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Zooplankton and microplastics interferences in the Douro and Lima estuaries across temporal and spatial differences

Maria Francisca Tavares Brochado Teixeira Espincho

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Maria Francisca Tavares Brochado Teixeira Espincho

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Orientador – Doutora Sandra Cristina da Costa e Silva Ramos
Categoria – Investigadora
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental
Co-orientador - Doutora Cristina Marisa Ribeiro de Almeida
Categoria – Investigadora
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental

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Abstract

Plastic pollution and particularly, microplastics (MPs), represent an emerging concern worldwide. Microplastics (MPs) are plastic debris with less than 5 mm in size, and their presence has been confirmed in many aquatic environments, including estuaries.

Estuaries are important transitional ecosystems between rivers and oceans, and these environments provide important ecosystem services and play an important role for several species, including zooplankton.

Smaller marine organisms, such as zooplankton, demand increased attention regarding contaminants, since they are particularly vulnerable to environmental threats, and they play an important base role in the marine food web. In fact, a perturbation in zooplankton's well-being may condition proper ecosystem functioning and trophic chain interactions. MPs pose a risk to these organisms, since they can be mistaken as food and ingested. Upon ingestion, MPs may cause injuries and possibly death of these organisms. However, field studies regarding interactions between MPs and lower trophic organisms such as zooplankton are still scarce.

The present work aimed to evaluate MPs contamination of zooplankton from the Douro and Lima estuaries (NW Portugal). To achieve this, four surveys were conducted during 1-year to collect zooplankton and water samples from different sites in each estuary. In the laboratory, major zooplanktonic groups were quantified using a Bogorov chamber. MP presence in estuarine water and in two of the most abundant zooplankton groups (copepods and chaetognaths) was assessed, using dedicated protocols previously optimized. MPs retrieved from water samples and zooplanktonic organisms were characterized by size, shape and colour, and the polymer was identified by Fourier-transform infrared spectroscopy (FTIR) analysis. Results showed that MPs were present in all water samples, with the two estuaries presenting similar average MP concentrations (Lima: 2.4 ± 2.0 MPs m^{-3}; Douro: 2.3 ± 1.9 MPs m^{-3}). Zooplanktonic organisms were also contaminated with MPs. Chaetognaths exhibited higher MP contamination in both Lima (5.3 ± 5.2 MPs ind^{-1}) and Douro estuaries (5.9 ± 6.1 MPs ind^{-1}). On the other hand, copepods tended to have lower levels of MPs contamination (Lima: 2.4 ± 2.1 MPs ind^{-1}; Douro: 3.7 ± 4.1 MPs ind^{-1}). Such differences between MP concentration of these two zooplankton groups could indicate the possibility of MP trophic transfers at the lower levels of the food web. Another important finding was the fact that MPs found in zooplanktonic organisms did not reflect the MPs present in the surrounding water. Small size blue fragments were the most common MP among zooplanktonic organisms, while in estuarine waters, fibers were the most

common MP, and a variety of colours were observed. Such results may indicate a potential MP selection by zooplanktonic organisms. Overall, the present study showed MP contamination of estuarine environments and zooplankton organisms, highlighting the possibility of MP trophic transfers between copepods and chaetognaths.

Resumo

A poluição por plásticos e particularmente, por microplásticos (MPs), representa uma preocupação emergente a nível mundial. Os microplásticos (MPs) são detritos plásticos com menos de 5 mm de dimensão e cuja presença foi confirmada em muitos ambientes aquáticos, incluindo estuários.

Os estuários são importantes ecossistemas de transição entre rios e oceanos, e estes ambientes fornecem importantes serviços aos ecossistemas e desempenham um papel importante para muitas espécies, incluindo o zooplâncton.

Os organismos marinhos pequenos, como o zooplâncton, exigem uma atenção acrescida no que respeita a contaminantes, uma vez que são particularmente vulneráveis a ameaças ambientais e desempenham um importante papel na base da cadeia alimentar marinha. De facto, uma perturbação no bem-estar do zooplâncton pode condicionar o bom funcionamento do ecossistema e interações da cadeia trófica. Os MPs representam um risco para estes organismos, uma vez que podem ser confundidos com alimentação e ingeridos. Após a ingestão, os MPs podem causar lesões e até a morte destes organismos. No entanto, estudos de campo sobre interações entre MPs e organismos de nível trófico inferior, como o zooplâncton, ainda são escassos.

O presente trabalho teve como objetivo avaliar a contaminação por MPs do zooplâncton dos estuários do Douro e do Lima (NW Portugal). Para o efeito, foram realizadas quatro campanhas durante um ano para recolha de amostras de zooplâncton e de água em diferentes locais de cada estuário. No laboratório, os principais grupos de zooplâncton foram quantificados utilizando uma câmara de Bogorov. A presença de MPs na água estuarina e em dois dos grupos de zooplâncton mais abundantes (copépodes e quetógnatas) foi avaliada, utilizando protocolos previamente otimizados. Os MPs recuperados das amostras de água e de amostras de zooplâncton foram caracterizados por tamanho, forma e cor, e o polímero foi identificado por análise de espetroscopia no infravermelho por transformado de Fourier (FTIR). Os resultados mostraram que MPs estavam presentes em todas as amostras de água, com os dois estuários a apresentarem concentrações médias semelhantes de MPs (Lima: 2,4 ± 2,0 MPs m^{-3}; Douro: 2,3 ± 1,9 MPs m^{-3}). O zooplâncton estava também contaminado com MPs. As quetógnatas apresentaram uma maior contaminação por MPs nos estuários do Lima (5,3 ± 5,2 MPs ind^{-1}) e do Douro (5,9 ± 6,1 MPs ind^{-1}). Por outro lado, os copépodes tenderam a apresentar níveis mais baixos de contaminação por MPs (Lima: 2,4 ± 2,1 MPs ind^{-1}; Douro: 3,7 ± 4,1 MPs ind^{-1}). Estas diferenças entre a concentração de MP nestes dois grupos de

zooplâncton podem indicar a possibilidade de transferências tróficas de MP nos níveis inferiores da cadeia alimentar.

Outra descoberta importante foi o facto de os MPs encontrados no zooplâncton não refletirem os MPs presentes na água. Fragmentos azuis de pequenas dimensões foram os MPs mais comuns entre o zooplâncton, enquanto que nas águas estuarinas as fibras foram o MPs mais comuns, tendo sido observada uma variedade de cores. Estes resultados podem indicar uma potencial seleção de MPs pelo zooplâncton. De um modo geral, o presente estudo revelou a contaminação por MPs de ambientes estuarinos e zooplâncton, salientando a possibilidade de transferências tróficas de MPs entre copépodes e quetógnatas.

Scientific outputs

I. Espincho, F., Pereira, R., Rodrigues, S. M., Silva, D. M., Almeida, C. M. R. and Ramos, S. (2023) Assessing microplastic contamination in zooplanktonic organisms from two river estuaries (submitted to Marine Pollution Bulletin).

II. Rodrigues, S.M., Espincho, F., Elliott, M., Almeida, C. Marisa R., Ramos, S. (2023) Methodology optimization to quantify microplastics present in planktonic organisms (submitted to MethodsX).

III. Espincho, F., Pereira, R., Rodrigues, S. M., Silva, D. M., Almeida, C. M. R. and Ramos, S. (2023) Microplastics contamination of zooplankton from the Douro and Lima estuaries. Poster presentation in BTC – Bue Think Conference, CIIMAR - Interdisciplinary Center of Marine and Environmental Research, Terminal de Cruzeiros de Leixões, Av. General Norton de Matos, Matosinhos - 13th September 2023.

IV. Espincho, F., Pereira, R., Rodrigues, S. M., Silva, D. M., Almeida, C. M. R. and Ramos, S. (2023) Microplastics contamination of zooplankton from the Douro and Lima estuaries. Oral presentation in IJUP – Investigação Jovem na Universidade do Porto, Reitoria da Universidade do Porto, Praça Gomes Teixeira, Porto - 11th May 2023.

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List of Abbreviations

ANOVA - One-way analysis of variance

BCP - Biological Carbon Pump

BPA - bisphenol A

MP - microplastic

PE – polyethylene

PET - polyethylene terephthalate

PP - polypropylene

PS – polystyrene

PVC - polyvinyl chloride

PUR - polyurethanes

PoTSs - potentially toxic substances

OECD - Organization for Economic Cooperation and Development

Chapter 1- General Introduction

1) Plastic production

Plastics are, by definition, synthetic polymers usually manufactured with the use of fossil fuels and, on a lesser scale, biomass such as cellulose, salt and renewable compounds (e.g. sugar cane, maize or plant oils) (UNEP, 2016). They are often divided into two categories: *thermoplastic*, which can be re-moulded and plastically altered when heated, and *thermoset* plastic, which can not be re-shaped by heat once formed (UNEP, 2016).

From the first development of plastics in the 19th century until current modern times, the use of plastic has grown exponentially to the levels we see today, in which plastic is a major indispensable component in everyday life, and in most industry sectors. In European countries, plastic is mostly used in everyday packaging, the construction and automotive sectors, as well as in electrical and electronic pieces (Plastics Europe et al., 2022). Several characteristics of plastics have dictated their popularity and widespread in society, such as their durability, strength, resistance to corrosion, thermal and electric insulation, versatility and low production cost (Food and Agriculture Organization of the United Nations, 2017).
It is estimated that plastic production in 2021 reached 390.7 million metric tons worldwide, with fossil fuels-based plastics representing 90% of total production (Figure 1) (Plastics Europe et al., 2022). The most commonly produced plastics are, in their majority, thermoplastic, such as polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), however, polystyrene (PS) and polyethylene terephthalate (PET) are also highly produced (Hahladakis et al., 2018).

However, plastic waste management still has not developed to the standard of plastic production and the short lifespan of plastic use in society (e.g. single-use plastic objects) and the persistence of plastic in the environment when discarded. The Organization for Economic Cooperation and Development (OECD) estimates that only 9% of plastic is recycled worldwide, in opposition to 57% of plastic ending in landfills, 29% incinerated and around 6% of plastic is mismanaged and uncollected (OECD, 2022). Due to the longevity of plastic, it is predicted that, with the exception of incinerated plastic waste, all plastic produced since the start of mass plastic production persists in the environment (Food and Agriculture Organization of the United Nations, 2017). The plastic that is incinerated or deposited in a landfill (amounting to the majority of plastic managed) also imposes environmental and economic setbacks, proving to be unsustainable long-term solutions (Erdle & Eriksen, 2023). Plastic in landfills may escape or release contaminants into the soils and groundwater if improperly managed (Hahladakis et al., 2018). On the other hand, incinerated plastic also presents environmental consequences, due to the release of CO_2

and toxicants such as dioxins and small hazardous particles upon combustion (Hohn et al., 2020).

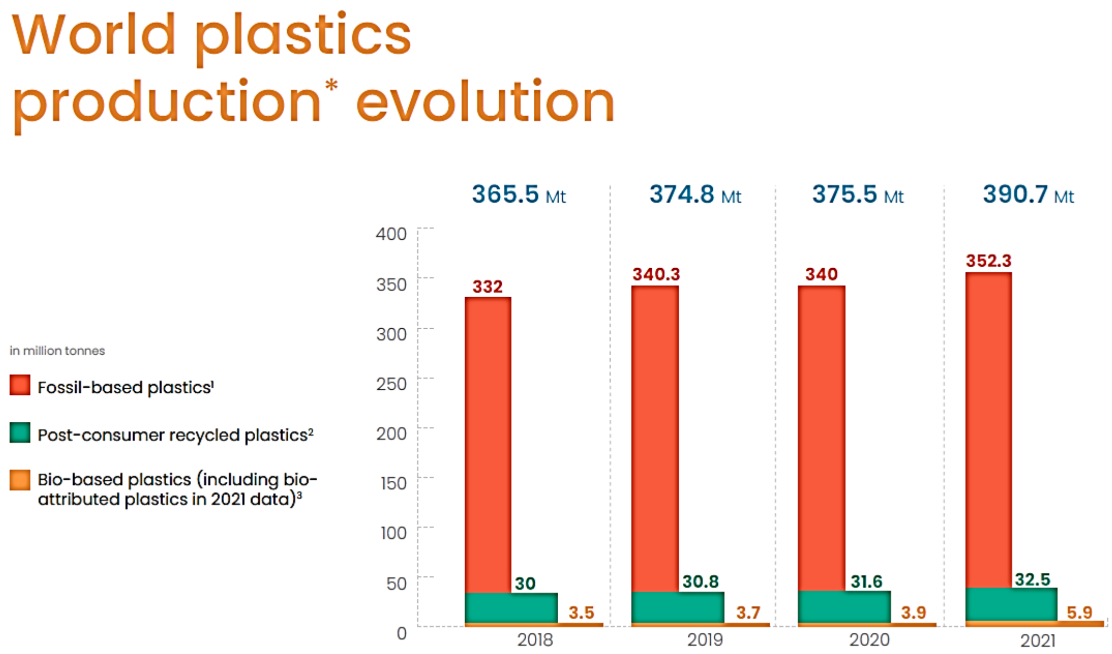

Fig.1 Plastic production, in million tones, worldwide.

