincialis*	Spain	NO

articles	**stages**	**polymer**	**shape**	**size**	**leachate analyses**	**endpoint organisational level (effect /** no effect**)**	**mc (µg/L)**	**med (d)**	**species**	**location**	**filtration**
Seuront et al., 2021	adults	PP	pellets	MP	YES	**organism**	12000000	< 1	*Mytilus edulis*	France	YES
									Mytilus galloprovincialis	South Africa	
									Perna perna		
									Chloromytilus meridionalis		
Shang et al., 2021a	adults	PS	spheres	MP	NA	**molecular; organism**	2,5	21	*Mytilus coruscus*	China	NO
Shang et al., 2021b	adults	PS	spheres	MP	NA	**molecular**	NA	14	*Mytilus coruscus*	China	NO
Silva et al., 2016	larvaes	PP	pellets	NA	NA (new and aged)	**organism**	NA	2	*Perna perna*	Brazil	YES
Sui et al., 2022	adults	PS	spheres	MP	NA	**organism**	NA	21	*Mytilus coruscus*	China	NO
Sui et al., 2023	adults	PS	spheres	MP	NA (washed)	**organism**	NA	28	*Mytilus coruscus*	China	NO
Sun et al., 2023	adults	PMMA	spheres	MP	NA	**molecular; cellular**	NA	14	*Mytilus galloprovincialis*	China	NO
Tang et al., 2022	adults	PS	spheres	MP	NA	**molecular; cellular; organism**	200	3	*Mytilus coruscus*	China	NO
		PS + ***Prorocentrum lima* algae**									
		***Prorocentrum lima* algae**	ø	Ø							
Trestrail et al., 2021	adults	PS	spheres	MP	NA	**molecular**	NA	7	*Mytilus galloprovincialis*	Australia	NO
		PE									
Uguen et al., 2022	juveniles	PP	pellets	MP	YES	**organism**	102000	< 1	*Mytilus edulis*	France	YES
Van Cauwenberghe et al., 2015	adults	PS	spheres	MP	NA	**molecular**	NA	14	*Mytilus edulis*	Belgium	NO
von Hellfeld et al., 2022	adults	PS	spheres	MP	NA	molecular; cellular; tissues & organs	0,05	3	*Mytilus galloprovincialis*	Spain	NO
		PS + **B[a]P**									
		PE + **Cadmium**									
		B[a]P	Ø	Ø							
		Cadmium									
von Moos et al., 2012	adults	HDPE	irregular	MP	NA ("additives free")	molecular; **cellular; tissues & organs;** organism	2500000	4	*Mytilus edulis*	Germany	NO
Wang et al., 2020	adults	PS	spheres	MP	NA	**molecular**	NA	14	*Mytilus coruscus*	China	NO
Wang et al., 2021	adults	PS	spheres	MP	NA (washed)	**molecular; organism**	NA	14	*Mytilus coruscus*	China	NO
				NP							
Wang et al., 2022	adults	PS	spheres	NP	NA (washed)	**molecular; cellular; organism**	500	7	*Mytilus galloprovincialis*	China	NO
Wang et al., 2023a	adults	PS	NA	MP	NA	molecular; tissues & organs	5,5	4	*Mytilus galloprovincialis*	China	YES
		PS + **Benzo(a)Pyrene**	ø	ø		**molecular; tissues & organs**					NO
		Benzo(a)Pyrene									
Wang et al., 2023b	adults	PS	spheres	NP	NA (washed)	**molecular**	500	7	*Mytilus galloprovincialis*	China	NO
Webb et al., 2020	adults	PE	spheres	MP	NA	**organism**	500000	2	*Perna canaliculus*	New Zealand	NO
		PE + **Triclosan**									
		Triclosan		Ø							
Wegner et al., 2012	adults	PS	spheres	NP	NA	**organism**	100000	< 1	*Mytilus edulis*	Netherlands	NO
Wei et al., 2021	adults	HDPE	NA	MP	NA	**molecular**	50000	4	*Mytilus galloprovincialis*	China	NO
		PS									
Yang et al., 2021	adults	PS	NA	MP	NA	tissues & organs	NA	21	*Mytilus coruscus*	China	NO
Yap et al., 2020	adults	PVC	irregular	MP	NA ("additives free")	organism	1500	35	*Mytilus galloprovincialis*	Australia	NO
Yu et al., 2022	adults	PS	spheres	MP	NA	**molecular; organism**	250	28	*Mytilus coruscus*	China	NO
		PS + **Carbamazepine**									
		Carbamazepine	ø	Ø							

CHAPTER II

THE TOLERANCE OF A KEYSTONE ECOSYSTEM ENGINEER TO EXTREME HEAT STRESS IS HAMPERED BY MICROPLASTIC LEACHATES

The tolerance of a keystone ecosystem engineer to extreme heat stress is hampered by microplastic leachates

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Abstract

Plastic pollution and ongoing climatic changes exert considerable pressure on coastal ecosystems. Unravelling the combined effects of these two threats is essential to management and conservation actions to reduce the overall environmental risks. We assessed the capacity of a coastal ecosystem engineer, the blue mussel *Mytilus edulis*, to cope with various levels of aerial heat stress (20, 25, 30 and 35°C) after an exposure to substances leached from beached and virgin low-density polyethylene pellets. Our results revealed a significant interaction between temperature and plastic leachates on mussel survival rates. Specifically, microplastic leachates had no effect on mussel survival at 20, 25 and 30°C. In turn, mussel survival rates significantly decreased at 35°C, and this decrease was even more significant following an exposure to leachates from beached pellets; these pellets had a higher concentration of additives compared to the virgin ones, potentially causing a bioenergetic imbalance. Our results stress the importance of adopting integrated approaches combining the effects of multiple environmental threats on key marine species to understand and mitigate their potential synergistic effects on ecosystem dynamics and resilience in the face of the changing environment.

1. Introduction

Plastic and its associated chemicals have become a global concern from both scientific and societal perspectives. The ever-increasing plastic production and poor waste management has led to a permanent contamination of the biosphere by plastic items (Barnes et al., 2009; Geyer et al., 2017; Patil et al., 2022). Coastal systems are some of the most heavily plastic-polluted ecosystems (Serra-Gonçalves et al., 2019; Wu et al., 2022). Plastics may pose a serious threat to organisms not only through physical damage (Derraik, 2002; Gall & Thompson, 2015), but also through the far less studied consequences of their chemical content (Delaeter et al., 2022; Seuront et al., 2022). Plastic debris may noticeably act as vectors for numerous chemical compounds that can be hazardous depending on the nature of the chemicals and their concentrations (Diepens & Koelmans, 2018; Koelmans et al., 2016), which can vary during the lifetime of the plastic. Virgin plastics are essentially composed of plastic polymers to which a range of additives are incorporated during their manufacture in order to improve their performances (Thompson et al., 2009), with *e.g.* phthalates making up 10 to 60% of the PVC weight (Net et al., 2015). Then, when released in the environment, a range of environmental contaminants, potentially more toxic than additives (*e.g.* heavy metals, pesticides), may be adsorbed on the surface of plastic particles and accumulate (Rochman, 2015; Thevenon et al., 2015), at concentrations up to 6 orders of magnitude higher than the environment (Mato et al., 2001) and then be desorbed (Delaeter et al., 2022). These sorption/desorption phenomena noticeably increases as particle size decreases (Mattsson et al., 2015). As such, plastics in general and microplastics in particular (*i.e.* particles size from 1 µm to 5 mm) may act both as a sink and a source of contaminants, exposing organisms to complex and potentially toxic cocktails of chemical compounds (Delaeter et al., 2022; Lynch et al., 2022; Seuront et al., 2022).

As climate change progresses, the effects of extreme weather conditions, including the increased frequency, intensity, and duration of heat stress, are expected to become more pronounced (Della-Marta et al., 2007; IPCC, 2022; Sun et al., 2019). In intertidal ecosystems, these events are particularly stressful, *e.g.* the body temperature variation experienced by mussel specimens between emersion and immersion can exceed 20°C (*e.g.* Galil et al., 2022; Raymond et al., 2022; Seuront et al., 2019; Zippay & Helmuth, 2012) and can cause damages and mass mortality episodes, especially in marine invertebrates such as mussels (Galil et al., 2022 ; Seuront et al., 2019 ; Zippay & Helmuth, 2012). Noticeably, the temperature tolerance of organisms is dependent on their health status, which can further be compromised by the presence of contaminants in seawater (Lavergne et al., 2015; Patra et al., 2007; Sokolova & Lannig, 2008). Even though plastic pollution and heat stress *de facto* frequently coincide, only a handful of studies have explored their combined repercussions. These essentially include adverse effects on survival, physiological processes, and cellular functions (Fonte et al., 2016; Gomez et al., 2021; Kratina et al., 2019; Wen et al., 2018) though exceptions exist (*e.g.* Ferreira et al., 2016; Hoffschröer et al., 2021; Plafcan and Stallings, 2022). Despite the plethora of studies that assessed the effects of microplastic leachates on various aspects of the biology and ecology of marine organisms (Delaeter et al., 2022), to the best of our knowledge the combined impact of plastic leachates and heat stress has not been addressed. Given the potentially high impact of these two stressors on intertidal ecosystems, the objective of this study was to assess the impact of microplastic leachates on the ability of a key intertidal ecosystem engineer (Buschbaum et al., 2009), the blue mussel *Mytilus edulis*, to survive aerial heat stress. Leachate solutions were consistently prepared from low-density polyethylene pellets that were either virgin (*i.e.* raw commercially available pellets) or found stranded on the beach, and aerial heat stress was simulated at 4 different temperatures (*i.e.* 20, 25, 30 or 35°C). We hypothesised that plastic leachate exposure alters the mussel ability to withstand an aerial heat stress event and that leachates from beached pellets will have a more deleterious impact than virgin ones because they have the potential to accumulate additional toxicants when dispersed in the environment.

2. Material and Methods

Study organisms

A total of 720 specimens of the blue mussel *M. edulis* (3 – 4 cm in shell length) were collected in September 2022 from the intertidal rocky shore at Pointe aux Oies (Wimereux, France; 50°47’08.3"N, 1°36’03.9"E) along the French coast of the eastern English Channel. Prior to the experiments, mussels were acclimated in the laboratory for 24 h in 85 L tanks filled with oxygen saturated natural seawater (Uguen et al., 2022) representative of *in situ* conditions (T = 20°C; S = 33‰).

Microplastics and leachate solution preparation

Microplastic leachate (MPL) solutions were prepared from either (i) commercially available (Materialix Ltd., London, United Kingdom) low-density spheroidal polyethylene pellets (with typical longest and shortest axes respectively 4.14 ± 0.21 mm and 1.89 ± 0.10 mm in length; V-MPL treatment) or (ii) cylindrical beached pellets (typically 4.04 ± 0.56 mm in width and 3.03 ± 0.82 mm in height) collected from the high-tide mark sediment surface of the nearby beach. Beached pellets were consistently identified as low-density polyethylene using Fourier transform infrared (FTIR) analysis with an Aldrich FTIR database search set at 97.5% correlation setting. GC-HRMS analyses showed that virgin pellets were composed of 5 additives, including 4 plasticisers and 1 antioxidant. By contrast, beached pellets contained 7 additives, 4 plasticisers and 3 antioxidants and in a quantity typically 2 to 4-fold higher than in virgin pellets. For more details about the polymer and chemical content identification and the method used, see Supplementary Material, S2.

Each type of pellet was mixed with natural aerated seawater for 24h (Seuront et al., 2021; Uguen et al., 2022, 2023) at 20°C at a concentration of 10 g of pellets per litre (*ca*. 400 pellets per litre, or equivalently 20 mL of pellets per litre; Uguen et al., 2023). After 24 h, the experiment was performed using the solution alone (*i.e.* without any pellets). A control solution (Control) was prepared by incubating aerated seawater for 24 h at 20°C. Consistent oxygen saturation and seawater renewal were ensured for each treatment during every immersion cycle.

Experimental design

The experiment was designed to mimic the temperature experienced by *M. edulis* during a typical tidal cycle at the sampling site (*i.e.* 6 h immersion/emersion with a sea surface and air temperature of 20°C; see SOMLIT 2022 data for Wimereux in September; https://www.somlit.fr/visualisation-des-donnees/), with a one-off emersion heat stress event representative of observed air temperature at the study site during the summer 2022 (25, 30 and 35°C; air temperature record = 39.6°C; Sorel et al., 2022). A previous study conducted at our study site, using robomussels, revealed that mussel body temperatures occasionally exceeded 35°C, reaching a maximum recorded temperature of 41.7°C, while the seawater temperature remained around 15°C, resulting in a delta temperature of over 20°C (Seuront et al., 2019).

Specifically, the 72-hour experiment consisted of 6 successive immersion-emersion cycles that were conducted (i) under naturally occurring seawater and air temperatures at our study site in September (*i.e.* 20°C) as a control, and (ii) under a 6-hour aerial heat stress event of 25, 30 or 35°C after one immersion-emersion-immersion cycle at 20°C and followed by 4 immersion-emersion recovery events at 20°C (Figure 23).

For each experimental treatment (Temperature × Solution), 20 mussels were used in triplicates (*i.e.* $N = 60$ mussels for each of the 12 experimental trials). During the immersion phase, the mussels were incubated in a 1.5 L glass jar containing 1 L of Control, V-MPL, or B-MPL solutions. Immersions lasted for 6 hours at a temperature of 20 °C (Figure 23). The emersion consisted of placing the mussels on natural seawater saturated paper towels in sealed 1.5 L glass jar to maintain around 95% relative humidity to avoid desiccation or evaporation (Seuront et al., 2019). For the aerial heat stress, the jars were placed in incubators (MIR-154, Panasonic, Japan; temperature resolution ± 0.3 °C) heated either at 20, 25, 30 or 35°C for 6 h (Figure 23). Relative humidity and air temperature experienced by mussels inside the jar were monitored using Hygro Buttons 23 (Proges-Plus, resolution 0.5°C and 1%) at 5 min interval (Supplementary Materials, S3).

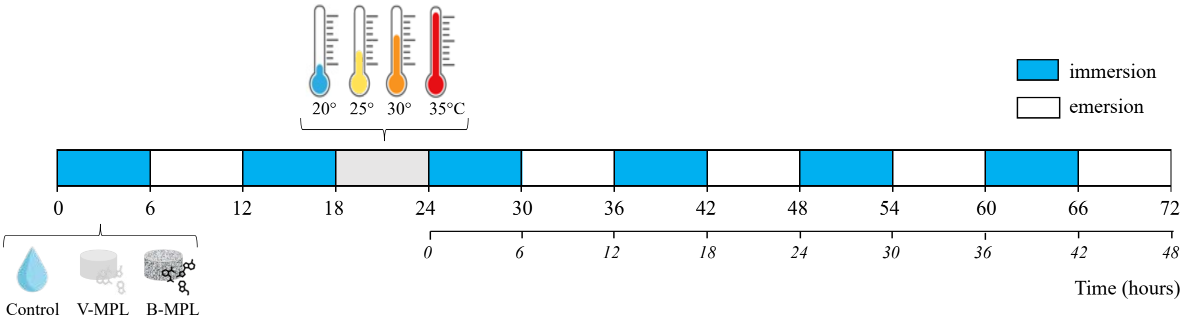


Figure 23: Schematic diagram of the immersion-emersion experiments. Mussels were immerged (blue rectangles) during 6 hours in Control, Virgin microplastic leachate (V-MPL) or Beached microplastic leachate (B-MPL) solutions and emerged (white rectangles) during 6 hours consistently at an aerial temperature of 20°C (control temperature) except during the second emersion (grey rectangle) where the mussels were exposed to a low (25°C), moderate (30°C) or high (35°C) aerial heat stress event. The numbers indicate the time in hours after the beginning of the microplastic leachate stress (normal font) or after the thermal stress (italic font).

Endpoint and Statistical analyses

After each immersion and emersion, the status of each mussel was visually checked. Opened mussels that did not respond to foot probing, *i.e.* no valve closure, were discarded and recorded as dead (Seuront et al., 2019; Martin et al., 1993; Waller et al., 1995; Wildridge et al., 1999).

To study the effects of the factors ‘Temperature’, ‘Solution’ and their interaction ‘Temperature × Solution’, data were analysed using a 2-way ANOVA with Solution (Control, V-MPL, B-MPL) and Temperature (20 °C, 25 °C, 30 °C, 35 °C) as fixed factors and percentage of survived mussels at the end of the experiment as the dependent factor. Significant effects were examined using Tukey-HSD post-hoc test. The data met the assumption required for the ANOVAs; homogeneity of variances and dispersion of the residuals were checked using the package ‘DHARMa’ (Hartig, 2022).

Lethal times at 50% of mortality (LT50; in hours) and their lower and upper fiducial confidence limits (CL) for each solution at 35 °C — the only temperature where a significant mortality was recorded — were estimated using a binomial generalised linear model with a probit link function using the package ‘ecotox’ (Finney, 1971; Hlina et al., 2021; Robertson et al., 2007; Wheeler et al., 2006). To determine the existence of differences between the LT50 at 35 °C of each treatment, a ratio test was used (Wheeler et al., 2006). Exact *p* values are given in Supplementary Materials S4, S5 and S7. All statistical analyses were performed using the software R Core Team (2022).

3. Results

Survival rates

Survival rates significantly decreased only after an aerial heat stress exposure at 35°C for all solutions, *i.e.* Control (48.3 ± 14.4%), V-MPL (53.3 ± 7.6 %) and B-MPL (26.7 ± 7.6%); Temperature × Solution, $p < 0.01$; S5; Tukey test: $p < 0.001$; Figure 24). Specifically, at 35°C, significantly more mussels died when exposed to B-MPL compared to those exposed to V-MPL and Control seawater (Temperature × Solution, $p < 0.01$; S5, Tukey test: $p < 0.01$ and $p < 0.05$, respectively; Figure 24). No significant differences in mussel mortality were found between V-MPL and Control seawater treatments at any of the aerial heat stress temperature tested (Temperature × Solution, $p < 0.01$; S5; Tukey’s test: $p > 0.05$; Figure 24).

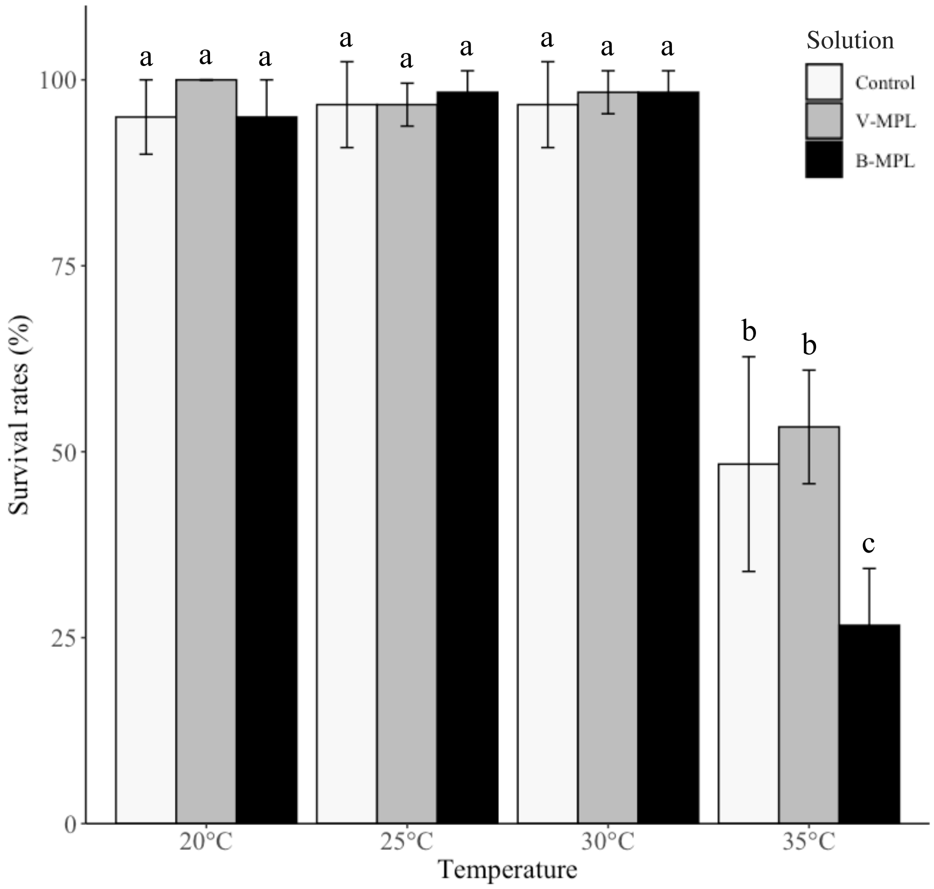


Figure 24: Survival rates (%; mean ± Standard Deviation) of *Mytilus edulis* at the end of the 72 h experiment after an exposure to control seawater (white), virgin (grey) and beached (black) MP leachate solutions combined with an aerial HS event at different temperatures. Letters depict significant differences among Temperature × Solution treatments ($p < 0.05$; Two-way ANOVA, Tukey post-hoc comparison). For details about the exact *p-values*, please refer to the Supplementary Materials S4 and S5.

Lethal time after a 35°C aerial heat stress

When exposed to a 35°C aerial heat stress, the LT50 (CL) of mussels exposed to control seawater, *i.e.* 69.5 h (64.4 – 78.0 h), was not significantly different from the LT50 recorded following exposure to V-MPL, *i.e.* 71.1 h (67.3 – 77.0 h; ratio test: $p > 0.05$, S6, S7). By contrast, mussels exposed to B-MPL were less resistant to a 35°C aerial heat stress, as LT50 was reached in significantly less time, *i.e.* 59.4 h (57.2 – 62.0 h), than the other two treatments (ratio test: $p < 0.001$, S6, S7).

4. Discussion

We provide the first evidence of the combined effect of temperature and plastic leachates on the survival rate of the keystone ecosystem engineer, *M. edulis*. At temperatures ranging from 20 to 30°C, the presence of leachates, either from virgin or beached microplastics, did not affect the mortality rates of *M. edulis*. In sharp contrast, under conditions of high thermal stress (35°C), and in agreement with previous studies (Jones et al., 2009; Seuront et al., 2019), there was a significant decline in survival rates for all treatments, especially when mussels were exposed to leachates from beached microplastics. These results raise concern, given that thermal events with mussel body temperature reaching more than 35°C during summer emersion at the study site occurred *ca* 11 times over a 84-day time series (Seuront et al., 2019), a scenario expected to intensify under future climate projections (IPCC, 2022). Sub-lethal synergistic effect of temperature and contaminant, *e.g.* (Andrade et al., 2022; Bordalo et al., 2023; Leite et al., 2020; Lopes et al., 2022; Moleiro et al., 2022), including plastics, *e.g.* (Gomez et al., 2021; Lenz, 2016; Pittura et al., 2022; Weber et al., 2020), have previously been documented in mussels under conditions of immersion. This present work, however, provides the first evidence of an enhanced thermal mortality through an exposure to microplastic leachates.

The adverse synergistic effect observed here is likely due to a bioenergetic imbalance, *i.e.* the energy costs exceed the available energy supply. Leachates originating from various plastics such as car tyre rubber, polypropylene, polyethylene terephthalate, polystyrene, polyvinyl chloride, and noticeably even bioplastics are capable of harming mussel cells by interfering with processes like lysosomal function, neurotransmission, oxidative stress and antioxidant defences; see (Capolupo et al., 2021a, 2023) for details on leachates composition. Under low and moderate thermal stress conditions, the detoxification and repair cost may be low enough to supply basal maintenance (Sokolova, 2013; Sokolova & Lannig, 2008). In turn, at high temperatures, the mussel heat shock response cost and cell damages caused by aerial heat stress (Nicastro et al., 2023b) are added to those of plastic leachates. In this context, our results suggest that the processes used to counteract the cellular damage caused by these combined stressors may become too costly and create a bioenergetic deficit, leading to high mortality (Sokolova, 2013; Sokolova & Lannig, 2008).

Noticeably, *M. edulis* survival rates following a 35°C aerial heat stress varied depending on the pellet's history, with beached microplastic leachates (from pellets collected in the environment and used at a locally realistic concentration; Seuront, 2018) being significantly more harmful than virgin microplastic leachates (from commercially available pellets). The more severe effect of B-MPL compared to V-MPL has previously been shown in *Perna perna* mussel embryos (Gandara e Silva et al., 2016) and also in a wide range of other species, *e.g.* in sea urchins (*Paracentrotus lividus;* Cormier et al., 2021; Rendell-Bhatti et al., 2021), jellyfish (Cormier et al., 2021), gastropods (Seuront, 2018) and even in dune plants (Menicagli et al., 2022), although exceptions exist such as in zebrafish (Cormier et al., 2021), copepods and another sea urchin (*Lytechinus variegatus;* Nobre et al., 2022, 2015).

The strongest effect observed for beached microplastic leachates compared to virgin ones after the high aerial heat stress is likely related to the pellet chemical content. Indeed, virgin pellets contained less additives, which were 2- to 4-fold less concentrated than in beached ones. The additives characterised were mainly phthalate plasticisers (*i.e.* Dimethyl phthalate, DMP; Diethyl phthalate, DEP; Di-n-butyl phthalate, DBP and Diisobutyl phthalate, DIBP) and Bisphenol S antioxidant, which are known to have various detrimental effects on marine invertebrates, for reviews see (Burgos-Aceves et al., 2021; Liu et al., 2021; Oehlmann et al., 2009). In addition, due to their permanence in the natural environment, beached pellets are also likely to be loaded with a harmful cocktail of environmental contaminants such as Persistent Organic Pollutants and heavy metals (Khalid et al., 2021; Rodrigues et al., 2019b). This is in sharp contrast to virgin pellets which are essentially composed of functional additives (*e.g.* flame retardants, plasticisers, colourants (Hermabessiere et al., 2017)). Although mussel survival rates were noticeably not affected at low temperatures when combined with either type of plastic leachates, this does not rule out the existence of sublethal effects such as behaviour, growth and reproduction which are likely to have longer term detrimental effects on the biology, ecology and ultimately survival (Sokolova, 2013). The resolution of these specific issues lies beyond the scope of the present work, but warrants the need for further studies.

5. Conclusion

Thermal stress and plastic pollution are prominent challenges in the Anthropocene. In the context of the anticipated rise in frequency, severity and duration of extreme heat events (IPCC, 2022), coupled with the growing production of plastic and the subsequent accumulation of plastic waste in the environment (Geyer, 2020), our results raise concerns and highlight the critical need for multi-stress studies to fully comprehend the impacts of these stressors on organisms. In turn, increase in temperature has been shown to enhance the leaching of plastic associated chemical compounds (Dhavamani et al., 2022; Kida & Koszelnik, 2021; Kida et al., 2022) and hence their potential toxicity, highlighting the need for further studies to understand the fate of plastic-associated chemicals in warming ecosystems. Considering the engineering role of the species used in this study, the combined impact of environmentally realistic temperature (Seuront et al., 2019), as well as the type and concentration of plastic pellet (Seuront, 2018), could pose a significant threat not only to the species itself but also to the overall ecosystem that relies on these species for sustenance.

Supplementary Materials

S2: Identification protocol and main results of the polymer and additives of the virgin polyethylene and beached pellets used.

Polymers & additives:

Methods

The identification of the additive content of both virgin and beached pellets was carried out using a CDS Pyroprobe 6150 pyrolyser (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% dimethylsiloxane 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 additives (i.e. plasticisers, flame retardant, antioxidants and UVs stabilisers; Table 1). The 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.

We subsequently identified the polymeric composition of the beached pellets and quantified the elemental composition (*i.e.,* C, H2, O, and heavy metals) adsorbed on their surface as well as their additive content. To identify pellet polymer types, we used Fourier transform infrared (FTIR) with an Aldrich FTIR database search set at 97.5% correlation setting.

Table 1. List of the additive scanned in the present study.

Function	N°	Molecules	Abbreviation	CAS
Plasticisers	1	Dimethyl phthalate	**DMP**	**131-11-3**
	2	Diethyl phthalate	**DEP**	**84-66-2**
	3	Di-allyl phthalate	**DAlP**	**131-17-9**
	4	Diisobutyl phthalate	**DIBP**	**84-69-5**
	5	Di-n-butyl phthalate	**DBP**	**84-74-2**
	6	Tributyl Acetyl Citrate	**ATBC**	**77-90-7**
	7	Di-n-hexyl phthalate	**DHP**	**84-75-3**
	8	Benzyl butyl phthalate	**BBP**	**85-68-7**
	9	Bis-2-Ethylhexyl Adipate	**DEHA**	**103-23-1**
	10	Diisoheptyl phthalate	**DIHP**	**71888-89-6**
	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**
	14	Diisononyl hexahydrophthalate	**DINCH**	**166412-78-8**
	15	Di-n-octyl phthalate	**DIOP**	**117-84-0**
	16	Diisononyl phthalate	**DINP**	**68515-48-0**
	17	Di-nonyl phthalate	**DNP**	**84-76-4**
	18	Diisodecyl phthalate	**DIDP**	**68515-49-1**
Flame retardants	19	Triethyl Phosphate	**TEP**	**78-40-0**
	20	Tripropyl Phosphate	**TPP**	**115-86-6**
	21	Tributyl Phosphate	**TBP**	**126-73-8**
	22	2,4,6-Tribromophenol	**2,4,6,TBP**	**118-79-6**
	23	Tris(2-Chloroethyl)Phosphate	**TCEP**	**115-96-8**
	24	Tris(2-Chloroisopropyl)Phosphate	**TCPP**	**13674-84-5**
	25	2,4,4'-Tribromodiphenyl ether	**BDE-28**	**41318-75-6**

	26	Tris(1,3-Dichloro-2-Propyl)Phosphate	**TDCPP**	**13674-87-8**
	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**
	35	2,2’,4,4’,5,6’-Hexabromodiphenyl ether	**BDE-154**	**207122-15-4**
	36	2,2’,3,4,4’,5’,6-Heptabromodiphenyl ether	**BDE-183**	**207122-16-5**
	37	1,2-Bis (2,4,6 Tribromophenoxy) ethane	**BTBPE**	**37853-59-1**
Antioxidants	38	6,6'-di-tert-butyl-2,2'-thiodi-p-cresol	**Irganox® 1081**	**90-66-4**
	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 – plasticisers - stabilisers	48	4-Tert-Octylphenol	**4-t-OP**	**140-66-9**
	49	Nonylphenol	**NPs**	**84852-15-3**
	50	4-nonylphenol	**4-NP**	**104-40-5**
	51	Nonylphenol Monoethoxylate	**NP1EO**	**27986-36-3**
	52	Bisphenol F	**BPF**	**620-92-8**
	53	4-Nonylphenol Monoethoxylate	**4-NP1EO**	**104-35-8**
	54	Bisphenol A	**BPA**	**80-05-7**
	55	Bisphenol B	**BPB**	**77-40-7**
	56	Nonylphenol diethoxylate	**NP2EO**	**N/A**
	57	Bisphenol S	**BPS**	**80-09-1**

Results

Virgin polyethylene pellets were composed of 5 additives (Figure 1), including 4 plasticisers (Dimethyl phthalate, DMP; Diethyl phthalate, DEP; Di-n-butyl phthalate, DBP; Diisobutyl phthalate, DIBP), 1 antioxidant (Bisphenol S, BPS).

In contrast, beached pellets contained 7 additives (Figure 1), the same as virgin pellets with two additional antioxidants, *i.e.,* Butylated hydroxytoluene (BHT) and nonylphenol (NPs). In addition, the quantity of additives in Beached pellets was higher than that measured in virgin pellets, *i.e.,* 2-fold for Bisphenol S (BPS), Diisobutyl phthalate (DIBP) and Dimethyl phthalate (DMP), 3-fold for Diethyl phthalate (DEP) and 4-fold for Di-n-butyl phthalate (DBP).

