UNIVERSIDADE D
COIMBRA

José Luís Clemente Marques

MICROPLASTICS IN THE MONDEGO ESTUARY AND ADJACENT COASTLINE: OCCURRENCE, CHARACTERISATION AND ASSOCIATED BACTERIAL COMMUNITIES

Dissertação no âmbito do Mestrado em Bioquímica,
orientada pela Doutora Filipa Bessa e pela Doutora Joana Costa,
apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências
e Tecnologia da Universidade de Coimbra.

Julho de 2021

José Luís Clemente Marques

MICROPLASTICS IN THE MONDEGO ESTUARY AND ADJACENT COASTLINE: OCCURRENCE, CHARACTERISATION AND ASSOCIATED BACTERIAL COMMUNITIES

Dissertação no âmbito do Mestrado em Bioquímica, sob orientação científica da Doutora Ana Filipa Silva Bessa (MARE) e co-orientação da Doutora Joana Cardoso Costa (CEF), apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Julho de 2021

Agradecimentos

Não poderia de deixar de começar pelo meu Pai. A ti, obrigado por teres confiado em mim ao longo de todo este percurso de seis anos e me teres proporcionado esta oportunidade. Ao longo deste periodo, pelo meio houve uma mudança de curso e de cidade, houve incerteza e houve muitas dúvidas e rumos incertos em relação ao meu futuro, mas a tua confiança em mim permaneceu sempre. És a minha referência. Obrigado Pai!

Agradecer à Dra. Filipa Bessa e à Dra. Joana Costa por toda a sua orientação, ensinamentos e ajuda prestada ao longo deste último ano e por terem partilhado comigo esta aventura repleta de desafios.

À Dra. Aitana Ares pelo processamento e análise dos dados da sequenciação e por todos os ensinamentos e ajuda prestada.

À equipa do MAREFOZ, em especial à Cátia Marques e à Irene Gutierrez pela ajuda na recolha das minhas amostras em trabalho de campo.

À Prof. Dra. Paula Marques e ao Prof. Dr. Luís Carvalho pela formação, ajuda e ensinamentos prestados no laboratório de espectroscopia vibracional do QFM-UC.

À malta do Ramo de baixo, Camponês, Vítor, Edu, Márcio e Catarina, obrigado por toda a vossa amizade e por me ajudarem a manter a manter a sanidade mental ao longo deste ano tão dificil marcado pela pandemia.

À malta da melhor residência de Coimbra, Santos Rocha, em especial ao melhor amigo que Coimbra me deu, Eduardo, obrigado por toda a amizade, música e “má vida” e por me ajudares a manter a sanidade mental ao longo deste último ano tão complicado.

Abstract

Microplastic (<5 mm) contamination is considered nowadays ubiquitous in the environment and even in an extremely ambitious future scenario (with no further emissions) will continue to increase. Therefore, it is important to evaluate the potential ecological risks and consequences that might outcome not only for today's environmental microplastic contamination levels but also for possible future levels. One of the current concerns is related to the possibility of microplastics harbour different bacterial communities than those found in their environment and the potential for these particles to act as vectors of transmission and spread of key bacterial groups, such as pathogens. Yet, a relatively low number of studies have investigated the nature of microplastics associated bacterial communities, the so-called "Plastisphere", in transitional coastal ecosystems, such as sandy beaches or estuarine areas. Taken that into account, two sampling campaign events were conducted in two transitional ecosystems in November-December 2020. Samples of water samples on the Mondego estuary and sand samples on its adjacent coastline were (Figueira da Foz, Portugal) collected for microplastic analysis. After manual sorting of particles under sterile conditions, DNA extraction and 16S rRNA amplicon high throughput sequencing was used to profile the bacterial communities on the surface of microplastics and from those found on the samples of water and sediments from the transitional ecosystems. All particles were characterised according to type, colour and size, and the chemical nature of the particles was inspected by μ-FTIR spectroscopy after DNA extraction.

A total of 89 particles were isolated and the majority of microplastics extracted from the estuarine environment were fibres (87%) and were identified as Polyacrylonitrile (PAN) and polypropylene (PP), while on the beach environment the majority of microplastics were fragments and foams (85%) and were identified as Polyethylene (PE) and Polystyrene (PS) microplastics. Although no significant differences were detected between the bacterial communities from distinct samples and between the α-diversity indexes from microplastics and their surrounding environments, data and community structure analyses showed the occurrence and abundance of typical marine-associated bacterial genera on estuarine microplastics that were scarce or absent from estuarine waters. Overall, the bacterial communities in the estuarine microplastics were more related to the beach samples than with the estuarine waters. These observations suggest the existence of a substantial contribution of a sea-river trajectory to the overall estuarine microplastic contamination. Furthermore, it was also observed the occurrence of unique and higher abundance of key bacterial groups on microplastics, such as pathogens (e.g., *Pseudoalteromonas*, *Flavobacterium*, *Lactococcus*, *Staphylococcus*, *Acinetobacter*, *Mycobacterium*, or *Shewanella*). The presence of these members might suggest a wastewater treatment plant (WWTP) or sewage origin but further research is required to assess this possibility. These results highlight the concern for these particles to act as vectors of transmission

and spread in transitional ecosystems. This study also highlights the importance of the study of microplastic-associated bacterial communities to the comprehension of microplastic environmental contamination. Although this study provides new insights into this recent scientific topic, further research will be required to increase our understanding of these topics.

Keywords: Microplastics, Plastisphere, bacterial communities, transitional coastal ecosystems, Mondego estuary, sandy beaches.

Resumo

A contaminação por microplásticos (<5 mm) é considerada hoje em dia como ubíqua no ambiente e mesmo num cenário futuro extremamente ambicioso (sem mais emissões) irá continuar a aumentar. Assim, é importante avaliar os potenciais riscos e consequências ecológicas que possam advir não só dos níveis atuais de contaminação ambiental por microplásticos, como também em possíveis cenários futuros. Uma das preocupações atuais está relacionada com a possibilidade dos microplásticos abrigarem comunidades bacterianas diferentes das encontradas nos seus ambientes e o potencial para estas partículas atuarem como vetores de transmissão e propagação de grupos bacterianos chave, como agentes patogénicos. No entanto, apenas um número relativamente baixo de estudos investigou a natureza das comunidades bacterianas associadas aos microplásticos, a chamada "Plastisfera", em ecossistemas de transição costeiros, como praias arenosas ou áreas estuarinas. Tendo isto em conta, foram realizadas duas campanhas de amostragem em dois ecossistemas de transição de Novembro a Dezembro de 2020 onde foram recolhidas amostras de água do estuário do Mondego e de areias da linha costeira adjacente (Figueira da Foz, Portugal) para a análise de microplásticos. Após a extração manual das partículas em condições estéreis, a extração de DNA e a sequenciação do gene *16SrRNA* por *amplicon high throughput* foi utilizado para traçar o perfil das comunidades bacterianas da superfície dos microplásticos e das encontradas nas amostras de água e sedimentos dos ecossistemas de transição. Todas as partículas foram caracterizadas de acordo com o tipo, cor e tamanho e a natureza química das partículas foi inspecionada por espectroscopia μ-FTIR após a extração do DNA.

Foram isoladas um total de 89 partículas, a maioria dos microplásticos extraídos do ambiente estuarino foram fibras (87%) e foram identificadas como fibras de Poliacrilonitrilo (PAN) e de Polipropileno (PP), enquanto no ambiente de praia a maioria dos microplásticos foram classificados como fragmentos e espumas (85%) e identificados como microplásticos de Polietileno (PE) e de Poliestireno (PS). Apesar de não terem sido detetadas diferenças significativas entre as comunidades bacterianas e os índices de diversidade α dos microplásticos e dos seus ambientes circundantes, a análise dos dados e da estrutura das comunidades mostraram a ocorrência e abundância de géneros bacterianos tipicamente marinhos nos microplásticos estuarinos que eram escassos ou ausentes das águas estuarinas. Foi ainda identificada uma maior proximidade entre as comunidades bacterianas dos microplásticos estuarinos e as amostras das praias do que com as águas estuarinas. Estas observações sugerem a existência de uma contribuição substancial da trajetória mar-rio para a contaminação global por microplásticos nos estuários. Para além disso, foi também observada a ocorrência e uma maior abundância de grupos bacterianos únicos em microplásticos, tais como agentes patogénicos (p.e. *Pseudoalteromonas*, *Flavobacterium*, *Lactococcus*, *Staphylococcus*, *Acinetobacter*,

Mycobacterium, e *Shewanella*). A presença destes membros pode sugerir uma origem de estações de tratamento de águas residuais ou de esgotos, mas serão necessários mais estudos para avaliar esta possibilidade. Estes resultados sublinham a possibilidade de estas partículas poderem atuar como vetores de transmissão e disseminação em ecossistemas de transição. Este estudo revela também a importância do estudo das comunidades bacterianas associadas a microplásticos na compreensão da contaminação ambiental por microplásticos. Apesar de proporcionar novas perspetivas serão necessários mais estudos para aumentar a compreensão sobre este tópico científico recente.

Palavras-chave: Microplásticos, Plastisfera, comunidades bacterianas, ecossistemas costeiros de transição, Estuário do Mondego, Praias arenosas.

Table of Contents

1 Introduction

1 Introduction

1.1. An overview on plastics

1.1.1. The (a)rise

Plastics have been around for more than a century, providing numerous solutions for humans. The term "plastic" is derived from the Greek words "*plastikos*" and "*plastos*", meaning "fit for moulding" and "moulded", respectively, with both terms referring to the material's malleability or plasticity during its manufacture (Millet et al., 2019). Although it is largely known that plastics are a modern invention, 'natural polymers', such as amber, tortoiseshell, horn, baleen and ivory, are present in nature (Amaral-Zettler et al., 2020; Millet et al., 2019). The discovery of the first semisynthetic plastic materials, such as cellulose nitrate, started in the 1850s and arise from the necessity of replacing the use of those limited natural materials (Amaral-Zettler et al., 2020).

In 1907, Leo Baekeland (who coined the term plastic later on), discovered Bakelite, the first synthetic plastic material (Baekeland, 1909). In 1912, polyvinyl chloride (PVC) and polyvinyl acetate (PVA) were discovered by Fritz Klatte (Millet et al., 2019). The following year, Jacques E. Brandenbergen, invented Cellophane, a clear, flexible and waterproof packaging material (Millet et al., 2019). Later, in 1927, Waldo Semon found a way to plasticise PVC, converting it into a flexible material that could be used for flooring, pipes, electrical insulations and roofing membranes (Millet et al., 2019). In the 1930s, polyamide polymer (known commercially as Nylon™) was introduced (Millet et al., 2019). This was the first synthetic fibre, which becomes immensely popular at the time, especially in stockings, and of great utility during World War II, as well as polymethyl methacrylate (Plexiglas™), that replaced glass in aircraft windows (Amaral-Zettler et al., 2020). World War II meant a boost for the production and further development of plastics (Millet et al., 2019). A wide variety of pioneering polymers, which are still used today, were invented during the wartime period, such as polyethylene (PE), polystyrene (PS), polyester, polyethylene terephthalate (PET), silicones and many more (Millet et al., 2019).

After World War II, many of these polymers found their way to the general public in the form of low-cost, disposable, single-use items, inspiring the term "throwaway living" (coined by *Life Magazine* in 1955) that remains part of the public mindset today (Amaral-Zettler et al., 2020). The 1950s saw the growth of plastics for domestic use (Millet et al., 2019). In the same period, plastics also became a major force in the clothing industry, with the incorporation of Polyester, Nylon™ and Lycra™ fabrics that were easy to wash, needed no ironing and often were cheaper than their natural alternatives (Millet et al., 2019). Synthetic polymers even have a life beyond Earth since a plastic (polyamide) flag was planted on the moon surface in 1969 (Amaral-Zettler et

al., 2020). By the 1970s, plastics had become the most widely used materials in the world, with notable exceptions being the materials used extensively in the construction sector, such as steel and cement (Amaral-Zettler et al., 2020; Geyer et al., 2017). Polymer materials have played key roles in economic expansion, innovation and the production of low-priced goods in the emerging world market, particularly in the 1990s, and continues to be a growing industry (Amaral-Zettler et al., 2020). A world without plastics, or synthetic organic polymers, seems unimaginable today and in the future of our societies.

1.1.2. Production

Global production of plastic resins and fibres increased whoppingly from 2 Mt (million tonnes) in 1950 to around 438 Mt in 2018 (Geyer et al., 2017; PlasticsEurope, 2020; Textile Exchange, 2020). To put in context, 438 Mt represents around 56 Kg of plastic materials produced in a single year for each of the approximately 7,8 billion humans. The total amount of virgin plastics manufactured from 1950 through 2015 was estimated at 8300 Mt (Geyer et al., 2017).

The vast majority of monomers used to make plastics, such as ethylene and propylene, are derived from fossil hydrocarbons (Geyer et al., 2017). In fact, the production of plastics accounts for 4-6% of global oil consumption (PlasticsEurope, 2017). Research and innovation are ongoing to diversify the raw material base to produce plastics, derived from renewable resources, the so-called bio-based plastics (Millet et al., 2019). One of the solutions includes the use of similar polymers to those produced from crude oil, but with monomers being produced from biomass (Millet et al., 2019). For example, sugar cane can serve for the production of ethylene and consequently, polyethylene (Millet et al., 2019). Other solution includes new polymers derived from new monomers (Millet et al., 2019). For example, starch can be used to produce polylactic acid (PLA) (Millet et al., 2019). However, in 2018, the global production capacity of bio-based plastics was only 2.11 Mt, a negligible value in the 368 Mt of plastic resins produced in the same period (European Bioplastics, 2019). Noteworthy, that bio-based plastics do not mean the same as 'bioplastics'. According to European Bioplastics, plastic material is defined as bioplastic if it is either bio-based, biodegradable, or features both properties (European Bioplastics, 2019). Plastic biodegradation is the microbial conversion of all its organic constituents into carbon dioxide, new microbial biomass and mineral salts under oxic conditions, plus methane under anoxic conditions (SAPEA, 2020).

Different types of plastics can be grouped into two main polymer families, thermoplastics and thermosets (Millet et al., 2019). Thermoplastics are a family of plastics that can be melted when heated and hardened when cooled, in a reversible process (Millet et al., 2019). This means that it can be reheated, reshaped and hardened repeatedly, making them mechanically recyclable (Millet et al., 2019). Thermoplastics represent almost 80% of the plastic demand (Millet et al.,

2019). This category includes all types of polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), expanded polystyrene (EPS) and polyethylene terephthalate (PET) (Millet et al., 2019). On the other hand, thermosets are a family of plastics that undergo a chemical change when heated, creating a three-dimensional network (Millet et al., 2019). After being heated and formed these plastics cannot be re-melted and reformed (Millet et al., 2019). This category includes epoxy resins and polyurethanes (PURs) (Millet et al., 2019). The largest groups in total plastic resins production are PE (36%), PP (21%), PVC (12%), followed by PET, PUR and PS (≤10% each) (Geyer et al., 2017), while Polyester, most of with is PET, accounts for 70% of all synthetic fibres production (Geyer et al., 2017). Together, these seven groups account for 92% of all plastics ever made (Geyer et al., 2017).

1.1.3. Usage

Global plastics demand can be categorized into 8 major segments by its end-use markets and product lifetime distributions: packaging; building and construction; textiles; consumer and institutional products; transportation; industrial machinery; and other sectors (includes medical and agriculture) (Geyer et al., 2017). The average time plastics are used before they're discarded ranges from less than 6 months, for packaging, to 35 years, for building and construction (Geyer et al., 2017).

Packaging is plastics largest market, an application whose growth was accelerated by a global shift from reusable to single-use containers, with approximately 42% of all plastic resins ever manufactured being used for this purpose, which is predominantly composed of PE, PP and PET (Geyer et al., 2017). Flexibility, strength, lightness, stability, impermeability, versatility, ease of sterilization and resource-efficiency are the main features of plastics that contribute significantly to its commercial success for this application (Millet et al., 2019). Although less known, these features confer plastic packaging environmental benefits when compared with alternative packaging materials (Pilz & Brandt, 2011). If food was packaged using materials other than plastics, it would take around twice as much as related energy consumption, resulting in 2.7 times more greenhouse gas (GHG) emissions over their lifetime and in 3.6 times more packaging mass (Pilz & Brandt, 2011). Similar environmental benefits can be applied to other plastic markets, such as in building and construction (e.g., plastic insulation) and in transportation (car and aviation) (Pilz & Brandt, 2011). The problem with plastic packaging resides in the prevailing single-use and linear economy mindset, excessive plastic packaging use, and foremost and transversal to all plastic markets, its end-use fate.

1.1.4. Fate

Packaging represents nearly half of all plastic waste generated globally (Geyer et al., 2017). The share of plastics in municipal solid waste (by mass) has increased from less than 1% in 1960 to more than 10% by 2005 in middle- and high-income countries (Jambeck et al., 2015), while at the same time the solid waste generation itself has grown steadily over time (Hoornweg et al., 2013). The increasing amount of post-consumer plastic waste generation generally follows three different fates: it can be recycled, incinerated (with or without energy recovery) or discarded, either in contained managed systems, such as sanitary landfills or left uncontained in open dumps and/or in the natural environment (Geyer et al., 2017). Between 1950 and 2015, the cumulative plastic waste generation was estimated at 6300 Mt (Geyer et al., 2017). Of this, around 60% of all plastics ever produced, 4900 Mt, were discarded and are accumulating in landfills or the natural environment, 12% have been incinerated and only 9% have been recycled (Geyer et al., 2017).

The properties that make plastics so versatile in innumerable applications – durability, chemical stability and resistance to degradation – are the same that make these materials so difficult for nature to assimilate (Geyer et al., 2017). The fact that none of the mass-produced plastics worldwide biodegrade in any meaningful way, the whopping growth of plastics production (and waste generation) in the past 70 years and the current management strategies for end-use plastics makes plastic waste a source of growing and near-permanent contamination of the natural environments. At the same time, public awareness of plastic pollution in the environment has increased in the last decades (Amaral-Zettler et al., 2020). This increasing public awareness has been translated into rising public pressure and legislation to dampen the input of plastic debris into natural environments (Amaral-Zettler et al., 2020).

1.1.5. Plastic litter in aquatic ecosystems

Marine litter is regarded as “any persistent, manufactured or processed solid material discarded, disposed or abandoned in the marine and coastal environment including all materials discarded into the sea, on the shore, or brought indirectly to the sea by rivers, sewage, storm water, waves, or winds” (UNEP & NOAA, 2012). It didn’t take long for plastics to become the most common form of marine debris since they become available to the general consumer public around 70 years ago. Despite the first reports of plastic pollution in the oceans appear in the scientific literature in the early 1970s (Carpenter & Smith, 1972), plastic litter in marine ecosystems still presents an important and growing global pollution problem. Today, plastic debris constitutes approximately 60-90% of the litter that accumulates in marine environments (Andrady, 2015; Pham et al., 2014). Due to its durability, low-recycling rates, poor waste

management and maritime use, a significant portion of the plastics produced worldwide enters and persists in aquatic ecosystems (Lebreton et al., 2017).

The release of plastics into aquatic environments occurs through a variety of pathways. Land-based sources are considered the dominant input of plastics into aquatic ecosystems, representing 80% of marine plastic litter (GESAMP, 2015). This includes transport *via* runoff into rivers and sea, leakage from waste-collection systems, illegal dumping, beach littering and atmospheric transportation (Chin & Fung, 2019; Lebreton et al., 2017). In 2015, it was estimated that the 192 coastal countries (93% of the global population) generated 2.5 billion tonnes of municipal solid waste in the year 2010, with 11% representing plastic waste (275 Mt) (Jambeck et al., 2015). Of this, 4.8 to 12.7 Mt was estimated to enter the oceans, equivalent to 1.7% to 4.6% of the total plastic waste generated in those countries (Jambeck et al., 2015). A more recent study estimated that 1.15 to 2.41 Mt of plastic waste currently enters the ocean every year from rivers (Lebreton et al., 2017). Additionally, and especially in developing countries, mismanaged landfills could lead to the displacement of plastic waste by winds or during natural hazards such as tsunamis and hurricanes, which can result in large flushes of plastic entering rivers and seas of coastal areas (Jambeck et al., 2015). On the other hand, aquatic-based sources can be summarized as originating from aquaculture, shipping, fishing and recreational activities (GESAMP, 2015). It is estimated that 0.64 Mt of fishing gear alone are discarded in the sea every year (Good et al., 2010).

Contamination of freshwater, estuarine systems, beaches and shorelines with plastic litter has also been widely reported all over the world (Chin & Fung, 2019). Beaches and shorelines represent the transition between marine ecosystems and terrestrial ecosystems. The presence and abundance of plastic litter in these areas can be directly associated with the land-based and ocean-based sources aforementioned, population densities and the amounts of tourism and industrial activities present in nearby areas, or indirectly associated with those areas, brought by the action of currents, waves, winds or other meteorological phenomena (Chin & Fung, 2019). A recent report predicted that a large part (66.8%) of all the buoyant macroplastic (>0.5 cm) released into the marine environment since the 1950s is stored by the world's shoreline as stranded, settled and/or buried debris, undergoing episodes of capturing and resurfacing, with an estimated weight of 46.7-126.4 Mt of macroplastics (Lebreton et al., 2019). On the other hand, estuaries represent the transition between freshwater and marine ecosystems and are influenced by both (Stothra Bhashyam et al., 2021). These dynamic ecosystems are one of the most productive ecosystems on Earth often described as biodiversity hotspots and nursery grounds for both aquatic and terrestrial species (McLusky & Elliott, 2010; Stothra Bhashyam et al., 2021). However, these ecosystems are also vulnerable to a multitude of anthropogenic stressors such as waste disposal land reclamation, aquaculture, fishing activities and pollution (Stothra Bhashyam et al., 2021). Estuaries are both hotspots and pathways for plastics pollution, capturing and

transferring plastics and microplastics from rivers and anthropogenic sources to marine ecosystems (Bessa et al., 2018; Naidoo et al., 2015; Stothra Bhashyam et al., 2021). Their semi-enclosed nature is responsible for retaining plastic and microplastic litter within a water body (Bessa et al., 2018; Stothra Bhashyam et al., 2021).

As the distribution of plastic litter varies among different ecosystems and environments, different organisms have been reported to contain and/or ingest plastics (Chin & Fung, 2019). A recent review noted that over 690 species, including marine mammals, seabirds and turtles, have been reported to ingest plastic (Provencher et al., 2017). Furthermore, several marine animals, such as seabirds, crustaceans, pinnipeds and other mammals are prone to plastic entanglement, with most of the entanglement incidents being ascribed to plastic fishing materials such as fishing gear (Chin & Fung, 2019).