*including plastics production from polymerization and production of mechanically recycled plastics. Polymers that are not used in the conversion of plastic parts and products (i.e., for textiles, adhesives, sealants, coatings, etc.) are not included.

1. Includes fossil-based thermoplastics, thermosets and polyurethanes (PUR)
2. Data on post-consumer recycled plastics had been developed in 2018, data for other years are estimations
3. Including bio-attributed plastic in 2021 data. Source: nova-institute 2022; data for bio-based structural polymers, preliminary estimations for 2021

(Adapted from Plastics Europe, 2022)

Plastic items are the most abundant type of marine debris (UNEP, 2016). The presence of plastic pollution was first reported on seabirds in 1962 by Rothstein (1973) and floating in marine environments by Carpenter & Smith Jr (1972) in the 1970s. A study conducted by Jambeck et al. (2015) estimated that between 4.8 to 12.7 million metric tons of plastic entered the ocean in 2010. Plastic contamination in the oceans is partly located on the ocean surface - Eriksen et al. (2014) estimated that 5.25 trillion plastic particles could be floating on the ocean surface. However, recent studies also highlight contamination in the deep sea, reporting unprecedented concentrations of plastic being deposited on the seafloor (Ding et al., 2022; Pabortsava & Lampitt, 2020).

Jambeck et al. (2015) predicted that mismanaged plastic waste entering aquatic environments will substantially increase by an order of magnitude by 2025, with predictions for the cumulative sum of plastic debris from 2010 to 2025 to reach aquatic environments to be between 100 and 250 million MT. This emerging problem is more severe in areas with high population density, and without proper waste management solutions, consequently, plastic can easily accumulate in unprecedented quantities in landfills and aquatic environments such as rivers, lakes and oceans (Hoornweg et al., 2013). Other land-based sources are also associated with plastic contamination of the oceans, such as littering, harbour activities and tourism, or overflow of sewage systems – overall, land-based sources account for approximately 90% of marine debris entering the oceans, and rivers and estuaries can often have a role in the transport of the plastic debris from land to the oceans (Pinheiro et al., 2021).

Plastic pollution in marine environments can also derive directly from marine-based sources and activities such as fishing boats, recreational activities, ships and off-shore platforms (Food and Agriculture Organization of the United Nations, 2017).

Different types of plastic have different characteristics that shape their behaviour in the marine environment. Characteristics like density affect plastic's buoyancy, which determines if plastic floats in the water column or sinks. Their composition may also determine how and at what rate a piece of plastic will degrade over time, eventually turning brittle, losing its mechanical integrity and fragmenting into smaller pieces. The degradation agent may be physical, such as high temperatures or UV light sources; chemical, such as oxidation; ionic radiation; or hydrolysis. Some air pollutants can also influence the fragmentation and degradation of plastic, such as carbon monoxide (CO), sulphur dioxide (SO_2), or ozone (O_3) (Hahladakis et al., 2018). Along the degradation process of plastic, certain characteristics may change, like the density of the polymer, which can then affect the buoyancy capability, leading a previously sinkable plastic to float to the surface water.

Furthermore, plastics can have chemical compounds, either intentionally added to plastic during the manufacturing process, or by absorption of chemical compounds present in the aquatic environments. During the manufacturing process, "additives" such as flame retardants, antioxidants, light and thermal stabilizers, and pigments, can be added to plastics to enhance performance, functionality and the ageing processes (Hahladakis et al., 2018). Several of these additives, as well as chemical compounds that plastic can absorb from the environment, are classified as potentially toxic substances (PoTSs), such as phthalates, bisphenol A (BPA), lead, tin, cadmium, formaldehyde and acetaldehyde, benzene and other volatile organic compounds (Hahladakis et al., 2018). Plastics can act as transport vectors of those chemical compounds, even increasing their environmental persistence (Ivar do Sul & Costa, 2014; UNEP, 2016). All these chemical compounds pose an additional threat to organisms that ingest/ absorb those plastics.

2) Microplastics (MPs)

Microplastics (MPs) have been receiving increased attention from the scientific community in recent years, mostly associated with their ubiquity occurrence and their impact on aquatic environments. MPs are commonly defined as plastic debris with a diameter below 5 mm at their longest diameter (Arthur, 2009). They are often categorized as primary MPs or secondary MPs, depending on their size at the time of their manufacturing. Primary MPs are intentionally manufactured in small sizes (<5 mm), and are generally produced with industrial applications, such as 'scrubbers', plastic powders used for moulding, or plastic nanoparticles; or in cosmetic formulations such as cleansers and exfoliating products (Arthur, 2009; Fendall & Sewell, 2009). Secondary MPs result from the breakdown of larger plastic items, either during the handling of the products or after they were discarded and released into the environment (Arthur, 2009). The breakdown and degradation of plastic, through factors such as UV radiation, abrasion, water and wind movements, leads to the loss of mechanical integrity, embrittlement and fragmentation into smaller pieces, forming MPs. The constant exposure to these factors can degrade MPs as well, leading to the appearance of even smaller MPs, and even nanoplastics (Rodrigues et al., 2019a; UNEP, 2016).

MPs are disseminated all over the globe in distinct ecosystems. Regarding aquatic environments, MPs have been detected in several of these environments, such as beaches, rocky shores, seabed sediments, estuaries, salt marshes, coral reefs, mangroves, seagrass, as well as wastewater effluents, surface waters, and freshwater systems (Figure 2) (Karbalaei et al., 2018). The concentration of MPs in these environments is variable and depends on several factors, such as wind and current conditions, geographical properties and the presence of urban areas and shipping trade routes.

Regarding MP presence in oceans, prevailing winds and surface ocean currents are considered as the main drivers of plastic transport from their source to other areas (Alfonso et al., 2021). On shorelines and beaches, MP debris is a mix of materials from local sources and debris transported by the wind or waves (Horton et al., 2017). Depending on several factors, these spots may act as permanent accumulation zones of debris, or zones that enable the transport of MPs to other areas (UNEP, 2016). MPs are also often found on coastal waters, due to the pressure from fisheries, aquaculture, shipping and other marine activities (Zhang et al., 2021; Higgins & Turner, 2023). In other remote marine areas, the appearance of MPs occurs mostly due to ocean bottom currents and wind movements, and the MPs found tend to present higher density, larger size and a higher degree of ageing and weathering (Y. Ding et al., 2022)

The accumulation of MPs in aquatic environments is more susceptible to occur in enclosed or semi-enclosed areas, where wind and water currents are less influential, and the enclosed nature enhances the accumulation of MPs. For example, the Mediterranean Sea is an example of such an area where a series of factors join together to create a prime spot for MP contamination: it has a heavily populated coastline and several major shipping routes that prove as sources for MP contamination, and an enclosed geography with low fluxes of water circulation (Gérigny et al., 2022; Ourmieres et al., 2023).

MPs in aquatic environments are also exposed to organisms such as algae, invertebrates, bacteria and other microorganisms, which can then grow and develop on the surface of the MPs, forming a biofilm. These biofilms may influence the density and sinking capacity of the MP, shifting its place in the water column – a high-density biofilm will sink the MP deeper in the water column, while a low-density biofilm can turn a MP buoyant and closer to the ocean surface (Nguyen et al., 2020). Due to the durability and travelling capacity of MPs, the biofilms that form around MPs could also contribute to the dispersion of invasive species to different areas of the globe, even remote coastal areas (Gregory, 2009).

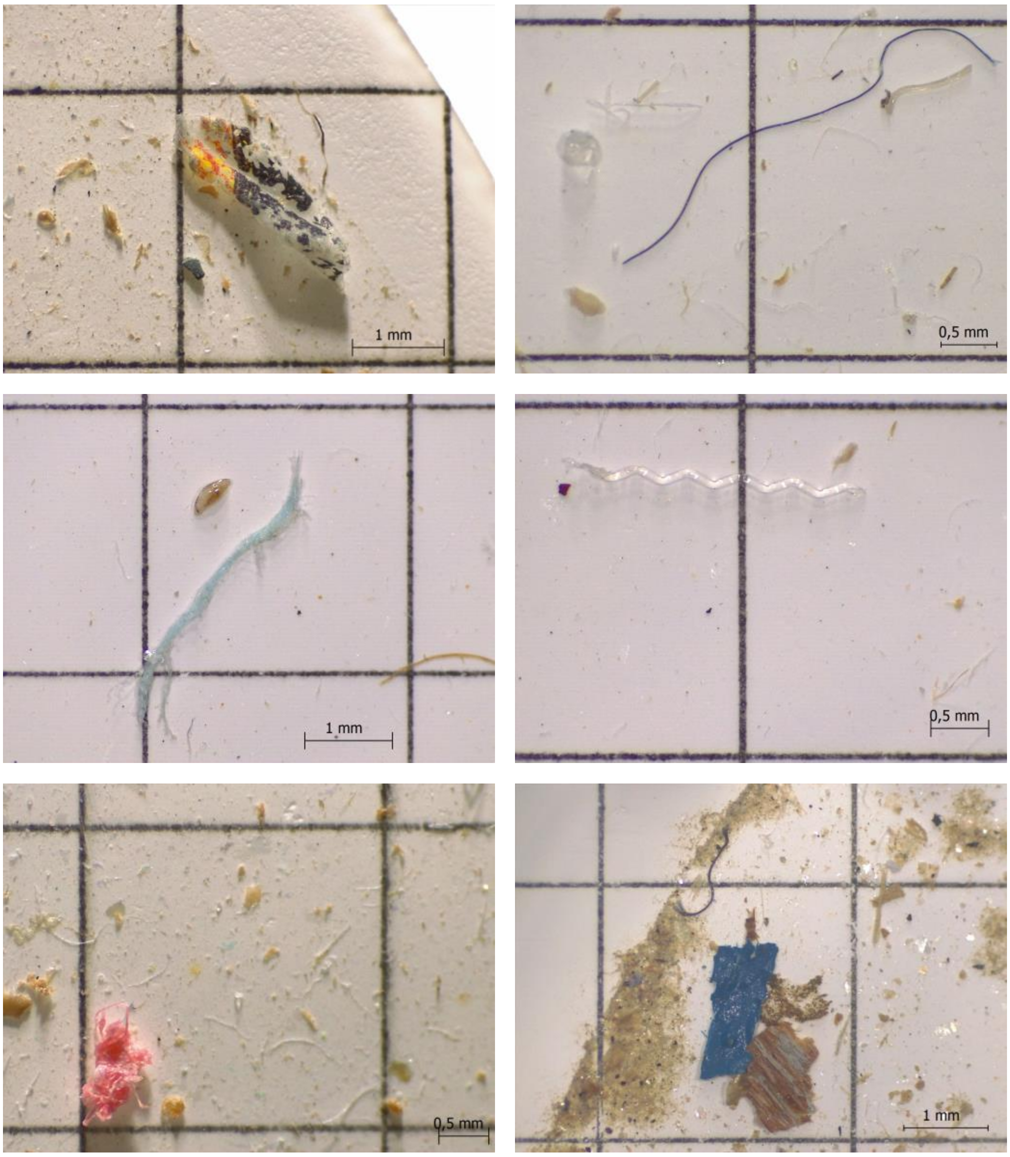

Fig. 2 Examples of microplastics commonly found in aquatic environments (collected on the Douro and Lima Estuaries).

3) Estuaries

Estuaries are transitional ecosystems between rivers and oceans, and are classified as threatened ecosystems, being often subjected to different types of human pressures, namely plastic pollution (Gray et al., 2018; Rodrigues et al., 2019a). These areas are of critical importance, due to several important goods and services they provide (Cunha et al., 2021). Several estuaries worldwide have reported the presence of MPs in their waters

(Defontaine et al., 2020; Hitchcock & Mitrovic, 2019; Rodrigues et al., 2019a) and also in their sediments (Alava et al., 2021; Almeida et al., 2023; Frère et al., 2017). These emergent contaminants may be posing additional environmental risks to estuarine communities, even compromising fundamental ecological functions of these ecosystems (Gray et al., 2018; Ramos et al., 2023).

4) Zooplankton

Zooplankton are the animal component of plankton, often defined as organisms that are carried by tides and currents, and are not able to swim or move willingly against them (Figure 3). They are generally microscopic in size, and represent an important part of ecosystems and food webs, due to their diverse communities and high abundance levels (NOAA, 2021; Richardson, 2008). They can be defined as *holoplankton*, when they spend their entire life in the water column as permanent members of the plankton community, or *meroplankton*, when they are temporary members of the plankton community (Slotwinski et al., 2014). Zooplankton provides plenty of ecosystem services, such as:

- Provisioning services, such as feeding in aquaculture settings (Abate et al., 2015) or materials for biomedical and chemical research (Zimmer, 2002);

- Regulating services, such as climate regulation by sequestering carbon in the deep sea, bioremediation of waste by reduction of nitrogen and phosphorus concentrations (Dinesh Kumar et al., 2016), and life cycle maintenance;

- Supporting services, such as nutrient cycling (Jónasdóttir et al., 2015), food sources, and larval recruitment in fisheries (Botterell et al., 2019).

