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Beatriz Agante Luís

USING MUSSELS AS BIOINDICATORS OF MICROPLASTIC CONTAMINATION IN COASTAL AND ESTUARINE WATERS

Dissertação no âmbito do Mestrado em Ecologia orientada pela Dra. Ana Filipa da Silva Bessa (Departamento de Ciências da Vida da Universidade de Coimbra e MARE - Centro de Ciências do Mar e do Ambiente) e coorientação do Dr. Tiago Verdelhos (Departamento de Ciências da Vida da Universidade de Coimbra e MARE - Centro de Ciências do Mar e do Ambiente) apresentada ao Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Declaro que esta tese foi elaborada e escrita por mim e confirmo que não foi submetida previamente, no todo ou em parte, para obtenção de outro grau académico. Confirmo que o trabalho descrito foi em sua maioria realizado por mim, e outras contribuições são claramente reconhecidas no texto com as devidas citações ou referências. Nomeadamente, este trabalho científico foi apoiado pelo projeto i-plastic - From the land to the sea, the fate of micro and nanoplastics (MICROPLAST/0003/2018).

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Beatriz Agante Luís
Departamento de Ciências da Vida
Faculdade de Ciências e Tecnologia
Universidade de Coimbra
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Abstract

Microplastic contamination (< 5 mm) is one of the most threatening current environmental issues, tightly related to human use and consumption behaviors, and affecting aquatic ecosystems. Despite the importance of transitional aquatic ecosystems as a vector of microplastic contamination between rivers and the ocean, there are still few studies focusing on these environments. This study focuses on the ingestion of microplastics by mussels (*Mytilus* sp.) in the Mondego Estuary (Portugal) and adjacent coastal areas, as well as the presence of microplastics in surrounding surface waters, to assess their potential use as bioindicator or sentinel species of microplastic contamination in these ecosystems. Mussels (n= 30 per station) and surface water (10l) samples were collected in six stations (Buarcos, Cova, Embocadura, Braço Sul, Marina and Braço Norte) in November 2021. In the laboratory, these samples were subjected to digestion with potassium hydroxide (KHO) 10% and hydrogen peroxide (H_2O_2), filtered and stored in Petri dishes for further characterization. The content of the filters were then observed and suspected microplastics extracted and characterized (size, shape and color) using a stereomicroscope.

Microplastics were found in all stations, in 151 (83.8%) of the collected mussels (n = 180). In total, 554 microplastics were registered in mussels with varying shell sizes. Significant differences regarding microplastic abundance in mussels were only observed between stations within the study site - higher concentrations were found at "Braço Sul" station of the estuary, presenting a mean value of 6.167 ± 5.867 microplastics ingested per mussel (p(perm) > 0.05). Fibers were the most common type of microplastics ingested by mussels (75.94%) in all locations, and blue the most common color found (40.76%). A pattern was detected regarding microplastic abundance and mussel size as mussels in Braço Sul had an overall higher weight, averaging 104 ± 35.245 grams per individual. In surface waters, 159 microplastics were recovered from all the samples, with an average of 1.804 ± 1.637 mm in size. Fibers were also the most abundant type of microplastic found (92.45%), and blue the most common color (55.34%). Contrary to mussel samples, the water samples with the highest abundance values were recorded at Marina and Braço Norte. Therefore, being unable to correlate the abundance of microplastic contamination

between mussels and surface waters as well as recognize a clear pattern across the sampling site.
These results suggest that microplastic contamination occurs within the estuary and adjacent coastal areas, and that mussels are ingesting these particles. Therefore, they can be considered sentinel species of microplastic pollution. The spatial scale resolution of this study did not allow a clear inference about their correlation with the water surface so in the future a wider scale should be tested as well as other water compartments (water column) to infer if they can truly be used as bioindicators in these ecosystems.

Keywords: Microplastic pollution, Bioindicators, Mussels, Mondego Estuary

Resumo

A contaminação por microplásticos (< 5 mm) é uma das questões ambientais mais ameaçadoras da atualidade, intimamente relacionada ao uso humano e aos comportamentos de consumo, afetando os ecossistemas aquáticos, mas também. Apesar da importância dos ecossistemas aquáticos de transição como vetor de contaminação de microplásticos entre os rios e o oceano, ainda são poucos os estudos com foco nesses ambientes. Este estudo incide sobre a ingestão de microplásticos por mexilhões (*Mytilus* sp.) no estuário do Mondego (Portugal) e zonas costeiras adjacentes, bem como a presença de microplásticos nas águas superficiais circundantes, de forma a avaliar o seu potencial uso como bioindicador ou espécie sentinela da contaminação de microplásticos desses ecossistemas. Amostras de mexilhões (n= 30 por estação) e águas superficiais (10l) foram coletadas em seis estações (Buarcos, Cova, Embocadura, Braço Sul, Marina e Braço Norte) em novembro de 2021. Em laboratório, as amostras foram submetidas à digestão com hidróxido de potássio (KHO) 10% e peróxido de hidrogênio (H_2O_2), filtradas e armazenadas em placas de Petri para posterior caracterização. Os filtros foram então observados e os microplásticos suspeitos extraídos e caracterizados (tamanho, forma e cor) usando um estereomicroscópio.

Foram identificados microplásticos em todas as estações de amostragem, em 151 (83,8%) dos mexilhões coletados (n = 180). No total, foram registados 554 microplásticos em mexilhões com tamanhos variados. Diferenças significativas em relação à abundância de microplásticos ingeridos pelos mexilhões foram apenas observadas entre as estações dentro do local de estudo - maiores concentrações foram encontradas na estação "Braço Sul" do estuário, apresentando um valor médio de 6.167 ± 5.867 microplásticos por mexilhão (p(perm) > 0,05). As fibras foram o tipo de microplástico mais comum ingerido pelos mexilhões (75.94%), em todos os locais, e o azul foi a cor mais comum encontrada (40.76%). Foi detetado um padrão relativamente à abundância de microplásticos e tamanho dos mexilhões uma vez que os organismos no Braço Sul registaram, em geral, pesos maiores, com uma média de 104 ± 35.245 gramas por indivíduo. Em águas superficiais, foram recuperados 159 microplásticos de todas as amostras com tamanho médio de 1.804 ± 1.637 mm. As fibras também foram o tipo de microplástico mais abundante (92.45%) e o azul a cor mais comum (55.34%). Contrariamente às amostras de mexilhão, as amostras de água com maiores valores de abundância foram registadas na Marina e Braço Norte. Portanto, não se encontrou uma correlação entre a abundância de microplásticos entre mexilhões e águas superficiais, nem um padrão claro nos diferentes locais de amostragem.

Esees resultados sugerem que existe contaminação por microplásticos no estuário e áreas costeiras adjacentes e que os mexilhões ingerem essas partículas. Podem, assim, ser consideradas espécies sentinelas da poluição por microplásticos. A resolução em escala espacial deste estudo não permitiu uma inferência clara sobre sua correlação com as águas superficiais, portanto no futuro uma escala maior deve ser testada assim como outros compartimentos hídricos (coluna d'água) para inferir se eles podem realmente ser usados como bioindicadores nestes ecossistemas.

Palavras-chave: Poluição por microplásticos, Bioindicador, Mexilhões, Estuário do Mondego

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Chapter I – Introduction

1.1 Plastic pollution in oceans and transitional ecosystems

Marine and coastal environments serve as productive zones that connect several subsystems. They encompass 71% of the earth's surface and are complex habitats with abundant biodiversity (Thushari & Senevirathna, 2020). Freshwater lotic systems connect to oceans and seas, creating unique transitional aquatic environments such as lagoons and estuaries. Diverse ecosystem services, such as provisioning of resources, regulation (carbon sequestration, water quality maintenance, climate regulation), support (photosynthesis, nutrient cycling, nursery and breeding grounds, oxygen production), and cultural functions (spiritual and cultural importance, recreation, and tourism) are provided by these settings (Lillebø et al., 2017). These are both ecologically and economically significant. Terrestrial and aquatic ecosystems are inextricably linked - changes in one system can affect the other. Various factors, including anthropogenic activity, have stressed marine ecosystems throughout the years, including pollution and physical deterioration of the environment (Dailianis, 2011). Due to unsustainable development, debris and trash have been accumulated, posing a serious threat to these systems. Plastic debris are more persistent in ocean basins than other types of litter due to its unique properties (Thushari & Senevirathna, 2020). As a result of non-sustainable management, five trillion pieces of plastic debris, weighing more than 260,000 tons, are floating over the world's ocean surface (Thushari & Senevirathna, 2020).

Global plastic production has increased at a high rate since its industrial mass production in the 1950s (Fig. 1) (Dowarah et al., 2020). At this time, only 1.7 million tons were generated annually over the world (Erikson et al., 2014), but since then, yearly production drastically increased, reaching 368 million tons in 2019 and expected to reach 540 million tons in 2040 (PlasticsEurope, 2020; van Cauwenberghe et al., 2015). In Europe alone, this value was 58 million tons (PlasticsEurope, 2020). This roughly equates to the mass of two-thirds of the world's population (Geyer et al., 2017).

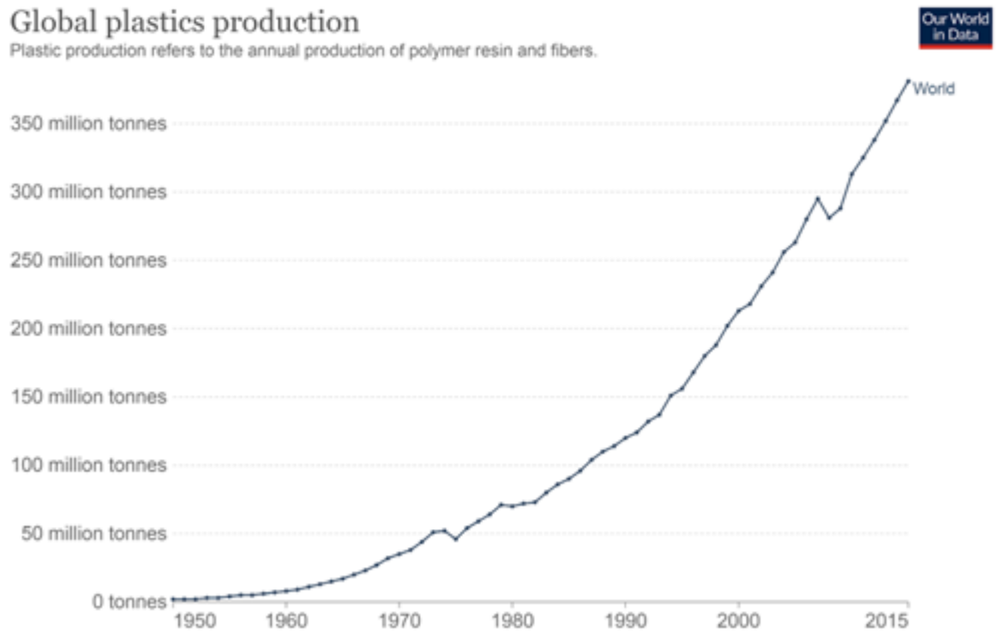

Figure 1 - Global plastic production throughout the years, between 1950 and 2015. Adapted from Geyer et al.'s study in 2017 and published by Our World In Data.

Attractive characteristics of plastic materials like low-cost production and molding facility contributed to their popularization. However, its long-lasting properties and overuse contribute to the accumulation of discarded plastic in various ecosystems (Kahlert & Bening, 2020; Rodrigues et al., 2019). Plastic accumulates in the environment due to increased productivity and slow biotic degradation is the main reason behind negative consequences in aquatic habitats (Issac & Kandasubramanian, 2021). These can endure hundreds of thousands of years before being disintegrated by mechanical and photochemical processes and/or additional and synergistic effects (Issac & Kandasubramanian, 2021).

Before 1980, waste management was based on recycling, and incineration of plastic was scarce. Rates climbed on average by around 0.7% a year between 1980 and 1990 when incineration and recycling emerged (Geyer et al., 2017). The estimated percentages of discarded, incinerated, and recycled plastic waste created globally in 2015 were 55%, 25%, and 20%, respectively (Geyer et al., 2017).

The industries generating the most plastic waste were the packaging sector, with 42% of all plastics entering the use phase (Geyer et al., 2017), followed by building and construction with 19% (Geyer et al., 2017). However, the manufacturing of primary plastic does not correspond to the generation of plastic garbage. The type of polymer used and the lifespan of the finished product both have an impact on plastic waste. When it comes to use, packaging, for instance, has a very short lifespan (typically around 6 months or less). In contrast, the plastic used in buildings and constructing industries has a typical lifespan of 35 years (Geyer et al., 2017). As a result, packaging is the main source of plastic waste globally, accounting for about half of the total number (Ncube et al., 2021). Both marine and land-based sources of plastic can end up in our oceans. When plastic pollution from marine sources is mentioned, it refers to the pollution brought on by fishing fleets that drop off abandoned boats as well as fishing nets, lines, and ropes (Kibria, 2017). It is estimated that 20 to 30 percent of ocean plastics originate from marine sources, with the remaining 70 to 80 percent coming from land-based sources globally (Macfadyen et al., 2009; Lebreton et al., 2018). The United Nations Environment Programme (UNEP) validates this data and has stipulated that fishing gear makes up around 10% of all ocean plastics (Macfadyen et al., 2009). While this represents the relative input of ocean plastics as a whole, the relative contributions of various sources will change based on context and geographic location. Intensified fishing activity in the Pacific Ocean is likely the biggest cause behind the "Great Pacific Garbage Patch (GPGP)", with its gear making up 52% of the plastic debris (Lebreton et al., 2018).

Surface currents and wind patterns have a significant impact on the dispersion and build-up of ocean plastics that stay afloat. Plastics tend to go towards the center of ocean basins after entering the oceans from coastal regions. As a result, they frequently gather in marine gyres (Erikson et al., 2014). The surface waters of the planet are thought to contain more than 5 trillion plastic particles (Eriksen et al., 2014). The Northern Hemisphere's basins contain the most plastic overall (Ritchie & Roser, 2018). Given that the vast majority of people on earth reside in the Northern Hemisphere close to the coast, this is to be expected. But even though it is less than in the Northern Hemisphere, the amount of plastic that has collected in the Southern oceans is also large. This is surprising given the fewer people living near the ocean and overall plastic inputs (Erikson et al., 2014). These findings imply that plastic pollution is migrating across oceanic gyres and basins more quickly than previously thought.

