NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF ENVIRONMENTAL SCIENCES AND ENGINEERING

MICROPLASTICS IN A PORTUGUESE COASTAL AREA: DISTRIBUTION PATTERNS ON SURFACE WATERS AND SEDIMENTS, INGESTION BY WILD MARINE FISH, AND RELATIVE CONTRIBUTION AS A CONTAMINATION PATHWAY

DIANA DUARTE RODRIGUES

Master in Marine Ecology

DOCTORATE IN ENVIRONMENT AND SUSTAINABILITY

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Microplastics in a Portuguese coastal area: distribution patterns on surface waters and sediments, ingestion by wild marine fish, and relative contribution as a contamination pathway

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"Much of the necessary knowledge is now available but we do not use it. We train ecologists in our universities and even employ them in our governmental agencies but we seldom take their advice." (…) "As man proceeds towards his announced goal of the conquest of nature, he has written a depressing record of destruction, directed not only against the earth he inhabits but against the life that shares it with him" (…) "*Is* anything being done? *Can* anything be done? Can *I* do anything?"

(In Silent Spring by *Rachel Carson*)

ABSTRACT

Five decades have passed since the first detection of microplastics (MPs) in the marine environment. However, research on this "invisible" pollution was only triggered in the beginning of the XXI century. Despite the remarkable progress, there are still questions remaining and, most important, preventive measures to be taken. Regarding the evaluation of MPs pollution in the Portuguese coast, the assessment of temporal changes (supported by regular sampling) has rarely been performed and subtidal data scarcely collected. In order to address the lacking data and to better understand the consequences of the anthropogenic pressures resulting from this coastal nation, this thesis primary tasks consisted of collecting and analyzing baseline data from the nearshore subtidal at a segment of the Portuguese west coast, both from water surface and sediments. Findings obtained from the study area, which comprises the Sado river estuary and Arrábida marine Park (where multiple socioeconomic activities take place), intend to trigger and support the improvement of waste management in the region. Moreover, owing to the fish samples (*Boops boops* (Linnaeus, 1758)) collected concurrently with the previous tasks, an evaluation of this species potential to be used as an indicator of MPs pollution in this coastal area, was conducted, consisting of this thesis third objective. Subsequently, with the purpose of understanding the effects occurring in the wild, due to MPs ingestion, a short-term experimental assay was conducted with larvae of *Sparus aurata* L., 1758 exposed to environmentally realistic conditions: besides being fed with MPs, they were reared in artificial seawater contaminated with nonylphenol (an endocrine disrupting compound). Additional aims, accomplished throughout the thesis, were to raise awareness about plastic pollution and to disseminate main findings with society. Most initiatives were conducted with local citizens and stakeholders from the municipalities located near the study area: Setúbal and Sesimbra.

Keywords: pollution, subtidal, ingestion, effects, raise awareness, preventive measures

RESUMO

Passaram cinco décadas desde a primeira deteção de microplásticos (MPs) no ambiente marinho. No entanto, a investigação sobre esta poluição "invisível" só se desencadeou no início do século XXI. Apesar do progresso notável, ainda existem muitas incertezas e, mais importante, medidas preventivas por tomar. Quanto à avaliação da poluição por MPs na costa portuguesa, a investigação de variações temporais (suportada por amostragens regulares) foi raramente efetuada, assim como amostragens no subtidal. De forma a responder às lacunas identificadas e para melhor compreender as consequências das pressões antropogénicas resultantes desta nação costeira, definiram-se como tarefas prioritárias desta tese a recolha e análise de dados do subtidal de um segmento da costa oeste portuguesa, tanto da superfície da água como dos sedimentos. Pretende-se que os resultados obtidos sobre a área de estudo, que compreende o estuário do rio Sado e o parque marinho da Arrábida (onde decorrem múltiplas atividades socioeconómicas), possam desencadear e fundamentar o melhoramento da gestão de resíduos da região. Adicionalmente, devido à recolha de amostras de peixes (*Boops boops* (Linnaeus, 1758)) efetuada em simultâneo com as amostragens anteriores, foi possível avaliar o potencial desta espécie como indicador de poluição por MPs nesta área costeira, consistindo no terceiro objetivo desta tese. Posteriormente, com o objetivo de compreender os efeitos que ocorrem na natureza devido à ingestão de MPs, foi realizado um ensaio experimental de curta duração com larvas de *Sparus aurata* L., 1758 expostas a condições ambientalmente realistas: além dos MPs incluídos nas dietas, foram mantidas em água do mar artificial contaminada com nonilfenol (um composto disruptor endócrino). Ao longo do desenvolvimento da tese procurou-se contribuir para a sensibilização da sociedade sobre esta problemática e divulgar as principais conclusões. A maioria das iniciativas foi direcionada aos cidadãos e stakeholders locais, dos municípios próximos à área de estudo: Setúbal e Sesimbra.

Palavras chave: poluição, subtidal, ingestão, efeitos, sensibilização, medidas preventivas

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ACRONYMS

MPs	Microplastics
UNSDG	United Nations Sustainable Development Goal
WWTP	Wastewater Treatment Plants
BPA	Bisphenol A
NP	Nonylphenol
PBDEs	Polybrominated diphenyl ethers
POPs	Persistent organic pollutants
NGO	Non-governmental organization
COVID-19	Coronavirus disease 2019
FTIR	Fourier Transformed Infrared Spectroscopy
ICNF	Instituto da Conservação da Natureza e das Florestas
GIT	Gastrointestinal tract
FAO	Food and Agriculture Organization of the United Nations
BW	Body weight
GITW	Gastrointestinal tract weight
SL	Standard Length
K	Fulton condition factor
FO%	Frequency of occurrence
KOH	Potassium hydroxide

CAT	Catalase
GST	Glutathione S-transferase
VTG	Vitellogenin
DMSO	Dimethyl sulfoxide
ROS	Reactive oxygen species
EPPO	Estação Piloto de Piscicultura em Olhão
UHMWPE	Ultra-high molecular weight polyethylene
PE	Polyethylene
PP	Polypropylene
PS	Polystyrene
PVA	Polyvinyl alcohol
PUR	Polyurethane
PAA	Poly(acrylic acid)
PA	Polyamide
PET	Polyethylene terephthalate
PAN	Polyacrylonitrile
PMMA	Poly (methyl methacrylate)
PVC	Polyvinyl chloride
PVAc	Polyvinyl acetate
LDPE	Low-Density Polyethylene
SMI-unit	Sediment-Microplastic Isolation device
$ZnCl_2$	Zinc chloride
PS-S	Polystyrene sulfonate
ClNaO	Sodium hypochlorite
Na2S2O3	Sodium thiosulfate pentahydrate
PDMS	Polydimethylsiloxane

SBSE	Stir bar sorptive extraction
PTFE	Polytetrafluoroethylene
GC-MS	Gas chromatography–mass spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
BSA	Bovine Serum Albumin
Na_2HPO_4	Disodium phosphate
KCl	Potassium chloride
EDTA	Ethylenediamine Tetraacetic Acid
DTT	Dithiothreitol
PBS	Phosphate-buffered saline
CDNB	1-Chloro-2,4-dinitrobenzene

1

GENERAL INTRODUCTION

1.1 Plastic Pollution: A Brief Review

Plastic pollution is globally recognized as a threat to the marine environment. Despite being frequently addressed in the political agenda [examples: Directive (EU) 2019/904, which aims to prevent and reduce the impacts of single-used plastics on the environment; "Reduce marine pollution" as the Sustainable Development Goal 14.1 (UNSDG 2030)[1]; implementation of government policies, as bans and taxes (Knoblauch & Mederake, 2021)], and covered by the media, in awareness campaigns or documentaries (Males and Van Aelst, 2020), this anthropogenic pressure has been difficult to tackle, contributing to weaken an already fragile ecosystem due to overfishing, climate change, ocean acidification and habitat destruction. Considering that plastic pollution is widely known to result from plastics high global demand, massive production (Cole, Lindeque, Halsband, & Galloway, 2011; Thompson et al., 2004) (Figure 1.1), excessive consumption, poor waste management, incorrect disposal and slow degradation rate (Barnes, Galgani, Thompson, & Barlaz, 2009), reducing this environmental problem will depend on the commitment of all sectors of society.

[1] https://sdgs.un.org/goals/goal14

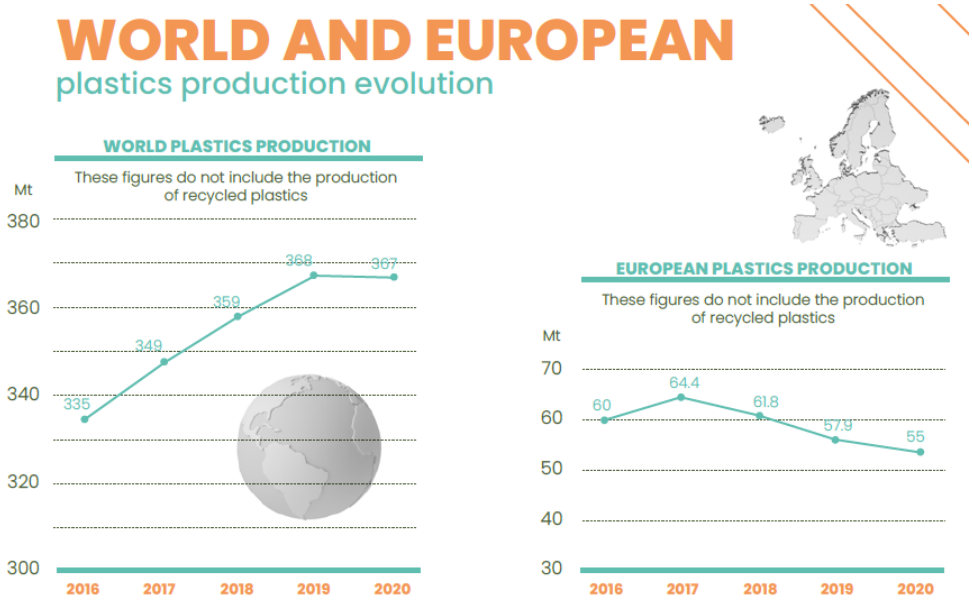

Figure 1.1 – World and European plastics production. Source: (Plastics Europe, 2021)

Plastic, as a low cost, lightweight, versatile, inert, and resistant material, occurs as a valuable commodity in our consumer society, being used at a daily basis, in domestic, medical, industrial, technological, transports and many other applications. Immediately after the first polymer invention (Bakelite, named after its inventor: Leo Baekeland) in 1907, the demand on plastics started to increase, being greatly impelled by the Second World War in the 1940s, with the manufacture of parachutes and airplane parts (Parker, 2019; Worm, Lotze, Jubinville, Wilcox, & Jambeck, 2017). Since then, many other polymers were invented and, currently, the industry of plastics offers innumerous options with different properties.

Despite such polymer diversity, the majority of plastics found in the ocean are Polyethylene and Polypropylene (Erni-Cassola, Zadjelovic, Gibson, & Christie-Oleza, 2019). These are the most produced polymers, being extensively used in food packaging (Geyer, Jambeck, & Law, 2017) (Figure 1.2) as single-used items. The enduring of such unsustainable consumption era, being celebrated in an article published in *Life* magazine in 1955, along with a fast discard and reduced recycling rates, highly contributed to an extremely high generation of waste over the years.

Not surprisingly, continuous inputs of plastic ended up occurring in the marine environment. In fact, this early perception about the widespread occurrence of plastic debris contributed for the elaboration of the regulations included in Annex V of the MARPOL International Convention, signed by more than 150 countries in 1973, which intended to prevent marine pollution from garbage being discarded from ships.

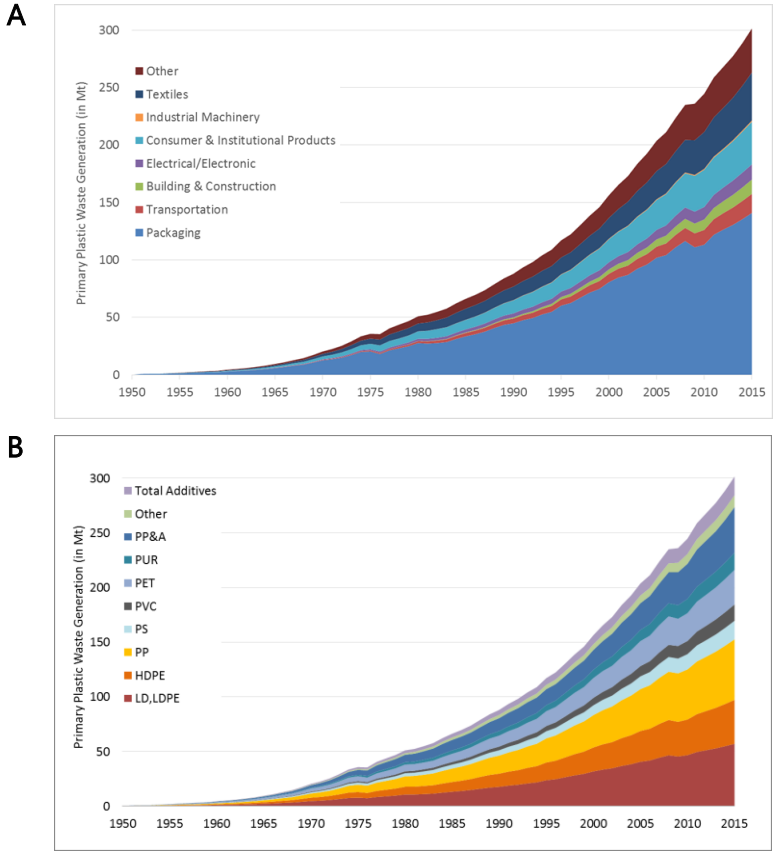

Figure 1.2 - Global primary plastics waste generation (in million metric tons) according to industrial use sector (A) and to polymer type (B); from 1950 to 2015; Source: (Geyer et al., 2017).

From that point, a considerable number of pioneer investigations contributed to disclose the distribution patterns of large items (macroplastics) on the pelagic (Day & Shaw, 1987; Lecke-Mitchell & Mullin, 1992; Morris, 1980; Venrick et al., 1973), seabed (Bingel, Avsar, & Ünsal, 1987; F. Galgani, Burgeot, et al., 1995; F. Galgani, Jaunet, Campillo, Guenegen, & His, 1995; Galil, Golik, & Türkay, 1995; Hess, Ribic, & Vining, 1999; Kanehiro, Tokai, & Matuda, 1995; Stefatos, Charalampakis, Papatheodorou, & Ferentinos, 1999) and shoreline (Corbin & Singh, 1993; Cundell, 1973; Lucas, 1992; Merrell, 1980; Walker, Reid, Arnould, & Croxall, 1997) compartments.

Soon, the aesthetics (Barnes et al., 2009) and safety issues associated with such ubiquity of debris were pointed out as socioeconomic impacts affecting mostly the tourism and nautical sectors (Cole et al., 2011). In addition, the transport of opportunistic colonizers (Barnes, 2002; Gregory, 2009) and the physical injuries caused on marine wildlife (emblematic, ecologically, and commercially important species), such as entanglement and chocking (Laist, 1987, 1997), became frequently reported. Then, in 1997, the discover of the Great Pacific Garbage Patch in North Pacific Subtropical Gyre by Captain Charles Moore (Andrady, 2011; Melanie Bergmann et al., 2017; Moore, Moore, Leecaster, & Weisberg, 2001), contributed to demonstrate the long-range transport of floating plastic, via wind and ocean currents.

Recently, owing to several robust models being developed, the quantities of waste generated so far could be estimated and some even show, according to different scenarios, the amount of waste predicted for the near future. While estimations based on 2010 data, published by Jambeck et al. (2015), show that between 1.1 to 8.8 million tons (Mt) of mismanaged plastic waste were produced by each country, Lebreton and Andrady (2019) estimated between 60 and 99 Mt of mismanaged plastic waste produced globally in 2015; and Borrelle et al. (2020) conclude that between 19 to 23 Mt of plastic waste, generated in 2016, has entered aquatic ecosystems.

Considering all the knowledge acquired in the last decades, even though there are still topics pending clarification, this scientific area is no more in its infancy. It has been intensively studied and there is enough up-to-date information to define realistic strategies. It's time to act and we are several decades late.

1.2 Microplastics

1.2.1 Definition and Categorization

The very first reports of microplastics being collected from the ocean, referred to as pre-production plastic pellets with 5 mm (E. J. Carpenter & Smith, 1972; Edward J. Carpenter, Anderson, Harvey, Miklas, & Peck, 1972), occurred several decades before the term being coined. In the following years, despite studies being mostly focused on large debris, there were some exceptions which contributed to provide further insights about smaller plastic pieces (for example: Day, 1980; Day and Shaw, 1987; Laist, 1987; Day et al., 1990; Shaw and Day, 1994). Only later, in the beginning of the XXI century, as a result of the growing concern and rapidly increase of research about this environmental problematic it became clear that, in addition to the larger items, the small sized particles – designated as microplastics - were also widespread from the water surface to the seabed (Thompson et al., 2004). Although being consensually accepted as items smaller than 5 mm since 2009 (defined by Arthur et al.), microplastics lower size limit, 1 µm, was only recently established (J.P.G.L. Frias & Nash, 2019; Gigault et al., 2018).

Owing to the enhanced perception about the diversity of microplastics (hereafter MPs) available in the oceans, it became clear that they could be assigned to two main categories (Andrady, 2011; Cole et al., 2011; Hidalgo-Ruz, Gutow, Thompson, & Thiel, 2012) and multiple types (shapes). The primary MPs, occurring as industrial pellets, microbeads from personal care products and industrial abrasives, were defined as items originally manufactured with small size. Conversely, the secondary MPs category, should be attributed to particles resulting from fragmentation of larger objects, as a consequence of mechanical processes, prolonged exposure to UV light and microbial-mediated biodegradation

(Andrady, 2003, 2011). Some examples are filaments, fibers and fiber bundles resulting from the degradation of fishing gear and textile shedding during washing (Mark Anthony Browne et al., 2011; Napper & Thompson, 2016), the irregular fragments and films generated with the deterioration of packaging items, paint flakes from nautical coating and dust from vehicle tires.

1.2.2 Sampling and Extraction Protocols

Despite the early evidence of the accumulation of MPs in seabed sediments, research become biased towards the sea surface compartment for a considerable period of time. This was related with the potential of samples primarily collected for zooplankton monitoring studies to be additionally used to assess MPs pollution. Also, it was strongly associated with the effectiveness and suitability of plankton nets for the purpose of estimating MPs pollution (Cole et al., 2013; J.P.G.L. Frias, Otero, & Sobral, 2014; Miller, Kroon, & Motti, 2017).

Yet, the growing understanding regarding the 100-fold discrepancy between the estimates of all the plastic waste input in the oceans and the estimates of the global load of floating debris being reported (Cozar et al., 2014; Eriksen et al., 2014; Lindeque et al., 2020) triggered an increase of research focused at sedimentary matrices. The easy access and low-cost sampling at beaches (Barnes et al., 2009; Pagter, Frias, & Nash, 2018; I. R. Santos, Friedrich, & Ivar do Sul, 2009; Van Cauwenberghe, Devriese, Galgani, Robbens, & Janssen, 2015) explained the shift into the investigation of intertidal sediments, mainly in sandy shores.

Despite all the knowledge being acquired, and scientists willingness to contribute, the lack of methods standardization and the use of different reporting units was preventing the comparison of results (Hidalgo-Ruz et al., 2012; Van Cauwenberghe, Claessens, Vandegehuchte, Mees, & Janssen, 2013). One of the first documents addressing this problem (François Galgani et al., 2013) was published in the scope of the Marine Strategy Framework Directive (MSFD, 2008/56/EC), yet, many others were compelled to improve and develop new extraction protocols that could prevent, for instance, the underestimation of high-density polymers (Claessens, Van Cauwenberghe, Vandegehuchte, & Janssen, 2013; Coppock, Cole, Lindeque, Queirós, & Galloway, 2017; J. Frias et al., 2018; Imhof, Schmid, Niessner, Ivleva, & Laforsch, 2012; Nuelle, Dekiff, Remy, & Fries, 2014). This critical progress was also essential for the investigation of MP pollution at subtidal sediments, which was still poorly addressed (J.P.G.L. Frias, Gago, Otero, & Sobral, 2016; Hidalgo-Ruz et al., 2012; Ling, Sinclair, Levi, Reeves, & Edgar, 2017; Nuelle et al., 2014; Yao et al., 2019).

Nowadays, when handling sediment, seawater and biota matrices, an effort should be made to follow, respectively, the main guidelines available in the following reports: Frias et al., (2018), Gago et al. (2018) and Bessa et al. (2019).

1.2.3 Sources, Pathways and Sinks

There are multiple pathways for MPs to reach the marine environment, though it is widely accepted that their sources occur predominantly on land (Rochman, 2020) (Figure 1.3). Industrial and domestic mismanaged waste may be transported through untreated sewage, stormwater runoff, river discharge, wind transport and tidal cycles (Andrady, 2011; Jambeck & Johnsen, 2015; Ryan, Moore, van Franeker, & Moloney, 2009). In what concerns the role of wastewater treatment plants (WWTP), though previously suggested to be inefficient in fibers and microbeads retention (Mark Anthony Browne et al., 2011; Gregory, 1996), recent studies have shown the opposite (Conley, Clum, Deepe, Lane, & Beckingham, 2019; Mason et al., 2016; Mintenig, Int-Veen, Löder, Primpke, & Gerdts, 2017; Murphy, Ewins, Carbonnier, & Quinn, 2016). However, it is argued that the reduced amount of MPs being released per liter in the effluent is still substantial and, thus, should be considered as a significant source. Moreover, MPs successfully retained in WWTP sludge end up spread again on land due to the use of the sludge as a fertilizer in agriculture fields (Harley-Nyang, Memon, Jones, & Galloway, 2022; Zubris & Richards, 2005).