1.2. Micro-plastics: A Macro-problem?

1.2.1. Size classification

Plastic debris can be characterised according to its origin (e.g., land, fishing-related or sewage-related debris), size, type, colour, polymer type or original usage (Thompson & Napper, 2019). One of the commonly used classifications is size (Thompson & Napper, 2019). Microplastics are commonly defined as being plastic particles smaller than 5 mm in diameter, although there is no consensus for a unified definition of microplastics size boundaries. Some authors, such as Hartmann et al. (2019), define the upper boundary as 1 mm. On the other hand, the lower boundary is often set on 1 μm, however, on-field studies are generally determined by operational constraints such as the mesh size of the nets used to sample surface water or the sieves used in sampling beach sand in field studies (Thompson & Napper, 2019). Macroplastic debris are often sufficiently recognizable to be categorized according to their original usage, however, attributing the source of microplastics is more challenging (Thompson & Napper, 2019).

1.2.2. Sources

Microplastics in the environment can be classified as primary or secondary concerning their source. The distinction is based on whether the particles were intentionally manufactured within the microplastic size range (primary) or whether they have resulted from the fragmentation of larger plastics (secondary) (Thompson & Napper, 2019).

Primary microplastics can be produced for direct use, such as plastic microbeads in cosmetics and personal care products and as air-blasting media or as building blocks for the production of larger plastic products, such as plastic pallets and plastic powders (Thompson & Napper, 2019). It has been estimated that up to 94 500 microbeads could be released from a

personal care product in a single-use (Napper et al., 2015). These enter household wastewater and some will escape the wastewater treatment into the environment (Browne et al., 2011; van Wezel et al., 2016). Some uses, such as in cosmetic products, are now beginning to be regulated. Until 2019, 9 countries (Netherlands, Australia, Canada, Italy, South Korea, New Zealand, Sweden, United Kingdom and United States) have placed restrictions on the use of microbeads in cosmetics and personal care products (Plastic Soup Foundation, n.d.). In this category, the production volumes can be used to provide estimates of potential inputs to the environment (Thompson & Napper, 2019).

Secondary microplastics are the result of the fragmentation of larger plastics and represent the main source of microplastics in the environment (Thompson & Napper, 2019). This degradation and fragmentation occur directly in the marine environment from larger macroplastic debris (e.g., plastics packaging, lost fishing nets) as a consequence of different mechanisms, such as weathering, photodegradation (U.V light), thermal degradation (visible light), thermal oxidation (infrared radiation), biodegradation and mechanical forces such as turbulence, abrasion and wave action (Amaral-Zettler et al., 2020; Thompson & Napper, 2019). A recent study estimates that 32.3% of all the buoyant macroplastics released into the marine environment since the 1950s to 2015 may already have degraded into microplastics, representing between 22.6-61.1 Mt of microplastics only in this subcategory of secondary microplastics (Lebreton et al., 2019).

Secondary microplastics are also generated from the abrasion caused by the usage of large plastic items on land entering the marine environment directly as microplastic particles (Boucher & Friot, 2017). This includes tires, textile fibres that detach from synthetic fibres, road markings, industrial abrasives and city dust (Boucher & Friot, 2017). It has been estimated that microfibres released during washing range from 124 to 308 mg for kg of washed synthetic fabric depending on the type of washed garment, corresponding to several microfibres ranging from 640 000 and 1 500 000 (De Falco et al., 2019).

Despite some studies indicates extremely high capture rates (>95%) of plastic particles in wastewater treatment plants, given the large volumes of influent daily, even low loss rates could result in considerable concentrations of these microplastics in the environment (Murphy et al., 2016). It has been estimated that wastewater treatment plants could release 65 million microplastic particles every day (Murphy et al., 2016).

1.2.3. Distribution and abundance

The presence of microplastics in the environment is considered ubiquitous (SAPEA, 2020). Microplastics have already been reported in all continents, oceans and seas, from the equator to the polar areas, from the deepest locations on earth to its highest mountains (Fig. 1.1).

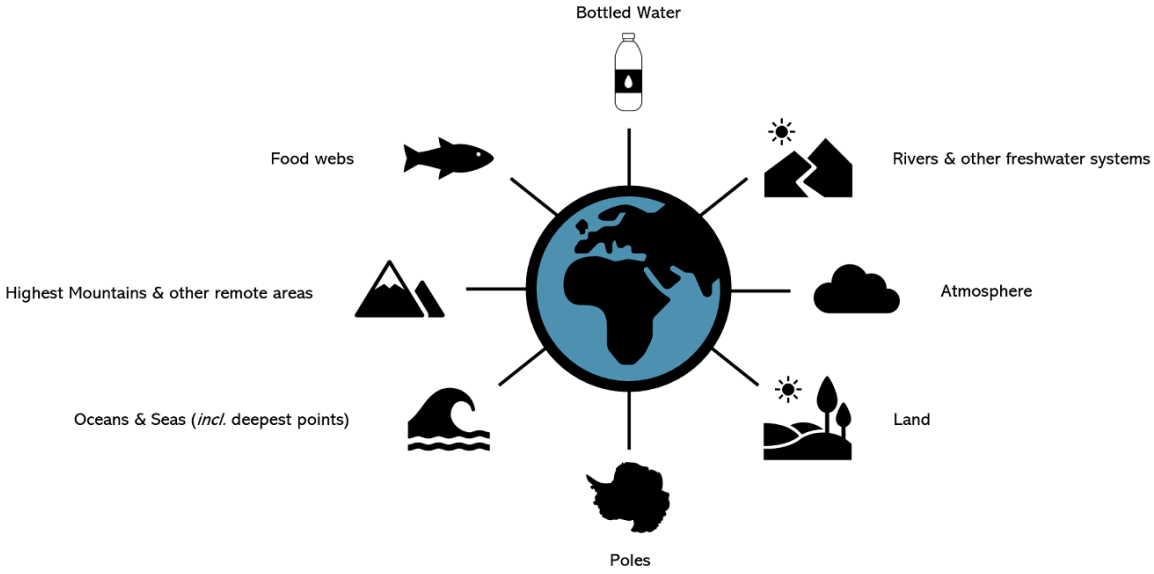


Figure 1.1: Environments and commodities (Bottled water) reported with microplastic contamination.

Several studies have attempted to estimate the abundance and accumulated weight of microplastic particles. Van Sebille et al. (2015) estimated that the accumulated number of microparticles in the ocean surface ranges from 15 to 51 trillion particles, weighing between 93 and 236 thousand tonnes. Eriksen et al. (2014) estimated more conservative values placing those numbers in more than 4.8 trillion microplastic particles weighing 35.5 thousand tonnes. Furthermore, Isobe et al. (2015) study estimated that, on average, every km^{-2} of open seas has more than 60 000 microplastics floating on the surface of the water. However, these estimates are restricted to the oceanic surface layer and based on the assumption that buoyant microplastic particles remain at the ocean surface. Lebreton et al. (2019) predicted that just the secondary microplastics, resulted from degradation and fragmentation of macroplastic debris in the marine environment over time, which does not account for direct input of microplastics from terrestrial sources, represents 0.28 to 0.75 million tons, far more than the previously mentioned estimates, suggesting that a substantial part of the microplastics have disappeared from the ocean surface layer. The explanations for this apparent disappearance of microplastics from the ocean surface are the following: (1) degradation and fragmentation, resulting in particles that are too small to quantify due to limitations in sampling and analysis techniques (Amaral-Zettler et al., 2020); (2) shoreline deposition by the action of currents and waves (Lebreton et al., 2019); (3) biofouling of the floating microplastics (colonisation by microorganisms and biofilm formation) that may result in loss of buoyancy in seawater ultimately ending up below the ocean surface sinking to deeper waters, with eventual seafloor deposition or resurface repeating the same cycle of events (Lebreton et al., 2019); (4) advection and other hydrodynamic processes responsible for vertical transport of microplastics (Lebreton et al., 2019; Maximenko et al., 2012); (5) entering in the marine food webs via ingestion by zooplankton and larger marine organisms and incorporation of

microplastics into phytodetrital aggregates and faecal material (Pabortsava & Lampitt, 2020); (6) biodegradation – although plastics are a fairly new habitat for microorganisms, microbial hydrocarbon degradation activities have been known for some time and thus the 10^{29} microbial inhabitants of the ocean with their metabolic diversity might be responsible for degradation of plastic debris (Amaral-Zettler et al., 2020).

According to these, some studies have also been focusing on the vertical distribution of microplastics in the ocean water column, rather than just the horizontal distribution in the ocean surface (Choy et al., 2019; Pabortsava & Lampitt, 2020). Pabortsava & Lampitt (2020) estimated that the combined mass of just the three most-littered plastics (PE, PP and PS) of 32-651 µm size-class suspended in the top 200 m of the Atlantic Ocean is 11.6-21.1 Mt. In a similar study, Choy et al. (2019) examined the distribution of microplastics at water column depths ranging from 5 to 1000 m and the highest concentrations were present at depths between 200 and 600 m. Furthermore, the presence of microplastics in deep-sea sediments is considered ubiquitous, with studies reporting its presence even in remote locations such as the deep-sea sediments of the Arctic (Tekman et al., 2020) and Southern Oceans (Cunningham et al., 2020). These reports and evidences suggest that both inputs and stocks of ocean microplastics may be much higher than previously reported (Pabortsava & Lampitt, 2020). Noteworthy that both the horizontal and vertical abundance and distribution appear to be subjected to strong heterogeneity (Pabortsava & Lampitt, 2020).

Apart from the open seas, the occurrence of microplastics has been widely reported on beaches and shorelines all over the world (Chin & Fung, 2019). Some studies showed that surface microplastic concentration has a statistically significant correlation with human population densities and the intensity of tourism activities (Browne et al., 2011; Fok & Cheung, 2015). However, in opposition other studies have suggested that the concentration of microplastics on beaches and shorelines is rather influenced by natural factors, such as seasonal variation, natural hazards or winds and currents (Chin & Fung, 2019).

Although much of the focus has been on the marine environment, including beaches and shorelines, a wide variety of freshwater systems, such as rivers and lakes, have been globally reported with microplastic contamination (Auta et al., 2017). Rivers represent a major contributor pathway for plastics to the ocean, being responsible for the flush of microplastic particles to the oceans (Dris et al., 2015; Eerkes-Medrano et al., 2015; GESAMP, 2015). The differences in microplastic concentrations observed between sampled rivers, resulting in different rates of river-based microplastic inputs into the ocean, is explained by the following factors: population densities, levels of urbanization and industrialization, and rainfall rates within catchment areas; port activity; tributaries; the presence of agriculture along the river course; the presence of

wastewater treatment plants; and the presence of artificial barriers (e.g., dams and weirs) (Lebreton et al., 2017) (Fig. 1.2).

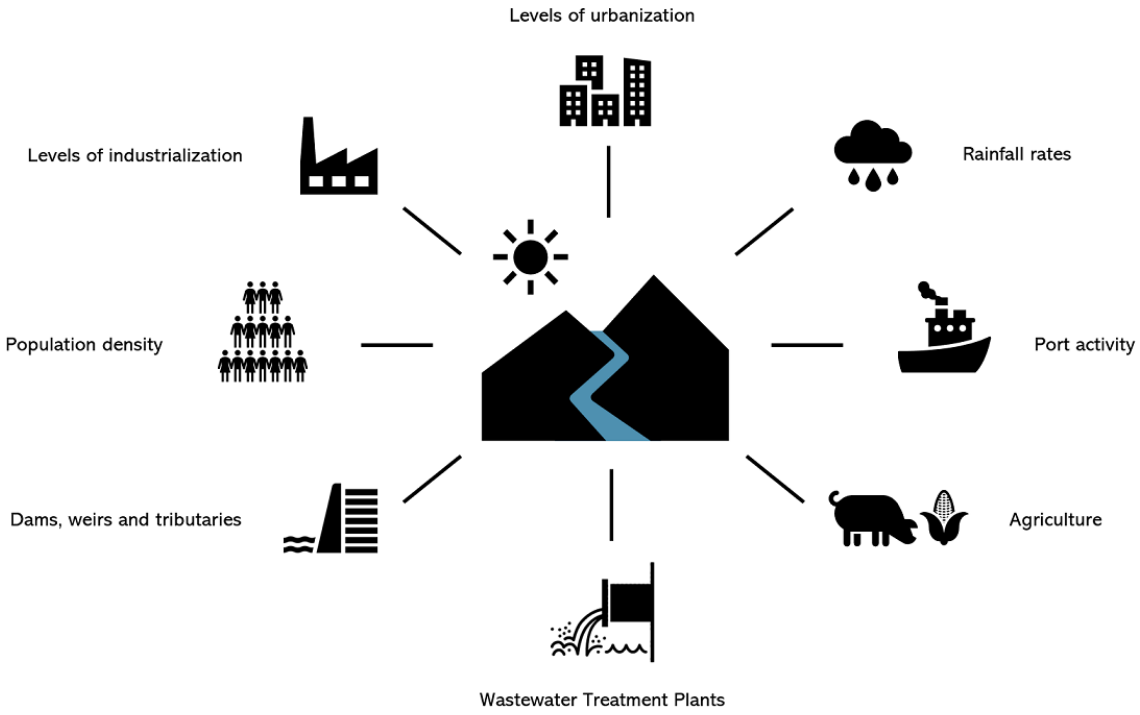


Figure 1.2: Possible emission sources and sinks of microplastics in a riverine and estuarine system.

In the freshwater systems, microplastic contamination levels appear to be subjected to temporal variations, with studies reporting different orders of magnitude between microplastics concentration measured at different periods (Lebreton et al., 2017). Seasonal variations, specifically dry and wet weather events, were the main explanation for this phenomenon, implying that run-off plays an important role in the transport of microplastics into freshwater systems (Lebreton et al., 2017).

Furthermore, even within the same river, different microplastic concentrations observed in different sampling locations are the result of the presence of significant sources (e.g., wastewater treatment plant, tributaries) and sinks (e.g., weirs) along the river course (Lebreton et al., 2017).

1.2.4. Impacts

Although the impact of meso- and macroplastics are more prominent by eye, therefore, is often subjected to a greater focus in scientific research and media coverage, the effects of microplastics in the aquatic ecosystems have recently received more attention, unravelling a variety of ecological consequences in the environment (Thompson & Napper, 2019). These

ecological consequences can be divided into four interconnected areas: ingestion; transport of non-native species by microbial colonisation and biofilm formation (including potential pathogens and resistant bacteria); acting as vectors for potentially harmful chemicals, and unknown impacts on biodiversity and aquatic food webs and in food security (especially seafood and aquaculture) (Fig. 1.3).

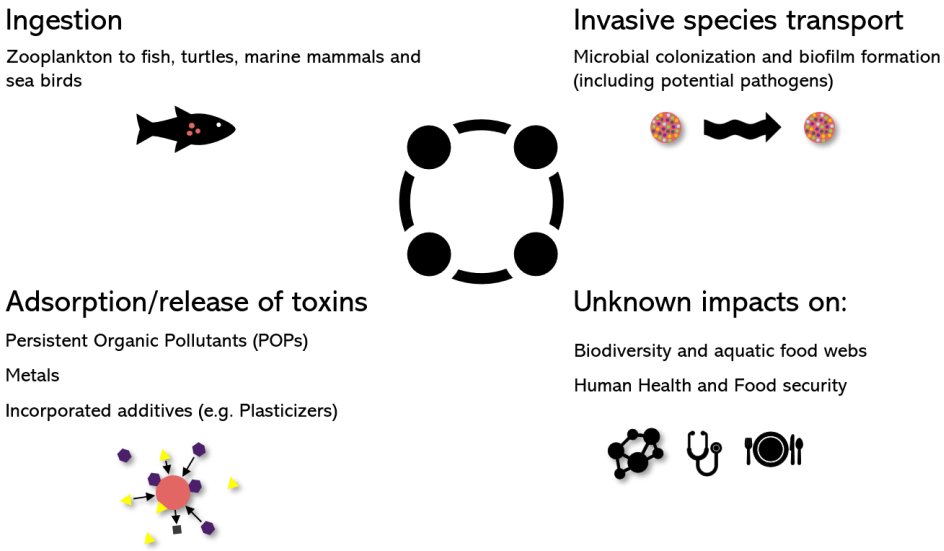


Figure 1.3: Ecological impacts of microplastic contamination in the environment and the potential to human health risk.

The small size of microplastic particles and their ubiquity in the aquatic environments means that they can interact with a very wide variety of organisms from all sizes and trophic levels (Cole et al., 2013, 2015; Gall & Thompson, 2015). Therefore, the ingestion of microplastics has been observed in different trophic levels ranging from microscopic organisms such as zooplankton to fish, marine mammals and seabirds (Worm et al., 2017). For example, Lusher et al. (2013) showed that of 504 pelagic and demersal fish from the English Channel, from 10 species, over one-third had microplastics in their digestive tract. Similar findings were reported by Bessa et al. (2018) in the Mondego Estuary (Portugal), with 38% of the 120 individuals, from 3 commercial fish species, having microplastics isolated from their gastrointestinal tracts. Organisms at lower trophic levels have also been reported to ingest and accumulate microplastics, which can be transferred to higher trophic levels within the food webs (Thompson & Napper, 2019). Furthermore, there is the potential risk of uptake across the cell membrane and gut epithelium for very small plastics particles (including micro- and nano-plastics), but still little is known about this possibility and its associated impacts. Additionally, the ingestion of microplastics is also likely to be influenced by their properties such as type, density and colour (Thompson & Napper, 2019). For example, buoyant microplastics are potentially more ingested by pelagic feeders and high-density microplastics by benthic feeders (Thompson & Napper, 2019). In fact, benthic organisms

such as blue mussels, lugworms, amphipods and sea cucumbers have been reported to ingest microplastic particles (Besseling et al., 2013; Tourinho et al., 2010; Van Cauwenberghe & Janssen, 2014). However, the influence of other properties, such as type and colour, in the ingestion of microplastics is yet not well established and is expected to be restricted to photic zones.

Several studies have shown the impacts of microplastics ingestion: physical effects including physiological stress responses in fish and invertebrates; compromises the ability of planktonic organisms to feed; the ability of marine worms and fish to gain energy from their food; and reproductive disruption in oysters, which could have associated population-level consequences (Thompson & Napper, 2019). However, most of the studies set to examine the effects of microplastics ingestion are based on laboratory manipulative experiments, using higher concentrations than those commonly found in the environment (Lenz et al., 2016), only providing information of thresholds for future levels of contamination rather than providing clear evidence of current environmental consequences (Thompson & Napper, 2019).

There is also the concern of microplastics transfer microorganisms between locations (Thompson & Napper, 2019). Plastic debris lasts much longer than most natural substrates such as macroalgae, feathers or wood, representing a novel type of pelagic substrate for microbial colonisation and transportation that can travel over long distances and contaminate different environmental compartments (Zettler et al., 2013). Microplastics collected in numerous surface waters and sediments locations have been reported to be colonized by a variety of microorganisms including bacteria, cyanobacteria, diatoms, ciliates and radiolaria (Thompson & Napper, 2019). However, the relative importance and the differences in the microbial communities between plastic debris (including microplastics) and those found in water or on other transport vectors (e.g., macroalgae, feathers or wood) is still not well established.

Furthermore, microplastics can adsorb/leach harmful chemicals from/to the environment (e.g., water) that can facilitate the transfer of chemicals to organisms directly as a consequence of ingestion or indirectly via release to the waterbodies (Thompson & Napper, 2019). Microplastics present a large surface area to volume ratio and a hydrophobic surface that makes them susceptible to adsorb and accumulate contaminants such as persistent organic pollutants (especially hydrophobic organic pollutants) and metals at concentrations several orders of magnitude higher than the surrounding water (Thompson & Napper, 2019). Additive chemicals are incorporated into plastics during their manufacture or processing to enhance plastic durability and corrosion resistance or to act as stabilizers, plasticizers or flame retardants used at high concentrations (10-50%) that can be leached out to the environment (Thompson & Napper, 2019). However, recent analysis, such as from Diepens & Koelmans (2018) suggested that microplastics would not lead to increasing concentration of a substance in the tissues of tolerant organisms in the food webs. The effect of microplastics on chemical uptake into biota indicates that it doesn't

provide a substantial contribution when compared with other exposure pathways (e.g., food, pray or ambient water), although it can be substantial in locations where abundances of plastic debris are higher, or in the future with the increase in plastic abundances (SAPEA, 2019). Further research and investigation are required to continue to access and explore the effects of microplastics, its interactions with chemical pollutants and its incorporated additives on biota (including humans). A very recent study by Tian et al. (2021) discovered that the reason behind the death of coho salmon in urban streams of the U.S Pacific Northwest, where up to 90% of the adults migrating up certain streams to spawn would suddenly die after rainstorms, comes from an additive chemical (6PPD-quinone) widely used to protect tyres, which are composed of 24% synthetic polymers (U.S Tire Manufacturers Association, n.d.), from ozone (reactive atmospheric gas) that leaches out of the particles tyres shed onto the pavement (Stokstad, 2020). This highlights the importance of further research on the ecological impacts of the chemical additives incorporated into plastic or plastic containing products.

1.3. Microplastics associated bacterial communities

1.3.1. Relevance, study evolution and experimental approaches

For 45 years that is known that plastic debris, including microplastics, in the aquatic environments are carriers of microbial communities (Stanier, 1975). This microbial life associated with plastic debris would be later coined by Zettler et al. (2013) as the "Plastisphere". However, only recently the role of microbial interactions with microplastics in aquatic environments has been investigated in more detail (Oberbeckmann & Labrenz, 2020). Although anthropogenic debris has been entering the aquatic environments for centuries, within the last decades microplastics became ubiquitous and the numerically dominant form of marine debris and are primarily colonized by bacteria and other microscopic life (Amaral-Zettler et al., 2020). The recognition that this novel human-made substrate in the environment can facilitate microbial dispersal and affect all aquatic ecosystems, has raised the interest in the microbial ecology associated with plastic debris (Amaral-Zettler et al., 2020). Microplastics are lightweight, small and resistant particles that provide a stable, durable and hydrophobic substrate that can be colonized by microorganisms, transported over long distances and supports the growth of microbial biofilms, which means that they can contaminate several environmental compartments over time. Mincer et al. (2019) previously estimated that the microbial life associated with plastic debris is approximately 0.01-0.2% of the total microbial biomass in open ocean surface waters, but given the recent fact that we can only account for 1% of the plastic debris that is released in the marine environment (Lebreton et al., 2019), this biomass is likely to be substantial (Amaral-Zettler et al., 2020).