The "Great Pacific Garbage Patch" (GPGP), which is located in the North Pacific, is the most well-known example of plastic accumulation in surface waters. This is related to the region's high coastal plastic inputs and intensive fishing in the North Pacific Ocean. With

1.8 trillion floating pieces, plastics make up 99.9% of the total floating debris in the GPGP (Lebreton et al., 2018). The concentration of surface plastics in the area has reportedly increased exponentially in recent decades (De Bhowmick et al., 2021). Around 52% of plastics in the region derive from fishing activities (fishing lines, nets, and ropes), and 47% originate from hard plastics like sheets and films (Lebreton et al., 2018).

The plastics accumulated in the ocean basins can be predominantly classified into five categories based on size: macroplastics (5–50 cm), mesoplastics (0.5–5 cm), microplastics (1 µm–5 mm), and nano plastics (<1 µm) (Table 1). Their dispersion in the water column is also influenced by their density (Liu et al., 2018).

Table 1 - Categorization of plastic types according to their size.

Plastic type	Size
Macroplastic	5 - 50 cm
Mesoplastic	0.5 - 5 cm
Microplastic	1 µm - 5 mm
Nano plastic	< 1 µm

When it comes to ecosystems like rivers, while some hotspots are located in East Africa and the Caribbean, Asia is home to the majority of the world's most polluted rivers when it comes to plastic waste (Meijer et al., 2021). Poor management practices (plastic debris enters rivers and the ocean due to mismanagement of debris); having a city nearby (paved surfaces where both water and plastic can drain into river outlets); high precipitation rates (meaning plastics washed into rivers, and the flow rate of rivers to the ocean was high); and proximity to the coast are the common characteristics associated with the largest emitting rivers (Meijer et al., 2021). Despite being drained by relatively small rivers, cities like Manila in the Philippines and Jakarta in Indonesia produce a significant amount of plastic pollution (Meijer et al., 2021). This demonstrates that to address plastic pollution issues, waste management must be improved.

Plastics are organic polymers derived from petroleum and can be classified into categories according to their constitution (Ahmed et al., 2018). These include polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), polyvinyl chloride (PVC), and polyester, of which PE and PP are the most common, accounting for approximately 18%

of global production and ranking first and second, respectively, in the global market (Stock et al., 2021; Issac & Kandasubramanian, 2021). PET is the third most manufactured plastic and is primarily used as a packaging material, despite its lack of popularity compared to polyethylene and polypropylene (Leng et al. 2018; Issac & Kandasubramanian, 2021). PET is used as a packaging material because of its safety, lightweight, affordability, and low manufacturing cost and sinks quickly, becoming accessible to benthic species due to its density of 1.37–1.45 g cm^3 (Weber et al. 2018). While PET is resistant to weather variability, fragmentation mechanisms are not, and abiotic weathering in marine conditions is expected to occur through photo-oxidation and hydrolysis (Chamas et al., 2020). Because the pH of the ocean can change the chemical balance of microplastics by increasing or decreasing the rate of chemical leaching from their surface, PET, which is generally thought to be harmless, could become toxic in the near future (Piccardo et al. 2020).

More than 380 million tonnes of plastic that are produced worldwide each year end up in the environment and oceans. The majority of plastic waste is thought to wind up in landfills, and just around 3% makes its way into the sea (Jambeck et al., 2015). The majority of the plastic materials we create should float at the ocean's surface because they are less dense than water. However, the most accurate estimates of the amount of plastic floating at sea are much smaller than the amount of plastic that enters our oceans in a single year (Jambeck et al., 2015). This discrepancy is called the "missing plastic problem" (Lebreton et al., 2018).

A global model of ocean plastics from 1950 to 2015 was developed by Lebreton, Egger, and Slat (2019) to map both the quantity and age of plastic in various ocean ecosystems. The shoreline, coastal areas, and offshore settings were used to quantify where plastic builds up in the water. The study revealed that although a large portion of the macroplastic debris on our shorelines is older and predating the previous 15 years, it can persist for many decades without decomposing. Most macroplastics (79%) in coastal areas are recent, or less than 5 years old (Lebreton et al., 2019). Macroplastics from many years ago, even from the 1950s and 1960s, are still present in the environment (Lebreton et al., 2019). In offshore habitats, the majority of microplastics (75%) are from the 1990s and earlier, indicating that it can take several years for plastics to degrade (Lebreton et al., 2019).

According to Geyer et al. (2017), of the total manufactured plastics in the world, only 9% end up being recycled and 12% incinerated. Whereas 79% are left unattended in landfills, dumps, and natural ecosystems, including the oceans (Dowarah et al., 2020). Moreover, notwithstanding an increase in plastic production over the last decade, plastic waste management prevails as a global challenge and is currently one of the world's most serious threats to aquatic ecosystems (Bessa et al., 2018; Werner et al., 2016; Thushari & Senevirathna, 2020).

The fragmentation of large plastics brings other challenges to the presence of microplastics in aquatic environments. Microplastics, specifically, account for 92.4% of all plastic debris worldwide (Ghayebzadeh et al., 2020). When debris of macroplastics is degraded into smaller pieces by mechanical forces and ultraviolet (UV) light (Dowarah et al., 2020), these smaller particles are much easier for animals to consume or integrate into sediments.

This is crucial in addressing the plastic waste issue since it provides information on what to anticipate going forward. In a study by Lebreton et al. (2019), three scenarios were modeled, including one in which we stopped emitting any plastic into the ocean by 2020, another in which they increase until 2020 and then level off, and a third in which they continue to increase until 2050 at rates consistent with historical growth rates. The findings showed that macroplastics would continue to exist in our surface seas for many more decades even if we had completely stopped ocean plastic pollution by the year 2020 (Lebreton et al., 2019). In every scenario, the huge plastics already a part of our shorelines and surface waters will continue to break down, increasing the number of microplastics in our surface ocean. Furthermore, any additional plastics we use, will increase the problem.

This leads to the question: how do we act against plastic pollution? Besides trying to decrease plastic waste inputs into the oceans and rivers, efforts must be concentrated upstream on less use and consumption of microplastics to avoid leakage to the environment, as well as recapturing and eliminating plastic waste already present in our offshore waterways and shorelines. For that, it’s important to understand plastic pollution to a further extent and understand its spread across the trophic chain as well as through its dispersion pathways which include rivers, oceans, and transitional ecosystems like estuaries.

1.2 Microplastics: origin and effects

Microplastics (MPs) are more accurately defined as synthetic solid particles or polymer matrices insoluble in water, and of regular or irregular shape (Frias & Nash, 2019). And although some researchers recently set the maximum size restriction at 1 mm (Frias & Nash, 2019), they are often characterized as particles less than 5 mm (Browne et al., 2010; Vianello et al., 2013; Dekiff et al., 2014). Microplastics can be classified into two categories depending on their origin: primary and secondary (Antunes et al., 2018; Bessa et al., 2018; Issac & Kandasubramanian, 2021). Primary MPs are pre-existing polymers that were manufactured in a specific size and/or shape for distinct applications and goods. Secondary MPs, on the other hand, are obtained from larger pieces of plastic and can originate from the action of ultraviolet radiation (UV), mechanical abrasion, temperature, and even microbial activity (Iyare et al., 2020; Ghayebzadeh et al., 2020). The latter corresponds to the major cause of the formation of microplastics in aquatic environments. In addition to these two groups, microplastics can be subdivided according to their shape. These categorizations often include fragments, fibers, foam, nurdles, and microbeads (Bessa et al., 2019 - relatório). Smaller pieces of plastic that break off from larger pieces of plastic are known as fragments. Common examples include cutlery, lids, and single-use products. Fleece clothing, diapers, and cigarette butts are all sources of fibers. According to Hartline et al. (2016) wastewater treatment plants fail to filter out 40% of microfibres, other type of microplastic particles. Styrofoam usually takes part in food containers, coffee cups, and packing material. And, lastly, nurdles are small plastic pellets used for producing plastic goods such as container lids (Shahnawaz et al., 2019).

The main cause of primary microplastics immediate release from sewage systems into the environment, which contributes to accumulation in the ecosystem, is ineffectiveness in the elimination process due to their reduced size and low perceptibility (Issac & Kandasubramanian, 2021). This includes, for example, microplastic beads found in skincare and hygiene products (Kalková et al. 2017). Just 6 kg of synthetic clothing releases an average of 700,000 fibers in a single wash (Napper and Thompson 2016). Due to their role as a raw material for plastic goods, pellets used in industrial applications are also a source of microplastic discharge into the environment (Li et al. 2016). Dental and pharmaceutical carriers are the primary sources of microplastic contamination in the medical field (Issac & Kandasubramanian, 2021).

Due to their floatability and persistence in the environment, microplastics are common marine contaminants in aquatic systems and serve as a vehicle for the transfer of pollutants to aquatic animals (Eder et al., 2021). Because of their small size, a variety of organisms consume them, which disrupts their physiological systems and allows them to propagate down the food chain until it reaches humans (Issac & Kandasubramanian, 2021). Therefore, the negative impacts of microplastics are transferred up the food chain to higher trophic levels.

Microplastic pollution in aquatic systems can come from a variety of sources, including the production of plastic goods, water, sewage treatment facilities, industrial or agricultural waste, plastic weathering, fisheries, and aquaculture (Rezania et al., 2018). Plastic waste from households, businesses, and other sources is introduced to marine habitats, where it accumulates and negatively impacts aquatic life. A significant source of microplastic contamination is the use of microcapsule fertilizers, which are frequently employed in agriculture to minimize nitrate leaching into groundwater (Katsumi et al., 2020). Paddy field pathways allow these discharges to finally reach the ocean, where they create a significant flow of microplastic, especially during irrigation season. For instance, vehicle tires are one of the main sources of microplastic pollution in the oceans, according to recent studies (Kole et al., 2017). Elastomer, carbon black, fibers, and other organic and inorganic materials are used to make tires, which are then flown or delivered via other waterways directly to the sea. Other significant causes of microplastic contamination are offshore drilling, fish hatcheries, and fishing, which directly endanger the biota since secondary microplastics (MPs hereafter) result from long-term deterioration (Brandon et al., 2016; Lusher et al., 2017). Microplastics get into and are released directly into water resources, particularly freshwater systems since wastewater treatment plants are not equipped or prepared to filter these polymers (Li et al. 2018). Even though these particles are often resistant to biodegradation, some substances can cause gastrointestinal blockages by being passively or actively absorbed by consumers after disintegration (Lei et al. 2018). Knowing this, it comes as no surprise that microplastics have been found in practically all aquatic organisms and animals at all levels of the food chain. Even though this is true, the total amount of microplastics varies significantly even within the same region, depending on the organisms and the area in which they are found (Desforges et al. 2015; Walkinshaw et al. 2020).

Mainly, there are three potential major negative effects associated with the consumption of microplastics: (1) Physical and physiological effects, for instance, decreased development and alteration of feeding habits (the greater the number, the greater the risk associated); (2) Toxicity associated with the release of hazardous compounds - additives (including antioxidants, plasticizers, flame retardants, and pigments) incorporated during the production of the plastic can leach into tissues of the body, resulting in bioaccumulation or provoked changes; (3) Harmful reactions to potential pollutants imbibed in the microplastics, turning microplastics into carriers for contaminants into aquatic systems. Some common pollutants found in these polymers include polycyclic aromatic hydrocarbons, polychlorinated biphenyl (PCB), dichlorodiphenyltrichloroethane (DDT), organo halogenated pesticides, hexachlorocyclohexane, and chlorinated benzenes. POPs, like PBDE and PCB, have the ability to imitate natural hormones, being responsible for disorders related to reproduction (Issac & Kandasubramanian, 2021). Apart from direct consumption linked with dietary habits, humans get subjected to microplastics through cosmetics, dust particles found in the airway, and the usage of plastic products (Issac & Kandasubramanian, 2021).

The effects of microplastics in different organisms are multiple and the impacted functions include food uptake, feeding habits, growth, spawning, body weight, or even contribute to oxidative stress and affect the well-functioning of organs like the liver, heart, and intestines (Yu et al., 2020; Messinetti et al., 2019; Hossain et al., 2019; Banaee et al., 2020; Lo & Chan, 2018; Lei et al., 2018). Because there is currently minimal knowledge on the trophic transfer of chemicals included in these polymers, it is difficult to anticipate whether pollutants are eliminated or bioaccumulated inside the organism. Malnutrition is still the most common observed consequence in most species, and it has a deleterious impact on development and reproduction (Berglund et al., 2019). It is especially worrisome as these hazardous effects can be passed between levels in the trophic chain (Athey et al., 2020).

The effects of microplastics primarily relate to entanglement or ingestion (Gall and Thompson, 2015). Due to their size, shape, and color, microplastic particles are mistaken for food, contributing to their consumption (Schuyler et al., 2014). These polymers have been found in many animals living in aquatic systems and their effects vary according to their size and species. Some examples of the problem behind this type of pollution to marine biota include polystyrene microplastics having a deleterious influence on oyster reproduction and feeding. In this scenario studied by Sussarellu et al. in 2016, the yield and growth of offspring exposed to microplastics decreased by 41% and 18%, respectively: The accumulation of microplastic debris inside the guts of gentoo penguins, preventing them from consuming food and contributing to the absorption of hazardous compounds in the water, affecting their growth and development (Bessa et al., 2019); Impacts on zooplankton eating patterns, fertility, and function, such as copepods. Because of their size, large number of lipids, and opulence, these organisms are important prey for many fish larvae (Cole et al., 2015). Ingestion of microplastics has been shown to influence their overall health, resulting in a 40 percent loss in carbon biomass and a shortfall in energy, indicating rapid lipid consumption, impairing their growth, or contributing to their mortality. Long-term exposure also results in smaller eggs and lower hatching rates (Cole et al., 2015); Herbicides like glyphosate, for example, have different toxicity levels when microplastics are present in the environment (Zocchi & Sommaruga, 2019). Tests performed by Zocchi & Sommaruga (2019) using Daphnia magna, crustaceans used as food by many aquatic organisms, demonstrated a fatality rate increase of 53,3% and 30% when incorporated with microplastics for polyethylene beads and polyamide fibers, respectively. Contrasting with the 23.3% mortality rate of glyphosate not associated with microplastics (Zocchi & Sommaruga, 2019); Faecal impaction and malnutrition are caused by an increase in microplastic concentration inside mussels. Mussels play a crucial role in aquatic ecosystems. Large mussels absorb great quantities of water while storing primarily fibers. The bigger the mussels, the more microplastics can be found to be ingested (Berglund et al., 2019). Furthermore, microplastics have a high potential to attract contaminants, resulting in the absorption of hazardous compounds by mussels; Impacts on both the reproductive and immune systems of various shark species. Ingestion is the most common method of intake, which can be done directly or through food (mostly crustaceans and molluscs) (Germanov et al., 2019). Again, the presence of chemicals associated with microplastic is the utmost cause of these problems (Parton et al., 2020).