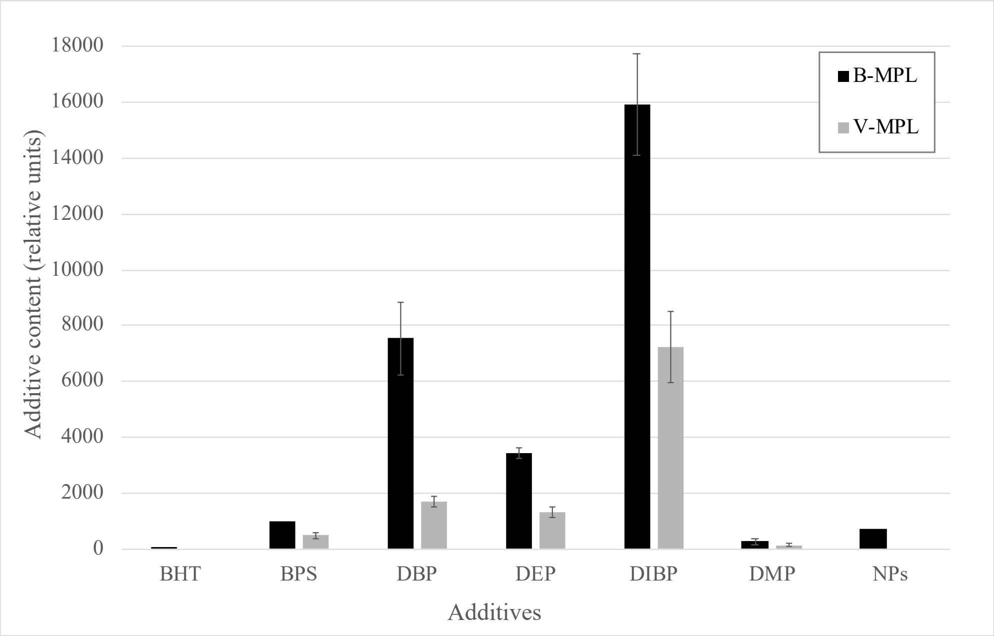
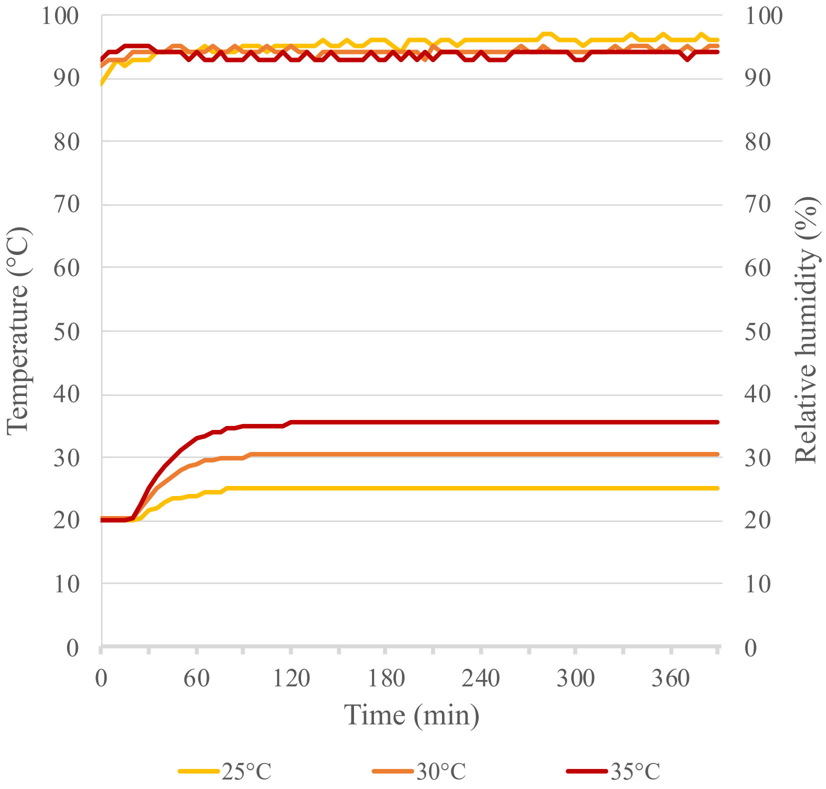


Fig. 1. Additive content of beached (black) and virgin (grey) low-density polyethylene pellets (mean ± standard deviation; n = 3). BHT: Butylated hydroxytoluene; BPS: Bisphenol S; DBP: Di-n-butyl phthalate; DEP: Diethyl phthalate; DIBP: Diisobutyl phthalate; DMP: Dimethyl phthalate; NPs: Nonylphenols.

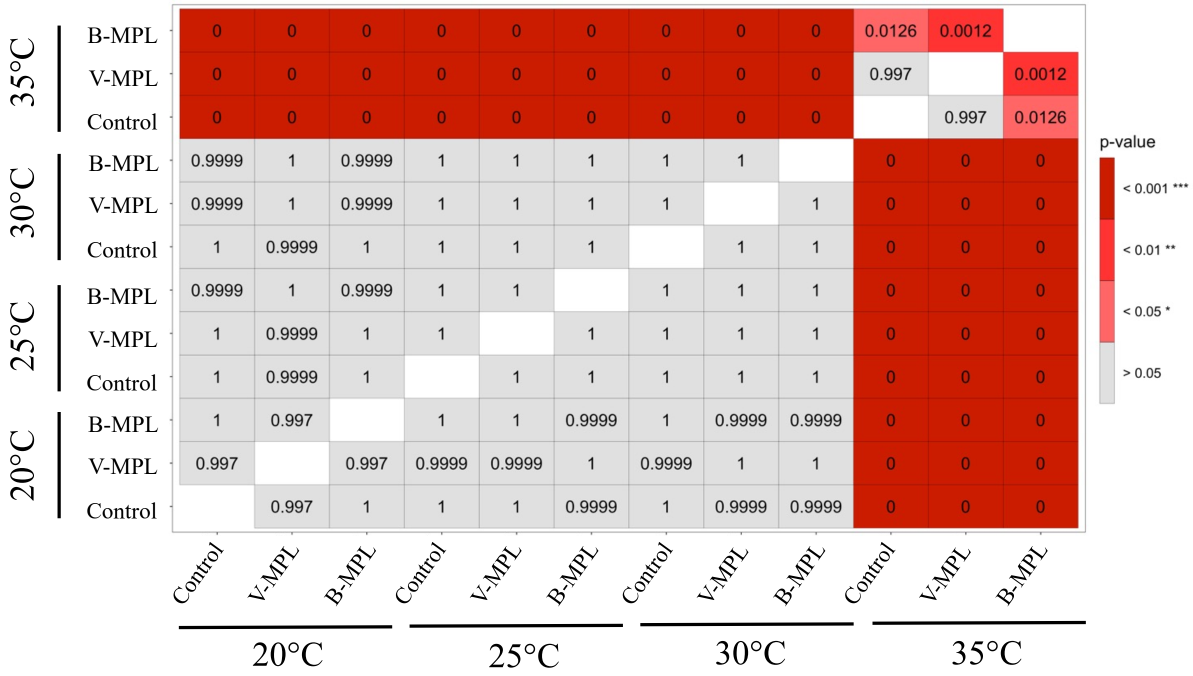
S3: Details of the data, i.e. temperature and relative humidity, recorded by the HygroButtons for a 6 h emersion at 25, 30 or 35°C from a temperature of 20°C. The relative humidity is around 95% for the 25, 30, 35°C emersion. The emersion temperature stabilises at 20°C after 80 min, at 25°C after 100 min and at 35°C after 120 min.



S4: Results of 2-way ANOVA applied to the mussel survival rates with Temperature (20, 25, 30, 35°C) and Solution (control seawater, virgin and beached MP leachates) as fixed factors.

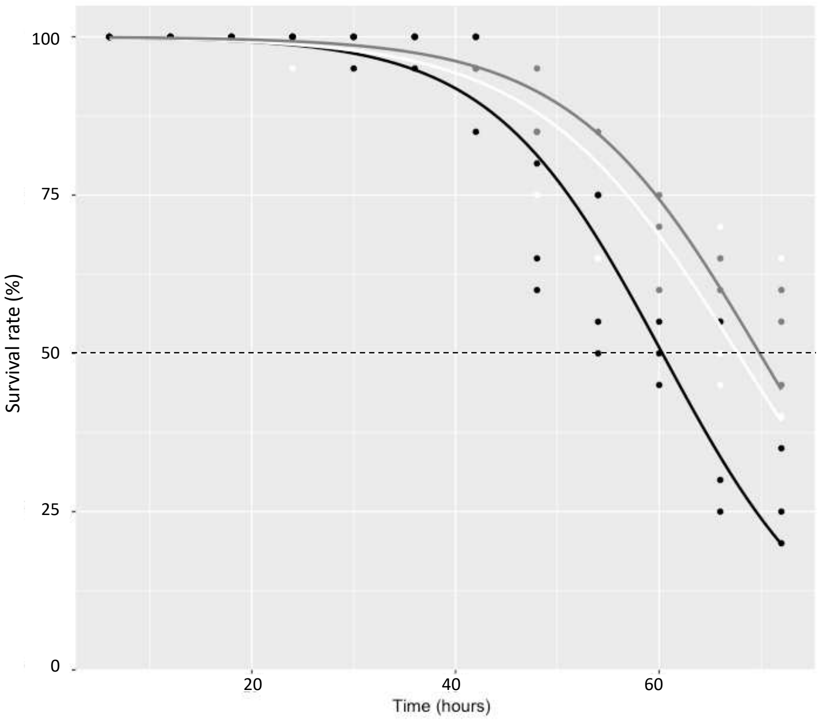
Source of variation	df	MS	*F*	p	
Temperature	3	6671	168.5	2.96e-16	***
Solution	2	172	4.33	0.02473	*
Temperature × Solution	6	154	3.9	0.00747	**
Residuals	24	40			

S5: Results of Tukey post-hoc test applied to the 2-way ANOVA significant interaction ‘Temperature × Solution’ (*see S3*). *P*-values are rounded to 4 decimal places.



		20°C			25°C			30°C			35°C		
		Control	V-MPL	B-MPL	Control	V-MPL	B-MPL	Control	V-MPL	B-MPL	Control	V-MPL	B-MPL
35°C	B-MPL	0	0	0	0	0	0	0	0	0	0.0126	0.0012	
	V-MPL	0	0	0	0	0	0	0	0	0	0.997		0.0012
	Control	0	0	0	0	0	0	0	0	0		0.997	0.0126
30°C	B-MPL	0.9999	1	0.9999	1	1	1	1	1		0	0	0
	V-MPL	0.9999	1	0.9999	1	1	1	1		1	0	0	0
	Control	1	0.9999	1	1	1	1		1	1	0	0	0
25°C	B-MPL	0.9999	1	0.9999	1	1		1	1	1	0	0	0
	V-MPL	1	0.9999	1	1		1	1	1	1	0	0	0
	Control	1	0.9999	1		1	1	1	1	1	0	0	0
20°C	B-MPL	1	0.997		1	1	0.9999	1	0.9999	0.9999	0	0	0
	V-MPL	0.997		0.997	0.9999	0.9999	1	0.9999	1	1	0	0	0
	Control		0.997	1	1	1	0.9999	1	0.9999	0.9999	0	0	0

S6: Observed (circles) and fitted (solid curves) trends of the *M. edulis* survival rate after a 35°C aerial HS when exposed to control seawater (Control, in white), virgin (V-MPL, in grey) and beached microplastic leachate (B-MPL, in black) solutions. Curves were estimated using a generalised linear model (glm) with a binomial distribution and a "loggit" link function.



S7: Results of the Ratio test to compare the LT50 estimated for the three solutions, *i.e.* control seawater, virgin microplastic leachates (V-MPL) and beached microplastic leachates (B-MPL) after a 35°C aerial HS.

Comparison	Standard error	Z-test statistic	p	
Control – V-MPL	0.00994	1.40	0.162	
Control – B-MPL	0.00856	7.09	1.32e-12	***
V-MPL – B-MPL	0.00850	8.77	1.73e-18	***

CHAPTER III

MICROPLASTIC LEACHATES DISRUPT THE CHEMOTACTIC AND CHEMOKINETIC BEHAVIOURS OF AN ECOSYSTEM ENGINEER (*MYTILUS EDULIS*)

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

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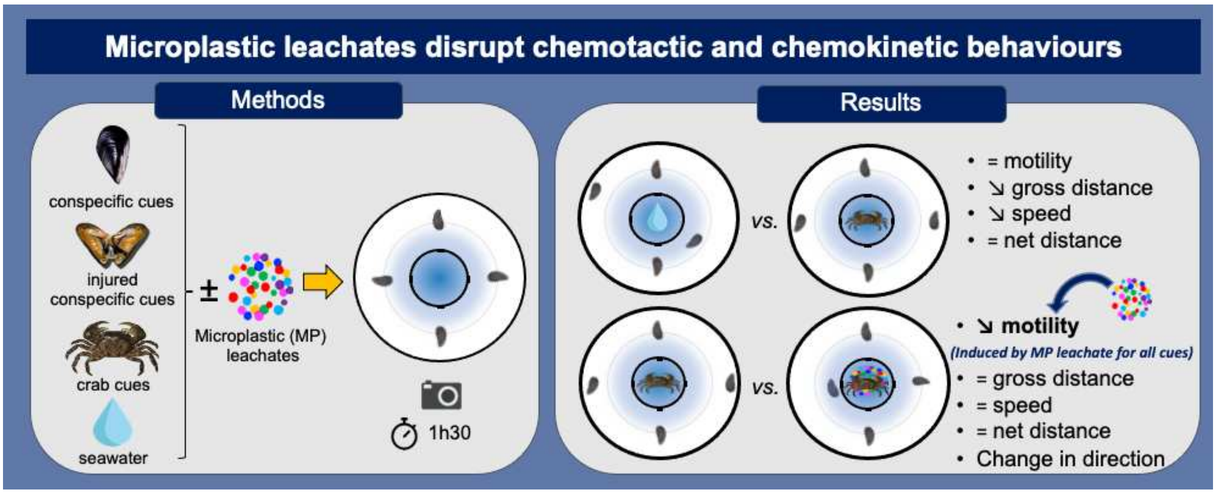
Graphical abstract



Abstract

The massive contamination of the environment by plastics is an increasing global scientific and societal concern. Knowing whether and how these pollutants affect the behaviour of keystone species is essential to identify environmental risks effectively. Here, we focus on the effect of plastic leachates on the behavioural response of the common blue mussel *Mytilus edulis*, an ecosystem engineer responsible for the creation of biogenic structures that modify the environment and provide numerous ecosystem functions and services. Specifically, we assess the effect of virgin polypropylene beads on mussels' chemotactic (*i.e.* a directional movement in response to a chemical stimulus) and chemokinetic (*i.e.* a non-directional change in movement properties such as speed, distance travelled or turning frequency in response to a chemical stimulus) responses to different chemical cues (*i.e.* conspecifics, injured conspecifics and a predator, the crab *Hemigrapsus sanguineus*). In the presence of predator cues, individual mussels reduced both their gross distance and speed, changes interpreted here as an avoidance behaviour**.** When exposed to polypropylene leachates, mussels moved less compared to control conditions, regardless of the cues tested. Additionally, in presence of crab cues with plastic leachates, mussels significantly changed the direction of movement suggesting a leachate-induced loss of their negative chemotaxis response. Taken together, our results indicate that the behavioural response of *M. edulis* is cue-specific and that its anti-predator behaviour as well as its mobility are impaired when exposed to microplastic leachates, potentially affecting the functioning of the ecosystem that the species supports.

1. Introduction

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

Intertidal mussels are considered key ecosystem engineers on both soft and hard substrates (Borthagaray & Carranza, 2007; Reise, 2002). Noticeably, these organisms are motile; they use their foot both to move and attach themselves to their substrate through the production of byssal threads, extracellular collagenous structure (Schneider et al., 2005; Waite et al., 2005). Both their motility and byssal production allow them to spatially self-organise into dense mono- and multi-layered beds, which decrease the vulnerability to predation, desiccation, heat, and wave dislodgement, while increasing fertilisation success and survival (Iwasaki, 2015; Nicastro et al., 2012; Zardi et al., 2021) .Through the formation of beds, intertidal mussels locally dominate rocky shores and operate a biogenic transformation of the habitat (Menge & Sutherland, 1987; Paine & Levin, 1981; Reise, 2002). These relatively stable and resilient structures enhance local biodiversity by facilitating the establishment and maintenance of a range of different species (Arribas et al., 2014; Borthagaray & Carranza, 2007; Palomo et al., 2007; Reise, 2002; Spilmont et al., 2018). Beyond their ecological role, mussels also play an important economic and heritage value through professional and recreational fishing, *e.g.* global aquaculture production for *Mytilus edulis* and *M. galloprovincialis* was 287,957 tons in 2016 (FAO Fishstat) with an estimated net worth ranging between US\$2.5 10^6 and US\$100 10^6 in the United States only (Zippay & Helmuth, 2012).

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

Chemotaxis (*i.e.* a directional change in movement in response to a chemical stimulus) and chemokinesis (*i.e.* a non-directional change in movement properties such as speed, distance travelled or turning frequency in response to a chemical stimulus) are key behavioural processes involved in the response to chemical cues (Bell & Tobin, 1982; Wilkinson, 1985). Only a few studies have addressed chemotaxis in bivalves and interactions with conspecifics, *i.e.* De Vooys (2003) with *M. edulis* and Huang et al. (2007) *with Tridacna squamosa*, and between a bivalve (*Montacuta ferruginosa*) and its commensal species (*Echinocardium cordatum*; Morton, 1962).

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

This issue is, however, particularly relevant given the growing awareness of the ubiquity and toxicity of plastic compounds and their leachates in marine systems (Gall & Thompson, 2015; Gunaalan et al., 2020; Jamieson et al., 2017). Plastic pollution is one of the main challenges of the 21st century through its deleterious physical and chemical effects on aquatic life (Anderson et al., 2016; Auta et al., 2017; Derraik, 2002; Gall & Thompson, 2015; Rochman, 2015; Sussarellu et al., 2016), leading to the death of millions of aquatic organisms annually (Ocean Conference United Nations, 2017). These effects can be due to either macroplastic (> 5 mm), microplastic (< 5 mm) and nanoplastic (< 100 nm) items either produced directly in the smaller form industrially or resulting from the breakdown of larger plastic items (Cole et al., 2011; Paul-Pont et al., 2018). Plastic pollution also has a far more pernicious effect on marine life through the release of various molecules that are either absorbed (i.e. intrinsically bounded) to the polymer during the manufacturing process (e.g. light and heat stabilisers, antioxidants, nucleating and antistatic agents, flame retardants, plasticisers and colourants) or adsorbed at the surface of a polymer such as persistent organic pollutants, which include polycyclic aromatic hydrocarbons, polychlorinated biphenyls, polybrominated diphenyl ethers, pesticides or heavy metals (Delaeter et al., 2022). Recent studies have shown that direct exposure to either microplastics (Crump et al., 2020) or microplastic leachates (Seuront, 2018) can interfere with the cognitive system, sensory perception, and thus behaviour of marine invertebrates. For instance, the intertidal mussel *M. edulis* behaviourally responds to microplastic leachates through an increase in aggregation rate and frequency (Seuront et al., 2021). However, despite the ever-increasing concern about the ecological impact of plastic leachates (Delaeter et al., 2022; Gunaalan et al., 2020), it is still unknown whether plastic leachates interfere with conspecific or prey-predator chemical signals in bivalves in general, and in intertidal mussels in particular.

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

2. Material and Methods

Study organisms

Both individual *Mytilus edulis* (1.5–2.0 cm in shell length; Supplementary Materials, S8) and *H. sanguineus* (1.4–2.4 cm in length; Supplementary Materials, S8) were sampled in March 2021 at low tide on a rocky intertidal reef of the infra-littoral zone of the Fort de Croy

(Wimereux, France; 50°45'52.3"N, 1°35’55.1"E) along the French coast of the English Channel.

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

Chemical cues

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

Conspecific cue seawater was prepared through the addition of 20 mussels which were placed for a 24 h period into 1 L of aerated seawater. Injured conspecific cue seawater was prepared through the addition of 20 injured mussels previously crushed with a stone, to 1 L of aerated seawater for a 24 h period. Crab cue seawater was prepared through the addition of 3 males and 3 females *Hemigrapsus sanguineus* (sex ratio of 1:1) into 1L of aerated seawater for a 24 h period. For more information about exact sizes, see Supplementary Materials, S8.

For the mixed cues experiments, commercially available polypropylene pellets (typically 3.3–4.7 mm in diameter; Pemmiproducts, Aachen, Germany) were added to the previous treatment (*i.e.* natural seawater, conspecific, injured conspecific and crab cues). They were left 24 h in aerated tanks at a concentration of 12 g of pellets per litre (ca. 600 MPs per L, or equivalently 20mL of MPs per L; Seuront, 2018; Seuront et al., 2021), in order to have four new solutions: seawater with leachates, conspecific cues with leachates, injured conspecific cues with leachates and crab cues with leachates. As a first approximation of the nature of the additive content of our polypropylene pellets, we ran a pyrolysis analysis coupled to a gas chromatography and a high-resolution spectrometer (see Supplementary Materials, S9), which led to the identification of 2 antioxidants (*i.e.* 4-tert-octylphenol, 4tOP, and Butyl hydroxytoluene, BHT) and 6 plasticisers (*i.e.* Diethyl phthalate, DEP, Diisobutyl phthalate, DIBP, Dibutyl phthalate, DBP, Bis(2-ethylhexyl)phthalate, DEHP, Di-n-octyl phthalate, DIOP, and Diisononyl phthalate, DINP).

Chemotactic and chemokinetic assay

Both acclimation and experiments were consistently carried out under the same controlled conditions. Temperature was measured at the beginning and at the end of the experiment. Before the start of each experiment, byssal threads were carefully cut with scissors to separate the mussels from each other and from the substrate. Behavioural experiments were conducted in 22 cm diameter glass arenas with smooth, featureless surfaces (Figure 25). To infer the presence of a chemotactic and/or chemokinetic response of the individual mussel to the different cues, their positions were recorded with respect to a point source. This point source was obtained by four equidistant holes (0.5 cm in diameter) drilled through the side of a clay pot (6.5 cm in diameter; Figure 25) which had a top central hole. Arenas were filled with 600 mL unfiltered seawater saturated in oxygen. Five milliliters (5 mL) of either control or treatment seawater was injected in a clay pot through the top hole at a rate of 0.7 mL s^{-1}. Thus, the concentration of MP leachates, which is the same for all solutions containing plastic leachates, that flows out of the clay pot, was estimated at 1.66 mL L^{-1} (50 MPs per litre). Once fully mixed in the arena, this concentration reached 0.166 mL L^{-1} (5 MPs per litre), a concentration consistent with those found in coastal waters, *i.e.* 3.5 ± 2.0 MPs L^{-1} with concentrations between 1 and 6.4 MPs per wild *M. edulis* (United Kingdom; Li et al., 2018). Immediately after the treatment or control injection, each specimen was placed individually at a distance of 4 cm from each side hole of the pot (n = 4 mussels; Figure 25). Video recording started immediately after the injection of water cue in the clay pot, at a rate of 1 frame per minute for 1h30 using a GoPro camera (GoPro HERO7 Black, GoPro Inc., San Mateo, California, USA) placed 40 cm above the experimental container. The choice of using four mussels per arena was made to minimise aggregations between mussels, which decreases with the distance between the mussels (Côté & Jelnikar, 1999; Nicastro et al., 2007).

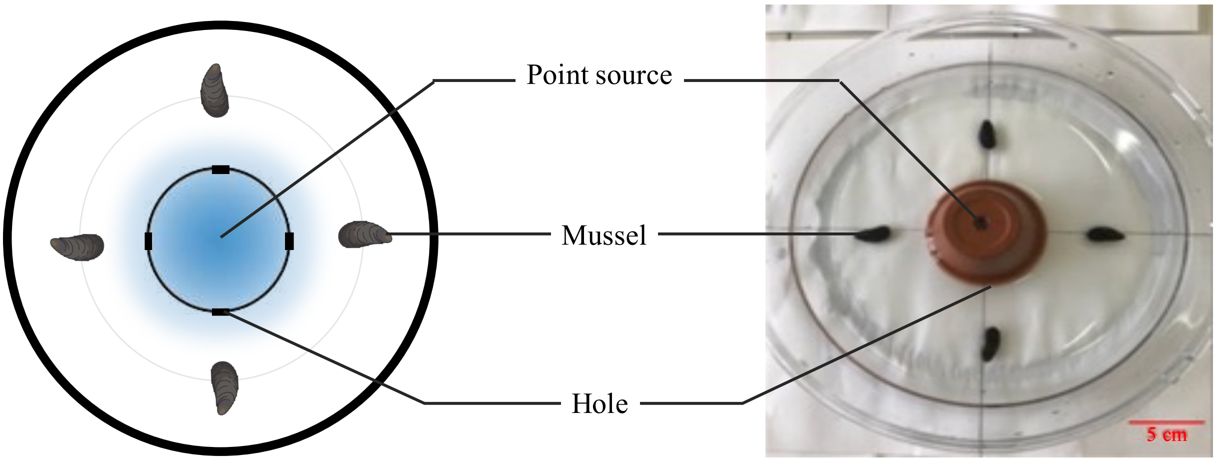
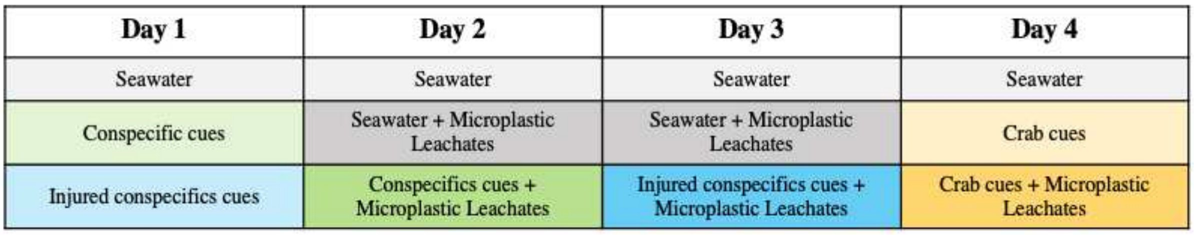


Figure 25: Experimental design of one arena of the chemotaxis and chemokinesis experiments. Mussels were placed at equidistant distances from each other at 4 cm from the holes in a glass arena. To see how the liquid diffuses from the point source clay pot, see Supplementary Materials, S10.

Each day, three treatments were run concurrently: seawater (control; n = 6 arenas) and two different treatments (n = 6 arenas for each treatment; Table 2; Figure 26). Six additional arenas using seawater as control were also performed. Thus, the sample size for each cue was: seawater n = 30 arenas; seawater with leachates n = 12 arenas and n = 6 arenas all other cues tested with and without plastic leachates (see the experimental plan; Table 2; Figure 26). An additional control was also performed. Each mussel was only used once and then discarded, no mussels showed injury or mortality signs during the experiment.

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



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

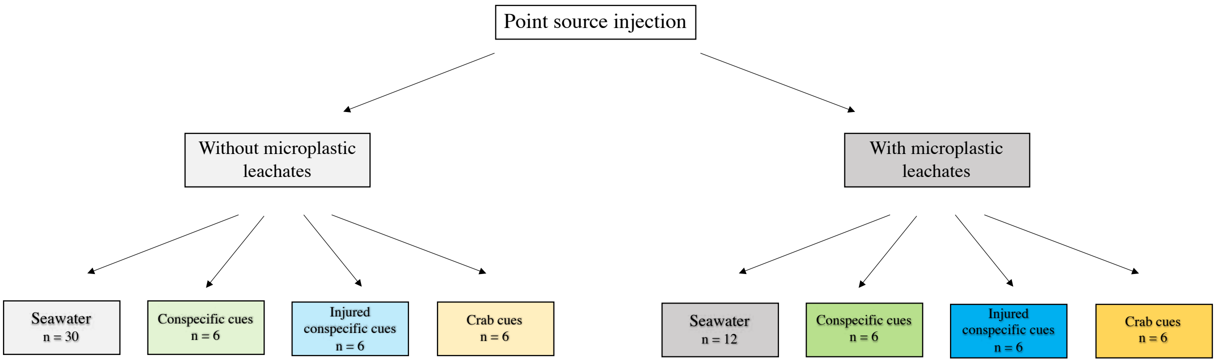


Figure 26: Flow diagram of the treatments and their replications. Stimuli without plastic leachates (light colour): (grey) Control, natural seawater, i.e. no stimulus; (green) stimuli from conspecifics Mytilus edulis; (blue) stimuli from injured M. edulis; (orange) stimuli from fed Hemigrapsus sanguineus; stimuli with microplastic (MP) leachates (dark colour).

Behavioural analysis

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

First, motility was assessed by comparing the proportion of motile and non-motile mussels, to have an average percentage of mussel motility per arena. Chemokinesis was subsequently assessed through mean speed, gross distance (*i.e.* the total distance covered by the mussel between its initial and final position), net distance and confinement index (*i.e.* the ratio between net distance and gross distance) per arena. These parameters were measured only for mussels moving a net distance above 1 cm (i.e. approximately half mussel's shell length) using the TrackMate plugin of ImageJ (Tinevez et al., 2017). Note that the choice of a net distance greater than 1cm was specifically made because distances less than 1 cm were assigned by TrackMate to mussels oscillating around their centre of mass without actually actively moving as their foot was never observed outside their valves (see Supplementary Material, S11). Chemotaxis was estimated by recording the final motile mussel position with reference to the source point, in order to have an average percentage of mussels with a negative chemotaxis (repulsion from the source point) per arena.

Statistical analyses

To compare differences among all treatments and to take into account the potential confounding effect of running treatments on different days, the motility, speed, gross distance, net distance, confinement index and chemotaxis of mussels, observed in the control seawater, were compared between days with an ANOVA (for more details, see Supplementary Material, S12). As no significant differences ($p > 0.05$; see Supplementary Material, S12) were found among control replicates run on different days, behavioural data for each treatment from controls and treatments were pooled across days for further analyses.

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

In order to measure the speed, gross distance, net distance, confinement index and chemotaxis, only mussels with a net distance greater than 1 cm (about half a body length) were considered, hence the sample size per treatment was: seawater $n = 27$, seawater with leachates $n = 12$ and $n = 6$ for the other treatments. The distribution of speed, gross distance, net distance and confinement index measurements were tested for normality (Shapiro's test, $p = 0.00005$, $p = 0.00004$, $p = 0.070$, $p = 0.406$, respectively) and homogeneity of variance (Levene's test, $p = 0.141$, $p = 0.616$, $p = 0.582$, $p = 0.060$, respectively). As all parameters showed a homogeneity of variance, but were not always normally distributed, parametric analyses were consistently used as described above. A 2-way ANOVA with treatment (control or microplastic leachates) and cues (seawater, conspecifics, injured conspecifics or crabs) as fixed factors was finally performed and significant effects were further examined using a Tukey-HSD post-hoc test.

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

3. Results

3.1. Chemokinesis

Motility

Polypropylene leachates inhibited significantly mussel motility for all cues tested (Treatment; p = 0.043; Figure 27, Table 3). Specifically, a significantly higher percentage of mussels moved in the control treatment (no leachate) than when they were exposed to microplastic leachates (Tukey's test: p = 0.049).

Table 3: Results of 2-way ANOVA applied to the percentage of motile mussels with Treatment (control or microplastic leachates) and Cue (seawater, conspecifics, injured conspecifics or crabs) as fixed factors.

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

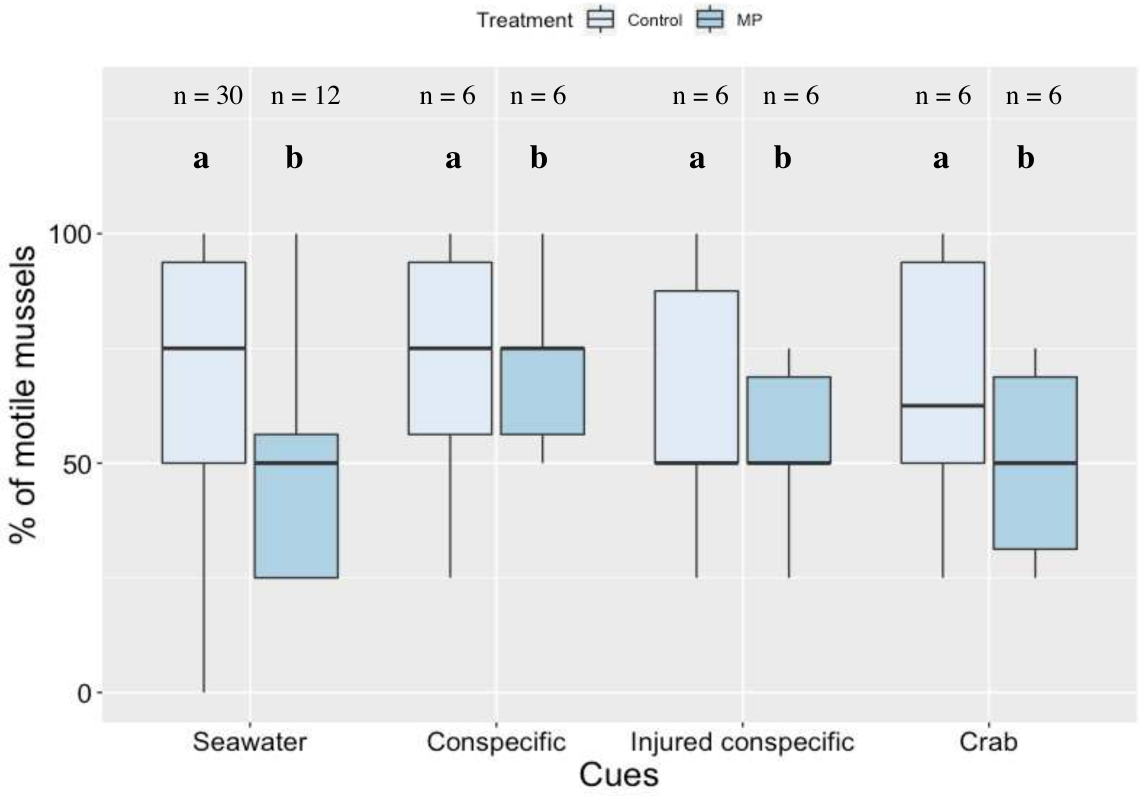


Figure 27: Percentage of motile *Mytilus edulis* by replicate in control seawater and natural cue treatments (light blue) and microplastic leachates treatments (dark blue). Significant differences ($p < 0.05$; Tukey-HSD) between treatments were identified by different letters and the number, n, of arenas used was also indicated.

Speed

There was a significant effect of Cue (Cue; $p = 0.005$; Figure 28, Table 4). Specifically, regardless of the treatment, mussels always moved significantly slower when exposed to crab cues than when exposed to conspecific cues or to control seawater (Tukey's test: $p = 0.043$ and $p = 0.002$ respectively). However, the speed of mussels exposed to conspecific cues, injured conspecific cues and control seawater did not significantly differ (Seawater; Tukey's test: $p > 0.05$).

Table 4: Results of 2-way ANOVA applied to the mean speed by replicate with Treatment (control or microplastic leachates) and Cue (seawater, conspecifics, injured conspecifics or crabs) as fixed factors.