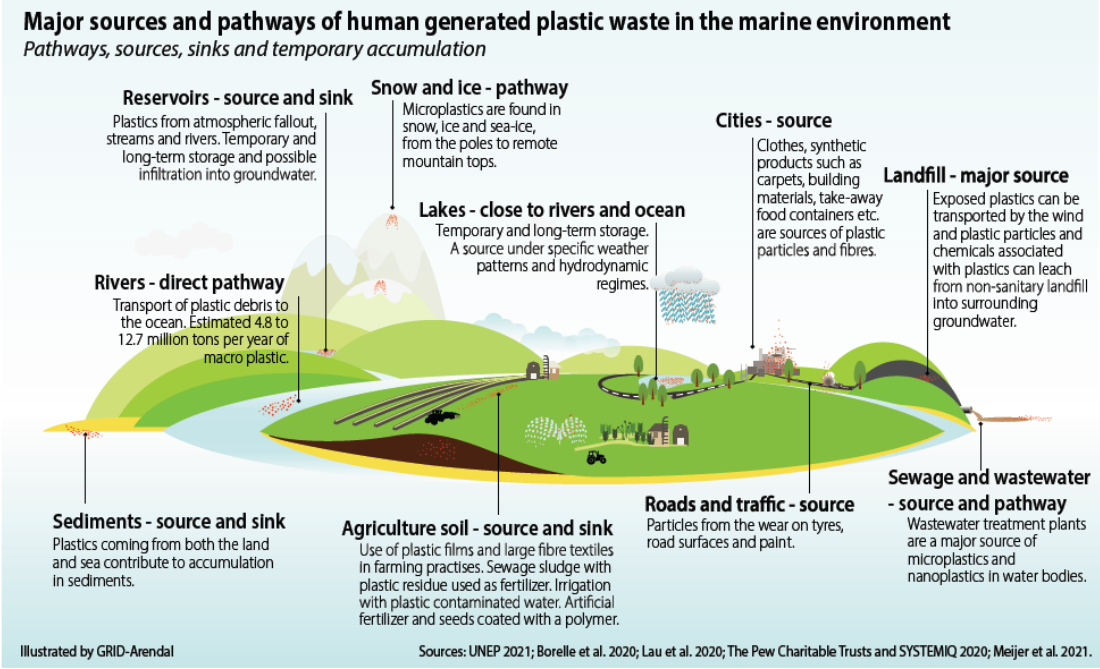

Figure 1.3 – Major land-based sources of plastics. Source (United Nations Environment Programme, 2021)

There are also several sea-based sources contributing for plastic inputs in the ocean (Figure 1.4), namely maritime traffic (either merchant, recreational, government and war vessels), offshore oil and gas platforms, aquaculture installations, fishing activities (L. Lebreton et al., 2018), coastal harbors, and accidental spillages during transportation (Mark Anthony Browne et al., 2011; GESAMP, 2016; Gewert, Ogonowski, Barth, & MacLeod, 2017; Jambeck & Johnsen, 2015; Rochman, 2020).

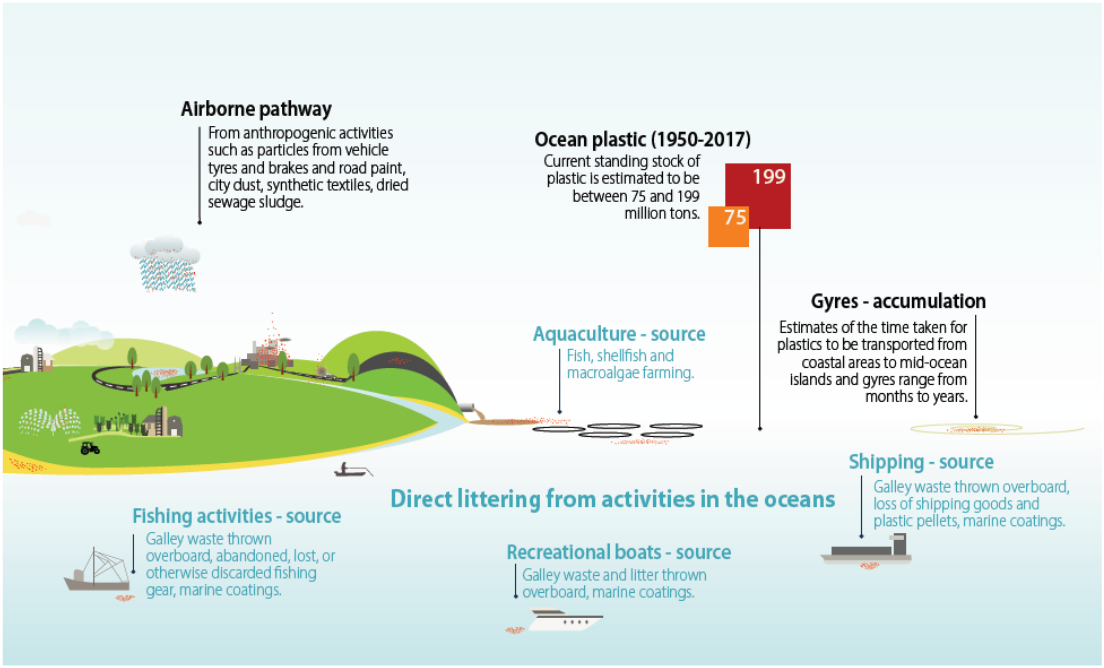

Figure 1.4 - Major sea-based sources of plastics. Source (United Nations Environment Programme, 2021)

Regardless of their origin, when virgin MPs reach the seawater (where density is approx. 1.02 g/cm^3), their position in the water column (vertical distribution) will take place according to the polymer's density: low-density polymers will float in seawater, high-density polymers will sink, and others will keep drifting at midwater due to their neutral buoyancy. However, since a microbial film (commonly referred to as Plastisphere) starts to cover the MPs surface within weeks after entering in the marine environment (Zettler, Mincer, & Amaral-Zettler, 2013), followed by the attachment of algae and invertebrates, this will affect particles buoyancy (Kaiser, Kowalski, & Waniek, 2017; Lobelle & Cunliffe, 2011; Ye & Andrady, 1991). In fact, besides contributing for the deposition of floating plastics in seabed, biofouling will increase plastics potential to be ingested by marine organisms as it provides them a more attractive smell (Savoca, Tyson, McGill, & Slager, 2017; Savoca, Wohlfeil, Ebeler, & Nevitt, 2016). Moreover, the sink of low density particles may also occur through incorporation into marine snow (Porter, Lyons, Galloway, & Lewis, 2018; Van Cauwenberghe, Vanreusel, Mees, & Janssen, 2013; Woodall et al., 2014) or into fecal pellets (Cole et al., 2016; Coppock et al., 2019).

The spatial distribution of MPs, either vertical or horizontal, is also influenced by hydrodynamic forces, mainly by wind (Kukulka, Proskurowski, Morét-Ferguson, Meyer, & Law, 2012; van Sebille et al., 2020), but also with tides, waves and thermohaline gradients (Zhang, 2017), which affect particles deposition and resuspension. In addition, both vertical and horizontal distribution may be influenced by biota migrations (Choy et al., 2019; Setälä, Lehtiniemi, Coppock, & Cole, 2018), through ingestion and egestion of MPs in different compartments of the water column and different locations.

Although the shoreline, when compared with the water column and seabed environments, has a high potential for the generation of secondary MP due to the high mechanical abrasion, temperatures, and exposure to UV radiation (Pegram and Andrady, 1989; Gregory and Andrady, 2005; Barnes et al., 2009; Andrady, 2011), the seabed sediments are in fact suggested to consist of major sinks of MPs (Rochman, 2020).

1.2.4 Associated Contaminants

The capacity of MPs to transport contaminants, early noticed by Carpenter et al. (1972) and Mato et al. (2001) in plastic pellets, quickly suggested that they could act as potential vectors of such contaminants to biota and between locations (Teuten, Rowland, Galloway, & Thompson, 2007; Teuten et al., 2009). Then, owing to the subsequent studies focused on the sorption capacity of plastics, it became clear that such affinity with contaminants depends on the pollutant and polymer type (Bakir, Rowland, & Thompson, 2014a; Rochman, 2015), weathering conditions (J. C. Antunes, Frias, Micaelo, & Sobral, 2013; Mato et al., 2001) and particle size (Cole et al., 2011).

Both the high surface area to volume ratio (enhanced by weathering (Mato et al., 2001)) and the hydrophobic surface of MPs are known to increase adsorption of the highly hydrophobic persistent organic pollutants (POPs) available in seawater (Andrady, 2011; Bakir, Rowland, & Thompson, 2012; Bakir et al., 2014a). In fact, according to Mato et al. (2001), such adsorbed contaminants may achieve concentrations of several orders of magnitude higher than in the surrounding seawater.

It has been also reported that other emerging contaminants, such as pharmaceuticals (Martín, Santos, Aparicio, & Alonso, 2022) and metals (Holmes, Turner, & Thompson, 2012; Rochman, Hentschel, & Teh, 2014), may also associate with MPs and affect different trophic levels. Lastly, besides being heavily contaminated with waterborne pollutants, plastics may also carry toxic additives (plasticizers, flame retardants, antimicrobials, dyes or UV-stabilizers), as phthalates, bisphenol A (BPA), nonylphenol (NP) and polybrominated diphenyl ethers (PBDEs), which are incorporated during manufacture (Teuten et al., 2009) (Figure 1.5).

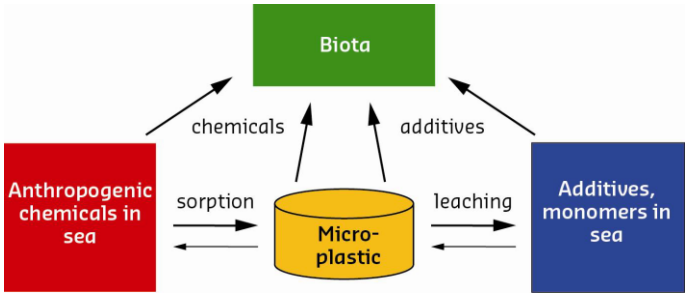

Figure 1.5 – Partitioning of chemicals between plastics, biota and seawater. Source: (Leslie, van der Meulen, Kleissen, & Vethaak, 2011)

1.2.5 Effects on marine biota upon ingestion

Owing to MPs small size, they can be ingested by marine organisms. Ingestion can occur intentionally, when MPs resemble natural preys (Ory, Sobral, Ferreira, & Thiel, 2017; Shaw & Day, 1994), accidentally, when there is a passive intake of MPs during foraging activities, or by trophic transfer, when predators feed on prey that already carry MPs (Farrell & Nelson, 2013; Fossi et al., 2018; Nelms, Galloway, Godley, Jarvis, & Lindeque, 2018; Neves, Sobral, Ferreira, & Pereira, 2015; Roch, Friedrich, & Brinker, 2020; Wright, Thompson, & Galloway, 2013) (Figure 1.6).

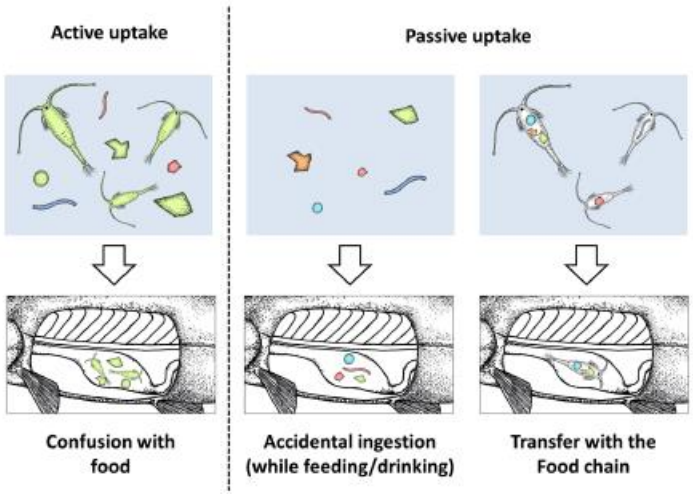

Figure 1.6 – Possible pathways of MPs ingestion. Source (Roch et al., 2020)

According to experimental studies, the ingestion of MPs may produce adverse effects. It can cause physical damage in tissues (Chen et al., 2022; Corinaldesi et al., 2021; Espinosa, Esteban, & Cuesta, 2019; Hsieh et al., 2021; Pedà et al., 2016; von Moos, Burkhardt-Holm, & Köhler, 2012) and/or induce toxicological responses (Mark Anthony Browne, Niven, Galloway, Rowland, & Thompson, 2013; Oliveira, Ribeiro, Hylland, & Guilhermino, 2013; Rochman, Hoh, Kurobe, & Teh, 2013; Rochman, Kurobe, Flores, & Teh, 2014; J. Wang et al., 2019). Yet, caution is needed when referring to toxicological effects because laboratorial trials have been rarely conducted under environmental realistic conditions and thus uncertainties remain (Law & Thompson, 2014). Also, the relevance of MPs as vectors of contaminants may change according to physiological conditions, as temperature, pH and salinity (Albert A. Koelmans, Besseling, Wegner, & Foekema, 2013).

As contaminants desorption largely depend on gut retention time and on the gradient of the chemical concentration between the plastic and the organism contaminant burden (Albert A Koelmans, 2015; Albert A Koelmans, Besseling, & Foekema, 2014), the contribution of other contamination pathways must be considered – which frequently failed in laboratorial trials. In fact, as argued by Gouin et al. (2011) and Koelmans et al. (2013, 2014a, 2016), if a dietary exposure of MPs contaminated with POPs also considers the ingestion of natural prey and dermal uptake of pollutants from seawater (two

important contamination pathways), the effects specifically resulting from MPs ingestion should be minimum. Also, MPs may in fact play a detoxification role (i.e., attenuate bioaccumulation in tissues (Albert A Koelmans, 2015)) if the chemical fugacity direction of the organic pollutants occur from the organism tissues to the MPs, which will ultimately be egested (Gouin et al., 2011).

On the other hand, if plastic additives are the toxic contaminants under analysis, effects from ingestion of MPs might be more significant (Albert A Koelmans, 2015) as, in this case, plastic act as a relevant source of contamination (Teuten et al., 2009). Also, since additives are not chemically bound to polymers, they are more susceptible to leach out to the surrounding environment (Thompson, Moore, vom Saal, & Swan, 2009).

Moreover, if the concentration of MPs provided in diets is not environmental relevant, the consequent detrimental effects observed will not be representative of what is occurring to marine wildlife (Cole, 2016; Albert A. Koelmans, Gouin, Thompson, Wallace, & Arthur, 2014; Neves et al., 2015). This highlights the urgency on collecting environmental data (MPs quantification and diversity) from subtidal coastal waters (which are scarce) to support realistic experimental designs. Also, it underlines the importance of prudence when interpreting and disseminating data to society, especially with alarmist results.

Either way, if contaminants are transferred across the marine trophic web (bottom-up cascade effects), bioamplification may follow (Batel, Linti, Scherer, Erdinger, & Braunbeck, 2016; Tosetto, Williamson, & Brown, 2017a), eventually causing detrimental impacts on human health due to seafood contamination. Therefore, it is critical to assess MP impacts in marine biota, especially in countries where seafood represents a relevant diet component, such as Portugal (FAO, 2010), in order to evaluate the risks of MP to food safety and public health and consequently establish appropriate preventive measures.

Another potential consequence from ingestion of contaminated MP is the eventual interference in the organisms behavior, such as altered activity rates and responses to chemical alarm cues (Mitchell, Chivers, McCormick, & Ferrari, 2015). However, impairment of chemical cues detection or interpretation by fish have been scarcely studied (de Sá et al., 2015).

1.2.6 Effects on human health

Human exposure to MPs mainly occurs through oral and inhalation routes (Wright & Kelly, 2017). It is widely recognized that atmospheric fallout is a relevant source of fibers (Dris, Gasperi, Saad, Mirande, & Tassin, 2016) and mainly in indoor air where fibers, released from textiles, do concentrate (Dris et al., 2017). It has been confirmed that fibers can be inhaled (Jenner et al., 2022; Pauly et al., 1998) and cause inflammation and pulmonary diseases, including lung cancer (Kremer, Pal, Boleij, Schouten, & Rijcken, 1994; Pauly et al., 1998).

The other important route of exposure is through diet, as MPs have been detected in seafood, honey, sugar, beer, table salt and drinking water (as reviewed by Cox et al., 2019), milk (Kutralam-Muniasamy, Pérez-Guevara, Elizalde-Martínez, & Shruti, 2020) and others.

Regarding consumption of seafood, while shellfish is eaten whole, i.e., including the gut, this is not the case for fish. However, smaller items are suggested to be able to enter in the circulatory system (observed in mussels; Browne et al., 2008) and accumulate in tissues which are then consumed by humans.

Although effects in human health from MPs ingestion are poorly understood and difficult to measure due to the lack of information about exposure doses, it has been suggested to, along with other factors, contribute to obesity (Baillie-Hamilton, 2002), due to the contaminants associated to MPs, namely plastic additives.

Another case of exposure worth to highlight occurs with infants, via ingestion through polypropylene feeding bottles due to particles released during sterilization (D. Li et al., 2020), and with developing fetus, via placenta (Ragusa et al., 2021).

While questions about health risks remain, there are reports assuring that MPs ingested are excreted through feces (Schwabl et al., 2019) and those inhaled, are expelled through mucociliary clearance (Wright & Kelly, 2017).

1.2.7 Actions Towards a Sustainable Society

As a typical hot topic, information about plastic pollution is emerging from all around the globe and quickly reaching society, through powerful images (Figure 1.7) and videos on social media, NGOs awareness campaigns, news, documentaries and petitions for plastic bans (Albert A. Koelmans et al., 2014; Law, 2017). The general public is inevitably becoming more aware about this problematic and associated impacts; however, an informed society does not necessarily translate into changes of mentalities and habits.

Figure 1.7 – Cover of June 2018 issue of National Geographic magazine.
Source[2]

Moreover, due to the COVID-19 pandemic, actions towards plastic pollution reduction have lost momentum in 2020. The once threatened plastic industries (due to an increasing society awareness to environmental crises), ended up adapting their businesses to face the exponential demand on personal protective equipment and other hygiene and medical plastic supplies. Nowadays, being the control phase of the pandemic apparently achieved, strategies on plastic waste reduction, waste management, and environmental recovery became imperative to be discussed and should be faced as the worthiest to invest time, resources, and money. In fact, since plastic production is highly dependent on fossil fuel and is strongly responsible for the emission of greenhouse gases to the atmosphere (as well as at the end of plastic life cycle, during incineration), it ends up contributing to climate change (Bauer et al., 2022; Ford et al., 2022). This link recently established is being increasingly addressed and highlights the importance of multidisciplinary research.

Moreover, society urgently needs to be reminded, explained, and clarified about the incredibly important role of recycling in waste management. Ideally, recycling should be mainly restricted to end-of-life plastic, which will consist of material (valuable resource) for new production (Thompson, 2015). That is why it is often mentioned in awareness campaigns that, before discarding items for recycling, we should rethink about our options and confirm if plastic goods may still be reused or repaired. However, by advising citizens to consider recycling as the least priority action, we may unintentionally be connoting recycling as a bad action. Also, while the scientific community is aware about the importance of recycling in a circular economy (Bora, 2020; Bucknall, 2020), we must recognize that, in parallel, citizens have been developing a sense of disbelief regarding recycling which, even if not based on valid

[2] https://nationalgeographicpartners.com/2018/05/planet-or-plastic/

and factual arguments, should be faced as a problem (and an opportunity) to improve ways to encourage and recover peoples' trust. Moreover, companies linked to waste collection and recycling, which have a fundamental role in a circular economy, should have more incentives from the government. This could, for example, reduce situations as overflowing garbage bins at critical periods of the year or even increase trash collection points (Law, 2017).

Nevertheless, there are numerous and relevant measures being taken by institutions, NGOs and industries. An encouraging example, occurring at a national level, is Pacto Português para os Plásticos[3] that acts as a platform to inform consumers about several initiatives from industries that have adopted sustainable practices, which may inspire other businesses to join the challenge. At the end, preventing plastic pollution needs to be a concern and a priority to both consumers and industries.

Also worthy to highlight is the critical outreach work being done by so many NGOs, both at a national and international level which, despite repeating the message over and over, still recognize that, at the end of the day, there is a lot of work left to do. It is tremendously grateful to watch words becoming actions but equally exhausting to face skeptical audiences. Environmental education, namely ocean literacy, should be recognized as an important matter to fully integrate scholar programs. Nevertheless, there is no doubt about the increasing fraction of society adopting a more sustainable behavior as consumers, as buying less and more consciously (Herrmann, Rhein, & Sträter, 2022).

Though there were several innovative options being developed due to the incredible demand on alternatives to plastic, the success of some of them is still debatable, because ultimately, they may instead consist of additional environmental problems. Biodegradable plastics are an example, as they will only degrade at industrial composting facilities (Ferreira-Filipe, Paço, Duarte, Rocha-Santos, & Patrício Silva, 2021; Thompson et al., 2009).

Another potential tool to sensitize society is through citizen science. By involving local communities in the scientific data collection, it increases their perception about the problematic and the hazards associated with plastics in the environment and potentiate the spread of sustainable habits. An example is the development of apps that allow citizens to easily contribute for marine debris databases (national: https://lixomarinho.app/; international: https://www.nationalgeographic.org/education/programs/debris-tracker/).

Despite the willing to change, demonstrated by all sectors of society, a massive commitment into plastic emissions reduction will be only achieved if government measures/regulations are established, taxes charged, rewards/incentives applied (tax breaks or subsidies (Worm et al., 2017)), public health problems arise or if the quality of subsistence activities is jeopardized. Therefore, gathering

[3] https://www.pactoplasticos.pt/

scientific data is essential to support successful measures that aim to decrease the amount of plastic reaching seas and oceans and to justify/support the requested changes to both consumers and industries.

As decreasing plastic production will simultaneous reduce fossil carbon demand (Thompson et al., 2009) it enhances the urgency of tackling such an environmental problem with more decisive and long term measures taken by policy makers (Albert A. Koelmans et al., 2014). Turning the tide on plastic pollution is proving to be a hard job, but it is critical to stay on this path, for the sake of our ocean health and future generations.

1.3 Microplastics in Portugal

1.3.1 Previous Studies

The quantification of MPs (namely pellets), along with the characterization of pollutants adsorbed, consisted of the primary focus of several studies conducted in beaches located on mainland Portugal (J. C. Antunes et al., 2013; J. Antunes, Frias, & Sobral, 2018; J. P. G. L. Frias, Martins, & Sobral, 2011; J.P.G.L. Frias, Sobral, & Ferreira, 2010; João P. G. L. Frias, Antunes, & Sobral, 2013; J. Martins & Sobral, 2011; Mizukawa et al., 2013).