Early studies of the “Plastisphere” identified morphologically distinct organisms, such as diatoms and filamentous bacteria through microscopy, especially SEM (scanning electron microscopy) (Amaral-Zettler et al., 2020; Stanier, 1975). The recent growing interest and research in the role of microbial interactions with microplastics in aquatic environments and the understanding of the microorganisms that inhabit these substrates has been achieved through the application of modern molecular methods, especially high-throughput DNA sequencing (C. De Tender et al., 2017) and also CLASI-FISH (combinatorial labelling and spectral imaging – fluorescence *in situ* hybridization) (Schlundt et al., 2020). The method of choice for comparative molecular ecology studies has been amplicon sequencing, initially via 454 pyrosequencing and later via Illumina MiSeq or HiSeq sequencing (Amaral-Zettler et al., 2020). The recent studies associated with this topic have been investigating the microorganisms that thrive on microplastics, the establishment of plastic-specific biofilms, community assembly and successions, enrichment of pathogenic bacteria (particularly members of the genus *Vibrio*), antimicrobial-resistance genes and metal-resistance genes, coupled to a vector function of microplastics, interactions within communities, their metabolic capacities (including the potential microbial degradation of plastic debris) and how communities affect their surrounding ecosystem (Amaral-Zettler et al., 2020; Oberbeckmann & Labrenz, 2020). Despite the current interest in this topic, still, only a limited number of studies on the “Plastisphere” have used high-throughput DNA sequencing (Amaral-Zettler et al., 2020). Most of the studies exploring the microbial communities associated with plastics focused on samples collected in surface open waters from Europe (especially in the North Sea and the Mediterranean Sea), Northwest Atlantic Ocean, Northwest and Northeast Pacific Ocean (Rogers et al., 2020). Data are lacking from below the water surface, coastal areas, freshwater systems, sediments, polar regions and in the Southern Hemisphere (Rogers et al., 2020).

The experimental approaches followed in the recent studies generally follow two pathways: examining microbial communities on environmental plastic debris (including microplastics) or incubation experiments with known polymer types. Incubation conditions include the suspension *in situ* within the natural water column of a selected environment, laboratory aquaria of various sizes with flowthrough seawater systems exposed to light or in the dark, static laboratory systems in containers of various sizes with water collected once from the aquatic system of interest and/or sediments (Amaral-Zettler et al., 2020). Most of the studies follow this experimental approach over environmentally collected microplastics. This might be due to the higher costs and technical complexity to obtain such samples from the environment (e.g., sea, estuaries, rivers, sediments), as well as in downstream processing, such as the identification of plastics resin and the post- or pre-identification DNA extraction of the microbial communities associated with the environmental collected microplastics (Amaral-Zettler et al., 2020). Furthermore, another challenge of working with environmental microplastic samples is the

low biomass available for DNA extractions and subsequent microbial profiling, which affects the success of producing amplifiable DNA (Amaral-Zettler et al., 2020). These challenges make it difficult to correlate microbial communities with polymer types (or substrates) using environmental collected samples, which incubation or *in situ* experiments overtake by using selected plastic types in controlled quantities, mainly "raw" plastics from known manufacturing sources. While incubation or in situ experiments overtake the challenges faced in studies using environmental microplastic samples, allow to study community assembly and analyse individual and isolate variables, such as time, substrate, geography or substrate, in community assembly and composition, they are static and thus fail to simulate the real conditions that microplastics are subjected in the environment. These small durable and resistant particles contaminate different environments over time (e.g., land, river, estuary, sea, sediments) with different and variable residence times, which shape its community assemblies, community compositions and biofilm formations.

1.3.2. Colonisation and community composition

Several microorganisms have been found attached to microplastics, such as fungi, diatoms, algae and most commonly, bacteria (Mammo et al., 2020). While some studies have pointed out that the microbial communities on (micro)plastics are different from other particles in the same environment, such as wood, cellulose or glass, and that certain microbial groups are consistently associated with plastics, this is still a subject of debate, as well as no agreement has yet been reached on whether microplastic-associated communities display an increased or decreased α-diversity when compared with natural particles and the surrounding water (Amaral-Zettler et al., 2020; Oberbeckmann & Labrenz, 2020). The conducted studies have pointed to geography-dependent, environmental-dependent, time-dependent and substrate-dependent differences (Amaral-Zettler et al., 2020).

Geography differences have been reported at various scales, from oceanic differences to regional differences on PET submerged in the North Sea coast of England less than 200 km apart (Amaral-Zettler et al., 2020). Furthermore, one meta-analysis compared studies from the Baltic Sea, North Sea and Yangtze Estuary (China) revealing that the average similarity between communities associated with microplastics from the Baltic Sea and Yangtze Estuary (both strongly influenced by rivers) was 12%, higher than the 7% similarity between communities from the Baltic and North Seas, highlighting the importance of biogeographical and environmental factors, such as salinity, on the community composition (Oberbeckmann & Labrenz, 2020). An incubation study in the North Sea showed differences in bacterial communities on microplastics during exposures in winter, spring and summer, but no significant differences were registered between communities on PET and glass (control) (Oberbeckmann et al., 2016). Some studies, however,

reported differences between communities associated with microplastics and natural particles such as cellulose, the particle-attached water fraction, or sediments (Oberbeckmann & Labrenz, 2020). One of those studies, an incubation experiment in the Baltic Sea reported differentiation between assemblages on polystyrene and polyethylene from assemblages on wood, model of a natural particle, but only in certain environmental conditions, highlighting the importance of the sampling area in the development of the microbial biofilm (Oberbeckmann & Labrenz, 2020).

The influence of the particle surface on its colonisation can be shaped by characteristics such as degradability, hydrophobicity, electric charge, roughness or indirectly via the formation of a conditioning film over the particle (Oberbeckmann & Labrenz, 2020). Within a given system, the polymer type and surface characteristics of the microplastics surface may influence what microorganisms attach, but the communities on different substrates appear to converge over time as their biofilms mature (Amaral-Zettler et al., 2020). One meta-analysis investigating β-diversity reported no significant differences in the bacterial communities associated with different polymer types, indicating that the plastic itself (i.e. polymer resin) is a minor factor determining microplastic-associated biofilms (Oberbeckmann & Labrenz, 2020). Instead, microplastic biofilms are shaped primarily by biogeographical and environmental factors (Oberbeckmann & Labrenz, 2020). Community differences on biofilms, aggregates of cells either attached or unattached to a substrate that grows within a matrix composed of extracellular polymeric substances (EPS), on microplastics and those from the surrounding water are expected since biofilm formation typically constitutes a considerable change in the lifestyle of a microorganism from a planktonic or motile state to a sessile state, whereby specific gene sets involved in chemotaxis, communication, adhesion and substrate transport are expressed to enable individual cells to form a matrix analogous to tissues as well as fluid channels that help distribute nutrients between cells (Amaral-Zettler et al., 2020). Like other bacteria that prefer an attached over a free-living lifestyle it can be assumed that, overall, most microplastic-biofilm members are opportunistic general colonizers (Oberbeckmann & Labrenz, 2020). Microorganisms can colonize plastic substrates within hours after immersion in an aquatic system (Amaral-Zettler et al., 2020). Early colonizers might be attracted not by the polymer surface itself but rather by the conditioning film, which presents advantages, such as increased access to limited nutrients (Oberbeckmann & Labrenz, 2020). For instance, the family Rhodobacteraceae, an abundant and commonly reported member of the bacterial communities associated with microplastics are also known for its early and abundant colonisation of a broad range of particle surfaces (Oberbeckmann & Labrenz, 2020).

Molecular data and SEM images from incubation studies reported early colonisation and domination by diatoms in the first week which then decrease in relative abundance, killed or grazed from the surface as bacteria attach and the community become more diverse (Amaral-Zettler et al., 2020). This includes bacteria such as *Rhodobacteraceae*, important for biofilm formation producing EPS that promotes the settlement of other bacteria, and *Rhodospirillaceae*,

which includes purple sulfur bacteria, many of which can fix N_2 attracting other community members, increasing the carrying capacity (Amaral-Zettler et al., 2020).

The most commonly reported bacterial communities attached to microplastics belong to the phylum Proteobacteria, classes Alphaproteobacteria and Gammaproteobacteria, irrespective of the type of aquatic environment (Mammo et al., 2020). In marine environments, Cyanobacteria are also commonly reported from microplastic biofilms (Mammo et al., 2020). Firmicutes are found in both freshwater and marine environments in microplastic biofilms (Mammo et al., 2020). On the other hand, highly dominant bacteria, such as *Candidatus* Pelagibacter found in both seawater and freshwater worldwide, tends to be scarcely reported on microplastics (Amaral-Zettler et al., 2020).

1.3.3. Pathogens

As plastic debris continues to increase and accumulate in the environment, an emerging concern is the potential for microplastics to act as vectors for pathogen transport (Bowley et al., 2021). Masó et al. (2003) was the first report about the attachment of harmful microbes to plastic debris, but the landmark was the study by Zettler et al. (2013) that highlighted the potential for marine microplastics to harbour distinct communities of microbes on their surfaces (Bowley et al., 2021). Since then the research on the microbial communities associated with (micro)plastics escalated and of particular concern are the increasing reports of numerous pathogenic bacteria on microplastic surfaces (Bowley et al., 2021). Various studies reported the presence of potentially pathogenic microorganisms from environmental microplastic samples such as *Vibrio* spp., *Aeromonas* spp., *Arcobacter* spp., *Pseudoalteromonas* spp., *Shewanella* spp., *Alteromonas* spp., *Tenacibaculum* spp., *Phormidium* spp., or *Leptolyngbya* spp. recovered from various locations worldwide, in both seawater and freshwater environments (Amaral-Zettler et al., 2020; Bowley et al., 2021).

The microplastics long-distance dispersal potential raises important questions as to whether the increasing amount of plastic waste in aquatic ecosystems provides greater opportunities for pathogens to be transported and transmitted to potential hosts, leading to increasing outbreaks of disease, compared to the opportunities provided by natural particles. Although some evidence pointed out that the total abundance of pathogenic bacteria on microplastics may be similar when compared to other natural particles (Oberbeckmann & Labrenz, 2020), there are several additional factors to consider such as (1) the attachment processes and microbial interactions (e.g., horizontal gene transfer) on microplastic particles; (2) the rate and distance transport of pathogen-colonized microplastics across different environments, and whether the bacterial communities change; (3) vertical transport processes through to the benthos, where ingestion and trophic transfer occurs; (4) the uptake and retention

of microplastics into aquatic organisms, especially commercially consumed species, and the likelihood of disease transfer occurring as a result and how this may pose a risk to human health (Bowley et al., 2021) (Fig. 1.4).

In comparison with seawater and natural particles, the microplastics bacterial communities show more significant rises in the metabolic pathways that contribute to infectious diseases (Bowley et al., 2021). Therefore, microplastics may act not only as a vehicle for pathogen dispersal but also as a pool of strains that have acquired pathogenicity islands and other antimicrobial properties through horizontal gene transfer (Bowley et al., 2021). In fact, it has been observed an increased frequency of plasmid transfer in bacteria associated with microplastics when compared with free-living bacteria or natural particles, which is proposed to aid in the spread of antimicrobial resistance, although the mechanisms underpinning this phenomenon are still unclear (Bowley et al., 2021). Heavy metals (e.g., aluminium, copper, zinc), as well as other pollutants (e.g., persistent organic pollutants), are shown to be sorbed onto the plastic surface which may influence selection processes and horizontal gene transfer within attached microbial communities (Bowley et al., 2021) (Fig. 1.4).

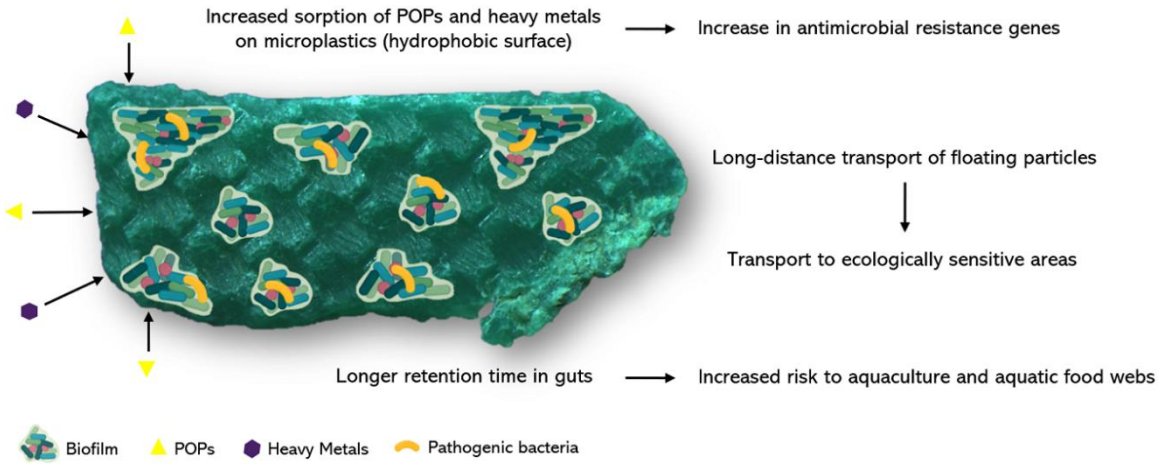


Figure 1.4: Microplastic bacterial colonisation, biofilm formation, heavy metals and persistent organic pollutants (POPs) sorption, and the risks of pathogen-plastic interactions adapted from Bowley et al. (2021).

Rivers are a major source of microplastics and pathogens in coastal waters (Bowley et al., 2021). A large number of riverine microplastics come from sewage effluents, and it has been reported that the attached bacterial communities differ from the organisms in the surrounding environment downstream of the effluent (Bowley et al., 2021). A recent study revealed a higher abundance of the family *Campylobacteraceae*, known to cause human gastrointestinal infections, attached to microplastics downstream from a sewage treatment plant (Amaral-Zettler et al., 2020; McCormick et al., 2014). This raises the question of the role of the world's rivers in transporting pathogens (Bowley et al., 2021). Current evidence suggests that the microplastics bacterial

communities adapt and change to prevailing conditions as there are transported through a riverine system (Bowley et al., 2021).

Furthermore, there is a concern that microplastics can act as a vector of pathogen transport in seafood species (Bowley et al., 2021). Microbial diseases in fish, crustaceans and molluscs are a major source of loss in the aquaculture industry, with *Vibrio* spp. being the most common pathogen of fish and shellfish aquaculture systems (Amaral-Zettler et al., 2020). Critical to elucidating this threat is the knowledge gap as to whether the ingestion of pathogen-contaminated microplastics can lead to disease transfer and, if so, the required exposure (Bowley et al., 2021). To date, there is only one study that directly demonstrated pathogen transfer via microplastics ingestion. Using green fluorescent protein (GFP)-tagged *E*. *coli* attached to the microplastic surface they visually demonstrated the transfer to the gut tissues of the northern star coral (Rotjan et al., 2019). Whether this occurs under natural settings in other aquatic organisms and the relevance to infection rates and human health outcomes is still unknown and further research is needed (Bowley et al., 2021).

2 Objectives

2 Objectives

Although the presence of small plastic fragments in the environment has been described in scientific literature since the 1970s, the landmark paper from Thompson et al. (2004) defined the term “microplastic” and reported that these particles have been contaminating and accumulating in the oceans since the 1960s levering research and concern on microplastic environmental contamination. Similar logic applies to the microbial life associated with plastics in the environment. Although the first mention of microbial life attached to plastic debris goes back to 1972 (Carpenter & Smith, 1972), it was not until Zettler et al. (2013) landmark paper, in which they coined the term “Plastisphere”, that the research on the microorganisms attached to (micro)plastics escalated. In the last few years, substantial contributions and advances have been made regarding this topic. However, there are still numerous questions and knowledge gaps that require further research to add to the worldwide studies performed to date. Considering the absence of studies in the North-east Atlantic coast, outside of the Celtic Sea and North Sea, and the reduced quantity of studies in brackish and freshwater systems, this work was performed in the Mondego Estuary (Portugal) and adjacent coastline and aimed to address the following objectives:

(I) Profile the bacterial communities and key bacterial groups associated with the microplastics and from their respective transitional ecosystems (estuarine and beach sand);

(II) Compare the bacterial community profiles between microplastics and their respective transitional ecosystems counterparts;

(III) Assess the potential of microplastics as vectors of transmission and spread of key bacterial groups in transitional ecosystems;

(IV) Contribute to the understanding and knowledge on the microplastic-related environmental pollution, on the “Plastisphere” and its potential ecological impacts, especially in transitional ecosystems.

3 Experimental Procedures

3 Experimental Procedures

3.1. Study area

This study was conducted at the Mondego estuary, a warm-temperate, polyhaline, intertidal system covering an area of 8.6 km^2 along the Atlantic coast of Portugal, Europe (40°08' N, 8°50' W)(Silva et al., 2021; Teixeira, 2016)(Fig. 3.1). The estuary comprises two arms, the north and the south, separated by an alluvium-formed island (Murraceira island), joining again near the mouth (Teixeira, 2016). The north arm is deeper, with depths between 4 to 8 m and a tidal range between 1 to 3 m, and mainly used as a navigation channel, presenting a higher hydrodynamic activity than the south arm (Silva et al., 2021). On the other hand, the south arm is shallower, with depths between 2 to 4 m and a tidal range between 1 to 3 m, and is characterised by large areas of intertidal mudflats, with almost 75% of the area exposed during the low tide (Nunes et al., 2011; Silva et al., 2021). The water flow on the south arm depends on the tides and freshwater input from the Mondego river and its main tributary, the Pranto river (Nunes et al., 2011). The river basin is occupied mainly by agricultural (32%) and forest (64%) areas, distributed throughout the basin, whereas urban (2.34%) and industrial (0.68%) areas are located mainly on the coastal strip (Teixeira, 2016).

This study was also conducted in the adjacent coastal line of the Mondego estuary, at three sandy beaches: Forte de Santa Catarina, Cabedelo and Quiaios. This coastal area presents a warm temperate Atlantic-Mediterranean climate and semidiurnal tides with a maximum amplitude of about 3.5 m (Gonçalves et al., 2009). Forte de Santa Catarina beach and Cabedelo beach are widely recognized as urban beaches since they are closer to the important tourist centre town of Figueira da Foz, presenting a high potential of recreational use and anthropogenic pressure (Bessa et al., 2014). Furthermore, Forte de Santa Catarina beach, embedded in the Mondego River mouth is strongly influenced by the river water and its dynamics, followed by the Cabedelo beach located south of the river mouth breakwater. Lastly, Quiaios beach, which is outside the influence of the Mondego river dynamics due to its distance and north positioning to the river mouth, is in a rural area with lower human beach use and, consequently, under lower anthropogenic pressure (Bessa et al., 2014).

3.2. Samples collection

The collection of samples (microplastics, water and sediments) was performed during two sampling campaigns: estuarine on November 25th, 2020 and sandy beaches on December 4th, 2020.

The estuarine sampling campaign was carried out by boat during high tide (with the maximum point at 11:42 a.m.) in the Mondego Estuary (Figueira da Foz, Portugal) where three representative sites were sampled: site E1 was located close to a recreational marina in the south arm of the estuary (40º7'47.116''N, 8º51'4.565''W), site E2 was located close to an effluent of the wastewater treatment plant in the north arm of the estuary (40º8'23.732''N, 8º48'52.858''W) and site E3 was located close to a thermoelectric power station upstream the bifurcation of the estuary into two arms (40º7'16.519''N, 8º46'17.899''W) (Fig. 3.1). Microplastic samples were collected at surface water by dragging a plankton neuston net (335 µm mesh, circular net opening of 0.5 m of diameter) against the stream of water for 10 minutes in each sampling site starting at the following time points: 10:15 a.m. for E1, 11:35 a.m. for E3 and 12:15 p.m. for E2. To quantify the water volumes filtered by the nets the variation in revolutions registered on the Mechanical Flow Meter (HYDRO-BIOS) was annotated for each sampling event. The collected filtered water samples were transferred into sterile 1L plastic bottles and stored in ice on board. For further DNA extraction of free-living (FL) and particle-attached (PA) communities present in the estuarine water 4L of surface water were also collected at each sampling site in sterile 1L plastic bottles and stored in ice on board (Fig. 3.2). Samples were stored in the laboratory at 4 ºC until further processing on the following day.

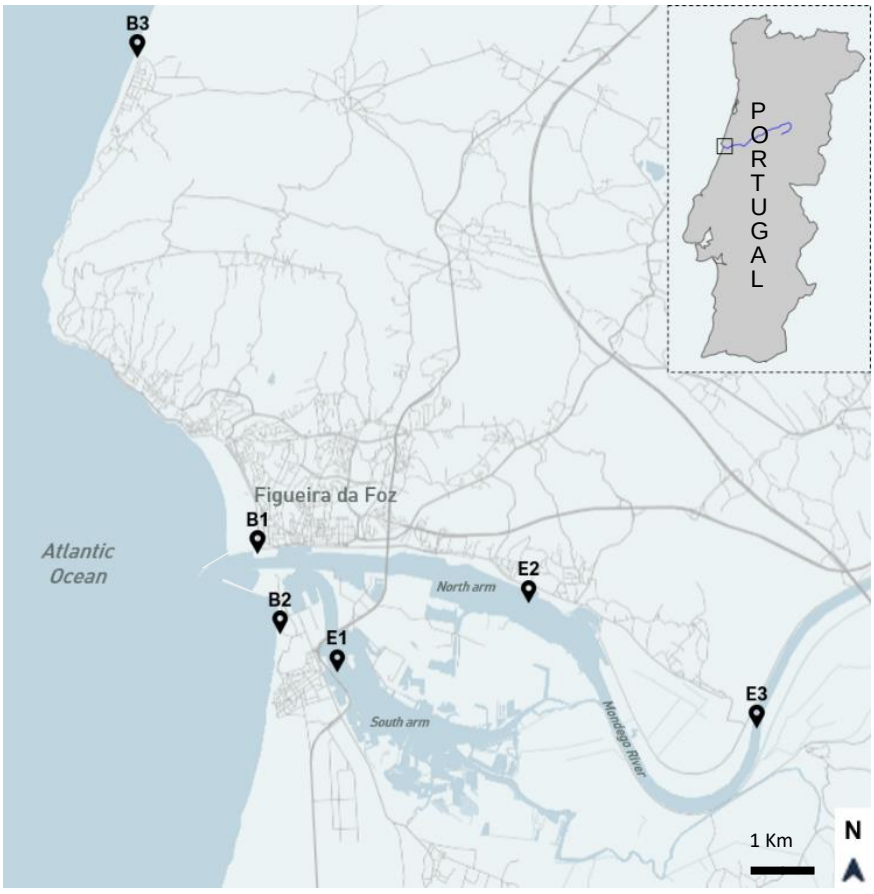


Figure 3.1: Overview of the Mondego estuary and adjacent coastline along the central coast of Portugal and the sampling sites: Estuarine (E1-E3) and Sandy beach (B1-B3).

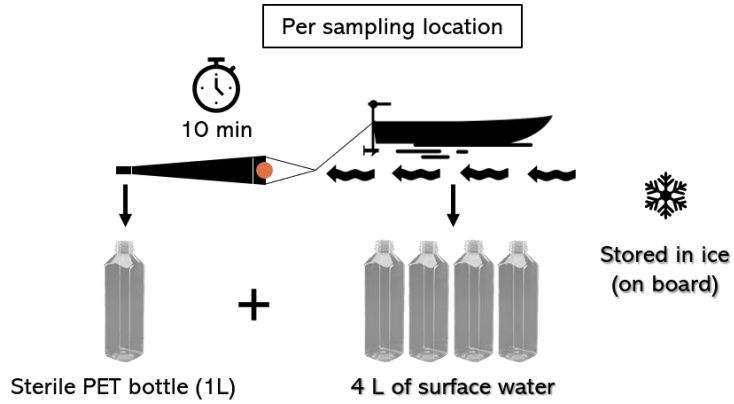


Figure 3.2: Schematic overview of the estuarine sampling campaign procedures.