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

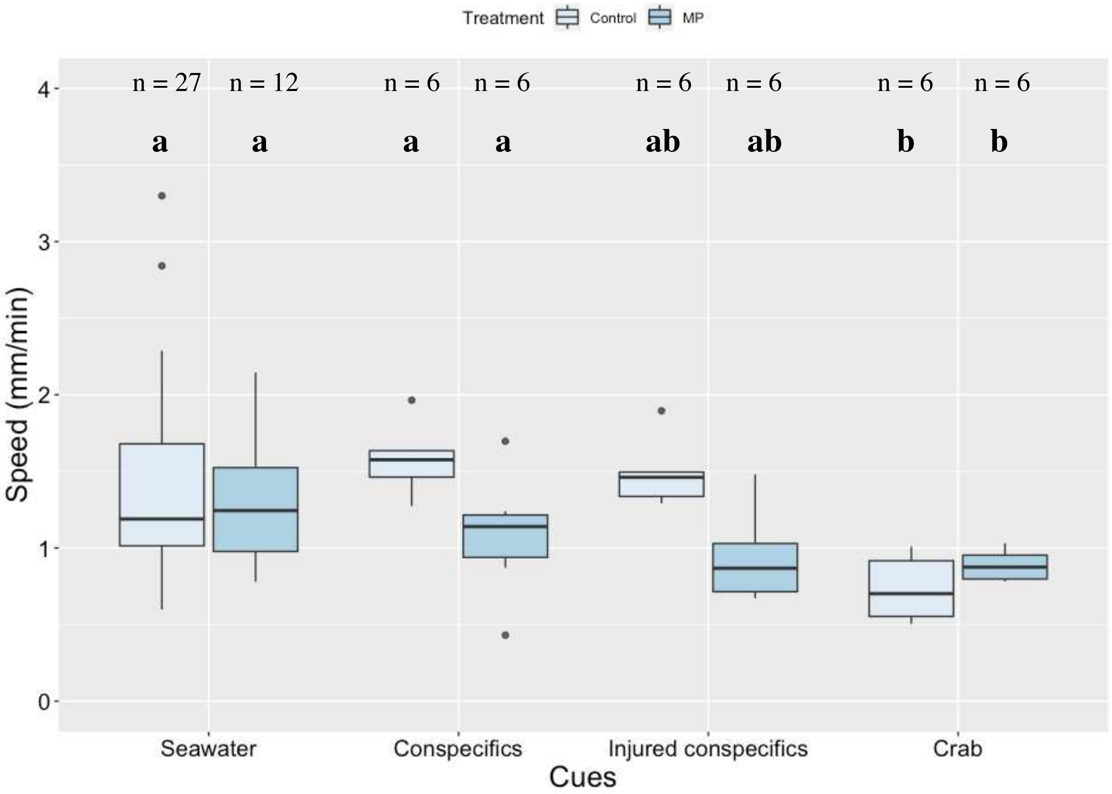


Figure 28: Mean speed of *Mytilus edulis* by replicate in control seawater and natural cue treatments (light blue) and microplastic leachates treatments (dark blue). Significant differences ($p < 0.05$; Tukey-HSD) between treatments were identified by different letters and the number of arenas used was also indicated (n =).

Gross Distance

The gross distance travelled by mussels varied significantly depending on the Cue (Cue; p = 0.0498; Figure 29, Table 5). Specifically, regardless of the presence of leachates, the gross distance was always lower in mussels exposed to crab cues than when exposed to sea water

(Tukey's test: $p = 0.032$ respectively). There were no significant differences for the mussel speed when exposed to conspecific cues, injured conspecific cues and the control (Seawater; Tukey's test: $p > 0.05$).

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

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

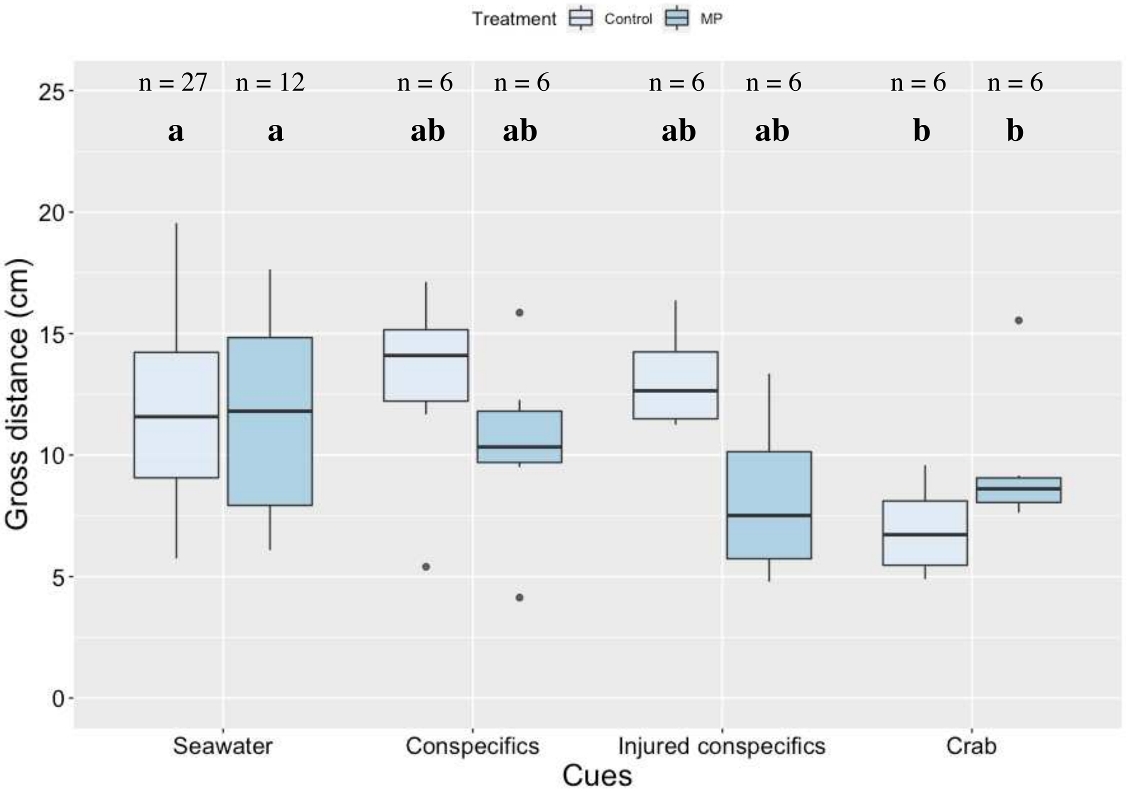


Figure 29: Mean gross distance travelled by *Mytilus edulis* by replicate in control seawater and natural cue treatments (light blue) and microplastic leachates treatments (dark blue). Significant differences ($p < 0.05$; Tukey-HSD) between treatments were identified by different letters and the number of arenas used was also indicated (n =).

Net Distance and Confinement index

There was a significant effect of the Treatment × Cue interaction (Treatment × Cue; p = 0.036; Figure 30, Table 6). However, the Tukey's test revealed no significant differences (p > 0.05) for the net distance traveled by mussels between every cue and treatment, even if a marginal difference was found between conspecifics with and without microplastic leachates (p = 0.094).

Table 6: Results of 2-way ANOVA applied to the mean net distance by replicate Treatment (control or microplastic leachates) and Cue (seawater, conspecifics, injured conspecifics or crabs) as fixed factors.

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

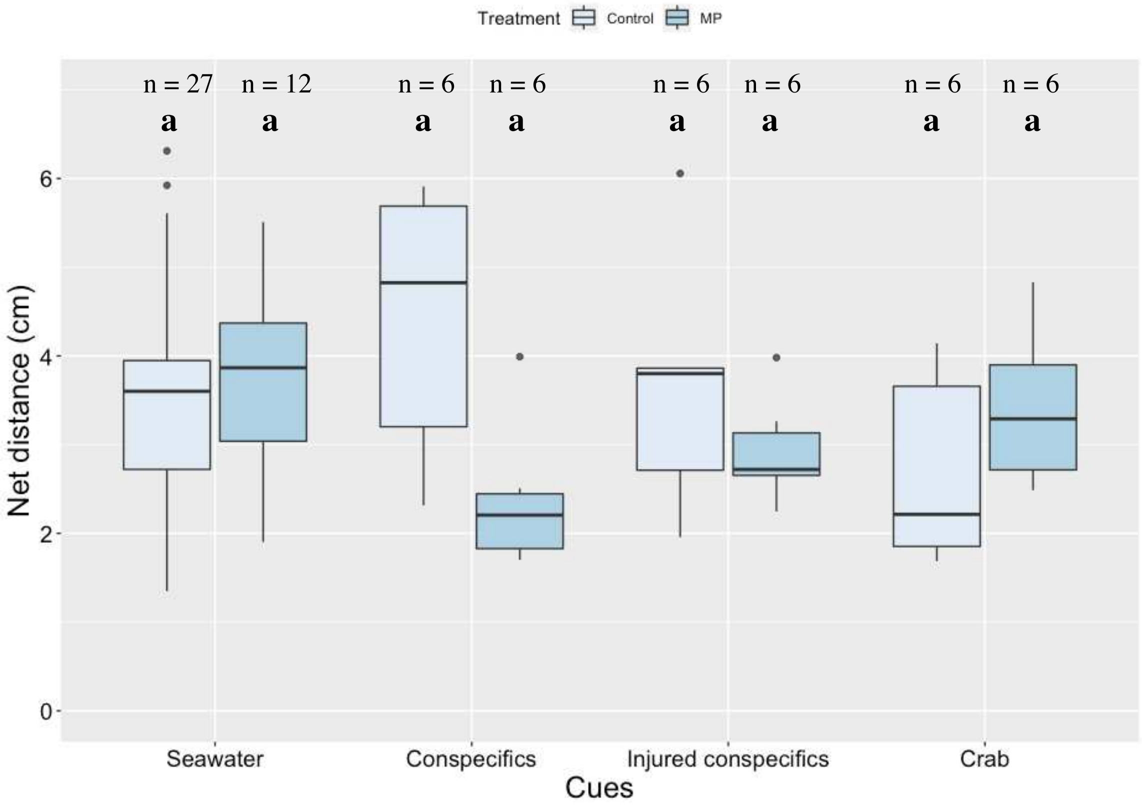


Figure 30: Mean net distance travelled by *Mytilus edulis* by replicate in control seawater and natural cue treatments (light blue) and microplastic leachates treatments (dark blue). Significant differences (p < 0.05; Tukey-HSD) between treatments were identified by different letters and the number of arenas used was also indicated (n =).

The confinement index did not differ among factors nor treatments (Table 7).

Table 7: Results of 2-way ANOVA applied to the mean confinement index by replicate with Treatment (control or microplastic leachates) and Cue (seawater, conspecifics, injured conspecifics or crabs) as fixed factors.

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

3.2. Chemotaxis

There was a significant effect of the Treatment × Cue interaction on the mussel chemotaxis (Treatment × Cue; p = 0.038; Figure 31, Table 8), as significantly more mussels showed a negative chemotaxis to crab cue without microplastic leachates than with microplastic leachates (Tukey's test: p = 0.047).

Table 8: Results of 2-way ANOVA applied to the percentage of mussels moving away from the source point with Treatment (control or microplastic leachates) and Cue (seawater, conspecifics, injured conspecifics or crabs) as fixed factors.

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

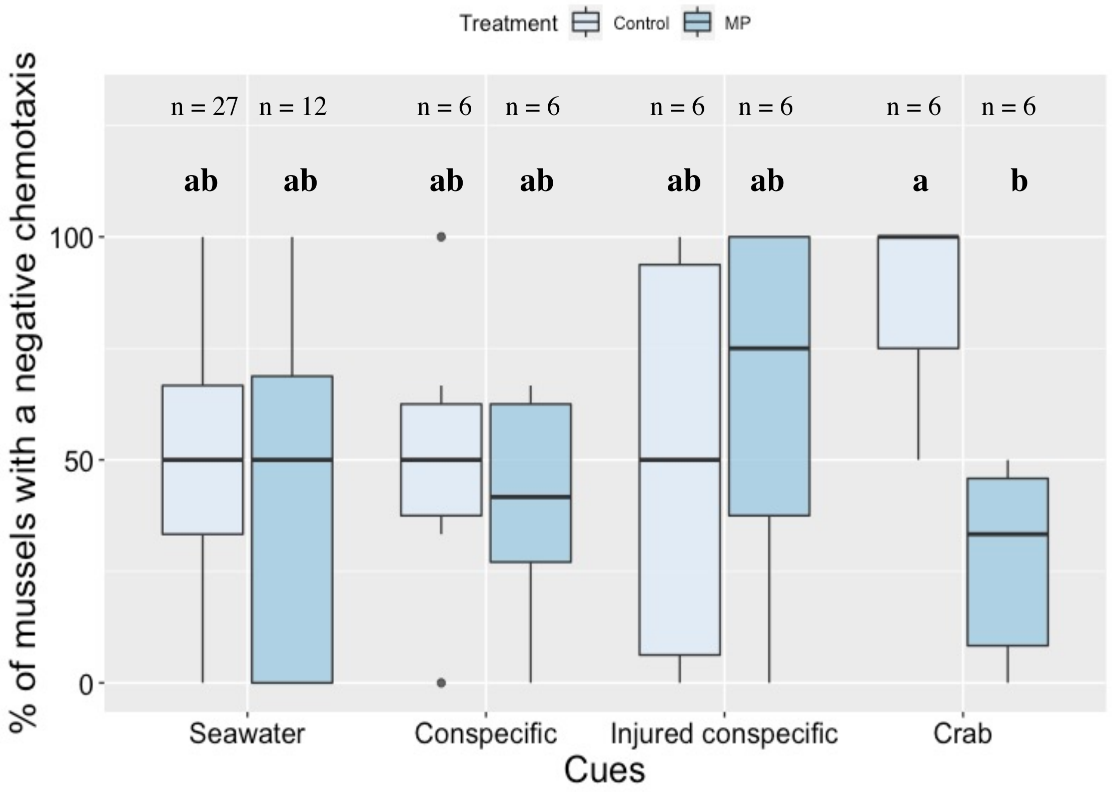


Figure 31: Percentage of *M. edulis* moving away from the point source by replicate in control seawater and natural cue treatments (light blue) and microplastic leachates treatments (dark blue). Significant differences ($p < 0.05$; Tukey-HSD) between treatments were identified by different letters and the number of arenas used was also indicated (n =).

4. Discussion

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

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

Lack of behavioural response towards conspecific cues

Mytilus edulis did not show any behavioural change in response to conspecific cues, in accordance with previous work on the effect of conspecific cues on *M. edulis* aggregation patterns (Commito et al., 2016). These observations are, however, in contrast with previous studies which showed that positive chemotaxis was the main driver of conspecific aggregation in bivalves (Huang et al., 2007) and in particular in *M. edulis* (de Vooys, 2003). These discrepancies may, however, be related to (i) potential behavioural divergence in *M. edulis* between those living on the bottom of a concrete ditch with running seawater or on beach groynes as seen in the Netherlands (de Vooys, 2003) with those living on natural rocky shores habitats as seen in France (present work) and/or (ii) the presence of a seasonal component in the behavioural response of *M. edulis* to conspecific cues (de Vooys, 2003). Though the identification of the mechanisms at the origin of the above-mentioned behavioural differences lies well beyond the scope of the present work, it warrants the need for further work.

Lack of behavioural response toward injured conspecific cues

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

Crab cues induce a chemokinetic and potentially a chemotactic response in Mytilus edulis

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

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

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

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

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

Microplastic leachates alter M. edulis chemokinetic behaviour

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

Thus, in seawater contaminated with leachates, mussels were significantly less motile than in the control seawater treatment. This contrasts with the behaviour of the mollusc *Littorina littorea*, which was not affected by the presence of leachates from virgin polypropylene microbeads, although leachates concentration was higher (*ca.* 10- to 100-fold higher) than in our study (Seuront, 2018). This observation highlights distinct species chemosensitivity to plastic leachates.

Microplastic leachates alter M. edulis response to conspecific cues

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

Microplastic leachates alter M. edulis response to injured conspecific cues

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

Microplastic leachates alter M. edulis response to crab cues

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

The impairments described above are in line with previous work showing that phthalates (DEHP, DBP, DIBP) can alter the neurological behaviour in nematodes (Tseng et al., 2013). Indeed, these are the dominant additives found in our polypropylene beads (Supplementary Materials, S9). Phthalates disrupt the nematode's antioxidant defence system, the morphology of some thermosensory neurons and the genes involved in this thermotaxis function (Tseng et al., 2013), altering their locomotion, *i.e.* body bends, head thrashes, and reversal frequencies (Tseng et al., 2013). In a similar fashion, these additives could induce a neurotoxicity in mussels, which would act on the neurosensory system and lead to an alteration of their movements and chemotactic capacities. A disturbance in *M. edulis* motility and neuro-sensory response could have consequences in aggregation rate and would impact the mussel growth, reproduction and anti-predator responses (Harger, 1968; Schneider et al., 2005; van de Koppel et al., 2008; Zardi et al., 2021). These impairments could lead to changes in mussel bed formations, to a decrease in mussel population and ultimately impact the ecosystem structure and functioning in *M. edulis* (Borthagaray & Carranza, 2007).

5. Conclusion

Taken together, our results show that *M. edulis* behavioural responses are cue-specific. Critically, exposure to plastic leachates altered mussel behavioural responses by altering their motility. The fitness of mussels is directly related to their motility through their ability to aggregate. The impact of microplastic leachates also altered the response of mussels to conspecific cues, which is a direct link to aggregation, and to their crab predator cues. This observation indicates that chemical communication among mussels may be disrupted by plastic leachates, suggesting that aggregation rate and bed formation and also prey-predator interaction may be impacted on the short-term, at least by polypropylene leachates and at the concentration tested here which is consistent to the contamination occurring in the marine environmental (*i.e.* 5 MPs L^{-1}). Prey-predator interactions are essential traits of the ecology of marine systems and variations in the ability to detect predation risk can significantly influence population and community structures (Ferrari et al., 2010). The changes caused by plastic leachates may have important effects on the bioengineering role of *M. edulis* and have cascading effects on the associated communities. Given the predicted increase in plastic pollution and the relative limited information on plastic leachates compared to plastic ingestion (Fauvelle et al., 2021), our work highlights effects of plastic leachates that may have fundamental knock-on effects on the functioning of intertidal ecosystems and the services provided to society. However, before extrapolating our results any further, it is crucial to look at the impact of plastic leachates on the predator behaviour, to see if its predatory performance is also affected or not in a plastic-contaminated environment.

Supplementary Materials

S8: Details of the mean measurements (mm) of the organisms used for the different cue treatments.

	Conspecific	Injured conspecifics	Conspecifics + Leachates	Injured conspecifics + Leachates	Crab
N1	22.26	17.12	20.50	22.20	
N2	23.03	17.23	21.03	22.41	
N3	22.45	22.74	22.50	22.39	
µ	22.56	19.03	21.34	22.33	18.41

S9: Additives identification

Samples preparation

Raw polypropylene pellets were cut using a scalpel under a binocular microscope (Olympus SZX16), to obtain fragments of ca. 500 µm^2. These fragments were subsequently inserted into pyrolysis quartz tubes.

Thermodesorption method for the detection of Organic Plastic Additives (OPAs) using Py-GC-HRMS (Pyrolysis coupled to a gas chromatography and a high-resolution mass spectrometer)

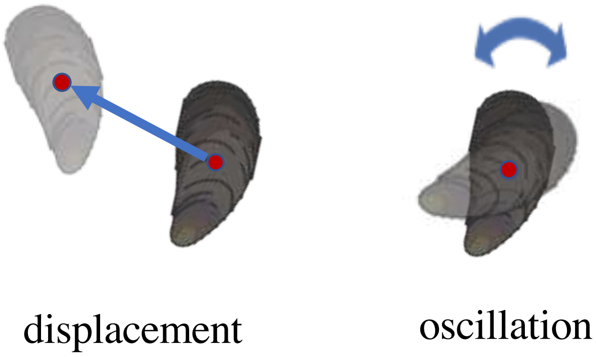
All analyses were carried out using a pyrolyser CDS Pyroprobe 6150 (CDS Analytical) followed by a GC-HRMS device (GC Trace 1310-MS Orbitrap Q exactive, Termo Fisher Scientific). With the aim to thermally desorb the chemicals additives potentially contained into the samples, they were heated into the pyrolyser at a temperature of 350 °C. Samples were subsequently separated on a GC column (Restek Rxi-5-MS capillary column, cross-linked poly 5% diphenyl-95% dimethylsiloxane, 30 m × 0.25 mm (i.d.) × 0.25 µm film thickness), with a split ratio of 1:5. The acquisition was performed on full-scan (FS) mode (m/z = 30.00000–600.00000). The resulting chromatograms were analysed using Xcalibur and TraceFinder softwares to identify OPAs among a selection of additives including plasticisers, flame retardants, antioxidants and UVs stabilizers. The respective additives were identified on the basis of the retention times, m/z, and specific ions after a comparison with the chromatograms of the respective standard solutions.

Identified additives

- 2 antioxidants (*i.e.* 4-tert-octylphenol, 4tOP, and Butyl hydroxytoluene, BHT)
- 6 plasticisers (*i.e.* Diethyl phthalate, DEP, Diisobutyl phthalate, DIBP, Dibutyl phthalate, DBP, Bis(2-ethylhexyl)phthalate, DEHP , Di-n-octyl phthalate, DIOP, and Diisononyl phthalate, DINP)

S10: Picture of a test made to visually show the dispersion of the solution emanating from the clay pot. This test was carried out by mixing water and ink, the amount of this mixture was latter re-evaluated so that the spray only reached half the radius of the arena.

S11: The difference between displacement movement (*i.e.* displacement of the centre of mass = motile mussels) and oscillation movement (*i.e.* no displacement of the centre of mass = non-motile mussel).



S12: Details of the normality after a Shapiro's test, homogeneity after a Levene's test and results of 1-way ANOVAs applied to the control mussel motility, speed, gross distance, net distance, confinement index and chemotaxis with days as fixed factors.

- **Motility**: Normality: p = 0.0157 & Homogeneity: p = 0.7798

```
            Df        Sum Sq   Mean Sq  F value   Pr(>F)
Day          4        2625     656.3    0.736     0.576
Residuals   25        22292    891.7
```

- **Speed**: Normality: p = 0.962 & Homogeneity: p = 0.04005
    - **Speed**: after normalization with log: Normality: p = 0.349 & Homogeneity: p = 0.6044

```
            Df         Sum Sq  Mean Sq  F value   Pr(>F)
Day          4         1.525   0.3812   2.805     0.0507
Residuals   22         2.991   0.1359
```

- **Gross distance:** Normality: p = 0.280 & Homogeneity: p = 0.2693

```
            Df         Sum Sq   Mean Sq  F value   Pr(>F)
Day          4         281.3    70.33    2.787     0.0517
Residuals   22         555.1    25.23
```

- **Net distance:** Normality: p = 0.482 & Homogeneity: p = 0.1592

```
            Df        Sum Sq    Mean Sq  F value   Pr(>F)
Day          4        15.87     3.968    2.67      0.0591
Residuals   22        32.69     1.486
```

- **Confinement index:** Normality: p = 0.179 & Homogeneity: p = 0.6909

```
            Df        Sum Sq    Mean S    F value  Pr(>F)
Day          4        0.0381    0.00953   0.606    0.663
Residuals   22        0.3460    0.01573
```

- **Chemotaxis:** Normality: p = 0.135 & Homogeneity: p = 0.4865

```
            Df         Sum Sq   Mean Sq  F value   Pr(>F)
Day          4         5336     1334     2.046     0.123
Residuals   22         14345    652
```

CHAPTER IV

SIZE-DEPENDENT RESPONSE OF THE MUSSEL COLLECTIVE BEHAVIOUR TO PLASTIC LEACHATES AND PREDATOR CUES

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

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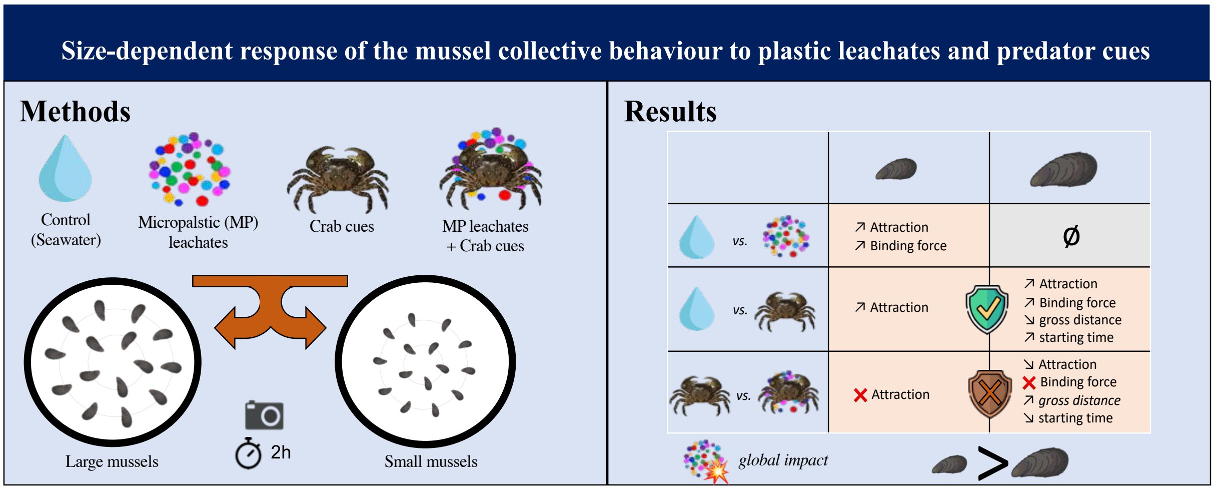
Graphical Abstract



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 implications 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.

1. Introduction

Ecosystems are strongly influenced by prey-predator interactions (Barbosa & Castellanos, 2005; Belgrad & Griffen, 2016; Lima & Dill, 1990). Predation exerts a strong selective pressure on prey, leading to the evolution of numerous anti-predator adaptations (Barbosa & Castellanos, 2005; Dawkins et al., 1979; Lima & 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 & Butman, 2000). Once a predator is detected, prey survival can be optimised by minimising predator encounters and attack success probabilities through gregarious behaviours and various forms of collective dynamics (Hamilton, 1971; Lehtonen & 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 & 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 & Jelnikar, 1999; Kobak et al., 2010; Kong et al., 2019; Nicastro et al., 2007; Reimer & 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 & Litvaitis, 2013) and predation risk (Brousseau et al., 2001; DeGraaf & 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

& 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 are 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, > 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 in fitness, cell, embryogenic development and behaviour, which could have repercussions at the community level (Capolupo et al., 2020, 2021a; 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

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 & Carranza, 2007; Buschbaum et al., 2009). In addition, the species also holds 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 preferred 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 & 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).

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, S13) 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 pellet 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 & 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 pellets 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, S14A. 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, S14B).

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., 2014, 2016; 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, S15). 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 the 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, S16; S17 for examples) placed 40 cm above the experimental arena.

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 nonlinear 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 & Malafaia (2021), de Faria et al. (2018), Freitas et al. (2023) and Guimarães et al. (2023) for further details and Supplementary Material, S18, 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, S19). 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, S20).

Statistical analyses

As the sample sizes were small ($n = 6$), the use of non-parametric tests was preferred (Abdulazeez, 2014; Gibbons & Fielden, 1993; Hoskin, 2012). The normality (Shapiro's test) and heterogeneity of variances (Levene's test) results are given in the Supplementary Material, S21 and S25. 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 mussels, 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 & 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 for the cluster score to avoid a bias as the same quadrat size was used for both small and large mussels (Supplementary Material, S19).

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 S21). 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, S22), 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 S21).

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, S21). 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; Figure 32B and 33B; Supplementary Material, S21). 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, S23, S24). 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.

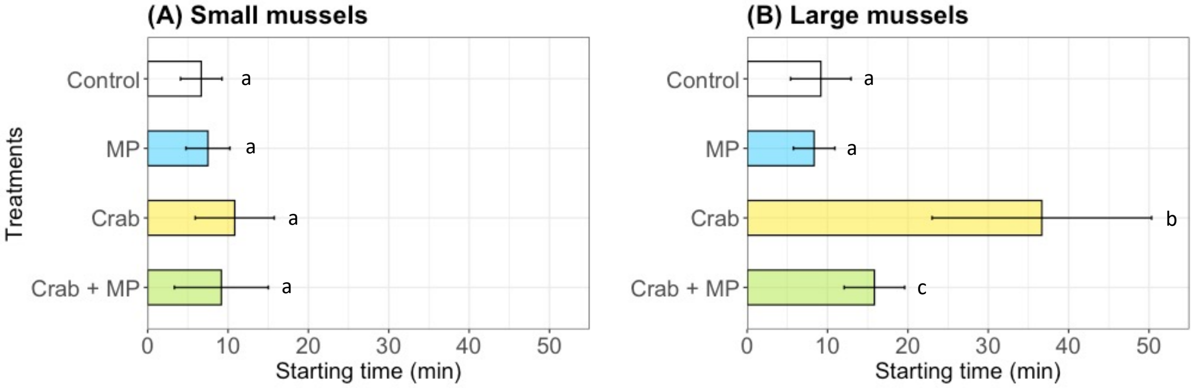


Figure 32: Starting time in minutes (min; mean ± SD) taken by (A) small and (B) large mussels to form the first aggregation in control seawater (white; n = 6), MP leachates (blue; n = 6), crab cues (yellow; n = 6), and 1 : 1 MP leachates and crab cues treatment mixture (green; n = 6). Letters depict significant differences ($p <$ 0.05; Conover's test) among treatments.

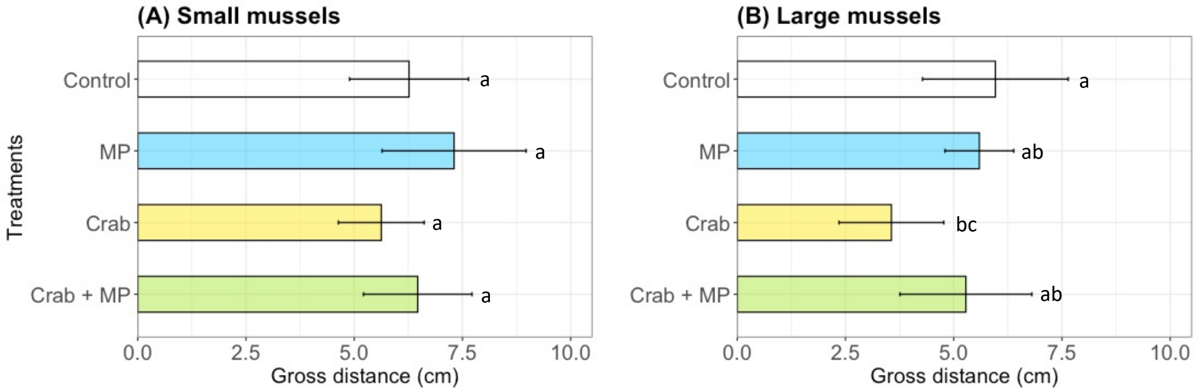


Figure 33: Gross distance in centimetres (cm; mean ± SD) travelled by (A) small and (B) large mussels in control seawater (white; $n = 6$), MP leachates (blue; $n = 6$), crab cues (yellow; $n = 6$), and 1 : 1 MP leachates and crab cues treatment mixture (green; $n = 6$). Letters depict significant differences ($p < 0.05$; Conover's test) among treatments.

Behavioural effect of crab cues

Hemigrapsus sanguineus cues had a significant size-specific effect on the collective behaviour of the blue mussel *Mytilus edulis* (Figures 32 and 33; Supplementary Material S21). 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, $p = 2.4\times10^{-5}$; Figure 32B). Similarly, the average mussel gross distance was significantly lower (*ca.* 2-fold) than in the control treatment (Conover's test, $p = 0.035$; Figure 33B). In contrast to what was 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 S24). Finally, the observed significant positive linear correlation between $A_{\max}$ and byssus thread production ($p = 0.013$; Supplementary Material S24) is indicative of a greater aggregation strength in the presence of crab cues compared to control seawater. In line with what was observed in large mussels, small mussels exhibited a significant (p = 0.028; Supplementary Material S23) 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.

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 (Figure 32B and 33B). 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; Figure 32B), they were highly significantly slower (*ca.* 2.3-fold) to form the first aggregate than when exposed to crab cues alone (Conover's test, p = 0.028; Figure 32B). 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; Figure 33B). 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 S24) 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.

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, S25), 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; Figures 33 and 35; Supplementary Material S25). 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, $p = 0.007$; Figure 32) to aggregate and, reached a significantly lower maximum aggregation ($A_{\max}$: 34.74 ± 12.08% *vs.* 72.63 ± 18.54%; WMW test: $p = 0.02$; Figure 34; Supplementary Material S25). 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: $p = 0.008$; Figure 35; Supplementary Material S25).

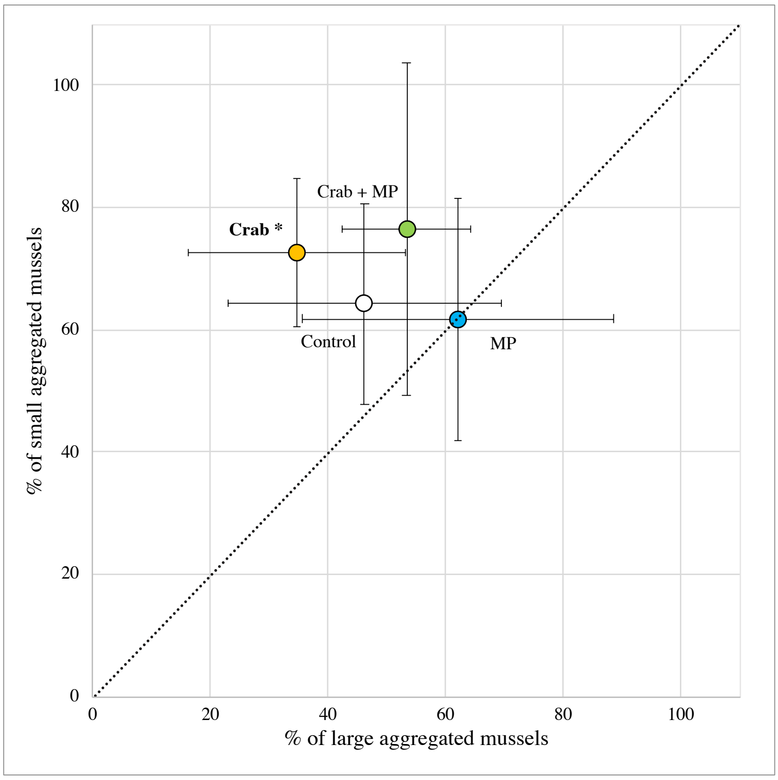


Figure 34: Maximal proportion of aggregation ($A_{\max}$; %) at the end of the 2 h experiment for small and large *Mytilus edulis* in control seawater (white), MP leachates (blue), crab cues (yellow) and 1 : 1 MP leachates and crab cues mixture (green). Treatment name in bold indicates the presence of 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.