Zooplankton can alter ecosystem dynamics through many different actions, as they are located in a strategic position in the food web. They act as grazers of algae and bacteria and contribute to the primary production of nutrients to phytoplankton by nutrient recycling, facilitating access to nutrients such as nitrogen and phosphorous (Vanni, 2002). On the other hand, they connect primary producers to consumers in higher trophic levels, bridging the gap between these two groups and ensuring the energy transfer across the food web (Richardson, 2008). Zooplankton is also essential to the effectiveness of the Biological Carbon Pump (BCP), a crucial mechanism for the upkeep of atmospheric carbon dioxide levels, due to the ability to control particles by grazing, changing particles sinking dynamic and transfer of particulate organic carbon from the surface to the depth (Cavan et al., 2017; Lomartire et al., 2021).

Fig 3. Zooplankton organisms, including chaetognaths and copepods (Peijnenburg & Goetze, 2013)

Changes in zooplankton biomass stocks greatly impact the food web and may induce significant changes in the biomass reserves of other organisms, from other types of plankton to organisms in higher trophic levels, such as fish (Wright et al., 2013). The relation between zooplankton and fish has been studied extensively since the 80s, namely in a study that showed a decrease in the abundance of zooplankton in the same time period as a decrease in commercial fish in the North Sea (Reid, 1984). Mackas et al. (2013) analysed zooplankton data over the course of 20 years (1990 – 2010) in the Strait of Georgia, registering a big fluctuation in zooplankton abundance during those two decades that may have affected the population of juvenile salmon and herring. These studies highlight the important role of zooplankton in the marine food web, and how disturbances in zooplankton can easily escalate to other species, some of them with high economic importance.

Zooplankton, inadvertently, is also a helpful bioindicator of changes in ecosystems, often acknowledged as “sentinels of environmental changes and pressures” (Lomartire et al., 2021). In fact, they are often sampled in long repeated time series to detect changes in the environment (Lomartire et al., 2021; Richardson, 2008). Several characteristics make zooplankton a particularly good bioindicator, namely: 1) they are extremely sensitive to temperature changes, 2) have small lifespans which allow for an effective analysis of disruptants versus zooplankton dynamics, 3) are free-floating, hence their distribution and population movements reflect changes in temperature and currents in a determined ecosystem. All these conditions assure that any disturbance will most likely be first recognized in zooplankton in a shorter response time than in higher trophic levels, which in turn may allow for a quicker, more effective intervention against ecosystem disruptions and threats (Lomartire et al., 2021; Ndah et al., 2022).

Two major zooplankton groups of key importance are copepods and chaetognaths (Figure 3). They are abundant among zooplankton communities and have key roles in the planktonic food webs. Copepods, from the subclass Copepoda, are generally considered the most abundant zooplankton group in aquatic environments and are among the most studied groups of marine zooplankton (Bucklin et al., 2021; Dang et al., 2015). They can be found in high abundance in plenty of aquatic environments, from freshwater systems such as rivers, lakes, groundwater and even hot springs, to marine environments. Copepods usually present a cylindrical segmented body and two setose caudal rami on the posterior end of the abdomen, and typically have an average size ranging from 0.5 to 2 mm (Dang et al., 2015; Williamson & Reid, 2001). Their life cycle starts as *nauplius*, and goes through several stages of development until reaching adulthood (Williamson & Reid, 2009). Copepods have extreme importance in the food web, connecting primary producers to higher trophic levels, and have diverse types of feeding, including grazers and suspension feeders, detritivores, omnivores, carnivores, as well as parasitic forms (Heuschele & Selander, 2014). Many studies report selectivity in copepods' feeding habits, although there is much uncertainty about the exact mechanisms of selectivity and their criteria (Kleppel, 1993). Through mechanical and/or chemical mechanisms, copepods seem to be able to distinguish prey by size, mostly large pieces of food which seem to be verified and selected, while smaller pieces do not suffer this screening (Isari et al., 2013). Food quality and concentration, and nutrient availability to copepods can have a major influence on their growth and reproduction, on the trophic transfer of nutrients across the food web and even on carbon cycling. In fact, limitations to copepods' growth and reproduction can lead to lower carbon conversion rates and its accumulation at the producer level in the food web, and subsequent export to the environment, instead of its transfer throughout the food web (Vargas et al., 2010).

Chaetognaths are second to copepods in terms of the total biomass of zooplankton, composing between 5-30% of the total biomass (Patuła et al., 2023). The phylum Chaetognatha is composed of nearly 200 species, and they are carnivorous, hermaphroditic holoplanktonic organisms (Patuła et al., 2023; Slotwinski et al., 2014). They are large organisms, typically measuring more than 5 mm, and have a long cylindrical body, often described as "worm-like". The head presents curved hooks, pigmented eyes and teeth. They are ambush feeders that forage on zooplankton, mainly feeding on moving organisms with sufficient size to be perceived by chaetognaths, but small enough to allow for ingestion

(Saito & Kiørboe, 2001). Prey is perceived by chaetognaths through hydromechanical signals detected by mechanoreceptors hairs on their body surface (Saito & Kiørboe, 2001). Chaetognaths are considered important energy transfer agents to higher trophic levels, such as commercially important fish species (Patuła et al., 2023; Wu et al., 2014), and often have a significant impact on their prey population. Copepods are among the most important food sources for chaetognaths, and therefore, these two organisms are heavily linked, and changes or disturbances in one of these populations could impact the other population (Baier & Purcell, 1997; Terazaki, 2004).

5) Interactions between zooplankton and microplastics

The presence of MPs in aquatic environments impacts not only the habitats (water, sediments) but also the aquatic organisms present in these environments. MPs pose a threat to organisms of small size, such as zooplankton, in a way that macroplastics do not. Ingestion of MPs by zooplankton has already been reported in several ecosystems worldwide (Aytan et al., 2022; Desforges et al., 2015; Klasios & Tseng, 2023). However, the association between MPs and zooplankton is still underrepresented when considering the number of publications regarding MPs (Rodrigues et al., 2021). Furthermore, a majority of the publications regarding ingestion of MPs by zooplankton are laboratory studies, rather than field studies (Rodrigues et al., 2021). The biological effects of MPs are primarily addressed in laboratory studies, while field studies tend to focus on concentrations of MPs in the organisms and surrounding environments (Botterell et al., 2019).

It is important to note that although laboratory studies are essential to understand the potential effects of MPs, they often use different conditions that do not always accurately reflect the field conditions, which compromises the extrapolating of laboratory-based results. Firstly, laboratory studies tend to use virgin MPs, which have not been exposed to and altered by environmental conditions. The type of MPs also can vary greatly between laboratory and field studies, with the laboratory studies using mostly beads and spherical MPs, while the most common MPs found in the field are fibers and fragments, usually with irregular shapes and rough textures, due to weathering and degradation by the environment (Rodrigues et al., 2021). It is also important to point out that MP concentrations are often higher in laboratory settings than what is typically found in the field, thus, biological or toxicological effects are not nearly as commonly observed in field studies, where MP contamination is generally lower.

Certain studies have also explored the possibility of increased ingestion of MPs that have been submitted to ageing and weathering action, and are therefore similar to typical food items for zooplankton. Vroom et al. (2017) verified that aged MPs had higher ingestion rates by copepods than pristine MPs, and concluded that this phenomenon could be either because of the irregular natural-looking shape of an aged MP, or due to the formation of a biofilm on its surface.

The ingestion of MPs by zooplankton may translate to the accumulation of these particles inside the organisms, and may lead to internal abrasions and physical blockages of the alimentary tract (Wright et al., 2013). In turn, these lesions may impair their feeding behaviour, leading to nutrient deficit, issues with reproduction and gene expression, reduced growth and increased mortality (Cole et al., 2013; He et al., 2022; Zhang et al., 2019). Furthermore, MPs can also carry other contaminants, such as persistent toxic chemicals, that may trigger toxicity reactions and/or accumulate in the organism's lipid reserves (Lima et al., 2014). MPs may also form biofilms where microorganisms and organic matter can accumulate, and be transported inside zooplankton upon the ingestion of the MP (He et al., 2022). Other effects of contamination have also been reported, such as reduction of enzymatic activity (Gambardella et al., 2017), changes in filtration capacity and swimming activity (Rodrigues et al., 2021), and endocrine disruption (Wright et al., 2013). The egestion of MPs through faecal pellets may also cause disturbances to the ecosystem. Through the incorporation into these pellets, MPs that were previously buoyant and remained near the surface of the water sink in the water column, changing their bioavailability (Sipps et al., 2022).

The large majority of studies seem to focus on the individual and biological effects and MP concentrations, while ecological effects are not as well studied. For, example very few studies have explored the trophic transfer of MPs via the food chain by zooplankton. A study by Setälä et al. (2014) stands out by providing evidence of the transfer of MPs by zooplankton to higher trophic levels through ingestion, in a laboratory setting. The impact of MPs on communities and ecosystems, and therefore on ecological functions such as photosynthesis, primary production and predator-prey interactions are underdeveloped topics as well (Rodrigues et al., 2021).

6) Thesis Objectives

MP pollution is an emergent environmental concern gaining increasing attention from the scientific community, government and environmental agencies, and even the general public. However, there are still significant gaps in scientific knowledge regarding the impact of MPs, namely in aquatic organisms of lower trophic levels, such as zooplankton. The lack of field studies and standardized methods for sample collection and MP quantification, as well as the ecological effects of MPs in zooplankton communities, are some of the most unexplored topics.

Estuaries are important transitional ecosystems, subjected to contamination and plastic pollution (Browne et al., 2010). The Douro and Lima river estuaries are still fairly unknown regarding their contamination by MPs, and studies correlating zooplankton and MP contamination are scarce. The main goal of this thesis is to investigate the MP contamination of estuarine zooplankton in two Portuguese estuaries, namely the Douro and Lima river estuaries (NW Portugal). The specific objectives are:

- Assess temporal and spatial patterns of MPs and zooplankton in the two distinct estuaries, through four sampling campaigns performed during one year (in February, May, August and November), with sampling points distributed across the horizontal gradient of each estuary, covering the lower, middle and upper sections;

- Study the effects of MPs in zooplanktonic communities through the ratio between the main zooplankton groups and MPs;

- Examine the occurrence of MPs in copepods and chaetognaths, two relevant groups of zooplanktonic organisms, to infer about MP concentration and characteristics of ingested MPs in zooplankton, and MP trophic transfers at the lower levels of the food web.

Chapter 2

Assessing microplastic contamination in zooplanktonic organisms from two river estuaries

(submitted to Marine Pollution Bulletin)

Francisca Espincho[1,2,*], Rúben Pereira[1,2], Sabrina M. Rodrigues[1,2], Diogo M. Silva[1,2], C. Marisa R. Almeida[2,3] and Sandra Ramos[2]

[1]ICBAS - Institute of Biomedical Sciences Abel Salazar, Porto University, Porto, Portugal;

[2]CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, Porto University, Matosinhos, Portugal;

[3]Chemistry and Biochemistry Department, Sciences Faculty, Porto University, Portugal

Abstract

Microplastics (MPs) are an emerging concern to aquatic environments and ecosystems, however, field studies regarding this topic and the impact of MPs on organisms of lower trophic levels such as zooplankton, namely MP contamination, are still scarce. The present work aims to evaluate MP contamination of zooplankton from the Douro and Lima estuaries (NW, Portugal). During 1-year, seasonal surveys were conducted to collect zooplankton and water samples from different sites in each estuary. Zooplankton was quantified and identified into major zooplanktonic groups. Dedicated protocols previously optimized by the team were used to assess MP presence in water samples and in two of the most abundant zooplankton groups (copepods and chaetognaths). Results showed the presence of MPs in all water samples, with similar MP concentrations in both estuaries (Lima: 2.4 ± 2.0 MPs m^{-3}; Douro: 2.3 ± 1.9 MPs m^{-3}). Chaetognaths exhibited higher MP contamination in Lima (5.3 ± 5.2 MPs ind^{-1}) and Douro estuary (5.9 ± 6.1 MPs ind^{-1}) than copepods, which tended to have lower levels of MP contamination (Lima: 2.4 ± 2.1 MPs ind^{-1}; Douro: 3.7 ± 4.1 MPs ind^{-1}). Zooplanktonic organisms were mostly contaminated by small size blue fragments, while in estuarine water fibers were the most common MPs and MPs of several colours were also observed. Such differences in MPs indicate a potential MP selection by zooplanktonic organisms. Results give insights of MPs contamination of zooplanktonic organisms from estuarine environments, highlighting the possibility of MP trophic transfers at the lower levels of the food web.