Microplastics disperse through various pathways such as wastewater discharges, river flows, extreme events (e.g., storms), winds, animals, maritime, and agricultural activities. These vectors cause the particles to enter different environments, including freshwater and transitional environments such as estuaries (Antunes et al., 2018). Because of the dangers that microplastics pose to marine biota, in order to reduce the possibility of MPs building up in the food chain, it is important to understand coastal hotspots of these particles in order to take action and monitor the excessive use of plastic additives, as well as implement regulations to control plastic contamination.

1.3 Microplastics in coastal and transitional ecosystems

Estuarine and coastal areas are among the most valuable aquatic ecosystems, admitting their richness in productivity and indispensable ecological and economic role (Barbier et al., 2011). They are also some of the most exploited and endangered natural systems worldwide (Lotze et al. 2006, Worm et al. 2006, Halpern et al. 2008). Anthropogenic action is rapidly and severely contributing to their degradation (Barbier et al., 2011). These environments provide a variety of goods and services such as food resources, coastal protection, temporary shelter, habitat for a wide diversity of species (not exclusively estuarine but also marine species), and partake in migration routes (Rodrigues et al., 2019). Among the services provided, estuaries are considered essential nursery habitats for fish in addition to sheltering a variety of seabirds, fish, and mammals (Whitfield, 2020; Costanza et al., 1997). According to Worm et al. (2006), the global decline of estuarine and coastal regions is known to have an impact on at least three ecosystem services: fisheries (which have declined by 33%), the provision of nursery habitats like oyster reefs, seagrass beds, and wetlands (which have declined by 69%), and the filtering and detoxification services offered by suspension feeders, submerged vegetation, and wetlands (63% decline). Biological invasions, diminishing water quality, and decreased coastal protection from flooding and storm events are all related to the loss of biodiversity, ecosystem services, and coastal vegetation (Braatz et al. 2007, Cochard et al. 2008, Koch et al. 2009).

Hydrodynamic forces (tides, waves, and wind) and physical characteristics of the microplastics (density, size, and shape) considerably influence the dispersal and consequential entrance of MPs into the marine environment (Zhang, 2017). Estuaries and coastal regions are vulnerable to plastic contamination and have been identified as microplastic hotspots as drainage systems like freshwater streams are significant vectors for the transportation of land-based plastics into the marine environment (Browne et al., 2010; Wright et al., 2013). Despite their involvement in the transmission of plastic to oceans, less research has been done on freshwater bodies than on marine ones, and there is currently a paucity of information on the consumption of microplastics by animals in transitional aquatic settings (e.g., Possatto et al.,2011; Vendel et al., 2017).

Previous research has found that plastic particles can be mistaken for food and swallowed accidentally due to their resemblance to prey appearance and shapes (Lehel & Murphy, 2021). They can also be directly ingested through prey that already contains microplastics (Egbeocha et al., 2018). Because plastic debris gets transferred to the sea via rivers, transitional systems like estuaries play a crucial role in transporting these particles from the land to the sea; and, because of the dynamic nature of these ecosystems, microplastics can linger in these habitats for long periods and get consumed by numerous aquatic organisms, although the amount ingested varies depending on the species (Dris et al., 2020; Egbeocha et al., 2018). Because of the dangers that microplastics can pose to marine biota, as addressed earlier, it's critical to act and monitor the excessive use of plastic additives, as well as implement regulations to control plastic contamination (SAPEA, 2019).

1.4 Bioindicator species in microplastic pollution

Mussels are an important component of benthic assemblages worldwide and are thought to act as ecosystem engineers via the occupation of primary space, filtration, and provision of secondary habitat (Browne et al., 2008). Many studies were done in vivo to investigate the deleterious effects of these particles on mussel species and their role in trophic transfer (Dowarah et al., 2020).

Investigating the biotic effects of exposure to microplastics should be done through biomonitoring. Bivalves are of specific interest for MPs presence analysis in ecosystems because of the filter-feeding habit that exposes them directly to MPs available in the environment (Pazos et al., 2020). Microplastics have also been detected in mussels and clams in recent years (Davidson & Dudas, 2016; Weber et al., 2020).

Mussels, already well-established biomonitors for environmental contaminants, are a suitable candidate for assessing MPs exposure in the environment (Li et al., 2019). Mussels can filter large volumes of water and actively filter, and trap suspended particulates such as algae and sediments (Catarino et al., 2018), which causes these organisms to ingest more microplastics (Setälä et al., 2016). Previous studies have suggested that mussels can act as bioindicator organisms regarding plastic pollution as these organisms are widely spread, susceptible to microplastic ingestion, closely connected to marine predators and human food (implying the entrance of MPs in the human body), and hold vital ecological niches (Li et al., 2019). In addition, bivalves are very popular among seafood consumers, therefore having high commercial values, and are thought to be the largest source of MPs from seafood to humans since they are consumed whole (Lusher et al., 2017). Although not all the characteristics of the MPs in mussels can exactly match those in their environment, quantitative correlations of abundance between the microplastics in mussels and nearby sea waters make it possible to estimate the levels of environmental microplastic pollution from the MPs in mussels (Qu et al., 2018), which is interesting given that the abundance of MPs in field-collected mussels is directly related to human activities. This increases their interest as species for the assessment of microplastic quantification. As a method, it’s effective in reducing or avoiding error rates and misinterpretation resulting from contingency in environmental medium because the concentration of pollutants, including microplastics, in mussels, tends to remain stable after obtaining a balance between intake, assimilation in tissues, and defecation or digestion (Setälä et al., 2016; Beyer et al., 2017).

Many *Mytilus* species (e.g., M. *edulis* and M. *galloprovincialis*) hold a high commercial value as seafood (Food and Agriculture Organization of the United Nations, 2017), hence the concern about trophic transfer and the possibility of MPs ingestion by humans. An estimated 11,000 MPs are consumed by European shellfish consumers each year, even though there is still no evidence of MPs ingestion by humans via trophic transfer. Furthermore, the European Food Safety Authority (EFSA) Panel for Contaminants in the Food Chain recently stated that the occurrence data in shellfish food items are inadequate, implying exposure levels are largely unknown (Catarino et al., 2018).

Additionally, microplastic levels in mussels have been added to European databases about pollutants of rising concern in seafood and microplastic uptake and accumulation in Belgian mussels have been chosen as a measure of the state of the maritime environment (De Witte et al., 2014; Vandermeersch et al., 2015). Mussels were also suggested as a bioindicator species to monitor marine microplastic pollution in a workshop on "Distribution, Source, Fate, and Impact of Marine Microplastics in Asia and the Pacific," which was sponsored by the IOC Sub-Commission for the Western Pacific (WESTPAC) (WESTPAC, 2017). Because of their abundant stocks for frequent sampling and capacity to reflect local conditions, OSPAR has selected mussels as excellent monitoring species (OSPAR, 2012). However, none of the regulatory agencies have yet provided any guidelines for standard monitoring practices (inc. OSPAR, MSFD, NOAA, UNEP).

Because of its wide geographic range, including intertidal and subtidal environments, and its importance concerning the diet of various predators (including human consumption), mussels can provide information on MPs contamination throughout numerous locations (Catarino et al., 2018; Dowarah et al., 2020). Hence, *Mytilus* sp. were selected as model organisms for this study.

Despite not being so common in estuarine areas due to their low tolerance to lower salinities, mussels exist associated with fixed structures, such as bridges, in the Mondego Estuary. Previous studies have recorded the survival of mussels in environments with salinity values ranging from 20 to 35 ‰, varying with temperature (Brenko & Calabrese, 1969).

1.5 Objectives of the study

The main goal of this study is to assess the levels of microplastic pollution in transitional aquatic systems along with adjacent coastal areas, more precisely:

1. Determine the levels of microplastic contamination in mussels (*Mytilus* spp.) and waters, using the Mondego Estuary as well as adjacent coastal areas as case study.
2. Assess the potential existence of a gradient of microplastic contamination from the estuary to the sea (identification and quantification of microplastics).
3. Evaluate the potential of *Mytilus* spp. to act as a bioindicator or sentinel species of microplastic pollution in transitional and coastal waters.

Chapter II – Materials and Methods

2.1 Study area and sample collection

This study was conducted in the Mondego River estuary and adjacent coastal areas in Figueira da Foz, Coimbra (Portugal). The sampling campaign was performed during low tide, carried out by boat within the estuarine sampling stations, and by foot on intertidal coastal areas, in two consecutive days in November 2021.

2.1.1 The Mondego Estuary - case study

The Mondego River, largest river under exclusive Portuguese administration, meets the Atlantic Ocean in a small mesotidal estuary (1600 ha) located on the western central coast of Portugal (40°080 N, 8°500 W) (Mantas et al., 2013) (Fig. 2). Its estuary drains a 6670 km² basin and sustains a population of nearly 885 thousand inhabitants. The terminal part (7 km long and 2–3 km at its widest part) is divided by Morraceira Island, which separates into two arms (North and South) with distinct hydrological features. The north arm is the most profound (8–12 m at high tide) and receives most of the freshwater arising from the river. The south arm is shallower (2–4 m at high tide) and receives fresh water from the Pranto River, a small branch of the Mondego. In adjacent coastal areas, sandy beaches and marine soft bottoms constitute most of the surrounding habitats (Gaspar et al., 2017). To the south of the estuary, thus receiving water directly from there, is Praia do Cabedelo. Facing north, there are Praia da Claridade and Praia de Buarcos, consecutively. The coast is under the prevailing northwest (NW) oceanic swell's influence, reaching values over 5 m in the winter when most frequent storms occur from WNW. The tidal range varies inside the estuary between 0.35 and 3.3 m, while water residence time goes from one day in winter to five days during summer, on the north arm, and three days in the winter to nine days in the summer, on the south arm (Gaspar et al., 2017). The retention time of water in the south arm of the Mondego Estuary is much bigger compared with the North Arm. This is because the main flow of water occurs in the North Arm. Therefore, the water moves and is replaced much faster in the latter, which can possibly influence the presence and distribution of microplastics in the estuary. The freshwater inflow is the main factor influencing the residence time in the Mondego Estuary and retention time is shorter in winter than in summer because of more intense freshwater inflows in the wintertime (Kenov et al., 2012).

Regarding ecological value, the estuary constitutes an important recognized nursery area for commercially valuable fish species and has an indispensable regional socio-economic value. In detail, the system supports industrial activities, mercantile and fishing harbors, salt extraction, aquaculture farms, and agriculture areas (Bessa et al., 2018; Gaspar et al., 2017). Consequently, the Mondego estuary has also undergone several anthropogenic pressures counting resource depletion and pollution with hydro morphological transformations over the last decades (Marques et al., 1997; Neto et al., 2010). The estuary is also under the influence of two wastewater treatment plants, Vila Verde, and S. Pedro, which is a possible source of MP contamination in this ecosystem.

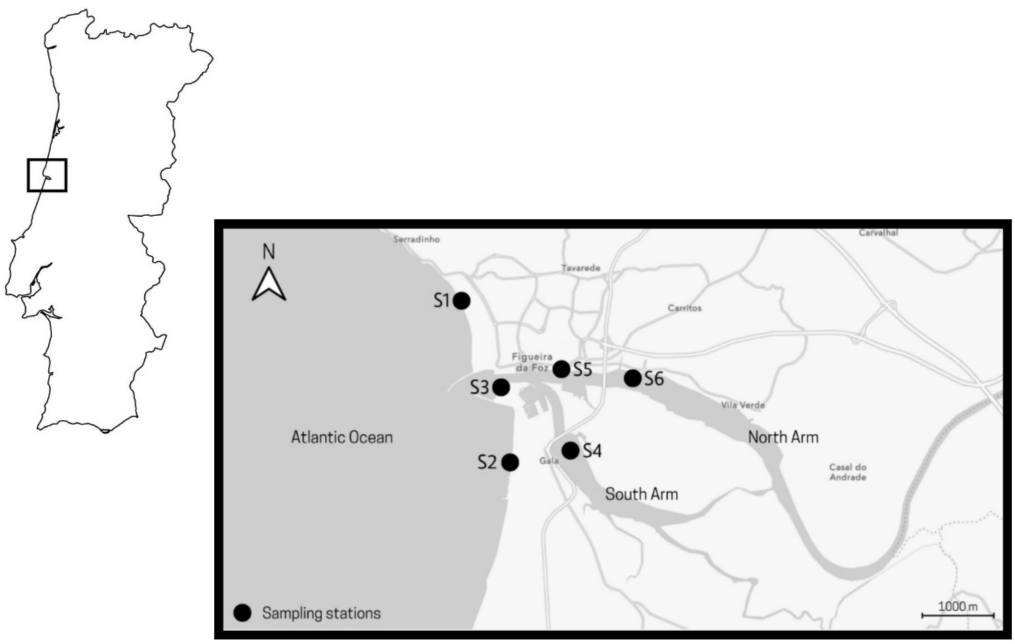

Figure 2 - The Mondego estuary (Portugal) with the location of the sampling areas S1 (Buarcos) and S2 (Cova) in coastal areas and S3 (Embocadura), S4 (Braço Sul), S5 (Marina), and S6 (Braço Norte) in the estuary.