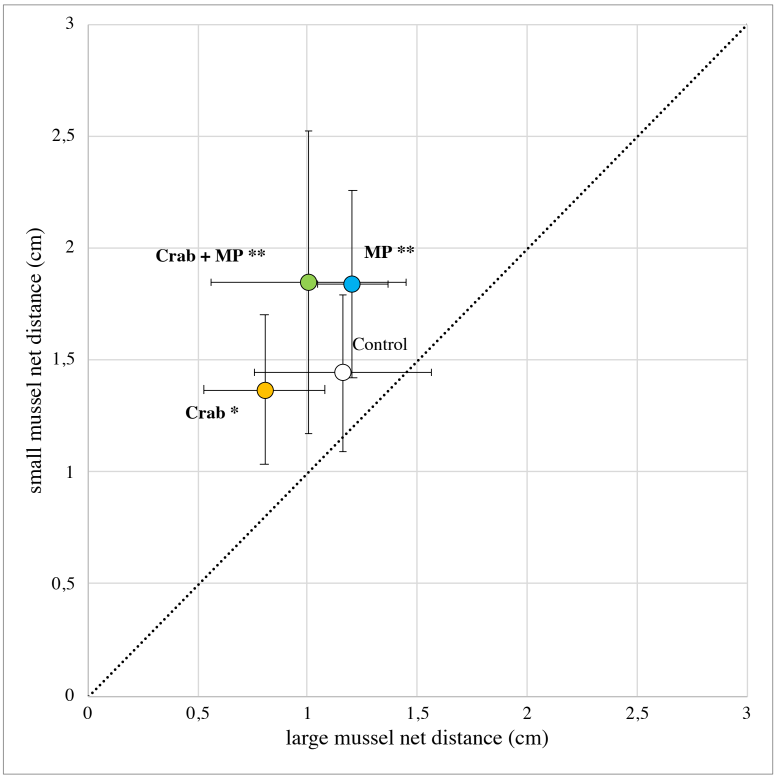


Figure 35: Net distance (cm) travelled by small and large *Mytilus edulis* in control seawater (white), MP leachates (blue), crab cues (yellow) and 1 : 1 MP leachate and crab cue treatment mixture (green). Treatment names in bold indicate the presence of significant differences between small and large 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

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 storms) 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., 2021a).

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 & Clements, 1996; Luan et al., 2019; Orr et al., 2022). This highlights the importance of the selected life stage to assess the 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 heatwave 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 makes them less resistant to stress (Tremblay et al., 1998). Thus, post-spawning weakness may also be responsible for a higher sensitivity to MP leachate 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 condition index and growth rate, and thus, despite an additional stress (Bertness & Grosholz, 1985; Commito et al., 2016; Cubillo et al., 2012; Kong et al*.,* 2019; van de Koppel et al., 2008). 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 & Tendengren, 1997; Côté & 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.

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 latter 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 & Litvaitis, 2013; Ishida & 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 & 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 to be 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 mussels when exposed to predator cues is in accordance with previous studies conducted on large *Mytilus* spp. (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 towards 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 & Jelnikar, 1999; Reimer & 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 & 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 & Jelnikar, 1999; Reimer & 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 & 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.

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 & Malafaia, 2021; Guimarães et al., 2023), tadpoles (da Costa Araújo & 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, a 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., 2021c). Additional experiments are nevertheless required to investigate these assumptions further 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 are more affected by MP leachate pollution than large mussels as showed by an inhibition of their anti-predator collective response. This could eventually cause 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 in 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 experiments and (iii) test their impact on predators and establish the overall ecological risk of these pollutants.

Supplementary Materials

S13: Image of virgin polypropylene microplastics used in the experiment.

S14: (A) Additives identification protocol adapted from Uguen et al., 2022 and (B) figure of the peak area of the identified additives in the polypropylene pellet before (black) and after (grey) the 24 h incubation in seawater.

A.

Samples preparation

Raw polypropylene pellets were cut using a scalpel under a binocular microscope (Olympus SZX16), to obtain fragments of ca. 500 µm². These fragments were subsequently inserted into pyrolysis quartz tubes.

Thermodesorption method for the detection of Organic Plastic Additives (OPAs) using Py-GC-HRMS (Pyrolysis coupled to a gas chromatography and a high-resolution mass spectrometer)

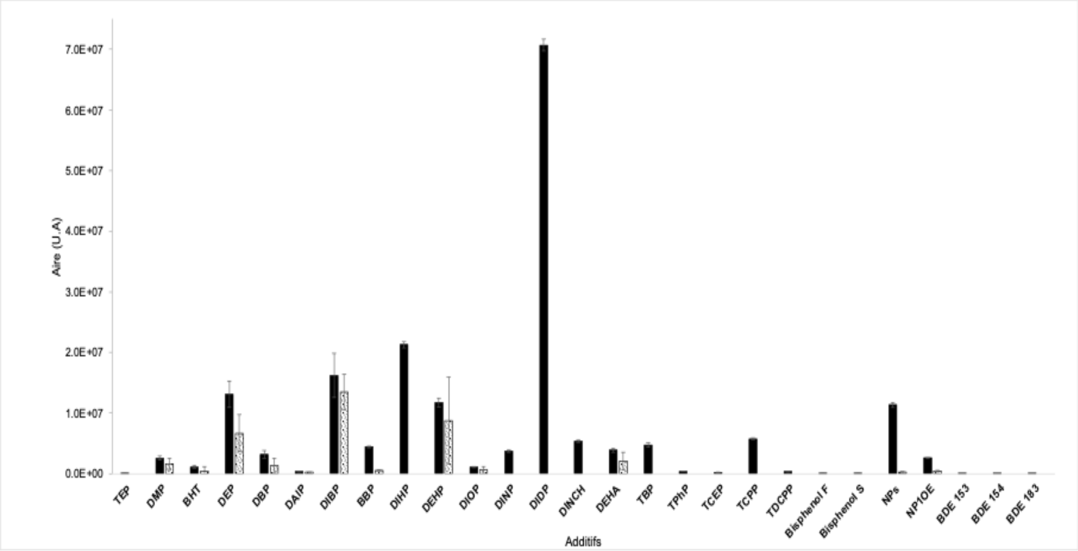
All analyses were carried out using a pyrolyser CDS Pyroprobe 6150 (CDS Analytical) followed by a GC-HRMS device (GC Trace 1310-MS Orbitrap Q exactive, Termo Fisher Scientific). With the aim to thermally desorb the chemicals additives potentially contained into the samples, they were heated into the pyrolyser at a temperature of 350 °C. Samples were subsequently separated on a GC column (Restek Rxi-5-MS capillary column, cross-linked poly 5% diphenyl-95% dimethylsiloxane, 30 m × 0.25 mm (i.d.) × 0.25 µm film thickness), with a split ratio of 1:5. The acquisition was performed on full-scan (FS) mode (m/z = 30.00000–600.00000). The resulting chromatograms were analysed using Xcalibur and TraceFinder softwares to identify OPAs among a selection of additives including plasticisers, flame retardants, antioxidants and UVs stabilisers. The respective additives were identified on the basis of the retention times, m/z, and specific ions after a comparison with the chromatograms of the respective standard solutions.

Identified additives

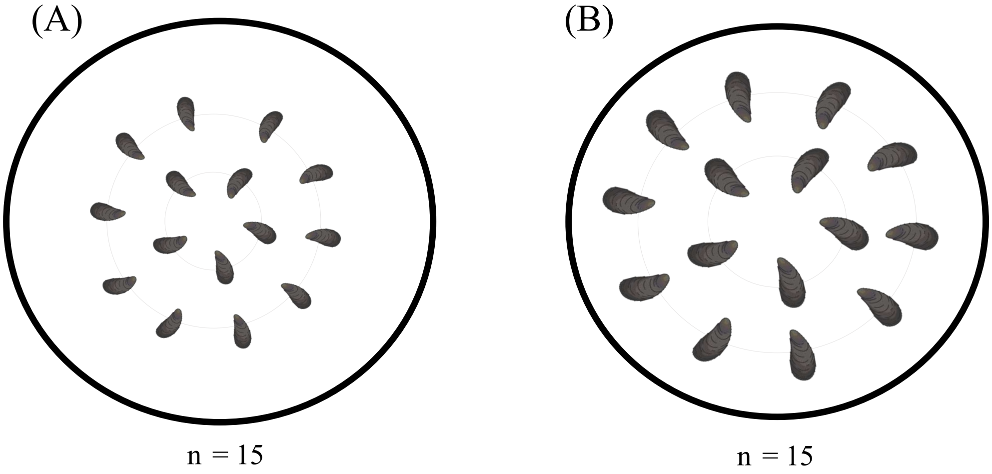
- 3 brominated flame retardants, *i.e.* 2,2',4,4',5,5'-Hexabromodiphenyl ether (BDE-153); 2,2',4,4',5,6'-Hexabromodiphenyl ether (BDE-154) and 2,2',3,4,4',5',6-Heptabromodiphenyl ether (BDE-183);
- 6 phosphorus flame retardants, *i.e.* Tributyl Phosphate (TBP); Triethyl Phosphate (TEP); Tris(2-Chloroethyl)Phosphate (TEPP); Tris(2-Chloroisopropyl)Phosphate (TCPP); Tris(1,3-Dichloro-2-Propyl)Phosphate (TDCPP) and Triphenyl Phosphate (TPhP)
- 5 antioxidants, *i.e.* Butylated hydroxytoluene (BHT); Bisphenol F (BPF); Bisphenol S (BPS); Nonylphenol (NPs) and Nonylphenol monoethoxylate (NP10E)

- 13 plasticisers, *i.e.* Dimethyl phthalates (DMP); Diethyl phthalate (DEP); Di-n-butyl phthalate (DBP); Di-allyl phthalate (DAIP); Diisobutyl phthalate (DIBP); Benzylbutyl phthalates (BBP); Diisoheptyl phthalate, (DIHP); Bis(2-Ethylhexyl) phthalate (DEHP); Diisononyl phthalate (DINP); Diisodecyl phthalate (DIDP); Diisononyl hexahydrophthalate (DINCH); Di-n-octyl phthalate (DIOP); Bis-2-Ethylhexyl Adipate (DEHA)

B.



S15: Schematics of the experimental design of one arena of the aggregation experiments for (A) small and (B) large mussels.



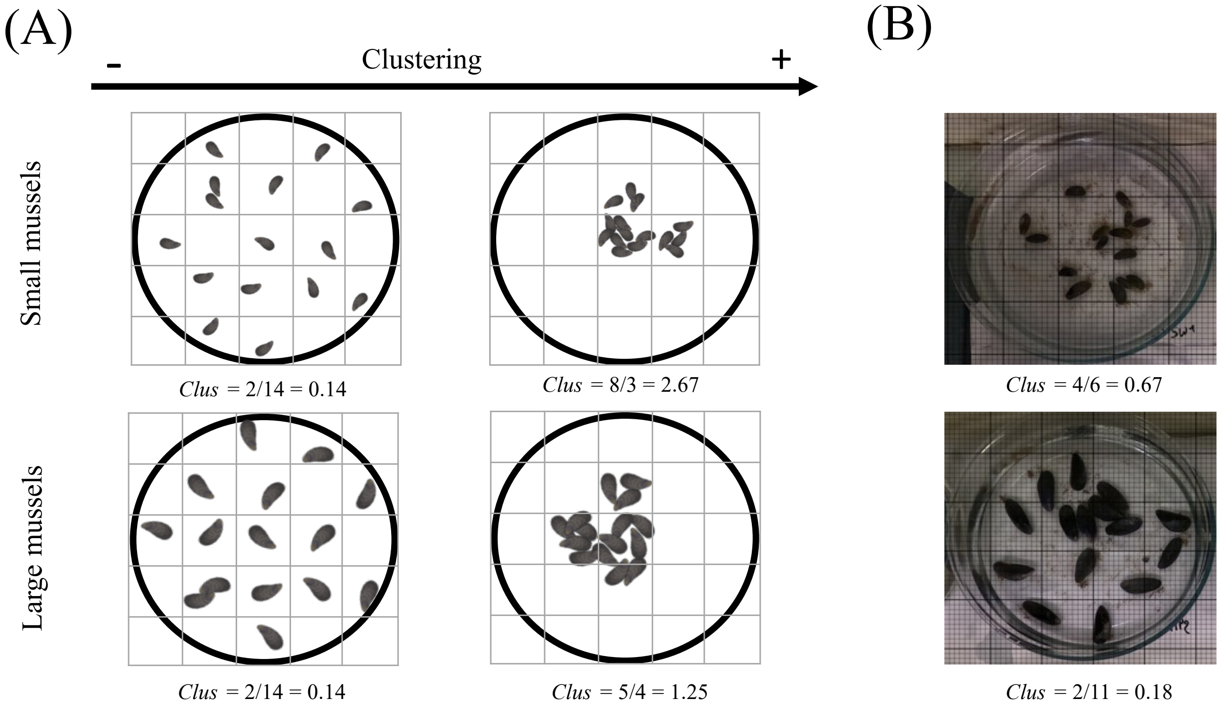
S16: Example of a time lapse for the small mussels with a picture every 0.1 seconds.

→ See the Supplementary Materials, S4 of the paper: Uguen, M., Gaudron, S. M., Nicastro, K. R., Zardi, G. I., Spilmont, N., & Seuront, L. (2023). Size-dependent response of the mussel collective behaviour to plastic leachates and predator cues. *Science of The Total Environment*, *888*, 164037. https://doi.org/10.1016/j.scitotenv.2023.164037

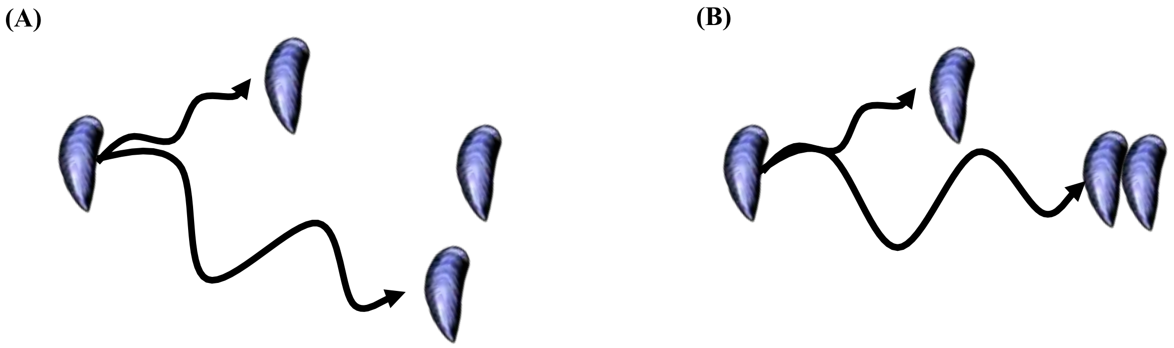
S17: Example of a time lapse for the large mussels with a picture every 0.1 seconds.

→ See the Supplementary Materials S4 of the paper: Uguen, M., Gaudron, S. M., Nicastro, K. R., Zardi, G. I., Spilmont, N., & Seuront, L. (2023). Size-dependent response of the mussel collective behaviour to plastic leachates and predator cues. *Science of The Total Environment*, *888*, 164037. https://doi.org/10.1016/j.scitotenv.2023.164037

S18: (A) Schematics representation of examples of the cluster score calculation for small and large mussels, adapted from da Costa Araújo & Malafaia (2021). (B) Example of representative images for the cluster score calculation for small and large mussels. When the same mussel is found in several quadrants, it is only counted in the quadrat where it is the most predominant.



S19: Schematics representation of (A) the lack of a significant correlation between the distance travelled and the maximum aggregation $A_{\max}$ (*i.e.* even if a mussel increases its displacement the number of aggregations remains the same) and (B) the presence of a significant positive correlation between the distance travelled distance and $A_{\max}$ (*i.e.* if a mussel increases its displacement the number of aggregations also increases).



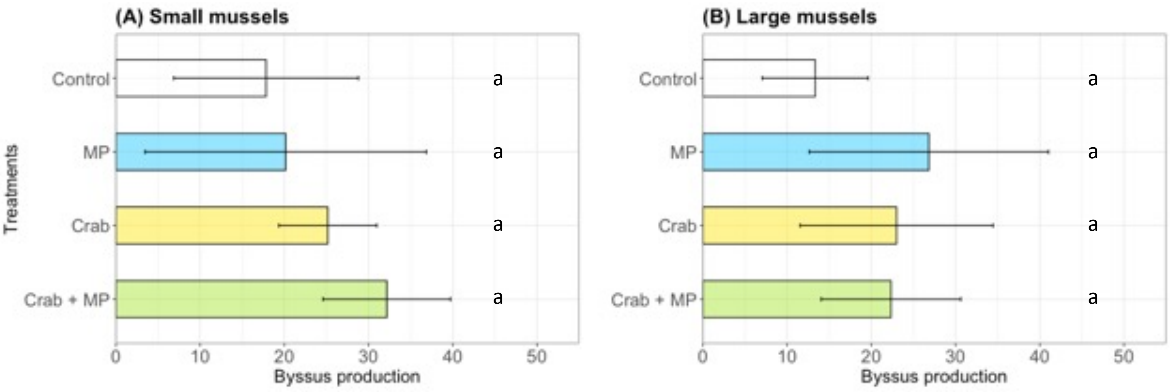
S20: Schematic representation of (A) the lack of a significant correlation between byssus thread production and the maximum aggregation $A_{\max}$ (*i.e.* when aggregation increases, the number of byssus thread produced remains steady) and (B) the presence of a significant positive correlation between thread byssus thread production and $A_{\max}$ (*i.e.* when both aggregation and the number of byssal threads produced increase).


(A) **(B)**

S21: Statistical assessment of the normality (Shapiro's test), heterogeneity of variance (Levene's test) and the differences between experimental treatments for each mussel size class and behavioural variables (* $p < 0.05$; *** $p < 0.001$; Kruskal-Wallis test) and main results after the post-hoc Conover test with Holm's correction.

Behavioural variable	Size	normality	heterogeneity	H-value	df	*p-value*		main results
Starting time	Large	0.0344	0.06396	17.224	3	**0.0006357**	***	Crab > Crab + MP > MP = Control
	Small	0.0123	0.5944	3.4292	3	0.3301		
Gross distance	Large	0.869	0.64	8.6067	3	**0.035**	*	Control > Crab
	Small	0.551	0.5714	3.7667	3	0.2878		
Net distance	Large	0.0265	0.5066	5.1667	3	0.16		
	Small	0.0183	0.8709	4.6667	3	0.1979		
Confinment index	Large	0.911	0.3782	3.0467	3	0.3845		
	Small	0.547	0.1642	1.7867	3	0.6178		
Byssus	Large	0.0805	0.7092	5.8402	3	0.1196		
	Small	0.826	0.139	5.6966	3	0.1273		
Amax	Large	0.744	0.591	4.8467	3	0.1834		
	Small	0.946	0.3953	1.9592	3	0.5809		
k	Large	0.755	0.06167	0.44667	3	0.9304		
	Small	0.000265	0.5803	3.14	3	0.3705		
Cluster score	Large	0.0833	0.4476	0.73692	3	0.8645		
	Small	0.0152	0.2951	3.0995	3	0.3765		

S22: Byssus thread production (mean ± SD) for (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). Same letters depict no significant difference (*p* > 0.05; Conover's test) among treatments.



S23: Correlation table for small mussels between behavioural variables for each treatment: (A) Control seawater, (B) Microplastic leachates, (C) Crab cues and (D) 1 : 1 Crab cues and Microplastic leachates treatment mixture. Bold numbers indicate a significant correlation between the two behavioural variables (*: *p* < 0.05; **: *p* < 0.01; Pearson correlation test).

(A) Control

	Gross distance	Net distance	Byssus
Net distance	0.46		
Byssus	-0.21	-0.24	
Amax	0.74	-0.08	-0.10

(B) Microplastic leachates

	Gross distance	Net distance	Byssus
Net distance	0.73		
Byssus	0.78	0.78	
Amax	**0.90***	**0.91***	**0.92****

(C) Crab cues

	Gross distance	Net distance	Byssus
Net distance	**0.83***		
Byssus	0.78	0.64	
Amax	**0.86***	0.55	0.46

(D) Crab cues + Microplastic leachates

	Gross distance	Net distance	Byssus
Net distance	0.74		
Byssus	-0.04	0.62	
Amax	0.34	-0.26	-0.65

S24: Correlation table for large mussels between behavioural variables for each treatment: (A) control seawater, (B) microplastic leachates, (C) crab cues and (D) 1 : 1 microplastic leachate and crab cue treatment mixture. Bold numbers indicate a significant correlation between the two behavioural variables (*: $p < 0.05$; Pearson correlation test).

(A) Control

	Gross distance	Net distance	Byssus
Net distance	0.74		
Byssus	0.10	-0.15	
Amax	0.09	0.59	0.31

(B) Microplastic leachates

	Gross distance	Net distance	Byssus
Net distance	0.72		
Byssus	0.48	0.68	
Amax	0.51	0.59	0.68

(C) Crab cues

	Gross distance	Net distance	Byssus
Net distance	0.76		
Byssus	0.81	0.58	
Amax	**0.88***	**0.87***	**0.90***

(D) Crab cues + Microplastic leachates

	Gross distance	Net distance	Byssus
Net distance	0.71		
Byssus	0.23	0.54	
Amax	0.69	**0.87***	0.48

S25: Statistical assessment of the normality (Shapiro's test), heterogeneity of variance (Levene's test) and of the differences between mussel size class, *i.e.* small and large mussels for each treatment and behavioural variable (*: $p < 0.05$; **: $p < 0.01$; Wilcoxon-Mann-Whitney test) and main results.

Behavioural variable	Treatment	normality	heterogeneity	W	*p-value*		main results
Starting time	Control	0.386	0.5995	25	0.2464		
	MP	0.00369	0.4475	21	0.6404		
	Crab	0.345	0.1314	35	**0.007051**	**	Large > Small
	Crab + MP	0.190	0.4257	30	0.05806		
Gross distance	Control	0.880	0.8811	15	0.6889		
	MP	0.873	0.1678	8	0.1282		
	Crab	0.329	0.9105	2	**0.01307**	*	Small > Large
	Crab + MP	0.273	0.6808	9	0.1735		
Net distance	Control	0.707	0.8182	12	0.3785		
	MP	0.411	0.2349	1	**0.008239**	**	Small > Large
	Crab	0.269	0.8039	3	**0.02024**	*	Small > Large
	Crab + MP	0.0715	0.7261	1	**0.008239**	**	Small > Large
Confinement index	Control	0.405	0.9767	9	0.1735		
	MP	0.468	0.1313	7	0.0927		
	Crab	0.829	0.828	13	0.4712		
	Crab + MP	0.399	0.4693	6	0.06555		
Byssus	Control	0.989	0.1816	12.5	0.4217		
	MP	0.217	0.5666	23	0.4712		
	Crab	0.118	0.5666	12	0.3768		
	Crab + MP	0.281	0.8535	6	0.06508		
Amax	Control	0.0225	0.7209	9	0.1735		
	MP	0.946	0.3534	18	1		
	Crab	0.214	0.8822	3	**0.02024**	*	Small > Large
	Crab + MP	0.596	0.1666	8	0.1282		
k	Control	0.411	0.7646	22	0.5752		
	MP	0.190	0.3539	20	0.8102		
	Crab	0.00722	0.4077	12	0.3785		
	Crab + MP	0.540	0.06134	17	0.9362		

CHAPTER V

MUSSEL AGGREGATION BEHAVIOUR: A REPRODUCTIVE STRATEGY IMPAIRED BY PLASTIC LEACHATES

Mussel aggregation behaviour: a reproductive strategy impaired by plastic leachates

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Abstract

Aggregation behaviour is a key process shared by a wide range of organisms. It provides benefits such as predator protection, resistance to abiotic stressors, and enhances reproductive success. In marine coastal ecosystems, mussel aggregations play a crucial role in increasing rocky shore biodiversity. Despite the well-documented ecological significance of mussel aggregations, many aspects of their temporal dynamics and their contribution to the species' reproductive success remain unexplored. Here, we first investigated the possibility that aggregation behaviour actually acts as a reproductive strategy by inferring the potential links between aggregation rate and reproductive maturity stages in the blue mussel *Mytilus edulis* over the course of a seasonal cycle. We demonstrated that the seasonality of *M. edulis* aggregation rate was significantly described as a sinusoidal function of the mean reproductive maturity stage. Specifically, aggregation increased during gametes maturation until the onset of spawning, and subsequently declined, following a cyclical pattern that supports a reproductive function of this aggregation behaviour in mussel. Given the ubiquity of plastics as marine contaminants and their known harmful effects on organisms, we subsequently assessed how the temporal patterns observed in aggregation behaviour may be impacted by an exposure to leachates from commercially available polypropylene pellets. We found that these leachates led to a loss of the cyclical pattern observed in control seawater, and discussed the observed modifications in the temporal patterns of aggregation in terms of a leachate-induced defence signal or a leachate-enhanced reproductive signal. Understanding these complex interactions is crucial, given the pivotal role mussels and their aggregations play in shaping marine ecosystems. In particular, the present work shed new light on the multifaceted relationship between aggregation behaviour, reproduction, and contaminants, offering insights into the resilience of these ecosystems in the face of contemporary challenges.

1. Introduction

Aggregation behaviour, *i.e.* the tendency of animals to group together, is a fundamental ecological process, which confers important benefits on individual fitness, such as reducing the predation risk, dampening the abiotic stressors and providing better access to resources and mates (Allee, 1931; Parrish & Edelstein-Keshet,, 1999). The benefits of aggregation may extend well beyond the individual level, as for some instances, group formation may lead to the completion and maintenance of entire ecosystems. Specifically, in the marine environment, the aggregations of certain species of bivalves, polychaetes, corals or sponges, generate complex three-dimensional biogenic reef structures, often referred to as “marine animal forests” (Rossi et al., 2017). These structures provide new habitats and alleviate physical stress, which often leads to changes in species assemblages, modification of the structure of the food web, and favour the establishment and sustainability of biodiversity hotspots (Jones et al., 1997; Romero et al., 2015; van der Zee et al., 2016).

Some of these engineer species, such as corals and sponges, typically aggregate during their settlement, and subsequently do not move during their adult life. In contrast, mussels do not lose their ability to move during their juvenile and adult stages after larval settlement. Instead, they use their foot and byssal gland complex to move, readjust their position, and firmly attach themselves to the substrate and form dynamic aggregations referred to as mussel beds (Commito et al., 2014; Seuront et al., 2021; Zardi et al., 2021). Beyond offering an optimal habitat to a wide range of species (Borthagaray & Carranza, 2007; Buschbaum et al., 2009), these aggregations protect mussels from predation (Côté & Jelnikar, 1999; Kong et al., 2019; Nicastro et al., 2007; Reimer & Tedengren, 1997), wave dislodgment (Zardi et al., 2021), and fluctuations in temperature and humidity (Nicastro et al., 2012). Aggregation behaviour has also been shown to be a reproductive strategy in numerous benthic broadcast spawners (*e.g.* Himmelman et al., 2008; Keesing et al., 2011; Marquet et al., 2018; Mendo et al., 2014;

Shepherd, 1986; Simon & Levitan, 2011). In broadcast spawners, such as mussels, the population density and proximity of individuals are commonly referred to as among the most important processes in fertilisation success (Levitan & Petersen, 1995; McEdward, 1995; Molloy et al., 2012), especially given that contact time between gametes required for successful fertilisation is only a few minutes in mussels (Liu, 2009). In this context, the ability of mussels to move and aggregate may also play a role in enhancing their reproductive success. As previously suggested for another broadcast spawner, the limpet *Patella vulgata* (Coleman et al., 2006), considering aggregation behaviour as a reproductive strategy would result in an increase in the tendency to aggregate when gametogenesis is completed.

The behavioural responses of mussels in particular, and marine organisms in general, however, are prone to be impaired by the wide range of anthropogenic contaminants present in the marine environment; see Melvin & Wilson (2013) for a review. Among them, plastic is the most common marine contaminant, with 4.8 to 12.7 million tonnes estimated to be released into the ocean each year (Jambeck et al., 2015). Plastics are associated with numerous chemical compounds, such as additives (*e.g.* phthalates, Bisphenol A, antioxidants), that can be leached out once in the environment and affect marine organisms; see Delaeter et al. (2022) for a review. Specifically, plastic leachates have been shown to disrupt the aggregation, locomotion, chemical perception and reproductive success of mussels (*e.g.* Capolupo et al., 2021a, 2023; Cozzolino et al., 2023; Gandara e Silva et al., 2016; Seuront et al., 2021; Uguen et al., 2022, 2023). Despite the range of observed responses following an exposure to plastic leachates, to the best of our knowledge, there is still no information on how plastic pollution may impact mussel aggregation behaviour over the course of a seasonal cycle.

Here, we choose the blue mussel *Mytilus edulis* as model organism. This gonochoristic species exhibits a seasonal reproduction, typically characterised by one or two spawning events usually triggered by an increase in temperature above 10–12°C when gametogenesis is completed (Boromtharanat et al., 1987). Despite extensive research on the reproduction of this species in the Atlantic Ocean since the mid-20th century (Lubet, 1959), there is still a critical lack of knowledge in *M. edulis* reproduction in the Eastern English Channel (EEC), where they hold economic, social and patrimonial importance (Dauvin, 2019). Moreover, the EEC is heavily impacted by various marine and land-based types of pollution (Dauvin, 2019; Tappin & Millward, 2015), including plastic (Gravier & Haut, 2020). For instance, in 2016, 8 tonnes of plastic pellets were spilt from a tanker truck on a highway located *ca.* 2.5 km from the shoreline, dispersed through the surrounding waterways, leading to significant shoreline contamination that persisted for several years after the accident (Gravier & Haut, 2020). However, the biological and ecological effects of plastic pollution in the EEC are still relatively unknown, despite recent efforts to bridge this knowledge gap (Seuront, 2018; Seuront et al., 2021; Uguen et al., 2022, 2023; Zardi et al., in press).

In this context, the aim of this paper was first to test the hypothesis that the aggregation behaviour of *M. edulis* can be considered as a reproductive strategy, in which case we would expect an increase in the tendency to aggregate when gametogenesis is completed. To do so, we combined behavioural assays and histological analyses over an eight-month study. Then, through an additional treatment, we aim to assess whether exposure to leachate of commercially available polypropylene pellets may impair the observed seasonal patterns in aggregation behaviour.

2. Material and Methods

Sampling

Every month, from March to December 2021 (with the exception of October and November 2021), adult individuals of *Mytilus edulis* (shell length: 2.5 – 3.5 cm, n = 200) were sampled from a rocky intertidal reef (Pointe aux Oies, Wimereux; 50°47'08.3"N, 1°36'03.9"E) along the French coasts of the eastern English Channel. Every month, mussels (*n* = 180) were acclimated for 24 hours in the laboratory in 85 L tanks filled with running aerated natural seawater representative of *in situ* conditions of temperature and salinity (see Supplementary Material, S26) before the behavioural assays took place. Concurrently, for the histological analysis, mussels (*n* = 20) were dissected and the gonads were fixed in 10% formaldehyde and stored at 4°C. As *M. edulis* is a gonochoric species and gametes spread outside of the gonad to the mantle lobes during the completion of the gametogenesis (Seed, 1969), to determine the sex of each specimen, a biopsy of the mantle was observed under a microscope (Motic BA210) during the dissection (Fraser et al., 2016).

Microplastic leachate solution

Mussels were exposed to either (i) control natural seawater or (ii) microplastic leachate solution (MPL). MPL was prepared from commercially available polypropylene pellets (3.80 ± 0.21 cm × 2.95 ± 0.50 cm; white colour; Pemmiproducts, Aachen, Germany) incubated for 24 h at 12°C in natural aerated seawater at a concentration of 20 mL of pellets (*ca.* 12 g; 400 pellets) per litre. The pellet incubation temperature was fixed at 12°C during the eight-month study to avoid any biases related to different desorption kinetic of additives due to temperature (Dhavamani et al., 2022; Kida & Koszelnik, 2021; Kida et al., 2022).

The additives contained in the polypropylene pellets before and after their 24 h-incubation were analysed in a previous study (Uguen et al., 2023), *i.e.* by using a pyrolysis analysis coupled to a gas chromatography and a high-resolution spectrometer (for further details in the method used, see Supplementary Material S27). A total of 27 additives were identified, including three brominated flame retardants, six phosphorus flame retardants, five antioxidants and 13 plasticisers (see Supplementary Material S27 for further details).

Aggregation behaviour assessment

Mussels (n = 15) were placed in a 22 cm glass arena, their narrow end facing the centre of the arena in two concentric circles, at *ca.* 1 body length from each other. The arenas (N = 12) were filled with 1.5 L of control seawater or MPL, each treatment was consistently replicated 6 times. Mussel position was recorded every minute for 2 h using a GoPro camera (GoPro HERO7 Black, GoPro Inc., San Mateo, California, USA; Uguen et al., 2023). To minimise stress, each mussel was only used once and byssal threads were carefully cut prior to the experiment. Experiments were conducted in the absence of any acoustic or mechanical disturbances in a temperature-controlled room. Temperature of the room was set to the mean temperature recorded *in situ* (where mussels were collected) ranging from a minimum of 8°C in March to a maximum of 19°C in September (see Supplementary Material, S26). At the end of the experiment, aggregation behaviour was quantified as the proportion of mussels (%) forming aggregates defined as two or more mussels with their shells in direct physical contact (Seuront et al., 2021; Uguen et al., 2023).