1. Introduction

Plastic is a major concern regarding marine environments, being the most abundant type of marine debris (UNEP, 2016). Microplastics (MPs) have been receiving increased attention from the scientific community in recent years since their presence is extremely significant in marine pollution, accounting for 92% of plastic debris found on the ocean surface (Eriksen et al., 2014). MPs are commonly defined as plastic debris with a diameter below 5 mm (Arthur, 2009), and are disseminated all over the globe, in distinct ecosystems such as beaches (Herrera et al., 2018), seabed sediments (Karlsson et al., 2017), estuaries (Defontaine et al., 2020; Sipps et al., 2022; Trindade et al., 2023), wastewater effluents (Murphy et al., 2016), surface waters and freshwater systems (Horton et al., 2017). The concentration of MPs in these environments is fairly variable and depends on several factors, such as wind and current conditions, geographical characteristics and the presence of urban areas and shipping trade routes (Barnes et al., 2009). MPs are more frequently found in enclosed or semi-enclosed sea areas, and on upper levels of the water column, near the surface water and shorelines. MPs can also sink and concentrate at the bottom of the aquatic environment due to modifications in their density and buoyancy (Barnes et al., 2009).

Estuaries are also areas contaminated with MPs. Estuaries are important aquatic ecosystems since they represent a transition zone between the sea and the freshwater streams, playing an important role for several species and providing important ecosystem services (Gray et al., 2018; Trindade et al., 2023). Estuaries also provide an important tool to understand the dispersion mechanisms of MPs (Defontaine et al., 2020). In recent years we have seen an increase in research focused on the presence and abundance of MPs in estuaries (Browne et al., 2010; Gray et al., 2018; Lima et al., 2014; Rodrigues et al., 2019a; Sipps et al., 2022; Taha et al., 2021; Trindade et al., 2023).

Estuarine communities are composed by few resident species and several migratory species that temporarily inhabit these areas, and the presence of MPs can affect all of them. Zooplankton represents the animal component of planktonic communities, and they are often defined as organisms that are carried by tides and currents, and are not able to swim or move willingly against them (NOAA, 2021). Zooplankton is present in a great variety of aquatic environments, and occupies a key place in the food web by connecting two trophic levels, primary producers to consumers (Havens, 2002; Sun et al., 2018a; Sun et al., 2018b). A perturbance on zooplankton biomass can disturb and impact biomass stocks of other types of plankton, and influence ecosystem services (Wright et al., 2013). As highlighted by Rodrigues et al. (2021), the association between MPs and plankton is still relatively underrepresented when considering the number of publications regarding MPs. Furthermore, several of these publications are laboratory studies, and comparisons between them and field studies must be done with caution, due to the differences in MP concentration, polymer type, shape and MP condition (laboratory studies tend to use virgin MPs - MPs which have not been exposed to environmental conditions) (Rodrigues et al., 2021). The ingestion of MPs by zooplankton has been verified in a few recent field studies, showing that zooplankton tends to ingest MPs that are present in their environment and are of similar size to their typical prey (e.g. (Klasios & Tseng, 2023; Sipps et al., 2022; Taha et al., 2021; Zavala-Alarcón et al., 2023). Laboratory studies report many biological impacts upon ingestion, such as internal injuries to body tissues and the alimentary tract (He et al., 2022; Wright et al., 2013), impaired feeding behaviour (Cole et al., 2015), reduced fecundity, and reduced energy levels leading to deficient growth (Cole et al., 2015; Zhang et al., 2019). In addition, waterborne pollutants are able to adhere to the plastic polymer of many MPs and may induce chemical toxicity to zooplankton (Cole et al., 2013; Lima et al., 2014). It has also been shown in laboratory studies that the ageing and weathering action MPs withstand during their long-term permanence in aquatic environments can lead to an increase in their uptake by certain organisms. Vroom et al. (2017) confirmed that aged MPs were ingested by more individuals and at faster rates than pristine MPs in copepods, and this higher ingestion could be due to differences in shapes and biofouling, that lead to a similarity between these MPs and the typical food items of these organisms. In terms of ecological effects, the number of studies is even lower. For example, Setälä et al. (2014) showed in a laboratory study that MPs ingested by zooplankton have the potential to be transferred to higher trophic levels along the food webs, through the ingestion of zooplankton by a predator. In addition, Sipps et al. (2022) stressed that MPs may be incorporated into faecal pellets, sinking into the water column and changing the bioavailability of otherwise buoyant MPs. But more research is needed, namely regarding field studies to take in consideration the real conditions to which the organisms are exposed to.

Attending to the emergent concern of MP pollution, it is important to increase the scientific knowledge of the real impacts of MPs on aquatic environments and organisms, particularly those from lower trophic levels as the zooplankton. Therefore, the main goal of this study is to assess MPs contamination of estuarine zooplankton, using two Portuguese estuaries as case study, namely the Douro and Lima estuaries (NW Portugal) to specifically : 1) assess temporal and spatial patterns of MPs and zooplankton ratios in the two distinct estuaries; 2) investigate the occurrence of MPs in two relevant groups of zooplankton organisms, namely copepods and chaetognaths, which can have an impact on MP trophic transfers at the lower levels of the food web.

2. Materials and methods

2.1 Study Area

Two distinct estuaries in the north of Portugal were selected as case study: the Douro river estuary and the Lima river estuary (Fig. 4). The Douro estuary is a salt-wedge estuary, and its upstream limit is defined by the Crestuma dam, located 21.6 km upstream of the river mouth (Azevedo et al., 2008). The Douro estuary can be divided into three distinct zones (Vieira & Bordalo, 2000): lower, middle and upper estuary. The Douro estuary is characterized by a strong urban presence, mostly in the last 8 km of its length, harbouring two major Portuguese cities (Azevedo et al., 2006). It is also heavily influenced by wastewater treatment plants' effluents and rivers/streams that drain into the estuary (Azevedo et al., 2006; Rodrigues et al., 2019a). The Lima estuary is a seasonally stratified estuary, and it is also divided into three areas: the narrow lower estuary located in the river mouth; the middle estuary which is classified as a shallow saltmarsh zone; and the upper estuary, characterized by a decrease in depth and the channel width (Ramos et al., 2010). The Lima estuary is less impacted by anthropogenic activities, but still with some urban pressure and a large commercial harbour in the lower estuary (Costa-Dias et al., 2010). This estuary still has natural banks and a large saltmarsh area located in the middle estuary, and upstream the Lima estuary receives urban and agricultural effluents (Ramos et al., 2015).

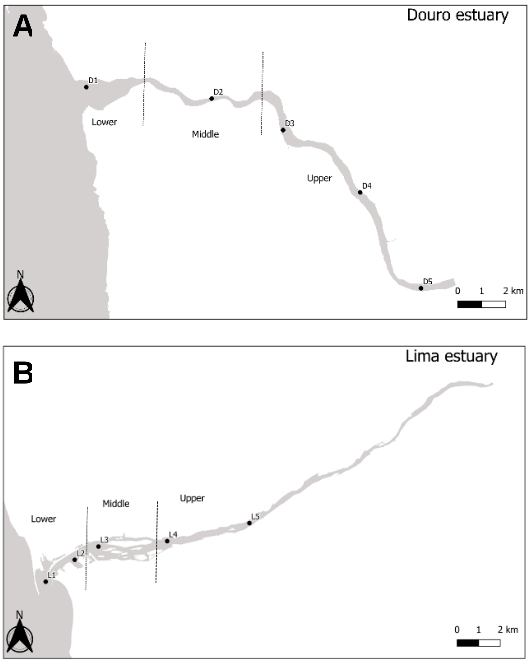

Fig.4. Location of the five sampling stations in the Douro Estuary (A) and Lima Estuary (B), distributed throughout the lower, middle and upper sections of the estuaries.

2.2 Sampling methodology

Sampling campaigns were conducted in 2022, in the following months: February (winter campaign), May (spring campaign), August (summer campaign) and November (autumn campaign). Due to logistic constraints with the vessel used in sampling surveys, it was not possible to do the summer campaign in the Lima Estuary. In each estuary, five sampling stations distributed across the horizontal gradient of each estuary were surveyed, covering the lower, middle and upper sections of each estuaries (Fig. 4). In the Douro estuary: D1 is located in the lower estuary near the river mouth; D2 in the middle estuary; and D3, D4 and D5 are located in the upper estuary. In the Lima estuary: L1 and L2 are located in the lower estuary; L3 is located in the middle estuary; and L4 and L5 are located in the upper estuary. Zooplankton and MPs in estuarine water were collected by means of a 150 µm mesh size planktonic net. At each sampling site, planktonic tows were performed for 1 minute near the surface of the water. The samples were immediately preserved with 70% ethanol until further laboratory analyses. The volume of filtered water was quantified with a flowmeter (Hydro-Bios) attached to the plankton net.

2.3 Zooplankton analysis - Quantification and identification of major zooplanktonic groups

Quantification and identification of major zooplanktonic groups in the samples were performed by sub-sampling 2 mL of the original sample in a Bogorov chamber and analysing it on a stereomicroscope. The procedure was done three times in total, each time sub-sampling 2 mL of each sample. The number of zooplankton organisms was standardized to the number of individuals per m^3 of filtered water. The major zooplanktonic groups considered were: copepod, nauplii, cladocera, oikopleura, crypsis larvae, tintinid, hidrozoa, chaetognata, fish egg, ostracoda, veliger, ichthyoplankton and polychaete larvae (Pereira et al., 2023). All preventive measures to prevent MPs contamination, detailed in the following section, were carried out in all zooplankton laboratorial analysis.

2.4 MPs analysis

2.4.1 Measures to prevent MP contamination

Prevention of MP contamination was of key importance throughout the course of this study. Several measures were taken to ensure no MP contamination of any samples: specific lab coats of cotton were always used during laboratory procedures; all laboratory material and supplies were thoroughly washed with deionized water and ethanol before use; for procedures on the stereomicroscope with open samples, an open petri dish with deionized water was placed near the stereomicroscope and inspected for MPs at the beginning and end of procedures.

2.4.2 MPs in water samples

MP analysis was executed through a protocol previously developed by the team, adapted from the NOAA protocol, described in Rodrigues et al. (2019b). In resume, samples were initially sieved through a 0.03 mm filter cloth and the solids were placed on a beaker, both previously washed with deionized water. Samples were left to dry overnight at 90 °C. The following day, 20 mL of 0.05 M Fe(II) solution and 20 mL of 30% H_2O_2 solution were added to each sample, which were then heated at 75°C. Twenty minutes after the occurrence of a chemical reaction (in the form of heat and the appearance of bubbles), another 20 mL of 30% H_2O_2 solution was added. After another waiting period of 20 minutes for a second chemical reaction to occur, 18 g of NaCl were added in order to increase the density of the solution. The samples continued to be heated for another 30 minutes. Later, the saturated solution was placed on a density separator and left overnight. The next day, the solids floating on the density separator were filtered and left to dry at room temperature.

All previously mentioned MP prevention contamination measures were carried out during the entire procedure.

2.4.3 MP in zooplanktonic organisms

MPs were retrieved from zooplankton organisms, using an adaption of a dedicated protocol developed to analyze MPs in planktonic organisms, including zooplankton (Rodrigues et al., submitted). The basis of the process consists in digesting the organic content of the organism with 30% H_2O_2 solution. The protocol was properly optimized and validated for zooplankton samples through several tests, such as: laboratorial tests to assess possible sources of contamination, tests with several types of common MP polymers (polyethylene (PE), polyvinyl chloride (PVC), polyethylene terephthalate /polyester (PET), cellulose acetate, Rayon, and polymethyl methacrylate (PMMA)), and tests to determine the ideal exposure time. It was concluded that the ideal exposure time to a 30% H_2O_2 solution was 7 hours (Rodrigues et al., submitted).

Two groups of zooplankton were selected to assess MP contamination in zooplanktonic organisms, namely copepods and chaetognaths. These two groups were chosen since they are typically frequent and abundant in temperate estuarine zooplankton communities. Also, they represent different trophic levels, as copepods are considered primary consumers, and chaetognaths are considered secondary consumers (Baier & Purcell, 1997; Terazaki, 1998). First, each individual was carefully separated from the original samples and inspected for any MPs or inorganic components on its exterior. After a thorough cleanse of each individual with deionized water, the organisms were placed in a clean glass flask. Three replicates were prepared for each sampling station from each sampling campaign. The number of zooplankton organisms selected per sample was 30 individuals. However, in some samples a lower number was available, due to different reasons, for example the absence of organisms in that specific time of the year; or the organisms were not in good preservation condition, probably due to sample preservation issues (February: L1=1 chaetognath; L2= 24 copepods; L3= 4 copepods; L4= 21 copepods; L5= 14 copepods; D3= 3 copepods, D4= 14 copepods; May: L2= 20 copepods; L3= 3 chaetognaths; L4= 9 copepods; L5= 19 copepods; D1= 10 chaetognaths, D2= 4 copepods; August: D1: 26 copepods; D1= 3 chaetognaths; D2= 2 copepods; D3= 1 copepod; D4= 1 copepod; D4= 1 chaetognath; D5= 1 copepod; D5= 1 chaetognath; November: L1: 7 chaetognaths; L2: 19 copepods; L2: 17 chaetognaths; L3: 23 copepods; L3: 10 chaetognaths, L4: 6 chaetognaths; L5= 1 copepod; L5= 2 chaetognaths; D2: 7 copepods;

D2: 27 chaetognaths; D3: 3 copepods; D3: 5 chaetognaths; D5: 1 copepod; D5: 1 chaetognath). Then, 2 mL of 30% H_2O_2 solution were added to each flask, and the flasks were placed at 65 °C for seven hours to ensure total digestion of the organic content. After, samples were filtered, and the filters were left to dry at room temperature.