Only one study has been published so far concerning microplastic contamination in the Mondego Estuary (Bessa et al., 2018), performed using two fish species (*Dicentrarchus labrax* and *Platichthys flesus*) and determined a frequency of occurrence of microplastic ingestion of 38% in these species. Particularly, the benthopelagic species *D. vulgaris* had the highest incidence (73%), with up to 14 microplastics retrieved from a single individual.

2.1.2 Sample collection

Mussel and surface water samples were collected in the Mondego Estuary during November 2021. In total, the samples were collected from 6 different locations: i) 3 beaches (Buarcos (S1), Cova (S3), and a third beach located in the mouth of the estuary, which we designated Embocadura (S2) that receives influence from the estuarine area; ii) 2 in the estuary (North arm (S6) and South arm (S5)); iii)1 in the harbor located closed to the mouth of the estuary (S4) (Fig. 1). In each station, 30 mussels were collected by hand, as well as 10 L of water. Water parameters (e.g., surface water temperature, salinity, pH, conductivity, dissolved oxygen (DO and DO%), and oxidation-reduction potential (ORP) were recorded using a multiparameter digital water quality sonde, in each station (Table 1).

Each mussel was wrapped individually in tin foil to avoid plastic contamination between the organisms, stored and kept in an icebox, and transported to the laboratory where they were frozen at - 20 ºC until further processing.

2.2 Sample processing

2.2.1 Mussels

In order to extract and analyze the presence of microplastics in the mussels, protocols and guidelines harmonized among studies in microplastic research for biota samples were followed (e.g., Bessa et al., 2019 and references therein). In total, 180 mussels were measured, dissected, and weighed - total weight (to 0.01 g) and total length (to 1 mm) were recorded for each individual, using a ruler and a precision balance, respectively. The organic content was taken from each of the shells, with the help of a scalpel and/or tweezers, and put inside glass beakers containing, approximately, three times the volume of the organic matter added in potassium hydroxide - samples were digested with a 10% potassium hydroxide (KOH) solution (analytical reagent grade, Fisher Chemical) (maximum 150ml) - as well as 5 mL of hydrogen peroxide (H_2O_2, 10%). The floating phase started 48 hours later after the organic matter was digested at room temperature. The liquid from each beaker was filtered using a vacuum bomb through a 1.2 µm pore (70 mm) Whatman GF/A glass microfiber filter paper. The filter was then sealed inside a properly identified Petri dish for further analysis.

2.2.2 Surface Waters

The 10L water samples from each station were directly filtered using a vacuum bomb through a 1.2 µm pore (70 mm) Whatman GF/A glass microfiber filter. The resultant filters from both processes were also placed inside a properly labeled Petri dish for subsequent analysis.

All laboratory materials used during the sample processing were cleaned with purified water filtered prior to use. Cotton laboratory coats were worn, and no plastic material was used during all procedures. The samples were analyzed in a laboratory with restricted access to ensure minimal airborne contamination during visual inspection and digestion of solutions. Three clean Petri dishes with filter papers were placed next to the sample during the inspection under a stereomicroscope as contaminant controls. No contamination was detected in the controls. Due to logistical constraints, it was not possible to determine the chemical composition of all extracted particles (polymer identification). For that reason, strict criteria were used. Criteria for visually identifying potential microplastics included: the absence of cellular or organic structures; a homogenous thickness across the particles; a homogenous color and gloss; and the Hot Needle Test, in which plastic particles change the structure or move when in contact with the needle (see De Witte et al. (2014)).

2.3 Observation and identification of microplastics

All suspected microplastic particles extracted after visual sorting were subsequently observed and photographed using a LEICA M80 (Leica Microsystems GmbH, Wetzlar, Germany) with image analysis system IC80 HD Camera with Leica Application Suite (LAS) software. Particles were classified and categorized by type according to their shape into fibers (elongated), fragments (angular and irregular pieces), films (thin and transparent), and their color (Bessa et al., 2018). Additionally, all particles were measured at their largest cross-section and categorized according to the following size classes: 2.5-2.9 mm, 3-3.4 mm, 3.5-3.9 mm, 4 mm - 4.4 mm, 4.5 mm - 4.9 mm, 5 mm - 5.4 mm, 5.5 mm - 5.9 mm, 6 mm - 6.4 mm, 6.5 mm -6.9 mm, 7 mm -7.4 mm and 7.5 mm - 8 mm.

2.4 Statistical analysis

To test for significant differences in the levels of microplastic ingestion between mussels in the Mondego estuary and adjacent coastal areas, a permutational multivariate analysis of variance and a posterior pairwise test (PERMANOVA; Anderson et al., 2008) was conducted, as data were not normally distributed (Kolmogorov-Smirnov: $p < 0.05$) and not homoscedastic (Levene's test $p < 0.05$). The similarity matrices were calculated using Euclidean distances for the number of microplastics per individual was employed (untransformed data). The design incorporated two factors: 1) Location (fixed), with two levels (Estuary and Coastal area), and Stations (nested in Location) with 6 levels (Cova, Buarcos, Embocadura, Marina, Braço Norte e Braço Sul). All statistical analyses were performed using PRIMER v.6 and its add-on package PERMANOVA+ (Anderson et al., 2008). Correlation analysis was used to ascertain the relationship between the abundance of microplastics in mussels and morphometric features like shell length and tissue weight. Pearson correlation coefficient was used to examine the association between microplastic load in surface water and mussels. Statistical tests were considered significant at p-values < 0.05. Results from mussels are presented here in microplastics per individual (MP/ind.) and microplastics per gram (MP/g) (w.w), and for surface waters, the abundance is measured per volume (MP/m^3).

Results

3.1 Analyses of the physicochemical properties of the surface water

The physicochemical properties of the surface water in all sampling locations were measured using a multiparametric probe. As can be seen in Table 2, there is an obvious salinity gradient across zones (from the riverine-estuarine area to the coastal zone). Water salinity was lower inside the estuary (lowest value of 5.67 in Braço Norte) and increases as it approaches the ocean and coastal areas (highest value of 32.95 recorded in Buarcos beach). Although the Embocadura sampling site is located on a beach near the river mouth, it is contained between seawalls on the southern part of the estuary and, therefore, its characteristics are similar to the water inside the estuary. This is verified by its salinity values, as at Embocadura was recorded a very similar value with the estuary and, in specific, the Braço Sul station (both with values of 19.06 and 20.76, respectively). Contrarily, both stations located in the northern arm (Marina and Braço Norte), also share similar salinity values, with salinity of Marina being recorded 11.52 at the time of its analysis. It's important to add that these parameters were measured in superficial waters and the tides vary depending on the hour and day, despite being measured at the same day it can receive influences from the mixing waters that occur in estuaries. Sampling locations like Braço Norte and Marina receive freshwater water from the river, which explains the low salinity values. Coastal areas, on another hand, are expected to receive less river water (especially Buarcos as it is located north of the river mouth and the water flows southward) and have more contact with water coming from the ocean. This is also corroborated by the salinity values obtained, which were higher in these zones (Table 2). Regarding the temperature, similar values were recorded in all sampling stations (14-15 ºC) and the content of oxygen in the water was also similar along the stations.

Table 2 - Characterization of the surface water samples collected at the Mondego estuary and adjacent coastal areas.

	Coast		Estuary			
	S1	S2	S3	S4	S5	S6
	Buarcos	Cova	Embocadura	Braço Sul	Marina	Braço Norte
Temperature (ºC)	14.5	16.4	15.6	14.8	15.4	15.5
DO% L	109.1	92	91.1	89.7	91	89.6
DO mg/L	9.15	6.62	8.15	7.96	7.57	8.69
Conductivity	4204	4609	25097	266644	15753	8094
Salinity	32.95	31.95	19.06	20.76	11.52	5.67
ORP	103.8	101.6	97.5	94.9	94.1	95.2
pH	7.98	7.91	7.93	8.02	7.58	7.48

3.2 Mussel samples

3.2.1 Occurrence of microplastics in *Mytilus* spp.

Of the total mussels collected in the Mondego Estuary and adjacent coastal areas (n= 180), microplastics were recorded in 151 (83.8%) individuals, with an average of 3.08 ± 3.65 (SD) particles per mussel. In total, 554 microplastics were identified from all 180 mussel samples, with a maximum of 28 particles being extracted from a single individual found at Braço Sul. The average weight of mussels and microplastics per gram were 55.00 ± 45.44 (SD) and 9.12E-05 ± 7.14E-05 (SD), respectively. And the average mussel shell size and load of microplastics per individual were 4.45 ± 1.13 cm (SD) and 3.08 ± 1.55 (SD). Plastic particle sizes ranged from 0.074 mm to 8.01 mm, averaging 7.181 ± 1.229 mm.

The mean abundance of microplastics in mussels from different sampling locations is presented in Fig. 6. Microplastics were detected in all locations. While there was variation in the number of microplastics in the sampling points, the differences among coastal zones and the Mondego estuary were not statistically significant (P(Perm) > 0.05).

However, when analyzing sampling stations, there is a clear increase in the number of microplastics in the mussels found at Braço Sul, with the highest values (6.167 ± 5.867

mm), while the lowest were found at Buarcos beach (2.267 ± 2.067). Furthermore, these microplastic load were statistically different (P(Perm) = 0.0001) among stations. Pairwise tests revealed that Braço Sul was statistically different from all other stations P(Perm) < 0.05).

On the other hand, abundance results of microplastics per gram of wet weight of mussels, differed from the values obtained as MP/ind. The highest and lowest abundances were from mussels collected in Marina and Embocadura (Fig. 5) with mean values of 0.104 ± 0.086 and 0.048 ± 0.054, respectively. Mussels from Braço Norte and Cova both had ingested 0.083 microplastics per gram (with slightly different values of standard deviations: 0.077 and 0.078) and those from Buarcos and Braço Sul ingested 0.069 ± 0.068 microplastics per gram of wet weight (Table 3).

In terms of mussel size, mussels gathered in Braço Sul were the largest, weighing an average of 104 ± 35.245 grams, while those collected in Marina and Braço Sul were the smallest, weighing 25 ± 5.226 and 23 ± 4.772 grams, correspondingly. Mussels from Embocadura measured an average of 94 ± 61.257 grams (high variability in sizes) and those from Buarcos and Cova, both coastal sites, had similar average weights of 34 ± 6.685 and 33 ± 7.137, respectively.

3.2.2 Characterization of microplastics from mussels (*Mytilus* spp.) according to shape, size, and color

Microplastics with various shapes, colors and sizes were detected in the content of the mussels collected from the Mondego Estuary and coastal areas. The two shapes primarily found can be sub-grouped into two categories: fibers and fragments, where fibers were the most ingested (75.94%) by mussels and fragments the least ingested (24.05%) (Fig. 4). Interestingly, a single aggregate or tangle of fibers was found inside one mussel collected from one of the coastal sampling sites (Cova). Regarding color distribution, in general, the most prominently identified was blue, with a frequency of 40.76%. green (15.05%), gray (8.57%), red (15.24%), black (14.10%), translucent (4.76%), white (0.57%), beige (0.38%), purple (0.38%), and brown (0.19%) were among the other particle colors identified in the samples (Fig. 7). Color, anyhow, is a subjective and ambiguous identifying parameter that is highly dependable on the observer. Hence, the results reported here are just intended to demonstrate the visual range of particles discovered.

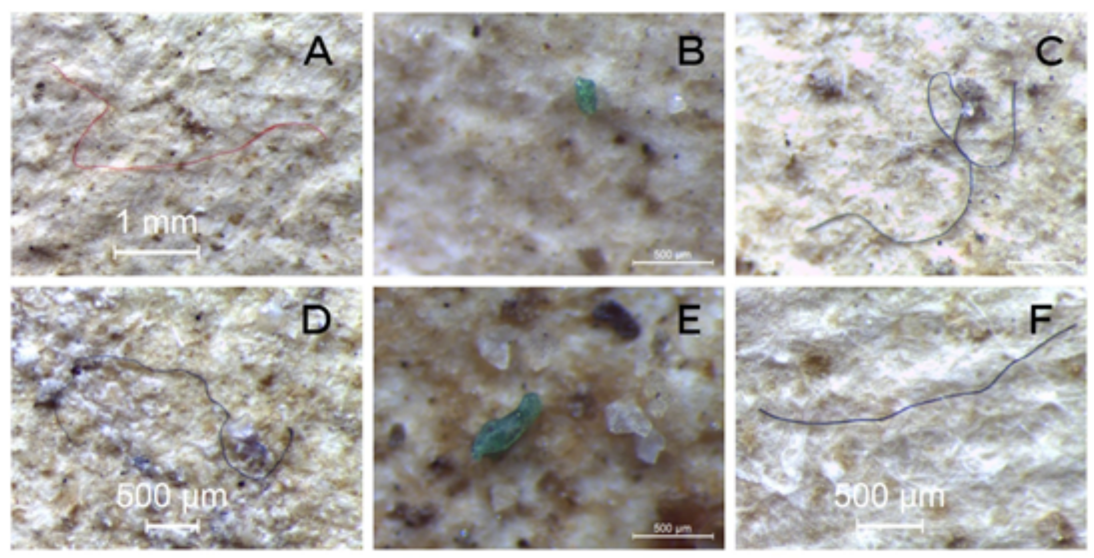

Figure 3 - Examples of microplastics found in the content of mussels in each of the sampling locations (A- red fiber from Braço Norte; B- green fragment from Braço Sul; C- blue fiber from Buarcos; D- black fiber from Cova; E- green fragment from Embocadura; F- blue fiber from Marina).

The mean number of fibers and fragments was similar in most sites but significantly higher in Braço Sul. Embocadura station exhibited a higher number of fragments compared to other sampling locations (Figure 4). Regarding color, green and red microplastics were the most recurrent in the south arm of the estuary, whereas blue particles were the most numerous in all sites, having a similar frequency of occurrence (Figure 7).

The size distribution of the microplastics found inside the mussels was bimodal. Most microplastics detected were sized between 3.5 and 3.9 mm, occupying 24.70% of the total numbers. Classes including sizes from 4 to 4.4 mm, 6 to 6.4 mm, and 6.5 to 6.9 mm also stick out with several particles superior to 50 and a percentage of occurrence of 15.78%, 13.04%, and 14.92% respectively (Figure 8). Microplastics ranging from 7 to 7.4 mm and 7.5 to 8 mm in size were the least common and represent only 2.22% and 0.17% of the samples obtained, respectively.