Reproductive maturity stages assessment

Gonads were dehydrated in an ethanol series (70–100%), cleared in Diasolv and impregnated in paraffin. Each sample was then sectioned at 5 µm using a microtome (Leica Ltd.), and stained with Haematoxylin and Eosin (HE; Mammone et al., 2020). Histological sections were examined under a microscope (Axioscope 5, Zeiss Ltd.) equipped with the software Zen (Zeiss Ltd.).

Specimen reproductive maturity stages of both male and female were assessed based on criteria established in previous studies (Auffret, 2003; Chipperfield, 1953; Choi et al., 2022; Seed, 1969; Supplementary Material, S28). Specifically, the reproductive maturity for each individual was divided into five stages (x_i) ranked from 1 to 5: undifferentiated (x_1), early development (x_2), late development (x_3), ripe (x_4), and partially spent (x_5). In addition, the mean reproductive maturity stage (μ) was calculated for each month as:

$$\mu = \frac{\sum n_i \times x_i}{N} \tag{1}$$

where n_i is the number of individuals in the development stage x_i, and N the total number of individuals (Choi et al., 2022; Supplementary Material, S28).

Statistical analyses

To assess seasonal variation in mussel aggregation behaviour, a Kruskal-Wallis test (KW test hereafter) was performed for each control and MPL treatment with time as factors (8 levels: March, April, May, June, July, August, September and December) and aggregation behaviour as dependent variable. The KW test was followed by the powerful Conover-Iman test with Holm's correction to identify distinct groups of measurements; the Conover-Iman test (Conover test hereafter) was used instead of the less powerful though more well-known Dunn test (Gilbert, 2019). To assess the presence of a cyclic relationship between mussel aggregation and reproductive stage, the aggregation rate (A(μ); %) observed in the control seawater was expressed as a function of the mean reproductive stage (μ), and fitted with a sinusoid of the form:

$$A(\mu) = a \cdot sin\,(\omega \cdot \mu + \phi) + b \tag{2}$$

The nonlinear least-squares method was used to estimate the parameters, *i.e.* amplitude (a), the angular frequency (ω), the phase shift (ϕ) and the baseline (b) of the sinusoidal wave. This method aimed to minimise the sum of squares residual (SSR) and maximise the coefficient of determination r^2. To assess the impact of microplastic leachates on this cyclic pattern, the proportion of aggregated mussels in MPL was compared with the nonlinear sinusoid equation fitted for control data by measuring the SSR and r^2.

3. Results

Mytilus edulis aggregation behaviour

In control seawater, time had a significant effect on the blue mussel *Mytilus edulis* aggregation behaviour (KW test, H = 31.363, df = 7, $p < 0.001$; Figure 36A). Specifically, the aggregation rate increased significantly until it reached a maximum in May (*i.e.* 81.1 ± 9.8 %; mean ± standard deviation, SD) and subsequently decreased significantly until a minimum in September (*i.e.* 25.6 ± 16.0 %), which was noticeably 3.2-fold lower than in May. The aggregation rate remained low in December (38.9 ± 5.0%) and was not significantly different from September (see Supplementary Materials S29 for the exact Conover *p* values).

M. edulis reproductive maturity

Reproductive maturity stages typically varied with time (Figure 36B**)**, with mussels in pre-spawning condition before May and where, from March to April, gametogenesis for both sexes was completed with fully-grown oocytes for females and the presence of spermatozoids in rosettes for male (Stage 4, ripe; Figure 36B; Supplementary Materials S28). May marked the onset of the main spawning period (*i.e.* with the appearance of partially spent specimens; Stage 5; Figure 36B; Supplementary Materials S28) which lasted for some mussels until August, when a second minor spawning event may have occurred. In some cases, specimens were completely empty already in July (Figure 36B; Stage 1; Supplementary Materials S28). In September, both male and female mussels were devoid of mature gametes and started a new cycle of reproductive development (Stage 1 & 2; Figure 36B; Supplementary Materials, S28). Finally, in December, females carried oocytes in vitellogenesis and males mostly spermatocytes (Stage 3; Figure 36B; Supplementary Materials, S28).

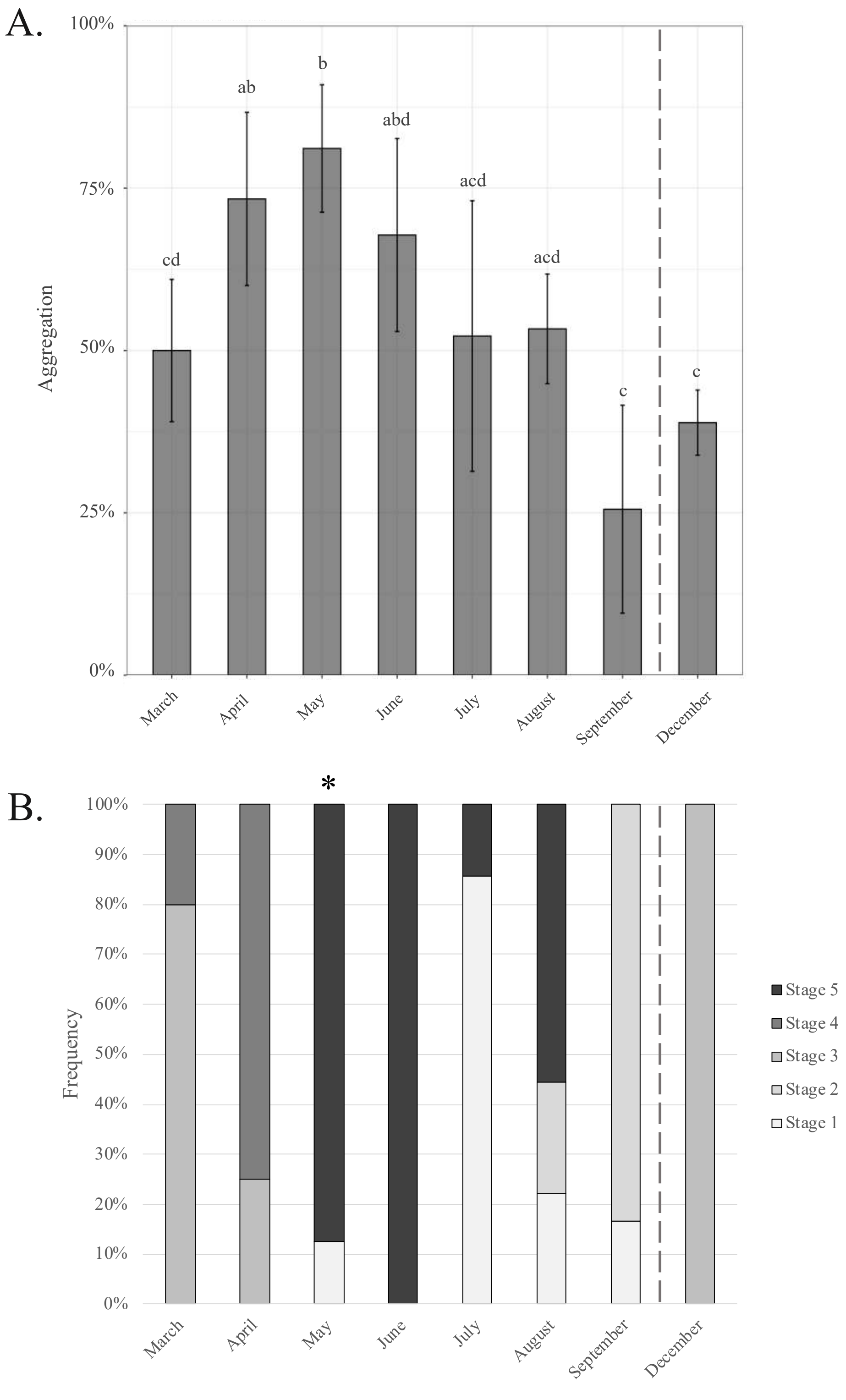


Figure 36: Monthly evolution of *Mytilus edulis* (A) aggregation (expressed as the proportion of aggregated individuals; %) at the end of the 2-h experiment (mean ± standard deviation) and (B) reproductive maturity stages, *i.e.* undifferentiated (stage 1), early development (stage 2), late development (stage 3), ripe (stage 4), and partially spent (stage 5). Dashed line indicates a two-month time gap with no sampling. Letters depict significant differences ($p < 0.05$; Conover test; see Supplementary Materials S29) among treatments. Asterisks indicate the start of the spawning period.

Aggregation behaviour and reproductive maturity

M. edulis aggregation behaviour varied cyclically according to its reproductive maturity stages (Figure 37). The relationship between mussel aggregation and reproductive stage was consistently highly significantly described by the sinusoidal equation: $A(\mu) = 31.04 \cdot \sin(1.63 \cdot \mu - 11.72) + 53.28$ (SSR = 163; $r^2 = 0.965$; $p < 0.001$). Specifically, aggregation increased with reproductive maturity until the start of spawning and then decreased until mussels entered a new reproductive cycle.

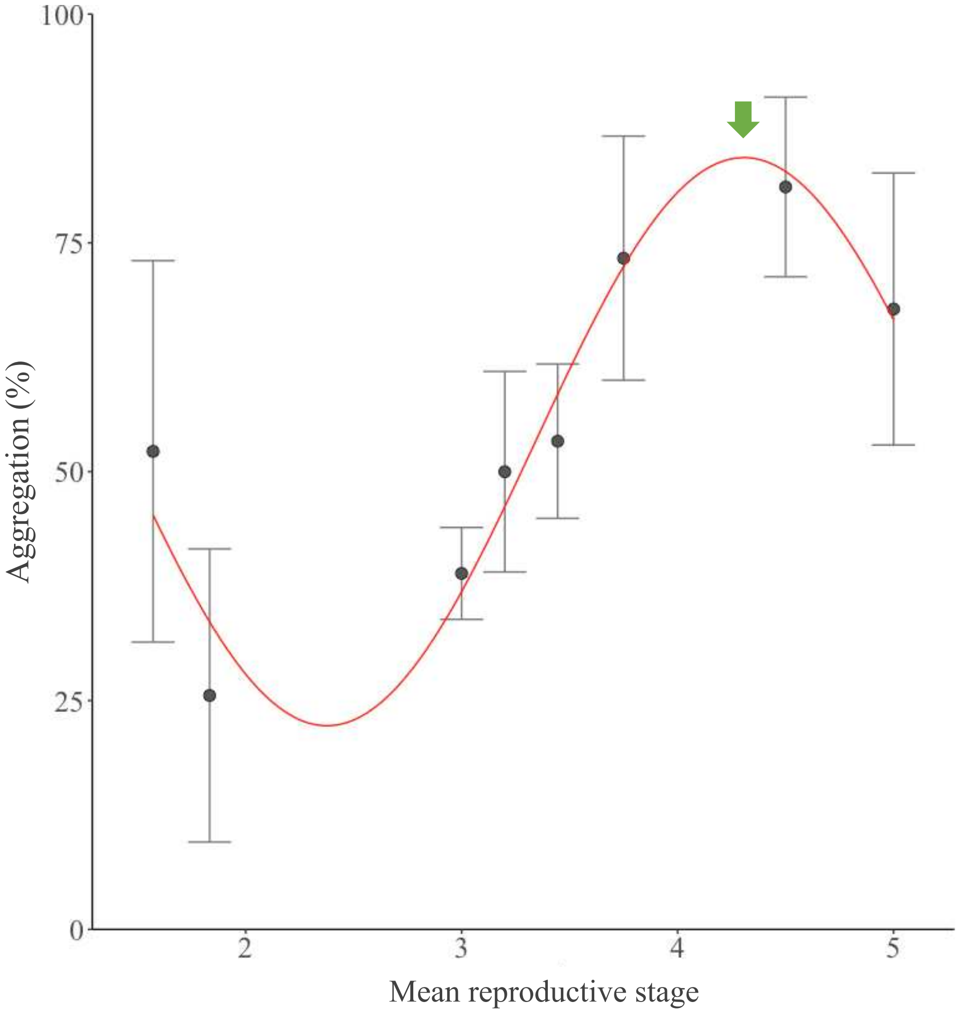


Figure 37: Relationship between the aggregation (expressed as the proportion of aggregated individuals; %) in *Mytilus edulis* at the end of the 2-h experiment (mean ± standard deviation) and its mean reproductive maturity stages, *i.e.* undifferentiated (stage 1), early development (stage 2), late development (stage 3), ripe (stage 4), and partially spent (stage 5). Red line represents the best sinusoidal fit between the aggregation (A) and the mean reproductive stage (μ), *i.e.* $A(\mu) = 31.04 \cdot \sin(1.63 \cdot \mu - 11.72) + 53.28$. Green arrow indicates the onset of the spawning period.

Microplastic leachates and aggregation pattern

When exposed to MPL, time had a significant effect on the aggregation behaviour of *Mytilus edulis* (KW test, H = 26.919, df = 7, $p < 0.001$; Figure 38**)**. However, this temporal pattern significantly differed from observations conducted following an exposure of *M. edulis* to control seawater; there is a lack of the peak in aggregation behaviour in May, at the onset of the spawning period (Figure 38). Mussel aggregation was high and relatively constant from March to July (with a minimum of 61.1 ± 20.0 % in June and a maximum of 76.7 ± 10.1 % in May), before significantly decreasing until a minimum in September (21.1 ± 6.5 %), and remaining low in December (40.6 ± 22.7 %) (see Supplementary Materials S30 for the exact Conover *p* values).

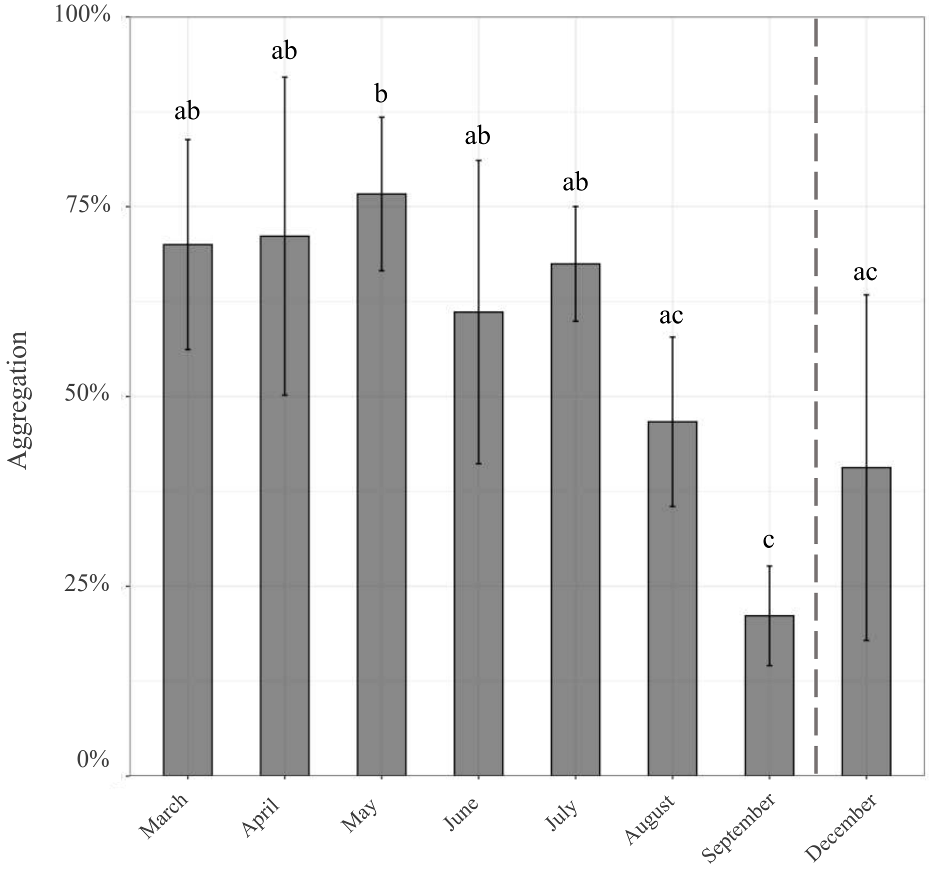


Figure 38: Monthly evolution of the aggregation (expressed as the proportion of aggregated individuals; %) in *Mytilus edulis* after exposure to microplastic leachate solution at the end of the 2-h experiment (mean ± standard deviation). Dashed line indicates a two-month time gap with no sampling. Letters depict significant differences ($p < 0.05$; Conover test) among treatments.

The cyclic pattern that linked mussel aggregation to reproductive maturity stages in the control was noticeably lost following MPL exposure. Indeed, when exposed to MPL, *M. edulis* aggregation could not be significantly described by the previous sinusoidal function of the mean reproductive stage found in control seawater ($p > 0.05$; Supplementary Materials, S31).

4. Discussion

Aggregation behaviour as a reproductive strategy?

Our results show that *Mytilus edulis* aggregation behaviour was consistently described as a sinusoidal function of its reproductive maturity stages. This cyclical pattern may indicate that aggregation behaviour —typically considered as an adaptive strategy to minimise predation and wave dislodgement risks (*e.g.* Côté & Jelnikar, 1999; Nicastro et al., 2007; Zardi et al., 2021)— may also play a role in the reproduction of this species, as aggregation rates were consistently highest when ripe gametes were present, hence when spawning event approaches. Reproduction is a key process of life, which is essential to the maintenance of populations and the evolution of species. In sharp contrast with organisms which reproduce via internal fertilisation, the reproductive success of broadcast spawners in general, and mussels in particular, have to face the dilution of gametes as male and female gametes are directly released into the water column (Mead & Epel, 1995; Serrao et al., 1996). To reduce this dilution effect and, given that the contact time between gametes required for successful fertilisation is only a few minutes (Liu, 2009), the formation of spawning aggregations has been frequently selected to maximise fertilisation success (Levitan & Petersen, 1995; Liu et al., 2011; McEdward, 1995; Molloy et al., 2012). This strategy has been described in various benthic invertebrates such as asterids (Himmelman et al., 2008; Keesing et al., 2011), ophiurids (Himmelman et al., 2008), echinids (Simon & Levitan, 2011), holoturids (Marquet et al., 2018), abalones (Shepherd, 1986), and scallops (Mendo et al., 2014), but also in a wide range of fish species; see *e.g.* Grüss et al. (2014) Robinson et al. (2008) and van Overzee & Rijnsdorp (2015) for reviews.

The hypothesis that the observed synchrony between *M. edulis* aggregation and reproductive maturity plays a role in the reproductive success of this species is supported by a range of abiotic and biotic factors. The seasonality of *M. edulis* aggregation has been observed *in situ*, with aggregation increasing toward summer when the abiotic conditions become benign and decreasing toward winter when abiotic conditions are more adverse (Capelle et al., 2023). This aggregation pattern is consistent with our *ex situ* experimental observations conducted under controlled conditions. Thus, as observed in Capelle et al. (2023), the aggregation patterns observed in the present work match a high food availability, in agreement with the typical seasonal dynamics of phytoplankton in the eastern English Channel (see *e.g.* Breton et al., 2021, 2022; Gentilhomme & Lizon, 1997; Schapira et al., 2008; Seuront et al., 2006, 2007; Seuront & Vincent, 2008). Yet, the highest aggregation rate we observed during the spring phytoplankton bloom is unlikely a response to maximise feeding. Aggregation is known to reduce mussel feeding under current velocity conditions lower than 0.4 m s^{-1} (Nielsen & Vismann, 2014). This was the case in our arenas where seawater was consistently still, but also, and more generally at our mussel collection zone (50°47’08.3”N, 1°36’03.9”E; see MARS3D hydrodynamical model, seabed data: https://marc.ifremer.fr). In turn, the higher aggregation rates observed during this period may be a response to maximise reproductive success, leading to a high production of planktotrophic larvae synchronised with the phytoplankton bloom. This would be consistent with the “match/mismatch” theory (Cushing, 1990), *i.e.* the recruitment success depends on the synchrony (match) or desynchrony (mismatch) in the time between the larval production and the production of their food source, a phenomenon previously suggested in *M. edulis* (Toupoint et al., 2012; Watson et al., 2003). Additionally, as mussel fertilisation success is enhanced both at lower water velocity, and when mussels aggregate in high-density patches that generate turbulence (Quinn & Ackerman, 2011), the synchronisation between mussel aggregation and benign abiotic condition (*i.e.* the phytoplankton bloom and the low hydrodynamic stress) suggests that the aggregation behaviour may be an adaptive strategy in order to enhance both reproductive and recruitment successes.

Mussels typically rely on olfactory cues to detect predators and conspecific, hence to move and form aggregates (*e.g.* de Vooys, 2003; Liu et al., 2011; Uguen et al., 2022, 2023). Specifically, the observed aggregation pattern may rely on sex pheromones released by individuals with ripe gametes. Indeed, in marine organisms, aggregation and pairing behaviour are stimulated by sex pheromones released by females and specifically by unfertilised mature oocytes (*e.g.* Boal et al., 2010; Gaudin-Zatylny et al., 2022; Gaudron et al., 2007; Painter et al., 1998), but can also be mediated by males (*e.g.* Marquet et al., 2018). To gain a deeper understanding of this behaviour, further research is needed to assess the precise source of these potential pheromones and to identify the specific molecules involved in this intricate chemical communication.

Gametes production is an exceedingly energy-demanding process and mussels expressed a loss of condition following spawning (Seed & Suchanek, 1992), which is consistent with the lower aggregation rate of our mussels at this period. A similar phenomenon has been observed in the *M. edulis* byssus thread quality, which exhibits a cyclical pattern linked to reproductive maturity, reaching its lowest point after the spawning season (Moeser et al., 2006). This has significant implications, such as increased mussel dislodging risk after spawning. In aggregations, mussels rely not only on their own byssal threads but also on attachments from other mussels (de Jager et al., 2017). Therefore, mussel resistance to dislodgment is linked to both the byssus quality and neighbour quantity (de Jager et al., 2017). Given that both of these parameters reach their lowest levels after spawning, mussel collective attachment strength — the overall force required to pull a mussel out of its mussel bed— is consequently diminished.

This observation is congruent with previous studies in the annual cycle of mussel collective attachment strength (Carrington, 2002; Carrington et al., 2015; Lachance, 2007), as opposed to individual strength (Nicastro et al., 2010; Zardi et al., 2007), which emphasised an increased risk of dislodgment during the post-reproductive period. Noticeably, the post-reproductive period coincides with the beginning of the storm season in the eastern English Channel (López Solano et al., 2022), hence further increase in dislodgment risk. This may lead to mass mortality events, as commonly reported at the study site at this period of the year (Seuront, pers. comm.). These events could result in an important loss of resources for both recreational and professional fishing, but also, given their bioengineering role, have ecological implications at the ecosystem level.

Microplastic leachates impair aggregation pattern

The aggregation pattern of *M. edulis* was significantly affected by leachates from virgin polypropylene pellets, with aggregation rates being consistently high and stable from March to July and close to the maximum observed in the control group in May. These results highlight a benefit-cost aggregation threshold that could be set at *ca.* 70%, a value which, if already reached in the control group, may mask the effect of additional stressors. This hypothesis may also explain the absence of impact of various stressors such as predator cues and plastic leachates on mussel aggregation behaviour (Commito et al., 2016; Seuront et al., 2021; Uguen et al., 2023).

While plastic leachates can impair mussel chemoreception, notably by hindering the ability of mussels to recognise their predator chemical cues (Uguen et al., 2022, 2023), they do not seem to impair the ability of *M. edulis* to aggregate. In turn, as mussels aggregate more than in the control experiment, the chemical compounds in the leachate solution might stimulate the release of aggregation pheromones, potentially as a defence rather than a reproductive signal (Wertheim et al., 2005). This hypothesis is consistent with the range of harmful effects plastic leachates cause in mussels from the cellular to the behavioural levels (Capolupo et al., 2020, 2021a, 2023; Cozzolino et al., 2023; Gandara e Silva et al., 2016; Seuront et al., 2021; Uguen et al., 2022, 2023). The response to plastic leachates may involve a dynamic adjustment of aggregation behaviour in *M. edulis* until an optimum, determined by a balance between its benefits and its costs, and the mussel energy budget available for such behaviour. As the increase in aggregation behaviour following an exposure to plastic leachates is not constant throughout the year, mussels may show a "window of sensitivity" to pollutants, in agreement with previous studies showing that seasonal physiological differences affect the sensitivity of species to contaminants (*e.g.* Dissanayake et al., 2011; Malanga et al., 2007; Ramos et al., 2014; Winner et al., 1990). This hypothesis is noticeably consistent with the energetic cost of aggregation behaviour, which may not be manageable following the spawning period to reach the *ca.* 70% optimum aggregation rate.

The observed aggregation patterns following an exposure to plastic leachate may also be related to an increase in the reproductive signal. Indeed, the main additives released by the polypropylene pellets used in this study are endocrine-disrupting chemicals (*e.g.* Bisphenols, Phthalates, Nonylphenols) that are known to lead to a range of perturbations in aquatic species, such as hormonal and reproductive impairments; see Burgos-Aceves et al. (2021), Oehlmann et al. (2009), Zala and Penn (2004) and Zhang et al. (2021) for reviews. More specifically, it has been shown that plastic additives, notably bisphenol and phthalates induced early spawning in *M. edulis* (Aarab et al., 2006). Thus, plastic leachates may act on mussels in an advanced stage of gametogenesis (stages 4 and 5), bringing them closer to their spawning event and potentially triggering the release of sexual aggregation pheromones. This would explain the observed patterns of increased aggregation behaviour during this specific period, to a threshold close to control aggregation at the start of the spawning event, while highlighting the absence of a similar response during the subsequent months when mussel gonads are in immature to partial gametogenesis stages (stages 1, 2 and 3).

Premature spawning may induce a mismatch between larvae production and optimal feeding conditions, leading to a reduced recruitment (Cushing, 1990). Plastic leachates have also been shown to disrupt gametes, fertilisation, and larvae development in various bivalves, *e.g. Magallana gigas, Pinctada margaritifera, Mytillus galloprovincialis, Perna perna* (Capolupo et al., 2020, 2023; Gardon et al., 2020; Tallec et al., 2022a). Our results further suggest that plastic leachates may act directly on the aggregation behaviour. Although this could be seen as a benefit in terms of protection against predation, wave dislodgment and temperature fluctuation (Côté & Jelnikar, 1999; Nicastro et al., 2007, 2012; Reimer & Tedengren, 1997; Zardi et al., 2021), this would reduce their access to food (Nielsen & Vismann, 2014). As aggregation is an energetically costly behaviour, the observed changes in aggregation rates following an exposure to plastic leachate may lead to a deficit in their energy budget and have repercussions on the species' fitness. These disturbances may have cascading effects on mussel population dynamics but also on the wide range of species associated with these aggregations.

5. Conclusion

We showed that mussel aggregation behaviour varies seasonally and seems related to the mussel reproductive cycle. Indeed, mussel aggregation increases with the gamete maturation and the proximity of the spawning event, decreasing afterwards. However, plastic leachates disrupt this seasonal aggregation pattern, primarily affecting the reproductive period by driving mussel aggregation levels to a threshold similar than in the control spawning conditions. By disturbing these key functions, plastic leachates may have implications not only on mussel fitness but also at larger ecological scales, by affecting the formation of the biogenic habitats of a wide variety of species. As such, our results warrant the need for further work in particular to decipher the role plastic leachates may play in mussel aggregation behaviour, *i.e.* as a trigger of a defence signal or an amplifier of a reproductive signal.

Supplementary Materials

S26: Mean and standard deviation of the sea surface temperature (°C) and Salinity (psu) for each month of 2021. Data were obtained from Coastal Coriolis data portal, for the MAREL Carnot station point (50°74’05”N, 1°56’77”E; https://data.coriolis-cotier.org/)

Month	Temperature	Salinity
Jan	7.62 (± 0.61)	32.26 (± 1.75)
Fev	6.58 (± 0.76)	32.71 (± 0.72)
Mar	7.75 (± 0.61)	33.93 (± 0.79)
Apr	8.87 (± 0.49)	33.53 (± 0.92)
May	11.99 (± 0.95)	30.90 (± 2.38)
Jun	15.67 (± 0.88)	30.29 (± 0.90)
Jul	17.95 (± 0.94)	32.13 (± 2.65)
Aug	18.7 (± 0.31)	31.66 (± 2.00)
Sep	18.72 (± 0.46)	33.38 (± 0.40)
Oct	15.42 (± 0.85)	33.64 (± 1.03)
Nov	12.33 (± 1.02)	34.21 (± 0.83)
Dec	8.89 (± 0.47)	33.09 (± 0.96)

S27: Additives identification protocol adapted from Uguen et al., 2023 (A) and results of the identified additives in the polypropylene pellet before and after the 24 h incubation (B)

A.

Samples preparation

Raw polypropylene pellets were cut using a scalpel under a binocular microscope (Olympus SZX16), to obtain fragments of ca. 500 µm^2. These fragments were subsequently inserted into pyrolysis quartz tubes.

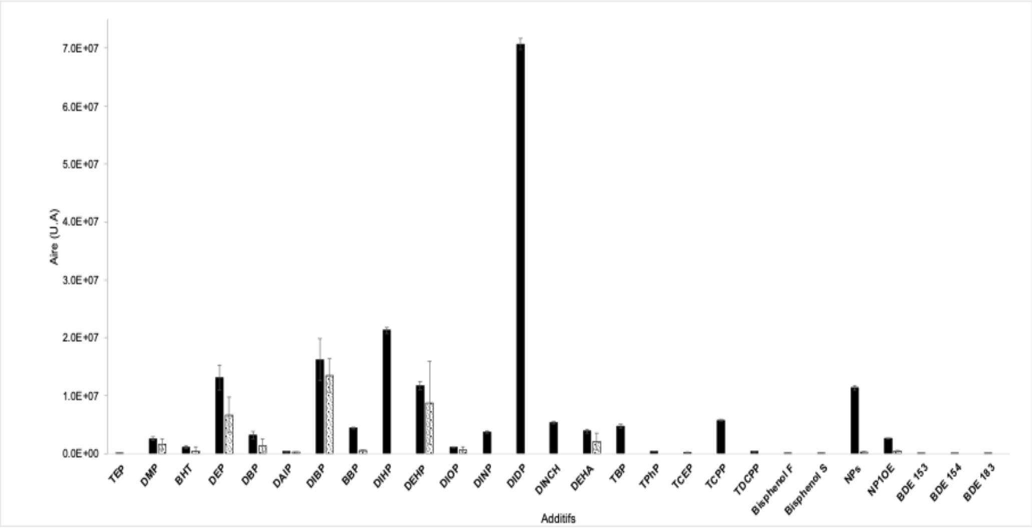
Thermodesorption method for the detection of Organic Plastic Additives (OPAs) using Py-GC-HRMS (Pyrolysis coupled to a gas chromatography and a high-resolution mass spectrometer)

All analyses were carried out using a pyrolyser CDS Pyroprobe 6150 (CDS Analytical) followed by a GC-HRMS device (GC Trace 1310-MS Orbitrap Q exactive, Termo Fisher Scientific). With the aim to thermally desorb the chemicals additives potentially contained into the samples, they were heated into the pyrolyser at a temperature of 350 °C. Samples were subsequently separated on a GC column (Restek Rxi-5-MS capillary column, cross-linked poly 5% diphenyl-95% dimethylsiloxane, 30 m × 0.25 mm (i.d.) × 0.25 µm film thickness), with a split ratio of 1:5. The acquisition was performed on full-scan (FS) mode (m/z = 30.00000–600.00000). The resulting chromatograms were analysed using Xcalibur and TraceFinder softwares to identify OPAs among a selection of additives including plasticisers, flame retardants, antioxidants and UVs stabilisers. The respective additives were identified on the basis of the retention times, m/z, and specific ions after a comparison with the chromatograms of the respective standard solutions.

Identified additives

- 3 brominated flame retardants, *i.e.* 2,2',4,4',5,5'-Hexabromodiphenyl ether (BDE-153); 2,2',4,4',5,6'-Hexabromodiphenyl ether (BDE-154) and 2,2',3,4,4',5',6-Heptabromodiphenyl ether (BDE-183);
- 6 phosphorus flame retardants, *i.e.* Tributyl Phosphate (TBP); Triethyl Phosphate (TEP); Tris(2-Chloroethyl)Phosphate (TEPP); Tris(2-Chloroisopropyl)Phosphate (TCPP); Tris(1,3-Dichloro-2-Propyl)Phosphate (TDCPP) and Triphenyl Phosphate (TPhP)
- 5 antioxidants, *i.e.* Butylated hydroxytoluene (BHT); Bisphenol F (BPF); Bisphenol S (BPS); Nonylphenol (NPs) and Nonylphenol monoethoxylate (NP10E)
- 13 plasticisers, *i.e.* Dimethyl phthalates (DMP); Diethyl phthalate (DEP); Di-n-butyl phthalate (DBP); Di-allyl phthalate (DAIP); Diisobutyl phthalate (DIBP); Benzylbutyl phthalates (BBP); Diisoheptyl phthalate, (DIHP); Bis(2-Ethylhexyl) phthalate (DEHP); Diisononyl phthalate (DINP); Diisodecyl phthalate (DIDP); Diisononyl hexahydrophthalate (DINCH); Di-n-octyl phthalate (DIOP); Bis-2-Ethylhexyl Adipate (DEHA)
-

B.



S28: Determination of stages of reproduction of both sex

The sexual maturity of all 20 specimens could not be achieved and a total of 105 specimens were assessed from March to December 2021, composed of 35 males, 66 females and 4 undifferentiated individuals, with at least 7 individuals by month (min= 7; max= 11; for details see Table 1). Five main stages are described following different criteria from previous studies (Chiperfield, 1951; Seed, 1969; Auffret at al., 2003; for details see Fig. 1, 2). For females, the mean oocytes Feret diameter was used in a final decision to attribute the stage of reproduction. The frequency of the different reproductive stages of *Mytilus edulis* sample in 2021 was thus obtained (Fig. 3).

Table 1: details about the individuals used to assessed the histological stage, *i.e.,* sex, stages, total number and measured mean sexual stage.