All previously mentioned MP prevention contamination measures were carried out during the entire procedure.

2.4.4 MPs characterization and polymer analysis

MPs recovered from each sample of water and zooplanktonic organisms were observed under the stereomicroscope, to be characterized and quantified by size, shape and colour. From those recovered from water samples, 82 MPs (6.7% of all MPs found in water samples) were submitted for further analysis by Fourier-transform infrared spectroscopy (FTIR), to verify the type of polymer. Polymer was not characterized in MPs of smaller dimensions due to technical limitations of the FTIR. The obtained spectra were compared with reference library spectra, and according to Rodrigues et al. (2020), matches with confidence levels of 75% and above were accepted, which amounted to 52 MPs.

2.5 Data Analysis

To investigate ecological impacts of MP contamination in zooplankton communities, a ratio between MP concentration in water samples and zooplankton abundance was calculated. The zooplankton abundance was transformed into $\log_{10}$ (“zooplankton abundance” +1) due to the large scale difference between the two data sets, following a similar method by Sun et al. (2018b).

One-way analysis of variance ANOVA were used to investigate significant differences in zooplankton abundance, MP concentration in estuarine waters and MP concentration in copepods and chaetognaths. These differences were investigated considering estuaries, sampling months (temporal variations) and sampling stations (spatial variations) as fixed factors. ANOVA assumptions were tested, namely homogeneity of variance was tested with the Cochran test. Whenever necessary, data was log-transformed to follow ANOVA assumptions. And, in the case when it was not possible to fulfill ANOVA assumptions, the non-parametric Kruskal-Wallis test was used. Post-hoc analyses were performed with Fisher LSD. A significance level of 0.05 was considered for all analyses. All tests were performed with TIBCO Statistica™ 14.0 software.

3. Results

3.1 Estuarine zooplankton communities

The total abundance of zooplankton was in average 7.7E+10 ± 8.8E+10 No m^{-3} in the Douro estuary, and 9.2E+10 ± 1.0E+11 No m^{-3} in the Lima estuary (Fig. 5A), without significant differences between the estuaries (1-way ANOVA: F= 0.12; $p \geq 0.05$). Also, the composition of the zooplankton community was similar between the two estuaries, with a total of thirteen zooplankton groups identified in both estuaries. In the Douro estuary, 10 groups were identified: copepods, naupili, oikopleura, cripsys larvae, tintinid, hidrozoa, chaetognath, fish egg, veliger and ichthyoplankton (Fig 5B); and in the Lima estuary 9 groups were identified: copepods, naupili, cripsys larvae, hidrozoa, chaetognaths, fish egg, ostracoda, veliger, polychaeta larvae (Fig. 5C). Overall, copepods were the most common group in all samples from the two estuaries. However, in May 2022 cladocera was the most abundant group observed in the Douro estuary (Fig.5B). Copepods made up between 74% and 90% of total zooplankton in the three sampling campaigns in Lima Estuary, and between 43% and 84% of total zooplankton in the four sampling campaigns in Douro Estuary. Naupili were generally the second most abundant group in both estuaries. Copepods and naupili were the only two groups found in all samples, while other groups varied among months or between estuaries. Oikopleura, cladocera and chaetognata were also frequently observed, while the remaining groups were less abundant and frequent.

Each estuary presented specific temporal and spatial patterns of zooplankton abundance. In the Douro estuary, there were no significant differences in temporal (1-way ANOVA: F= 0.84; $p \geq 0.05$) or spatial (1-way ANOVA: F= 1.36; $p \geq 0.05$) patterns (Fig. 5D). Although not significant, there was a tendency for the zooplankton abundance to be lower in the middle estuary and higher in the lower estuary. In November 2022, zooplankton abundance in Douro contradicted this general tendency, being higher in the upper estuary, mainly in sites D4 and D5. There was an increase in zooplankton abundance in site D5, in May, reaching the highest value observed in the Douro estuary.

In contrast, in the Lima estuary, zooplankton abundance varied significantly between sampling stations (1-way ANOVA: F= 5.47 $p<0.05$), but not between seasons (1-way ANOVA: F= 0.84 $p \geq 0.05$) (Fig.5E). Significantly lower zooplankton abundance was observed on the lower estuary in all sampling campaigns. And, the higher abundances were observed in the upper section, namely in site L4, with the highest zooplankton abundance in all campaigns.

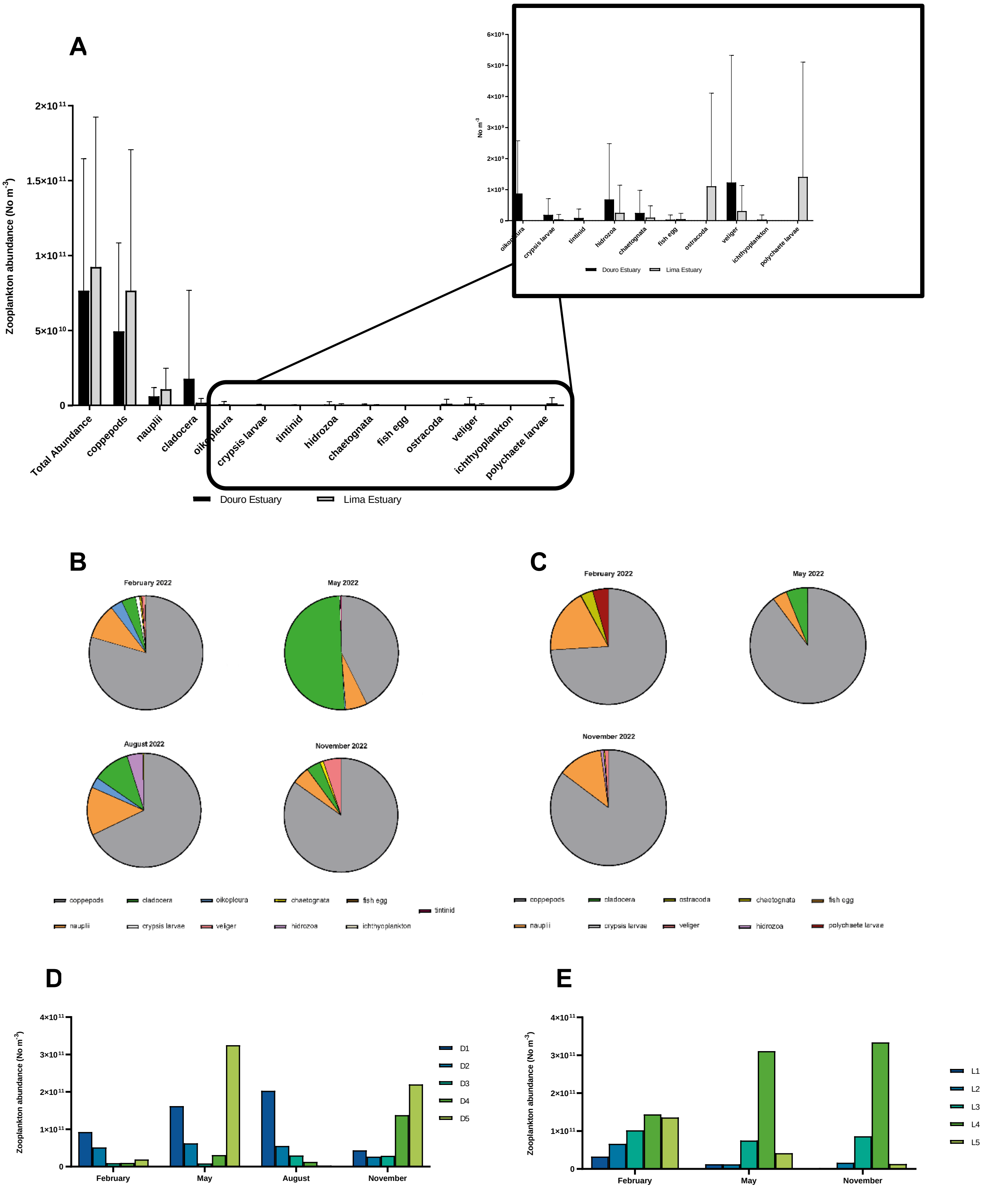

Fig.5 Zooplankton communities of the Douro and Lima estuaries: A- Total abundance (No m^{-3}) of zooplankton; B and C – monthly composition of the different zooplanktonic groups; D and E - temporal and spatial variation of zooplankton abundance in the Douro Estuary and Lima Estuary, respectively.

3.2 MPs in water samples

No MPs contaminations were observed when dealing with water samples.

3.2.1 Spatial and temporal variation of MPs in water samples

A total of 1222 MPs were retrieved from 20 water samples collected in the Douro estuary and 15 in the Lima estuary. MPs were present in all samples, with a mean concentration of 2.3 ± 1.9 MP m^{-3} in the Douro estuary, and 2.4 ± 2.0 MP m^{-3} in the Lima estuary. There were no statistical differences in MP concentration between the two estuaries (1-way ANOVA: F= 0.065; $p \geq 0.05$). In the Douro estuary, the maximum MP concentration was observed in November site D1, reaching 9.57 MP m^{-3} (Fig. 6A). However, MP concentration did not vary significantly between the different months (1-way ANOVA: F= 2.30; $p \geq 0.05$) or throughout the sampling stations (1-way ANOVA: F= 0.26; $p \geq 0.05$). In the Lima estuary, regarding variations in sampling stations, MP concentration did not vary significantly (Kruskal-Wallis: H_4=15, $p \geq 0.05$), however, the concentration of MPs across the sampling months varied significantly, with higher concentration in November than in February and May (1-way ANOVA: F= 6.77, $p<0.05$) (Fig.6B).

3.2.2 Characterization of MPs in water samples

The characteristics of MPs found were similar in both estuaries, with fibers being the most common shape of MPs retrieved from water samples, accounting for 61% of total MPs found in the Douro estuary, and 51% of total MPs collected in the Lima estuary (Fig. 6C). Fragments were the second most abundant, representing 27% and 41% of MPs collected in the Douro and Lima estuaries, respectively (Fig. 6C). On the other hand, films were less common, with only 11% in the Douro estuary and 8% in the Lima estuary (Fig. 6C).

Regarding MP colours, blue was the most common in both estuaries, representing more than 50 % of all MPs (65% in the Douro estuary and 61% in the Lima estuary) (Fig.6D). Ten more colours were detected in MPs of water samples (Fig. 6D), namely red (Douro=10%; Lima=4.4%), pink (Douro=1.4%; Lima=3.7%), black (Douro=1.8%; Lima=2.2%), orange (Douro=1.6%; Lima=5.8%), grey (Douro=3.0%; Lima=6.0%), and other colours with vestigial representation in the samples (Fig.6D).

Regarding MP size, smaller MPs (<1 mm) were the most abundant in both estuaries, representing 51% of all MPs found in the Douro estuary and 61% in the Lima estuary (Fig.6E). MPs measuring between 1 mm and 3 mm were the second most abundant size class, accounting for 41% of all MPs found in the Douro estuary, and 36% in the Lima estuary. Finally, MPs larger than 3 mm were less common (Douro=7.7%, Lima=2.9%) (Fig.6E).

A total of 7 different polymers were identified, namely polyethylene (PE) and polypropylene (PP), which were the two most common. Other examples of polymers identified were polyvinyl chloride (PVC), polyester terephthalate (PET), and poly(methyl methacrylate) (PMMA).