Table 3 - General data from the ingestion regarding the microplastic ingestion by mussels (*Mytilus* sp.) collected from the Mondego Estuary and adjacent coastal areas.

Location	Station	Frequency of occurrence (%)	Average mussel size (cm) (SD)	Average weight (g) (SD)	Average MP/ind (SD)	Average MP/g w.w. (SD)
Coast	Buarcos	12.274	4.157 (± 0.206)	34 (± 6.685)	2.267 (± 2.067)	0.069 (± 0.068)
Coast	Cova	14.621	4.070 (± 0.433)	33 (± 7.137)	2.700 (± 2.423)	0.083 (± 0.078)
Estuary	Braço Norte	11.011	3.544 (± 0.07)	25 (± 5.226)	2.033 (± 2.025)	0.083 (± 0.077)
Estuary	Braço Sul	33.394	6.222 (± 0.255)	104 (± 35.245)	6.167 (± 5.867)	0.069 (± 0.068)
Estuary	Embocadura	15.884	5.392 (± 0.211)	94 (± 61.257)	2.933 (± 3.85)	0.048 (± 0.054)
Estuary	Marina	12.816	3.309 (± 0.179)	23 (± 4.772)	2.367 (± 2.025)	0.104 (± 0.086)

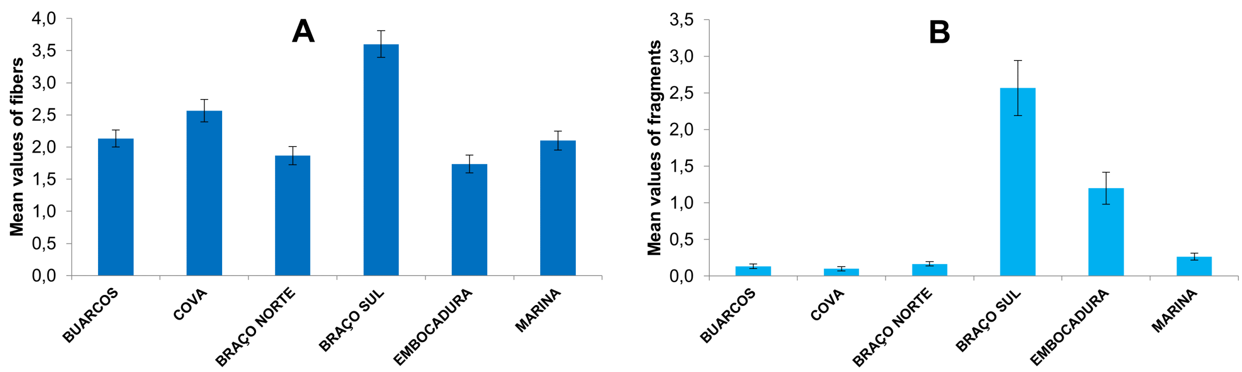

Figure 4 - Mean values of microplastics (± SE) categorized by shape (A- fibers; B- fragments) extracted from mussels along the Mondego estuary.

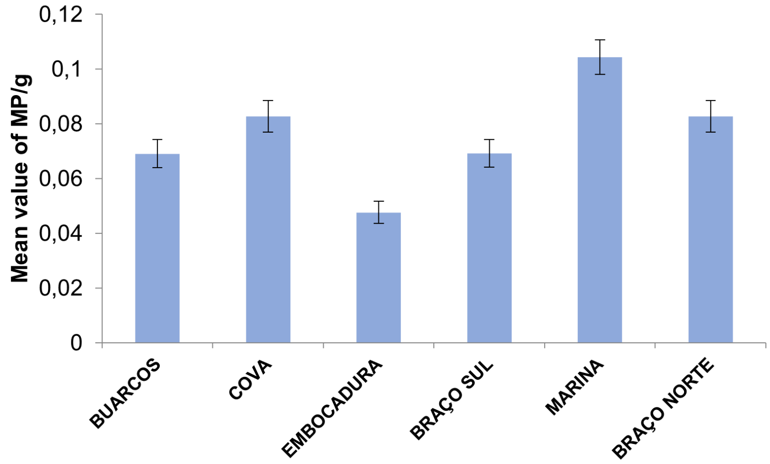

Figure 5 - Mean values of microplastics (± SE) extracted from mussels along the Mondego estuary per gram of wet weight.

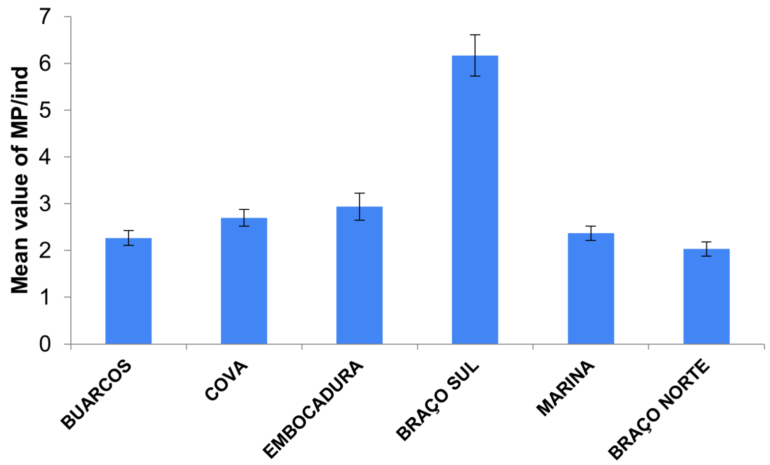

Figure 6 - Mean values of microplastics (± SE) extracted from mussels along the Mondego estuary per individual.

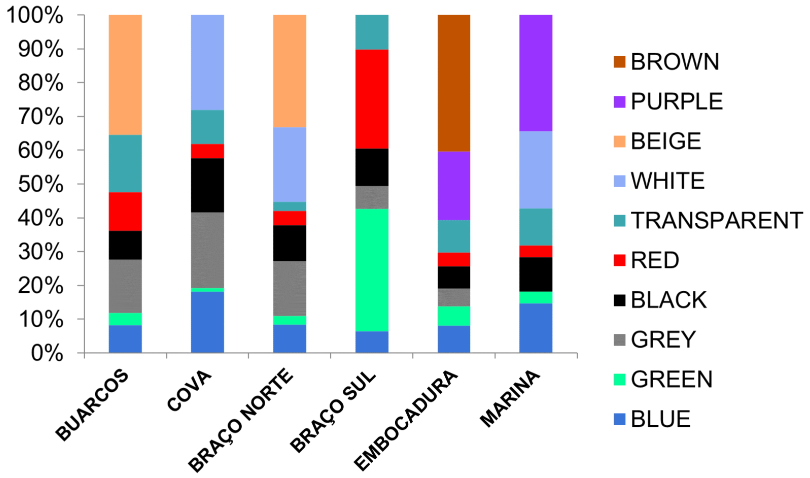

Figure 7 - Percentage of microplastics categorized by color extracted from mussels along the Mondego estuary.

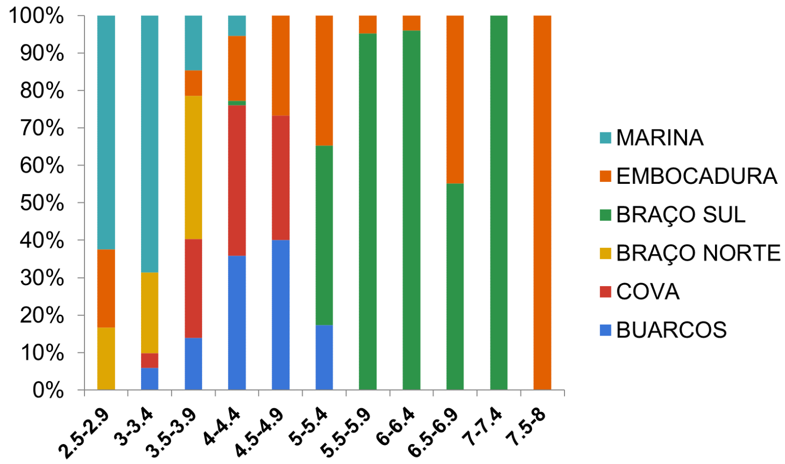

Figure 8 - Percentage of microplastics categorized by size (mm) extracted from mussels along the Mondego estuary and adjacent coastal areas.

Concentrations of plastics per gram were generally high in the north arm of the estuary, which includes Braço Norte (2.484) and Marina (3.091), as well as in Cova (2.423), one of the coastal sites (Table 3). Embocadoura was the sampling location with the least amount of microplastics per gram of mussel with a value of 0.955, the only with less than one microplastic per gram. Mussels from other locations included Buarcos, a coastal site, and Braço Sul, the south arm of the estuary, which had concentrations of 1.998 and 1.775 microplastics per gram of wet weight, respectively. Concerning the concentration of microplastics per individual, on another hand, Braço Sul stood out the most with a value of 6.167. All the other mussels from different stations had similar values between them. In coastal stations, mussels from Buarcos and Cova had 2.267 and 2.700 microplastics per individual, respectively, whereas, inside the estuary, Braço Norte had 2.033, Embocadura had 2.933, and Marina had 2.367 (Table 3).

3.3 Water samples

A total of 159 particles were identified from all water samples. Plastic particle sizes ranged from 0.060 mm to 9.649 mm, with an average of 1.804 ± 1.637 mm (Figure 11). The most common type of plastic found (92.45%) was fibers, followed by fragments (7.55%). Blue was the most prominent color (55.34%). Green (7.55%), gray (6.92%), black (18.87%), red (3.14%), translucent (5.66%), white (1.26%), brown (0.63%) and pink (0.63%) were among the other particle colors identified in the samples (Figure 10).
The mean number of fibers was particularly high in the northern arm of the estuary, which includes both Braço Norte and Marina. Fragments were not prevalent in any of the sampling locations. Nevertheless, they were also more common in Braço Norte and Marina (Figure 9).
Blue particles were higher in the north arm of the estuary, in accordance with the results obtained for shape. Yet, unlike what happened with mussels, the second most common color for microplastics in water was black (Figure 10).
Correlations between microplastic abundance in water and microplastic abundance in mussels per individual was negative and not very strong (-0.402). Values of MPs per gram of wet weight though had a stronger and positive correlation with contamination in surface waters (0.765).

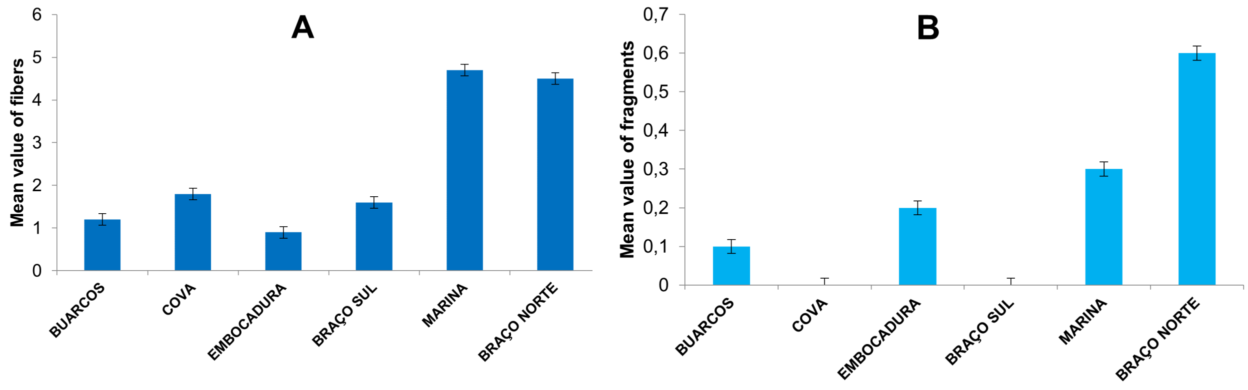

Figure 9 - Mean values of microplastics (± SE) categorized by shape extracted from mussels along the Mondego estuary's waters (A - fibers; B - fragments).

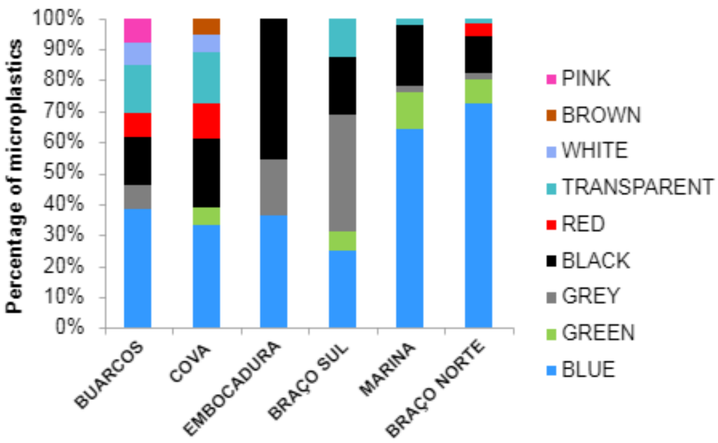

Figure 10 - Percentage of microplastics categorized by color extracted from mussels along the Mondego estuary's waters.

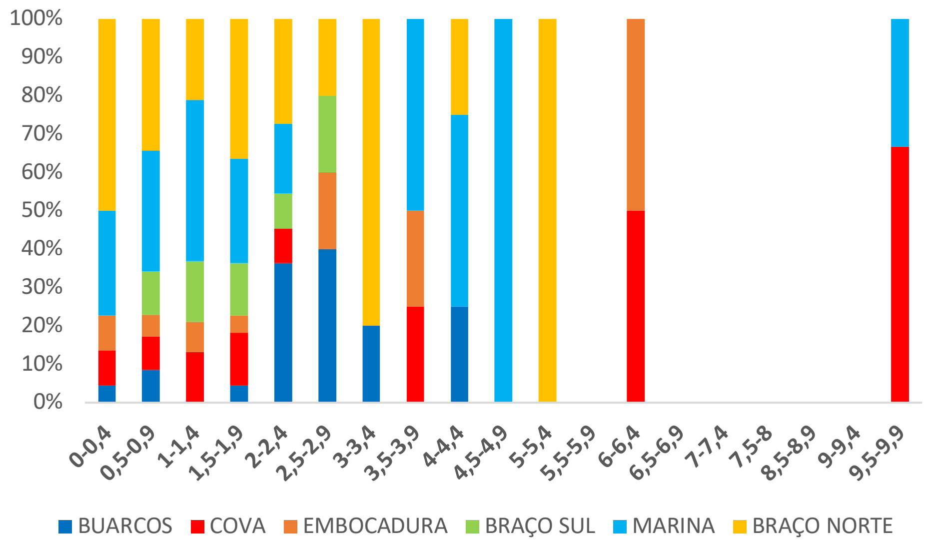

Figure 11 - Percentage of microplastics categorized by size (mm) extracted from waters along the Mondego estuary and adjacent coastal areas.