	Sex			Reproductive stage						
Month	Male	Female	Undiferentiated	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Total ind.	Mean stage
March	3	7				8	2		10	3.20
April	2	6				2	6		8	3.75
May	3	5		1				7	8	4.50
June	6	5						11	11	5,00
July	2	3	2	6				1	7	1.57
August	1	7	1	2	2			5	9	3.44
September	4	7	1	2	10				12	1.83
October	5	6		1	6	4			11	2.27
November	3	6			1	8			9	2.89
December	3	7				10			10	3.00

Ranking and criteria for female (Fig. 1)

Stage I: **Resting stage (Fig.1A&B)**

This is a transition stage where females have spawned all their oocytes, and the gonad is in a resting stage. Some acinus can be seen still with few oocytes but those are atretic being lysed with some anomalies in their membrane or in their cytoplasm. Most of acini are empty. Some pink colouration highlights special cells, with the adipogranular cells that accumulate protein and lipid, and the vesicular cells that are translucent that accumulate glycogen.

Stage II: Start of oogenesis (Fig.1C&D)

This stage is characterised by the proliferation of oogonia by mitosis, where oogonia are attached to the follicle membrane. There is a highly presence of reserve cells between follicles.

Stage III: Vitellogenesis (Fig.1E&F)

Vitellogenic oocytes are increasing in size but are still anchored into the follicle membrane. They look like pedunculated and a nucleolus is visible. The number of storage reserve is decreasing.

Stage IV: Maturation (Fig.1G&H)

Oocytes are fully-mature and free within the follicle, ready to be released. They have attained their optimal size (mean Feret oocyte diameter). Follicles fulfilled the gonad with the disappearance of reserve cells.

Stage V: Spawning stage (Fig.1I&J)

Most of follicles are empty but the spawning is partial and there are still follicles with oocytes at different stages (vitellogenic and mature oocytes). There is a reappearance of reserve cells.

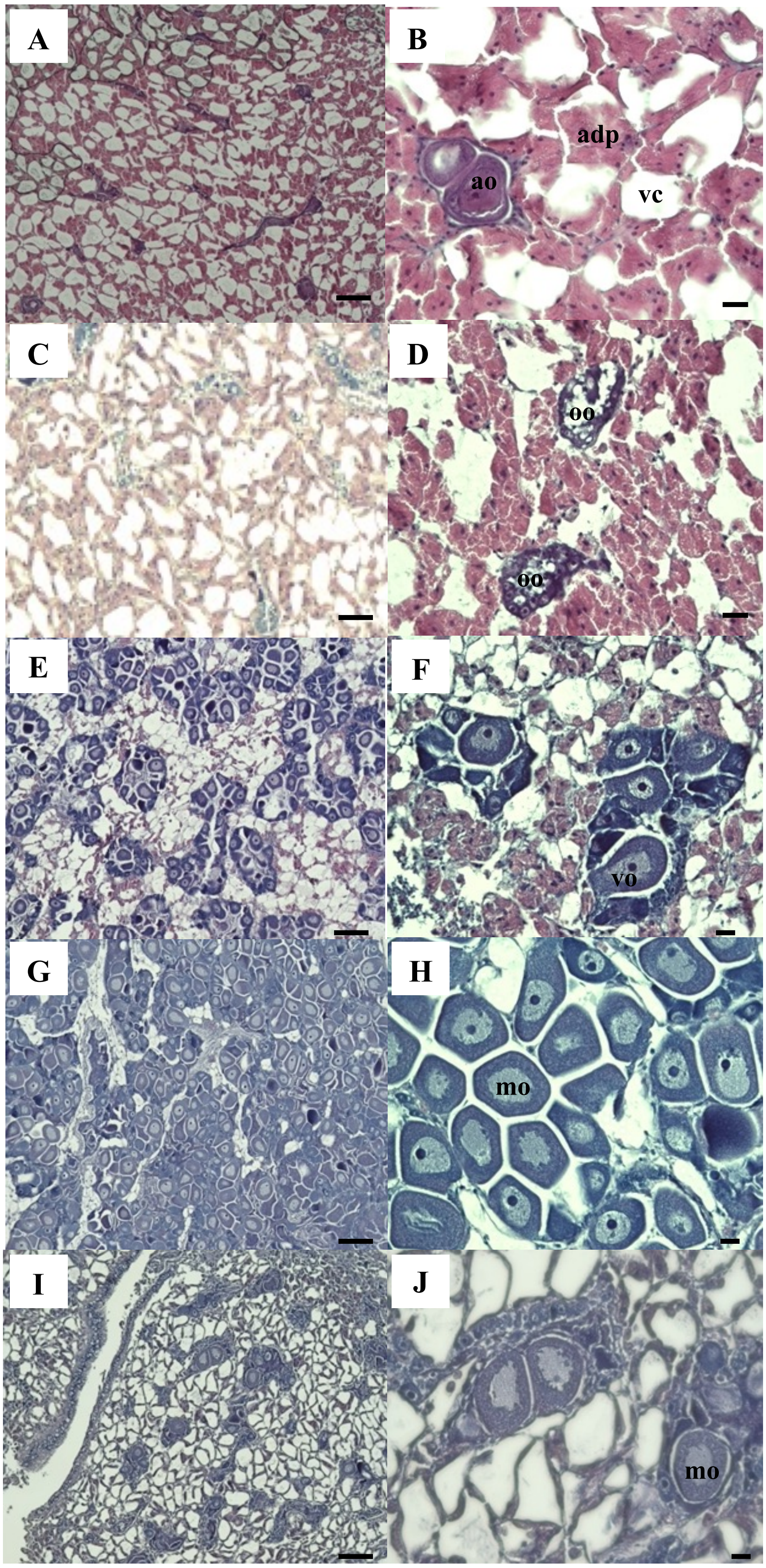


Figure 1: Histological sections through female gonad of *Mytilus edulis* at various stages of reproduction stained with Haematoxylin and Eosin. Resting stage (undifferentiated; A & B). Start of oogenesis (early development; C & D). Vitellogenesis (late development; E & F). Maturation (ripe; G & H). Spawning stage (spent; I & J). (ao) atretic oocytes; (adp) adipogranular cells; (vc) vesicular cells; (oo) oogonia; (vo) vitellogenic oocyte; (mo) mature oocyte. Scale bars are 100 µm for A, C, E, G & I. Scale bars are 20 µm for B, D, F, H & J.

Ranking and criteria for male (Fig. 2)

Stage I: **Resting stage (Fig.2A&B)**

This is a transition stage where males have spawned by releasing all the spermatozoids. The gonad is in a resting stage. Few germ cells are present in purple. Some pink colouration highlights special cells, with the adipogranular cells that accumulate protein and lipid, and the vesicular cells that are translucent that accumulate glycogen.

Stage II: Start of spermatogenesis (Fig.2C&D)

This stage is characterised by the proliferation of spermatogonia by mitosis, with some differentiation towards the centre of the follicle of spermatocyte. Islands of germinal tissue appeared at the base of the acinus like a branch. There is a highly presence of reserve cells between follicles.

Stage III: Growth and maturation (Fig.2E&F)

The number of follicles with male gametes has increased with the purple colouration starting to dominate in the histological section. Different stages of male gametes are visible (spermatogonia, spermatocytes and spermatozoids) with the dominance of spermatocytes. The number of storage reserve is decreasing.

Stage IV: Fully ripe (Fig.2G&H)

The pink colouration within the gonad has disappeared. The gonad is fulfilled with follicles dominated by spermatozoid arranged in rosettes with their flagella in the lumen of the follicle.

Stage V: Partial spawning stage (Fig.2I&J)

Some follicles are devoid of male gametes indicating a partial spawning event, but there are still follicles in ripe stages with spermatozoids. However, between these ripe follicles there is the reappearance of reserve cells.

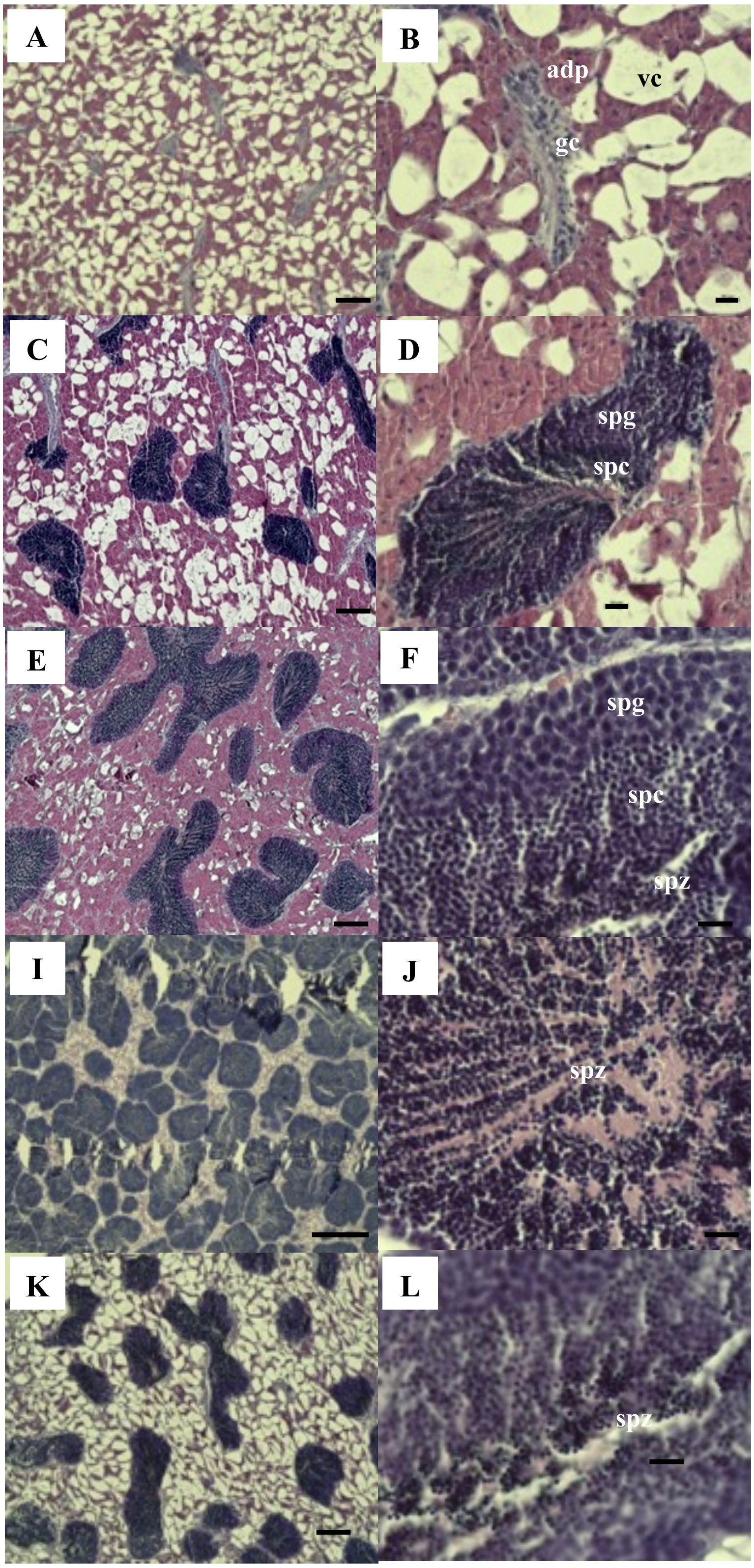


Figure 2: Histological sections of male gonad of *Mytilus edulis* at various stages of reproduction stained with Haematoxylin and Eosin. Resting stage (undifferentiated; A & B). Start of spermatogenesis (early development; C & D). Growth and maturation (late development; E & F). Fully ripe (ripe; G & H). Partial spawning stage (spent; I & J). (adp) adipogranular cells; (vc) vesicular cells; (gc) germinal cells; (spg) spermatogonia; (spc) spermatocyte; (spz) spermatozoid. Scale bars are 100 µm for A, C, E, G & I. Scale bars are 20 µm for B & D. Scale bars are 10 µm for F, H & J.

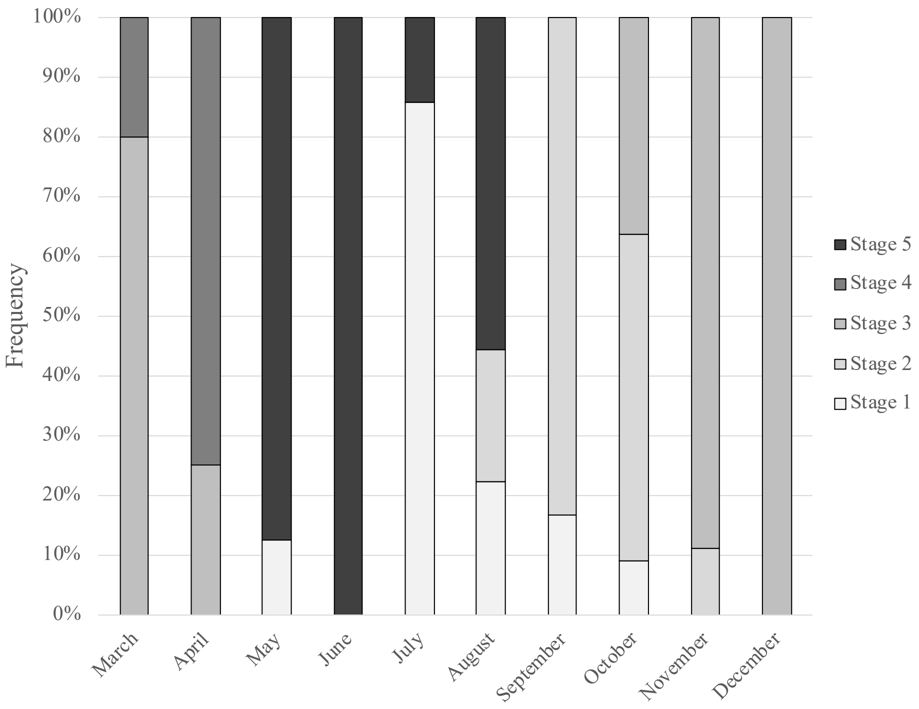
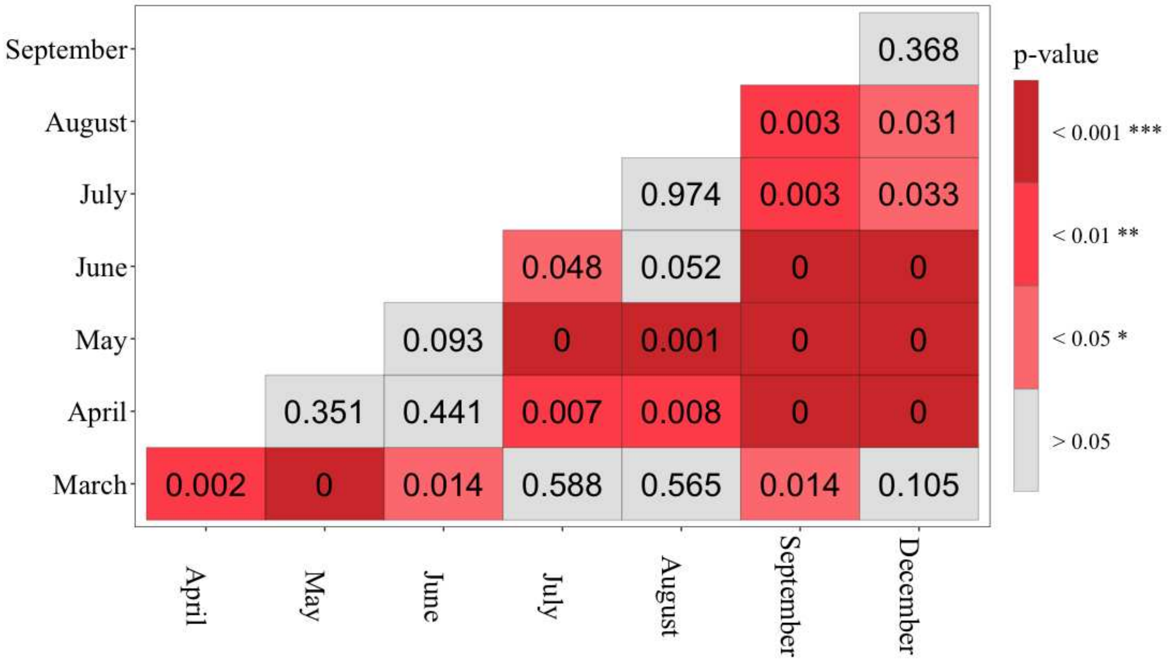


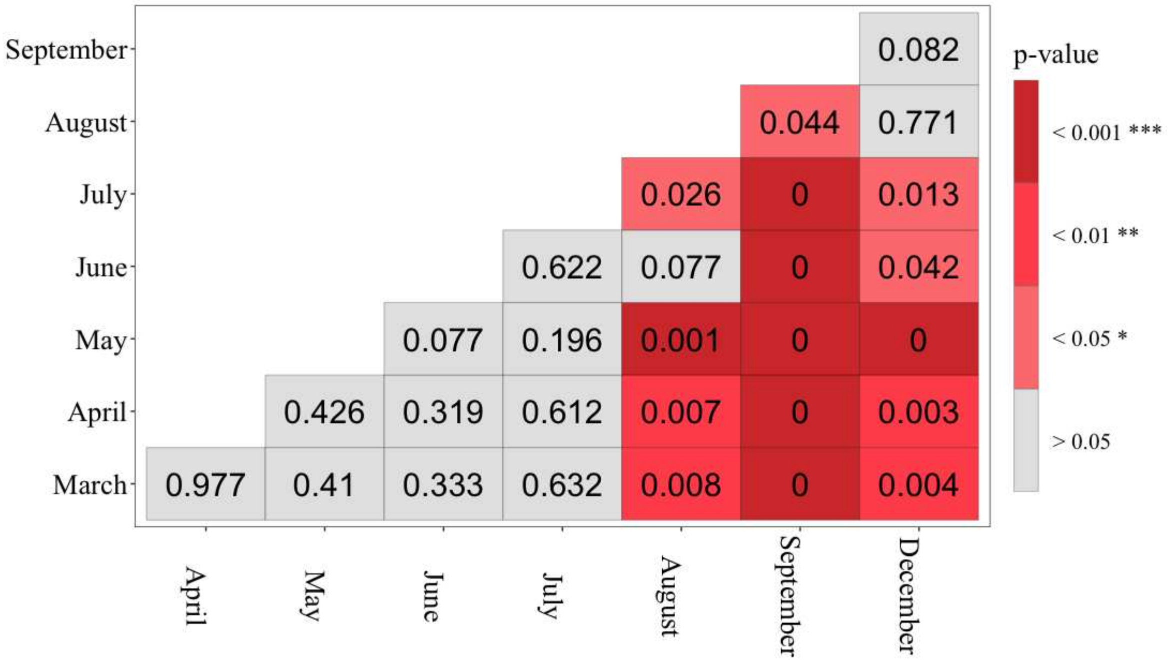
Figure 3: Frequency of the different reproductive stages of *Mytilus edulis* sample in 2021 from a rocky intertidal reef (Pointe aux Oies, Wimereux; 50°47’08.3"N, 1°36’03.9"E)

S29: Results of Conover post-hoc test with Holm’s correction for Control data. p-values are rounded to 3 decimal places.



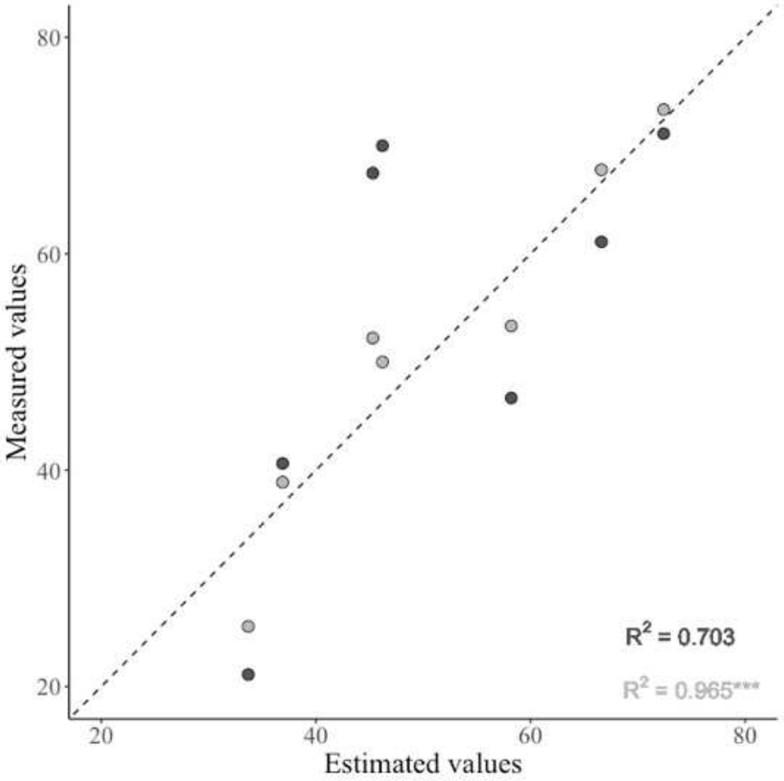
	April	May	June	July	August	September	December
September							0.368
August						0.003	0.031
July					0.974	0.003	0.033
June				0.048	0.052	0	0
May			0.093	0	0.001	0	0
April		0.351	0.441	0.007	0.008	0	0
March	0.002	0	0.014	0.588	0.565	0.014	0.105

S30: Results of Conover post-hoc test with Holm's correction for Microplastic leachate data. P-values are rounded to 3 decimal places.



	April	May	June	July	August	September	December
September							0.082
August						0.044	0.771
July					0.026	0	0.013
June				0.622	0.077	0	0.042
May			0.077	0.196	0.001	0	0
April		0.426	0.319	0.612	0.007	0	0.003
March	0.977	0.41	0.333	0.632	0.008	0	0.004

S31: Measured and estimated values for the aggregation rate for control (grey) and microplastic leachates data (black) and their respective correlation coefficient (R^2). The dashed line is the first bissectrix, i.e. Measured values = Estimated values. Estiamted values are determined by using the following equation: Agg(μ) = 31.04 · *sin*(1.63 · μ - 11.72) + 53.28; with μ the mean reproductive stage. *** = p-value < 0.001



CHAPTER VI

LOCATION-DEPENDENT EFFECT OF MICROPLASTIC LEACHATES ON THE RESPIRATION RATE OF TWO ENGINEERING MUSSEL SPECIES

Location-dependent effect of microplastic leachates on the respiration rate of two engineering mussel species

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Note: This project was conducted following the allocation of the "PhD initiative" grant from the INTEREST team provided by the laboratory UMR8187 LOG. This grant aimed to fund a project between two PhD students of the INTEREST team to foster initiative and independence from their supervisors in preparation for a postdoctorate.

Abstract

Microplastics are ubiquitous in the world's oceans and pose serious environmental concerns, including their ingestion and the release of potentially toxic mixtures of intrinsic and extrinsic chemical compounds (*i.e.* leachates; MPLs). Mussels, as key intertidal bioengineers and filter-feeders, are particularly susceptible to both exposure pathways. While the effects of microplastic ingestion have been widely investigated, research on the impacts of MPLs has only recently begun. This study examined the influence of MPLs derived from beached pellets collected in two separate regions, namely France and Portugal, on the respiration rates of two key ecosystem engineers, *Mytilus edulis* and *M. galloprovincialis*. Possibly due to distinct mixtures of leached chemicals, unlike Portuguese-MPLs, exposure to French-MPLs significantly decreased the respiration rate of both mussel species. This research provides new insights into the physiological impacts of MPLs on bioengineer species and highlights the relevance of MP sources and potential cascading effects at the ecosystem level.

1. Introduction

Plastic pollution is one of the major challenges of our time (Horton, 2022). Over the past decades, the growing demand for plastic has led to an exponential increase in production, which together with the global mismanagement of plastic waste disposal and the slow implementation of recycling strategies, has generated a dramatic accumulation of plastic waste in both terrestrial and aquatic environments (Cole et al., 2011; Law, 2017; Ostle et al., 2019). The extent of this accumulation is such that plastic now fundamentally jeopardises global biodiversity with negative implications for natural ecosystems, organisms and human well-being (Heidbreder et al., 2019; Smith et al., 2018; Thushari & Senevirathna, 2020).

Microplastics (*i.e.* plastic particles < 5mm; MPs; Law & Thompson, 2014) are of greater concern as they are the most abundant fraction of marine debris (Eriksen et al., 2014) and impact almost every level of the marine food web (Thushari & Senevirathna, 2020). They can be of primary (*i.e.* microbeads and industrial raw pellets) or secondary (*i.e.* derived from the fragmentation of larger plastic items; Cole et al., 2011; Hermabessiere et al., 2017) origin. Among primary MPs, the raw resin pellets used in the manufacture of plastic items are often accidentally leaked into the environment throughout their production chain or during their transportation (Gravier & Haut, 2020; OSPAR Commission, 2018). In the EU alone, it is estimated that the loss of plastic pellets ranges from approximately 17,000 to 165,000 tonnes annually, with over 40,000 tonnes ultimately reaching the oceans (Hann et al., 2018). In addition, localised spills often occur, causing extreme pollution events, as in Sri Lanka, where 70 billion plastic pellets were discharged following a shipwreck (Jayathilaka et al., 2022).

Once in the marine environments, MPs have the potential to leach a variety of functional additives incorporated during their production (*e.g.* flame retardants, plasticisers, stabilisers and metals; Hahladakis et al., 2018; Hermabessiere et al., 2017; Thompson et al., 2009), and also adsorb and desorb a range of anthropogenic chemical compounds, such as persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs) and heavy metals (Fries & Zarfl, 2012; Rodrigues et al., 2019b; Yeo et al., 2020). As a result of the sorption/desorption subtle dynamics between the surface of MPs and their surroundings—see *e.g.* Delaeter et al. (2022) for a review—the harm to organisms appears not to be only physical, that is due to MPs ingestion (Thushari & Senevirathna, 2020; Wright et al., 2013), but also chemical as a consequence of the leaching of potentially toxic chemical compounds (Delaeter et al., 2022; Lynch et al., 2022; Seuront et al., 2022).

As filter feeders, mussels are especially susceptible to various contaminants in the water column, including microplastics (Beyer et al., 2017) and their leachates (Jang et al., 2021). The ingestion of MPs, for example, has numerous negative effects on mussels (for a review, see Zhang et al., 2020), including alterations in their filtration and respiration rates (*e.g.* (Huang et al., 2022b; Rist et al., 2016; Tang et al., 2022; Wang et al., 2021). These are key physiological processes, closely related and interdependent. The mechanism that mussels use for filter-feeding, the movement of water over their gills, is also crucial for their respiration as it allows oxygen diffusion from seawater to the haemolymph (Gosling, 2008). Thus, any disruption or change in one process could impact the other, affecting mussels' survival and overall health. In this context, although exposure to microplastic leachates (MPLs) has recently been shown to alter reproductive success (Capolupo et al., 2020, 2023; Gandara e Silva et al., 2016), behavioural (Seuront et al., 2021; Uguen et al., 2022, 2023), and physiological (Cozzolino et al., 2023) mechanisms in mussels, their potential effects on respiration remain unexplored. This is critical not just for the organisms or species itself, but also potentially for the ecosystem, as mussels are key coastal ecosystem engineers in intertidal habitats (Suchanek, 1985). They form dense and dynamic beds that support local biodiversity (Borthagaray & Carranza, 2007; Buschbaum et al., 2009), providing essential ecological functions and services including water filtration (van Leeuwen et al., 2010), coastal protection (Ysebaert et al., 2019), and hold high economic value through food provision (Avdelas et al., 2021; Zippay & Helmuth, 2012). As a consequence, the impact of MPLs on mussel respiration can trigger adverse cascading effects and indirectly affect the well-being of other organisms, including humans.

On this basis, we investigated the effect of beached microplastic leachates (MPLs) on the respiration rate of two closely related intertidal mussel species, the blue mussel *Mytilus edulis*, and the Mediterranean mussel *Mytilus galloprovincialis.* These were selected as model species because they are dominant ecosystem engineers along the Atlantic European shore (Fly et al., 2015; Gosling, 1992; Hilbish et al., 2012; Skibinski et al., 1983) and are widely regarded as sentinel species (Beyer et al., 2017; Li et al., 2019). In addition, because the relative composition of contaminants carried by beached MPs can vary greatly, often exhibiting high spatial variability (Fisner et al., 2013; Ogata et al., 2009; Taniguchi et al., 2016; Vedolin et al., 2018), we tested the effect of MP pellets collected at two distinct geographical locations, namely Northern France and Southern Portugal, where each species respectively occurs. We hypothesised that (i) the respiration rate of both mussel species would have been impaired by MPLs and that (ii) distinct MPLs would have different effects on mussels' respiration.

2. Material and Methods

Study organism

Adult *Mytilus edulis* (*n* = 36; shell length: 4.67 ± 2.70 cm; mean ± standard deviation) and *Mytilus galloprovincialis* (*n* = 36; shell length: 4.26 ± 1.77 cm) were collected in January 2023 from an intertidal rocky reef at Pointe aux Oies (Wimereux, North of France; 50°47'08.0"N 1°35'59.0"E) and Praia de Faro (Faro, South of Portugal; 37°0'33.5''N -7°59'36.3''W), respectively. Mussels were manually cleared of epifauna and acclimated for 24 h in glass aquaria filled with 50 L aerated seawater representative of *in situ* conditions (*i.e.* S = 35.5 PSU, T = 15 °C for *M. galloprovincialis*; S = 32.5 PSU, T = 13 °C for *M. edulis*).

Microplastic leachate treatment

Microplastic pellets were collected from the shore of Northern France (Ambleteuse; 50°80'61.9"N 1°60'31.34"E; 4.04 ± 0.56 mm in height; >75% black;) and Southern Portugal (Praia do Castelejo; 37°10'02.60"N -8°94'51.92"W; 4.24 ± 0.42 mm in height; >75% white).

Three treatments were considered: (i) control (C) which was seawater as in acclimation conditions, (ii) French MPLs (FR-MPLs) and (iii) Portuguese MPLs (PT-MPLs). The MPL solutions were prepared at room temperature (*i.e.* 17 °C) by incubating low density polyethylene pellets collected at each location in aerated control seawater for 24 h at a concentration of 20 mL of plastic pellets per litre (Seuront, 2018; Uguen et al., 2023). Pellets were removed from the solution at the end of each incubation before the metabolic measurements took place.

Pyrolysis analysis coupled with gas chromatography and high-resolution mass spectrometry was used to assess the additive content of each batch of pellets before incubation. French pellets were associated to higher additive abundance, *i.e.* 10 additives, including 7 plasticisers, 2 antioxidants and 1 UV stabiliser, than Portuguese pellets, which contained the same additives with the exception of 1 antioxidant and 1 UV stabiliser. Overall, additive concentration was 2 to 18-fold higher in French pellets than in Portuguese ones. For a detailed list of additives composition and the method used, please refer to Supplementary Material, S32.

Respiration rate

For each species, mussels (*n* = 27) were individually placed into sealed glass respirometry chambers (V = 200 mL) filled with 100% O_2 saturated of either C, PT-MPLs or FR-MPLs sea water (*n* = 9 replicates per treatment). Blank chambers (*n* = 3) containing cleaned empty mussel shells were considered in each treatment. Oxygen concentration was recorded every 15 min for 1 hour using a non-invasive fibre optic oxygen measurement system (Fibox4, PreSens). Respiration rate (R, mgO$_2$ ind^{-1} h^{-1}) was calculated following Lampert (1984) as:

$$R = \frac{Ci_a - Cf_a}{T_a} - \frac{Ci_b - Cf_b}{T_b} \times V$$

where Ci_a is the initial O$_2$ concentration in the respiratory chamber with mussel (mg L^{-1}), Cf_a the final O$_2$ concentration in the respiratory chamber with mussel (mg L^{-1}), T_a the incubation time for the respiratory chamber with mussel (h), Ci_b the initial O$_2$ concentration for the blank (mg L^{-1}), Cf_b the final O$_2$ concentration for the blank (mg L^{-1}), T_b the incubation time for the blank (h) and V the volume of water considered in each respiratory chamber (L). Respiration rate was subsequently standardised to 45 mm shell length (R$_{std}$, mgO$_2$ ind^{-1} h^{-1}) following Bayne et al. (1987) as:

$$R_{std} = R\left(\frac{L_s}{L_m}\right)^b$$

Where L_s is the length of the mussel into the chamber (mm), L_m an arbitrary length fixed to 45 mm, and b the allometric coefficient between the size and the respiration rate. The value of the allometric coefficient is generally $ca.\ {}^3/_4$ in bivalves (Savina & Pouvreau, 2004) when it relates respiration rate to weight. Based on the assumption that weight scales with cubic length, this coefficient was recalculated to 2.25 to relate respiration rate to length.

Statistical analyses

To avoid potential biases in mussel respiration rates, individuals of both species were subjected to visual inspection for the presence of pea crabs (*Pinnotheres pisum*, as described by Bierbaum and Shumway, 1988). Specimens found to be infested were excluded from the analysis. Infestation was observed only in *M. edulis*, with two specimens in the C treatment and one each in the FR and PT-MPLs treatments, resulting in an unbalanced design for this species. Respiration rates obtained for *M. galloprovincialis* and *M. edulis* were normally distributed (Shapiro's test, $p = 0.775$ and $p = 0.567$, respectively) and homoscedastic (Levene's test, $p = 0.720$ and $p = 0.429$, respectively). One-way ANOVA was then used for each mussel species with "treatment" (C, PT-MPLs, FR-MPLs) as a fixed factor. Significant effects were examined using Tukey-HSD post-hoc test for balanced design (*i.e.* not infested *M. galloprovincialis* data) and Dunn post-hoc test with Bonferroni adjustment for unbalanced design (*i.e. M. edulis* data; Zar, 1999).