Later, marine subtidal data about the occurrence of MPs in the sea surface (J.P.G.L. Frias et al., 2014; S. M. Rodrigues, Almeida, & Ramos, 2020) and on sediments (J.P.G.L. Frias et al., 2016) became also available, being rapidly followed by two studies contributing with data about MP in estuarine waters (Bessa, Sobral, Borja, & Marques, 2018; S. M. Rodrigues et al., 2019). More recently, the ingestion of MPs by marine and estuarine biota, mainly fish, was also investigated (Bessa, Barría, et al., 2018; Cozzolino, de los Santos, Zardi, Repetto, & Nicastro, 2021; Neves et al., 2015; Pequeno, Antunes, Dhimmer, Bessa, & Sobral, 2021).

Despite consisting of valuable data, studies about MPs pollution in Portuguese coastal waters are scarce, preventing an in-depth evaluation of the extent of this threat, which will be essential to support the implementation of preventive measures at a national level.

This should be priority in Portugal, a coastal nation with one of the largest exclusive economic zone (EEZ) in Europe (1,727.408 km2) and awaiting validation from the UN Commission on the Limits of the Continental Shelf since 2009 (EMEPC, 2009) to extend the limits of the continental shelf beyond the 200 nautical miles, consequently achieving an area of 3,877.408 km^2.

Our country is expected to be aware of the strengths and weaknesses of such area; to take advantage of its resources but also to protect and preserve them from anthropogenic pressures.

1.3.2 This thesis contributions

In order to address the highlighted gaps, the research developed in the scope of this PhD thesis aimed to assess the level of MPs pollution occurring in the subtidal environment of a Portuguese coastal area, to evaluate the suitability of a fish species to be used as a local bioindicator of MPs and to understand the effects of fish larvae exposure to MPs and associated contaminants. The main findings of such research were disseminated in the region.

The study area selected to develop the mentioned objectives was comprised by the Sado river estuary and a marine protected area (Professor Luiz Saldanha Marine Park – PLSMP). Its selection was based on 1) the proximity to a highly industrialized littoral, 2) its importance for local communities (both from Setúbal and Sesimbra municipalities), because the subsistence of many families relies on artisanal fisheries and nature-based tourism activities, and 3) on the high biodiversity level reported for these waters, where the established conservation measures aim to protect species and habitats.

Thesis structure

This thesis workplan is comprised of 5 chapters. While 4 of them consist of scientific studies, the last one consists of a reflection about the dissemination events conducted locally. The objectives and contributions of each chapter are here described:

Chapters 2. and 3.

The spatiotemporal distribution patterns of MPs, occurring both at the water surface and at seabed sediments, were analyzed thoroughly according to MPs features (type and size), local pollution sources and hydrodynamism.

Both studies contribute with baseline data about MPs in the subtidal at the Portuguese coast, where such data is scarce. In addition, whereas the study focused on the water surface provides the MP:ichthyoplankton ratio for the first time, the study focused on sediments ensured the extraction of the usually underestimated denser polymers from the sample matrix, due to the use of a denser extracting solution.

Chapter 4.

The abundance and diversity of MPs ingested by a fish species (*Boops boops* (Linnaeus, 1758)) was evaluated and compared with MPs available in this species feeding grounds, which correspond to MPs collected in chapters 2. and 3.

Here we aimed to assess if the bogue could be a suitable bioindicator of MPs pollution in Portuguese waters, as suggested for the Mediterranean, by using a rarely used approach that robustly

evaluates the potential of a species for such role: the comparison of environmental and biota samples collected simultaneously.

Chapter 5.

The effects of MPs ingestion in *Sparus aurata* L., 1758 larvae exposed to other contamination pathways, as dermal uptake and prey ingestion, were analyzed, namely through biomarker responses.

This study was developed under an environmental relevant scenario, which was defined, whenever available, according to data reported for Portuguese coastal waters.

Chapter 6.

The science outreach activities, mainly conducted in Setúbal and Sesimbra municipalities with the purpose of communicating the main findings of the previous chapters to local citizens and stakeholders, were described in this chapter.

The effort put in such activities aimed to raise awareness about the topic, by sharing knowledge acquired from a familiar and important location to the audience.

The complete thesis workflow may be consulted in Figure 1.8.

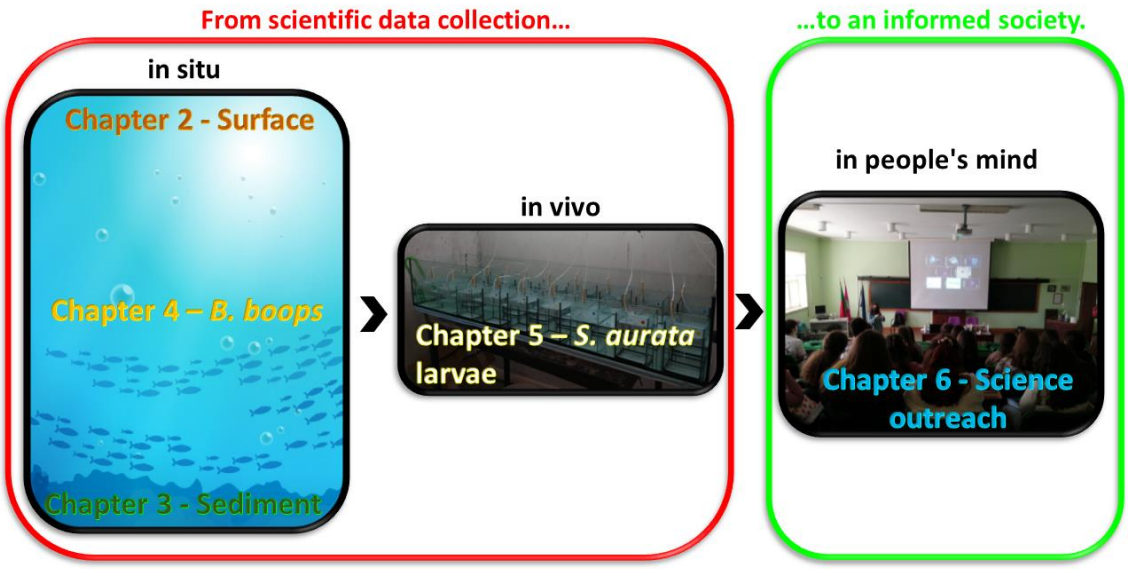

Figure 1.8 - Diagram of the PhD workplan.

2

DISTRIBUTION PATTERNS OF MICROPLASTICS IN SEAWATER SURFACE AT A PORTUGUESE ESTUARY AND MARINE PARK[4]

Manuscript #1 – Published in *Frontiers in Environmental Science*
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Abstract

Measuring local levels of marine pollution by microplastics (MP) and identifying potential sources in coastal areas is essential to evaluate the associated impacts to environment and biota. The accumulation of floating MP at the sea surface is of great concern as the neustonic habitat consists of a feeding ground for primary consumers (including filter-feeders) and active predators, which makes these organisms a relevant via of MP input into the marine trophic chain. Here, a baseline evaluation of MP accumulation at the sea surface was conducted with a neuston net (335 µm mesh) at the Arrábida coastal area, in Portugal. The study site encompasses a marine protected area and an estuary, both under strong anthropogenic pressures due to multiple activities taking place. A short-term investigation on local spatiotemporal distribution, concentration and composition of MP was performed for the first time, through the monthly collection (summer 2018 to winter 2019) of samples at 6 stations. All the neuston samples contained MP and their mean concentration was 0.45 ± 0.52 items m^{-3} (mean ± SD). Both the averaged MP:neuston and MP:ichthyoplankton ratios were higher in December, when concentrations of organisms decreased. Temporal distribution patterns followed expected trends, as MP concentration was clearly higher in winter months due to precipitation and runoff. Although mean MP concentrations did not vary significantly between sampling stations, there was a spatial distribution of MP in relation to particle shape and size. Fragments were the most abundant shape and MP belonging to 1-2 mm size class were dominant. Amongst a diversity of 10 polymers identified by FTIR analysis, polyethylene (PE), polypropylene (PP) and copolymer PP/PE were the most abundant. Potential links between local sources/activities and the different polymers were suggested. Altogether, the information provided in this study aims to raise awareness among the identified sectors and consequently to act towards the prevention of MP inputs in the region.

Keywords: microplastics, distribution, Sado estuary, marine park, MP:neuston ratio, MP:ichthyoplankton ratio, plastic polymers, Portugal

Introduction

Tackling marine plastic pollution became a major planetary challenge of the 21st century. Besides the worldwide scientific contribution to the topic for more than one decade and the increasing public awareness, governments have proven their commitment by implementing more sustainable measures and encouraging both initiatives and changes (European Commission, 2018; UNEP, 2018). Yet, although plastic production has recently decreased in Europe from 64.4 MT in 2017 to 61.8 MT in 2018, it has continued to grow at a global level, from 348 MT in 2017 to 359 MT in 2018 (Plastics

Europe, 2019). This tendency largely relies on the persistent high demand for such a low-cost, lightweight, versatile, and durable material (Barnes et al., 2009). Consequently, and adding to the excessive consumption of disposable items (Napper, Bakir, Rowland, & Thompson, 2015) and poor waste management (J.P.G.L. Frias et al., 2014), plastic pollution represents a significant threat to the marine environment (Laskar & Kumar, 2019). The latest estimations pointed out between 1.1 to 8.8 MT of mismanaged plastic waste being generated annually by land-based human activities at each country (Jambeck et al., 2015). From this waste amount, a considerable part ends up in the marine environment, mainly through wastewater treatment plants discharges, land runoff or transported by the wind, rivers and tides (Andrady, 2011; Jambeck et al., 2015). Despite the greater relevance of terrestrial sources, there are several sea-based activities, such as fishing, aquaculture, maritime traffic, offshore platforms and recreational uses, which may also be considered as additional sources of plastic pollution (Mark Anthony Browne et al., 2011; Gewert et al., 2017; Jambeck et al., 2015; UNEP, 2016).

Pioneer studies focused on plastic debris abundance and distribution in the marine environment inevitably verified that plastic pollution could act at a wide size range (from macro to nanoplastics), at a broad spectrum of impacts, as skin injuries or smothering from entanglement, gastrointestinal tract lesions or blockage from ingestion, and even act as vectors of pathogens and chemicals (Bowley, Baker-Austin, Porter, Hartnell, & Lewis, 2020; Kühn, Rebolledo, & van Franeker, 2015; Laist, 1987; Teuten et al., 2009). Indeed, the potential of smaller plastics to be ingested by marine biota (Barnes et al., 2009) and to be transferred throughout the trophic chain (Eriksson & Burton, 2003; Farrell & Nelson, 2013; Setälä, Fleming-Lehtinen, & Lehtiniemi, 2014), was rapidly recognized. This perception shifted the focus of investigation onto microplastics (hereafter MP; defined as particles between 1 μm and 5 mm (Arthur et al., 2009)) which developed into a new research topic addressed worldwide. In addition, the critical concern about the potential impacts of MP in human health through oral, dermal and inhalation exposure has triggered an increase of investigation on this subject although it remains poorly understood (Galloway, 2015; Revel, Châtel, & Mouneyrac, 2018; Thompson et al., 2009).

Regarding the origin of MP, it was considered to be either primary, if manufactured in microscopic size ranges (as industrial pellets and abrasives or microbeads from personal care products); or secondary, if resulting from fragmentation of larger objects (fishing gear, packaging, fibers from synthetic textile washing, paint flakes from nautical coating and dust from vehicle tires) (Cole et al., 2011; GESAMP, 2016; Hidalgo-Ruz et al., 2012; Rochman et al., 2019). The fragmentation of plastic may occur by photo-degradation, mechanical, chemical, and biological action (Andrady, 2011; Barnes et al., 2009; van Sebille et al., 2015). Regardless of its origin, an evident spatial distribution of MP in the water column occurs vertically, from the water surface to the seabed (Thompson et al., 2004). This mainly relies on polymers density and biofouling level, as both affect particles buoyancy (Gregory, 2009; Kaiser et al., 2017). Horizontal distribution of MP is also known to occur as a result of hydrodynamic forces,

mainly by wind (Kukulka et al., 2012), tides, waves and thermohaline gradients (Zhang, 2017). In addition, both vertical (Choy et al., 2019) and horizontal distribution may be influenced by biota, through ingestion and egestion of MP in different compartments of the water column and different locations.

Regarding the impacts on marine biota upon MP ingestion, besides physical harm (e.g. damage in the gastrointestinal tract with inflammatory responses (von Moos et al., 2012) or false sense of satiation (Kühn et al., 2015)), toxicological effects have also been reported (Rochman et al., 2013; Wright et al., 2013). These rely on potential load of harmful chemicals adsorbed from seawater onto plastic and on the toxic additives incorporated during manufacture (Teuten et al., 2009). As a result, MP are suggested to act as trophic vectors of contaminants (Garcia-Garin et al., 2020; Teuten et al., 2007), although their contribution for bioaccumulation (and bioamplification) in organisms tissues may not be as relevant as other contamination pathways, such as prey ingestion or dermal uptake (Albert Aart Koelmans et al., 2016).

Understanding the exposure of primary consumers to MP became essential to evaluate the consequent implications in the marine trophic chain (including eventual detrimental impacts on human health due to seafood contamination). This triggered an increase in research aiming at calculating encounter rates between MP and primary consumers, based on their concentrations and ratio (Collignon, Hecq, Galgani, Collard, & Goffart, 2014; Collignon et al., 2012; Hitchcock & Mitrovic, 2019). Yet, such research has been scarcely conducted in Portuguese waters (J.P.G.L. Frias et al., 2014; S. M. Rodrigues et al., 2019), being insufficient for a country where fisheries have a large cultural and social importance (FAO, 2017) and where seafood constitutes a very important diet component (Almeida, Karadzic, & Vaz, 2015; EUMOFA, 2020; FAO, 2010).

Both the Sado estuary and Professor Luiz Saldanha Marine Park, located at the Portuguese west coast, are important nursery areas for fish larvae (R. Borges, Vaz, Serrão, & Gonçalves, 2009; Rita Borges, Beldade, & Gonçalves, 2007) and constitute valuable artisanal fishing grounds (Batista et al., 2015; Horta e Costa, Batista, et al., 2013; Horta e Costa, Gonçalves, & Gonçalves, 2013). However, there are multiple anthropogenic activities taking place at this coastal zone, potentially contributing to local and regional MP pollution and thus posing a threat to this hotspot of biodiversity (A. H. Cunha et al., 2014). In this context, this study aims to contribute with baseline data on MP pollution at a Portuguese estuary and marine park by assessing temporal and spatial variations in concentration, distribution, and composition of MP particles. Two hypotheses are tested: (i) MP concentration decreases at sampling stations far away from the metropolitan area of Setúbal; and (ii) MP concentration increase in winter months when compared to summer and autumn months. Additionally, since these are important nursery areas for fish larvae, the ratio of MP to ichthyoplankton was calculated separately from the MP to neuston ratio, aiming to be useful either for comparing with other regions or as a simple and clear take-home message at science outreach activities.

Materials and Methods

Study area

The study area, located on the west coast of Portugal, encompasses the south-facing coastal area between the city of Setúbal and the village of Sesimbra (Figure 2.1). It comprises both the mouth of Sado estuary (designated as the transitional water body Sado-WB1 (ARH Alentejo, 2012)) and the Professor Luiz Saldanha Marine Park (from its eastern side - Figueirinha beach – until the buffer area contiguous to Sesimbra). The meso-tidal homogeneous Sado estuary has a mainly tidally driven flow (F. Martins, Leitão, & Neves, 2002) with an annual average flow of 40 m^3s^{-1} (Vale, Cortesão, Castro, & Ferreira, 1993). It is under considerable anthropogenic pressure due to numerous activities (mostly occurring in its northern margin), from urban and industrial (including maritime traffic), to agriculture and animal production, fisheries and tourism sectors (APA, 2016). Nevertheless, this estuary (Ramsar site no. 826) encompasses a Nature Reserve (ICNF, n.d.), where birds and habitats are, respectively, protected by a Special Protection Area (PTZPE0011; Birds Directive) and a site classified under the Habitats Directive (PTCON0011), both belonging to Natura 2000 network. Located outside these conservation areas, the Sado-WB1 is adjacent to the city of Setúbal (ca 119.000 inhabitants: Statistics Portugal 2013), being close to a multipurpose terminal port, ship repair yard and to the submarine outfall of Setúbal wastewater treatment plant. Along the Sado-WB1 margins, where two streams discharge (Comenda and Livramento), there are diverse nautical and tourist facilities, an important cement industry, an Orthopedic Hospital and beaches of high demand.

Established westward from the estuary, is Professor Luiz Saldanha Marine Park (hereafter marine park), a sheltered coastline from the prevailing north and north-west winds by the Arrábida mountain chain (Henriques, Gonçalves;, & Almada, 1999). The different protective measures established in this marine park aim to minimize the impacts of nautical, recreational and fishing activities on its biological and ecological patrimony (Henriques et al., 1999). The erosion of the adjacent cliffs of Arrábida (Costa, Erzini, Caselle, Folhas, & Gonçalves, 2013; E. J. Gonçalves, Henriques, & Almada, 2002) contribute to the complex substratum found in this subtidal rocky reef, which is expected to export MP to the adjacent marine environment due to the breakdown of larger items through physical abrasion on rocks (Cheshire et al., 2009; Eriksson & Burton, 2003). One of 2 submarine outfalls regularly used for effluent discharges of wastewater treatment plants of Sesimbra Municipality (ca. 50.000 inhabitants; Statistics Portugal 2013) is located within the study area.

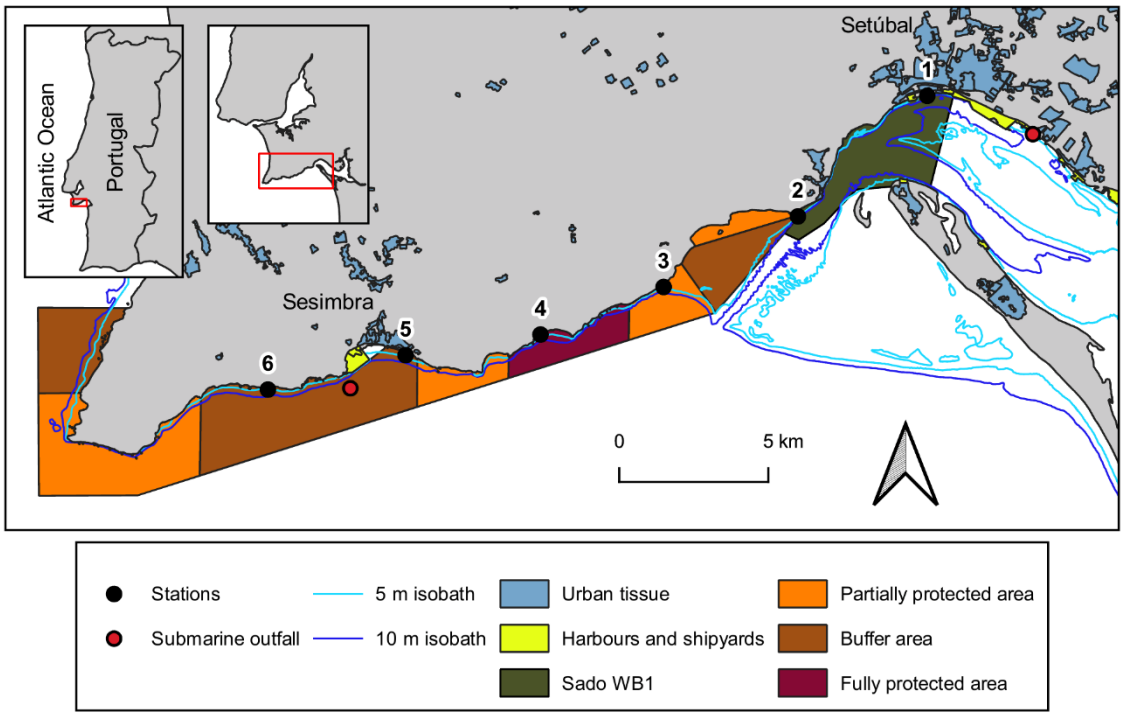

Figure 2.1 – Map of the study area with the location of sampling stations, at the coastal area between Setúbal city and Sesimbra village, at the Portuguese west coast. Urban tissue includes industry and tourist facilities. The isobath lines were provided by Instituto Hidrográfico and Águas do Sado (Setúbal WWTP) and SIMARSUL (Sesimbra WWTP). Map creation was based on 2 information layers: 1) land use and occupation of 2018[5] and 2) transitional surface water bodies of Portugal mainland[6], respectively developed by Direção-Geral do Território and by Agência Portuguesa do Ambiente. Marine Park borders and protection areas were depicted according to the POPNA spatial plan regulation (n. º 141/2005).

Sampling methods

Six sampling campaigns were conducted from August 2018 to February 2019 (summer to winter), at 6 stations (Table 2.1). These were located at the 5 m isobaths and distributed 5 km apart from each other, from the mouth of Sado estuary through the marine park (Figure 2.1).

[5]https://snig.dgterritorio.gov.pt/rndg/srv/por/catalog.search#/metadata/b498e89c-1093-4793-ad22-63516062891b
[6]https://snig.dgterritorio.gov.pt/rndg/srv/por/catalog.search#/metadata/0F67303C-5822-4D91-80F3-D217FD33667F

Table 2.1 – Name, distance from the estuary (km) and GPS (datum WGS-84) coordinates of each sampling station

Station	Name	Distance from the estuary (km)	LAT (º)	LON (º)
St1	Setúbal	0	38.51970	-8.89348
St2	Figueirinha beach	5	38.48294	-8.94286
St3	Portinho da Arrábida	10	38.46124	-8.99428
St4	Fully Protected area	15	38.44652	-9.04146
St5	Sesimbra	20	38.43987	-9.09325
St6	Mijona beach	25	38.42905	-9.14605

In each station, a 30 min neuston trawl (following Galgani et al., 2013) was performed in the E-W direction, at a constant speed of 1-3 knots. Initial and final GPS positions were registered and enabled trawl length and area calculations to allow posterior standardization of MP data (following Law et al., 2014). Sampling campaigns were specifically scheduled to days with calm weather conditions (Beaufort wind scale ≤3) and tows were performed out of the vessel wake zone (ca 25 m behind the vessel). Precautions intended to reduce vertical mixing of buoyant plastic particles and consequently increase the efficiency of the selected equipment (neuston net). The 3 m long neuston net (Aquatic Biotechnology) had a stainless steel 0.8 x 0.3 m (width x height) rectangular opening and a 335 µm polyamide mesh. Its floatation system assured that only half of the opening frame was submerged (therefore collecting MP floating in the top 15 cm of the water column). The flowmeter (Hydro-bios) attached to the lower third of the net opening enabled the calculation of the volume of filtered water. As only half of the net opening is submerged, the volume was calculated with the following formulae:

$$\text{Volume} = \frac{\text{net opening area}}{2} \times \text{Tow length, where}$$

$$\text{Tow length} = \text{flowmeter revolutions} \times \text{hydraulic pitch}$$

Following each tow, the content in the cod end container was thoroughly poured into a 250 µm stainless steel mesh sieve (where larger pieces of biological material as sticks, seagrass leaves and algae, were rinsed with filtered seawater before being discarded) and then stored in glass jars. A small aliquot (ca 50 ml) per sample was collected and preserved separately, in 100 ml of 70% ethanol, to allow the identification of neustonic organisms and the calculation of the MP:neuston and MP:ichthyoplankton ratios. The neuston samples (n=36) were transported in ice coolers to the laboratory and then frozen at -20°C.