The sandy beaches sampling campaign was carried out along the adjacent coastline of the Mondego estuary during low tide (the maximum point at 10:58 a.m.) where three selected beaches were sampled: Forte de Santa Catarina beach (B1) is embedded in the Mondego River mouth (40º8'49.436''N, 8º52'2.559''W), Cabedelo beach (B2) around 1.25 km south of the Mondego river mouth (40º8'7.601''N, 8º51'44.846''W) and site B3 located in the Quiaios beach around 10 km north of the Mondego river mouth (40º13'14.893''N, 8º53'30.913''W) (Fig. 3.1). Samples were collected in the line of the previous high tide (the maximum point at 4:55 a.m.). In each sampled beach the top 3 cm of sediment were collected into a sterile glass jar (total volume of around 1.5 L) with a sterile metal shovel (rinsed in 70% ethanol) in a 0.25 m^2 area (0.5 m wood square) and stored on ice upon arrival to the laboratory. For DNA extraction of the bacterial communities present in the sediment one sterile microtube was filled with sediment from each sampled beach, stored on ice upon arrival to the laboratory and then frozen at -80 ºC until DNA extraction.

3.3. Estuary samples processing

Estuarine water samples were processed after 24 hours upon their collection. These samples were filtered using MilliporeSigma™ Sterifil™ 47mm Aseptic Vacuum Filter Systems. The collected water samples were vacuum filtered through 1.2 μm Whatman GF/C microfiber filter papers being then individually transferred into Petri dishes (60 mm x 15 mm) with sterile forceps (flaming after ethanol rinsing) for the manual microplastics extraction process. The collected surface water samples were filtered through sterile 0.2 μm Pall filters being then transferred with sterile forceps into 50 mL Falcon tubes and stored at -80 ºC until DNA extraction of free-living (FL) and particle-attached (PA) communities present in the estuarine water.

3.4. Manual extraction

For the estuarine samples, the microplastics extraction from the water filters was performed manually using a dissecting microscope (Leica EZ4) in a laminar flow cabinet under sterile conditions with forceps systematically rinsed in ethanol and flamed between manipulations of each particle. Visually identified microplastic-like particles were characterised according to their type and colour (Fig. 3.3) and individually sorted into sterile microtubes filled with Milli-Q water to ensure that the microplastic-like particles stayed in the tubes and stored at -80 ºC until DNA extraction of the microplastic-associated bacteria.

Figure 3.3: Microplastics characterisation by type according to the classification provided by Gago et al. (2018a).

For beach sand samples, the content of the glass jar was poured into a white sterile tray and 6 to 7 microplastic particles detected by the naked eye were picked from the sediment of each sampled beach with sterile forceps (rinsed in 70% ethanol) into sterile microtubes and stored at -80 ºC.

To avoid sample contamination, specifically airborne fibre contamination in the laboratory, standard practices were followed, which included cleaning all equipment with prefiltered RO-water (Reverse Osmosis), limiting the use of plastic laboratory equipment and synthetic clothing, and performing all steps in a laminar flow cabinet.

3.5. DNA extraction

DNA extraction was performed on the microplastic-like particles (i) collected and isolated from the filtered estuarine surface water sampling points and (ii) collected and isolated from the sand of the sampling beaches for microplastics associated bacteria. Furthermore, DNA extraction was also performed on the environmental matrices samples, this is, (iii) on the 0.22 µm Pall filters used for the filtration of the collected estuarine surface water samples and (iv) on the sand samples collected from the sampling beaches for the bacterial communities naturally present in these environmental matrices. The DNA of microbial communities was extracted using Qiagen Powersoil DNA extraction kits (Qiagen GROUP) following manufacturer instructions. Before the extraction two intermediate samples preparation steps were carried out: 0.22 µm Pall filters used for the filtration of the collected estuarine surface water samples were macerated in sterile zipper

plastic bags with Milli-Q water to release the free-living (FL) and particle-attached (PA) communities attached to the filters; and the microtubes containing estuarine microplastic-like particles within Milli-Q water were poured into sterile Petri dishes and with the help of sterile forceps (glass bead sterilizer), under dissecting microscope, the microplastic-like particles were individually picked into the respective PowerBead Pro Tubes provided in the MoBio Powersoil DNA extraction kit. The extracted DNA was eluted in sterile DNA-Free PCR-Grade Water and stored at -20 ºC for further downstream applications.

3.6. Sample preparation and Illumina Sequencing

Samples were prepared for Illumina Sequencing by 16S rRNA gene amplification of the bacterial community. The DNA was amplified for the hypervariable V4 region with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. First PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit according to manufacturer suggestions, 0.3 µM of each PCR primer: forward primer 515F-Y (5'-GTGYCAGCMGCCGCGGTAA-3') and reverse primer 806rB (5'-GGACTACNVGGGTWTCTAAT-3') (Caporaso et al., 2011; McCormick et al., 2014) and 12.5 ng of template DNA in a total volume of 25 µL. The PCR conditions involved a 3 min denaturation step at 95 ºC, followed by 30 cycles of 98 ºC for 20 s, 64 ºC for 30 s and 72 ºC for 30 s and a final extension at 72 ºC for 5 min. Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to the manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

3.7. Processing sequences

The set of Illumina-sequenced paired-end fastq files, received without barcodes, were imported in R (version 4.04) and analysed, demultiplexed, primer sequences removed, chimaera-filtered and Amplicon Sequence Variants (ASVs) were obtained using DADA2 package (version 1.18) (Callahan et al., 2016). Following the package instructions, sequences quality was inspected by checking the quality plots, subsequently trimming of the last 20 bp for forward and allowing a max estimated error ("maxEE" option) higher than 2 per 100 bp for forward and reverse reads. Forward and reverse reads were truncated at position 240.

The ASVs were assigned with RDP Taxonomy 18 database, which provides quality-controlled, aligned and annotated Bacterial and Archaeal 16S rRNA sequences (Wang et al., 2007). The RDP Classifier tool was used with an 80% confidence cut-off. For species identification, the RDP Sequence Match tool was used and the sequences with 100% similarity were selected. Non assigned sequences, archaeal and eukaryotic sequences were removed. To ensure an equal sampling depth for all samples, the ASVs were rarefied to the same number (*n* = 23695) using the Phyloseq R package (version 3.3.3) (McMurdie & Holmes, 2013) and rarefaction curves were visualized using the ggplot2 R package (version 1.34) (Wickham, 2016). Raw sequence data were deposited in the Sequence Read Archive (SRA) database at the NCBI under BioProject accession number PRJNA706887.

3.8. Data analysis and statistics

Relative abundance graphs of the bacterial taxa mean abundances for all sample types (estuary microplastics, estuary water, beach microplastics and beach sand) were performed at all taxonomic levels (phylum, class, order, family and genus) using GraphPad Prism (version 8.0.1).

Alpha diversity based on the observed number of ASVs, species richness, Shannon, Simpson and Pielou diversity indices were calculated for each sample type in each sampling location using the R package Vegan (version 2.5.7) (R Core Team, 2016).

A principal coordinate analysis (PCoA) was performed using PAST program (version 4.02) (Hammer et al., 2001) to evaluate the differences in the microbial community compositions using the Bray-Curtis (BC) similarity index as an estimator of the taxonomic distance between sample types. The community structure was confirmed with a heatmap using GraphPad Prism (version 8.0.1). Hierarchical cluster, a cluster analysis method based on BC distances, was performed for all samples of each sample type. All analyses were performed using the ASVs frequency matrices at the genus level. Venn diagrams of the percentage and number of shared and unshared genus between sample types on both sampling environments (estuarine and beach sand) and between matrices (grouped microplastics, estuary water and beach sand) were generated using the R package Venn (R Core Team, 2016).

The bacterial communities statistical significance between sample types was performed by permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001). Also, the Kruskal-Wallis test ($p<0.05$) was used to find differences in alpha diversity indexes among the different sample types. A similarity percentage (SIMPER) analysis was performed to identify ASVs that accounted for the bacterial community differences between sample types. Both analyses were performed using PAST program (version 4.02) (Hammer et al., 2001).

3.9. Microplastics characterisation

Post DNA extraction particles were once again visually analysed, using a stereomicroscope LEICA M80 (Leica Microsystems GmbH, Wetzlar, Germany) with image analysis system IC80 HD Camera with Leica Application Suite (LAS) software. Particles type classification (Fig. 3.3) was reconfirmed, as well as their colours. In addition, all particles were measured at their largest cross-section and categorised by particle size range (<1 mm, 1-2 mm, 2-3 mm, 3-4 mm, 4-5 mm, and >5 mm).

The potential polymer type of the suspected microplastic particles (chemical characterisation) was analysed using micro-Fourier-transform infrared spectroscopy (μ-FTIR) (BRUKER HYPERION 2000) in the vibrational spectroscopy laboratory at the QFM-UC, Coimbra, Portugal. FTIR is a vibrational spectroscopy technique where infrared (IR) light interacts with molecular vibration providing a fingerprinting of the sampling material. These vibrations are measured by emitting an IR light from an IR source into a sample that absorbs some of the light according to the different vibrations it has and the detectors collect the transmitted or reflected light. This is simultaneously performed for all the wave lengths and the data will be converted by Fourier transformation to get the final spectrum (Fig. 3.4).

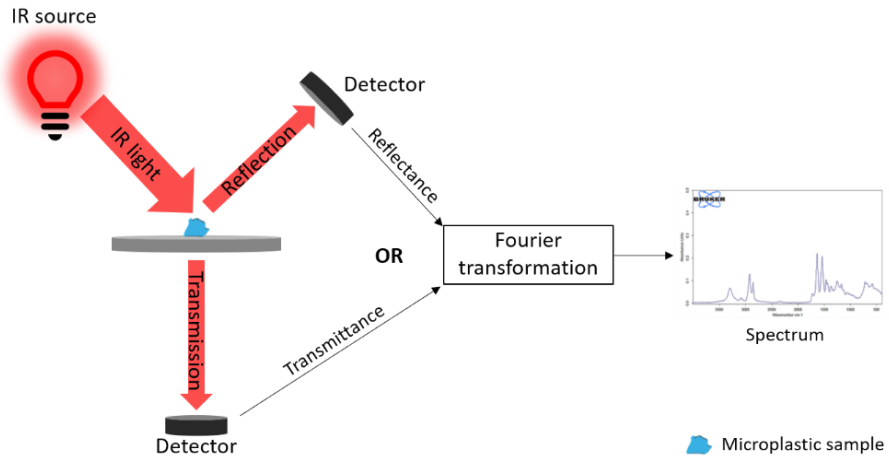


Figure 3.4: Simplified diagram of the Fourier-transform infrared spectroscopy (FTIR) spectra acquisition principles.

All the 20 collected and isolated sand particles were analysed by μ-FTIR. One infrared spectrum was acquired for each particle in a BRUKER HYPERION 2000 microscope (15× objective), with liquid nitrogen cooled Mercury Cadmium Telluride (MCT) detector, in reflectance mode (4000–600 cm^{-1}), with a resolution of 4 cm^{-1} and 128 scans.

On the other hand, the reduced size of the collected and isolated estuary particles (mostly microfibers) presented challenges in the process of chemical characterisation due to the difficulty

of individually particle manipulation into/in the FTIR equipment and the limited IR transparency of filters used in previous steps (microfiber filter papers). Therefore, were performed several attempts to overcome these challenges and optimise a protocol for the polymer identification of these particles of smaller dimensions. One solution was the placement of a calcium fluoride (CaF_2) disk between the used filters and the estuary microplastic-like particles. CaF_2 is limited in the mid-IR spectral range to about 900-1000 cm^{-1}, which in reflectance mode allowed to block the limited IR transparency of the used filters and still present clean spectra in the range between 1000 to 4000 cm^{-1}. However, due to the limited timeframe to the publication of this work and the use of the FTIR equipment, it was only possible to acquire spectra for 4 randomly selected estuary microplastic-like particles in reflectance mode (4000–600 cm^{-1}), with a resolution of 4 cm^{-1} and 256 scans.

Each measured FTIR spectrum was analysed using OMNIC software and compared with a commercial spectral library (Hummel Polymer Spectral Library, Thermo Fisher Scientific Inc.) and the BASEMAN library, which includes 326 reference spectra of plastics, as well as natural organic materials that can be misinterpreted as plastics, developed by Primpke et al. (2018). Only particles with matches greater than or equal to 60% were accepted and classified as “Synthetic polymers”, considering the polymer with the highest match value, while particles with less than 60% match were rejected and classified as “Unidentified”. However, for these particles, the spectra were individually inspected and interpreted based on the closeness of their absorption frequencies to those of chemical bonds of known synthetic particles and polymers, allowing us to infer its synthetic/polymeric nature but without scientific accuracy.

4 Results and Discussion

4 Results and Discussion

4.1. Microplastics abundance and characterisation

Microplastics were found in both environmental compartments (estuarine and beach sand) and in all sampling stations in the form of fragments, fibres, foams, pellets and films (Fig. 4.1). A total of 89 particles were collected from both environments.

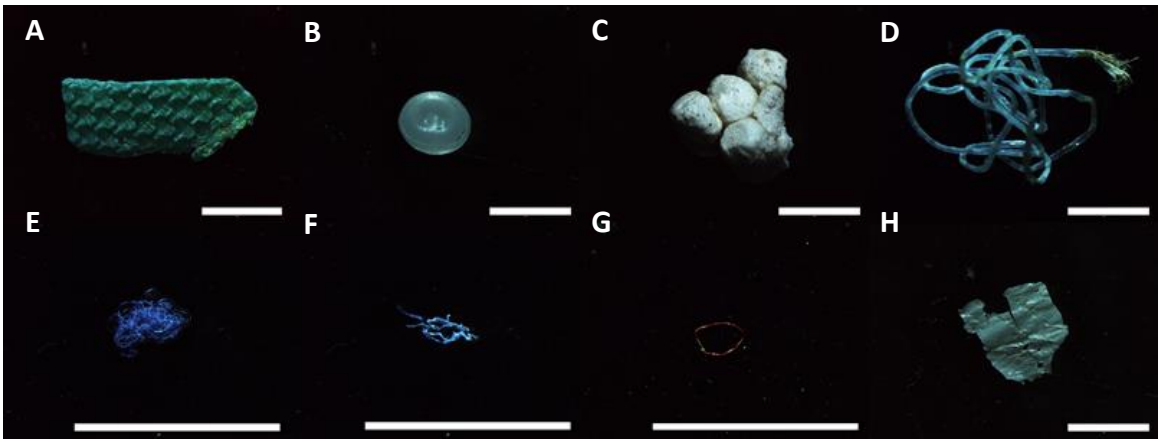


Figure 4.1: Representative microplastics isolated from the sandy beaches (A-D) and from the Mondego estuarine waters (E-H). Fragments (A); Pellets (B); Foams (C); Fibres (D-G) and Films (H). Scale bars = 5 mm.

Within the Mondego estuary, water samples were filtered and a total of 69 particles were isolated: 8 fibres, 1 film and 3 foam particles from the south arm (E1) (*n* = 12); 17 fibres, 1 film and 1 fragment from the north arm (E2) (*n* = 19); and 35 fibres, 2 films and 1 fragment from upstream (E3) (*n* = 38) (Fig. 4.2). Within the estuarine sampling sites, microplastics were mostly composed of fibres (87%) (Fig. 4.3). Despite the lack of studies addressing the presence and characterisation of microplastics in the Mondego river and estuary, the only study related to this topic, by Bessa et al. (2018), reported the occurrence of microplastics in commercial fish from the Mondego estuary, in which they were also mainly in the form of fibres (96%). In the rivers and estuaries of Portugal, microplastics have been directly isolated from water samples in the Douro estuary (Porto, North of Portugal) (Rodrigues et al., 2019) and in the Antuã river (Aveiro, Centre of Portugal) (Rodrigues et al., 2018), with both studies, however, reporting lower representativities (%) of fibres in their samples (35% and 23.25%, respectively) when compared with those in this study and the work of Bessa et al. (2018). Furthermore, in a study from the Sado estuary and Arrábida coastal area (Setúbal, South of Portugal) (Rodrigues et al., 2020), the Sado estuary sampling station had only around 3% of fibre representativity.

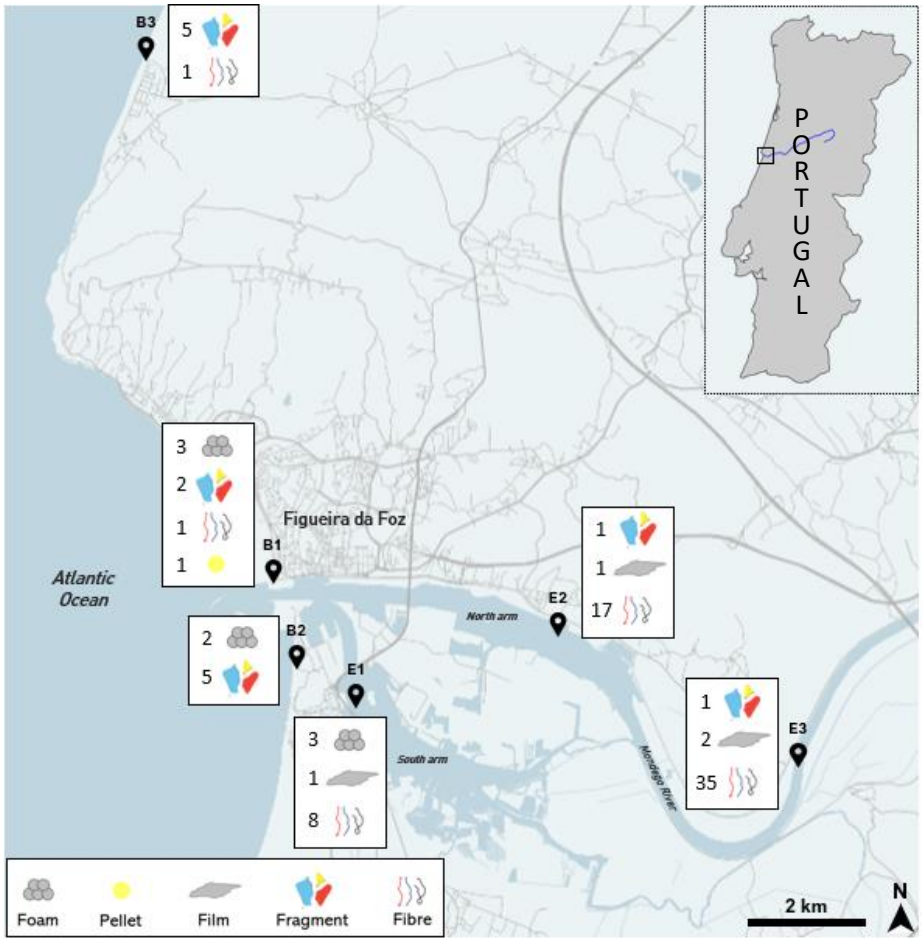


Figure 4.2: Overview of the estuary (E1-E3) and beach (B1-B3) sampling locations in the Mondego estuary and adjacent coastline and the respective microplastics abundances according to their shape.

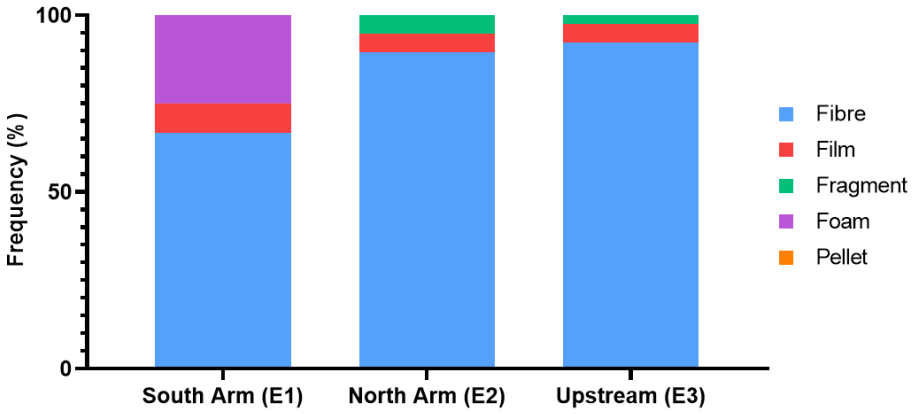


Figure 4.3: Occurrence frequency (%) of the microplastic types in the estuarine sampling locations.

The high percentage of fibres in the Mondego estuarine waters obtained in the present study are in line with reports that fibres are the most prevalent form of microplastics observed in aquatic environments (Browne et al., 2011; Gago et al., 2018b), including in estuarine systems worldwide (Gallagher et al., 2016; Lahens et al., 2018; Miller et al., 2017; Naidoo et al., 2015; Zhao et al., 2019). Furthermore, it supports the hypotheses indicated by Jabeen et al. (2017) and reinforced by Bessa et al. (2018) that freshwater and transitional systems are potentially more prone to fibre contamination than the marine environment because these systems are closely located to potential point source discharges of anthropogenic fibres, such as wastewater treatment plants (WWTPs). It is important to note however that animal and cellulosic fibres might be underrepresented in environmental pollution literature (Suaria et al., 2020) but although cellulosic fibres should not be considered synthetic, those man-made cellulosic fibres can contain chemicals, such as synthetic dyes, additives and flame retardants that may pose environmental threats, harbour different bacterial communities and take more time to degrade (than a natural-based particle). Despite the main sources and sinks of fibres in aquatic environments are not fully vetted, WWTPs effluents are often considered a significant point source of fibres emissions to the aquatic environments. Indeed, two WWTPs operate within the Mondego estuarine area, which provides only secondary water treatment and without the capacity to treat industrial wastewater (Teixeira, 2016), which might be important sources of fibres input into the Mondego riverine and estuarine waters. These fibres may also originate from the fragmentation of lost and discarded fishing gear and recreational sailing gear, as suggested by Bessa et al. (2018).

In the present work, an average particle concentration of 1.92 ± 1.24 particles m^{-3} (Mean ± SE) was obtained for the Mondego estuarine waters (particles >335 μm), which is in accordance with the mean value of 1.53 ± 1.04 particles m^{-3} reported from a study performed during one year (2017) along the Mondego estuary (Bessa et al., unpublished data). The aforementioned studies reported particle concentrations of 0.17 particles m^{-3} for the Douro estuary (Rodrigues et al., 2019), 58-1265 particles m^{-3} for the Antuã river (Rodrigues et al., 2018) and around 0.62 particles m^{-3} for the Sado estuary (Rodrigues et al., 2020). These substantial differences might be explained by the size range of the sampled and isolated particles, >550 μm for the Douro estuary and >55 μm for the Antuã river, but also the differences in the methodologies regarding the sampling devices, sampling mesh sizes and extraction protocols, and the temporal and spatial resolution of these studies. In addition, the method for microplastics extraction was adapted in the present work to preserve the integrity of the bacterial communities on their surfaces. Sample processing had to be performed fast and under sterile conditions, without the use of chemicals to degrade organic material, such as H_2O_2, and with manual extraction of the particles from the filters, used in the filtration of the trawl filtered estuarine waters, which had a high load of organic matter, difficulting the manual extraction of the particles increasing the risk of missing the detection of microplastics or to pick natural particles that look alike to synthetic polymers.