3. Results

The respiration rates of both *Mytilus galloprovincialis* and *Mytilus edulis* were significantly affected by MPLs (ANOVA, $p = 0.002$ and $p = 0.016$, respectively; Figure 39). Specifically, the respiration rates decreased significantly when exposed to FR-MPLs compared to the control for both *M. galloprovincialis* (Tukey test, $p = 0.002$; Figure 39A) and *M. edulis* (Dunn test, $p = 0.028$; Figure 39B). In contrast, for both species, no significant effects were observed in mussels exposed to PT-MPLs in comparison to the control group (*M. galloprovincialis*, Tukey test, $p = 0.055$; *M. edulis*, Dunn test, $p = 0.499$; Figure 39) or FR-MPLs (*M. galloprovincialis*, Tukey test, $p = 0.297$; *M. edulis*, Dunn test, $p = 0.630$, respectively; Figure 39).

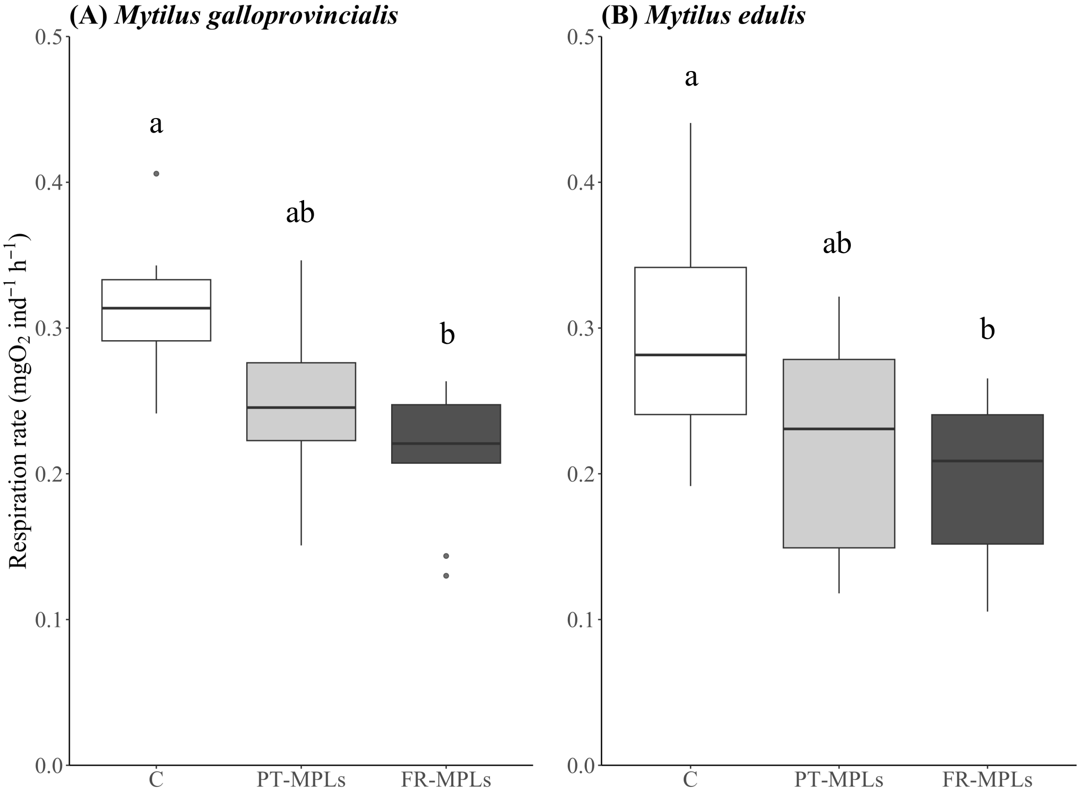

Figure 39: Respiration rate (mgO_2 ind^{-1} h^{-1}) of (A) *Mytilus galloprovincialis* and (B) *Mytilus edulis* when exposed to control seawater (C; white), Portuguese microplastic leachates (PT-MPLs; grey) and French microplastic leachates (FR-MPLs; black). Letters depict significant differences among treatments ($p < 0.05$; one-way ANOVA with (A) Tukey and (B) Dunn post-hoc test comparison).

4. Discussion

Microplastic leachates (MPLs) impaired the respiration rate of *Mytilus galloprovincialis* and *Mytilus edulis.* These findings are in accordance with previous research showing a significant decrease in clearance and respiration rates in the Pacific oyster *Magallana gigas,* during exposure to tyre leachates (Tallec et al., 2022b). In mussels, alterations in filtration, clearance and respiration rates were only observed during exposure to MP particles (*e.g.* Huang et al., 2022b; Rist et al., 2016; Sui et al., 2022, 2023; Tang et al., 2022; Wang et al., 2021). However, none of these studies conducted analyses on the chemical compounds leached from MPs, leaving uncertainty as to whether the alteration observed was due to the effects of the particles or the effects of their leachates.

Our work shows a significant decrease in the respiration rates of both mussel species during exposure to French microplastic leachates (FR-MPLs). PT-MPLs exhibited an intermediate effect, with no significant difference observed when compared to both control conditions and FR-MPLs. It is now well-established that the relative composition of contaminants can vary greatly between beached MPs and often exhibit high spatial variability (Fisner et al., 2013; Ogata et al., 2009; Taniguchi et al., 2016; Vedolin et al., 2018). The additive composition analysis performed in this study on a subsample of beached MPs revealed that both PT-MPs and FR-MPs carried a range of additives acknowledged for their detrimental effects on aquatic organisms (Burgos-Aceves et al., 2021; Liu et al., 2021; Oehlmann et al., 2009). Specifically, FR-MPs had higher additive abundance (n = 10) and concentration (2 to 18-fold higher) compared to PT-MPs (n = 8). We suggest that the variations observed between treatments were driven by their respective composition of additives (*e.g.* abundance and concentration). Similarly, it has been reported that the decrease in respiration rate of the oyster *M. gigas* was directly dependent on leachate concentration (Tallec et al., 2022b).

In addition, beached MPs often act as sources of heavy metals, making up complex toxic mixtures; see Cao et al. (2021) for a review. Recent findings also indicate that pollutants within such mixtures may contribute to the toxicity of MPLs, even when their concentrations are below their individual effect thresholds (Escher et al., 2020; Kortenkamp & Faust, 2018). Though we did not run further chemical analyses, it is nonetheless worth mentioning that beached pellets sampled in the eastern English Channel and in southern Portugal noticeably differ in their heavy metal content which has been recognised to impair movement and aggregation behaviour in both *M. galloprovincialis* (Cozzolino et al., 2023) and *M. edulis* (Zardi et al., in press). Indeed, the eastern English Channel has been widely acknowledged for the contamination of its sediment and coastal waters related to its history of metallurgical plants (Boughriet et al., 1994; Cuvillier-Hot et al., 2018; Kerambrun et al., 2012; Proix, 1989; Wartel et al., 1990), which is in contrast with the relatively pristine conditions characterising the coastal waters of southern Portugal (Mayer, 2022). Such differences are consistent with the changes observed in respiration rates, as heavy metals are known to affect oxygen uptake, filtration and heart rates, valve movement and gills structure in mussels (*e.g.* Brown & Newell, 1972; Curtis et al., 2000, 2001; Gregory et al., 2002; Martinović et al., 2015; Shen & Nugegoda, 2022; Sunila, 1988; Vijayavel et al., 2007). Here, we suggest that MPLs could trigger similar responses in mussels, limiting gas exchange, and, in turn, decreasing respiration rates. Finally, other chemical compounds such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and pesticides that are likely to be more diverse and abundant in the coastal waters of the eastern English Channel, which is highly impacted by terrestrial and riverine inputs, anthropogenic and marine traffic (Agence de l'eau Artois-Picardie, 2016; Dauvin, 2019; Tappin & Millward, 2015), may also have played a role in the observed changes in respiration rates and warrant the need for further work.

Considering the ecosystem engineering role of mussels, it is plausible that the ramifications of this work could extend beyond the direct effects of MPLs on mussel respiration and encompass more comprehensive effects at the ecosystem level. Mussel filtration, which is intrinsically linked to their respiration, enhances nutrient cycling and water quality and creates favourable conditions that increase species richness and local biodiversity (Karatayev et al., 2002; Lindahl et al., 2005; Newell, 2004). If the alterations in respiration here observed during short-term MPL exposure persist over extended periods, they could lead to energy depletion and impact overall fitness, as previously discussed by Sokolova and Lannig (2008) and Sokolova (2013). This would also have negative consequences on mussels' filtration, initiating a chain reaction that would affect water quality and nutrient cycling. Future research should delve into the potential impact of prolonged exposure to MPLs and define the physiological mechanisms leading to decreased respiration.

Supplementary Materials

S32: (A) Additives identification protocol and (B) results of the identified additives in the French and Portuguese MP.

(A) *Samples preparation and Py-GC-HRMS analysis*

Pellets were cut using a scalpel under a binocular microscope (Olympus SZX16), to obtain fragments of *ca*. 500 µm². These fragments were subsequently inserted into pyrolysis quartz tubes. Thermal desorption method for the detection of Organic Plastic Additives (*OPAs)* was performed using Py-GC-HRMS (pyrolysis coupled to gas chromatography and high-resolution mass spectrometry). All analyses were carried out using a pyrolyser CDS Pyroprobe 6150 (CDS Analytical) followed by a GC-HRMS device (GC Trace 1310-MS Orbitrap Q exactive, Termo Fisher Scientific). With the aim to thermally desorb the chemicals additives potentially contained into the samples, they were heated into the pyrolyser at a temperature of 350 °C. Samples were subsequently separated on a GC column (Restek Rxi-5-MS capillary column, cross-linked poly 5% diphenyl-95% dimethylsiloxane, 30 m × 0.25 mm (i.d.) × 0.25 µm film thickness), with a split ratio of 1:5. The acquisition was performed on full-scan (FS) mode (m/z = 30.00000–600.00000). The resulting chromatograms were analysed using Xcalibur and TraceFinder softwares to identify OPAs among a selection of additives including plasticisers, flame retardants, antioxidants and UVs stabilisers (Table S2). The respective additives were identified on the basis of the retention times, m/z, and specific ions after a comparison with the chromatograms of the respective standard solutions.

Table S2. List of screened additives.

Function	N°	Molecules	Abbreviation	CAS
Plasticisers	1	Dimethyl phthalate	**DMP**	**131-11-3**
	2	Diethyl phthalate	**DEP**	**84-66-2**
	3	Di-allyl phthalate	**DAlP**	**131-17-9**
	4	Diisobutyl phthalate	**DIBP**	**84-69-5**
	5	Di-n-butyl phthalate	**DBP**	**84-74-2**
	6	Tributyl Acetyl Citrate	**ATBC**	**77-90-7**
	7	Di-n-hexyl phthalate	**DHP**	**84-75-3**
	8	Benzyl butyl phthalate	**BBP**	**85-68-7**
	9	Bis-2-Ethylhexyl Adipate	**DEHA**	**103-23-1**
	10	Diisoheptyl phthalate	**DIHP**	**71888-89-6**
	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**
	14	Diisononyl hexahydrophthalate	**DINCH**	**166412-78-8**
	15	Di-n-octyl phthalate	**DIOP**	**117-84-0**
	16	Diisononyl phthalate	**DINP**	**68515-48-0**
	17	Di-nonyl phthalate	**DNP**	**84-76-4**

	18	Diisodecyl phthalate	**DIDP**	**68515-49-1**
Flame retardants	19	Triethyl Phosphate	**TEP**	**78-40-0**
	20	Tripropyl Phosphate	**TPP**	**115-86-6**
	21	Tributyl Phosphate	**TBP**	**126-73-8**
	22	2,4,6-Tribromophenol	**2,4,6,TBP**	**118-79-6**
	23	Tris(2-Chloroethyl)Phosphate	**TCEP**	**115-96-8**
	24	Tris(2-Chloroisopropyl)Phosphate	**TCPP**	**13674-84-5**
	25	2,4,4’-Tribromodiphenyl ether	**BDE-28**	**41318-75-6**
	26	Tris(1,3-Dichloro-2-Propyl)Phosphate	**TDCPP**	**13674-87-8**
	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**
	35	2,2’,4,4’,5,6’-Hexabromodiphenyl ether	**BDE-154**	**207122-15-4**
	36	2,2’,3,4,4’,5’,6-Heptabromodiphenyl ether	**BDE-183**	**207122-16-5**
	37	1,2-Bis (2,4,6 Tribromophenoxy) ethane	**BTBPE**	**37853-59-1**
Antioxidants	38	6,6'-di-tert-butyl-2,2'-thiodi-p-cresol	**Irganox® 1081**	**90-66-4**
	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 – plasticisers - stabilisers	48	4-Tert-Octylphenol	**4-t-OP**	**140-66-9**
	49	Nonylphenol	**NPs**	**84852-15-3**
	50	4-nonylphenol	**4-NP**	**104-40-5**
	51	Nonylphenol Monoethoxylate	**NP1EO**	**27986-36-3**
	52	Bisphenol F	**BPF**	**620-92-8**
	53	4-Nonylphenol Monoethoxylate	**4-NP1EO**	**104-35-8**
	54	Bisphenol A	**BPA**	**80-05-7**
	55	Bisphenol B	**BPB**	**77-40-7**
	56	Nonylphenol diethoxylate	**NP2EO**	**N/A**
	57	Bisphenol S	**BPS**	**80-09-1**

(B) *Additive content*

French MP were composed of 10 additives (Figure 1), including 7 plasticisers (Bis(2-EthylHexyl)Adipate, DEHA; Bis(2-Ethylhexyl)phthalate, DEHP; Diethyl Phthalate, DEP; Di-n-butyl Phthalate, DBP; Dimethylphthalate, DMP; Tri(2-ethylhexyl) phosphate, TEHPA; Diisobutyl phthalate, DIBP), 2 antioxidants (Butylated hydroxytoluene, BHT; Bisphenol S, BPS) and 1 UV stabiliser (2.4-di-tert-butylphenol).

Portuguese MP were composed of 8 additives (Figure 1), the same as French MP, except for the 2.4-di-tert-butylphenol UV stabiliser and the Bisphenol S antioxidant, which were absent. In addition, except for the Diisobutyl phthalate (DIBP) the quantity of additives in Portuguese MP was lower than that measured in French MP *i.e.* 18-fold for the Dimethylphthalate (DMP); 11-fold for the Tri(2-ethylhexyl) phosphate (TEHPA); 8-fold for the Bis(2-EthylHexyl)Adipate (DEHA); 6-fold for the Bis(2-Ethylhexyl)phthalate (DEHP); 3-fold for the Butylated hydroxytoluene (BHT), 2-fold for the Di-n-butyl Phthalate, (DBP) and Diethyl Phthalate (DEP).

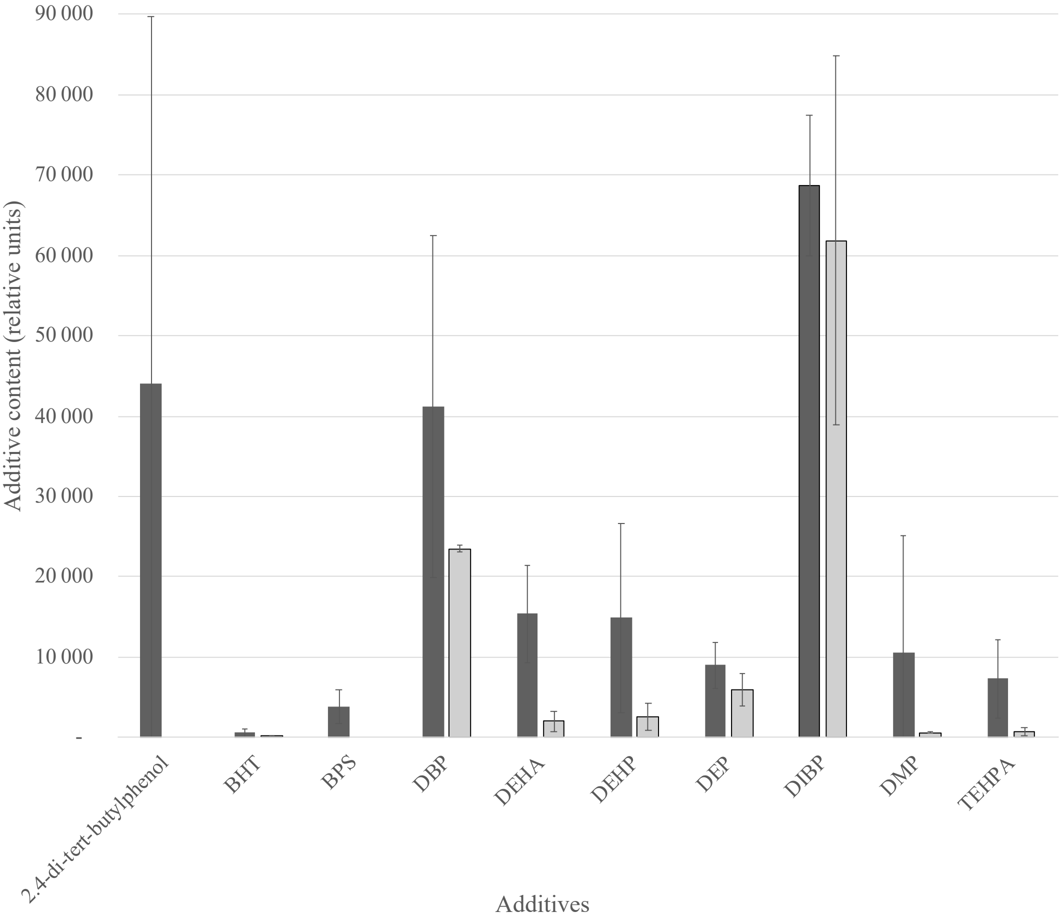

Figure 1. Additive content of French MP (black) and the Portuguese MP (grey), (mean ± standard deviation; n = 3). 2.4-di-tert-butylphenol; BHT: Butylated Hydroxytoluene; BPS: Bisphenol S (BPS); DBP: Di-n-butyl Phthalate; DEHA: Bis(2-EthylHexyl)Adipate; DEHP: Bis(2-Ethylhexyl)phthalate; DEP: Diethyl Phthalate; DIBP: Diisobutyl phthalate; DMP: Dimethylphthalate; TEHPA: Tri(2-ethylhexyl) phosphate.

GENERAL DISCUSSION

1. A contribution to the leachates impact literature on mussel

As shown in Chapter I, the existing research on the impact of plastic leachates on mussels is still limited. Indeed, prior to this thesis, only five studies have investigated this subject, three focusing on molecular, cellular, tissue, and organ responses, as well as mussel early-life stages (Capolupo et al., 2020, 2021a; Gandara e Silva et al., 2016; Wang et al., 2023a). The last one explored the behavioural responses of mussels, specifically in terms of aggregation, motility, and byssus production (Seuront et al., 2021). This thesis contributes to fill the literature gap on the impact of plastic leachates through mussel responses that have not yet been studied previously, notably the mussel thermal tolerance (Chapter II), the chemosensory sensitivity (Chapter III), the collective anti-predator responses (Chapter IV), the seasonal aggregation dynamic (Chapter V), and the respiration rate (Chapter VI; Figure 40). Our results showed that plastic leachates can disrupt each of the responses listed above (Figure 40), but also highlighted the complexity of the interaction between 'chemical', 'environment', and 'organism' in the response of mussels to plastic leachates. By going through the main results, we will examine how these three factors influence the mussel responses to plastic leachates, and the importance of considering them.

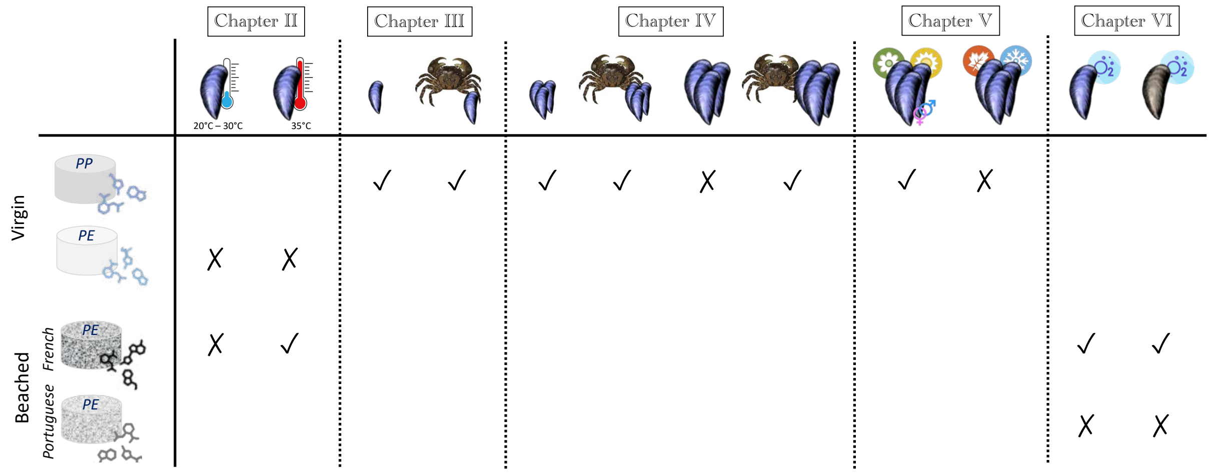

Figure 40: Summary of effect (✓) and no effect (✗) of microplastic leachates from virgin polypropylene (PP) and polyethylene (PE) pellets and beached polyethylene pellets collected in France or Portugal found on the different parameters studied in each Chapter. Globally, large mussel thermal tolerance (Chapter II), small mussel individual behaviour and antipredator response (Chapter III), small and large mussel collective behaviour and antipredator response (Chapter IV), sexually mature and immature large mussel collective behaviour dynamic (Chapter IV), large *Mytilus edulis* (blue) and *Mytilus galloprovincialis* (brown) mussel respiration (Chapter VI).

1.1. Mussel response to plastic leachates is chemical-specific

Four types of plastic pellets were used in this thesis: two virgin polymers, *i.e.* polyethylene (PE) and polypropylene (PP), and two beached pellets, *i.e.* collected from the coastlines of North of France and Southern Portugal, both identified as PE. In Chapters II and VI, we used each type of PE pellets to prepare the leachate solutions allowing for a pellet associated leachate toxicity comparison. First, virgin pellet leachates showed lower toxic levels (measured as reduced mortality rate; Chapter II; Figure 40) than those from French beached pellets. Second, leachates from beached pellets collected in polluted areas in northern France showed higher toxicity (measured as a lower respiration rate; Chapter VI; Figure 40) than those collected from more pristine beaches in southern Portugal. Taken together these results highlight that the mussel's response to plastic leachates was contingent on the plastic pellet used (Figure 40).

The chemical analyses conducted support these differences in toxicity. The leachate from French beached pellets, which had the highest toxicity, demonstrated a greater diversity and higher concentration of chemicals than the leachates from both virgin PE pellets and Portuguese beached pellets. We can also note that the composition of the French beached pellets was not identical in Chapters II and VI, indicating a temporal dimension in the chemical composition, as they were collected in the same place, but at different times. The sorption and desorption processes of chemicals follow chemical equilibrium kinetics (Lynch et al., 2022). Consequently, the chemical composition of the plastic (i) after its manufacture and (ii) after its time in the environment will influence the plastic leachate composition, and thus their toxicity. If a plastic particle is in a surrounding environment more polluted than the particle itself, it will accumulate pollutants. In contrast, when the plastic particle is in a less polluted environment than the particle itself, it will tend to release its pollutants. Indeed, plastic chemical composition can vary between (i) different virgin plastic polymer pellets (*e.g.* Capolupo et al., 2020; Gewert et al., 2021; Schiavo et al., 2021), (ii) same virgin plastic polymer pellets associated with different chemicals (*e.g.* Akoueson et al., 2023; Zimmermann et al., 2021), (iii) virgin and beached plastic pellets (*e.g.* Cormier et al., 2021; Gandara e Silva et al., 2016; Menicagli et al., 2022; Nobre et al., 2015, 2022; Rendell-Bhatti et al., 2021; Seuront, 2018; Chapter II), (iv) different beached plastic pellets according to their location (e.g. Fisner et al., 2013; Ogata et al., 2009; Taniguchi et al., 2016; Vedolin et al., 2018; Chapter VI) or time of collection (*e.g.* Balthazar-Silva et al., 2020; Jayasiri et al., 2015; Rochman et al., 2013b).

The high variability in the plastic chemical composition stresses the need to assess the chemical content of leachates in toxicological studies. As emphasised in Chapter I, only 8% of the 106 papers examining plastic impact on mussels conducted a chemical composition analysis, indicating a very low frequency of this practice. In addition, when the chemical composition is screened, it generally involves a qualitative assessment of specific chemical categories (*e.g.* plastic additives, trace elements, or PCBs) selected from a limited list of compounds (Seuront et al., 2022; Chapter I). This is typically the case in this manuscript, where for each of the pellet batch used, an analysis of plastic additives was conducted from a list of ‘only’ 57 additives, while it is know that more than 13,000 are associated with plastics during manufacture alone (UNEP, 2023). In addition, the beached pellets used in this manuscript probably contain a wide range of chemicals, including heavy metal contents, as they were found in beached pellets collected at the same two locations, in France and Portugal (Zardi et al., in press). Using beached plastics in toxicology studies is a good way to get closer to environmental realities, as they may have sorbed many chemical compounds from their surroundings. However, by multiplying the number of chemical compounds potentially found on its surface, the number of analyses to be carried out and consequently the cost of these analyses can be quite substantial and restrictive.

1.2. Mussel response to plastic leachates is environment-specific

In addressing the gaps identified in Chapter I, Chapter II aimed to assess the mussel response to the combined effects of plastic leachates and aerial temperature heat stress. A significant impact of leachates on mussel survival was only observed after a 35°C aerial heat stress exposure, with no effect at lower temperatures (20, 25 and 30°C; Figure 40). Thus, the mussel’s response to plastic leachates was found to be temperature-dependent. In addition, the interaction between temperature and plastic leachates was synergistic, indicating a combined effect stronger than the sum of the individual effects of each stressor (Crain et al., 2008).

These findings underline the importance of incorporating environmental factors into toxicological studies, which are typically conducted under optimal environmental conditions (Holmstrup et al., 2010). Indeed, using a single—optimal—temperature value, *e.g.* 20°C, would have led to the loss of crucial information concerning the potential risks posed by plastic leachates. In contrast, assessing a range of temperatures allowed for evaluating the synergistic effects between temperature and plastic leachates, ultimately resulting in significant mussel mortality. In addition, it is noteworthy that the temperatures used were environmentally relevant, *e.g.* a 35°C mussel body temperature is not uncommon during emersion at the experiment location and time of the year (Seuront et al., 2019). Thus, although the emersion factor has been overlooked in temperature studies (Chapter I), it can generate significant effects, which are accentuated by the leachates from plastics. It would be therefore advisable to give greater consideration to this factor in future studies.

Synergistic interactions are the most prevalent between environmental factors and toxicants (Holmstrup et al., 2010). Despite this, there is a scarcity of studies exploring multiple stressors involving plastic and other environmental stressors (Chapter I). Emersion temperature represents one of many factors in which, combined with plastic leachates, could lead to significant adverse impact on mussels. Notably, previous studies have shown synergistic effects on mussels involving toxicants and (i) immersion temperature (Andrade et al., 2022; Leite et al., 2020; Moleiro et al., 2022; Pittura et al., 2022), (ii) pH (Hu et al., 2017; Shang et al., 2018; Wu et al., 2018), (iii) salinity (Cuccaro et al., 2023; De Marchi et al., 2020; Sui et al., 2023), (iv) hypoxia (Wang et al., 2014, 2022, 2023) and (v) food availability (Gu et al., 2023; Shang et al., 2021a; Wang et al., 2021). To the best of my knowledge, no study has ever addressed how intertidal mussels respond when these stressors are combined with plastic leachates. In addition, these factors can act in combination on mussels, *e.g.* 'temperature + pH + leachates', and on the chemicals by influencing their nature and bioavailability, as shown for instance in the context of temperature on plastic sorption/desorption processes (Godoy et al., 2020; Guan et al., 2022; Khumalo et al., 2022; Kida et al., 2022; Chapter I). Thus, environmental factors can influence the toxicity of leachates through various processes, adding complexity to the challenges faced by mussels. Comprehensive investigations of the interactions of environmental factors and plastic leachates are necessary for accurately assessing the risk a species faces from a toxicant in its natural habitat. This is particularly crucial in light of current and future changes in our environment (IPCC, 2022).

1.3. Mussel response to plastic leachates is organism-specific

The Chapters IV and V assessed the impact of plastic leachates on mussels of different sizes and different sexual maturity stages, respectively. In Chapter IV, the results revealed a more pronounced effect of plastic leachates on the behaviour of small mussels than on larger ones (Figure 40). In Chapter V, we showed an impairment of the cyclical pattern of aggregation which is driven by a disruption of mussel aggregation when ripe gametes were present, closed to the spawning event (Figure 40). Maturity stage and size are two factors that can affect the mussel's sensitivity to toxicant, thus the mussel's response to plastic leachates was found to be dependent on the organism characteristics.

The vulnerability towards a contaminant can vary during the life cycle of an organism (Mohammed, 2013). Larger organisms, but also organisms with a higher condition index or nutritive status, are generally less sensitive to toxicants (González-Fernández et al., 2015; Chapter IV). This can be attributed to the "dilution by growth", *i.e.* exposure to the same contaminant concentration results in a higher internal concentration in smaller/lighter organisms due to their smaller volume (Jager, 2013). In addition, as numerous plastic additives are acknowledged to be endocrine disrupters, they may act differently depending on the sex of the mussel in addition to their sexual maturity. Indeed, a sex-specific effect was found in *Mytilus galloprovincialis* mussels when exposed to a plastic additive flame-retardant, the polybrominated diphenyl ethers (PBDEs; Ji et al., 2013). Invertebrate pollution impact studies, typically focused on organisms of a standard size, making their experiment at a given point of time without indicating the reproductive status or the organism sex (Burger, 2007; Chapter I).

However, our results show the importance of considering organisms of various characteristics, *e.g.* at different stages of their life cycle, to accurately assess the toxicity of a contaminant.

Additionally, in Chapter VI we showed an impact of plastic leachate on the respiration rate of two closely related mussel species, *i.e. Mytilus edulis* and *M. galloprovincialis* (Figure 40). While our results suggest a generally coherent respiratory response to plastic leachates among mussel species, it is important to note instances where this coherence does not hold true for other traits. For instance, the impacts of plastic leachates on Mytilidae aggregation and byssal production were previously shown to be species-specific (Seuront et al., 2021). In addition to interspecific variation, intraspecific sensitivity to plastic pollution can also occur, for instance between different *M. galloprovincialis* genetic lineages (Cozzolino et al., 2023), but for which evidence is still extremely scarce (Nicastro et al., 2023a). Further research is needed to deepen our understanding of these nuances in plastic pollution effects between and within species.

1.4. Mussel response to plastic leachate studies should be multifactorial

Paracelsus, the founding figure of toxicology, stated *ca.* 500 years ago "*Sola dosis facit venenum*", *i.e.* “only the dose makes the poison” (Paracelsus, dritte defensio, 1538). This concept indicates that any substance can be toxic at a specific concentration. Undeniably true, this concept upon which toxicology was built can be considered too simplistic (McCarty et al., 2020). Indeed, numerous studies on various chemicals, and specifically this manuscript on plastic leachates, showed that the toxic dose is contingent upon (i) the nature of the chemical substance, (i) the organism characteristics, and (iii) the environmental conditions of its surroundings (Figure 41). Thus, although single-stress studies offer valuable insights, they may underestimate the actual toxicity faced by an organism in the complex reality of its environment, where numerous confounding factors interact and shape the organism's response to contaminants. Even though the myriad of factors would represent a challenging task for future studies, they are essential for judiciously informing regulatory decisions concerning these pollutants.

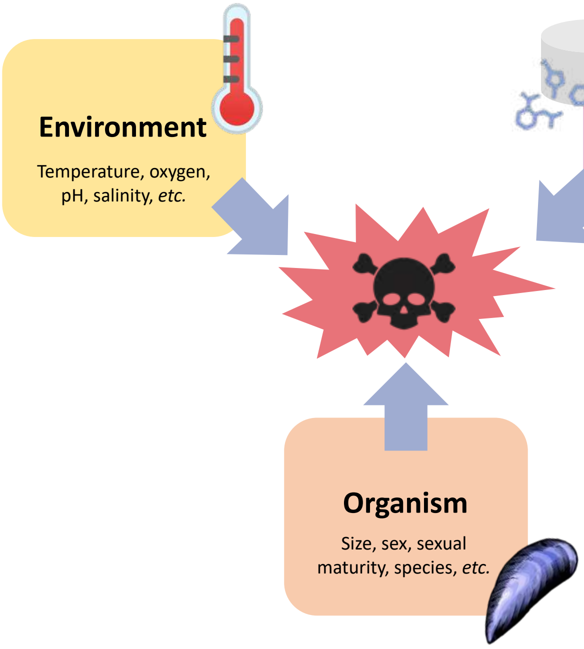

Figure 41: Summary of the factors that can affect the toxicity of plastic leachates on mussels.

2. The studied endpoints and what we learned from them

In the literature, the impact of plastics on mussels has been explored across various biological parameters, referred to here as endpoints, that have not concentrated the same research effort (Chapter I). Most of the research efforts on the impact of plastic, and plastic leachates in particular, on marine mussels focused on small-scale organisation level, encompassing molecular, cellular levels as well as tissue and organ impacts (Chapter I). The organisation level studied in this manuscript was the organism level. Specifically, the endpoints chosen were (i) a functional trait, mussel survival (Chapter II), (ii) a physiological trait, mussel respiration (Chapter VI) and (iii) a wide range of behavioural traits, including locomotion, aggregation and chemotaxis (Chapters III, IV and V). Given that a significant part of this manuscript focused on mussel behaviour, we will first examine what this parameter has taught us about plastic leachates impact on mussels. Then, in the last section, by gathering our main results on the different endpoints studied in this thesis, we will analyse the potential risks posed to ecosystems.