Laboratory Procedures

Sample processing and microplastics characterization

Due to the considerable volume of biological material present, samples were processed according to Gago et al. (2018). After thawing, the sample was transferred to a 2 L glass beaker where the biovolume was measured after 1 hour of sedimentation. Then, the organic content digestion was performed by adding a 10% KOH solution, with volume equivalent to at least 3 times the sample biovolume. Following the 48 hours of digestion at room temperature, density separation was conducted by adding 1 L of a hypersaturated NaCl solution (1.2 g cm^{-3}). After manual stirring, it was left to settle for 1 hour before filtration of the supernatant with a vacuum filtration system. After filtration of every 500 ml (approx.), the sample was stirred and allowed to settle again before the next filtration. Each filter (MFV2 glass fiber filter with 47 mm Ø and 1,0 µm pore; FILTER-LAB) was stored in a covered Petri dish until observation under a stereoscopic microscope (Leica MZ12.5) equipped with a camera (MOTICAM 10+). Particles were measured with the Motic Images Plus 3.0 software, considering the 0.335-5 mm size range (the lower limit corresponds to mesh size of the neuston net) and then attributed to one of the following size classes: 0.335-1, 1-2, 2-3, 3-4 and 4-5 mm. Characterization consisted of registering both color and shape. Particles were assigned to one of six shapes: fragment, film, foam, fiber, filament, and bead (Table 2.2; adapted from Lusher et al. (2017)). The particles selected to follow polymer identification were isolated in covered concave slides. MP concentration was reported as items m^{-3} and items km^{-2} to enable comparisons with similar studies.

Table 2.2 – Particle shape definition

Shape	Definition
Fragment	Hard or soft irregular particle
Film	Thin and malleable, flimsy particle
Foam	Lightweight, sponge-like particle
Fiber	Thin line, equally thick throughout its entire length, frequently curled
Filament	Thicker and straighter than fiber
Bead	Spherical particle

Polymer identification

Selection of particles for polymer identification, from all shapes (Table 2.3), was based on the best expert judgment according to similarity, texture, thickness, shine and reaction to touch (following Lusher et al., 2017).

Table 2.3 – Total of particles and number of MP selected for FTIR per shape

Shape	Total	FTIR
Fragment	1480	220
Film	557	26
Foam	638	6
Fiber	109	12
Filament	61	27
Bead	75	18
	2920	309

Polymer identification was achieved by Fourier Transformed Infrared Spectroscopy (FTIR). The majority of the particles (mainly between 1-5 mm) were analyzed in attenuated total reflectance (ATR) mode. Spectra were acquired using an Agilent Handheld 4300 FTIR Spectrometer with a DTGS detector, with controlled temperature and a diamond ATR sample interface; the analyses were performed at the sample surface. Spectra were acquired with a resolution of 4 cm^{-1} and 32 scans. For fibres and smaller particles (mainly at the 0.335-1 mm size range), analyses were carried out in a Nicolet Nexus spectrophotometer coupled to a Continuμm microscope (15x objective) with an MCT detector. Spectra were collected in transmission mode, with a resolution of 8 cm^{-1} and 128 scans. The spectra are shown here as acquired, without corrections or any further manipulations, except for the occasional removal of the CO_2 absorption at ca. 2300-2400 cm^{-1}. The identification of polymers was first made by searching in the extensive polymer spectral database of the Department of Conservation and Restoration (FCT NOVA) and the assignments were confirmed by analysis of the polymers characteristic bands (D. O. Hummel, 2002).

Quality assurance (QA) and quality control (QC)

The airborne contamination was analyzed by exposing wet filters to the air (procedural controls; blanks), both during field (inside a hanging open glass jar, at the boat deck, one per sampling campaign) and lab work, (inside Petri dishes, one at the left and one at the right of the working area, per group of 3 samples). All the fibers extracted from a sample which were similar to those found in the respective blanks (from field and lab work) were excluded from results. Sources of contamination were also minimized both during field and lab work by using glass, stainless steel, and aluminum materials. Samples were kept covered at all times, both cotton lab coat and nitrile gloves were always worn, and benches and equipment were rinsed before use with Milli-Q filtered water and ethanol.

MP:neuston and MP:ichthyoplankton ratios

The biovolume of neuston aliquots was registered after 1 hour of sedimentation in the graduated cylinders and then homogenized (manual stirring). Three subsamples of 2 ml each were analyzed under a stereomicroscope using a Bogorov counting chamber. Apart from insects, neuston organisms mainly consisted of zooplankton. Dominant groups (fish larvae and eggs, Mysidacea, Polychaeta, Chaetognata, Apendiculata, Bivalvia larvae, zoea and megalopa of Brachyura, Cladocera, nauplii of Cirripedia, Copepoda, Echinodermata larvae, Amphipoda, Isopoda and Insecta), rather than individual species or genera (Di Mauro, Kupchik, & Benfield, 2017), were counted with the support of a hand tally counter, enabling the calculation of each group abundance. Mean counts (all dominant groups were considered for MP:neuston ratio, whereas only fish larvae and eggs were considered for MP:ichthyoplankton ratio calculation) were extrapolated according to the aliquot and sample biovolume and then converted to individuals m^{-3}.

Statistical Analysis

To evaluate how the MP:neuston ratio varied temporally (along 6 months) and spatially (between the 6 stations), a Kruskal-Wallis test was performed. This non-parametric test, conducted after the invalidation of parametric assumptions, was followed by *posthoc* multiple comparisons with the Dunn's test. The same tests were applied for MP:ichthyoplankton ratio.

A two-way ANOVA without replication was performed to assess whether temporal (6 campaigns) and spatial (6 stations) variation occurred in MP concentration (dependent variable). This parametric test was used after Box-Cox transformation of original data to meet normality (Shapiro-Wilk test) and homogeneity of variances (Levene test) assumptions. *Posthoc* Bonferroni's test ($p<0.05$) were used to identify the sources of significant differences. Analysis were conducted in Statistica 13 (Statsoft) software.

The effect of campaigns and stations (fixed factors; with 6 levels each) in MP concentration of each particle shape (multivariate data) was tested by a permutational multivariate analysis of variance (PERMANOVA), with 999 permutations. Data were square-root transformed and the resemblance matrix between samples was calculated based on Bray-Curtis similarities. When differences were statistically significant, pair-wise comparisons among levels were analyzed. Then, to determine which particle shape most contributed to explain the dissimilarity amongst each pair of samples, the similarity percentages routine (SIMPER; with a cut-off percentage of 90% for low contributions) was conducted. These statistical procedures, which were conducted in the Primer 6 software with the Permanova+ add-on

(Anderson, Gorley, & Clarke, 2008; Clarke & Gorley, 2006), were similarly applied to understand the response of MP concentration of each size class to both factors (campaigns and stations).

Results

Presence and concentration of microplastics

From the total of particles (3317) extracted from the 36 neuston samples, 353 (11%) were discarded for being considered airborne contamination fibers and 44 (1%) were excluded after being identified as non-plastic particles by Fourier Transformed Infrared Spectroscopy. Therefore, the assessment of the temporal and spatial distribution of MP (size range 0.335 to 5 mm) was based on a total of 2920 particles. All samples contained MP, with a mean concentration of 0.45 ± 0.52 items m^{-3} (mean ± SD) and 40822.58 ± 43578.63 items km^{-2}. While the highest concentration per cubic meter was found in February at Figueirinha beach (St2; 2.06 items m^{-3}), the highest concentration per square kilometer was verified at Setúbal (St1; 203558.50 items km^{-2}). Conversely, the lowest concentration (0.04 items m^{-3} or 2068.85 items km^{-2}) was observed at Mijona beach (St6) in October (Figure 2.2). The number of MP ranged from 405 at St1 in February to 5 MP at St6 in October.

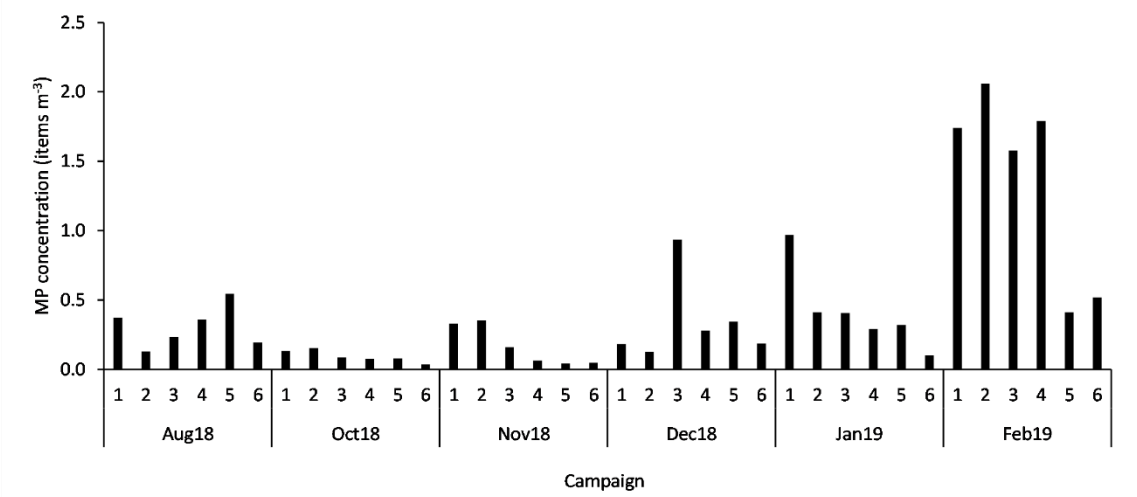

Figure 2.2 – MP concentration (items m^{-3}) in each sample (n=36; 1 to 6 stations; Aug18 to Feb19 campaigns).

Fourier Transformed Infrared Spectroscopy (FTIR) analysis

Among 265 particles confirmed as microplastics by FTIR analysis, a total of 10 polymers were identified (Table 2.4; Figure 2.3), including a Copolymer PP/PE. Despite the diversity of polymers identified, three of them (PE, PP and Copolymer PP/PE) represented more than 90% of the particles. Kaolin was also identified associated with PS and Copolymer PP/PE.

Table 2.4 – MP number and relative abundance (%) assigned to each polymer

MP	%	Polymer
176	66.42%	Polyethylene (PE)
48	18.11%	Polypropylene (PP)
25	9.43%	Copolymer PP/PE
5	1.89%	Polystyrene (PS)
3	1.13%	Polyvinyl alcohol (PVA)
3	1.13%	Rayon
2	0.75%	Polyester
1	0.38%	Polyurethane (PUR)
1	0.38%	Poly(acrylic acid) (PAA)
1	0.38%	Polyamide (PA)

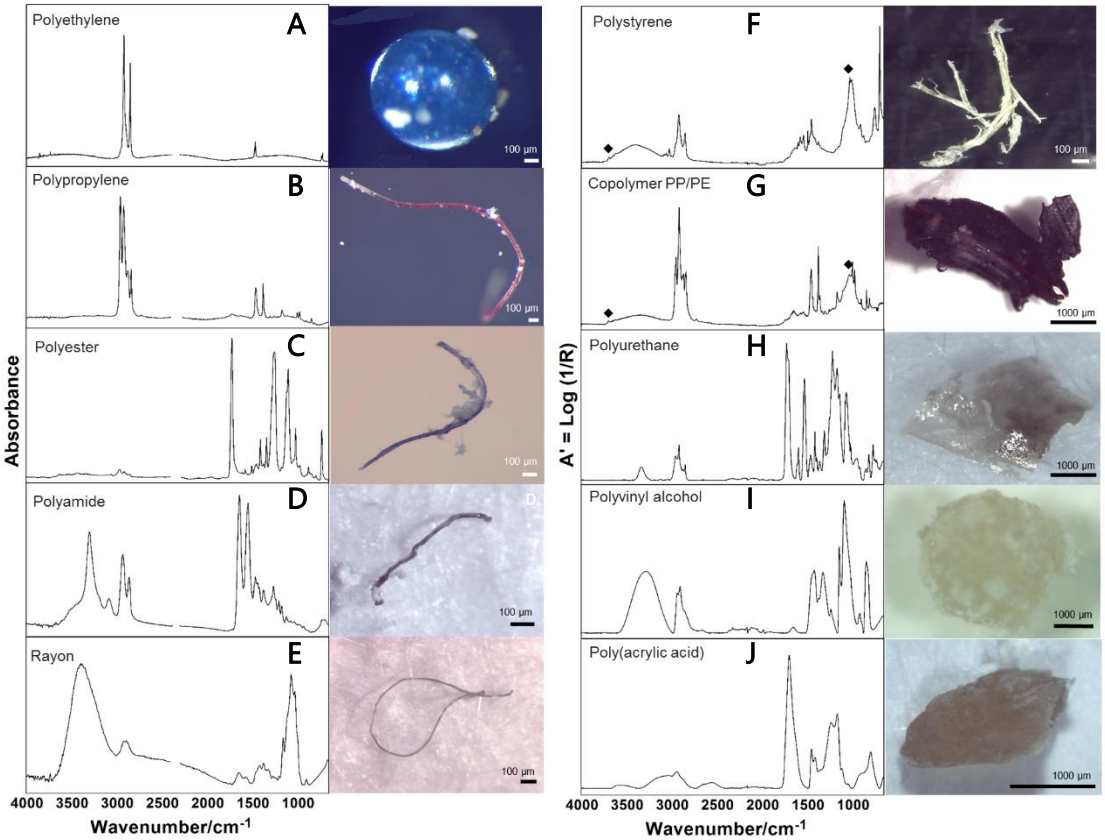

Figure 2.3 - Representative infrared spectra of the identified polymers, analyzed in transmission (left column) and ATR mode (right column); ◆ identifies the presence of kaolin. The image assigned to each spectrum corresponds to the particle analyzed by FTIR. (A; I) Bead; (B-E) Fiber; (F; G; J) Fragment; (H) Film shapes.

MP:neuston and MP:ichthyoplankton ratios

Considering all samples, the MP:neuston ratio was 0.0009 ± 0.0013, with the highest ratio 0.0059 (or 1:168.398) occurring in December (Figure 2.4), when neuston concentrations reached minimum levels (76.61 individuals m^{-3}). The average MP:ichthyoplankton ratio was 0.091 ± 0.146, with the highest ratio 0.773 (or 1:1.294) being observed in November. A statistically significant variation at the MP:neuston ($H=20.80$, $p<0.001$) and MP:ichthyoplankton ($H=17.32$, $p<0.05$) ratios was found between campaigns (Figure 2.5) but not between stations.

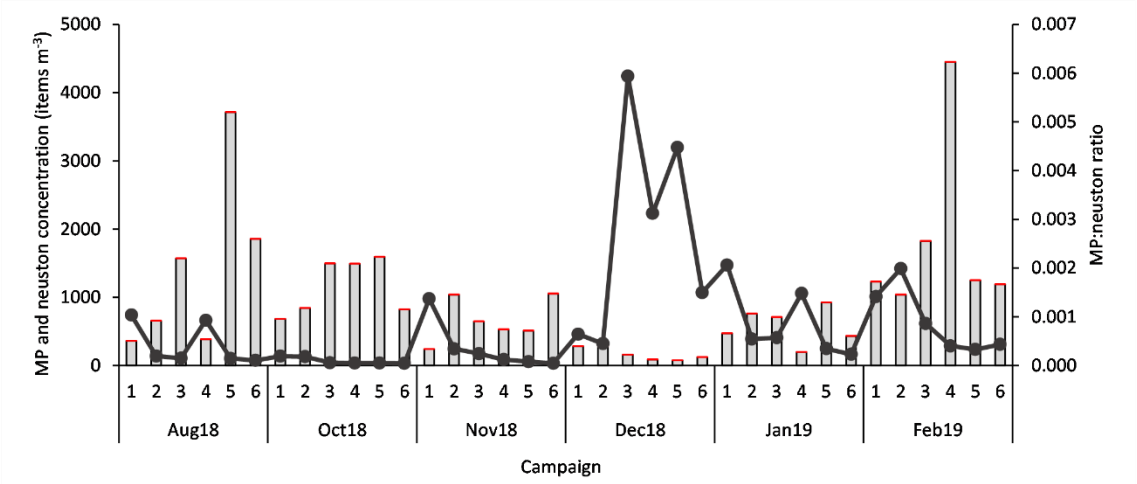

Figure 2.4 – MP concentration (items m^{-3}) (red bar), neuston concentration (items m^{-3}) (grey bar) and MP:neuston ratio (dark grey dots), in each sample (n=36; 1 to 6 stations; Aug18 to Feb19 campaigns).

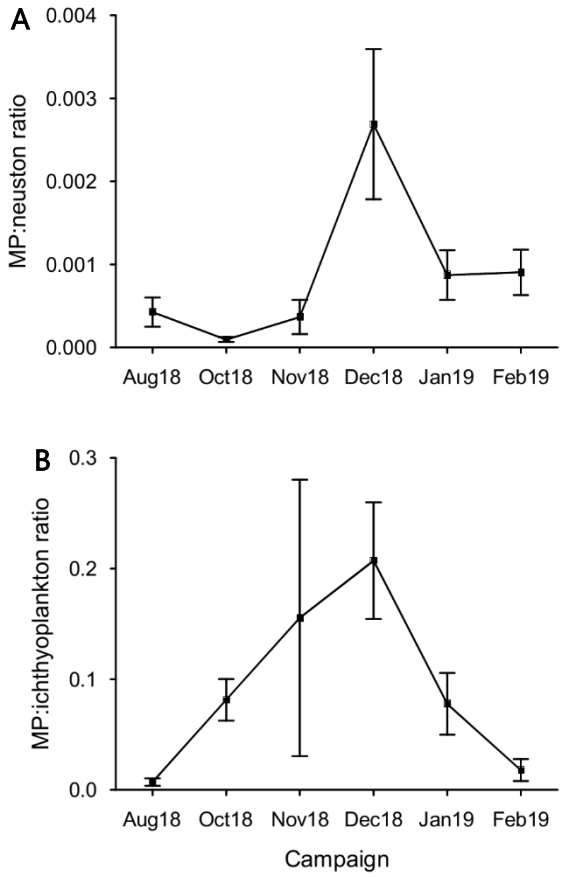

Figure 2.5 – Variation of the MP to primary consumers ratio in the sampling period. (A) MP:neuston ratio per campaign (mean ± SE, n=6); (B) MP:ichthyoplankton per campaign (mean ± SE, n=6).

Temporal and Spatial distribution

MP concentration in February was significantly higher than those found in all other campaigns (Figure 2.6A), except for January (Bonferroni test, $p<0.05$). In October, concentration was the lowest and significantly different from January. MP concentration did not vary significantly between stations ($p=0.06$; Figure 2.6B).

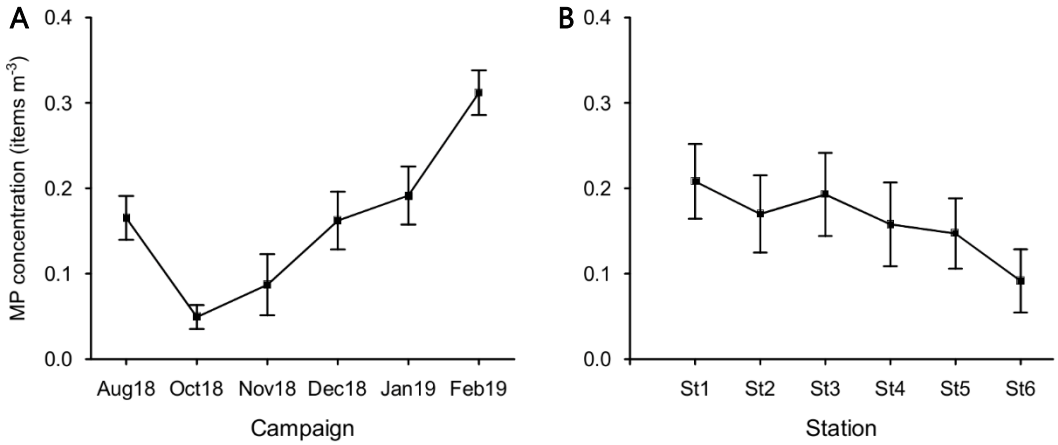

Figure 2.6 - Variation of MP concentration (items m^{-3}; mean ± SE) per campaign (A) and per station (B).

Distribution variations according to particle shape

The relative abundance of six MP shapes (Figure 2.7) had the following decreasing order: fragment (51%) > foam (22%) > film (19%) > fiber (4%) > bead (3%) > filament (2%). The PERMANOVA results showed significant differences in the MP concentration of each particle shape, between sampling campaigns (Pseudo-F = 6.57, P(perm) = 0.001) and stations (Pseudo-F = 2.11, P(perm) = 0.008). MP concentrations per particle shape differed mainly between October and February, but also between each of these 2 months and all the other campaigns. The combination of the 3 predominant shapes: fragments, films and foams contributed with more than 70% (cumulative percentage) for the dissimilarities between all pairs, with concentrations being always higher in February (Figure 2.8A). An additional result from pair-wise comparisons concerned the dissimilarities between November and January campaigns, which were based on the higher concentration of fragments, foams and beads (with contributions of 27.87%, 24.54% and 14.28%, respectively) found in January. Regarding spatial variation, the relatively higher foam and bead concentrations at St1 (estuary) explained dissimilarities found between this station and both St5 (Sesimbra; foam-24.82% and bead-14.00% contributions) and St6 (Mijona beach; foam-27.44% and bead-13.26% contributions) (Figure 2.8B). Moreover, at st6 concentrations of fragments were significantly lower than St3 (Portinho da Arrábida). No plastic pellets were collected in this study and all beads belonged to the smaller size class (0.335-1 mm).