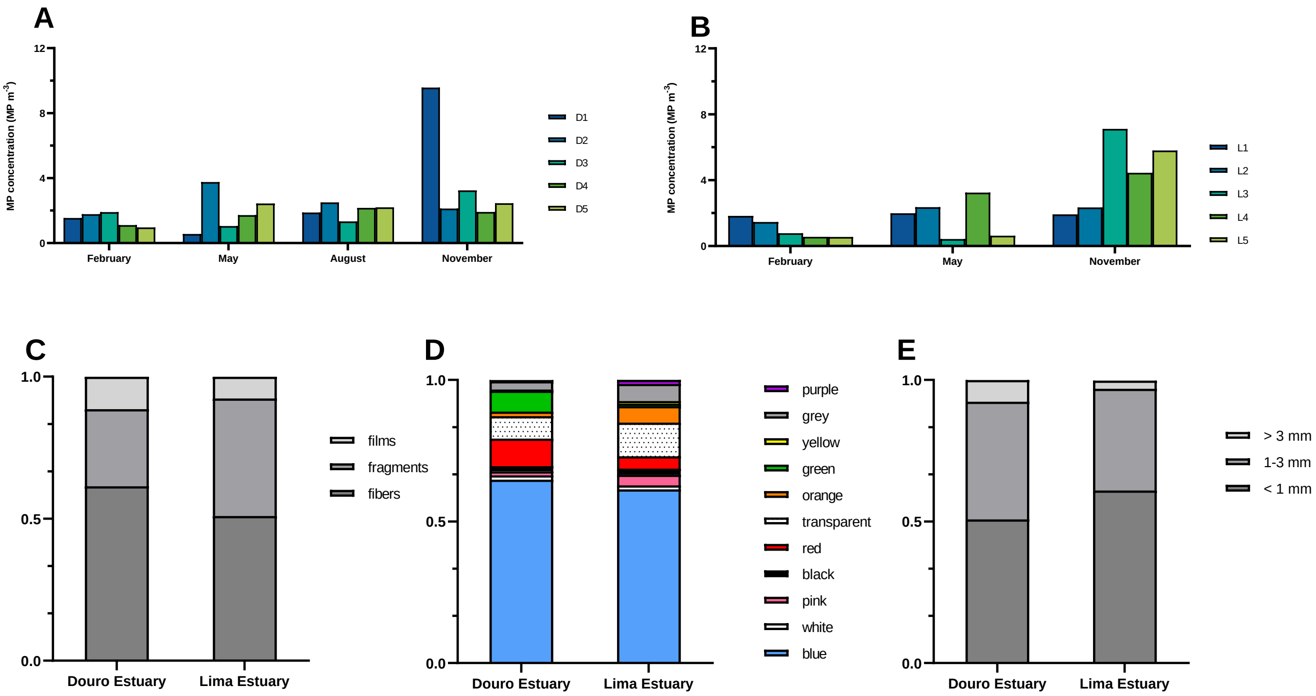

Fig.6 Temporal and spatial variation of MP concentration (MP m^{-3}) in water samples from the Douro Estuary (A) and Lima Estuary (B). (C), (D) and (E) represent the distribution of MPs by shape, colour and size, respectively, in the two estuaries.

3.3 MPs and zooplankton

3.3.1 MP/Zooplankton abundance ratio

Overall, the estimated water MPs: zooplankton ratio was 1:4.7 for the Douro estuary and 1:4.6 for the Lima estuary. In the two estuaries, there was a tendency for ratios to increase over time, reaching higher values in November (Fig. 7A). In fact, in November, the ratio reached 1:2.8 in the Douro estuary, and 1:2.5 in the Lima estuary, indicating an increase in MP concentration when compared to zooplankton abundance.

Each estuary exhibited a specific temporal and spatial pattern of MPs concentration and zooplankton abundance (Fig. 7B and Fig. 7C).

In the Douro estuary, although no significant temporal or spatial patterns were observed neither for zooplankton or MPs, zooplankton abundance tended to be lower in the middle estuary, while MPs decreased with the increase in distance from the river mouth (Fig.7B). We also verified a trend for higher MPs in November, although it did not translate into significant variances (Fig.7A).

In the Lima Estuary zooplankton abundance was significantly higher in the upper estuary, while MP concentration did not vary significantly along the estuary (Fig.7C). The temporal pattern revealed that MP concentration was significantly higher in November, while zooplankton abundance remained statically similar across all sampling months (Fig.7A).

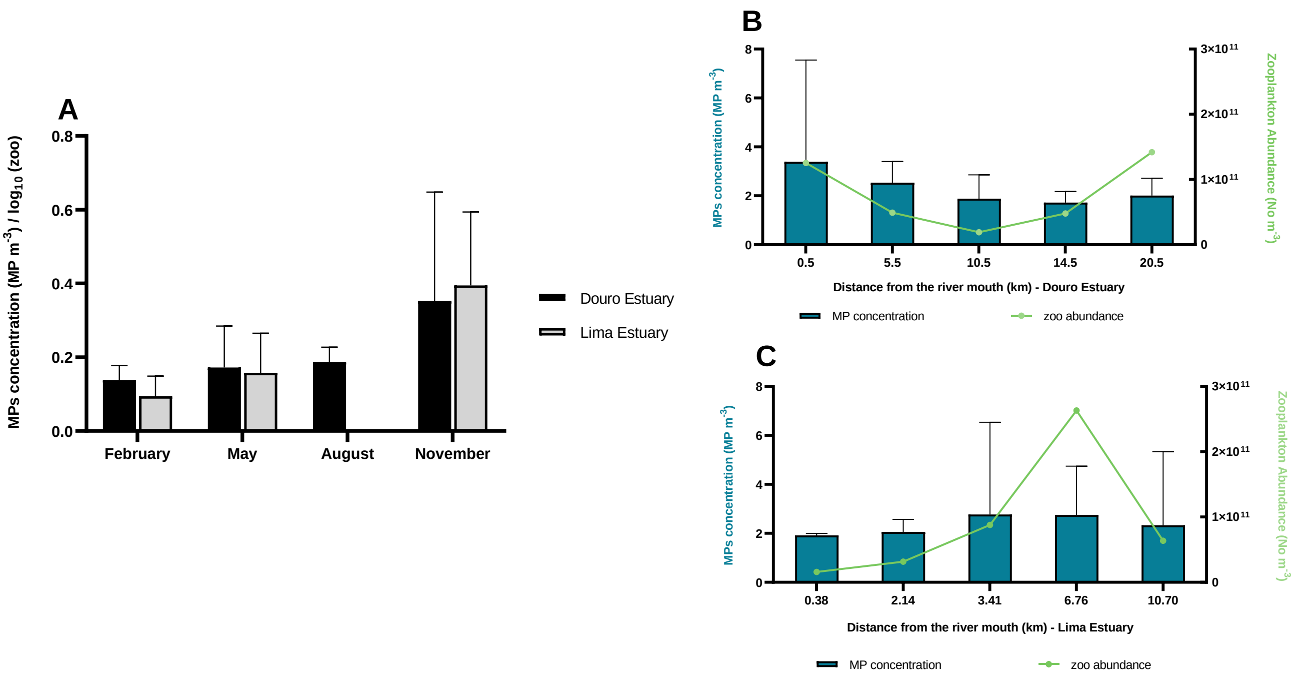

Fig.7 A- Ratio between MP concentration in water samples and zooplankton abundance in Douro and Lima estuaries. Spatial variation of mean concentration of MPs in water samples and mean abundance of zooplankton in Douro (B) and Lima estuaries (C).

3.3.2 MPs in zooplankton organisms

No MPs contaminations were observed when dealing with zooplankton organisms.

From a total of 958 zooplankton organisms analysed (779 copepods and 179 chaetognaths), 1474 MPs were retrieved. In copepods, MP contamination did not vary significantly between the two estuaries (Kruskal-Wallis: $H1=85$, $p \geq 0.05$), being on average 3.7 ± 4.1 MP ind^{-1} and 2.3 ± 2.1 MP ind^{-1} in the Douro and Lima estuaries, respectively. Similarly, MP contamination in chaetognaths did not vary significantly between the two estuaries (1-way ANOVA: $F= 0.0076$; $p \geq 0.05$), but higher values were estimated, namely 5.9 ± 6.1 MP ind^{-1} in the Douro estuary, and 5.3 ± 5.2 MP ind^{-1} in the Lima estuary.

While in the Lima estuary there were no significant differences in spatial (Kruskal-Wallis: $H4=43$, $p \geq 0.05$) (Fig. 8D) and temporal (Kruskal-Wallis: $H2=43$, $p \geq 0.05$) (Fig.8C) variations in MP contamination in copepods, in the Douro estuary significant differences were observed. MP contamination of copepods in Douro Estuary varied significantly among sampling stations (Kruskal-Wallis: $H4 = 42$, $p<0.05$), in which sampling station D2 presented higher MP concentrations (Fig.8B), and among seasons (1-way ANOVA: $F= 3.7165$, $p<0.05$), with concentration reaching its highest value in August (Fig. 8A).

Regarding chaetognaths, there were no spatial differences in MP concentration, neither in the Douro estuary (Kruskal-Wallis: $H4=18$, $p \geq 0.05$) (Fig.8B) nor in the Lima estuary (Kruskal-Wallis: $H4=18$, $p \geq 0.05$) (Fig.8D). In contrast, chaetognaths contamination varied significantly among sampling months in the two estuaries. In the Douro estuary, MP contamination reached the significantly highest values in August (1-way ANOVA: $F= 4.1125$; $p<0.05$) (Fig.8A). And in the Lima estuary, the significantly lowest concentration in chaetognaths was observed in November (Kruskal-Wallis: $H2=18$, $p<0.05$) (Fig. 8C).

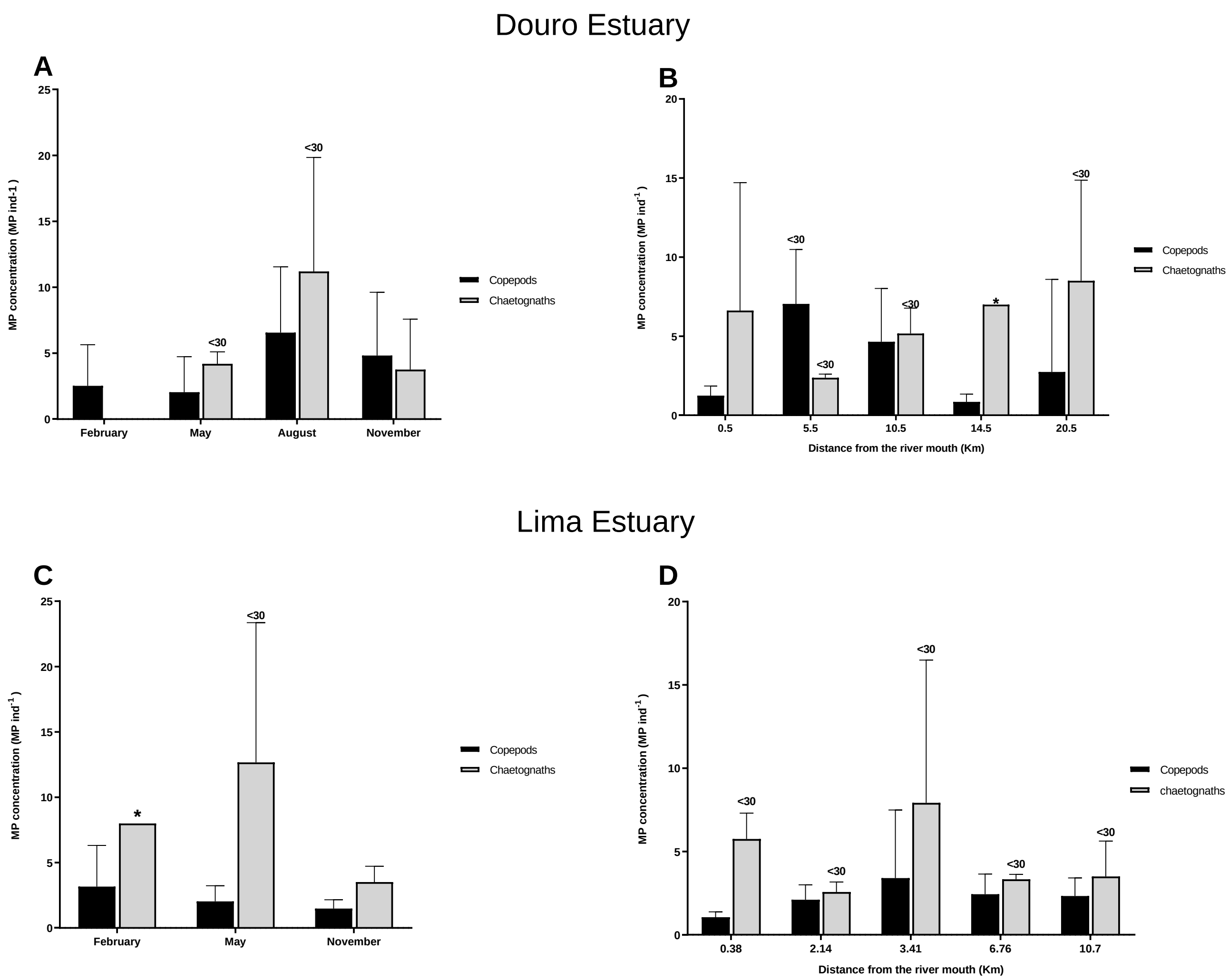

Fig.8 Concentration of MPs in zooplanktonic organisms (Coppepods and Chaetognata) from the Douro estuary in different sampling months(A) and sampling stations (B); and from Lima estuary in different months (C) and different sampling stations (D) (results presented as mean ± standard deviation; n=30 except when number of organisms in samples were lower; * indicates that only one individual (Chaetognata) was collected).