2.1. The behaviour, a sensitive endpoint providing important information

An important part of my thesis focused on mussel behaviour (Chapters III, IV, V), an overlooked though highly sensitive parameter. The paradox between research effort and sensitivity was highlighted in Chapter I. Indeed, among the endpoints studied at the organism level, behavioural traits received minimal research attention (16.6%), despite being the most sensitive, as 77% of the mussel behavioural traits were significantly impacted by plastic exposure (Chapter I). Behavioural studies can provide crucial insights into how contaminants affect the response of organisms, as well as the underlying mechanisms of these effects. This section delves into these aspects through the main behavioural insights of this thesis.

The behavioural studies conducted in this thesis highlighted the sensitivity of mussels to their chemical environment and the complex response that plastic leachates can elicit. Indeed, Chapters III and IV showed that despite the relatively short common evolutionary history between *Mytilus edulis* and its invasive crab predator *Hemigrapsus sanguineus* at our study site (Dauvin et al., 2009), mussels whether small or large, solitary or collectively, were able to adapt their behaviour accordingly. These chapters also showed an impairment in mussel antipredator behaviour when exposed to plastic leachates. The use of crab cues and the loss of negative chemotactic behaviour towards them in Chapter III allowed identifying the underlying mechanisms for these effects as an alteration in the mussel chemical communication, that linked a specific cue to a specific behavioural response.

Contaminants can act on different steps in the chemical communication chain, namely (i) the cue source, (ii) the cue in seawater, (iii) the cue reception and (iv) the signal transduction (Olsén, 2011; Schirrmacher et al., 2021; Figure 42).

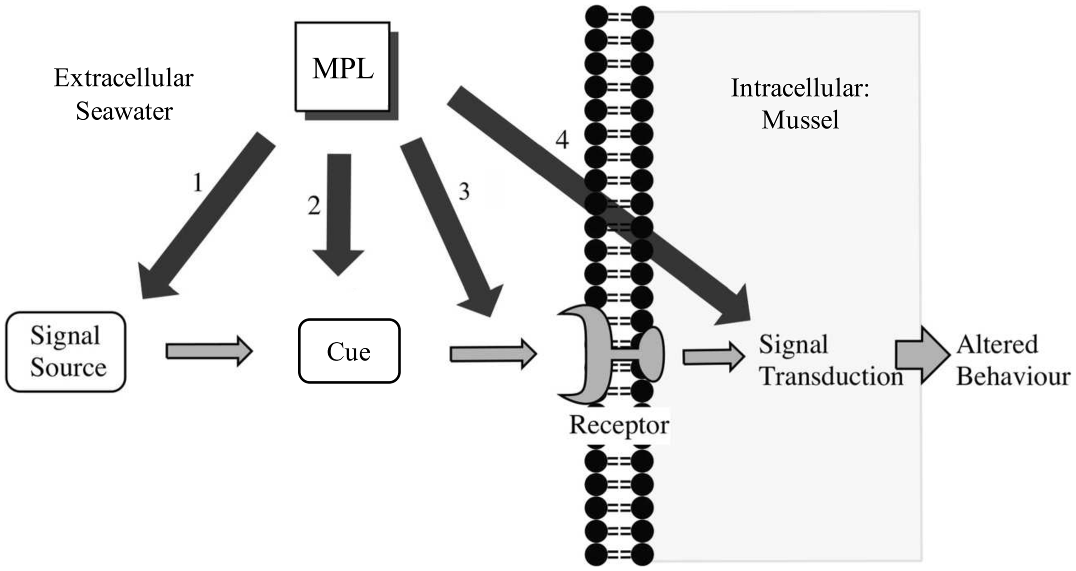

Figure 42: Visualisation of the possible mechanisms by which microplastic leachates (MPL) can alter the mussel response to cues. The signal transmission pathway from source to behavioural response is shown with light grey arrows. The potential mechanisms where MPL can interfere with this pathway are shown in dark grey and numbered. Thereby the MPL can affect the signal source (1), the signalling cue (2), the receptor or its interaction with the ligand (3) and the signal transduction (4). Adapted from Schirrmacher et al., (2021).

Here, as the crabs were not incubated with the plastic leachates, the impact on the cue source can be excluded. Thus, the prey's inability to recognise its predator's cues results in an impact of plastic leachates on the downstream part of the chemical communication chain, *i.e.* interactions with (i) crab cue molecules, (ii) mussel chemical receptors and/or (iii) through a transduction impairment. A recent study supports the latter hypothesis, showing a change in gene expression related to the mussel olfactory transduction pathway after a microplastic exposure (Qi et al., 2023). Another study, though conducted on goldfish, showed that olfactory behavioural responses were altered by microplastic exposure, that acted on cue reception, transduction, and induced olfactory bulb damage and neurotoxicity (Shi et al., 2021).

Neurotoxicity following plastic—but also plastic leachate—exposure was also described in mussels by changes in the neurotransmitter enzyme cholinesterase (Avio et al., 2015; Brandts et al., 2018; Capolupo et al., 2021a, 2021b; Choi et al., 2021, 2022; Pittura et al., 2022; Chapter I), which could impact the olfactory information processing.

However, while plastic leachates can impair the mussel's ability to detect chemical cues from its crab predator (Chapters III and IV), they do not seem to hinder its ability to detect conspecifics as shown in Chapters IV and V. Thus, plastic leachates do not seem to affect the perception of conspecific pheromones. Similarly, a recent study demonstrated that plastic leachates from beached pellets did not alter the mussel electro-osphradiogram, *i.e.* its chemosensory activity, in response to three amino-acids, the L-cysteine and L-leucine and proline (Cozzolino et al., 2023). Plastic leachates may interact with a specific type of cells or receptors, *e.g.* those responsible for crab cue recognition, and did not affect those responsible for conspecific or amino acid recognition. Although the activation and inhibition of specific cellular responses by specific cues and specific contaminants have been extensively studied in fish (for a review, see Shahriari et al. (2023)), such investigations are still scarce in molluscs. Indeed, even in gastropods—one of the most studied classes of molluscs in terms of olfactory capacity—the association between the numerous putative types of sensory cells and their functions remains unclear; see Wyeth (2019) for a review. Nevertheless, it is essential to understand how olfactory systems function to accurately analyse their interactions with contaminants and, consequently, to understand the resulting behaviours (or lack of it). However, that lack of knowledge of the underlying mechanisms involved in the behavioural responses described in this manuscript does not detract from the central point, *i.e.* their disruption by microplastic leachates.

Behaviour represents fast, sensitive and powerful tools in toxicology studies to assess the effect of contaminants (Bertram et al., 2022; Melvin & Wilson, 2013). Organism behaviour plays a critical role in fitness, influencing population dynamics, species interactions, and ecosystem functioning, making behaviour assessments ecologically meaningful (Ford et al., 2021). In addition, some behavioural responses are largely neglected in chemical effect assessment, although they can provide valuable information, *e.g.* collective behaviour and prey-predator interactions (Bertram et al., 2022). As discussed in Chapters III, IV and V, the disruption of such responses by plastic leachates raises significant concern about how these modifications might affect the mussel fitness and shape the communities. The importance of studying these behavioural responses is not negligible and could improve risk assessment, development of criteria and regulation of chemical contaminant (Ford et al., 2021).

2.2. Are mussels and their ecosystems threatened by plastic leachate pollution?

Our results displayed that plastic leachates disrupt the ability of the mussels to withstand two major natural stressors from the intertidal environment, *i.e.* aerial temperature and predation pressure (Seed & Suchanek, 1992). These disturbances caused by plastic leachate could have a direct impact on the survival of mussels. Although survival was clearly impacted by the combination of aerial heat events and plastic leachates (Chapter II), the anti-predator experiments (Chapters III and IV) did not have survival as an endpoint. Indeed, Chapters III and IV highlighted that plastic leachates impaired mussel's antipredator behaviour. Plastic leachates hinder mussels' ability to detect a predation risk, altering their predator avoidance and escaping behaviours. This increases the likelihood of mussels being consumed by crabs, especially small mussels, which are their preferred prey (Brousseau et al., 2001). However, to confirm the higher risk of mussel mortality due to crab predation in the presence of plastic leachate, it is necessary to study the impact of the same plastic leachates on the crab predatory performances, which to the best of our knowledge, has not yet been studied. There is however evidence of the lack of impact of plastic leachates from surgical masks on *Hemigrapsus sanguineus* behaviour (Delaeter et al., 2023). If plastic leachates alter the behaviour of the mussels towards its predator, and the predator is not affected by this pollutant, this could eventually lead to an increase of the mortality rate due to predation in natural habitats.

Plastic leachates also impaired mussel 'basal' responses. In small mussels, plastic leachates induced a decrease in their motility when solitary (Chapter III), and an attraction to conspecifics and greater aggregation strength when in group (Chapter IV). In large mussels, plastic leachates induced a decrease in mussel respiration rate (Chapter VI) and a loss of the cyclical pattern linking their aggregation to their reproductive stage, notably by enhancing aggregation when close to their spawning event (Chapter V). Although the increase in mussel attraction to conspecifics and aggregation could be seen as having more positive than negative aspects such as protection toward predation, wave dislodgment, temperature fluctuation, as well as potential increase in reproductive success (Côté & Jelnikar, 1999; Nicastro et al., 2007, 2012; Reimer & Tedengren, 1997; Zardi et al., 2021;Chapter V), this would increase competition and reduce their access to food (Koppel et al., 2005; Nielsen & Vismann, 2014). Aggregation is also an energetically costly behaviour, and if combined with a decrease in respiration rate as demonstrated in Chapter VI, these could lead to deficits in their energy budget and have repercussions on the species' fitness. Additionally, if as hypothesised in Chapter VI leachate may enhanced reproductive signal and induced spawning, this could result in (i) a mismatch between larvae production and optimal feeding conditions (Cushing, 1990), and (ii) strong damage to *Mytilus edulis* oocytes and larvae. The latter hypothesis is noticeably supported by experiments specifically conducted on the effects of these plastic leachates on *M. edulis* oocytes and larval development (Uguen et al., unpublished data). These two potential consequences of an exposure to plastic leachates are both likely to lead to a reduced recruitment.

Note that the *in situ* impact and ecosystem-level assumptions discussed here are derived from laboratory data obtained under controlled conditions, using mussels of specific characteristics, following short-term exposure to leachates of a specific polymer type and concentration. Therefore, caution should be exercised when extrapolating these findings to real-world scenarios. Nevertheless, the overview of the impacts of plastic leachates that this thesis provides, summarised above, shows that plastic leachates have an overall harmful effect on mussels. As temperature and predation respectively limit the upper and lower zonation of mussels (Seed & Suchanek, 1992), exposure to plastic leachates by impairing temperature tolerance and antipredator behaviour, could reduce the mussel distribution zone on the intertidal shores (Figure 43). In addition, the other effects found and their potential consequences on the energy budget and reproduction, described above, may have implications on mussel fitness. Due to the mussel's bioengineering role (Borthagaray & Carranza, 2007; Buschbaum et al., 2009), what this thesis work has shown at the species level could have cascading effects on the associated communities (Figure 43) and on various other services that mussels provide, such as coastal protection (Borsje et al., 2011), water quality (Newell, 2004; Timmermann et al., 2019) and food provision (Avdelas et al., 2021).

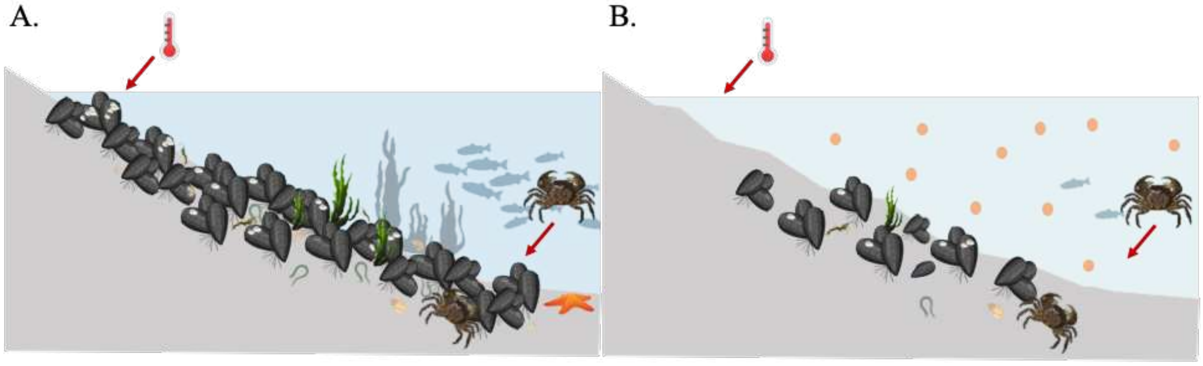

Figure 43: Visualisation of the (A) natural undisturbed mussel bed and its habitat provision and (B) the potential impact of plastic leachates on rocky intertidal ecosystems based on the extrapolation of this thesis results on mussels. The arrows indicate temperature and predation drivers and orange dots the microplastic leachates. It should be noted that a variety of other natural (*e.g.* competition, wave action) and anthropogenic stressors (*e.g.* acidification, overfishing) may also act on this system. Inspired from Kunze et al., 2021.

GENERAL CONCLUSION

This PhD thesis has provided novel insights into the impact of plastic leachates on a key ecosystem engineer species: the blue mussel *Mytilus edulis*. The impact of plastic leachates was observed across various mussel responses, showing adverse effects on notably the mussel thermal tolerance, its chemosensory abilities, collective anti-predator responses, seasonal aggregation dynamic, and respiration. These impacts could have larger-scale consequences due to the mussel key role as an ecosystem engineer and may influence the health of mussel beds, their associated high biodiversity and the great number of ecological and economic services they provide. These results raised particular concerns as the selected stressors (*e.g.* plastic, temperature, invasive crab predation) are environmentally coherent in the context of the Eastern English Channel, but could also aplly to other systems, as plastic pollution, extreme temperatures as well as introduction of invasive species are set to increase (IPCC, 2022; Lebreton & Andrady, 2019), underlining the urgency of addressing these environmental challenges to preserve the health and functioning of these ecosystems.

This thesis also highlights the complex interaction between the type of plastic pellet used, environmental conditions, and organism characteristics in determining leachate toxicity in mussels. By oversimplifying the experimental context of the impact of the contaminant, we may neglect the complex reality of natural ecosystems. Ecosystems are composed of multiple species, each consisting of individuals of different sizes, ages, sexes, and sexual maturities, and they undergo variations in their environment, such as fluctuating temperatures, pH, salinity, hydrodynamic stress and predation pressures. This richness of variables and their interactions that may shape the plastic leachate toxicity in mussels deserves to be taken into account in future toxicological studies. It is only through this holistic prism that we will be able to accurately measure the state of health of the mussels in their ecosystem and unravel the potential cascading effects to larger scales, opening up a wide range of perspectives that deserve to be explored.

PERSPECTIVES

A plethora of questions have emerged from this thesis work, and paved the way for a diverse range of research perspectives. These perspectives will be discussed hereafter for each section of this manuscript.

CHAPTER II

Emersion heat event combined with plastic leachates can lead to significant mussel mortalities. In addition to these short-term heat events, organisms are also subjected to heat events of longer duration, the so-called heatwaves. Heatwaves are anomalously warm events lasting at least 5 days, with temperatures warmer than the 90th percentile based on a 30-year historical baseline period (Hobday et al., 2016). Exposure to aerial heatwaves significantly decreases mussel thermal tolerance (Jones et al., 2009; Seuront et al., 2019; Sorte et al., 2011). In this context, the results of Chapter II stress the need to further understand the impact of the combined stress of plastic leachates and an aerial heatwave on mussel survival.

To address this point, a similar experimental design as in Chapter II could be tested, by repeating the aerial heat stress over several emersions on at least 5 consecutive days. Improvements could be made. For instance, it will be interesting to (i) consider several sizes of mussels in each treatment, mixing recruits (< 0.6 mm), small mussels (< 2 cm), and large mussels (> 4 cm) to approach the real composition of a mussel bed and to assess a potential size-dependence in their sensitivity, (ii) use mussels from the upper and lower shore zones to analyse their respective sensitivity, (iii) dissect the mussels at the end of the experiment to sex them and determine if males and females respond equally to this type of stress, (iv) conduct the experiment before and after the spawning event (*e.g.* in April and September) to take into account the potential impact of the reproductive stage.

In addition, by increasing extreme events, climate change acts not only in the form of heat events, but also as extreme cold spells that can also lead to bivalve mass mortalities; for a review, see Masanja et al. (2023). An assessment of the impact of the combined stress of plastic leachates and aerial cold spells on mussel survival would be needed to assess the effect of cold spells along the French shores of the Eastern English Channel, where the substrate temperatures as low as -8 to -10°C recorded in early February 2021 rip off an entire population of the high shore gastropod *Littorina saxatilis* (Seuront, unpublished data). The resolution of this issue would necessitate, first to deploy biomimetic sensors in the field to record extreme temperatures, as was done in summer in Seuront et al. (2019). Based on the resulting temperature records, similarly to the experiment proposed above, a series of realistic cold stress experiments could be carried out, providing a more complete overview of how mussels respond to plastic leachates in their environment.

CHAPTER III & IV.

The perspectives resulting from Chapters III and IV are grouped together as they are intrinsically linked to the mussel olfactory sensitivity to their predators and the subsequent risk of being predated. As previously shown, plastic leachates can act on different steps in mussel chemical communication chain, and it will be interesting to find out more about this process. However, as pointed out in the discussion, little is still known about the olfactory sense of mollusc in general, and mussels in particular.

A first step would be to know where to look. The osphradium is a chemosensory organ shared by the vast majority of molluscs, including mussels (Lindberg & Sigwart, 2015; Lucas, 1931; Morton, 1962). However, a recent review suggests that the osphradium in gastropods, although chemosensitive, would not be involved in controlling the direction of movement relative to odour sources (Wyeth, 2019). Its author suggests that the ability to track an odour source would be linked to other chemosensory organs (Wyeth, 2019). While conducting experiments described in Chapters III and IV, I observed that mussels tend to stretch their foot as if ‘tasting’ the seawater for cues before initiating a movement, a behaviour also described in other studies (*e.g.* Morello and Yund, 2016). Thus, the mussel foot could potentially act as a second chemosensory organ, a hypothesis which, to our knowledge, has not been explored further. Notably, in bivalves, the nervous system is composed of three pairs of ganglia inter-connected, *i.e.* the visceral, cerebropleural and pedal ganglia, pedal ganglia which specifically innervates the foot (Kotsyuba et al., 2020). To confirm or refute this hypothesis, we could use an electrophysiological technique, the electro-olfactogram, developed by Rato et al. (2023) for oysters, and confirmed as working on mussels osphradium by Cozzolino et al., (2023). Once the chemosensitive zones will be identified, it could be interesting to conduct a histology analysis to identify whether there are different types of sensory cells, as in gastropods or fish (Shahriari et al., 2023; Wyeth, 2019), and to identify the cues to which they are sensitive to. Then to determine the cellular transduction pathway, a protocol such as the one used by Dew et al. (2014) on fish could be adapted for mussels.

In order to determine whether the predation probabilities on mussels are higher in the presence of plastic leachates, it is necessary to understand how the crab's predation abilities are affected by plastic leachates. To achieve this, an experiment would be specifically designed to test the crab's abilities to detect and handle mussels under a plastic leachate exposure similar to that used in this manuscript (pellet type, concentration). To do so, first we would have to test the chemotaxis of crabs exposed to plastic leachates towards mussel cues by adapting the design used in Chapter III for this species, *e.g.* by using a T-maze approach similar to what has been implemented to assess the chemosensory abilities of the intertidal snail *Littorina littorea* (Seuront & Spilmont, 2015). Subsequently, to investigate crab handling capacity, crabs could be placed in plastic leachate solution and be provided with mussels from various size classes and after a defined duration, the number of mussels of each size class could be counted and compared to the control.

In addition, an important aspect of chemical communication that has not been tested in this manuscript is related to the possibility that plastic leachates impact the ability of the source (*i.e.* crab and mussel) to release their cues. To do so, in the proposed experiment above, it would be interesting to make two different 'mussel cues + plastic leachates' treatments, *i.e.* one by incubating the mussels directly with the plastic pellets, and the other by incubating the pellets and mussels separately and mixing them afterwards. Additionally, it would be valuable to repeat one or both of the experiments from Chapters III or IV by incubating the crabs directly with the plastic pellets.

CHAPTER V

The mussel aggregation rate was found to increase during gamete maturation until the onset of spawning, and then to decrease, following a cyclical pattern. To achieve this behaviour, mussels may rely on sex pheromones released by individuals with ripe gametes.

To test this hypothesis, the experimental design of Chapter I could be used to assess the chemotactic response of mussels to different cues around the spawning season (*i.e.* April, May). Following the approach of Marquet et al. (2018) on the sea cucumber, several cues could be tested to identify what mediates aggregation, *i.e.* seawater conditioned by males, seawater conditioned by females, male gonad homogenates, female gonad homogenates, male spawning water and female spawning water. Each mussel used in this experiment would need to be dissected following the chemotaxis assay to know its sex and thus provide clarity in attributing the observed responses to specific cues and help identify the key factors influencing mussel aggregation patterns.

It would also be interesting to test whether this pattern is found in other mussel species and *in situ*. This could be assessed in mixed mussel beds composed of morphologically different mussel species, *e.g. Mytilus galloprovincialis* and *Perna perna* in South Africa or *M. edulis* and

M. trossulus on the eastern coast of Canada. Following Nicastro et al. (2008) digital pictures of several quadrats could be taken monthly in the field. Subsequently, the monthly distribution of each conspecific aggregation pattern could be studied.

Additionally, as plastic leachates were found to disrupt the cyclical pattern linking aggregation and reproductive stage by enhancing aggregation rates around the mussel spawning event, the plastic leachates may induce an early spawning event (Aarab et al., 2006). A subsequent question would be to assess if the plastic leachates tested in Chapter V induce early spawning in *M. edulis*. To investigate this, mussels in an advanced state of sexual maturity, *i.e.* around April, could be subjected to long-term incubation with plastic leachates. The incubation duration required before the onset of spawning could then be compared to the control group to determine whether the plastic leachates have triggered premature spawning.

CHAPTER VI

The respiration rate of both *Mytilus edulis* and *M. galloprovincialis* was found to be impacted by plastic leachates. However, other parameters, such as mussel aggregation and byssus production, were found to be impacted differently by plastic leachates, depending on the species (Seuront et al., 2021). The question of the effects of other parameters between different mussel species remains.

Mussel sensitivity to thermal stress is species-dependent, *e.g. M. galloprovincialis* exhibits a higher tolerance temperature than *M. edulis* (Thomas & Bacher, 2018). In addition, these two species hybridise (Coustau et al., 1991; Bierne et al., 2003), and the sensitivity to heat stress of their offspring is dependent on the species of the female, *i.e. M. galloprovincialis-M. edulis* hybrid larvae from *M. galloprovincialis* females exhibit lower sensitivity to heat stress than those from *M. edulis* females (Mlouka et al., 2020). Thus, it would be interesting to assess the sensitivity of these two species and their hybrids to a combined exposure ‘temperature × plastic leachates’.

In Chapter VI, the pellets used to prepare the plastic leachate solutions were collected from the shores of two different locations. Although the additive composition was analysed, no information is available regarding the additive composition they contained before entering the environment and that could also differ greatly. Improvements could be made to allow us to know the contamination of each site. To do so, a batch of virgin polymers with a known composition could be divided into several parts and incubated in the desired locations for a specified duration before being used for the experiment.

CHAPTER I & GENERAL DISCUSSION

These two sections emphasised the disparities in research efforts and the gaps to be addressed regarding the impact of plastic, and especially plastic leachates, on mussels. There are numerous disparities, and so are the perspectives that arise from them. It would be interesting to use different combinations of various (i) types of chemicals, (ii) organisms, and (iii) environmental conditions, and to focus on endpoints that have barely been considered. For instance, it would be advisable to enhance the research effort in (i) nanoplastics, and plastics fibres and their leachates, (ii) mussel species from Africa and Central America, (iii) mussel behavioural responses, (iv) mesocosms or *in situ* experiments, and assess the impacts on associated species. Conducting multigenerational exposure experiments is also an important aspect since, to the best of my knowledge, it has never been studied in mussels. Finally, considering multiple environmental factors, notably those linked to the Anthropocene (*e.g.* heat stress, acidification, hypoxia), is crucial to accurately fostering a comprehensive understanding of the ecological consequences of plastic pollution, and to promote effective conservation strategies to ensure the health and sustainability of our oceans in our changing world.

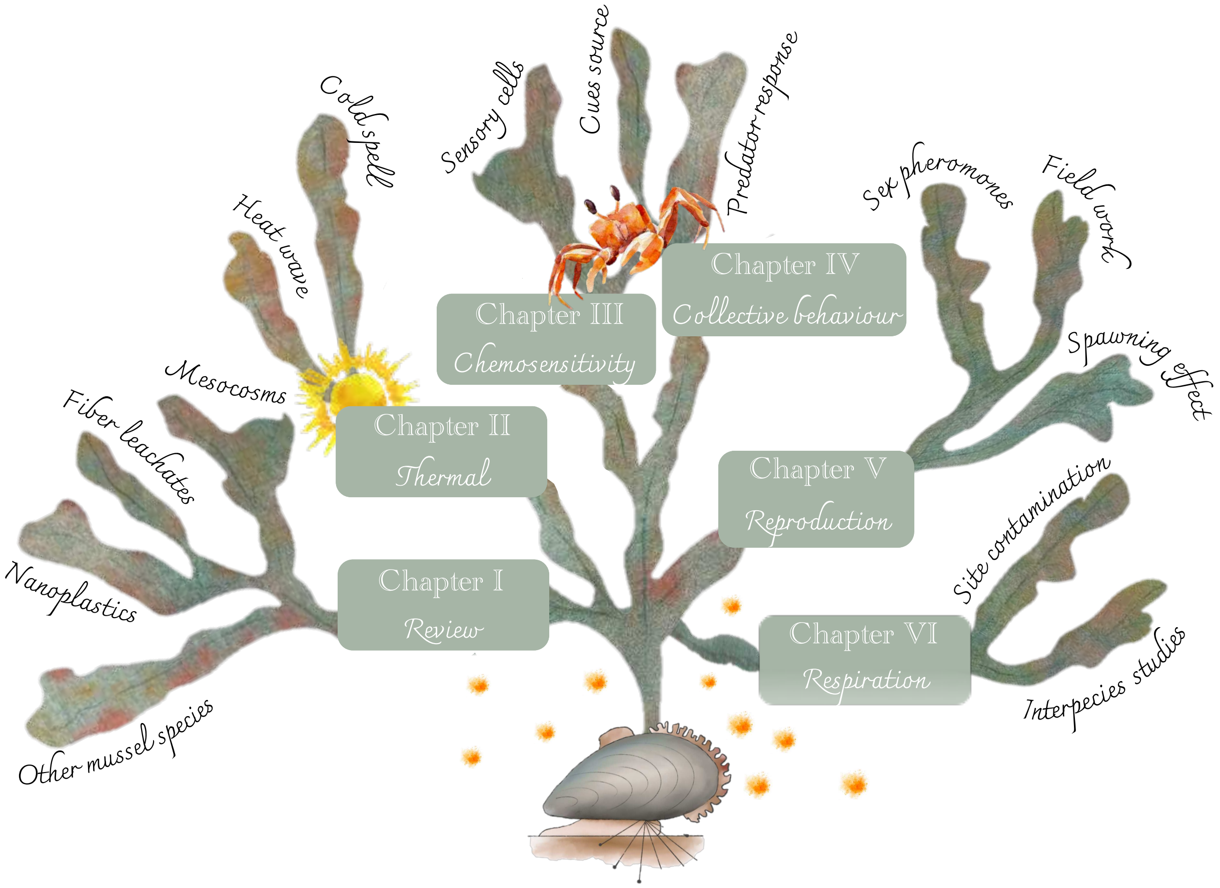

Figure 44: Thesis perspectives © Solène Henry

PERSONAL REFLECTION

While plastic-associated chemicals and their consequences have been gaining attention only recently, over half a century ago, Carpenter et al. (1972) described for the first time the presence of plastics in coastal waters, their ability to accumulate toxic substances and to be ingested by marine organisms. Regrettably, their research came to an abrupt halt due to pressure from the *Society of the Plastics Industry,* as revealed in a Plastisphere Interview cited in Andrady (2022). Thus, the significant time lapse between the early recognition of the link between plastics and toxic compounds in the marine environment and the surge in the toxicological studies on plastic leachates affecting marine fauna, which began around 2015 (Delaeter et al., 2022), highlights the influence of plastic industry lobbyists. Although the weight of the plastics industry lobbies still exists, *i.e.* it is still extremely challenging to access the lists of additives in plastic items from any manufacturer, studies in the plastic leachate field are now proliferating.

Moreover, citizens and policymakers are becoming increasingly engaged in addressing this global issue. The Global Plastics Treaty serves as evidence of a concerted effort to address plastic pollution on a global scale. With 175 countries candidates for ratification, the negotiation of this treaty which will end in 2024, marks the first international agreement on plastic pollution, encompassing the entire life cycle of plastics, *i.e.* production, consumption, and waste management. However, the last negotiations that took place from 13 to 19 November 2023, in Nairobi, Kenya, depict the confrontation of two blocks. On the one hand, 50 countries, including France, advocate for an ambitious treaty to end plastic pollution by 2040, establishing a legally binding international instrument, notably through acting on plastic production and consumption. On the other hand, countries with vested interests in the plastic economy, *e.g.* the United States, Russia, China and Saudi Arabia, are advocating for action on plastic recycling rather than reducing production.

However, recycling alone is not a sufficient solution. Plastic pollution extends far beyond being a mere waste problem; it is a broader challenge linked to its entire life cycle. Consequently, efforts should focus on multiple fronts, including reducing plastic production, promoting the reuse of plastic, and reevaluating the societal model that has prevailed over the last few decades and created this need for plastic consumption. In essence, the most effective strategy to combat plastic pollution is to act at its source and turn off the ‘plastic tap’.

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New insights into the ecology of a key ecosystem engineer, the blue mussel *Mytilus edulis*, in an era of global change: focus on the effects of plastic leachate pollution

Abstract

The massive contamination of the environment by plastics is an increasing global scientific and societal concern. While research on microplastics is currently flourishing, the study of their leachates, *i.e.* the cocktail of chemicals that may have been added during the manufacture process to enhance plastic performances, or environmental contaminant adsorbed during their life cycle, remains relatively unexplored. However, plastic leachates have the potential to cause detrimental effects, which becomes especially serious when they impact key species. Thus, this thesis aimed to assess the effects of microplastic leachates on a marine species of major economic and ecological importance, the mussel *Mytilus edulis* within the Eastern English Channel ecosystem. First, a state-of-art of the research efforts and their disparities in plastic impact on marine mussels was established. Then, through laboratory experiments plastic leachates from virgin or beached pellets were shown to impair (i) the survival of mussels after an emersion heat stress, (ii) the small mussel chemotactic and chemokinetic behaviours, (iii) the mussel collective anti-predator behaviour to the crab *Hemigrapsus sanguineus*, (iv) the mussel seasonal aggregation dynamics linked to its reproductive stages, and (v) the mussel respiration rate. These results also highlight the complex interaction between the plastics used, the environmental conditions, and the organism-specific characteristics that can influence the leachate toxicity in mussels. Given the key role of mussels as ecosystem engineers, these impacts could have larger-scales consequences. By altering the health of mussel beds, plastic leachates could trigger cascading effects with repercussions on associated biodiversity and the numerous ecological and economic services they provide.

Keywords

Marine mussel, contaminant, intertidal ecology, microplastic, bioengineering species, ecotoxicology

Nouvelles perspectives sur l'écologie d'un acteur clé de l'écosystème, la moule bleue *Mytilus edulis*, à l'ère du changement global : focus sur les effets de la pollution par les lixiviats de plastiques

Résumé

La problématique de la pollution plastique constitue une préoccupation majeure de notre temps. Alors que les recherches sur les microplastiques sont actuellement florissantes, l'étude du cocktail de composés chimiques qui leur sont associés, incluant des additifs intégrés lors de la fabrication des plastiques pour améliorer leurs performances, ainsi que des contaminants environnementaux adsorbés à leur surface, demeure peu explorée. Cependant, cette menace chimique peut s'avérer particulièrement sérieuse lorsqu’elle impacte des espèces clefs. L'objectif de cette thèse était d'évaluer les effets des lixiviats de microplastiques sur une espèce marine d'importance économique et écologique majeure, clef des écosystemes intertidaux, la moule commune, *Mytilus edulis*, dans l'écosystème de la Manche-Est. Tout d’abord, un état de l’art des efforts de recherche et de leurs disparités en matière d'impact du plastique sur les moules marines a été dressé. Par la suite, des expérimentations en laboratoire ont permis de montrer l’impact des lixiviats de plastique sur (i) la survie des moules à un stress thermique à l’emersion, (ii) leur capacité chimiotactique et chimiocinétique, (iii) leurs réponses collectives anti-prédateurs au crabe *Hemigrapsus sanguineus*, (iv) la dynamique saisonnière de leur agrégation liée à leur stade de reproduction, (v) ainsi que sur leur respiration. Ces résultats mettent également en lumière l'interaction complexe entre les plastiques utilisés, les conditions environnementales et les caractéristiques propres à l'organisme qui peuvent influencer la toxicité des lixiviats pour les moules. Ces effets néfastes engendrés par les lixiviats de plastiques sont particulièrement préoccupants en raison du rôle clef de la moule en tant qu'espèce ingénieure. En altérant la santé des moulières, les lixiviats de plastiques pourraient déclencher des effets en cascade ayant des répercussions sur la biodiversité associée et les nombreux services écologiques et économiques qu'elles fournissent.

Mots clefs

Moule marine, contaminant, écologie intertidale, microplastique, espèce ingénieure, écotoxicologie