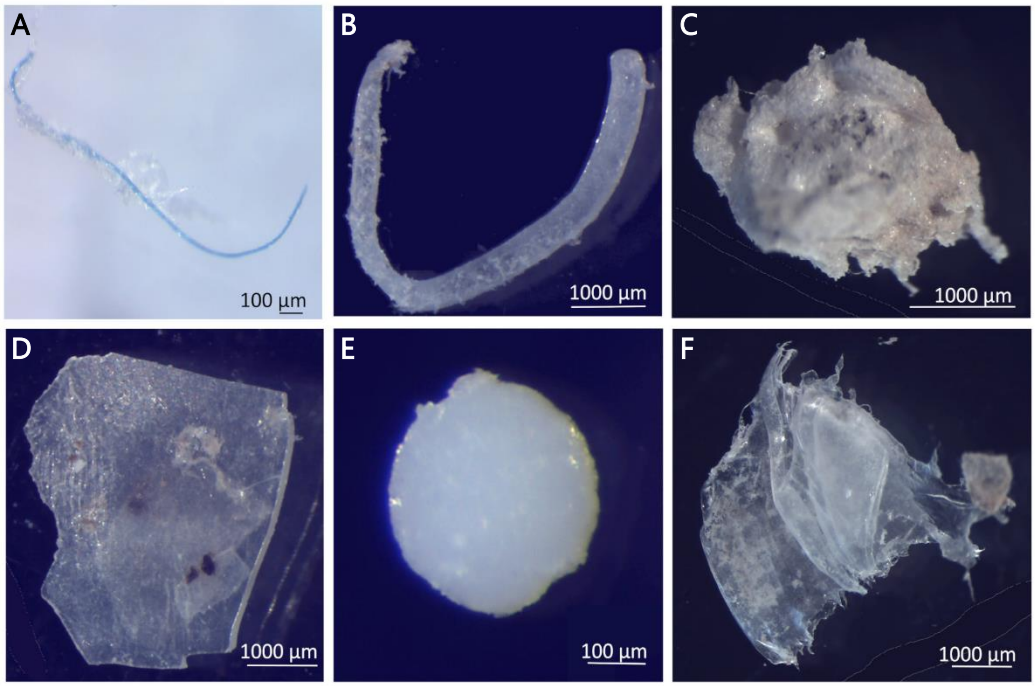

Figure 2.7 – Selected microplastics from each particle shape, in neuston samples from the Sado estuary and Professor Luiz Saldanha Marine Park. (A) Fiber; (B) Filament; (C) Foam; (D) Fragment; (E) Bead; (F) Film.

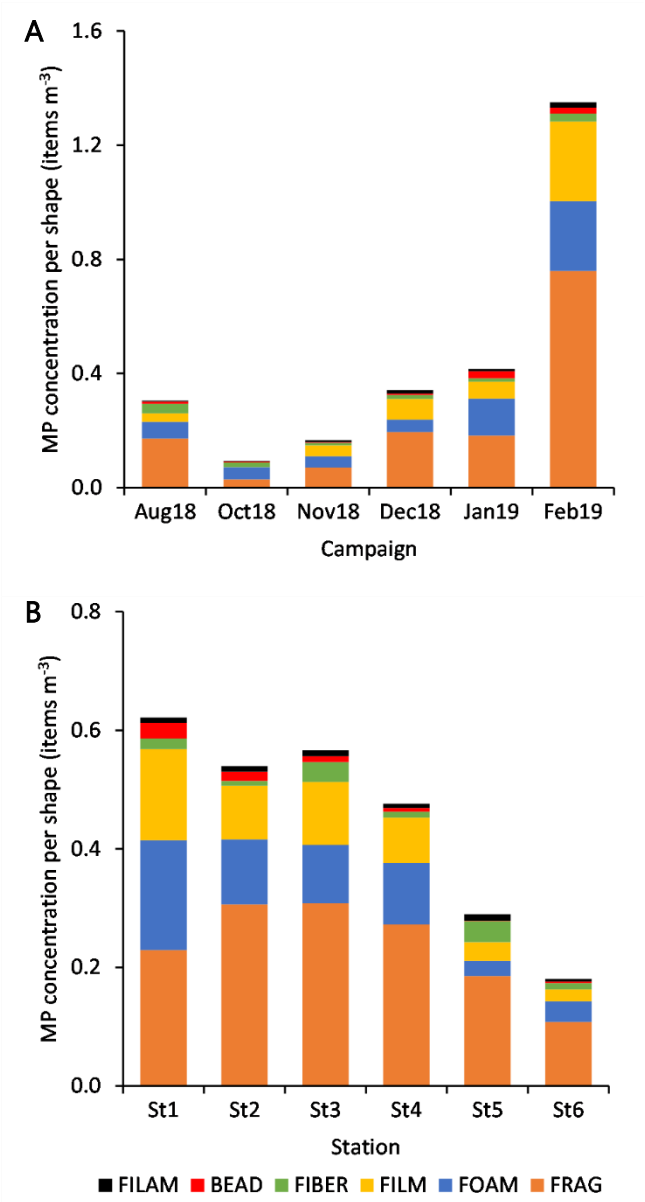

Figure 2.8 – Variation of MP concentration (mean items m^{-3}, n=6) per particle shape, by campaign (A) and by station (B)

Distribution variations according to size class

By decreasing order, the relative abundance of each size class (mm) was: 1-2 (36%) > 2-3 (24%) > 3-4 (16%) > 0.335-1 (15%) > 4-5 (9%). According to PERMANOVA results, MP concentration varied according to size class between campaigns (Pseudo-F = 7.69, P(perm) = 0.001) and stations (Pseudo-F = 2.55, P(perm) = 0.005). MP belonging to the 1-2 and 2-3 mm size classes explained (with more than 46% of cumulative contribution) the dissimilarities found between February and all the other campaigns and also between January and both November and October months (Figure 2.9A). In addition, while the 0.335-1 mm size class largely contributed (ca. 29%) to distinguish August from October (being more

represented in August), the higher concentration of MP at the 1-2 and 3-4 mm class sizes in December, compared to October, contributed more than 48% for their differences. The particle size range at St6 (the furthest station from the estuary) was distinct from all the others, mainly due to its low concentration of MP belonging to the 3-4 mm size (contributions between 21-25%) and particularly different from st1 and st4 due to the smaller concentration of MP at the 4-5 and 1-2 mm size ranges, respectively (Figure 2.9B).

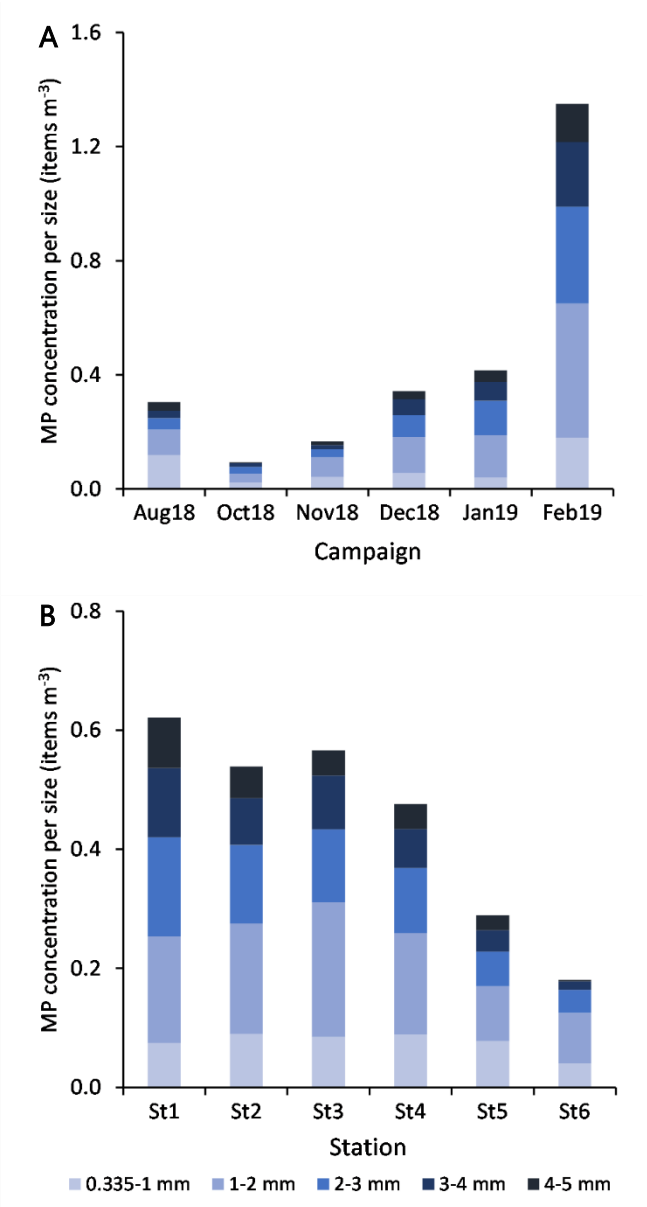

Figure 2.9 – Variation of MP concentration (mean items m^{-3}, n=6) per size class, by campaign (A) and by station (B)

Discussion

Presence and mean concentration of microplastics – comparison with other studies

The presence of MP in all coastal samples collected in this study is in accordance to reported MP pollution levels close to shore and to estuaries, either at Portuguese (Frias et al., 2014), European (Pedrotti et al., 2016; Frère et al., 2017), Gulf of Mexico (Di Mauro et al., 2017) or Indonesian (Germanov et al., 2019) waters. The mean MP concentration found in this study (0.45 ± 0.52 items m^{-3}) was higher than levels found in other Portuguese locations such as the Douro estuary (0.17 ± 0.16 items m^{-3}; Rodrigues et al. (2019)) and others (Aveiro: 0.002 ± 0.001 items m^{-3}; Lisboa: 0.033 ± 0.021 items m^{-3}; Costa Vicentina: 0.036 ± 0.027 items m^{-3}; Algarve: 0.014 ± 0.012 items m^{-3}; Frias et al. (2014)), but was lower than values reported by Bessa et al. (2018) for the Mondego estuary (1.53 ± 1.04 items m^{-3}).

In addition, if compared with surface waters of estuaries and contiguous coastal areas from other countries, our study area presents higher MP concentrations than those quantified by Lima et al., (2014) at the Goiana estuary in Brazil (0.26 items m^{-3}). Conversely, mean MP concentration at Arrábida was more than one order of magnitude lower than the 3 estuaries in Australia east-coast investigated by Hitchcock and Mitrovic (2019) (with a range of 23 – 198 items m^{-3} at the Clyde estuary, the one with the lowest MP concentration). However, comparisons must be performed cautiously as local environmental conditions, levels of anthropogenic pressure, and methodologies applied may differ among studies (Lima et al., 2014). In fact, the lack of methodologies standardization has been often highlighted (Gago et al., 2018; GESAMP, 2019) and remains a current challenge.

MP:neuston and MP:ichthyoplankton ratios

The accumulation of floating MP at the seawater surface layer leads to concerns about the exposure of neustonic organisms, such as zooplankton (including ichthyoplankton), to these synthetic particles and, consequently, of their active predators and filter-feeding biota (Collignon et al., 2012).

As expected, the increasing tendency of MP concentration observed in winter months and the simultaneous decline of zooplankton and larval fish abundance (M. E. Cunha, 1993; Primo, Azeiteiro, Marques, & Pardal, 2011) increased both MP:neuston and MP:ichthyoplankton ratios in this time of the year. Regarding the MP:ichthyoplankton ratio, although MP have never exceeded ichthyoplankton in number at any sample, their similar proportions suggest a higher potential for MP to be ingested either by fish larvae or by ichthyoplankton's predators (crustaceans: crabs, shrimps, euphausiids, amphipods, and copepods; ctenophores; fishes; medusae (Bailey & Houde, 1989; Paradis, Pepin, & Brown, 1996)).

As a consequence, it would be expected to find critical variations in the following spring at these important nursery areas. Further studies focused on MP:ichtyoplankton ratio and on MP ingestion by wild fish larvae would be essential to confirm possible impacts, as their survival largely influence fish recruitment success and population fluctuations (Houde, 1987).

The average MP:neuston ratio verified in this work (0.0009) was low when compared to other studies: 0.002 at the Bay of Calvi (Collignon et al., 2014) and 0.2 at the Ligurian Sea (Pedrotti et al., 2014). To our knowledge, besides the MP to fish larvae ratio (1.5:1.0; fish eggs excluded) found in Douro river, Portugal (S. M. Rodrigues et al., 2019), no studies have assessed the proportion between MP and ichthyoplankton alone (0.091 in average; 1:1.294 maximum), as it has been pooled together with all other zooplankton organisms.

Temporal and Spatial distribution

Both temporal and spatial distribution variations were verified for MP concentration in our study site. As expected, MP concentrations increased significantly in winter months, achieving a maximum in February. This is in agreement with the reported increase of MP concentrations in marine coastal waters after storms and heavy rainfall, typically frequent in winter season for Mediterranean-type climatic conditions (J. A. Santos, Corte-Real, & Leite, 2005), which induces frequent floods and increase river discharges (Gündoğdu, Çevik, Ayat, Aydoğan, & Karaca, 2018; Hitchcock, 2020; Veerasingam, Mugilarasan, Venkatachalapathy, & Vethamony, 2016). Regarding the spatial distribution, it was anticipated a clear seaward decrease in MP concentration at stations further away from the metropolitan area of Setúbal (Sado estuary), with an eventual increase at the station close to Sesimbra. Instead, MP pollution level found at stations located between Setúbal and Sesimbra municipalities kept similar orders of magnitude, although with a slight decrease tendency. Such retention of MP, which might be related with the shelter provided by Arrábida mountain chain against the prevailing north and north-west winds, may impact the high biodiversity of this Marine Park. Therefore, the continuous input of MP in the estuary (at St1, the closest station to the urban area of Setúbal) is suggested to partially accumulate in the sheltered Arrábida nearshore area.

Further explanations could rely on the hydrodynamics at the Arrábida rocky reef which may potentially enhance fragmentation of both MP or even larger items, by mechanical action against rocks (Cheshire et al., 2009; Eriksson & Burton, 2003) contributing for the increase of secondary MP. Subsequently, the continuous exportation of these MP by local currents could explain the considerable concentration of particles at st5, despite being distant from the estuary. Concentrations calculated at this station may also result from Sesimbra village input of MP yet, due to the fragmentation potential at the

sandy surf zone of this sheltered bay, particles may easily achieve sizes which are not retained by the neuston net.

Lastly, fragmentation enhanced during retention at the Arrábida nearshore may also contribute to export MP in the coastal drift, explaining the unexpected high concentration of MP reported further south by Frias et al. (2014) at Costa Vicentina.

Distribution variations according to particle shape

Bead and foam shapes presented distinct patterns in their distribution at the study area, unlike the other MP shapes. Both were predominantly collected in station 1 (Setúbal), contrasting with station 5 and 6 (Sesimbra and Mijona beach), with concentrations being higher in the January and February campaigns. The preponderance of foam shape (expanded polystyrene) in the estuary is potentially related to fisheries activities, consisting of secondary MP from the breakdown of buoys and cooler boxes for bait and catches, which despite the decrease of fishery activities during winter (DGRM, 2018) are frequently kept close to the seashore and left exposed to adverse weather conditions till the next fishing season. Conversely, beads (primary MP) are suggested to enter in the marine ecosystem by wastewater treatment plant (WWTP) effluents after domestic use (Fendall & Sewell, 2009), as these particles may not be retained in the treatment processes.

The predominance of fragments in this study is in line with results from a similar study performed in Portugal, in the Douro estuary (S. M. Rodrigues et al., 2019) and in Australia, at Clyde, Bega and Hunter estuaries (Hitchcock & Mitrovic, 2019). This suggests that secondary sources of MP prevail, rather than primary sources, and are related with the diverse activities taking place in the nearby urban area, including littering.

Our findings differ from studies reporting fibers as the predominant shape detected (Beer, Garm, Huwer, Dierking, & Nielsen, 2018; Bessa, Sobral, et al., 2018), usually attributed to fishing ropes degradation (Ramos, Barletta, & Costa, 2012) and to the inefficient retention of fibers from textile laundry by the WWTP (Mark Anthony Browne et al., 2011). In fact, fibers represented only 4% of the total of MP found in our study, after the exclusion of airborne contamination (11%) from the original MP amount. The small abundance of fibers reported here may rely on the retention efficiency of treatment processes of WWTW (Gies et al., 2018) at both Setúbal (advanced secondary treatment) and Sesimbra (tertiary treatment) Municipalities, or be related to the sampling method applied, as neuston nets are suggested to underestimate the concentrations of fibers when compared with other methods (Barrows, Neumann, Berger, & Shaw, 2017; Green et al., 2018).

Distribution variations according to size class

Distribution patterns of MP according to their size were noticed both in time and space. In fact, the predominance of bigger sized MP (3-4 and 4-5 mm size) inside the estuary, the abundance increase of MP in December (beginning of winter), particularly MP belonging to the 3-4 mm size class and the high concentration of MP from intermediate size classes (1-2 and 2-3 mm) at january and mostly in February, suggest that MP inputs in this Portuguese region occur mostly close to Setúbal and mainly consists of larger particles which undergo fragmentation over time.

The preponderance of 1-2 mm sized particles among the 5 size classes, instead of the expected smallest size class (0.335-1 mm), according to Fredrik Norén (2007) and Kang et al. (2015) findings, may be essentially related with the sampling method used here. As mentioned before, the use of neuston nets may underestimate fibers concentrations, which are more malleable and easier to escape through the net mesh, explaining the low concentrations of fibers collected here and in particular those belonging to our smallest size class (0.335-1 mm). Secondly, it could be related with the retention time spent at sheltered stations, which could enhance biofouling levels and consequently cause smaller particles to sink (Kaiser et al., 2017) or to be ingested, as biofilms are suggested to increase MP palatability (Vroom, Koelmans, Besseling, & Halsband, 2017).

As several studies have already highlighted (Lenz, Enders, & Nielsen, 2016; Song et al., 2014), MP concentrations at surface waters are potentially underestimated due to the lower size limit of the range considered for monitoring, usually *ca.* 330 µm (net mesh used). Consequently, as there is a tendency over time for continuous fragmentation of plastic and permanent input to the marine ecosystem, studies are missing the size fraction which is potentially more abundant and easily ingested by primary consumers (Cole et al., 2013). Therefore, the selection of a sampling method that efficiently collects smaller MP in further studies would be required to clarify the abundance patterns found at this coastal area.

Polymer diversity

Polymer identification of particles in plastic pollution studies is essential to confirm visual identification processes (François Galgani, Hanke, & Maes, 2015), to characterize the diversity of polymers available and to assist in identifying potential local sources, as it will empower authorities and stakeholders to tackle this global concern by implementing efficient prevention measures. The high polymer diversity (10 polymers) detected mirrors the diverse activities performed in the area, both on land (domestic, commercial, industrial and tourism) and at sea (fishing and recreational activities, intense maritime traffic to shipyards).

As expected, polyethylene (PE) and polypropylene (PP) showed higher percentages, since they are widely used in many applications (mainly packaging of consumer goods and single-use items). Nevertheless, there was also a considerable amount of particles identified as copolymer PP/PE, which occurs as an industrial way to recycle both PE and PP by giving origin to other high demanding applications and expanding market options (Graziano, Jaffer, & Sain, 2019), as containers, outdoor decking or sack bags (Aumnate, Rudolph, & Sarmadi, 2019). Polystyrene (PS) particles, can be related to fragmentation of disposable cutlery, cups and Styrofoam® items (expanded and extruded PS, EPS and XPS), which are currently used in fishing activities, in food trays and other disposable items (Farrelly & Shaw, 2017). A note should be mentioned regarding the presence of kaolin in PS and copolymer PP/PE particles, which is used as a filler to improve the strength of the plastic material. Particles of polyvinyl alcohol polymer (PVA), considered of low environmental impact, may have been originated from medical and sanitary devices, as well as from food packaging. In fact, this polymer is considered appropriate for orthopedic applications (Baker, Walsh, Schwartz, & Boyan, 2012) potentially linked to the Orthopedic Hospital located close to St2.

Fibers were identified as Rayon, a cellulose-based semi-synthetic fiber frequently found in similar studies (Comnea-Stancu, Wieland, Ramer, Schwaighofer, & Lendl, 2017); polyester, widely used in packaging, textile, automotive, medical, electronic, and construction sectors (Camlibel, 2018); and polyamide (PA), predominantly used in fishing nets, but also used in the automotive sector and as a bone tissue scaffold in the medical sector (Atayeter & Atar, 2018; Winnacker, 2017). Polyurethane (PUR) is widely used in coating epoxy resins to protect boat hulls from deterioration and used as rigid foams to insulate boats from extreme temperatures and noise, besides biomedical, construction, and automotive applications (Akindoyo et al., 2016). Finally, polyacrylic acid (PAA) is used in the manufacture of household cleaning products, but also for enhancing the mechanical properties of hydrogels used as biological glues in the medical and tissue engineering sector (GVR - Grand View Reasearch, 2017).

Conclusion

As expected, MP pollution in this study was higher during the winter months, co-occurring with the usual decrease of primary consumers abundance in this season. The consequent increase of both MP:neuston and MP:ichthyoplankton ratios suggests therefore a critical time period for marine biota feeding in the neustonic habitat. Regarding MP spatial distribution, instead of a clear decreasing gradient from the estuary (area with higher human impact) to further coastal stations, a slight decline in concentrations was observed, suggesting a retention effect close to the Arrábida shore. Although fragments were the dominant shape, only foam and beads presented distinct variation in space, according to the

location of their potential sources (fishing harbor and WWTP submarine outfall). The predominance of particles at the 1-2 mm size range instead of the smaller size range (0.335-1 mm), is suggested to be related with the sampling method used, although further studies would be required to clarify this hypothesis. The diversity of polymers reflects the multiple activities occurring in the estuary and in the marine park, highlighting the urgent need to disseminate findings locally, namely on fishing communities and in tourism, industrial and marine traffic sectors. Sharing scientific findings with society aims to increase public awareness about MP pollution and to inspire actions towards the prevention and reduction of plastic entering the marine environment.

Author Contributions

DR conducted fieldwork sampling, laboratory procedures (MP extraction and characterization, neuston identification), statistical analysis and wrote the manuscript. JA performed FTIR analysis, collaborated in the discussion and selection of the best method for MP extraction from neuston samples, provided assistance with laboratory procedures and at reporting FTIR analysis, and results. VO performed micro - FTIR analysis, assisted in the interpretation of spectra, and at reporting FTIR analysis and results. PS coordinated the study, discussed results, gave important contributions to the writing and to the English review of the text. MHC reviewed and made important contributions to the text. All authors contributed to the article and approved the submitted version.