Discussion

4.1 Surface waters

Rivers, estuaries, and lakes are primarily known to be responsible for transporting plastic debris from land to sea (Meijer et al., 2021), therefore assessing the levels of microplastics in these systems is as relevant as studying microplastic abundance in the oceans. There's also a high correlation between microplastic abundance in mussels and human activity, and mussels from locations with heavy human activity tend to have much greater microplastic concentrations (Li et al., 2016). High abundance of fibers within the northern arm of the estuary, which include Marina and Braço Norte stations, can be a consequence of discharges done to the Mondego River, as freshwater and transitional systems are more susceptible to fiber contamination than the marine environment due to their proximity to point sources of fiber discharges (Jabeen et al., 2017). The influence of freshwater in these stations is concurrent with salinity values obtained when characterizing the area, which were low in these two stations. Regarding color, blue particles were higher in both Marina and Braço Norte. Xu et al. (2021) has recorded a higher abundance of transparent and blue microplastic particles in surface waters due to fishery activities and human domestic sewage. These values can also be associated with discharges occurring in the Mondego Estuary like the influence of one wastewater treatment plant (WWTP) situated in the north arm.

4.2 Mussels

Due to their dynamic conditions, estuaries hold a considerable interspecific variability in microplastic ingestion rates by their resident species. This variability is affected by various factors (e.g., tide, wind, residence time) comprising sample site location and anthropogenic disturbance. Despite the lack of evidence on the consequences and mechanistic processes involved, the number of MPs identified in estuarine species in all aquatic environments poses a serious threat to their health.

Despite the separation of sampling locations by which zones were affected by seawater or freshwater the most (Coast and Estuary), statistical analysis showed no relevant differences between them (P(PERM) > 0.05). The only significant difference in microplastic abundance in mussels was recorded between stations, in Braço Sul, where a significantly higher concentration of microplastics was recorded - mean value of 6.17 ± 5.87 particles per individual (P(PERM) > 0.05). Water retention time in the southern arm of the estuary is bigger (about 9 days) than the one in the north arm (about 2 days) and coastal areas, as mentioned before, meaning the pollutants may stay in this area for a longer time. There are also indicators that microplastics can accumulate through time. Karlsson et al. (2017) recorded significantly higher concentrations in mussels (37 items/kg dry weight) compared to surrounding sediment (48 items/kg dry weight) and seawater (27 items/L). Therefore, there's a higher probability for mussels to ingest these particles, as they become more easily accessible to be filtered by these animals. There is also a higher number of fragments in zones that receive water from the south arm (Embocadura and Braço Sul), with an average of 2.6 and 1.6 particles per mussel. As plastic debris persists in these areas, there's a higher chance for them to degrade into fragments that end up in the water column, turning easily accessible to get ingested by mussels.

Colorwise, mussels showed no pattern when it came to particles ingested. Overall, almost all colors expressed themselves in all sampling locations with no significant differences, showing very heterogeneous results. There was a higher percentage of green and red microplastics in Braço Sul (accounting for 70.89% and 57.50% of all the green and red particles found, respectively). Embocadura was the only sampling location with brown microplastics, and the two stations located in the north arm (Marina and Braço Norte) as well as Cova were the only stations exhibiting white microplastics.

Regarding biometry, size discrepancy between mussels from all stations might have affected the results obtained as there was no correlation between mussel size or weight and their microplastic abundance. Taking abundance in mussels as items/g.w and abundance in waters as items/L into account, previous research revealed a positive and quantitative association of microplastics in mussels and their surrounding waterways, with similar values of abundance between the two (Van Cauwenberghe et al., 2015; Karlsson et al., 2017; Li et al., 2018; Qu et al., 2018). This directly contradicts the results of this study. However, this is not an isolated occurrence as one prior study also did not show the quantitative correlation between microplastics in mussels and their surrounding waters (Li et al., 2018). They associated these results to the rapid translocation of smaller compared to larger plastic particles in mussels. The apparent ability of mussels to retain smaller sizes of microplastics was supported by its findings that mussels contained more (44%–83%) of the smaller sizes of microplastics (less than 250 µm) compared to waters with only 30%–40%. This hypothesis does not really match our results, as microplastics did not differ much in size between surface waters and mussels and were, on average, larger in mussel tissues (1.918 ± 1.229 mm). In this study, despite trying to collect individuals in the same size range, mussels differed greatly across sampling locations. Mussels collected from Braço Sul were significantly bigger with an average size of 6.22 cm whereas those collected in the north arm (Marina and Braço Norte) were the smallest at 3.31 and 3.54 cm, respectively. Habitat can be a cause of size variability between mussel communities (Atkinson et al., 2012). As shown in Bråte et al. (2018), bigger mussels are potentially able to filter more pollutants, including microplastics. This could be the main reason behind a spike in abundance in Braço Sul, where the mussel communities had higher sizes. We also cannot disregard human error in the identification of the microplastics as it is a process totally dependable on the observer and some microplastics can be hard to detect.

So far, there has only been one study conducted in the Mondego Estuary regarding microplastic contamination (Bessa et al., 2018). Regarding morphological characteristics, fibers were the most common type of microplastic swallowed by fish inside the estuary, accounting for 96% of the total incidence. Microfibers of synthetic origin, specifically, accounted for the vast majority (> 80%) of microplastics detected in the marine environment and consumed by biota. These results are compatible with those collected from mussels in this study, as the type of microplastic with the highest abundance in their tissues was also fibers (75.94 %). The color, length, and polymer type of the fibers recovered from fish in the Mondego estuary also differed. Blue, transparent, and black were the most common colors. In this study, blue was the most ingested color of microplastic (40.76%). On another hand, the second and third highest frequencies for color were red (15.24%) and green (15.05%). It was hypothesized that the color of these particles, as well as their resemblance to food, contributed to their ingestion by fish. Two possible explanations given for the high frequency of blue and transparent fibers found in the estuary were trawl nets or the breakdown of lost gears. Jabeen et al. (2017) proposed freshwater and transitional systems to be more susceptible to fiber contamination than the marine environment due to their proximity to point sources of fiber discharges. These results coincide with this theory. Although the principal sources and sinks of fibers in aquatic environments are not yet well established, wastewater treatment plant (WWTP) effluents are frequently pinpointed as a substantial point supply of fibers to the environment. WWTP effluents from home laundry emissions and fragmentation of fishing gear (e.g., ropes and nets) can be the root source of many fibers found in the Mondego estuary, as there are two different stations discharging to the river. Additional sources of fibers have been identified as lost and discarded fishing and leisure sailing gear. Welden and Cowie (2017) studied the breakdown of regularly used polymer ropes (polypropylene, polyethylene, and nylon) and estimated that the rate of microfiber release in benthic habitats, referring simply to gear losses (mass of microplastic created), is around 3968 tons per month. Furthermore, according to Dris et al. (2017), air fiber shedding is also a significant route for the propagation of microfiber pollution into the environment and should be considered when evaluating fiber contamination in aquatic ecosystems. Qu et al. (2018) also stated that while other types of microplastics (e.g., beads) are more readily swallowed by mussels over shorter periods of time, fibers in mussels are the product of long-term accumulation in the marine environment and other types could also be digested more quickly than fibers if consumed. Therefore, a higher abundance of fibers in stations where water time retention is shorter (Braço Sul) follows what was expected.

4.3 Mussels as bioindicators of microplastic contamination

Regarding microplastic abundance, stations located in the north arm of the estuary (Marina and Braço Norte) were the only ones significantly more microplastic contamination compared to other locations when it came to surface waters (P(PERM) > 0.05). In mussels, on another hand, the only location which had significant discrepancy with the rest was Braço Sul, on the south arm of the estuary (P(PERM) < 0.05). Therefore, it wasn't possible to correlate the abundance of microplastic contamination between mussels and surface waters.

Concerning morphological characteristics of the microplastics recovered, there was also no apparent direct correlation between surface waters and those inside mussels. In surface waters, there was a large percentage of blue microplastics found in the north arm of the estuary. The percentage of blue particles in these two stations only accounted for 78.4% of all the blue microplastic debris found. In other stations the color distribution was more heterogeneous and did not display clear differences. Coastal stations had slightly higher percentages of blue microplastics as well, but the stations influenced by water in the south arm showed higher values of black and gray microplastics. Specifically, 54.55% of the gray particles found in surface waters were collected in Braço Sul and Embocadura displayed an abundance of 45.45% of black microplastics.

There are a few reasons that could explain these results, which are not consistent with similar past studies where correlation between the two could be seen (Qu et al., 2018). Because the water is constantly moving and locations are influenced by tides as well as discharges made to the Mondego River, the water collected for analysis is but a momentary representation of the contamination the estuary is subjected to. Mussels, on another hand, are sessile filter-feeding animals, therefore the time of residence of pollutants is expected to be much bigger inside their tissues compared to surface waters. The total volume of water collected for samples was also a somewhat low and, consequently, might have not been the ideal amount, as it was only 10 L. Considering the samples were also collected during low tide and there is influence of freshwater from the river mainly in the north arm of the estuary (confirmed by similar salinity values in the two stations), the contaminants present are likely to include those from WWTP and other anthropogenic sources related to industrial activities, mercantile and fishing harbors, salt extraction, aquaculture farms, and agriculture areas as demonstrated in Bessa et al.,2018 (Gaspar et al., 2017). Wastewater treatment plants are responsible for the discharge of primary microplastics and, specifically, a high number of microplastic fibers from the breakthrough of synthetic fabrics to freshwater systems and transitional ecosystems (Browne, 2015; Napper and Thompson, 2016), which aligns with the results obtained in this study. The concentration of pollutants inside mussels, including microplastics, has also been shown to tend to stabilize after obtaining a balance between intake, assimilation in tissues and defecation or egestion (Li et al., 2019). The results obtained here also corroborated the information of Bråte et al. (2018) on mussel biometry and its correlation to abundance and size of microplastics ingested. Sampling locations in the north arm of the estuary had smaller mussels with an average size lower than 4 cm (3.31 and 3.54 cm, respectively), whereas the mussels from Braço Sul were measured 6.22 ± 0.57 cm average in size. Having this in consideration, it makes sense that most of the microplastics ingested by mussels from Braço Norte and Marina measured only between 2.5 and 3.5 cm whereas most of those in Braço Sul technically surpassed the microplastic size range of 5 cm, measuring between 5.5 and 7.5 cm. A weak negative correlation was seen between mussel size and microplastic contamination within surface waters whereas this value was stronger compared to ingestion of microplastic per gram of wet weight. Differences in correlation results between abundance in surface waters and indicators like MP/ind and MP/g w.w., and especially negative ones, might indicate microplastic ingestion isn't directly associated with mussel size or weight. Even so, mussel communities were diverse, and a standardized mussel size range wasn't possible to obtain. It's likely that this fact linked with filtering and digestion/egestion patterns in mussels as well as difference in residence time might have contributed to these results.

4.4 Final remarks and future steps

This study represented a first approach in testing the ability of using mussels as potential bioindicators of microplastic pollution in transitional ecosystems such as estuaries. With the results provided in this study, we can infer that indeed microplastic pollution is widespread in both estuarine and coastal areas and that mussels are ingesting those particles in all stations selected from the Mondego estuary (Portugal). It is however, not clear if they can act as indicators of microplastics pollution instead of sentinels of microplastic ingestion due to several reasons: This study represented a snapshot and a temporal scale would be important to test if these patterns are consistent through time; The sampling stations inside the estuary are relatively close to each other which could influence these results with no clear variation among the stations and samples; Since this species are intolerant to low salinities it was not possible to increase the number of stations upstream, but it was clear that there is an influence of the riverine input of microplastics in the estuary and that mussels can incorporate microplastics and potentially accumulate in their organs (differences in the color detected).

For future assessments and studies, it is, therefore, advised on the increase of spatial and temporal scales, the water sampling effort, and the chemical analyses of microplastics.

References

Ahmed, T., Shahid, M., Azeem, F., Rasul, I., Shah, A. A., Noman, M., ... & Muhammad, S. (2018). Biodegradation of plastics: current scenario and future prospects for environmental safety. Environmental science and pollution research, 25(8), 7287-7298. https://doi.org/10.1007/s11356-018-1234-9

Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. PRIMER-E Ltd., Plymouth, UK.

Antunes, J., Frias, J., & Sobral, P. (2018). Microplastics on the Portuguese coast. Marine Pollution Bulletin, 131, 294–302. https://doi.org/10.1016/j.marpolbul.2018.04.025.

Arthur, C., Baker, J. E., & Bamford, H. A. (2009). Proceedings of the International Research Workshop on the Occurrence, Effects, and Fate of Microplastic Marine Debris, September 9-11, 2008, University of Washington Tacoma, Tacoma, WA, USA. https://doi.org/10.1111/j.1399-3054.1984.tb04608.

Athey, S. N., Albotra, S. D., Gordon, C. A., Monteleone, B., Seaton, P., Andrady, A. L., ... & Brander, S. M. (2020). Trophic transfer of microplastics in an estuarine food chain and the effects of a sorbed legacy pollutant. Limnology and Oceanography Letters, 5(1), 154-162. https://doi.org/10.1002/lol2.10130.

Atkinson, C. L., Julian, J. P., & Vaughn, C. C. (2012). Scale-dependent longitudinal patterns in mussel communities. Freshwater Biology, 57(11), 2272-2284. https://doi.org/10.1111/fwb.12001

Banaee, M., Gholamhosseini, A., Sureda, A., Soltanian, S., Fereidouni, M. S., & Ibrahim, A. T. A. (2021). Effects of microplastic exposure on the blood biochemical parameters in the pond turtle (Emys orbicularis). Environmental Science and

Pollution Research, 28(8), 9221-9234. https://doi.org/10.1007/s11356-020-11419-2.