The occurrence of microplastics in the Mondego estuary might be explained not only by the fibres sources aforementioned but also by the anthropogenic dynamics in the Mondego river basin. The river basin is occupied by urban (2.34%) and industrial (0.68%) areas, with two of its most populated cities, Coimbra and Figueira da Foz, growing along the river margins (Teixeira, 2016), and so these areas might play an important role in the microplastic contamination, as urban and industrial areas reduce soil permeability, which may cause the runoff of urban/industrial (micro)plastic contaminated waters to the river. Also, agricultural areas represent 32% of the river basin area (Teixeira, 2016) which might also contribute to microplastic emissions to the waters that are drained to the riverine and estuarine waters. Furthermore, the Mondego estuary system supports mercantile and fishing harbours, salt-extraction, aquaculture farms and wastewater treatment plants (WWTPs) (Bessa et al., 2018), which may also contribute to the occurrence of microplastics in this system.

In the adjacent coastal sandy beaches, a total of 20 microplastics were isolated from the sand samples: 3 foam particles, 2 fragments, 1 fibre and 1 pellet from the Forte de Santa Catarina beach (B1) (*n* = 7); 2 foam particles and 5 fragments from the Cabedelo beach (B2) (*n* = 7); and finally, 5 fragments and 1 fibre from the Quiaios beach (B3) (*n* = 6) (Fig. 4.2). Within the sandy beaches, microplastics were mostly fragments and foams (85%) (Fig. 4.4). The most common type of microplastic reported along the Portuguese coast was in the form of fragments, foams and pellets (Prata et al., 2020). Indeed, a study by Antunes et al. (2018), which analysed the occurrence and characterisation of microplastics along the Portuguese coast, reported that pellets represented 79% of all the microplastic particles, followed by fragments (14%) and foams (6%). However, this latter has study sampled beaches with industries proximity, while in the present work the sampled sandy beaches had lower industries proximity, which might explain the difference in pellet representativity with the one here obtained (5%).

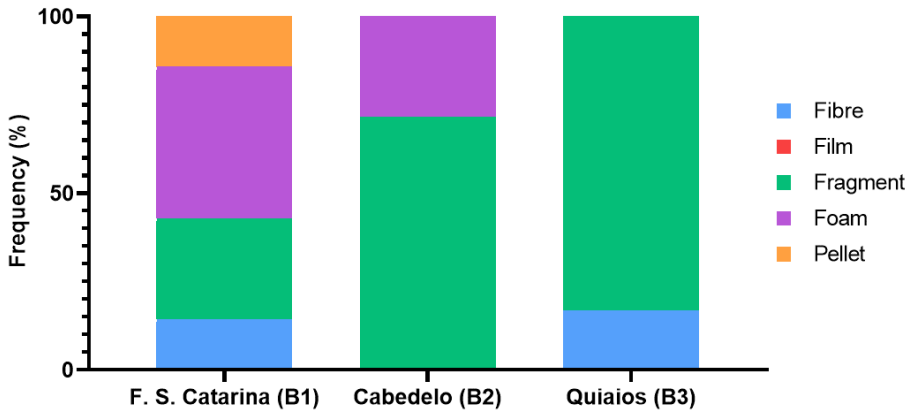


Figure 4.4: Occurrence frequency (%) of the microplastic types in the sandy beaches sampling locations.

In the present work, an average particle concentration of 26.67 ± 1.09 particles m^{-2} was obtained for the selected/analysed beaches. Despite only a few studies have reported microplastic occurrence on the coast of Portugal, the average particle concentration on beaches described in continental Portugal and the Azores is 25 particles m^{-2} (Prata et al., 2020). However, the highest concentrations of microplastics are found in beaches in the centre, region of the study area of this work, with the closest sandy beaches with the reported microplastic occurrence, Mira and Vieira de Leiria, presenting concentrations of 148 ± 161 particles m^{-2} and 590 ± 622 particles m^{-2}, respectively (Antunes et al., 2018). These values are far superior to ones obtained in the present study of 26.67 ± 1.09 particles m^{-2}. However, the mentioned beaches are influenced by high industrial activity in the surrounding areas. The particle concentration here reported reflects only one sampling campaign that occurred during the winter, being just a snapshot of the microplastics contamination in the sampled sandy beaches. These, present a high potential of recreational use and anthropogenic pressures, especially during the bathing season, which is intimately related to the potential higher levels of microplastic contamination in the summer and lower during the winter (Bessa et al., unpublished data). Future research should account for spatial and temporal variations to obtain a more representative frame of the microplastic contamination in these sandy beaches.

Regarding the characterization of all particles recovered, blue was the most common colour (46.07%) among the total 89 collected particles and the five most represented colours (blue, red, white, transparent and black) accounted for over 92% of all the particles (Fig. 4.5). These results are in concordance with the 47% blue particles reported in commercial fish from the Mondego estuary, by Bessa et al. (2018) and with the colour classification criterion by Gago et al. (2018a), based on the most common microplastic colours reported in peer-reviewed publications, in which the first five colours are the same that the most abundant colours reported here.

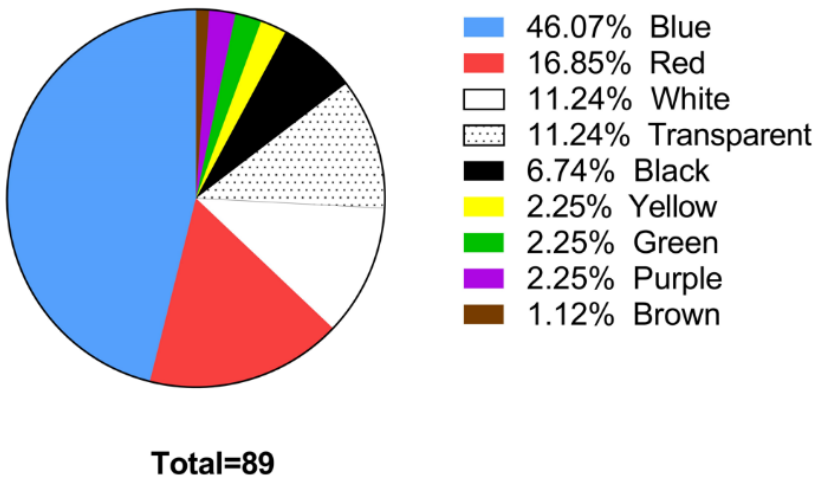


Figure 4.5: Colour distribution (%) of all collected and isolated microplastic particles (estuary and sandy beaches).

Furthermore, the average particle size of the collected and isolated particles in estuarine waters was smaller, 2.21 ± 0.18 mm (size range between 0.39 mm and 7.34 mm), than in sandy beaches sediment, 9.58 ± 0.69 mm (size range between 3.63 mm and 15.15 mm) (Fig. 4.6). Although the average particle size in sandy beaches and that over 25% of all particles (both environments) were bigger than the microplastic upper boundary considered here (5 mm), these particles still presented reduced dimensions when compared with the following size category, mesoplastics (5 to 200 mm), and were still considered in the analyses as the main purpose/goal of this work was the characterisation of the microplastics bacterial communities, since it was been reported that the size, and therefore, the surface area does not appear as the main factor in shaping microplastic bacterial communities (Frère et al., 2018).

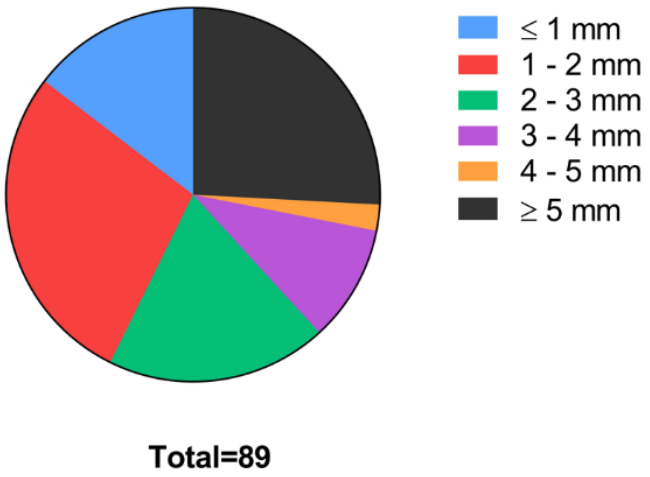


Figure 4.6: Size distribution of all collected and isolated microplastic particles (estuary and sandy beaches).

As for chemical characterisation, all isolated particles from the sandy beaches sediment were analysed by µ-FTIR, with spectra acquisition, and a posterior software spectral analysis revealed that eleven particles could be classified as "Plastic polymers", meaning they presented matches higher than 60%, with six particles being identified as Polyethylene (PE) and five as Polystyrene (PS) (Fig. 4.7 and 4.8). The remaining nine particles presented matches lower than 60%, being rejected and classified as "Unidentified" (Fig. 4.8). This selected threshold is within the match range described by Cowger et al. (2020), in which most of the reviewed studies count spectra with a percentage match greater than 60%, up to 90%. A recent meta-analysis identified PE, PP and PS as the most abundant polymers in the marine environment (Erni-Cassola et al., 2019). Although no PP particle has been identified here for the sandy beaches, meaning that no particle obtained a match higher than 60% for PP, the "Unidentified" particles spectra were individually inspected and interpreted based on the closeness of their absorption frequencies to those of chemical bonds of known synthetic particles and polymers, allowing to identify five likely

PP particles. This means that the results here reported are in concordance with the reporting PE, PP and PS as the most abundant polymers in marine environments (Erni-Cassola et al., 2019).

On the other hand, only five fibres of the isolated estuary particles had their spectra acquired, with two fibres being classified as “Plastic polymers”: one as Polypropylene (PP) and the other as Polyacrylonitrile (PAN), while the remaining three being classified as “Unidentified” (Fig. 4.7 and 4.8). The remaining 64 estuary particles were not analysed and were classified as “Not evaluated” (data not shown). This situation is explained by the logistical and technical constraints previously mentioned (see section 3.9 of the Experimental procedures). Also, fibres characterisation still presents challenges, uncertainties and controversy. These particles can be of natural origin, semi-synthetic or synthetic. A recent study by (Suaria et al., 2020) compiled a global dataset from 916 seawater samples collected in six ocean basins, characterising approximately 2000 fibres by µ-FTIR, revealed that only 8.2% of oceanic fibres were synthetic, with most being cellulosic (79.5%) or of animal origin (12.3%). However, it is extremely challenging to distinguish between natural and man-made (which can contain synthetic dyes, additives and flame retardants) cellulosic fibres by FTIR techniques. In the same study, PP fibres represented 4.3% of all identified synthetic fibres while acrylic and nylon fibres represented 8.6%, which supports the finding of a PP fibre and an acrylic fibre (PAN) here reported for the estuarine waters.

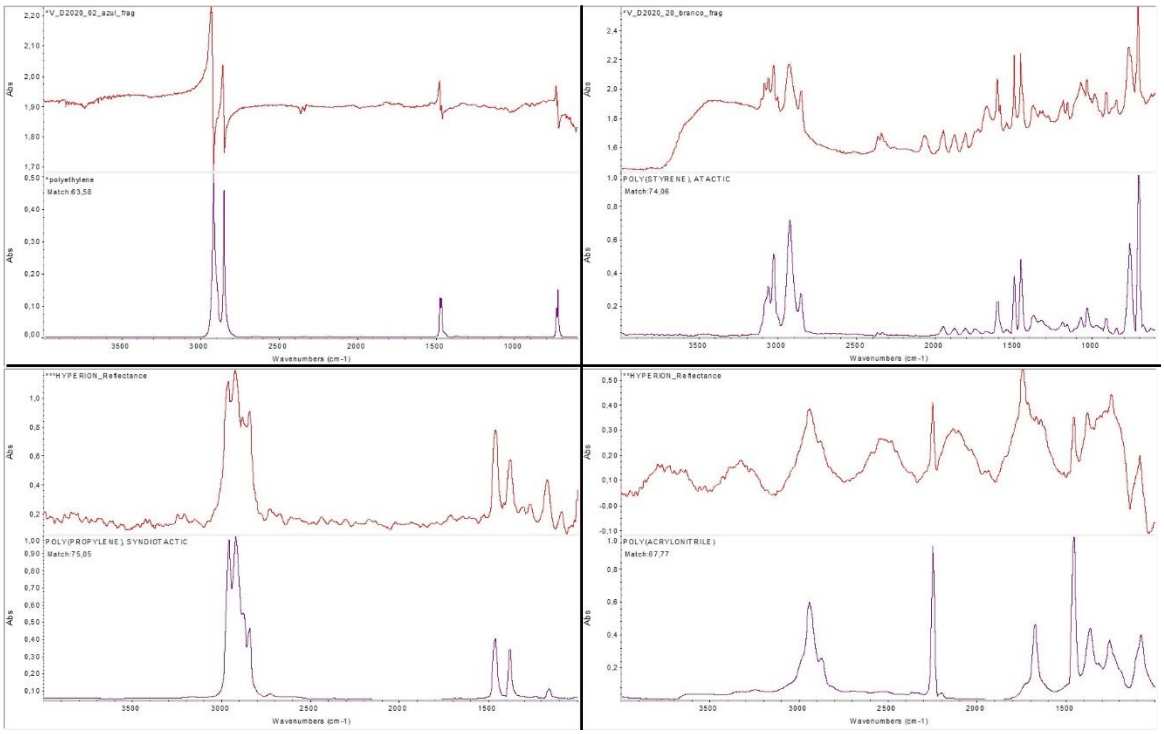


Figure 4.7: Representative spectra of the isolated microplastics from the sandy beaches (top) and from the Mondego estuarine waters (bottom). Red spectra represents microplastic particle spectrum and the purple spectra represents best reference spectrum match. Selected particles represent the different identified polymers: Polyethylene (top left) (63.58% match), Polystyrene (top right) (74.06% match), Polypropylene (bottom left) (75.05% match) and Polyacrylonitrile (bottom right) (67.77%).

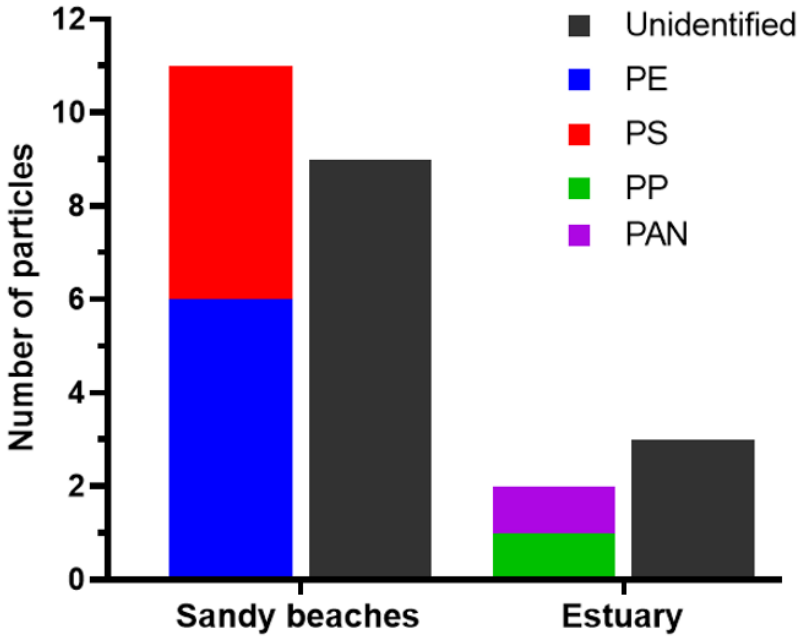


Figure 4.8: Chemical characterisation of the analysed isolated particles (spectra acquisition and analysis): all the 20 sandy beach particles and 5 out of the 69 estuary particles. PE – Polyethylene; PS – Polystyrene; PP – Polypropylene; PAN – Polyacrylonitrile.

Noteworthy that the chemical characterisation process by μ-FTIR was performed after the DNA extraction of the particles, which may have sorbed the chemicals used in the DNA extraction kit, difficulting or interfering in the spectral analysis, which might explain the lower matches obtained comparing to other studies and the need to define/adjust the match threshold here applied in the lower limit of 60%. For further research, it may be appropriate to wash the particles right after the DNA extraction process.

4.2. Associated bacterial communities

4.2.1. Bacterial communities structure

Before quality filtering an average of 88,046 reads per sample were recovered (an average of 93,726.3 reads in estuary microplastics, 113,572.7 reads in estuary water, 75,317.3 reads in beach microplastics and 69,567.6 reads in beach sand) and an average of 62,403.6 reads was used afterwards (an average of 60,701.6 reads in estuary microplastics, 87,316 reads in estuary water, 53,516.6 reads in beach microplastics and 48,080.3 reads in beach sand). Therefore, Illumina MiSeq sequencing analysis resulted in a total of 633,757 good quality reads with an average of 256 bp after chimaeras removal. The sequence reads in the different steps of quality control are shown in Table 4.1. All samples presented rarefaction curves with a stationary phase indicating

sufficient depth of sequencing to account for most of the taxa amplified in both the microplastics and environmental matrices (estuary water and beach sand) (Fig. 4.9).

Table 4.1: Number of sequence reads in the different steps of quality control for each sample. (E1MP-E3MP) Estuary microplastics; (E1W-E3W) Estuary water; (B1MP-B3MP) Beach microplastics; (B1S-B3S) Beach sand.

Samples	Input	Filtered	Denoised	Merged	Tabled	Nonchim
E1MP	125138	79451	78323	75454	75454	74404
E2MP	73032	50241	48527	45708	45708	45027
E3MP	83009	52413	50912	48239	48239	47700
E1W	128374	99843	93082	85280	85280	83975
E2W	81761	62626	57395	52782	52782	52206
E3W	130583	99479	91820	85376	85376	84039
B1MP	71063	49550	44489	39406	39406	39227
B2MP	83526	58486	52994	47057	47057	45950
B3MP	71363	52514	49969	46067	46067	45367
B1S	86736	59012	54543	49322	49322	48913
B2S	57483	40617	35895	31068	31068	30322
B3S	64484	44612	41049	36960	36960	36627

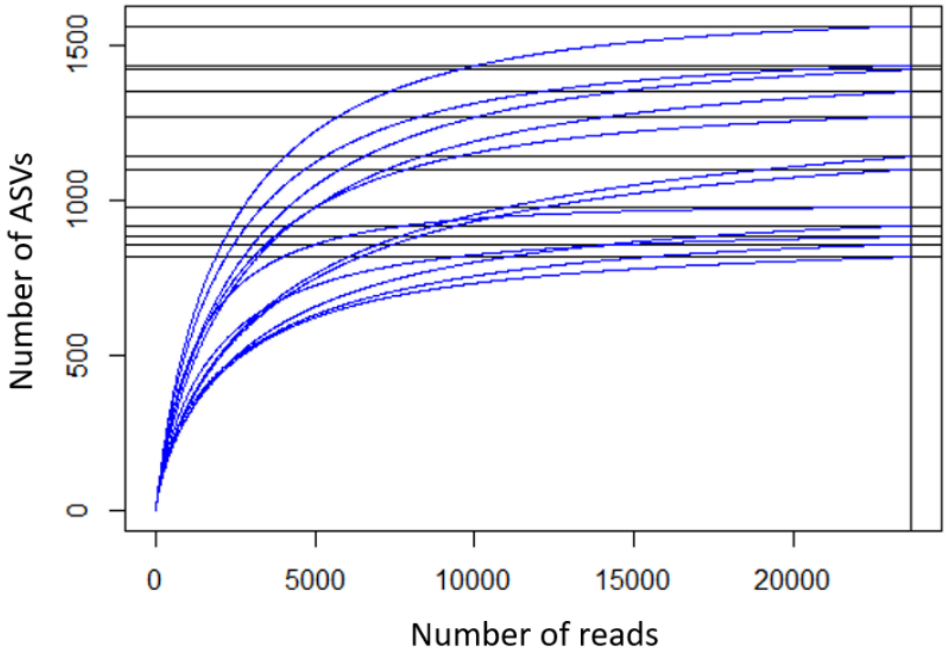


Figure 4.9: Rarefaction curves for all samples. The horizontal lines represent the stationary phase for each sample. The vertical line represent the ASVs rarefaction to an even number of reads per sample (n = 23695) to ensure an equal sampling depth for all samples.

Good quality reads were taxonomically classified using The Ribosomal Database Project (RDP), recovering Archaea (477 ASVs), Eukaryotes (37 ASVs) and Bacteria (9486 ASVs) taxa. Reads assigned to *Cyanobacteria*/*Chloroplasts* were detected, mainly in estuarine waters, accounting for 12% on average of the total relative abundance. No mitochondrial reads were detected.

After the first classification, Archaea and Eukaryotes reads were removed from the analyses, since the goal was the study of the bacterial communities, and the bacterial reads were rarefied into the minimum sequencing depth. From each sample, 23,695 sequences were retrieved and taxonomically annotated, revealing 8,999 different ASVs; of which 3,279 belonged to 36 phyla, 84 classes, 151 orders, 313 families, and 818 genera. The remaining 5,720 ASVs were considered unassigned at different levels (sequences with <80% similarity): 2,402 at the phylum level, 974 at the class level, 869 at the order level, and 1,475 at the family level.

At the phylum level, the bacterial communities present in all samples (estuarine microplastics, estuarine water, beach microplastics and beach sand) were dominated by *Proteobacteria* (48.67% to 50.95%) and *Bacteroidetes* (22.31% to 30.01%). Together, both phyla represented between 73.26% and 80.81% of the relative abundance in all samples (Fig. 4.10). Furthermore, estuarine microplastics presented a higher abundance of *Firmicutes* (>8%) when compared with the other samples (0.70 - 1.34%). These three phyla, *Proteobacteria*, *Bacteroidetes* and *Firmicutes*, are frequently the main phyla detected in microbial communities of microplastic biofilms from aquatic environments (Delacuvellerie et al., 2019; Dussud et al., 2018; Frère et al., 2018; Gong et al., 2019; Jiang et al., 2018; Kirstein et al., 2019; Zettler et al., 2013), and despite the *Proteobacteria* and *Bacteroidetes* being indeed the most abundant phyla in microplastic samples here they were also the most abundant phyla in the bacterial communities of the environmental matrices in a very similar extent. On the other hand, *Firmicutes* was only abundant in the estuarine microplastic samples. Typical sewage-associated microorganisms belong predominantly to the phylum *Firmicutes* (e.g., *Streptococcus*, *Lactobacillus*, *Blautia*, *Lachnospiraceae*, *Enterococcus*, *Ruminococcus*) (Oberbeckmann et al., 2015). This association suggests that the high abundance of *Firmicutes* on estuarine microplastics might be related to an WWTPs microplastic input, which can harbour these communities. Furthermore, *Cyanobacteria /Chloroplast* had a similar abundance in microplastic samples (2.11-3.63%), higher abundance in estuarine waters (13.73%), and residual abundance in beach sand (0.23%). The phyla *Planctomycetes* and *Acidobacteria* had higher representativity on beach samples than in the estuarine samples. On the opposite direction, the phylum *Campilobacterota* had higher representativity in estuarine samples. The extremophilic phylum *Deinococcus-Thermus* was present on the estuarine microplastics (>1%) but nearly absent from estuarine waters.