Regarding the shape of MPs retrieved from copepods, fragments were the most common type, both in the Douro estuary (83%) (Fig.9A) and in the Lima (78%) (Fig.9D). Similarly, fragments were also the most frequent MP retrieved from chaetognaths from the Douro estuary (82%) (Fig.9A) and the Lima estuary (75%) (Fig.9D). Fibers were the second most common shape of MPs retrieved from zooplanktonic organisms, with percentages ranging from 17% of MPs retrieved in copepods in Douro estuary and 18% in chaetognaths in Douro estuary (Fig.9A), to 21% of all MPs found in copepods in Lima estuary and 24% in chaetognaths in Lima estuary (Fig.9D). Films were rarely observed, and the highest numbers were registered in the Lima estuary, representing 0.65% of MPs observed in copepods, and 0.37% of MPs retrieved from chaetognaths (Fig.9D). In the Douro estuary, no films were retrieved from copepods, and only 0.24% of MPs observed in chaetognaths were films (Fig.9A).

Blue was the most common colour of MPs found in all zooplankton samples – 78% of MPs found in copepods and 81% of MPs in chaetognaths, in the Douro Estuary, were blue (Fig.9B); while in Lima estuary, 80% of MPs found in copepods and 76% of MPs in chaetognaths had the colour blue (Fig.9E). The remaining MPs were distributed between seven other colours (orange, green, red, black, white, grey and transparent MPs).

In terms of size, the majority of MPs found were from the smallest size class (smaller than 0.5 mm). In the Douro Estuary, this size class represented 92% of all MPs found in copepods and 91% of MPs in chaetognaths (Fig.9C); while in the Lima estuary, 86% of MPs found in copepods and 87% in chaetognaths were in the smallest size class (Fig. 8F). As the size of MPs increases, their percentage in samples decreases, with larger MPs (> 3 mm) accounting for only 1.8% of MPs retrieved from copepods and chaetognaths from the Douro estuary (Fig.9C), and 0.85% of MPs found in copepods and 0.79% of MPs in chaetognaths in Lima estuary (Fig.9F).

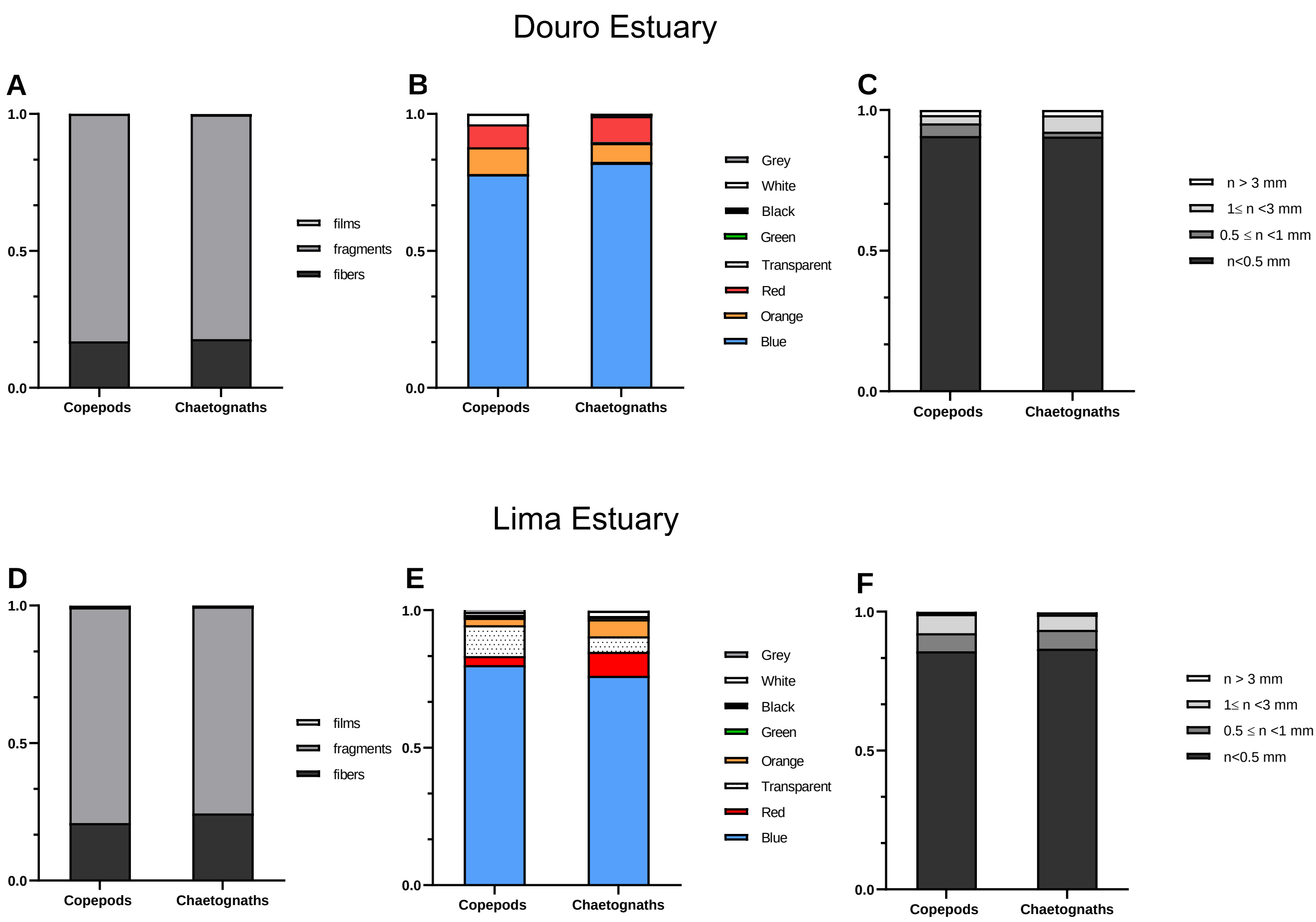

Fig.9 Characterization of MPs by type (A and D), colour (B and E) and size (C and F) in copepods and chaetognaths, in the Douro Estuary (A to C) and in the Lima Estuary (D to F).

4. Discussion

MP contamination is an emergent concern in aquatic environments, and although research on this topic has been increasing recently, there is still missing information from field studies on MP contamination levels and how they impact environments and wildlife, in particular organisms on lower trophic levels of the food web, such as plankton. In Portugal, namely in the Douro and Lima Estuary, there are still few studies regarding MP contamination, namely Rodrigues et al. (2019a) and Prata et al. (2021) in Douro and Almeida et al. (2023) in the Lima Estuary.

The present study is the first to investigate a possible relationship between MP and zooplankton in two distinct estuaries, the Douro estuary and the Lima estuary, and provide important field insights into zooplankton ingestion of MPs and the possible trophic transfer of MPs in the food web.

4.1 MP contamination in zooplankton in the Douro and Lima Estuary

This study is one of the first to show MP contamination of both copepods and chaetognaths in the Douro and Lima estuary. MP concentration in copepods and chaetognaths in both estuaries had the same order of magnitude. Overall, field studies regarding MPs in copepods and chaetognaths are scarce, and concentrations found in our study were generally higher than other values found in other studies with field samples. For example, Kosore et al. (2018) found chaetognaths to have ingested 0.46 particles ind^{-1} and copepods 0.33 particles ind^{-1}; while Sipps et al. (2022) reported concentrations between 0.30–0.82 MP individual^{-1} for three different species of copepods. It is important to note that each of these studies used different methodologies, and therefore, comparisons should be made cautiously. Furthermore, Sipps et al. (2022) note in their study that the nitric acid used for isolation of ingested MPs could cause depolymerisation and fragmentation of certain polymers, undervaluing the total value of MPs digested. Due to the optimization and validation of our protocol for MPs in zooplankton organisms (Rodrigues et al., submitted), we can ensure that we did not underestimate MP values, because our protocol enables the proper degradation of the zooplankton organisms while maintaining the polymerization and physical integrity of all MPs in samples.

Results showed that, in both estuaries, chaetognaths exhibited higher MP contamination levels than copepods. Such differences between MP concentration in copepods (Douro= 3.7±4.1 MP ind^{-1}, Lima= 2.3±2.1 MP ind^{-1}) and in chaetognaths (Douro=

5.9±6.1 MP ind^{-1}, Lima= 5.3±5.2 MP ind^{-1}) can be a consequence of the higher body size of chaetognaths, and therefore, a higher concentration of MPs could be expected. However, it is important to take into consideration the trophic levels of these two types of zooplankton and their distinct feeding habits: copepods are typically primary consumers, feeding on phytoplankton and protists, while chaetognaths are considered secondary consumers, feeding on zooplankton such as copepods (Baier & Purcell, 1997; Kosore et al., 2018; Terazaki, 1998). Therefore, the present results indicate a potential MP transfer between these two trophic levels, as chaetognaths may feed on contaminated copepods. Similar hypotheses were noted by Goswami et al. (2023) and Sun et al. (2018b) that report zooplankton in a higher trophic level to be more susceptible to accidental MP ingestion or accumulation due to contaminated prey. Hence, the present results support MP transfer along the trophic chain, highlighting the fact that it can start at the lower levels of the trophic chain.

Regarding the characterization of the MPs found in zooplankton organisms, we can detect some interesting differences when compared to the MPs typically found in water samples. For instance, while in water samples, the most common type of MP found was fibers, in zooplanktonic organisms fragments made up the majority of MPs found. Aytan et al. (2022) registered a similar result, reporting fibers as the most common MP in the water column, but fragments as the primary MP ingested by copepods. Such results indicate that both copepods and chaetognaths selectively ingested fragments from all the MPs available in the surrounding water. In fact, fragments are more similar to the typical zooplankton prey, therefore explaining this selection. Although copepods can include species with different feeding habitats (e.g. filter feeding, herbivory, predation), the present results show that these organisms tended to select the second most common MP in the surrounding water. In fact, typical filter feeders such as mussels or oysters tend to be contaminated with the same type of MPs of the surrounding water (e.g. Bom & Sá, 2021; J. Ding et al., 2022), which was not observed in our study. In fact, some studies, mainly performed in laboratory settings, have tried to seek out answers for possible feeding selectivity in copepods, many reporting size and shape as a major selectivity aspect (Coppock et al., 2019; Meyer et al., 2002), while others report unselective feeding (Djeghri et al., 2018). The present study reinforces the need for further field studies to increase the scientific knowledge of how organisms are contaminated by MPs in realistic conditions.

It is worth noting that, although there has been a recent upsurge of studies on the ingestion and impacts of MPs in smaller marine organisms, such as zooplankton, the majority of these studies are performed in laboratory conditions, often using virgin MPs in higher concentrations than the ones commonly registered in field studies. Despite the importance of these studies in understanding the biological impacts of MP ingestion on zooplankton health, such as internal injuries (He et al., 2022; Wright et al., 2013; Zavala-Alarcón et al., 2023), impaired feeding behaviour and reduced fecundity (Cole et al., 2015), it is critical to recognize that the damages reported in these laboratory settings may not accurately reflect the impacts of MPs in zooplankton in the field, and that field studies regarding this topic are often limited and in less number than their laboratory counterparts. Our study aimed to assess the concentration of MPs in zooplankton organisms in their natural environment – either by direct ingestion of MPs or by the ingestion of prey contaminated by MPs. Hence, the adaptation of laboratory studies to realistic environmental conditions is extremely important, for example in using different types of MPs common in the environment, in order to properly compare the ingestion of different types of MPs by zooplankton.

In terms of MP colours, blue was the most common colour found in copepods and chaetognaths, with blue MPs composing a large majority of the MPs found in these two types of zooplankton. This might be a consequence of the fact that MPs from the surrounding water were also mostly blue, and also their resemblance with typical food colours. Several studies also reported blue as the most common colour, such as Goswami et al. (2023) that accounts blue MPs as representing 50% of all MPs found in zooplankton. In terms of size, there was also a tendency for the majority of MPs found in zooplanktonic organisms to be from the smallest size class considered, i.e. below 0.5 mm. Several other studies, such as Klasios & Tseng (2023) register the same tendency for smaller fragments to be more bioavailable for zooplankton.

The present study indicates that the zooplanktonic community of the two estuaries might be under similar pressure posed by MPs, since MP contamination of zooplanktonic organisms values were similar between the two estuaries. Moreover, a similar average ratio between MPs versus zooplankton was observed. The two estuaries also showed a similar temporal tendency for these ratios to increase in autumn (November sampling), associated with increasing MP contamination in water. Due to the importance of zooplankton to the food web and complex ecosystems such as estuaries, it is important to study threats and interferences to these organisms. The ingestion of MPs by copepods may be one of the entry points of MPs in the food web, and one of the first transfers of MPs through trophic levels, from primary consumers (copepods) to secondary consumers (chaetognaths) that feed on contaminated copepods.