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3

DISTRIBUTION PATTERNS OF MICROPLASTICS IN SUBTIDAL SEDIMENTS FROM THE SADO RIVER ESTUARY AND THE ARRÁBIDA MARINE PARK, PORTUGAL[7]

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Abstract

Understanding local accumulation patterns of microplastics (MPs) in subtidal sediments is crucial to assess how available such particles are for ingestion by benthic feeders and to identify the potential pollution sources in the region upon which is urgent to act. The coastal urban centers of Setúbal and Sesimbra (Portugal) and the multiple activities taking place at the contiguous Sado estuary and in the sheltered waters of Professor Luiz Saldanha Marine Park make this a relevant case study about MPs pollution in the seabed. Here, a short-term investigation assessed the spatiotemporal distribution, abundance, and composition of MPs on the nearshore seabed. Sediment samples were monthly collected from summer 2018 to winter 2019, in six stations. Despite the differences observed in rainfall between campaigns, no distinct patterns were detected in the accumulation of MPs throughout the sampled months. Yet, strong variations occurred among stations. The abundance of MPs in the Sado estuary (1042.8 ± 430.8 items kg^{-1}) was higher in comparison to all the stations located along the marine park (52.9 ± 31.9 items kg^{-1}). Fragments comprised 70% of particles found in estuarine sediments, while fibers were the predominant type in marine sediments. The majority of MPs collected in the estuary shared the same size class as the best represented grain size fraction: 0.250-0.500 mm. On average, the ratio between the abundance of MPs and the abundance of meiofauna organisms was higher in the estuary, suggesting more encounter rates, by both meiofauna and their predators, with MPs. The distribution of MPs throughout the study area was moderately correlated with sediment sorting and organic matter content. Also, the distinct mineralogical content of each station indicates a reduced sediment transit between stations and consequently a weak exportation of MPs from the estuary. The majority of the polymers identified by Fourier Transform Infrared Spectroscopy was denser than seawater. Polyethylene terephthalate (PET) represented 41% of the items analyzed and was mostly assigned to fibers and fiber bundles. Unveiling the distribution patterns of MPs along this segment of the Portuguese west coast enabled to identify a high-risk area where the implementation of preventive measures is urgent.

Keywords: pollution, accumulation, microplastics to meiofauna ratio, granulometry, organic matter content, rainfall, mineralogical content, sewage discharge

Introduction

Marine sediments are long-term sinks for microplastics (MPs) (Coppock et al., 2021; Cozar et al., 2014; Pohl, Eggenhuisen, Kane, & Clare, 2020; Zhang, 2017). Though firstly demonstrated by Thompson et al. (2004), such evidence was actually in accordance with several previous studies

reporting marine sediments as the ultimate fate of larger plastic debris (Bingel et al., 1987; Galgani et al., 1995a, 1995b, 2000; Kanehiro et al., 1995). However, despite this perception, plastic pollution research has been mostly focused on seawater surface, as argued by Lusher et al. (2014), van Sebille et al. (2015), Porter et al. (2018) and Yao et al. (2019). The shift on scientific interest towards sedimentary matrices was triggered with the growing understanding about the 100-fold discrepancy between the estimates of all the plastic waste input in the oceans and the lower estimates of the global load of floating debris being reported (Cozar et al., 2014; Eriksen et al., 2014; Lindeque et al., 2020).

The lack of methodological standardization in pioneer studies about MPs in the marine substrate compelled the improvement and development of new extraction protocols (Rochman, Regan, & Thompson, 2017) that could prevent, for instance, the underestimation of high-density polymers (Imhof et al., 2012; Claessens et al., 2013; Nuelle et al., 2014; Coppock et al., 2017; Pagter 2018; Frias et al., 2018). The baseline data being subsequently acquired confirmed that, besides the particles exceeding seawater density (>1.02 g cm^{-3}), the low-density MPs would also end up reaching the seafloor (Frias et al., 2016; Martin et al., 2017; Coppock et al., 2021) as earlier reported for larger items (Hess et al., 1999; Holmström, 1975; Kanehiro et al., 1995; Stefatos et al., 1999). Deposition of low density particles into benthic substrates will depend on biofouling processes (Holmström, 1975; Pegram and Andrady, 1989; Ye and Andrady, 1991; Andrady, 2011), incorporation into marine snow (Porter et al., 2018; Van Cauwenberghe, Vanreusel, et al., 2013; Woodall et al., 2014) or into fecal pellets (Cole et al., 2016; Coppock et al., 2019).

Regardless of their density, MPs in the marine environment may have multiple origins. Besides those resulting from the fragmentation of larger plastic (Ryan et al., 2009; Thompson et al., 2004), which strongly occurs at shorelines due to the higher mechanical abrasion, temperatures, and exposure to UV radiation (Andrady, 2011; Barnes et al., 2009; Gregory & Andrady, 2005; Pegram & Andrady, 1989); others come from land, namely through wastewater treatment plants (WWTP) effluents (Mark Anthony Browne et al., 2011; Fendall & Sewell, 2009; Gregory, 1996; Murphy et al., 2016), sewage discharges and urban (stormwater) runoff (Piñon-Colin, Rodriguez-Jimenez, Rogel-Hernandez, Alvarez-Andrade, & Wakida, 2020; Werbowski et al., 2021); other pathways of plastic transport from land include wind, rivers and tides (Jambeck et al., 2015; McCormick, Hoellein, Mason, Schluep, & Kelly, 2014). Additionaly, several sea-based activities may also contribute to MPs pollution, such as fishing, aquaculture, maritime traffic, offshore platforms and recreational (Andrady, 2011; Jambeck et al., 2015; UNEP, 2016).

The high potential for MPs to accumulate in coastal sediments is therefore related with both the proximity to the multiple pollution sources and the propensity of particles to sink, independently of their polymeric composition. As a consequence, the interactions of MPs with bottom-dweller organisms (Bour, Avio, Gorbi, Regoli, & Hylland, 2018; Graham & Thompson, 2009; Murray & Cowie, 2011;

Van Cauwenberghe, Claessens, Vandegehuchte, & Janssen, 2015), as meiofauna (Gusmão et al., 2016) and/or their predators (Bellas, Martínez-Armental, Martínez-Cámara, Besada, & Martínez-Gómez, 2016; A. L. Lusher, McHugh, & Thompson, 2013), end up occurring more frequently at coastal areas. Furthermore, as both estuarine and coastal marine sediments are known to accumulate high concentrations of organic and inorganic pollutants (Bellanova et al., 2022; Castro & Vale, 1995; Lacorte, Guillamón, Martínez, Viana, & Barceló, 2003; Vieira et al., 2021), the MPs settling in these areas are expected to be highly associated to such contaminants (Bakir, Rowland, & Thompson, 2014b).

Despite the growing concern regarding the ecotoxicological risk faced by sediment biota upon MPs ingestion (including commercial species) and the increase of research about this topic, the identification of the adverse effects is yet to be fully accomplished and far from being consensually accepted. In fact, as argued by several researchers (Burns & Boxall, 2018; Lenz et al., 2016; Phuong et al., 2016; Rochman & Boxallz, 2014), detrimental impacts being reported in experimental studies have frequently resulted from testing conditions which greatly exceed those considered as environmentally relevant. Thus, assessing realistic levels of exposure to MPs faced by biota associated to subtidal sediments (defined as those permanently submerged and extending from the low tide mark to about 200 m depth) is critical, particularly in Portugal, a coastal nation, where studies on this topic are scarce. So far, the available data about MPs pollution on the seafloor at Portuguese coastal waters relies on the research conducted by Frias et al. (2016), about sediments collected in Algarve, from depths lower than 25 m.

Hence, here we aim to provide baseline data about MPs accumulation in subtidal sediments from the Portuguese west coast, namely from the Professor Luiz Saldanha Marine Park and the Sado river estuary, where conservation measures with more than two decades aim to protect habitats and species. In particular, besides investigating if temporal and/or spatial patterns occur in MPs abundance, distribution, and composition in this region, we also estimated (to the authors knowledge, for the first time) the MPs to meiofauna ratio in each sample. By comparing their abundances we intended to identify the areas where meiofauna organisms would face higher risk of exposure to MPs. In addition, we aimed to assess potential relationships between MPs abundance and rainfall, sediment organic matter and granulometric parameters, which might be usefull in further monitoring studies. Lastly, the analysis of the mineral content of sediment samples was also conducted to determine, together with polymers identification, possible links to potential pollution sources in this region.

Materials and Methods

Study Area

The study area, comprised by the Sado estuary and the Professor Luiz Saldanha Marine Park (Figure 3.1), is subject to multiple anthropogenic pressures. These have been described in a preceding study focused on MPs occurring at these surface waters (Rodrigues et al., 2020), which shares the same fieldwork period and location as this study. Sediment samples were thus collected in six sampling campaigns, as described in the previously mentioned study (between August 2018 and February 2019), at the same 6 nearshore sampling stations (located at the 5 m isobaths). The 6 sampling campaigns occurred every 30 days (approximately) whenever the weather conditions allowed. As we had to ensure a Beaufort wind scale ≤3 to properly collect the floating MPs with neuston trawls, the scheduling of sampling campaigns required adjustments which prevented an entirely consecutive sequence of months.

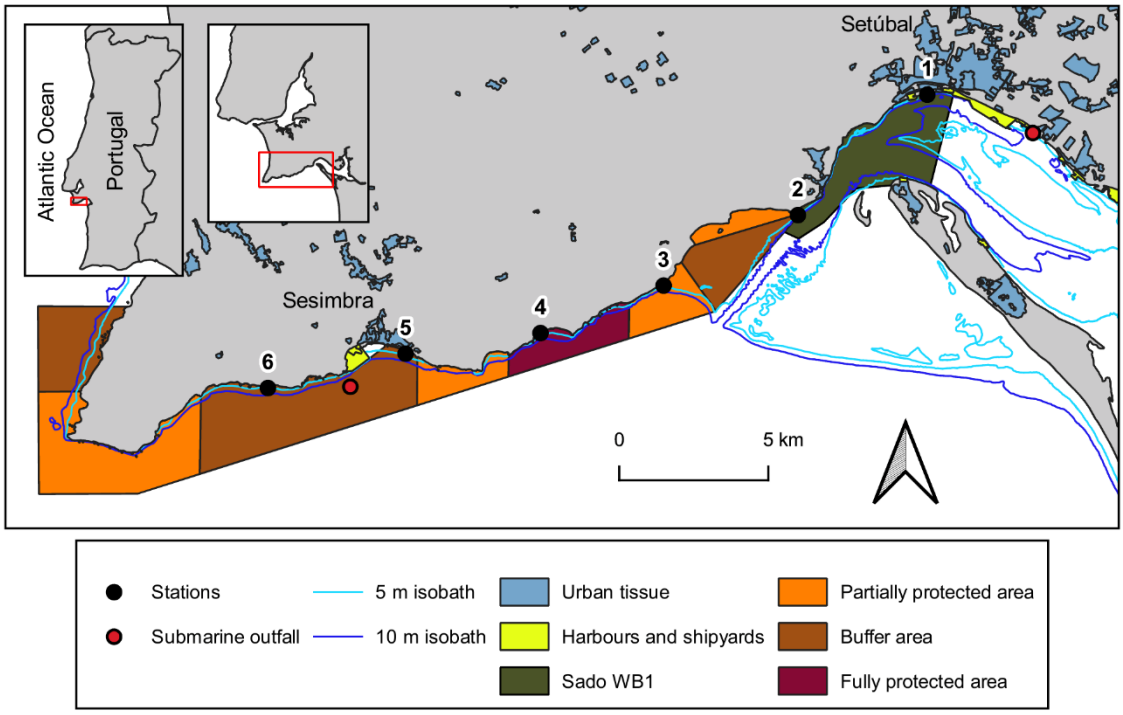

Figure 3.1 - Map of the study area (developed in QGIS) with the location of the 6 sampling stations (black dots) distributed along the coastal area of Setúbal and Sesimbra, on the Portuguese west coast. The complete information about the map layers as well as the list with the GPS coordinates of each station are provided at the map source: Rodrigues et al. (2020).

Sampling methods

Two replicate sediment samples (R1 and R2) were collected at each station with a Wildco® Petite Ponar benthic grab. This grab collects sediments from the seabed superficial layer and has a

sample area of ca 15x15 cm. After retrieving the grab from the water, its load was laid directly into a stainless-steel tray (34x24 cm) and the excess water was discarded. Sediment was then transferred with a wooden spoon to a 500 ml glass jar, up to its maximum capacity. Separately, a small aliquot was collected from one of the replicates for meiofauna analysis, stored in a 250 ml glass jar and fixed in 70% ethanol. Sediment samples (72 in total) were transported in ice coolers and kept frozen at -20 °C until analysis.

Laboratory Procedures

MPs extraction and characterization

MPs extraction was carried out at the laboratory, after thawing samples at room temperature. Approximately 250 g (wet weight) of each replicate was transferred into Ø150 mm glass petri dishes, manually homogenized and placed in the oven at 60 °C for 48 hours. The content left in the jars was frozen again until further analysis.

Three sub-replicates of 50 g each (dry sediment) were directly weighed in 1 L beakers. In order to remove the organic matter content, 150 ml of 10% hydrogen peroxide (J. Frias et al., 2018) was added to each beaker. The content was mixed with a glass rod for 1 minute and left for 24 hours in a fume hood, at room temperature. Each sub-replicate was subsequently poured into a 63 μm sieve and rinsed with distilled water. Then, it was transferred into a Sediment-Microplastic Isolation device (SMI-unit; designed by Coppock et al., 2017) where a magnetic stir bar (45 x 8 mm) was previously added. The SMI-unit was topped up with $ZnCl_2$ solution (1.5 g cm^{-3}; APC Pure®) until a volume of 700 ml was achieved, and the sediment was mixed as described in the protocol of Coppock et al. (2017). All samples were left to settle for 2 hours, except for those collected from st1 (at the estuary) which, due to higher silt/clay fractions, needed a longer period (20 h).

When the settling period was over, the valve was closed and the headspace content was vacuum filtered through a glass microfiber filter placed on the stainless-steel screen support of the glass filtration base (filter: MFV2 FILTER-LAB 47mm Ø with 1 μm pore; filtration base: XX1014732, Millipore). To recover the MPs eventually left in the internal surface of the SMI-unit, the top part was rinsed thoroughly with $ZnCl_2$ and filtered a second time. Finally, the $ZnCl_2$ solution at the bottom part was also filtered, ensuring the solution reuse in subsequent samples. Since the solution density could slightly decline with the continuous use, two batches of $ZnCl_2$ solution were prepared to ensure a similar MPs extraction efficiency among all samples (5L each; one for R1 samples and another for R2 samples). The extraction of MPs from R2 replicates was only performed after procedures for R1 group were completed.

Filters from each sub-replicate, were stored individually in glass petri dishes and observed under a stereomicroscope (Leica® S8APO) equipped with a camera (Motic® MOTICAM 10+). Particles were classified according to color and type, counted, and measured with the Motic® Images Plus 3.0 software. Only MPs belonging to the 0.063-5 mm size range were considered, being all categorized to one of the following size classes: 0.063-0.125 mm, 0.125-0.250 mm, 0.250-0.500 mm, 0.500-1 mm, and 1-5 mm; these size classes were selected to match the grain size fractions considered in granulometric analysis. Also, each microplastic was assigned to one of seven types: fragment, film, fiber, fiber bundles, filament, glitter, and bead, as described in Table 3.1 (adapted from Lusher et al. (2017), Rochman et al. (2019) and Rodrigues et al. (2020)). All particles similar to shavings (Total = 621) were excluded from analysis because they were considered to result from the degradation of the SMI valve made of Polyvinyl chloride (Nel, Krause, Sambrook Smith, & Lynch, 2019). Particles selected for polymer identification were isolated in covered concave slides. The abundance of MPs per sample consisted of the average of counts from the 6 sub-replicates of 50 g (3 from R1 and 3 from R2), which were then normalized to a constant weight and reported as items per kg of dry sediment (items kg^{-1}).

Table 3.1 – Definition of each particle type

Type	Definition
Fragment	Hard or soft irregular particle
Film	Thin and malleable, flimsy particle
Filament	Thicker and straighter than fiber
Fiber	Thin line, equally thick throughout its entire length, frequently curled
Fiber bundle	Several fibers tightly wound together in a knot-like formation
Bead	Spherical particle
Glitter	Shiny/metalized and flat particle, usually hexagonal

Polymer identification

About 8% (186 items; Table 3.2) of the total of particles was selected for polymer identification. The selection was conducted after discarding fibers considered airborne contamination and was based on the best expert judgment according to similarity, texture, thickness, and shine. All particles with a 1-5 mm size range, except for fibers, were analyzed by Fourier Transform Infrared Spectroscopy in attenuated total reflectance (FTIR-ATR), using a Perkin Elmer® Spectrum Two spectrometer. For smaller particles (0.063-1 mm) and fibers, analyses were carried out on a µ-FTIR spectrometer (Perkin Elmer® Spotlight 200i Microscope System), with microscope aperture 100 × 100 µm, using a strong Norton-Beer apodization. All spectra were acquired at room temperature under reflectance mode with a resolution of 4 cm^{-1} and 1 cm^{-1} wavenumber intervals, within 4000–500 cm^{-1}. The analysis was performed on the sample surface, sometimes in more than one point, when results were dubious. A background scan

was performed before any analysis series. Polymer identification relied on a match over 80% (Pequeno et al., 2021) between the sample and a referenced database (Primpke, Wirth, Lorenz, & Gerdts, 2018). The assignments were confirmed with the analysis of the polymers characteristic bands (Arshad, Naraghi, & Chasiotis, 2011; D. O. Hummel, 2002; Jung et al., 2018; Kausar, 2015; Marković et al., 2009; Xiao, Chen, Zhou, & Zhong, 2002).

Table 3.2 – Number (N) of particles per type and respective amount of items selected for FTIR. Total provided in bold.

Type	N	FTIR
Fragment	1427	76
Film	141	18
Filament	30	16
Fiber	473	36
Fiber bundle	44	15
Bead	120	13
Glitter	118	12
	2353	**186**

Quality Assurance and Quality Control

To assess airborne contamination, control filters (blanks) were exposed to the air, both during sampling (inside a hanging open glass jar at the boat deck, one blank per sampling campaign) and throughout lab work (in petri dishes, one per replicate). Thus, all the fibers extracted from samples which were similar to those found in respective blanks were excluded from results. Other contamination sources were minimized, both during field and laboratory work, by using glass, stainless-steel and wooden materials. At the laboratory, samples were kept covered at all times, a cotton lab coat and nitrile gloves were always worn and working surfaces were rinsed before use with Milli-Q water and ethanol. Moreover, all prepared solutions and rinsing liquids were filtered before use.

MPs to meiofauna ratios

After staining sediment aliquots with Rose Bengal for 1 hour, the content of each jar was transferred into a 38 μm sieve, in order to discard the ethanol. Next, from the sediment retrieved on the sieve, 6 sub-aliquots of 5 mL were collected with a measuring spoon. Whereas the average abundance of meiofauna was quantified in 3 out of the 6 sub-aliquots, the other 3 allowed the conversion of the 5 mL

volume into dry weight. The former group of sub-aliquots was placed separately into 1 L beakers and the other group was pre-weighed in Ø100 mm glass petri dishes. The meiofauna extraction protocol, adapted from Somerfield and Warwick (2013), consisted of adding 200 mL of filtered tap water to each beaker, being their content stirred and decantated onto a 63 μm sieve. This was repeated 4 times, except for st1 samples due to the higher silt/clay fraction (6 times). The meiofauna (size range: 63 to 500 μm; (Giere, 2009)) retained by the sieve was washed back into a Bogorov counting chamber and counted under the stereomicroscope with the support of a hand tally counter. In what concerns the sub-aliquots kept in petri dishes, these were placed uncovered in the oven at 60 °C to dry (for ca 12 hours) and then weighed; the mean of the 3 weight measurements was calculated. Finally, the abundance of meiofauna in sub-aliquots was extrapolated to a standard (dry) sediment weight (kg) and expressed as individuals per kg of dry sediment (ind kg^{-1}), to match the reported units used for MPs (items kg^{-1}) and to enable the calculation of the MPs to meiofauna ratio.

Sediment characterization

One replicate per sample was randomly selected to run granulometry and loss-on-ignition procedures. After defrosting, approximately 100g (wet weight) of sediment, from each sample, was transferred to a Ø150 mm glass petri dish, manually homogenized and oven-dried at 105°C for 24 h.

For granulometric analyses (protocol adapted from Pagter et al. (2018)), 50 g of each sample (dry sediment), was weighed in 1 L beakers, to the nearest 0.01 g. Then, to remove the organic matter, 200 mL of H_2O_2 (6%) was added to each beaker. After manually stirring, samples were left to digest until there was no sign of reaction (2 days on average; 50 mL of H_2O_2 were added per each extra day). Samples were subsequently poured onto a 63 μm sieve, rinsed with distilled water, and washed back into the 1 L glass beaker till a volume of 400 mL was obtained. To cause dispersion and to disaggregate fine-grained particles, 225 mL of a 4.2% Calgon solution (35 grams of sodium hexametaphosphate and 7 grams of sodium carbonate in 1 liter of distilled water (Kaur & Fanourakis, 2016)) was added to each beaker, stirred and left overnight. Then, samples were poured again into a 63 μm sieve and rinsed with distilled water. After that, they were transferred into a Ø150 mm petri dish and oven-dried at 105 °C for 24 hours. After cooling, each sample was weighed and dry sieved in an automated column shaker (RETSCH AS 200 basic) through a series of graduated sieves (2 mm, 1 mm, 0.500 mm, 0.250 mm, 0.125 mm, and 0.063 mm) for 10 minutes. The weight of the sediment retained in each sieve was registered. To determine the weight of the <63 μm fraction (clay and silt fractions combined), the sum of all weighed fractions was subtracted to the initial weight of sediment. Finally, the sediment grain size distribution, mean grain size and sorting, according to Folk and Ward (1957) geometric graphical measures, was determined from weight of the 6 fractions using the freeware Gradistat® (Version 9.1). Grain size

fractions were classified according to the Udden-Wentworth grade scale (Udden, 1914; Wentworth, 1922).