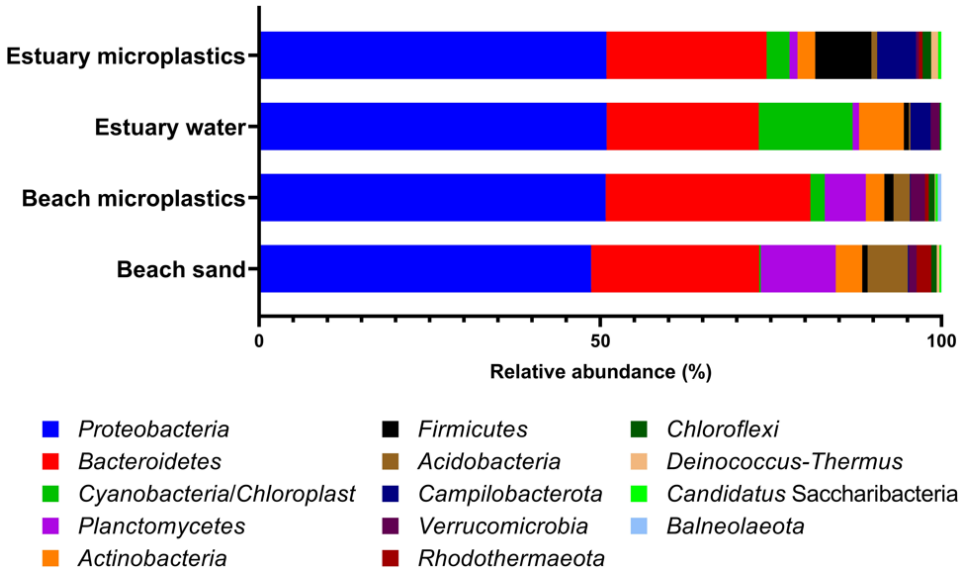


Figure 4.10: Relative abundance of bacterial phyla in the analysed samples. Phyla representing less than 0.1% are not represented.

At the class level, *Gammaproteobacteria*, *Alphaproteobacteria* and *Flavobacteriia* represented the major bacterial classes in all samples, with a combined representativity between 63.94% and 78.88% of relative abundance (Fig. 4.11). Members of the *Gammaproteobacteria* have been described as a dominant pioneer bacterial community on plastic biofilm formation, decreasing in relative abundance over time, being replaced by members of the *Alphaproteobacteria*, *Betaproteobacteria* and *Flavobacteria*, which increase their relative abundance (De Tender et al., 2017). In general, early pioneer bacterial communities in marine and estuarine microplastic biofilms belong to members of the *Gammaproteobacteria* and *Alphaproteobacteria* (Lee et al., 2008; Oberbeckmann et al., 2015). Furthermore, one *in situ* colonisation experiment revealed that diatoms (*Chloroplast*) dominated the plastic biofilms after one week, and by week two, many of the diatoms had been killed or grazed from the surface as other organisms attach, as *Cyanobacteria* and associated heterotrophic bacteria, and the community becomes more diverse (Amaral-Zettler et al., 2015). Therefore, the slightly lower relative abundance of *Gammaproteobacteria* and *Chloroplast* and the higher relative abundance of *Cyanobacteria* on the estuarine microplastics when compared with the beach microplastics might suggest a more recent microbial colonisation and community succession for the latter. The classes *Deltaproteobacteria* and *Bacilli* had higher abundances on the estuarine microplastics than in their water counterparts. In addition, the class *Planctomycetacia* had higher representativity in beach samples than in the estuarine samples. On the opposite, the classes *Cyanobacteria* and *Campylobacteria* had higher representativity on the estuarine samples.

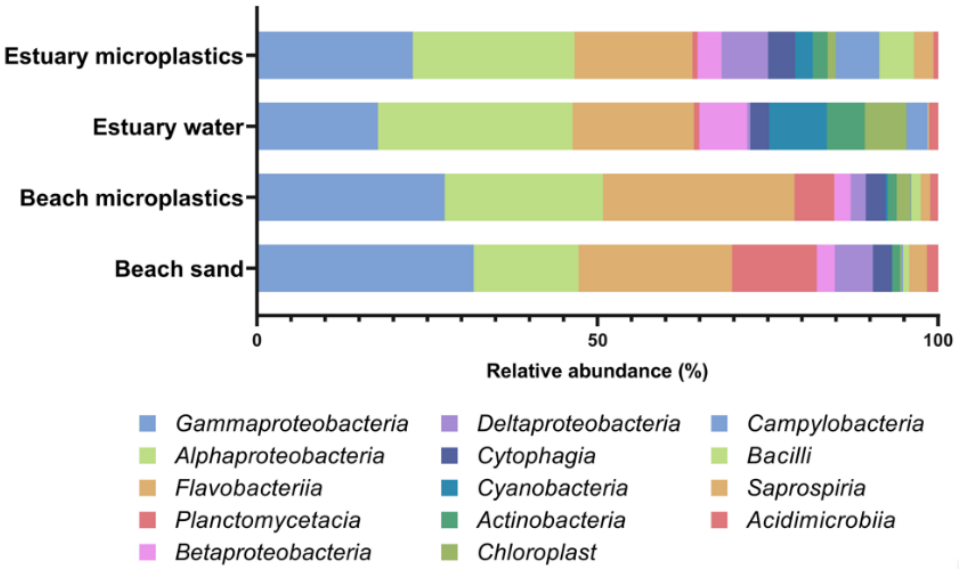


Figure 4.11: Relative abundance of bacterial classes in the analysed samples. Classes representing less than 1% are not represented.

At the order level, *Flavobacteriales* and *Rhodobacterales* presented high abundances in all samples, with 21.72% to 34.37% and 13.10% to 14.52% of relative abundance, respectively (Fig. 4.12). Once again, it has been reported that members of the order *Rhodobacterales* (*Alphaproteobacteria*) increased in relative abundance over time on plastic biofilms, while members of the order *Oceanospirillales* and *Alteromonadales* (*Gammaproteobacteria*) decrease (Dang et al., 2008; Wright et al., 2020). Therefore, the slightly higher relative abundance of *Rhodobacterales* and lower of *Oceanospirillales* and *Alteromonadales* on the estuarine microplastics when compared with the beach microplastics, might also again indicate a more recent microbial colonisation and community succession for the latter (as reported at the class level). Furthermore, the orders *Rhodobacterales*, *Sphingomonadales* and *Rhizobiales*, which have been reported to represent important microbial associations within the microbial communities of the "Plastisphere" (Jiang et al., 2018), had higher relative abundances on microplastics than in their environmental matrices. Members of the order SAR11, which are known to dominate the surface waters of the world's oceans (Schattenhofer et al., 2009), were highly abundant in the estuarine waters but nearly absent from the remaining samples. The orders *Oceanospirillales*, *Alteromonadales* and *Burkholderiales*, present in all samples, have been reported to be more abundant in plastic surfaces than in control biofilms (e.g., wood, cellulose, or glass) across multiple studies and environments and to have potential plastic biodegrading members (Ogonowski et al., 2018; Wright et al., 2020). Also, the orders *Bacillales*, *Desulfobacterales*, *Pseudomonadales* and *Saprospirales* had higher abundances on the estuarine microplastics than in the estuarine waters,

while Family VIII had lower abundance than their estuarine water counterparts (Fig. 4.7). In addition, orders *Chromatiales* and *Pirellulales* had higher representativity in beach samples than in the estuarine samples. On the opposite, the order *Campylobacterales* presented higher representativity in the estuarine samples.

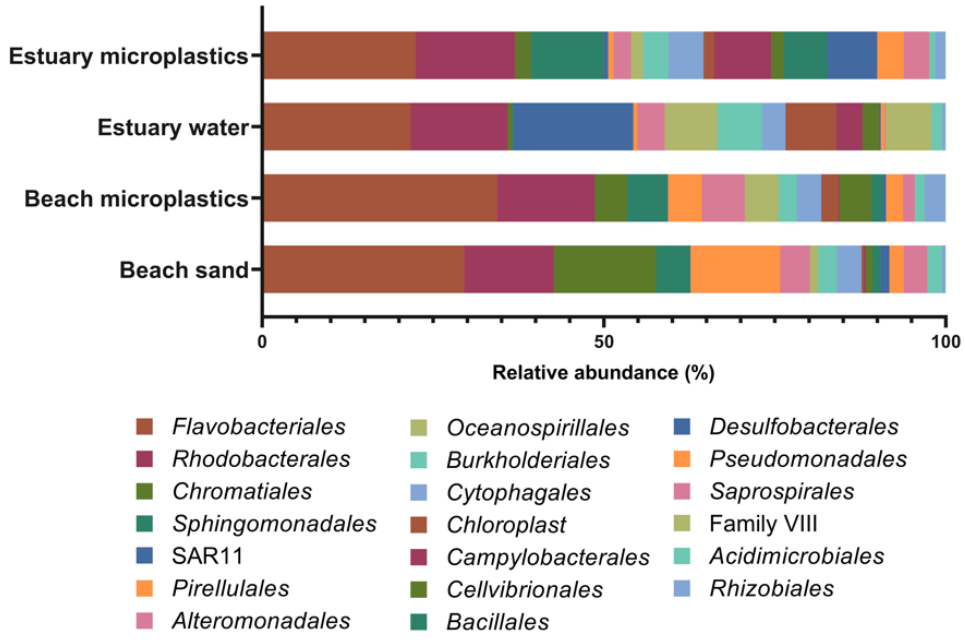


Figure 4.12: Relative abundance of bacterial orders in the analysed samples. Orders representing less than 1% are not represented.

At the family level, all samples presented a high abundance of *Flavobacteriaceae* (12.33% to 42.41%) and *Rhodobacteraceae* (17.17% to 19.30%) (Fig. 4.13). One meta-analysis reported that members of these two families are core members of the bacterial communities associated with PE microplastics (Oberbeckmann & Labrenz, 2020). Another study, an *in situ* colonisation experiment by Amaral-Zettler et al. (2015), reported *Flavobacteriaceae* as a microbial group that increases its abundance on plastics over time and *Rhodobacteraceae* as increasing its abundance on plastics until the second week, followed by a decreasing, although Pinto et al. (2019) reported that both *Flavobacteriaceae* and *Rhodobacteraceae* were abundant during the later stages of the microplastics colonisation. However, it is important to note that the family *Rhodobacteraceae*, here reported as abundant in microplastics is well known for its early and abundant colonisation of a broad range of particle surfaces (e.g., wood, cellulose, or glass) (Oberbeckmann & Labrenz, 2020). Once again, family SAR11, which are known to dominate the surface waters of the world's oceans (Schattenhofer et al., 2009) and linked to ocean anoxia and nitrogen loss processes (Tsementzi et al., 2016), was highly abundant in the estuarine waters but nearly absent from the

remaining samples. *Erythrobacteraceae*, a common marine plastic-colonising family, has been identified on different types of plastic and found on both macro- and microplastics (Rogers et al., 2020). Furthermore, *Erythrobacteraceae* was identified as containing potential hydrocarbon degraders with a higher abundance on plastics than water or sediments (Wu et al., 2020), which is in concordance with the results observed here. The families *Alteromonadaceae* and *Xanthomonadaceae* were substantially abundant on the estuarine microplastics but nearly absent from estuarine waters. These families have been identified as containing potential hydrocarbon degraders and to be more abundant in plastics than in other samples (Wright et al., 2020). In the case of *Xanthomonadaceae*, this family is also known for harbour pathogenic bacteria that can infect different hosts, including humans, animals and plants (Assis et al., 2017). Families *Cryomorphaceae*, Family VIII and *Cytophagaceae* had higher abundances on the estuarine microplastics than in the remaining samples. Also, the families *Thiovulaceae*, *Hymenobacteraceae* and *Desulfobacteraceae* within the estuarine samples had a higher representativity on microplastics than on their water counterparts (Fig. 4.8). Furthermore, the family *Pirellulaceae* had higher representativity in the beach samples than in the estuarine samples. On the opposite, families *Chloroplast* and *Comamonadaceae* presented higher representativity in the estuarine samples.

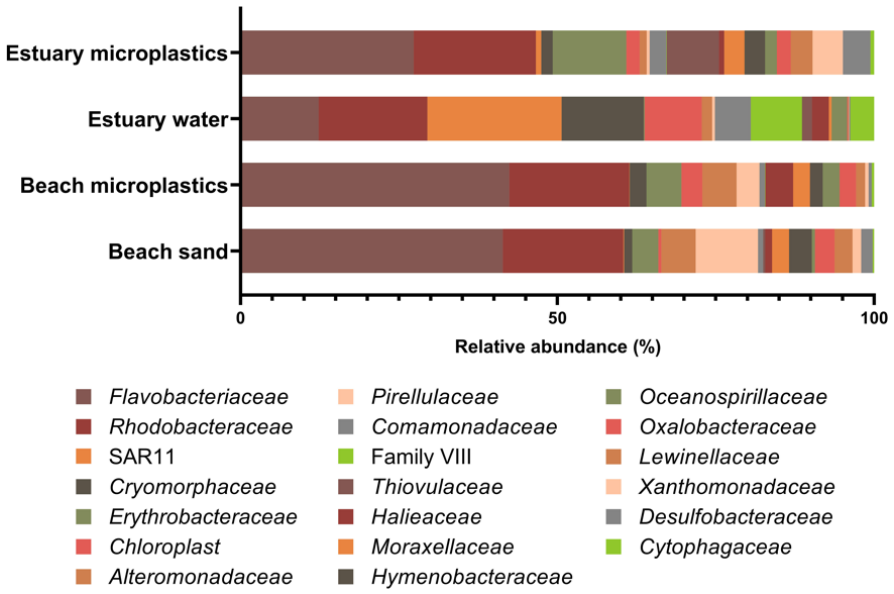


Figure 4.13: Relative abundance of bacterial Families in the analysed samples. Families representing less than 1% are not represented.

At the genus level, although estuarine microplastics, beach microplastics and beach sand presented relatively even genera relative abundance distributions, on the estuarine waters only four genera, *Candidatus* Pelagibacter, *Litoreibacter*, *Bacillariophyta* and *Foliisarcina*, represented nearly 72% of the relative genera abundance. Overall, high heterogeneity in genera relative abundances was observed between samples, especially between estuarine samples (water and microplastics). Although in the estuarine waters the genera *Candidatus* Pelagibacter, *Litoreibacter*, *Bacillariophyta* and *Foliisarcina* had a representativity of nearly 72%, in the estuarine microplastics they only accounted for just 10% of the relative abundance (Fig. 4.14).

The genus *Candidatus* Pelagibacter, which is known to dominate marine open waters but tend to be scarce on plastics debris (Amaral-Zettler et al., 2020), was also highly abundant in the estuarine waters but scarce in the estuarine microplastics and nearly absent from the beach samples (Fig. 4.14 and 4.15). The same pattern was observed for the genus *Litoreibacter*, which has been reported as commonly detected member on microplastics (Frère et al., 2018). The genus of the algae group *Bacillariophyta* is an abundant member of biofilms on many surfaces in aquatic ecosystems and a typic initial colonizer, is also reported in plastic biofilms (Oberbeckmann et al., 2018). Here, this genus was abundant in all samples except in the estuarine microplastics, where it was scarce. On the opposite, the genus *Foliisarcina* was highly abundant in the estuarine waters but practically absent from the remaining samples. In fact, this genus is not usually associated with microplastics in the scientific literature.

In addition, the genera *Erythrobacter*, *Paracoccus*, *Maribacter*, *Pseudoalteromonas*, *Winogradskyella* and *Gramella* were more abundant on microplastics than in their environmental matrices. The genus *Erythrobacter* is one of the most common and abundant members of the "Plastisphere", and it was demonstrated that members of this genus can degrade hydrocarbons in microplastic biofilms (Curren & Leong, 2019). Here, this genus was more abundant on microplastics, especially in the estuary, where a high abundance was observed (17.91%) but practically absent from the estuarine waters, reinforcing the observation that this genus is a core member of the "Plastisphere". The observation that the genus *Paracoccus* had a high abundance on microplastics but was scarce in the environmental matrices is an interesting result since this genus had not been previously associated with microplastics. Members of this genus are important components of the microbiomes of different pristine and polluted environments, with many *Paracoccus* strains been isolated from soil, brines and marine sediments, sewage, and biofilters (Lasek et al., 2018). Furthermore, the genus *Maribacter*, which had only been associated with plastics in a marine *in situ* incubation study (Oberbeckmann et al., 2016), had a higher abundance on microplastics, especially in the estuarine samples where it had a substantial abundance (12%) but completely absent from the water. This genus has been isolated from marine habitats and species, such as seawater, sediments and algae (Zhang et al., 2020). Since microplastics are usually reported to have a river-sea trajectory and this genus has only been

reported on marine habitats, this raises the question of how these estuarine microplastics have acquired these bacterial genera. Interestingly, the estuarine microplastics with the highest abundance of *Maribacter* was from the furthest estuarine sampling location from the river mouth. A reasonable explanation for this might be the saltwater intrusion into the estuary during tides. In fact, the estuarine sampling campaign occurred close to the maximum point of high tide and during the autumn, in which the influence of the saltwater intrusion extends further than the furthest estuarine sampling location from the river mouth. This occurrence suggests the existence of a substantial contribution of a sea-river trajectory to the overall estuarine microplastic contamination. The genus *Pseudoalteromonas* has been reported to harbour hydrocarbon degraders and potential pathogens and for being a commonly detected member on PP as well as on PE plastics but not in the background waters (Bowley et al., 2021; Oberbeckmann et al., 2015), which is in concordance with the reported higher abundance of this genus on microplastic samples in the present study. Additionally, this observation and the potential for this genus to harbour pathogens highlights the concern that microplastics may act as vectors of transmission and spread of these members in these transitional ecosystems. The genus *Winogradskyella*, which is known to be specific to sedimentary plastics and PE plastics and to harbour hydrocarbon degraders (Delacuvellerie et al., 2019; Kirstein et al., 2019), had also a higher relative abundance on microplastics when compared with the environmental matrices. The genus *Gramella*, which has been reported to be enriched on PE-associated biofilms and to be able to degrade polymeric carbon sources and hydrocarbons (Delacuvellerie et al., 2019), had a three-fold higher abundance on the beach microplastics than in the beach sand and was also present in the estuarine microplastics but absent from the estuarine waters.

The genus *Psychrobacter*, which has been reported in marine environments (Guern et al., 2014) and in biofilms associated with different marine plastics (Zettler et al., 2013), was indeed present in both beach samples (microplastics and sand). However, in the estuary, this genus was also substantially abundant on microplastics (7.25%) but scarce in the estuarine waters (0.21%). Once again, this genus is typically associated with marine environments with a high relative abundance on the estuarine microplastics, which reinforces the aforementioned suggestion of the existence of a substantial sea-river trajectory to the overall estuarine microplastic contamination. The genus *Zeaxanthinibacter*, which is reported to be hydrocarbon degraders and to be overexpressed in the presence of hydrocarbons (Delacuvellerie et al., 2019), had an expressive abundance in the beach sand (21.36%) and a substantial contribution in the beach microplastics (4.61%) but practically absent from the estuarine samples. The genus *Sulfitobacter*, which is commonly reported in marine environments and has been identified as primary surface colonizers in different coastal waters of the world (Dang et al., 2008), was indeed abundant in both beach samples (12.5% for sand and 6.19% for microplastics). However, this genus, which is also reported to be a common member of the microplastic biofilms (Basili et al., 2020; Delacuvellerie et al.,

2019), was also present in the estuarine microplastics (2.02%). The genus *Flavobacterium*, which can harbour fish pathogens and be reported to be abundant in PE microplastic biofilms (Gong et al., 2019), was found on both microplastic samples and in the estuarine samples, with the latter having a three-fold higher relative abundance on microplastics than in the estuarine waters. Most members of the genus *Massilia* have been isolated from soil, while others have been isolated from air, drinking water, rock surface, ice-core, glacier permafrost and human clinical samples (Dahal et al., 2021). Here, this genus was abundant to a similar extent on both beach samples and in the estuarine microplastics (5.32-6.08%) but was absent from the estuarine waters. This evidence might suggest a possible origin of some estuarine microplastics from agricultural areas present in the river basin, which can harbour these communities (soil). Lastly, the genus *Altererythrobacter*, which have been reported on marine environments, estuarine waters and in freshwater microplastics (Di Pippo et al., 2020; Lee, 2019), had a similar abundance in the beach samples but, interestingly, had also a high relative abundance (9.05%) in the estuarine microplastics and was absent from the estuarine waters.

The remaining unmentioned twelve genera had not been previously described as being associated with microplastics in the consulted scientific literature.

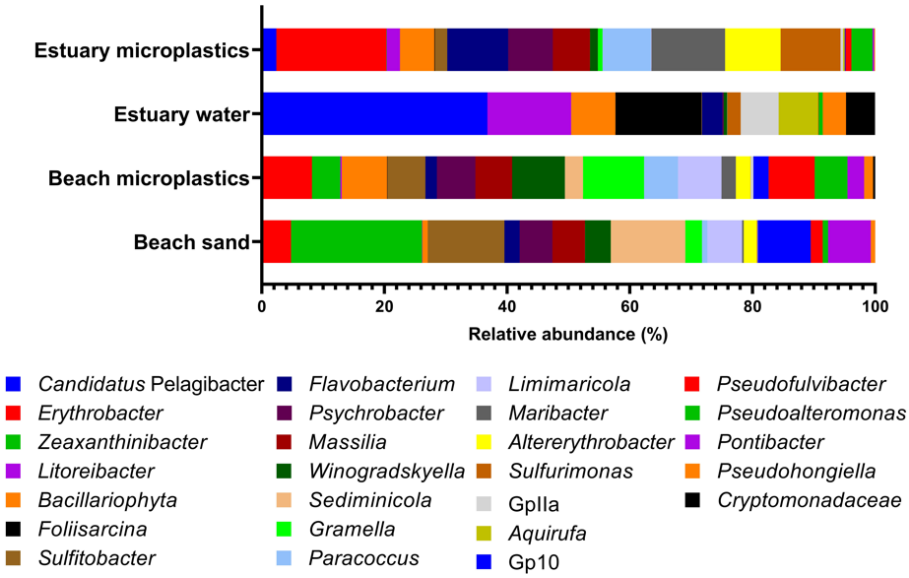


Figure 4.14: Relative abundance of bacterial genera in the analysed samples. Genera representing less than 1% are not represented.