Barbier, E. B., Hacker, S. D., Kennedy, C., Koch, E. W., Stier, A. C., & Silliman, B. R. (2011). The value of estuarine and coastal ecosystem services. Ecological monographs, 81(2), 169-193. https://doi.org/10.1890/10-1510.1.

Berglund, E., Fogelberg, V., Nilsson, P. A., & Hollander, J. (2019). Microplastics in a freshwater mussel (Anodonta anatina) in Northern Europe. Science of the total environment, 697, 134192. https://doi.org/10.1016/j.scitotenv.2019.134192.

Bessa, F., Barría, P., Neto, J. M., Frias, J. P. G. L., Otero, V., Sobral, P., & Marques, J. C. (2018). Occurrence of microplastics in commercial fish from a natural estuarine environment. Marine Pollution Bulletin, 128, 575–584. https://doi.org/10.1016/j.marpolbul.2018.01.044.

Bessa, F., Ratcliffe, N., Otero, V., Sobral, P., Marques, J. C., Waluda, C. M., ... & Xavier, J. C. (2019). Microplastics in gentoo penguins from the Antarctic region. Scientific reports, 9(1), 1-7. https://doi.org/10.1038/s41598-019-50621-2.

Beyer, J., Green, N. W., Brooks, S., Allan, I. J., Ruus, A., Gomes, T., ... & Schøyen, M. (2017). Blue mussels (*Mytilus edulis* spp.) as sentinel organisms in coastal pollution monitoring: a review. Marine environmental research, 130, 338-365. https://doi.org/10.1016/j.marenvres.2017.07.024.

Braatz, S., Fortuna, S., Broadhead, J., & Leslie, R. (2007). Coastal protection in the aftermath of the Indian Ocean tsunami: What role for forests and trees?. RAP Publication (FAO).

Brandon, J., Goldstein, M., & Ohman, M. D. (2016). Long-term aging and degradation of microplastic particles: comparing in situ oceanic and experimental weathering patterns. Marine pollution bulletin, 110(1), 299-308. https://doi.org/10.1016/j.marpolbul.2016.06.048

Bråte, I. L. N., Hurley, R., Iversen, K., Beyer, J., Thomas, K. V., Steindal, C. C., ... & Lusher, A. (2018). *Mytilus* spp. as sentinels for monitoring microplastic pollution in Norwegian coastal waters: A qualitative and quantitative study. Environmental Pollution, 243, 383-393. https://doi.org/10.1016/j.envpol.2018.08.077

Brenko, M., & Calabrese, A. (1969). The combined effects of salinity and temperature on larvae of the mussel *Mytilus edulis*. Marine Biology, 4(3), 224-226. https://doi.org/10.1007/bf00393897.

Brown, C. J., Fulton, E. A., Hobday, A. J., Matear, R. J., Possingham, H. P., Bulman, C., ... & Richardson, A. J. (2010). Effects of climate-driven primary production change on marine food webs: implications for fisheries and conservation. Global Change Biology, 16(4), 1194-1212. https://doi.org/10.1111/j.1365-2486.2009.02046.

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M., & Thompson, R. C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). Environmental Science and Technology, 42(13), 5026–5031. https://doi.org/10.1021/es800249a.

Browne, M. A., Galloway, T. S., & Thompson, R. C. (2010). Spatial patterns of plastic debris along estuarine shorelines. Environmental science & technology, 44(9), 3404-3409. https://doi.org/10.1021/es903784e.

Catarino, A. I., Macchia, V., Sanderson, W. G., Thompson, R. C., & Henry, T. B. (2018). Low levels of microplastics (MP) in wild mussels indicate that MP ingestion by humans is minimal compared to exposure via household fibres fallout during a meal. Environmental Pollution, 237, 675–684. https://doi.org/10.1016/j.envpol.2018.02.069.

Chamas, A., Moon, H., Zheng, J., Qiu, Y., Tabassum, T., Jang, J. H., ... & Suh, S. (2020). Degradation rates of plastics in the environment. ACS Sustainable Chemistry & Engineering, 8(9), 3494-3511. https://doi.org/10.1021/acssuschemeng.9b06635

Cochard, R., Ranamukhaarachchi, S. L., Shivakoti, G. P., Shipin, O. V., Edwards, P. J., & Seeland, K. T. (2008). The 2004 tsunami in Aceh and Southern Thailand: a review on coastal ecosystems, wave hazards and vulnerability. Perspectives in Plant Ecology, Evolution and Systematics, 10(1), 3-40. https://doi.org/10.1016/j.ppees.2007.11.001.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., & Galloway, T. S. (2015). The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus. Environmental science & technology, 49(2), 1130-1137. https://doi.org/10.1021/es504525u.

Costanza, R., d'Arge, R., De Groot, R., Farber, S., Grasso, M., Hannon, B., ... & Van Den Belt, M. (1997). The value of the world's ecosystem services and natural capital. nature, 387(6630), 253-260. https://doi.org/10.1038/387253a0.

Dailianis, S. (2011). Environmental impact of anthropogenic activities: the use of mussels as a reliable tool for monitoring marine pollution. Mussels: Anatomy, Habitat and Environmental Impact; McGevin, LE, Ed, 1-30. https://doi.org/10.1016/j.marenvres.2020.104919.

Davidson, K., & Dudas, S. E. (2016). Microplastic ingestion by wild and cultured Manila clams (Venerupis philippinarum) from Baynes Sound, British Columbia. Archives of Environmental Contamination and Toxicology, 71(2), 147-156. https://doi.org/10.1007/s00244-016-0286-4.

De Bhowmick, G., Sarmah, A. K., & Dubey, B. (2021). Microplastics in the NZ environment: Current status and future directions. Case Studies in Chemical and Environmental Engineering, 3, 100076. https://doi.org/10.1016/j.cscee.2020.100076

De Witte, B., Devriese, L., Bekaert, K., Hoffman, S., Vandermeersch, G., Cooreman, K., & Robbens, J. (2014). Quality assessment of the blue mussel (*Mytilus edulis*):

Comparison between commercial and wild types. Marine pollution bulletin, 85(1), 146-155. https://doi.org/10.1016/j.marpolbul.2014.06.006.

Dekiff, J. H., Remy, D., Klasmeier, J., & Fries, E. (2014). Occurrence and spatial distribution of microplastics in sediments from Norderney. Environmental Pollution, 186, 248-256. https://doi.org/10.1016/j.envpol.2013.11.019.

Desforges, J. P. W., Galbraith, M., & Ross, P. S. (2015). Ingestion of microplastics by zooplankton in the Northeast Pacific Ocean. Archives of environmental contamination and toxicology, 69(3), 320-330. https://doi.org/10.1007/s00244-015-0172-5.

Dowarah, K., Patchaiyappan, A., Thirunavukkarasu, C., Jayakumar, S., & Devipriya, S. P. (2020). Quantification of microplastics using Nile Red in two bivalve species Perna viridis and Meretrix meretrix from three estuaries in Pondicherry, India and microplastic uptake by local communities through bivalve diet. Marine Pollution Bulletin, 153. https://doi.org/10.1016/j.marpolbul.2020.110982.

Dris, R., Gasperi, J., Mirande, C., Mandin, C., Guerrouache, M., Langlois, V., & Tassin, B. (2017). A first overview of textile fibers, including microplastics, in indoor and outdoor environments. Environmental pollution, 221, 453-458. https://doi.org/10.1016/j.envpol.2016.12.013.

Dris, R., Tramoy, R., Alligant, S., Gasperi, J., & Tassin, B. (2020). Plastic debris flowing from rivers to oceans: the role of the estuaries as a complex and poorly understood key interface. Handbook of Microplastics in the Environment, 1-28. https://doi.org/10.1007/978-3-030-10618-8_3-1

Eder, M. L., Oliva-Teles, L., Pinto, R., Carvalho, A. P., Almeida, C. M. R., Hornek-Gausterer, R., & Guimaraes, L. (2021). Microplastics as a vehicle of exposure to chemical contamination in freshwater systems: Current research status and way forward. Journal of Hazardous Materials, 417, 125980. https://doi.org/10.1016/j.jhazmat.2021.125980.

Egbeocha, C. O., Malek, S., Emenike, C. U., & Milow, P. (2018). Feasting on microplastics: ingestion by and effects on marine organisms. Aquatic Biology, 27, 93-106. https://doi.org/10.3354/ab00701.

Eriksen, M., Lebreton, L. C., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., ... & Reisser, J. (2014). Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. PloS one, 9(12), e111913. https://doi.org/10.1371/journal.pone.0111913.

Frias, J. P., & Nash, R. (2019). Microplastics: Finding a consensus on the definition. Marine pollution bulletin, 138, 145-147. https://doi.org/10.1016/j.marpolbul.2018.11.022

Gall, S. C., & Thompson, R. C. (2015). The impact of debris on marine life. Marine pollution bulletin, 92(1-2), 170-179. https://doi.org/10.1016/j.marpolbul.2014.12.041.

Gaspar, R., Marques, L., Pinto, L., Baeta, A., Pereira, L., Martins, I., Marques, J. C., & Neto, J. M. (2017). Origin here, impact there—The need of integrated management for river basins and coastal areas. Ecological Indicators, 72, 794–802. https://doi.org/10.1016/j.ecolind.2016.09.013.

Germanov, E. S., Marshall, A. D., Hendrawan, I. G., Admiraal, R., Rohner, C. A., Argeswara, J., ... & Loneragan, N. R. (2019). Microplastics on the menu: plastics pollute Indonesian manta ray and whale shark feeding grounds. Frontiers in Marine Science, 679. https://doi.org/10.3389/fmars.2019.00679.

Geyer, R., Jambeck, J. R., & Law, K. L. (2017). Production, use, and fate of all plastics ever made. Science advances, 3(7), e1700782. https://doi.org/10.1126/sciadv.1700782.

Ghayebzadeh, M., Aslani, H., Taghipour, H., & Mousavi, S. (2020). Contamination of the Caspian Sea Southern coast sediments with microplastics: A marine environmental problem. Marine Pollution Bulletin, 160. https://doi.org/10.1016/j.marpolbul.2020.111620.

Halpern, B. S., Walbridge, S., Selkoe, K. A., Kappel, C. V., Micheli, F., d'Agrosa, C., ... & Watson, R. (2008). A global map of human impact on marine ecosystems. science, 319(5865), 948-952. https://doi.org/10.1016/j.heliyon.2020.e04709

Hartline, N. L., Bruce, N. J., Karba, S. N., Ruff, E. O., Sonar, S. U., & Holden, P. A. (2016). Microfiber masses recovered from conventional machine washing of new or aged garments. Environmental science & technology, 50(21), 11532-11538. https://doi.org/10.1021/acs.est.6b03045

Hossain, M. S., Sobhan, F., Uddin, M. N., Sharifuzzaman, S. M., Chowdhury, S. R., Sarker, S., & Chowdhury, M. S. N. (2019). Microplastics in fishes from the Northern Bay of Bengal. Science of the Total Environment, 690, 821-830.

Issac, M.N., Kandasubramanian, B., 2021. Effect of microplastics in water and aquatic systems. Environmental Science and Pollution Research 28, 19544–19562.. https://doi.org/10.1007/s11356-021-13184-2

Iyare, P. U., Ouki, S. K., & Bond, T. (2020). Microplastics removal in wastewater treatment plants: a critical review. Environmental Science: Water Research & Technology, 6(10), 2664-2675. https://doi.org/10.1039/d0ew00397b.

Jabeen, K., Su, L., Li, J., Yang, D., Tong, C., Mu, J., & Shi, H. (2017). Microplastics and mesoplastics in fish from coastal and fresh waters of China. Environmental Pollution, 221, 141-149. https://doi.org/10.1016/j.envpol.2016.11.055.

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., ... & Law, K. L. (2015). Plastic waste inputs from land into the ocean. Science, 347(6223), 768-771. https://doi.org/10.1126/science.1260352.

JAMP [Joint Assessment and Monitoring Programme] Guidelines for Monitoring Contaminants in Biota OSPAR Commission (2012), p. 122 ref. no. 99–02e

Kahlert, S., & Bening, C. R. (2020). Plastics recycling after the global pandemic: resurgence or regression?. Resources, conservation, and recycling, 160, 104948. https://doi.org/10.1016/j.resconrec.2020.104948.

Kalčíková, G., Alič, B., Skalar, T., Bundschuh, M., & Gotvajn, A. Ž. (2017). Wastewater treatment plant effluents as source of cosmetic polyethylene microbeads to freshwater. Chemosphere, 188, 25-31. https://doi.org/10.1016/j.chemosphere.2017.08.131.

Karlsson, T. M., Vethaak, A. D., Almroth, B. C., Ariese, F., van Velzen, M., Hassellöv, M., & Leslie, H. A. (2017). Screening for microplastics in sediment, water, marine invertebrates and fish: method development and microplastic accumulation. Marine pollution bulletin, 122(1-2), 403-408. https://doi.org/10.1016/j.marpolbul.2017.06.081.

Katsumi, N., Kusube, T., Nagao, S., & Okochi, H. (2020). The role of coated fertilizer used in paddy fields as a source of microplastics in the marine environment. Marine Pollution Bulletin, 161, 111727. https://doi.org/10.1016/j.marpolbul.2020.111727.

Kenov, I. A., Garcia, A. C., & Neves, R. (2012). Residence time of water in the Mondego estuary (Portugal). Estuarine, Coastal and Shelf Science, 106, 13-22. https://doi.org/10.1016/j.ecss.2012.04.008.

Kibria, G. (2017). Plastic waste, plastic pollution–a threat to all nations. Project Report. https://doi.org/10.1036/1097-8542.br0802171.

Koch, H. M., & Calafat, A. M. (2009). Human body burdens of chemicals used in plastic manufacture. Philosophical Transactions of the Royal Society B: Biological Sciences, 364(1526), 2063-2078. https://doi.org/10.1098/rstb.2008.0208.

Kole, P. J., Löhr, A. J., Van Belleghem, F. G., & Ragas, A. M. (2017). Wear and tear of tyres: a stealthy source of microplastics in the environment. International journal of environmental research and public health, 14(10), 1265. https://doi.org/10.3390/ijerph14101265.