To determine the organic matter content of sediment (Cambardella, Gajda, Doran, Wienhold, & Kettler, 2001), three replicates of 1 g per sample (dry sediment) were weighed to the nearest 0.0001 g and transferred to labelled and pre-weighed crucibles. These were placed in a muffle furnace at 450 °C for 4 hours and, at the end of this period, moved into a desiccator for 1 hour and weighed again. The organic matter content, expressed as percentage, was calculated from weight loss on ignition i.e., from the difference between the sediment weight before and after ignition.

Lastly, approximately 2 g per sample (dry sediment) were transferred into individual plastic bottles and taken to the Geobiotec/Aveiro University laboratory facilities to identify the mineral content of sediment samples. Qualitative and semi-quantitative mineralogical analyses were carried out by X-ray diffraction (XRD) using a Philips®/Panalytical X'Pert-Pro MPD, Kα Cu (λ= 1,5405 Å) radiation. All samples (total sample) were ground in an agate ring mill to obtain a finer granulometry and were analyzed on random-oriented powders, X-ray scanned in the 2º to 40º 2θ interval at 1°2θ/min goniometer speed. The identification of the different mineral phases followed the criteria recommended by Schultz (1964), Thorez (1976), Mellinger (1979), Brindley and Brown (1980) and Pevear and Mumpton (1989).

Rainfall

Measurements from 4 meteorological stations (obtained from SNIRH[8]) located near the sampling site were considered: Comporta (23E/01C), Vila Nogueira de Azeitão (22C/02UG), Águas de Moura (22E/01UG) and Montevil (23F/01UG). Except for the first sampling campaign (Aug18), the mean rainfall (mm) assigned to each campaign was based on daily measurements collected from the 4 stations, registered uninterruptedly from the day after the last sampling until the day before sampling (about 30 days in total). In what concerns the Aug18 campaign, the mean rainfall calculation has only considered measurements from 3 stations, since data from Comporta was completely unavailable. In addition, the data obtained from those 3 meteorological stations was exclusively comprised by measurements registered during the 10 days which preceded the August sampling (because it was the only data available). Still, since the August month of 2018 was extremely dry (according to The Portuguese Institute for Sea and Atmosphere; IPMA; (IPMA, 2018)), we may assume that the missing data would not significantly change the mean rainfall calculation assigned to this specific campaign.

[8] https://snirh.apambiente.pt/index.php?idMain=

Statistical Analysis

Data was analyzed through non-parametric tests whenever parametric assumptions (normality by Shapiro-Wilk test and homogeneity of variances by Levene test) were not met. Spearman correlations assessed if rainfall, sediment organic matter, mean grain size and sorting could interfere with MPs abundances. To evaluate if and how the previous variables (except for sediment organic matter) changed temporally (along 6 months) and/or spatially (among the 6 stations), Kruskal-Wallis tests were performed, being post-hoc multiple comparisons followed with Dunn's test. The same analysis was conducted with the MPs to meiofauna ratio and with the Carbonate/Siliciclastic index. The variance of sediment organic matter (log(x+1) transformed), among stations and campaigns, was analyzed by a two-way ANOVA followed by Tukey post-hoc test for pairwise comparisons. The same test procedure was applied to assess the variance of meiofauna abundances (log(x+1) transformed). To analyze if fragments (log(x+1) transformed) were spatially distributed according to their mean size, a one-way ANOVA was conducted; the same test was performed with fibers (though with raw data). All the previously mentioned tests were performed with TIBCO Statistica™ 14.0.0 software and the level of significance was set at a p-value ≤ 0.05.

A univariate permutational analysis of variance (PERMANOVA), with 999 permutations, was performed to detect significant differences in MPs abundances, between stations and campaigns (fixed factors; with 6 levels each). Data were square-root transformed and the resemblance matrix between samples was calculated based on Bray-Curtis similarities. When differences were statistically significant, pair-wise comparisons among levels were analyzed and non-metric Multi-Dimensional Scaling (nMDS) plots were created. Additionally, whereas a multivariate PERMANOVA tested the effect of campaigns and stations in the abundance of each type of MPs in sediments, another focused on the response of MPs abundance according to size classes. Subsequently, to determine which type or size class most contributed to explain dissimilarities, the similarity percentages routine (SIMPER; with a cut-off percentage of 90% for low contributions) was conducted. Similarly, a multivariate PERMANOVA was applied to assess temporal or spatial patterns in the granulometric fractions of the sediment samples. All PERMANOVA analyses were developed in the Primer 6 software with the Permanova+ add-on (Anderson et al., 2008; Clarke & Gorley, 2006).

Results

Presence and abundance of MPs

From the total amount of particles extracted from sediment samples (4,060), 1,603 (40%) fibers were discarded for being considered airborne contamination during field and lab work. Also, 104 particles (belonging to several types) were excluded due to one of the following FTIR results: non-plastic particle, inconclusive match, or match under 80%. Therefore, the assessment of temporal and spatial distribution patterns of MPs in sediments was based on a total of 2353 particles (0.063 to 5 mm size range). Although 80% of this amount were MPs extracted from estuarine sediments (st1), all samples contained MPs (Figure 3.2A). The abundance of MPs in sediments collected at st1 was 1042.8 ± 430.8 items kg^{-1} (mean ± SD), whereas in sediments from st2 to st6 was 52.9 ± 31.9 items kg^{-1} (Figure 3.2B). The lowest MPs abundance (23.3 ± 29.3 items kg^{-1}) was observed in Sesimbra bay (st5) in Feb19 (winter), while the highest (2170 ± 1157.1 items kg^{-1}) was found in Oct18 (autumn), at Setúbal closest station (st1).

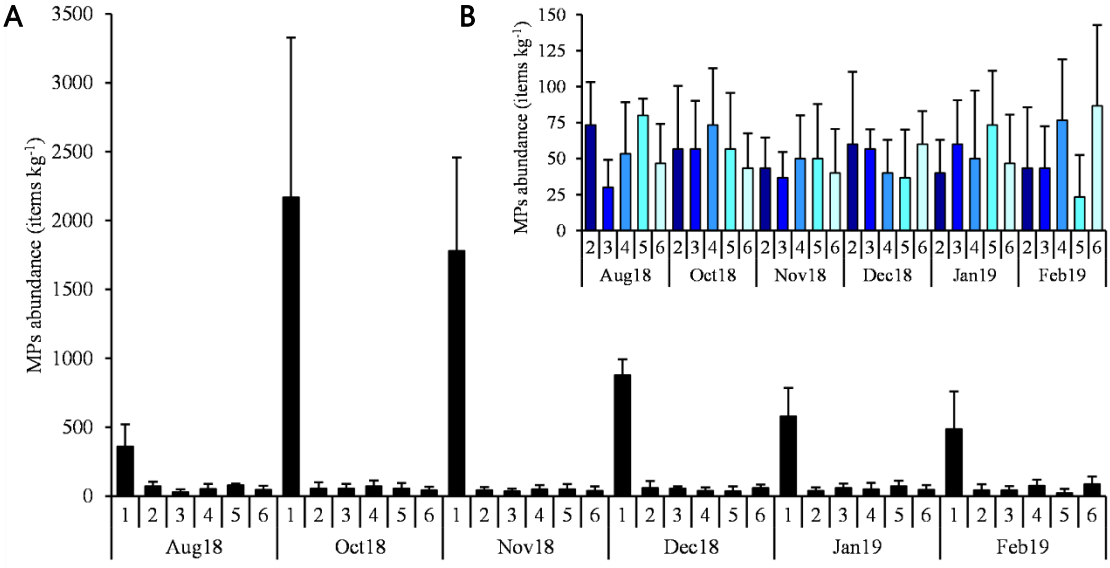

Figure 3.2 - MPs abundance (items kg^{-1}; mean ± SD) per sample. Whereas all samples (n = 36) are depicted in (A), only samples from stations 2 to 6 (n=30) are represented in (B).

Fourier Transformed Infrared Spectroscopy (FTIR) analysis

Among the 82 particles confirmed as plastic by FTIR analysis, a total of 11 polymers, including the Copolymer PP/PE, were identified (Table 3.3; Figure 3.3). Despite the high diversity of polymers, the majority of particles were identified as PET (41%), mostly assigned to fibers or fiber bundles (ca 65%; Figure 3.4).

Table 3.3 - MPs number and relative abundance (%) of each polymer

Polymer	N	%
Polyethylene terephthalate (PET)	34	41
Polyethylene (PE)	18	22
Polyacrylate (PAN[a], PMMA[b], others)	9	11
Polypropylene (PP)	8	10
Polystyrene (PS)	5	6
Copolymer PP/PE	2	2
Polyvinyl chloride (PVC)	2	2
Polyurethane (PUR)	1	1
Polyvinyl acetate (PVAc)	1	1
Polyamide (PA)	1	1
Polystyrene sulfonate (PS-S)	1	1

[a] Polyacrylonitrile

[b] Poly (methyl methacrylate)

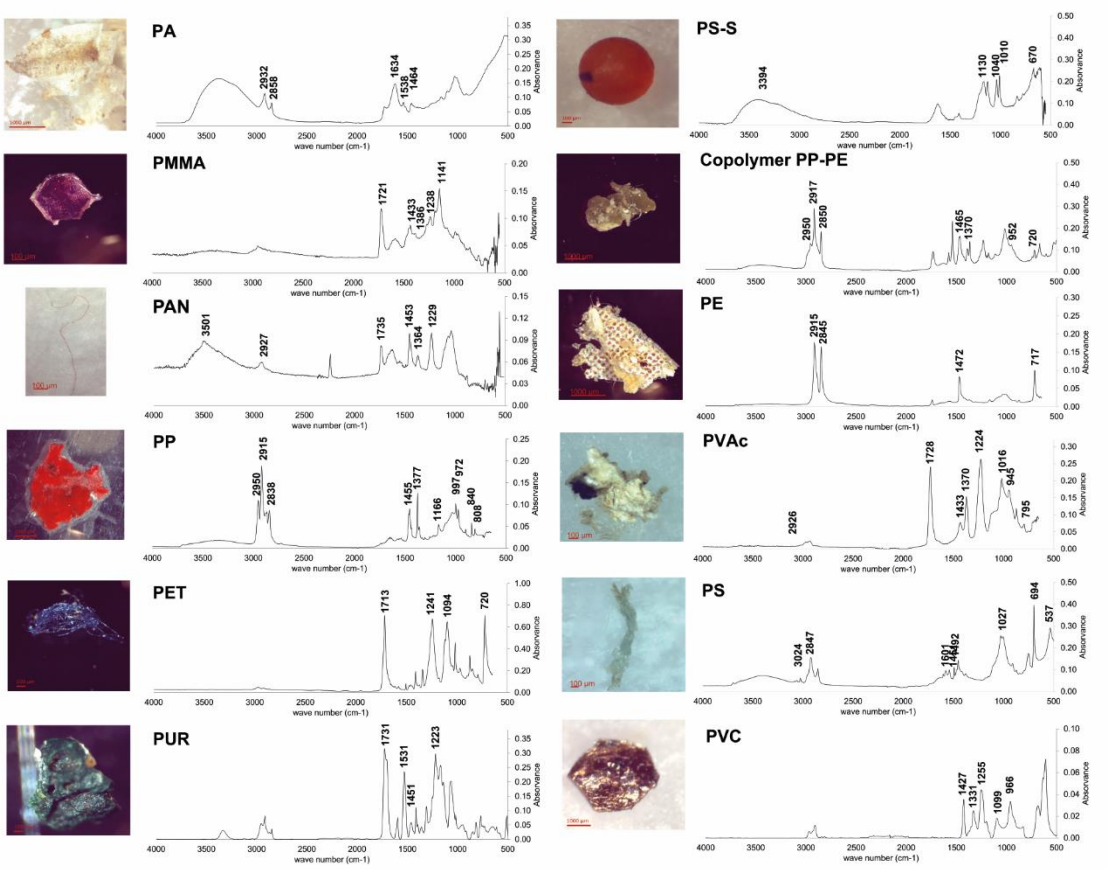

Figure 3.3 - Representative infrared spectra of the 11 identified polymers, with Polyacrylates represented by two items: PMMA and PAN. The image assigned to each spectrum corresponds to the MPs analyzed. The 12 MPs depicted in this figure belong to the following types: PA and PE - film; PS-S - bead; PMMA and PVC - glitter; Copolymer PP-PE, PP, PVAc and PUR - fragment; PAN - fiber; PS – filament; PET – fiber bundle.

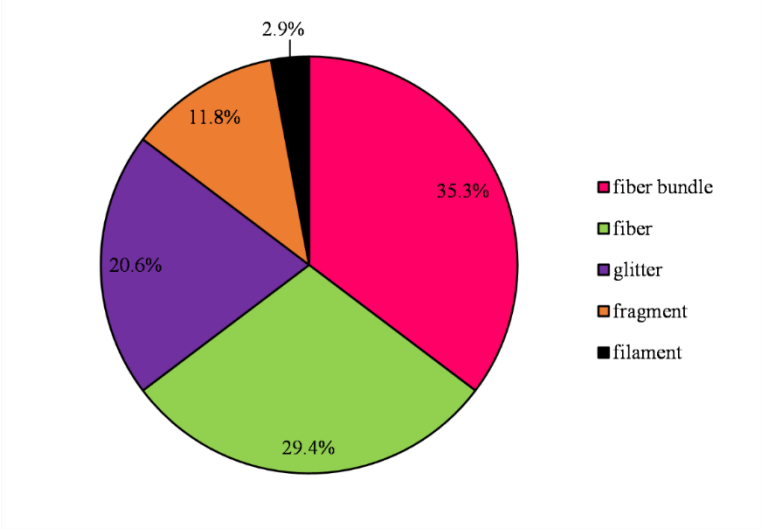

Figure 3.4 – Diversity of items identified as PET by FTIR, according to type of MPs.

Meiofauna abundance and MPs to meiofauna ratios

Fluctuations in meiofauna abundance were only significant between stations ($F_{(5,25)} = 5.941$, $p = 0.0009$; Figure 3.5B), being undoubtedly higher in the estuary (23444.1 ± 16614.9 individuals kg^{-1}; mean ± SD) than in all the other stations ($p < 0.05$). The lowest meiofauna abundance (3411.5 ± 2014.5 individuals kg^{-1}; mean ± SD) was registered at st6 (the furthest station from the estuary). The ratio between MPs and meiofauna differed significantly between stations ($H(5) = 13.95$, $p = .02$;Figure 3.5B), namely between st1 and st3, but not between campaigns ($H(5) = 4.64$, $p = .46$; Figure 3.5A). The highest ratio observed in st1 (estuarine sediments) was 0.065, or 1 microplastic to 15.3 meiofauna organisms, where both MPs and meiofauna abundances reached maximum levels; the average ratio in st1 was 0.047 ± 0.013 (mean ± SD). The lowest ratio among all samples was registered in st3 (0.003 or 1 microplastic to 383.1 meiofauna organisms).

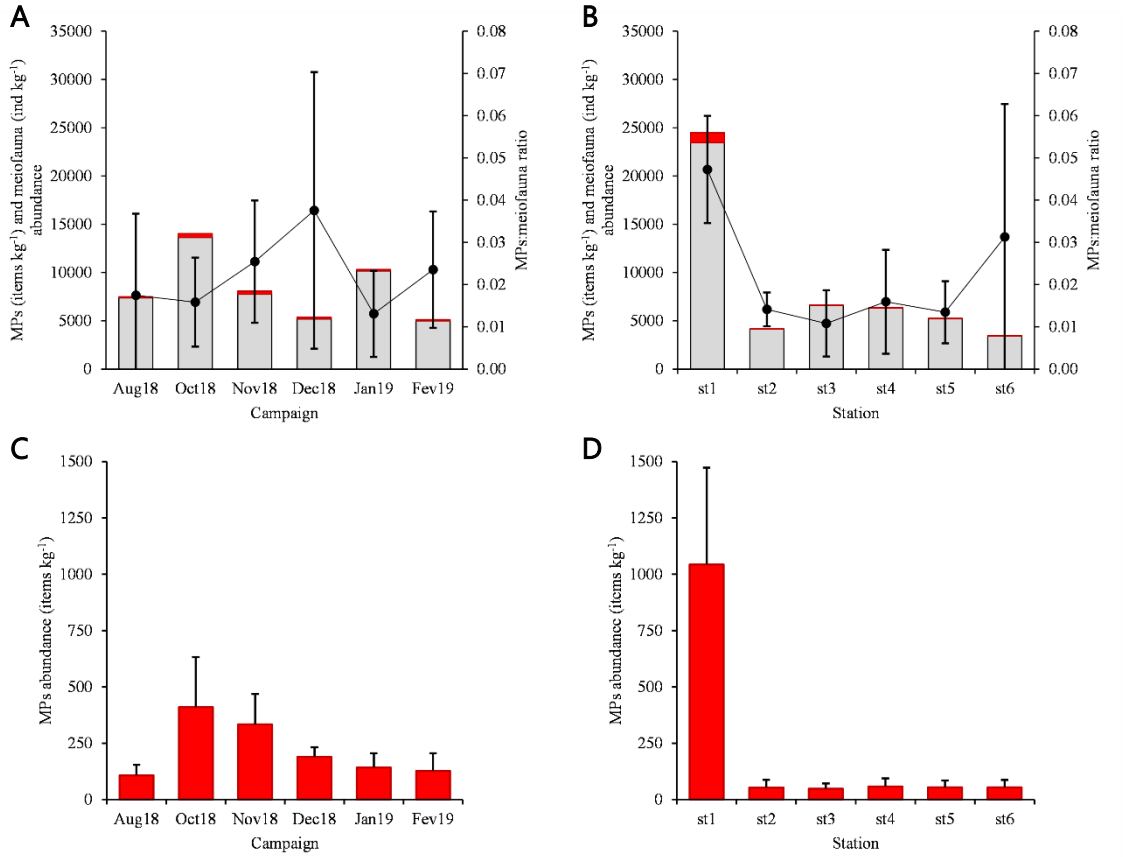

Figure 3.5 – MPs abundance (items kg^{-1}) (red bar), meiofauna abundance (individuals kg^{-1}) (grey bar) and MPs to meiofauna ratio (black dots), per campaign (A) and per station (B). MPs abundance (items kg^{-1}; mean ± SD; n = 6) per campaign (C) and per station (D).

Temporal and spatial distribution patterns of MPs

In contrast to the absence of significant temporal variations in MPs abundances among the monthly campaigns (Pseudo-F = 0.61, P(perm) = 0.823; Figure 3.5C), a significant spatial distribution pattern was observed (Pseudo-F = 31.22, P(perm) = 0.001; Figure 3.5D), consisting of a higher abundance of MPs in estuarine sediments (st1), in comparison with all the other stations (Figure 3.6 A to D). SIMPER results show that this dissimilarity (between st1 and all the other stations) mainly relies on the predominance and particular accumulation of fragments in the estuary (43 to 45 % contribution for the differences; Figure 3.6C). Furthermore, all the other types, except for fibers, were also mostly available in the estuary, though considerably less represented than fragments (Figure 3.6E). Conversely, fibers residually contributed (max 8%, according to SIMPER results) for the mentioned dissimilarity, as their abundances were similar throughout the study area (Figure 3.6C). While fragments predominated in estuary sediments (st1), fibers were the prevalent type found in marine sediments (i.e., at all the other 5 stations; Figure 3.6E). Moreover, dissimilarities between stations were also based on particle size

(Figure 3.6 B and D), particularly on MPs belonging to the 0.250-0.500 mm size class (with 26 to 27 % contribution to the differences found; Figure 3.6D). While MPs accumulated in the estuarine station were mostly assigned to the 0.250-0.500 mm, followed by the 0.500-1 mm size classes, at the ocean exposed stations, the majority of MPs belonged to 0.500-1 mm and 1-2 mm size classes (Figure 3.6F).

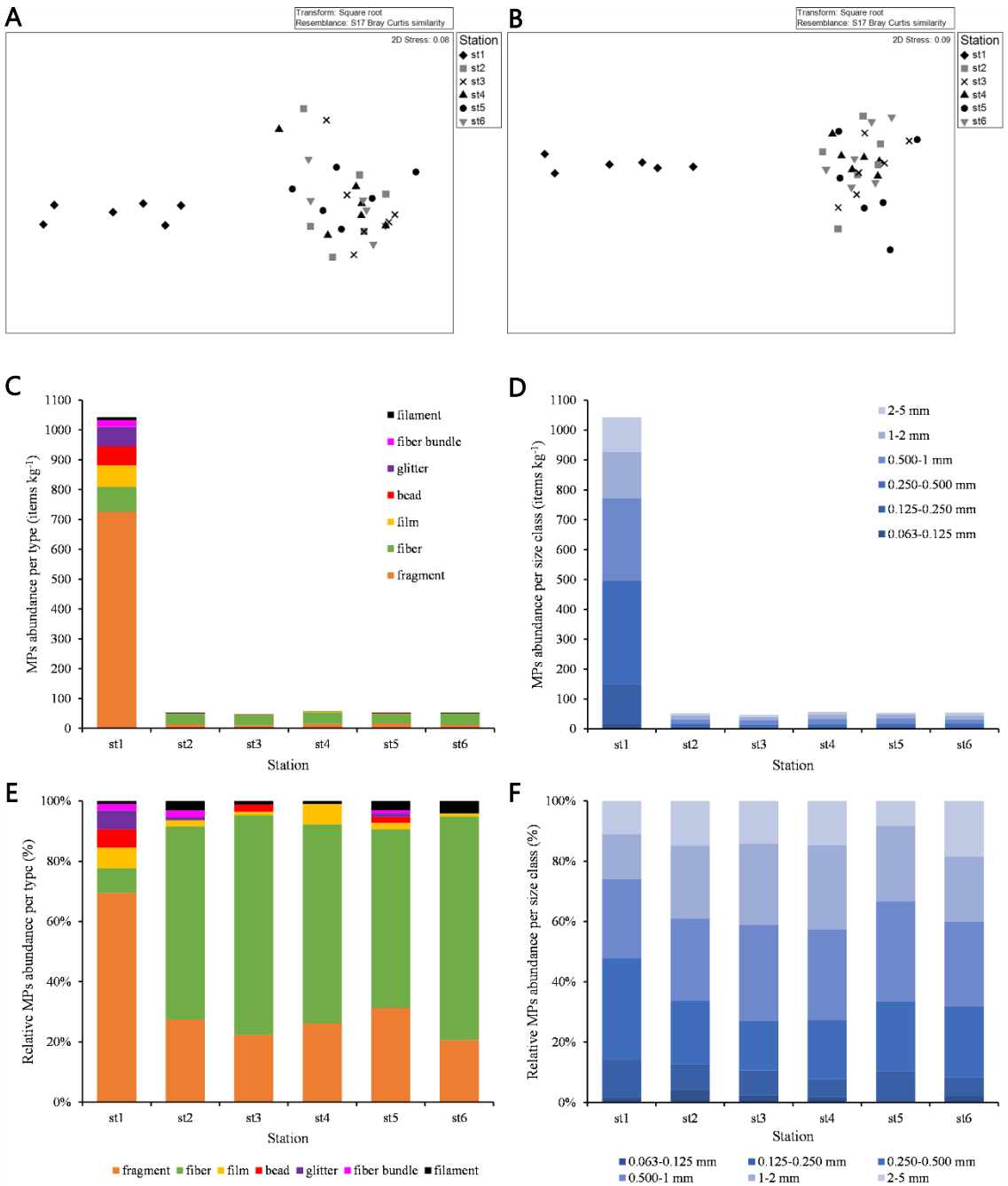

Figure 3.6 - Multidimensional scaling plot based on the Bray-Curtis distance between samples of the different stations according to type of MPs (A) and to MPs size class (B). Spatial variation of mean MPs abundance (items kg^{-1}) per particle type (C) and per size class (D). Relative proportion of particle types, per station (E). Relative distribution of particles size class, per station (F).