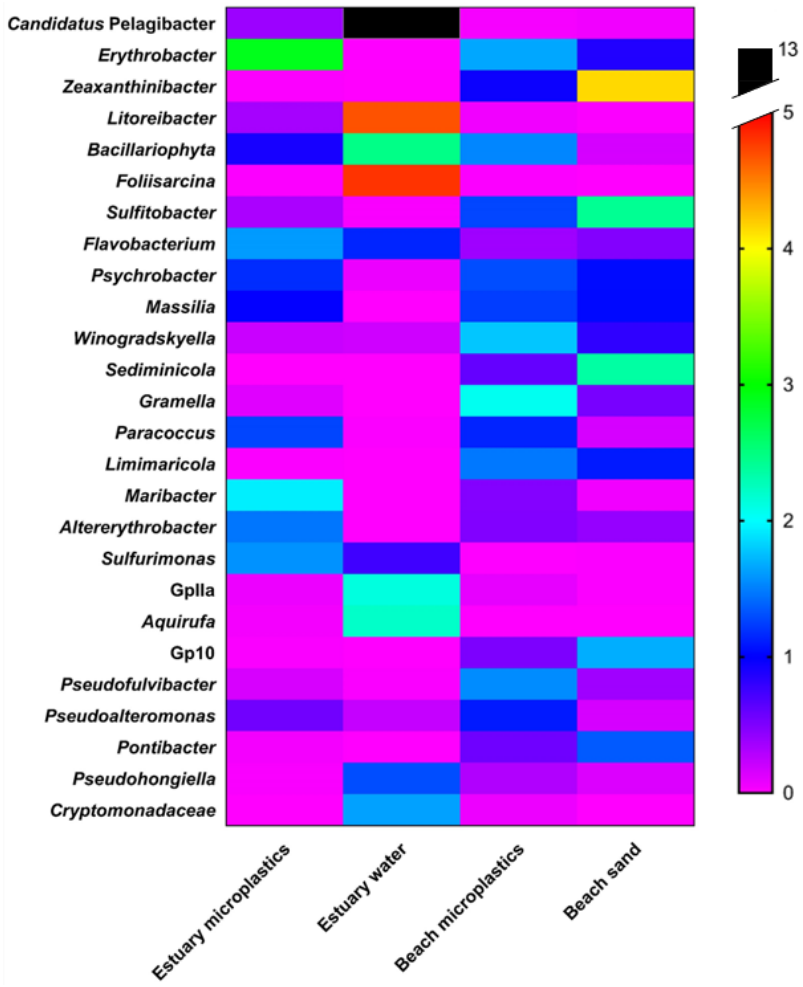


Figure 4.15: Heat map showing absolute abundances (mean values) of the most abundant bacterial genera (accumulated abundance >1%) across the sample types (estuary microplastics, estuary water, beach microplastics and beach sand).

4.2.2. Bacterial communities diversity

No significant differences in bacterial communities at the genus level were found between all samples (PERMANOVA Test, $p > 0.05$) (Table 4.2).

Table 4.2: P-values from the PERMANOVA Pairwise comparisons of bacterial communities between sample groups.

	Estuary microplastics	Estuary water	Beach microplastics	Beach sand
Estuary microplastics		0.1055	0.1002	0.0987
Estuary water	0.1055		0.1072	0.0969
Beach microplastics	0.1002	0.1072		0.2921
Beach sand	0.0987	0.0969	0.2921	

In both environments, the Shannon diversity and Pielou evenness indexes were higher in microplastics than in the environmental matrices (estuarine water and beach sand) (Table 4.3). However, the Kruskal-Wallis tests showed that these differences were not statistically significant (results not shown, *p* values > 0.05).

Table 4.3: Values of Shannon diversity index, Simpson diversity index, Pielou evenness index across samples. (Mean ± SE).

	Shannon	Simpson	Pielou
Estuary microplastics	5.90 ± 0.17	0.99 ± 0.00	0.83 ± 0.01
Estuary water	5.33 ± 0.14	0.98 ± 0.00	0.77 ± 0.00
Beach microplastics	6.20 ± 0.63	0.99 ± 0.00	0.86 ± 0.04
Beach sand	6.02 ± 0.27	0.99 ± 0.00	0.87 ± 0.02

No agreement has yet been reached on whether microplastic-associated bacterial communities present an increased or decreased diversity compared with their counterparts on natural particles or their environmental matrices. While some studies from aquatic ecosystems have reported similar or even higher α-diversities on microplastics, other studies have postulated the opposite (Oberbeckmann & Labrenz, 2020). On the other hand, It has been reported that microplastic biofilms are shaped primarily by biogeographical and environmental factors, such as salinity and nutrient concentration (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2018). Although significative differences were not observed in bacterial communities nor in bacterial community diversity indexes between samples within the same environment (estuarine and sandy beach), this might be an expected result since both bacterial communities (microplastics and environmental matrix) are under the influence of the same environmental factors at the time of the sampling events and there is no information regarding their residence time on these locations. On the other hand, the absence of significant differences in bacterial communities between microplastics (estuarine and sandy beaches) might also be an expected result not only by the fact that both microplastics samples were under relative geographical proximity, with an average distance between sandy beaches and estuarine sampling locations of 7.269 ± 1.443 km, but also by the aforementioned possibility of a sea-river microplastics trajectory.

4.2.3. Comparison of bacterial communities between samples

Although we did not observe significant differences in the diversity indexes between samples, we proceed to the analysis of genera identity and its comparison between samples. Venn diagrams revealed a higher percentage of unique genera in the total microplastics samples (36.9%) when compared with the unique genera found in the environmental matrices (10% for

estuarine water and 3.1% for beach sand). Also, the shared genera between all samples accounted for only 15% of all the observed diversity (Fig. 4.16A).

The higher proportion of shared genera between beach microplastics and beach sand (46.6%) than between estuarine microplastics and estuarine waters (34.7%) suggests that the surrounding beach sand had likely contributed with more bacterial members to the microplastic bacterial communities than the surrounding estuarine waters to the estuarine microplastics (Fig. 4.16B and 4.16C). The local environment was already suggested to serve as a bacterial source for plastic biofilm organisms. This means that most likely microplastics have a longer residence time on sandy beaches than in the estuarine environment. However, it is important to note that the dynamics of both environments are very different, contributing to biofilm formation in distinct ways. Furthermore, the unique genera found in both samples within the same environment (microplastics and environmental matrix) followed opposite directions. The beach microplastics had a substantially higher percentage of unique genera (42%) than their estuarine counterparts (18.8%). This might suggest that although beach microplastics probably had a longer residence time on sandy beaches they had most likely crossed other environments to acquire the unique genera that they presented. Finally, the estuarine water had a substantially higher percentage of unique genera (46.5%) than the beach sand (11.4%). This might be explained by the fact that many of the bacterial members on beach sand are natural biofilm formers and prefer a particle-attached lifestyle, similar to the bacterial communities found on microplastics, while on estuarine waters the bacterial members prefer a free-living over a particle-attached lifestyle.

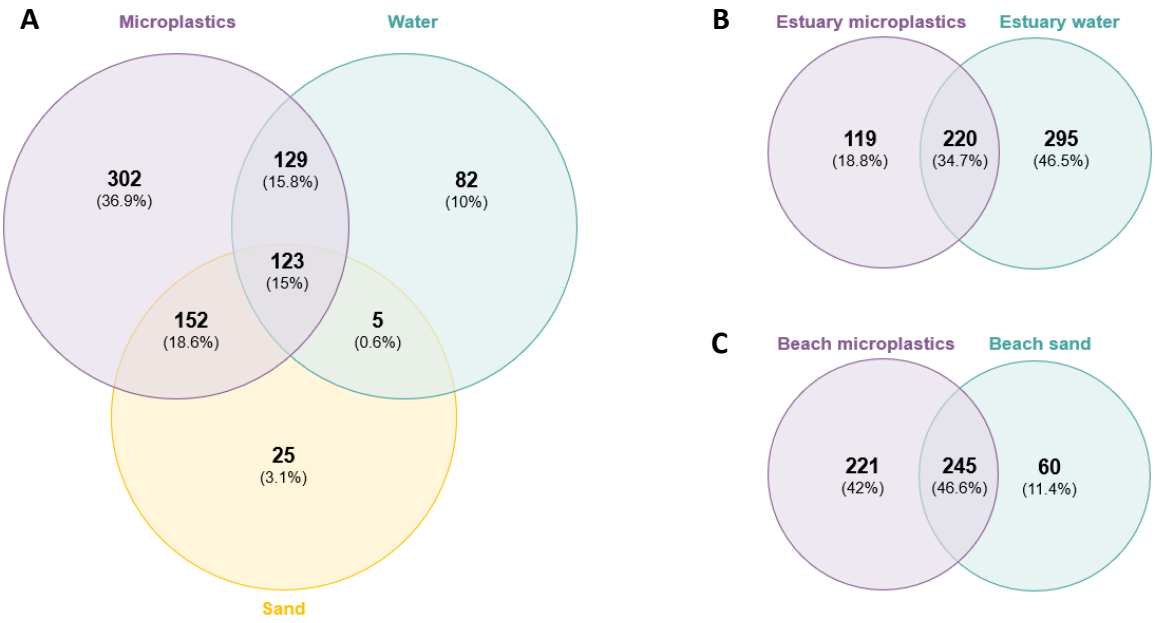


Figure 4.16: Venn diagrams representing the number and percentage (%) of unique and shared genera between: (A) Grouped microplastics, Estuary water and Beach sand; (B) Sandy beaches samples (Microplastics and Sand); (C) Estuary samples (Microplastics and Water).

To establish a potential correlation between bacterial communities of different samples, a Principal Coordinates Analysis (PCoA) was performed. The PCoA revealed that all samples were distinct, despite some relation between beach microplastics and beach sand (Fig. 4.17). The estuarine water bacterial communities were very distinct from the ones found in estuarine microplastics by both components 1 and 2, which explained 36.42% and 16.59% of the variance, respectively (Fig. 4.17).

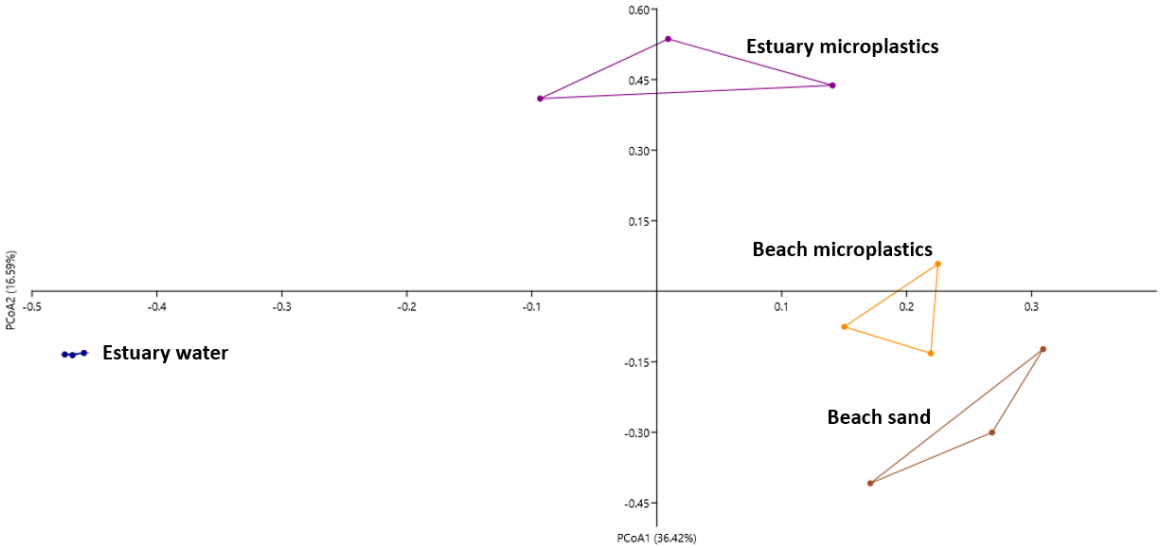


Figure 4.17: Principal coordinate analysis (PCoA) of the microbial communities based on Bray-Curtis (BC) similarity index as an estimator of taxonomic distance within and between sample types. PCoA1 (36.42%); PCoA2 (16.59%).

The previously described results were confirmed by the hierarchical clustering visualization (Fig. 4.13). Estuarine water bacterial communities clustered very close between sampling locations, presenting a high level of similarity (70%) and clustered away from all the remaining samples (estuarine microplastics, beach sand and beach microplastics) with only 20% similarity (Fig. 4.13), which shows that the bacterial communities on the estuarine microplastics presented higher similarity with the ones found on sandy beach samples (microplastics and sand) than with the ones found on estuarine waters. Furthermore, within the beach environment, the bacterial communities on the sand and on the microplastics were very close, appearing interspersed in the analysis (Fig. 4.18).

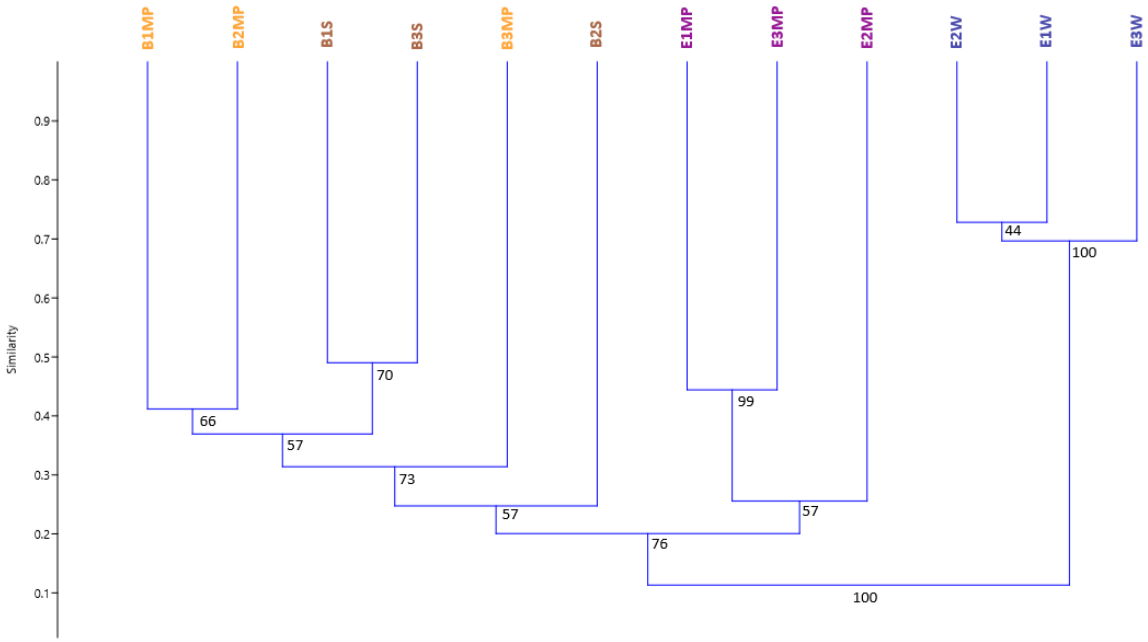


Figure 4.18: Hierarchical clustering based on Bray-Curtis distances of all samples. (E1W-E3W) Estuary water; (E1MP-E3MP) Estuary microplastics; (B1S-B3S) Beach sand; (B1MP-B3MP) Beach microplastics. Cluster displays similarity between samples.

SIMPER analysis revealed a higher overall average dissimilarity between the bacterial communities on the estuarine microplastics and those found in their environmental matrix (estuarine waters) (84.02%) than between beach microplastics and beach sand (68.15%). Furthermore, the dissimilarity between the bacterial communities found in both microplastic samples (estuarine microplastics and beach microplastics) was 76.85%, while the dissimilarity between bacterial communities found in both environmental matrices (estuarine and beach sand) was the highest, with 93.08% dissimilarity.

The top 50 contribution (percentage) responsible for the dissimilarity between estuarine samples (microplastics *vs* water) accounted for twenty-one genera, with *Candidatus* Pelagibacter presenting the highest contribution, 13.23%. In this environment, the genera *Maribacter*, *Altererythrobacter*, *Exiguobacterium*, *Hymenobacter* and *Dulcicalothrix* were present in the estuarine microplastics but absent from estuarine waters. The genus *Exiguobacterium* have already been reported to be present on microplastic-associated biofilm communities but not in the surrounding surface waters and have also been reported to be capable of degrading polystyrene (Chauhan et al., 2018; G. Yang et al., 2021; Y. Yang et al., 2015).

The top 50 contribution (percentage) responsible for the dissimilarity between beach samples (microplastics *vs* sand) accounted for twenty-eight genera. The contributions to the dissimilarity (%) presented high homogeneity, with all genera presenting low contributions to the

dissimilarity (<6%). The genus *Flavimarina* was the only genus from the twenty-eight to be present in beach microplastics yet absent from the beach sand.

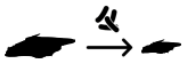
Finally, the top 50 contribution (percentage) responsible for the dissimilarity between microplastic samples (estuarine microplastics *vs* beach microplastics) accounted for forty genera and also presented low percentages of contribution to the dissimilarity (<3%). The genera *Clostridium*, *Dulcicalothrix*, *Pseudoxanthomonas*, *Halospirulina*, *Veillonella*, *Desulfobacterium* and *Comamonas* were present only on the estuary microplastics, while *Aurantimonas* and *Sediminicola* were only present on the beach microplastics. Members of the genus *Clostridium*, which was only present on estuarine microplastics, includes several relevant human and animal pathogens, such as *Clostridium botulinum*, *Clostridium perfringens* and *Clostridium difficile* (Gibbs, 2009). Members of the genus *Comamonas*, which was only found on estuarine microplastics, includes potential pathogens (Martínez-Campos et al., 2021).

The results observed on the PCoA, hierarchical clustering and SIMPER analysis revealed higher proximity and similarity between the bacterial communities on the beach microplastics and those found in beach sand than between the estuarine samples (microplastics and water). Interestingly, they also revealed that the bacterial communities on the estuarine microplastics had a closer proximity and similarity with the bacterial communities from the beach samples (microplastics and sand) than with those found on their environmental matrix (estuarine waters). This is in concordance with the aforementioned observation of the occurrence and high abundance of typical marine-associated bacterial genera on the estuarine microplastics that were scarce or even absent from estuarine waters. Therefore, this evidence further supports the suggested existence of a substantial contribution of a sea-river trajectory to the overall estuarine microplastic contamination.

Furthermore, beyond the previously mentioned bacterial taxa with potential pathogenic members, WWTPs/sewage-associated or potential hydrocarbon/plastic degrading members, were found other unique genera on the microplastic samples from both transitional ecosystems (estuarine and sandy beaches) that presented one or more of the aforementioned characteristics (Table 4.2). The presence of potentially pathogenic genera unique to microplastics highlights the potential for these particles as vectors of dissemination of key bacterial groups in transitional ecosystems. This poses potential ecological risks in these environments, as well as for human health, that requires further attention and research. For instance, in sandy beaches with high potential for recreational use, especially during the bathing season where the human presence is higher, this can increase the risk of human exposure to these potential pathogens through microplastics but also for seabirds and/or other species that are commonly found feeding on the seashore. In addition, the presence of WWTPs/Sewage-associated genera on microplastics in transitional ecosystems such as the estuary might indicate an entry point of some of those

particles in the riverine/estuarine system through WWTP/Sewage. Finally, although it has been observed the presence of potential hydrocarbon/plastic degrading genera on microplastics, which can be important for bioremediation processes, the time and efficiency of these members to biodegrade plastics is still unclear and they might play a negligible role in the biodegradation of microplastics in natural conditions (Oberbeckmann & Labrenz, 2020).

Table 4.2: Other key bacterial genera unique to microplastics on both environments (Estuary and Sandy beaches); WWTP – Wastewater Treatment Plants.

 Potential pathogenic genera	 WWTP/Sewage-associated genera	 Potential plastic/hydrocarbon degrading genera
	Estuary	
Lactococcus	*Aquabacterium*	*Aquabacterium*
Staphylococcus	*Blautia*	*Staphylococcus*
	Lactobacillus	
	Lactococcus	
	Prosthecobacter	
	Reyranella	
	Iamia	
	Staphylococcus	
	Sandy beaches	
Acinetobacter	*Acinetobacter*	*Acinetobacter*
Mycobacterium	*Fluviicola*	*Alcanivorax*
Shewanella	*Mycobacterium*	*Glaciecola*
Staphylococcus	*Paludibacter*	*Gracilimonas*
	Reyranella	*Hyphomonas*
	Staphylococcus	*Idiomarina*
	Streptococcus	*Neptuniibacter*
		Neptunomonas
		Oceaniserpentilla
		Oleispira
		Staphylococcus
		Stappia
		Thalassospira

5 Conclusions and Future Perspectives

5 Conclusions and Future Perspectives

This study aimed to profile the bacterial communities of microplastics and compare them with their respective environmental compartments (water and sediments) from transitional ecosystems, more specifically in estuarine and sandy beach environments, and to address the potential role of these particles as vectors of dissemination of key bacterial groups, such as pathogens. In general, the present work provides new insights into the comprehension of the microplastic contamination in estuarine systems. The occurrence of typical marine-associated bacteria on estuarine microplastics and its absence and/or scarcity from estuarine waters, as well as overall higher proximity of the bacterial communities on estuarine microplastics with the beach samples than with the estuarine waters, suggests the existence of a substantial contribution of a sea-river trajectory to the overall estuarine microplastic dynamics. Furthermore, this might suggest the possibility of some microplastics engaging in a river-sea-river cycle, leading to microplastic retention in the estuarine systems. Additionally, this also suggests the existence of an overestimation of the contribution of the river-sea trajectory to the estuarine microplastic contamination and highlights the importance of the study of the microplastic-associated bacterial communities as a "storyteller" factor in the microplastic environmental contamination.

Furthermore, the "Plastisphere" analysed in the present study revealed the occurrence of key bacterial groups, such as potential pathogens, WWTPs/sewage-associated and potential hydrocarbon/plastic degraders, that were unique to microplastics or were found in higher abundances than in the studied transitional ecosystems. This evidence reinforces the concern that microplastics can act as vectors of transmission and spread of these bacterial groups and their potential ecological consequences in these ecosystems as well as for human health.

Reports suggesting that even in an extremely ambitious scenario (no further emissions of plastics) the level of microplastics in the environment will continue to increase, highlighting the necessity of further research on the "Plastisphere" and its potential ecological and human health risks. This is of particular relevance in estuarine and sandy beach transitional environments that represent important human activities and ecological services. This work provided new insights and possibilities about these topics but further research is required to answer the open questions left here: how the tidal range and seasonality affects the microplastic-bacterial communities in transitional ecosystems and the sea-river trajectory of microplastics into estuarine systems? Are the bacterial communities from seawater microplastics closer to the estuary similar to the ones found on estuarine water microplastics? Is there a sea-river-sea microplastics cycle? How similar/different are the estuarine microplastics-bacterial communities from the bacterial communities of known microplastic sources, such as WWTPs/sewage effluents? Does ingested microplastics by organisms such as fish or seabirds also harbour these key bacterial groups? If so,

does this translate into a higher risk of infections or disease for the individual or the community? Does this occur in estuarine commercial fish and does it present any risk for human health? Does the current concentration of microplastics, which can harbour or be “hotspots” of potential pathogens, in beaches presents any risks for human health? If not, is there a concerning threshold level?