Lebreton, L., Slat, B., Ferrari, F., Sainte-Rose, B., Aitken, J., Marthouse, R., ... & Reisser, J. (2018). Evidence that the Great Pacific Garbage Patch is rapidly accumulating plastic. Scientific reports, 8(1), 1-15. https://doi.org/10.1038/s41598-018-22939-w.

Lehel, J., & Murphy, S. (2021). Microplastics in the food chain: food safety and environmental aspects. Reviews of Environmental Contamination and Toxicology Volume 259, 1-49. https://doi.org/10.1007/398_2021_77.

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., ... & He, D. (2018). Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode Caenorhabditis elegans. Science of the total environment, 619, 1-8. https://doi.org/10.1016/j.scitotenv.2017.11.103.

Leng, Z., Padhan, R. K., & Sreeram, A. (2018). Production of a sustainable paving material through chemical recycling of waste PET into crumb rubber modified asphalt. Journal of cleaner production, 180, 682-688. https://doi.org/10.1016/j.jclepro.2018.01.171

Li, J., Green, C., Reynolds, A., Shi, H., & Rotchell, J. M. (2018). Microplastics in mussels sampled from coastal waters and supermarkets in the United Kingdom. Environmental pollution, 241, 35-44. https://doi.org/10.1016/j.envpol.2018.05.038.

Li, J., Lusher, A. L., Rotchell, J. M., Deudero, S., Turra, A., Bråte, I. L. N., ... & Shi, H. (2019). Using mussel as a global bioindicator of coastal microplastic pollution.

Environmental pollution, 244, 522-533. https://doi.org/10.1016/j.envpol.2018.10.032.

Li, J., Qu, X., Su, L., Zhang, W., Yang, D., Kolandhasamy, P., ... & Shi, H. (2016). Microplastics in mussels along the coastal waters of China. Environmental pollution, 214, 177-184. https://doi.org/10.1016/j.envpol.2016.04.012.

Lillebø, A. I., Pita, C., Rodrigues, J. G., Ramos, S., & Villasante, S. (2017). How can marine ecosystem services support the Blue Growth agenda?. Marine Policy, 81, 132-142. https://doi.org/10.1016/j.marpol.2017.03.008.

Liu, M., Lu, S., Song, Y., Lei, L., Hu, J., Lv, W., ... & He, D. (2018). Microplastic and mesoplastic pollution in farmland soils in suburbs of Shanghai, China. Environmental Pollution, 242, 855-862. https://doi.org/10.1016/j.envpol.2018.07.051.

Lo, H. K. A., & Chan, K. Y. K. (2018). Negative effects of microplastic exposure on growth and development of Crepidula onyx. Environmental Pollution, 233, 588-595. https://doi.org/10.1016/j.envpol.2017.10.095

Lotze, H. K., Lenihan, H. S., Bourque, B. J., Bradbury, R. H., Cooke, R. G., Kay, M. C., ... & Jackson, J. B. (2006). Depletion, degradation, and recovery potential of estuaries and coastal seas. Science, 312(5781), 1806-1809. https://doi.org/10.1126/science.1128035.

Lusher, A., Hollman, P., & Mendoza-Hill, J. (2017). Microplastics in fisheries and aquaculture: status of knowledge on their occurrence and implications for aquatic organisms and food safety. FAO.

Macfadyen, G., Huntington, T., & Cappell, R. (2009). Abandoned, lost or otherwise discarded fishing gear. https://doi.org/10.4060/cb8071en.

Mantas, V. M., Pereira, A. J. S. C., Neto, J., Patrício, J., & Marques, J. C. (2013). Monitoring estuarine water quality using satellite imagery. The Mondego river estuary (Portugal) as a case study. Ocean & Coastal Management, 72, 13-21. https://doi.org/10.1016/j.ocecoaman.2011.06.013.

Marques, J. C., Pardal, M. A., Nielsen, S. N., & Jørgensen, S. E. (1997). Analysis of the properties of exergy and biodiversity along an estuarine gradient of eutrophication. Ecological Modelling, 102(1), 155-167. https://doi.org/10.1016/s0304-3800(97)00099-9.

Meijer, L. J., van Emmerik, T., van der Ent, R., Schmidt, C., & Lebreton, L. (2021). More than 1000 rivers account for 80% of global riverine plastic emissions into the ocean. Science Advances, 7(18), eaaz5803. https://doi.org/10.1126/sciadv.aaz5803.

Messinetti, S., Mercurio, S., Scarì, G., Pennati, A., & Pennati, R. (2019). Ingested microscopic plastics translocate from the gut cavity of juveniles of the ascidian Ciona intestinalis. The European zoological journal, 86(1), 189-195. https://doi.org/10.1080/24750263.2019.1616837.

Napper, I. E., & Thompson, R. C. (2016). Release of synthetic microplastic plastic fibres from domestic washing machines: Effects of fabric type and washing conditions. Marine pollution bulletin, 112(1-2), 39-45. https://doi.org/10.1016/j.marpolbul.2016.09.025.

Ncube, L. K., Ude, A. U., Ogunmuyiwa, E. N., Zulkifli, R., & Beas, I. N. (2021). An overview of plastic waste generation and management in food packaging industries. Recycling, 6(1), 12. https://doi.org/10.3390/recycling6010012

Neto, J. M., Teixeira, H., Patrício, J., Baeta, A., Veríssimo, H., Pinto, R., & Marques, J. C. (2010). The response of estuarine macrobenthic communities to natural-and human-induced changes: dynamics and ecological quality. Estuaries and Coasts, 33(6), 1327-1339. https://doi.org/10.1007/s12237-010-9326-x.

Parton, K. J., Godley, B. J., Santillo, D., Tausif, M., Omeyer, L., & Galloway, T. S. (2020). Investigating the presence of microplastics in demersal sharks of the North-East Atlantic. Scientific reports, 10(1), 1-11. https://doi.org/10.1038/s41598-020-68680-1.

Pazos, R. S., Spaccesi, F., & Gómez, N. (2020). First record of microplastics in the mussel Limnoperna fortunei. Regional Studies in Marine Science, 38. https://doi.org/10.1016/j.rsma.2020.101360.

Piccardo, M., Provenza, F., Grazioli, E., Cavallo, A., Terlizzi, A., & Renzi, M. (2020). PET microplastics toxicity on marine key species is influenced by pH, particle size and food variations. Science of The Total Environment, 715, 136947. https://doi.org/10.1016/j.scitotenv.2020.136947

PlasticsEurope, E. P. R. O. (2020). Plastics—the facts 2020. An analysis of European plastics production, demand and waste data, 15-17.

Possatto, F. E., Barletta, M., Costa, M. F., do Sul, J. A. I., & Dantas, D. V. (2011). Plastic debris ingestion by marine catfish: an unexpected fisheries impact. Marine pollution bulletin, 62(5), 1098-1102. https://doi.org/10.1016/j.marpolbul.2011.01.036.

Qu, X., Su, L., Li, H., Liang, M., & Shi, H. (2018). Assessing the relationship between the abundance and properties of microplastics in water and in mussels. Science of the total environment, 621, 679-686. https://doi.org/10.1016/j.scitotenv.2017.11.284.

Rezania, S., Park, J., Din, M. F. M., Taib, S. M., Talaiekhozani, A., Yadav, K. K., & Kamyab, H. (2018). Microplastics pollution in different aquatic environments and biota: A review of recent studies. Marine pollution bulletin, 133, 191-208. https://doi.org/10.1016/j.marpolbul.2018.05.022.

Ritchie, H., & Roser, M. (2018). Plastic pollution. Our World in Data.

Rodrigues, S. M., Almeida, C. M. R., Silva, D., Cunha, J., Antunes, C., Freitas, V., & Ramos, S. (2019). Microplastic contamination in an urban estuary: Abundance and distribution of microplastics and fish larvae in the Douro estuary. Science of the Total Environment, 659, 1071–1081. https://doi.org/10.1016/j.scitotenv.2018.12.273.

SAPEA. (2019). Science Advice for Policy by European Academies. A Scientific Perspective on Microplastics in Nature and Society.

Schuyler, Q., Hardesty, B. D., Wilcox, C., & Townsend, K. (2014). Global analysis of anthropogenic debris ingestion by sea turtles. Conservation biology, 28(1), 129-139. https://doi.org/10.1111/cobi.12126.

Setälä, O., Norkko, J., & Lehtiniemi, M. (2016). Feeding type affects microplastic ingestion in a coastal invertebrate community. Marine pollution bulletin, 102(1), 95-101. https://doi.org/10.1016/j.marpolbul.2015.11.053.

Shahnawaz, M., Sangale, M. K., & Ade, A. B. (2019). Plastic waste disposal and reuse of plastic waste. In Bioremediation technology for plastic waste (pp. 21-30). Springer, Singapore. https://doi.org/10.1007/978-981-13-7492-0_3

Stock, V., Laurisch, C., Franke, J., Dönmez, M. H., Voss, L., Böhmert, L., ... & Sieg, H. (2021). Uptake and cellular effects of PE, PP, PET and PVC microplastic particles. Toxicology in Vitro, 70, 105021. https://doi.org/10.1016/j.tiv.2020.105021

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M. E. J., ... & Huvet, A. (2016). Oyster reproduction is affected by exposure to polystyrene microplastics. Proceedings of the national academy of sciences, 113(9), 2430-2435. https://doi.org/10.1073/pnas.1519019113.

Thushari, G. G. N., & Senevirathna, J. D. M. (2020). Plastic pollution in the marine environment. Heliyon, 6(8), e04709. https://doi.org/10.1016/j.heliyon.2020.e04709.

Training Workshop on Distribution, Source, Fate and Impacts of Marine Microplastics in Asia and the Pacific, Phuket, Thailand, 20-22 September (2017)

van Cauwenberghe, L., Claessens, M., Vandegehuchte, M. B., & Janssen, C. R. (2015). Microplastics are taken up by mussels (*Mytilus edulis*) and lugworms (Arenicola marina) living in natural habitats. Environmental Pollution, 199, 10–17. https://doi.org/10.1016/j.envpol.2015.01.008.

Vandermeersch, G., Lourenço, H. M., Alvarez-Muñoz, D., Cunha, S., Diogène, J., Cano-Sancho, G., ... & Robbens, J. (2015). Environmental contaminants of emerging concern in seafood–European database on contaminant levels. Environmental Research, 143, 29-45. https://doi.org/10.1016/j.envres.2015.06.011.

Vendel, A. L., Bessa, F., Alves, V. E. N., Amorim, A. L. A., Patrício, J., & Palma, A. R. T. (2017). Widespread microplastic ingestion by fish assemblages in tropical estuaries subjected to anthropogenic pressures. Marine Pollution Bulletin, 117(1-2), 448-455. https://doi.org/10.1016/j.marpolbul.2017.01.081.

Vianello, A., Boldrin, A., Guerriero, P., Moschino, V., Rella, R., Sturaro, A., & Da Ros, L. (2013). Microplastic particles in sediments of Lagoon of Venice, Italy: First observations on occurrence, spatial patterns and identification. Estuarine, Coastal and Shelf Science, 130, 54-61. https://doi.org/10.1016/j.ecss.2013.03.022.

Walkinshaw, C., Lindeque, P. K., Thompson, R., Tolhurst, T., & Cole, M. (2020). Microplastics and seafood: lower trophic organisms at highest risk of contamination. Ecotoxicology and Environmental Safety, 190, 110066. https://doi.org/10.1016/j.ecoenv.2019.110066.

Weber, A., Jeckel, N., Weil, C., Umbach, S., Brennholt, N., Reifferscheid, G., & Wagner, M. (2021). Ingestion and toxicity of polystyrene microplastics in freshwater bivalves. Environmental Toxicology and Chemistry, 40(8), 2247-2260. https://doi.org/10.1002/etc.5076.

Welden, N. A., & Cowie, P. R. (2017). Degradation of common polymer ropes in a sublittoral marine environment. Marine pollution bulletin, 118(1-2), 248-253. https://doi.org/10.1016/j.marpolbul.2017.02.072.

Whitfield, A. K. (2017). The role of seagrass meadows, mangrove forests, salt marshes and reed beds as nursery areas and food sources for fishes in estuaries. Reviews in Fish Biology and Fisheries, 27(1), 75-110. Desforges, J. P. W., Galbraith, M., & Ross, P. S. (2015). Ingestion of microplastics by zooplankton in the Northeast Pacific Ocean. Archives of environmental contamination and toxicology, 69(3), 320-330. https://doi.org/10.1007/s11160-016-9454-x.

Woodall, L. C., Sanchez-Vidal, A., Canals, M., Paterson, G. L., Coppock, R., Sleight, V., ... & Thompson, R. C. (2014). The deep sea is a major sink for microplastic debris. Royal Society open science, 1(4), 140317. https://doi.org/10.1098/rsos.140317.

Worm, B., Barbier, E. B., Beaumont, N., Duffy, J. E., Folke, C., Halpern, B. S., ... & Watson, R. (2006). Impacts of biodiversity loss on ocean ecosystem services. science, 314(5800), 787-790. https://doi.org/10.1126/science.1132294.

Wright, S. L., Thompson, R. C., & Galloway, T. S. (2013). The physical impacts of microplastics on marine organisms: a review. Environmental pollution, 178, 483-492. https://doi.org/10.1016/j.envpol.2013.02.031.

Yu, Y., Chen, H., Hua, X., Dang, Y., Han, Y., Yu, Z., ... & Li, H. (2020). Polystyrene microplastics (PS-MPs) toxicity induced oxidative stress and intestinal injury in nematode Caenorhabditis elegans. Science of the Total Environment, 726, 138679. https://doi.org/10.1016/j.scitotenv.2020.138679.

Zhang, H. (2017). Transport of microplastics in coastal seas. Estuarine, Coastal and Shelf Science, 199, 74-86. https://doi.org/10.1016/j.ecss.2017.09.032.

Zocchi, M., & Sommaruga, R. (2019). Microplastics modify the toxicity of glyphosate on Daphnia magna. Science of the Total Environment, 697, 134194. https://doi.org/10.1016/j.scitotenv.2019.134194.