4.2 MP's presence in estuarine water

The present study confirmed the contamination of the Douro and Lima Estuaries with MPs, with similar contamination levels in the same order of magnitude (2.3 ± 1.9 MP m^{-3} in Douro and 2.4 ± 2.0 MP m^{-3} in Lima estuaries). A previous study by Rodrigues et al. (2019a) also characterized MPs in water samples from the Douro Estuary, and found levels of 0.17 MPs m^{-3} , while Prata et al. (2021) found a median MP concentration of 0.23 MP m^{-3} among three different sampling areas in the Douro estuary: a countryside area; a wastewater treatment effluent release zone; and an area in proximity to a boat dock and maintenance station. In the Lima Estuary, Almeida et al. (2023) found MP concentrations of estuarine water ranging from 0.010 MP m^{-3} to 0.20 MP m^{-3}. Among other estuaries and enclosed water forms, our results are within the same range found at the river mouth of the Black Sea (3.3 ± 2.0 particles m^{-3}) by Aytan et al. (2022), and at the Adour Estuary, in France (Defontaine et al., 2020). However, other studies showed MP concentrations in higher orders of magnitude, such as Taha et al. (2021), which retrieved 1687 particles m^{-3} in Terengganu estuary, in Malaysia; or Trindade et al. (2023) in a heavily populated bay in Brazil (5180 items m^{-3}). Likewise, lower MP concentrations have also been reported, namely by Lima et al. (2014) (0.2604 items m^{-3}), or Sun et al. (2018a) that reported MP concentrations of 0.13 ± 0.20 items m^{-3} in the Yellow Sea. It is important to highlight that differences in sampling methods, such as different net sizes, water pumps, and depth at which the sample is retrieved, as well as the wide range of different protocols used for processing MPs, can influence results, and should be taken into consideration when drawing comparisons.

When comparing both estuaries in our study, we initially hypothesized that Douro estuary would present higher MP contamination than Lima estuary, since Douro estuary is more impacted (Ramos et al., 2015), namely by a heavier urban pressure than Lima, due to the presence of two major cities in the vicinity of the estuary (Porto and Vila Nova de Gaia). Several studies (Desforges et al., 2015; Sun et al., 2018b; Tibbetts et al., 2018) relate the proximity to big urban centers and hotspots of human activity as a major source of MP contamination. However, results showed that MP concentration in water samples of both estuaries was similar. Gray et al. (2018), when comparing two South Carolina estuaries, also faced the same results, in which the estuary with the lowest surrounding population revealed to be the most contaminated with higher MP concentration. This outcome could be related to the features of the total area and the drainage area of the estuaries, such as anthropogenic pressure and industrial activities, showcasing that these factors could be more prone to influence MP concentration than the immediate surrounding population and subsequent human activity (Gray et al., 2018). In fact, our results showed that in November

MP concentration increased in both estuaries, and although the highest contamination value was observed in the Douro estuary (D1), in average the Lima estuary registered higher contamination than the Douro estuary. Such an increase in MP contamination could have been associated with higher precipitation values, typical of this time of the year (heavy rain in the days prior to our sampling campaigns), that might have transported MPs from upstream locations and river banks to the estuaries (Rodrigues et al., 2019a).

Regarding the characterization of MPs found in the water samples, we verified similar results between estuaries, with fibers being the most common type of MP, followed by fragments and films. Rodrigues et al. (2019a) in the Douro estuary and Almeida et al. (2023) in the Lima estuary also reported fibers as the most prevalent type of MPs in water samples. Several studies link the presence of fibers in aquatic environments to domestic sewage and proximity to wastewater treatment plants (WWTP) (Gray et al., 2018; Napper & Thompson, 2016), and remnants of fishing gear and other maritime activities (fishing lines, nets, ropes) (Goswami et al., 2023). Although different, the two estuaries are exposed to important fibers sources, namely WWTP and touristic maritime activities in the Douro estuary (Rodrigues et al., 2019a) and maritime activities such as maritime transport, fishing and aquaculture facilities in the Lima estuary. A variety of different coloured MPs was also observed, with a higher diversity of colours than the ones reported in this study for copepods and chaetognaths. Blue represented the most common colour, both in the Douro Estuary and Lima estuary. Overall, blue is regarded as one of the most common colours of MPs detected in aquatic environments (Trindade et al., 2023). The noticeable presence of polyethylene in both estuaries might be associated with the proximity to urban centers and areas with heavy touristic pressure, since polyethylene is a commonly used material to produce containers, wrappings and plastic bags. The second most common polymer found in our study was polypropylene, that despite all other potential domestic sources, can be associated with maritime activities, namely fishing gear and netting, rope and bottle caps (Coyle et al., 2020; GESAMP, 2016).

5. Conclusions

The present study showed the presence of MPs in water and zooplankton organisms in two Portuguese estuaries, Douro and Lima estuary, with an average ratio of 1 MP:4.7 zooplankton organism for the Douro estuary, and 1 MP:4.6 zooplankton organism in the Lima Estuary. Copepods and chaetognaths from the two estuaries were contaminated with MPs, with similar values between estuaries, and higher concentrations in chaetognaths than copepods, possibly indicating trophic transfer of MPs throughout the food web, by the ingestion of contaminated copepods by chaetognaths. Results also indicate that zooplankton organisms were contaminated by a specific type of MPs, blue fragments of small size, indicating some selectivity for MPs similar to zooplankton food. Overall, our study allowed a further understanding of MP contamination in estuarine environments, and gave important insights about the ingestion of MPs by copepods and chaetognaths. Results show a tendency for these organisms to ingest a certain type of MP. We can also account for a possible trophic transfer of MPs in the food web. Our study highlights the need to further investigate the ingestion of MPs by zooplankton and its impact on these organisms, as well as the impact on the ecosystems they inhabit.

Chapter 3

Final Considerations

The topic of MPs has been gathering attention from scientific researchers and the general public in recent times. Reports of contamination of aquatic environments by these emerging pollutants have been steadily appearing in the last few years, showcasing a worldwide presence of MPs in oceans, rivers, lakes and estuaries.

The MP contamination in estuaries poses a serious threat because these systems represent transitional sites between the ocean and freshwater streams, provide important ecosystem services and are of major importance to several species, including zooplankton.

Zooplankton, which comprises the animal component of the plankton, provides many services and is responsible for important ecological functions. They are located in a strategic position in the food web, connecting primary producers to higher trophic levels, enabling energy and nutrient transfer across the food web. Several studies have already confirmed the occurrence/ ingestion of MPs by zooplanktonic organisms, as well as the adverse effects of MPs on these organisms (e.g. (Aytan et al., 2022; Kosore et al., 2018; Rodríguez-Torres et al., 2020)). However, very few studies have explored the possibility of a trophic transfer of MPs in the food web, particularly in its lower levels. This study explores MP contamination of zooplankton in two Portuguese estuaries, through seasonal surveys conducted during one year in the Douro and Lima river estuaries.

Through the samples retrieved in these surveys, we were able to quantify zooplankton abundance and identify the major zooplankton groups present in the two estuaries, which included copepods and chaetognaths.

MP presence was first assessed in water samples, through a protocol previously optimized by our team (Rodrigues et al., 2019b). The protocol allowed to confirm the presence of MPs in all water samples collected in the estuaries, and revealed similar MP concentrations in both estuaries (Lima: 2.4 ± 2.0 MPs m^{-3}; Douro: 2.3 ± 1.9 MPs m^{-3}), that tended to increase in November, in both estuaries, which could be associated with higher precipitation values, typical of that time of the year. Overall, and according to the objectives of our study, the present study allowed us to develop a more complete understanding of MP contamination in estuarine waters, namely the spatial (through the use of varied sampling sites) and temporal (through sampling campaigns in four different months across a year) patterns of contamination in the Lima and Douro river estuaries.

The ratios between MPs and zooplankton organisms showed similar ratios for both estuaries, with an average ratio of 1 MP:4.7 zooplankton organism for the Douro estuary, and 1 MP:4.6 zooplankton organism in the Lima Estuary.

In a second step, MP presence was assessed in two different groups of zooplankton, copepods and chaetognaths. For that, we utilized a protocol optimized by our team (Rodrigues et al. (2023), submitted). In resume, zooplanktonic organisms are exposed to 30% H_2O_2 at a specific temperature, during a certain period of time, ensuring the degradation of all the organic matter, while keeping MP integrity in terms of type, size, colour and polymer. This protocol ensures that MP concentrations are not underestimated, and that the physical integrity of the MPs is not compromised during the laboratory process.

Results showed that chaetognaths exhibited higher MP contamination in both Lima (5.3 ± 5.2 MPs ind^{-1}) and Douro estuaries (5.9 ± 6.1 MPs ind^{-1}) than copepods, which tended to have lower levels of MP contamination (Lima: 2.4 ± 2.1 MPs ind^{-1}; Douro: 3.7 ± 4.1 MPs ind^{-1}). Copepods and chaetognaths occupy different trophic levels, i.e. copepods are mostly filter feeders and considered first consumers, connecting primary producers to higher trophic levels (Heuschele & Selander, 2014), while chaetognaths are secondary consumers, feeding on zooplankton and in particular, on copepods (Kosore et al., 2018). Therefore, in this context, such different values in contamination levels between these two zooplankton groups could indicate a trophic transfer of MPs in the lower levels of the food web (from copepods to chaetognaths). Hence the present study gives important field evidence that MPs are transferable along the lower trophic levels of planktonic communities.

Another important finding of the present study was the fact that the most common type of MP found differed between water and zooplanktonic organisms. Copepods and chaetognaths were mostly contaminated with small blue fragments, while fibers were the most common MP found in water samples. Such results could indicate a selectivity from these organisms towards MPs that resembled their typical food.

The conclusions reached by this study emphasize the need for further investigation of several aspects regarding the topic. Firstly, we highlight the need for more field studies regarding interactions between MPs and zooplankton, to complement the knowledge assessed with laboratory studies. Laboratory studies often present several characteristics that set them apart from real-life conditions, such as the type of MP or the concentration utilized, compromising the extrapolation of results and prediction of contamination patterns of ecosystems. Therefore, it will be important to gather more field evidence of MP effects on planktonic organisms and functioning.

Even when comparing results between different field studies, the major differences regarding the sampling procedures and protocols imply the need for caution when comparing MP concentrations, both in water samples and in zooplanktonic samples. Therefore, the standardization of MP protocols in these two types of samples should be also furthered in future research.

Due to the important ecological, economic and social role of estuarine ecosystems, their conservation and further understanding are extremely important. Estuaries should be monitored for the presence and impact of MPs, to identify possible sources of contamination, from fishing, industrial or touristic and recreational activities, which could be an important step towards implementing effective conservation measures for these environments. Additionally, preventing MP contamination in estuaries could also be an important measure to reduce MP contamination in the ocean, since estuaries are considered as major contamination sources of the ocean.

Furthermore, the integration of MP pollution data in ecological models could help to understand the effects on the estuarine ecosystem (Rodrigues et al., 2021). Interactions between MPs and organisms on the lower levels of the food web are still underrepresented, and information regarding this particular topic is still scarce. From the results of this study, the trophic transfer of MPs and zooplankton selectivity of MPs seem to be the topics that should gain more attention in future work. Regarding the trophic transfer of MPs in zooplankton, few studies have focused on this topic. The study of Setälä et al. (2014), performed in laboratory settings, is one of the few studies focused on MP trophic transfer in zooplankton, and is the first to show the transfer of plastic microparticles in zooplankton from one trophic level to a higher level. Future field studies should explore this transfer in zooplankton levels of the food web, in diverse zooplankton communities and diverse aquatic environments. Additionally, studies covering the transfer of MPs through multiple levels of the food web and the biomagnification effect of these contaminants could be extremely important for a deeper understanding of MP contamination.

Our study highlighted the discrepancy between the most common type of MPs in zooplanktonic organisms (fragments) and in the surrounding waters (fibers). Overall, copepods and chaetognaths seemed to select a certain kind of MP, namely blue particles of small size. Feeding selectivity, especially in copepods, is still a topic under study: while some studies show feeding selectivity towards characteristics such as size and shape (Coppock et al., 2019; Meyer et al., 2002), other studies report no feeding selectivity (Djeghri et al., 2018). Thus, future studies are necessary to explore this zooplankton selectivity towards MPs, and how feeding strategies and limitations of these organisms can impact them and in consequence, higher trophic levels.

MPs are causing more and more negative impacts on the natural environment and in diverse communities and ecosystems. The results derived from this thesis provide important scientific knowledge regarding the contamination of estuarine waters by MPs, and the interactions between MPs and zooplankton, showcasing the need for action to preserve the natural environments in our planet.

Chapter 4
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