To answer all these questions further research will be required to improve and depict the major challenges related to this recent scientific research area.

References

References

Amaral-Zettler, L. A., Zettler, E. R., & Mincer, T. J. (2020). Ecology of the plastisphere. In *Nature Reviews Microbiology*. https://doi.org/10.1038/s41579-019-0308-0

Andrady, A. L. (2015). Persistence of plastic litter in the oceans. In M. Bergmann, L. Gutow, & M. Klages (Eds.), *Marine Anthropogenic Litter* (Springer, pp. 57–72). https://doi.org/10.1007/978-3-319-16510-3_3

Association, U. S. T. M. (n.d.). *What's in a tyre*. U.S. Tire Manufacturers Association. Retrieved March 10, 2021, from https://www.ustires.org/whats-tire-0

Auta, H. S., Emenike, C. U., & Fauziah, S. H. (2017). Distribution and importance of microplastics in the marine environmentA review of the sources, fate, effects, and potential solutions. In *Environment International*. https://doi.org/10.1016/j.envint.2017.02.013

Baekeland, L. H. (1909). Original papers: The synthesis, constitution, and uses of bakelite. *Industrial and Engineering Chemistry*. https://doi.org/10.1021/ie50003a004

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*. https://doi.org/10.1016/j.marpolbul.2018.01.044

Bessa, F., Gonçalves, S. C., Franco, J. N., André, J. N., Cunha, P. P., & Marques, J. C. (2014). Temporal changes in macrofauna as response indicator to potential human pressures on sandy beaches. *Ecological Indicators*, *41*, 49–57. https://doi.org/10.1016/j.ecolind.2014.01.023

Besseling, E., Wegner, A., Foekema, E. M., Van Den Heuvel-Greve, M. J., & Koelmans, A. A. (2013). Effects of microplastic on fitness and PCB bioaccumulation by the lugworm Arenicola marina (L.). *Environmental Science and Technology*. https://doi.org/10.1021/es302763x

Boucher, J., & Friot, D. (2017). Primary microplastics in the oceans: A global evaluation of sources. In *Primary microplastics in the oceans: A global evaluation of sources*. https://doi.org/10.2305/iucn.ch.2017.01.en

Bowley, J., Baker-Austin, C., Porter, A., Hartnell, R., & Lewis, C. (2021). Oceanic Hitchhikers – Assessing Pathogen Risks from Marine Microplastic. In *Trends in Microbiology* (Vol. 29, Issue 2, pp. 107–116). Elsevier Ltd. https://doi.org/10.1016/j.tim.2020.06.011

Browne, M. A., Crump, P., Niven, S. J., Teuten, E., Tonkin, A., Galloway, T., & Thompson, R. (2011). Accumulation of microplastic on shorelines woldwide: Sources and sinks. *Environmental Science and Technology*. https://doi.org/10.1021/es201811s

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, *108*(Supplement 1), 4516–4522. https://doi.org/10.1073/PNAS.1000080107

Carpenter, E. J., & Smith, K. L. (1972). Plastics on the Sargasso sea surface. *Science*. https://doi.org/10.1126/science.175.4027.1240

Chin, L. W., & Fung, T. H. (2019). Plastic in Marine Litter. In *Issues in Environmental Science and Technology*. https://doi.org/10.1039/9781788013314-00021

Choy, C. A., Robison, B. H., Gagne, T. O., Erwin, B., Firl, E., Halden, R. U., Hamilton, J. A., Katija, K., Lisin, S. E., Rolsky, C., & S. Van Houtan, K. (2019). The vertical distribution and biological transport of marine microplastics across the epipelagic and mesopelagic water column. *Scientific Reports*. https://doi.org/10.1038/s41598-019-44117-2

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 and Technology*. https://doi.org/10.1021/es504525u

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., & Galloway, T. S. (2013). Microplastic ingestion by zooplankton. *Environmental Science and Technology*. https://doi.org/10.1021/es400663f

Comeau, A. M., Douglas, G. M., & Langille, M. G. I. (2017). Microbiome Helper: a Custom and Streamlined Workflow for Microbiome Research. *MSystems*, *2*(1). https://doi.org/10.1128/msystems.00127-16

Cunningham, E. M., Ehlers, S. M., Dick, J. T. A., Sigwart, J. D., Linse, K., Dick, J. J., & Kiriakoulakis, K. (2020). High Abundances of Microplastic Pollution in Deep-Sea Sediments: Evidence from Antarctica and the Southern Ocean. *Environmental Science and Technology*. https://doi.org/10.1021/acs.est.0c03441

De Falco, F., Di Pace, E., Cocca, M., & Avella, M. (2019). The contribution of washing processes of synthetic clothes to microplastic pollution. *Scientific Reports*. https://doi.org/10.1038/s41598-019-43023-x

De Tender, C., Schlundt, C., Devriese, L. I., Mincer, T. J., Zettler, E. R., & Amaral-Zettler, L. A. (2017). A review of microscopy and comparative molecular-based methods to characterize “Plastisphere” communities. In *Analytical Methods*. https://doi.org/10.1039/c7ay00260b

Diepens, N. J., & Koelmans, A. A. (2018). Accumulation of Plastic Debris and Associated Contaminants in Aquatic Food Webs. *Environmental Science and Technology*. https://doi.org/10.1021/acs.est.8b02515

Dris, R., Gasperi, J., Rocher, V., Saad, M., Renault, N., & Tassin, B. (2015). Microplastic contamination in an urban area: A case study in Greater Paris. *Environmental Chemistry*. https://doi.org/10.1071/EN14167

Eerkes-Medrano, D., Thompson, R. C., & Aldridge, D. C. (2015). Microplastics in freshwater systems: A review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. In *Water Research*. https://doi.org/10.1016/j.watres.2015.02.012

Eriksen, M., Lebreton, L. C. M., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., Galgani, F., Ryan, P. G., & 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*. https://doi.org/10.1371/journal.pone.0111913

European Bioplastics. (2019). *Bioplastics – Facts and Figures*.

Fok, L., & Cheung, P. K. (2015). Hong Kong at the Pearl River Estuary: A hotspot of microplastic pollution. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2015.07.050

Frias, J., Pagter, E., Nash, R., O’Connor, I., Carretero, O., Filgueiras, A., Viñas, L., Gago, J., Antunes, J., Bessa, F., Sobral, P., Goruppi, A., Tirelli, V., Pedrotti, M. L., Suaria, G., Aliani, S., Lopes, C., Raimundo, J., Caetano, M., & Gerdts, G. (2018). *Standardised protocol for monitoring microplastics in sediments*. https://doi.org/10.13140/RG.2.2.36256.89601/1

Gall, S. C., & Thompson, R. C. (2015). The impact of debris on marine life. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2014.12.041

GESAMP. (2015). *Sources, fate and effects of microplastics in the marine environment: a global assessment* (P. Kershaw (ed.)).

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

Gonçalves, S. C., Anastácio, P. M., Pardal, M. A., Cardoso, P. G., Ferreira, S. M., & Marques, J. C. (2009). Sandy beach macrofaunal communities on the western coast of Portugal - Is there a steady structure under similar exposed conditions? *Estuarine, Coastal and Shelf Science*, *81*(4), 555–568. https://doi.org/10.1016/j.ecss.2008.12.004

Good, T. P., June, J. A., Etnier, M. A., & Broadhurst, G. (2010). Derelict fishing nets in Puget Sound and the Northwest Straits: Patterns and threats to marine fauna. *Marine Pollution Bulletin*.

https://doi.org/10.1016/j.marpolbul.2009.09.005

Hammer, D. A. T., Ryan, P. D., Hammer, Ø., & Harper, D. A. T. (2001). Past: Paleontological Statistics Software Package for Education and Data Analysis. In *Palaeontologia Electronica* (Vol. 4, Issue 1). http://palaeo-electronica.orghttp//palaeo-electronica.org/2001_1/past/issue1_01.htm.

Hartmann, N. B., Hüffer, T., Thompson, R. C., Hassellöv, M., Verschoor, A., Daugaard, A. E., Rist, S., Karlsson, T., Brennholt, N., Cole, M., Herrling, M. P., Hess, M. C., Ivleva, N. P., Lusher, A. L., & Wagner, M. (2019). Are We Speaking the Same Language? Recommendations for a Definition and Categorization Framework for Plastic Debris. *Environmental Science and Technology*, *53*(3), 1039–1047. https://doi.org/10.1021/acs.est.8b05297

Hoornweg, D., Bhada-Tata, P., & Kennedy, C. (2013). Waste production must peak this century. In *Nature*. https://doi.org/10.1038/502615a

Illumina. (n.d.). *IMPORTANT NOTICE This document provides information for an application for 16S Metagenomic Sequencing Library Preparation Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System*.

Isobe, A., Uchida, K., Tokai, T., & Iwasaki, S. (2015). East Asian seas: A hot spot of pelagic microplastics. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2015.10.042

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

Lebreton, L. C. M., Van Der Zwet, J., Damsteeg, J. W., Slat, B., Andrady, A., & Reisser, J. (2017). River plastic emissions to the world's oceans. *Nature Communications*. https://doi.org/10.1038/ncomms15611

Lebreton, L., Egger, M., & Slat, B. (2019). A global mass budget for positively buoyant macroplastic debris in the ocean. *Scientific Reports*. https://doi.org/10.1038/s41598-019-49413-5

Lenz, R., Enders, K., & Nielsen, T. G. (2016). Microplastic exposure studies should be environmentally realistic. In *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1606615113

Lusher, A. L., McHugh, M., & Thompson, R. C. (2013). Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2012.11.028

Mammo, F. K., Amoah, I. D., Gani, K. M., Pillay, L., Ratha, S. K., Bux, F., & Kumari, S. (2020). Microplastics in the environment: Interactions with microbes and chemical contaminants. In *Science of the Total Environment* (Vol. 743, p. 140518). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2020.140518

Masó, M., Garcés, E., Pagès, F., & Camp, J. (2003). Drifting plastic debris as a potential vector for dispersing Harmful Algal Bloom (HAB) species | Los plásticos flotantes son potenciales vectores de dispersión de especies formadoras de proliferaciones algales nocivas. *Scientia Marina*, *67*(1), 107–111. https://doi.org/10.3989/scimar.2003.67n1107

Maximenko, N., Hafner, J., & Niiler, P. (2012). Pathways of marine debris derived from trajectories of Lagrangian drifters. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2011.04.016

McCormick, A., Hoellein, T. J., Mason, S. A., Schluep, J., & Kelly, J. J. (2014). Microplastic is an abundant and distinct microbial habitat in an urban river. *Environmental Science and Technology*. https://doi.org/10.1021/es503610r

McLusky, D. S., & Elliott, M. (2010). The Estuarine Ecosystem. In *The Estuarine Ecosystem*. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780198525080.001.0001

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Millet, H., Vangheluwe, P., Block, C., Sevenster, A., Garcia, L., & Antonopoulos, R. (2019). The Nature of Plastics and Their Societal Usage. In *Issues in Environmental Science and Technology*. https://doi.org/10.1039/9781788013314-00001

Mincer, T. J., Zettler, E. R., & Amaral-Zettler, L. A. (2019). Biofilms on Plastic Debris and Their Influence on Marine Nutrient Cycling, Productivity, and Hazardous Chemical Mobility. In *Handbook of Environmental Chemistry*. https://doi.org/10.1007/698_2016_12

Murphy, F., Ewins, C., Carbonnier, F., & Quinn, B. (2016). Wastewater Treatment Works (WwTW) as a Source of Microplastics in the Aquatic Environment. *Environmental Science and Technology*. https://doi.org/10.1021/acs.est.5b05416

Naidoo, T., Glassom, D., & Smit, A. J. (2015). Plastic pollution in five urban estuaries of KwaZulu-Natal, South Africa. *Marine Pollution Bulletin*, *101*(1), 473–480. https://doi.org/10.1016/j.marpolbul.2015.09.044

Napper, I. E., Bakir, A., Rowland, S. J., & Thompson, R. C. (2015). Characterisation, quantity and sorptive properties of microplastics extracted from cosmetics. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2015.07.029

Nunes, M., Marchand, P., Vernisseau, A., Bizec, B. Le, Ramos, F., & Pardal, M. A. (2011). PCDD/Fs and dioxin-like PCBs in sediment and biota from the Mondego estuary (Portugal). *Chemosphere*, *83*(10), 1345–1352. https://doi.org/10.1016/j.chemosphere.2011.02.081

Oberbeckmann, S., & Labrenz, M. (2020). Marine Microbial Assemblages on Microplastics: Diversity, Adaptation, and Role in Degradation. In *Annual Review of Marine Science*. https://doi.org/10.1146/annurev-marine-010419-010633

Oberbeckmann, S., Osborn, A. M., & Duhaime, M. B. (2016). Microbes on a Bottle: Substrate, Season and Geography Influence Community Composition of Microbes Colonizing Marine Plastic Debris. *PLOS ONE*, *11*(8), e0159289. https://doi.org/10.1371/journal.pone.0159289

Pabortsava, K., & Lampitt, R. S. (2020). High concentrations of plastic hidden beneath the surface of the Atlantic Ocean. *Nature Communications*. https://doi.org/10.1038/s41467-020-17932-9

Pham, C. K., Ramirez-Llodra, E., Alt, C. H. S., Amaro, T., Bergmann, M., Canals, M., Company, J. B., Davies, J., Duineveld, G., Galgani, F., Howell, K. L., Huvenne, V. A. I., Isidro, E., Jones, D. O. B., Lastras, G., Morato, T., Gomes-Pereira, J. N., Purser, A., Stewart, H., … Tyler, P. A. (2014). Marine litter distribution and density in European seas, from the shelves to deep basins. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0095839

Pilz, H., & Brandt, B. (2011). The impact of plastic packaging on life cycle energy consumption and greenhouse gas emissions in Europe. In *Denkstatt*.

Plastic Soup Foundation. (n.d.). *Beat the Microbead*. Retrieved February 22, 2021, from https://www.beatthemicrobead.org/

PlasticsEurope. (2017). *Plastics – The Facts 2017: An Analysis of European Plastics Production, Demand and Waste Data*.

PlasticsEurope. (2020). *Plastics – The Facts 2020: An Analysis of European Plastics Production, Demand and Waste Data*.

Provencher, J. F., Bond, A. L., Avery-Gomm, S., Borrelle, S. B., Bravo Rebolledo, E. L., Hammer, S., Kühn, S., Lavers, J. L., Mallory, M. L., Trevail, A., & Van Franeker, J. A. (2017). Quantifying ingested debris in marine megafauna: A review and recommendations for standardization. In *Analytical Methods*. https://doi.org/10.1039/c6ay02419j

R Core Team. (2016). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.r-project.org/

Rogers, K. L., Carreres-Calabuig, J. A., Gorokhova, E., & Posth, N. R. (2020). Micro-by-micro interactions: How microorganisms influence the fate of marine microplastics. *Limnology and Oceanography Letters*. https://doi.org/10.1002/lol2.10136

Rosner, B., Glynn, R. J., & Lee, M. L. T. (2006). The Wilcoxon signed rank test for paired comparisons of clustered data. *Biometrics*, *62*(1), 185–192. https://doi.org/10.1111/j.1541-0420.2005.00389.x

Rotjan, R. D., Sharp, K. H., Gauthier, A. E., Yelton, R., Baron Lopez, E. M., Carilli, J., Kagan, J. C., & Urban-Rich, J. (2019). Patterns, dynamics and consequences of microplastic ingestion by the temperate

coral, Astrangia poculata. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1905). https://doi.org/10.1098/rspb.2019.0726

SAPEA, S. A. for P. by E. A. (2019). *A Scientific Perspective on Microplastics in Nature and Society*. https://doi.org/10.26356/microplastics

SAPEA, S. A. for P. by E. A. (2020). *Biodegradability of plastics in the open environment*. https://doi.org/10.26356/biodegradabilityplastics

Schlundt, C., Mark Welch, J. L., Knochel, A. M., Zettler, E. R., & Amaral-Zettler, L. A. (2020). Spatial structure in the "Plastisphere": Molecular resources for imaging microscopic communities on plastic marine debris. *Molecular Ecology Resources*. https://doi.org/10.1111/1755-0998.13119

Silva, D. C. C., Neto, J. M., Nunes, C., Gonçalves, F. J. M., Coimbra, M. A., Marques, J. C., & Gonçalves, A. M. M. (2021). Assessment of seasonal and spatial variations in the nutritional content of six edible marine bivalve species by the response of a set of integrated biomarkers. *Ecological Indicators*, *124*, 107378. https://doi.org/10.1016/j.ecolind.2021.107378

Stanier, R. Y. (1975). Sieburth, J. M. 1975. Microbial seascapes. A pictorial essay on marine microorganisms and their environments. University Park Press, Baltimore. Unpaged. $9.50. *Limnology and Oceanography*. https://doi.org/10.4319/lo.1975.20.6.1059

Stokstad, E. (2020). Why were salmon dying? The answer washed off the road. *Science*. https://doi.org/10.1126/science.370.6521.1145

Stothra Bhashyam, S., Nash, R., Deegan, M., Pagter, E., & Frias, J. (2021). *Microplastics in the marine environment: Sources, Impacts & Recommendations*. http://research.thea.ie/handle/20.500.12065/3593

Teixeira, Z. F. G. (2016). *Resilience to land use induced impacts*. http://hdl.handle.net/10316/29520

Tekman, M. B., Wekerle, C., Lorenz, C., Primpke, S., Hasemann, C., Gerdts, G., & Bergmann, M. (2020). Tying up Loose Ends of Microplastic Pollution in the Arctic: Distribution from the Sea Surface through the Water Column to Deep-Sea Sediments at the HAUSGARTEN Observatory. *Environmental Science and Technology*. https://doi.org/10.1021/acs.est.9b06981

Textile Exchange. (2020). *2020 Preferred Fiber and Materials Market Report*.

Thompson, R. C., & Napper, I. E. (2019). Microplastics in the Environment. In *Issues in Environmental Science and Technology*. https://doi.org/10.1039/9781788013314-00060

Thompson, R. C., Olsen, Y., Mitchell, R. P., Davis, A., Rowland, S. J., John, A. W. G., McGonigle, D., & Russell, A. E. (2004). Lost at Sea: Where Is All the Plastic? *Science*, *304*(5672), 838 LP – 838. https://doi.org/10.1126/science.1094559

Tian, Z., Zhao, H., Peter, K. T., Gonzalez, M., Wetzel, J., Wu, C., Hu, X., Prat, J., Mudrock, E., Hettinger, R., Cortina, A. E., Biswas, R. G., Kock, F. V. C., Soong, R., Jenne, A., Du, B., Hou, F., He, H., Lundeen, R., … Kolodziej, E. P. (2021). A ubiquitous tire rubber–derived chemical induces acute mortality in coho salmon. *Science*. https://doi.org/10.1126/science.abd6951

Tourinho, P. S., Ivar do Sul, J. A., & Fillmann, G. (2010). Is marine debris ingestion still a problem for the coastal marine biota of southern Brazil? *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2009.10.013

UNEP, & NOAA. (2012). *The Honolulu Strategy – A Global Framework for Prevention and Management of Marine Debris*. https://marinedebris.noaa.gov/sites/default/files/publications-files/Honolulu_Strategy.pdf

Van Cauwenberghe, L., & Janssen, C. R. (2014). Microplastics in bivalves cultured for human consumption. *Environmental Pollution*. https://doi.org/10.1016/j.envpol.2014.06.010

Van Sebille, E., Wilcox, C., Lebreton, L., Maximenko, N., Hardesty, B. D., Van Franeker, J. A., Eriksen, M., Siegel, D., Galgani, F., & Law, K. L. (2015). A global inventory of small floating plastic debris. *Environmental Research Letters*. https://doi.org/10.1088/1748-9326/10/12/124006

van Wezel, A., Caris, I., & Kools, S. A. E. (2016). Release of primary microplastics from consumer products

to wastewater in the Netherlands. *Environmental Toxicology and Chemistry*. https://doi.org/10.1002/etc.3316

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07

Wickham, H. (2016). *ggplot2*. Springer International Publishing. https://doi.org/10.1007/978-3-319-24277-4

Worm, B., Lotze, H. K., Jubinville, I., Wilcox, C., & Jambeck, J. (2017). Plastic as a Persistent Marine Pollutant. *Annual Review of Environment and Resources*. https://doi.org/10.1146/annurev-environ-102016-060700

Ye, S., & Andrady, A. L. (1991). Fouling of floating plastic debris under Biscayne Bay exposure conditions. *Marine Pollution Bulletin*. https://doi.org/10.1016/0025-326X(91)90249-R

Zettler, E. R., Mincer, T. J., & Amaral-Zettler, L. A. (2013). Life in the “plastisphere”: Microbial communities on plastic marine debris. *Environmental Science and Technology*. https://doi.org/10.1021/es401288x

Supplementary Data

S1. DNA extraction protocol

MoBio Powersoil DNA extraction kit (MoBio Laboratories, CA, USA). Adapted from DNeasy® PowerSoil® Pro Kit Handbook (http://www.qiagen.com/dk/), Debeljak et al., 2016 and McCormick et al., 2014.

Procedure

1. To the provided tubes add either the microplastics or the plastic fragments. Gently vortex to mix.
2. Add 60 µL of Solution C1 and invert several times or vortex briefly.
3. Add 10 µL of Ready Lyse Lysozyme (diluted to 1000 U/µL) to each tube and invert several times or vortex briefly.
4. Incubate at 37ºC for 30 minutes.
5. Bead-beat for 7 minutes using bead-beating apparatus.
6. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature.
7. Transfer the supernatant to a clean 2 ml collection tube.
8. Add 250 µL of Solution C2 and vortex for 5 seconds. Incubate at 4ºC for 5 minutes.
9. Centrifuge the tubes at 10,000 x g for 1 minute at room temperature.
10. Avoiding the pallet, transfer up to, but no more than, 600 µL of supernatant to a clean 2 ml collection tubes.
11. Add 200 µL of Solution C3 and vortex briefly. Incubate at 4ºC for 5 minutes.
12. Centrifuge the tubes at 10,000 x g for 1 minute at room temperature.
13. Avoiding the pallet, transfer up to, but no more than, 750 µL of supernatant into a clean 2 ml collection tubes.
14. Shake to mix Solution C4 before use. Add 1200 µL of Solution C4 to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µL onto a Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µL of supernatant to the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature.
16. Add 500 µL of Solution C5 and centrifuge at 10,000 x g for 30 seconds at room temperature.
17. Discard the flow through.
18. Centrifuge again at 10,000 x g for 1 minute at room temperature.
19. Carefully place the Spin Filter in a clean 2 mL collection tube. Avoid splashing any Solution C5 onto the Spin Filter.
20. Add 40 µL of DNA-Free PCR Grade Water to the center of the white filter membrane.
21. Centrifuge at 10,000 x g for 30 seconds at room temperature.
22. Discard the Spin Filter. The DNA is in the tube and ready for any downstream application.