Among fragments collected from the estuary (st1; representing 70% of MPs in this station), the color green (34%) was the prevalent one, followed by blue (20%) and white (13%) (Figure 3.7A). Regarding fibers (similarly distributed among the 6 stations), black was the most frequent color (17%), followed by transparent (15%) and red (14%) (Figure 3.7B).

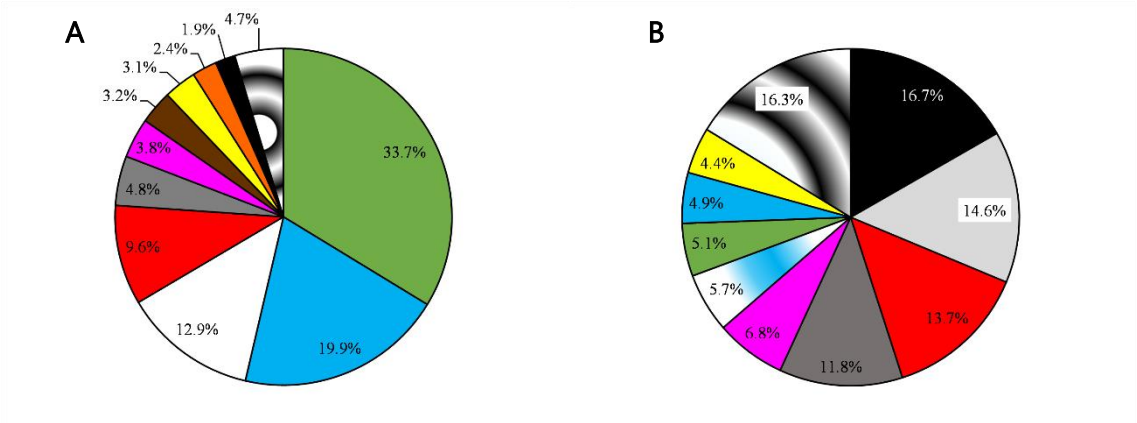

Figure 3.7 – Color composition of fragments collected at st1 (A) and of fibers collected from the 6 stations (B). The black & white slice corresponds to a pool of other colors. The light grey slice in (B) represents transparent fibers.

Neither fragments ($F_{(5,30)} = 2.18$, $p = 0.08$; Figure 3.8A) nor fibers ($F_{(5,30)} = 1.05$, $p = 0.41$; Figure 3.8B) showed significant differences in their mean size among the 6 stations. Mean size of fragments (486.8 ± 247.4) was 2.6 times smaller than fibers (1244.1 ± 243.6).

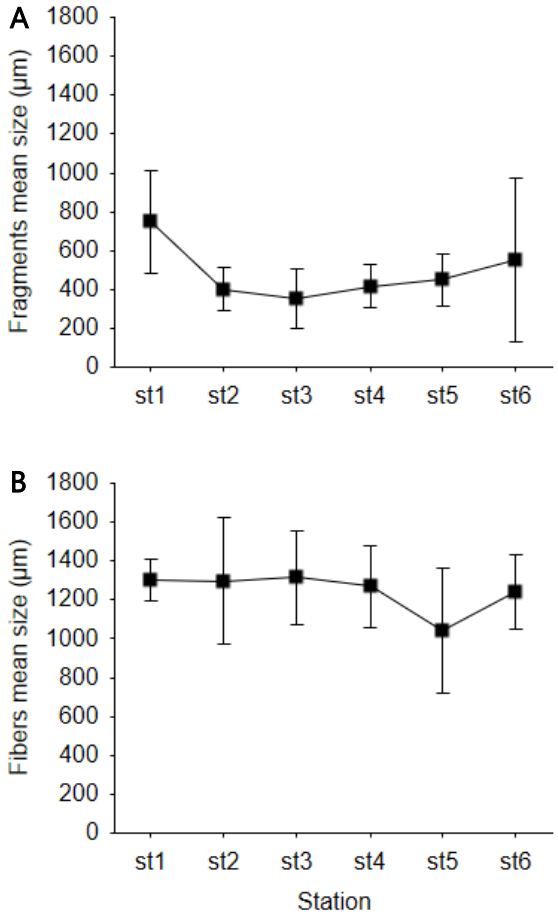

Figure 3.8 - Spatial variation of fragments (µm; mean ± SD) (A) and fibers (µm; mean ± SD) (B) mean size.

Granulometric parameters and organic matter content

In what concerns sediment granulometry, significant differences were observed between stations (Pseudo-F = 13.44, P(perm) = 0.001; Figure 3.9A), but not among sampling campaigns (Pseudo-F = 0.33, P(perm) = 0.95). The prevalence of the 0.125-0.250 mm (fine sand) and 0.250-0.500 mm (medium sand) grain size fractions in stations 1, 4 and 5 largely contributed (with a 35 to 51 cumulative percentage range) to distinguish them from the other 3 stations. Yet, st4 (located at the fully protected area of the marine park) significantly differed from st5 (at Sesimbra bay) due to the lower representation of the 0.500-1 mm (coarse sand) size fraction. Conversely, although coarse sand was the predominant grain size fraction (Figure 3.9B) in stations 2, 3 and 6, the sediments collected from the mouth of the estuary (st2) and from the closest station to Portinho da Arrábida (st3) were considered distinct due to the significantly higher content of the 1-2 mm size fraction (very coarse sand) in st2. Lastly, despite the generally low representation of the fine grain fraction (silt/clay) in all stations, it was slightly higher in

st1. Gradistat® categorized sediments from stations 1, 4 and 5 as medium sand, and the other three stations as coarse sand (following the Udden-Wentworth classification).

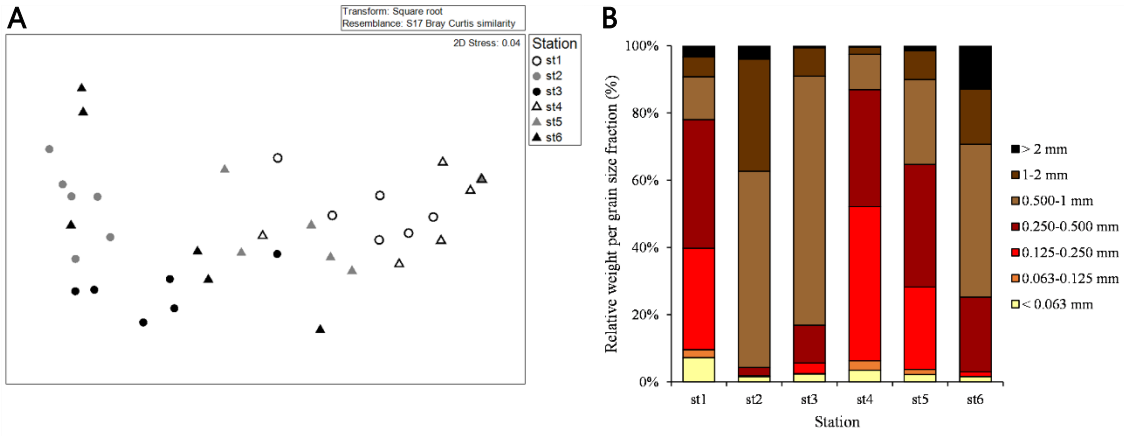

Figure 3.9 – Multidimensional scaling plot based on the Bray-Curtis distance between samples of the different stations according to grain size fractions (A) and relative distribution of grain size fractions per station (B).

Mean grain size ($H(5) = 25.40$, $p < .001$), sediment sorting ($H(5) = 25.78$, $p < .001$) and organic matter content ($F_{(5,25)} = 5.42$ $p < 0.05$) significantly differed between stations, but not between campaigns. Whereas mean grain size of both st1 (335.3 ± 131.4 µm; mean ± SD) and st4 (277.3 ± 99.7 µm) was considered distinct ($p < 0.05$) from st2 (918.7 ± 84.9 µm), only st4 differed significantly from st6 (863.2 ±384.4 µm) (Figure 3.10A). The poorly sorted sediments in st1 (2.49 ± 0.28 µm) were significantly different from the moderately well sorted sediments from station 2 (1.60 ± 0.07 µm) and 3 (1.51 ± 0.19 µm) ($p < 0.05$; Figure 3.10B); also, sediment sorting at st3 was significantly distinct from st5 (1.96 ± 0.21 µm). The organic matter content of sediments at st1 (1.84 ± 0.88 %) was significantly higher than st2 and st5 ($p < 0.05$; Figure 3.10C). Conversely, sediments of st2 had significantly lower organic matter content (0.57 ± 0.25 %) than all the other stations ($p < 0.05$), except from st5. Significant correlations (positive, though moderate) were detected between MPs abundances and both organic matter content ($r_s(34) = .37$, $p = .028$) and sorting ($r_s(34) = .43$, $p = .009$), but no correlation was detected with mean grain size ($r_s(34) = -.32$, $p = .055$).

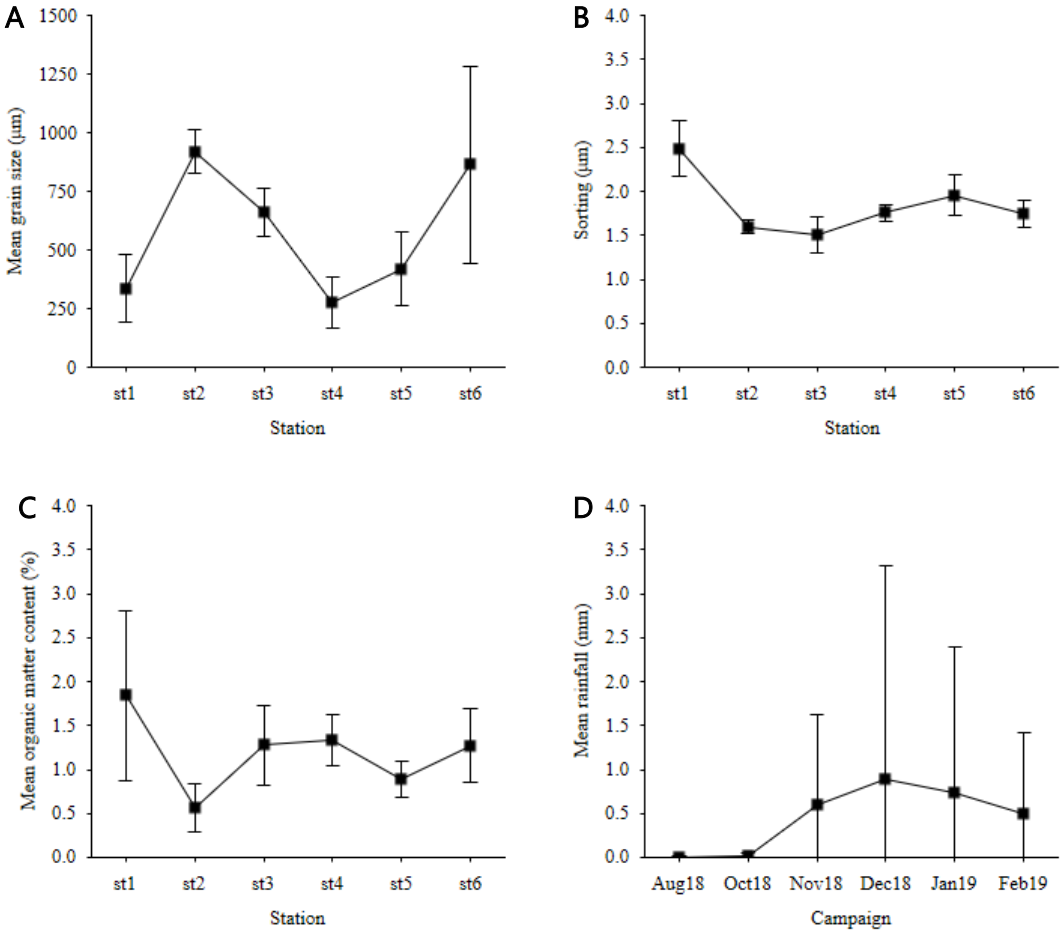

Figure 3.10 - Spatial variation of (A) mean grain size, (B) sediment sorting and (C) organic matter content. Temporal variation of rainfall (D). Data is provided as mean ± SD.

Rainfall

No relationship was observed between mean MPs abundance and rainfall ($r_s(4) = .26$, $p = .623$), despite the significant differences found in rainfall among campaigns ($H(5) = 102.50$, $p < .001$; Figure 3.10D). Rainfall registered in Aug18 and Oct18 was significantly lower than that registered during all the other sampling campaigns ($p < 0.001$).

Mineralogical content

The mineral composition of sediment samples mainly consisted of quartz, calcite, and dolomite. Proportions of aragonite (assumed to have origin in seashells), potassium feldspars and halite were residual and relatively random, being therefore not considered for the calculation of the Carbonate/Siliciclastic index. Mineral proportions changed significantly among stations ($H(5) = 28.11$, $p = .00$; Table

3.4), but not between campaigns ($H(5) = 0.67$, $p = .98$). The index calculated for st2 (the lowest, due to the absence of carbonates) was significantly different from the index obtained for st4 and st6, where the carbonate proportions were considerably higher.

Table 3.4 - Average proportion of each mineral and Carbonate/Siliciclastic index displayed per station. Stations were ordered from West to East.

Proportion (%)	st6	st5	st4	st3	st2	st1
Quartz	65.2	76.8	55.3	74.2	88.6	87.2
Calcite	2.2	4.1	21.8	4.0	0.0	5.3
Dolomite	28.3	3.2	13.5	7.8	0.0	1.1
Other	4.3	16.0	9.4	14.0	11.4	6.5
Carbonate/Siliciclastic index	0.47	0.09	0.64	0.16	0.00	0.07

Discussion

Abundance of MPs and comparison with other studies

Evident patterns were identified in the spatial distribution of MPs in subtidal sediments of the Portuguese west coast. While the Sado estuarine sediments presented extremely high abundances of MPs (1042.8 ± 430.8 items kg^{-1}), marine sediments from the Arrábida Marine Park, in comparison, accumulated 20-fold less MPs (52.9 ± 31.9 items kg^{-1}). This conspicuous difference, observed throughout the 6 monthly campaigns (as shown in Figure 3.2A), suggests a pronounced contribution from (i) the Sado river, which is expected to transport MPs resulting from the diverse industrial and artisanal fishing activities taking place in the Setúbal municipality (ca 123,000 inhabitants; according to INE Statistics Portugal, 2021[9]), mostly at Sado north margin; (ii) the sewage and stormwater discharges into the Livramento stream, which joins the estuary at the st1 location; and (iii) the effluent of Setúbal WWTP (submarine outfall) located east of st1. Contrarily to the station located inside the estuary, all the others - though located at a sheltered coastline from the prevailing north and north-west winds by the Arrábida mountain chain (Henriques, 1999) - are ocean exposed. Such marine sediments are thus expected to face higher turbulence levels which prevent the accumulation of higher abundances of MPs, as observed inside the estuary. Our findings corroborate the classification of estuaries and urban coastal areas as MPs hotspots (Maes et al., 2017; Wright et al., 2013) and show how urgent is to implement

[9] https://www.ine.pt/scripts/db_censos_2021.html

local preventive measures that engage the different society sectors into the decrease of MPs inputs in this coastal environment.

Comparatively with other estuaries, the abundance of MPs found in Sado sediments was only lower than levels reported for Durban Bay in South Africa (111,933 ± 29,189 items kg^{-1}; Preston-Whyte et al., 2021). However, it was higher than Densu delta in Ghana (4.0 ± 0.82 items per 10 g; Blankson et al., 2022), Warnow in Germany (379 ± 28 items kg^{-1} at the S10 station; Enders et al., 2019), Miri in Borneo Island (456.2 ± 33.6 items kg^{-1} in S5; Liong et al., 2021), Sebou in Morocco (187 items kg^{-1} in E1; Haddout et al., 2021) and both Dalio (ca 400 items kg^{-1}; Xu et al., 2020) and Changjiang (where the higher abundance was 150 items kg^{-1}; Peng et al. (2017)) in China. Conversely, the mean abundance of MPs in marine sediments of the Arrábida coast is at an intermediate position, considering what has been reported from other marine coastal areas. While slightly lower values were found in the Polish zone of the Southern Baltic Sea (range: 0–27 particles kg^{-1}; Graca et al., 2017), Algarve coast (10 ±1 items kg^{-1}; Frias et al., 2016) and Gdansk Bay (34 ± 10 items kg^{-1}; Zobkov and Esiukova, 2017), there are reports of higher MPs pollution levels in marine sediments at the Galway Bay (73 items kg^{-1}; Pagter et al., 2020), Park of Telaščica bay in Croacia (range: 32.3 ± 20.2 and 377.8 ± 18.8 items kg^{-1}; Blašković et al., 2017), Belgian coast (91.9 ± 21.9 items kg^{-1} in BCS Coast (S1–S3); Claessens et al., 2011), Aeolian Archipelago, Italy (range: 151.0 ± 34.0 and 678.7 ± 345.8 items kg^{-1}; Fastelli et al., 2016) and Southern North Sea (421 items kg^{-1}; Maes et al., 2017). Even though it is assumed that local pollution sources and hydrodynamic conditions differ between the mentioned regions, such comparisons (only possible due to the harmonized reporting units) are important to provide the big picture of the addressed topic.

Distribution patterns according to types and sizes of MPs

Regarding the representativeness of MPs types, fragments were by far the most abundant one in the estuarine sediments (st1), in agreement with findings from other studies (Haddout et al., 2021; Liong et al., 2021; Talley, Venuti, & Whelan, 2020; Vianello et al., 2013). Conversely, fibers were less abundant, but consistently spread throughout the six stations. Here it is important to highlight the potential for the fiber type to be commonly underestimated (Rummel et al., 2016) and therefore conceal serious scenarios, especially in sediments where this type of MPs is usually better represented (Marques Mendes, Golden, Bermejo, & Morrison, 2021; Martin et al., 2017). The considerable number of fibers discarded in this study (1603) for resembling those found in airborne contamination controls, may explain the reduced representation of this type of MPs in our study area. Even though consisting of a critical precaution step conducted before data analysis, this discarding process may wrongly exclude fibers which, despite being coincidently similar to those from controls, did not result from airborne contamination. Nevertheless, running this conservative step is preferable than to absolutely exclude all

fibers from reports/studies. An additional explanation for the reduced number of fibers in this study was the potential loss of fibers that may have occurred onboard, during sampling, when the water excess of each sediment sample was discarded (mentioned in the sampling methods section). This step was carried out because we could not assure this water source: if it was sediment pore water, bottom seawater adjacent to the sediment surface ("fluff layer"; Queirós et al., 2019)) or from the water column. Yet, despite such constraints, fibers were the prevalent type occurring in marine sediments, i.e. from st2 to st6, as reported elsewhere (Claessens et al., 2011; J.P.G.L. Frias et al., 2016; Graca et al., 2017; Martin et al., 2017; Pagter et al., 2020; Zobkov & Esiukova, 2017). This type of MPs can thus be considered the most available one for ingestion by marine benthic foragers occurring in this study area. Lastly, concerning all the other types of MPs (beads, glitter, films, filaments, fiber bundles), they were mainly found inside the estuary, similar to the accumulation pattern described for fragments.

Possibly, the occurrence of such distribution according to the type of MPs could be related with the higher surface area to volume ratio of fibers, in comparison to fragments and other irregular or voluminous types (Khatmullina & Isachenko, 2017; Pohl et al., 2020; Shin & Koch, 2005). As a result, due to a slower sinking process and easy resuspension from seabed, even with weak currents (Herzke, Ghaffari, Sundet, Tranang, & Halsband, 2021), fibers end up being transported/deposited further away from their potential source - sewage and/or WWTP discharges. Although an inefficient retention of fibers was reported to occur in WWTP (Browne et al., 2011), recent studies have shown that wastewater treatment processes are indeed highly efficient (Conley et al., 2019; Mason et al., 2016; Mintenig et al., 2017; Murphy et al., 2016). However, the authors argue that the reduced amount of MPs being released per liter in the effluent is still substantial and, thus, should be considered as a significant source. In what concerns our study area, besides the sewage discharge located close to st1, there are two WWTP discharge points, one at each extremity (depicted in Figure 3.1), which may be contributing for fibers ubiquity among all stations. Regarding the marginally higher amount of fibers observed in st1, it may possibly result from the weak hydrodynamic conditions occurring at this location, comparatively with the ocean exposure at the Sesimbra WWTP outfall (located between st5 and st6), where MPs should easily disperse. Lastly, such evident spatial distribution patterns, influenced by MPs type, may be also determining the distribution of MPs according to their size. This interpretation is not only based on the absence of significant changes in the mean size of both fragments and fibers among stations, but also on the distinct size displayed by these two types of MPs. Fibers mean length was almost 3 times higher than mean fragments size (strongly related with fibers elongated shape). We therefore assume that the prevalence of smaller MPs in st1 (between 0.250 and 1 mm) was due to fragments high abundance in this station, while the prevalence of bigger MPs (between 0.500 and 2 mm) in all the other stations, resulted from fibers predominance.

Granulometric parameters and organic matter content

According to the granulometric profile of sediments, whereas stations 1, 4 and 5 could be considered as depositional areas (relatively protected, low energy environments) due to the predominance of smaller grain size fractions, the conversely larger grain size fractions prevailing in stations 2, 3 and 6, indicate higher energy environments (Kersten & Smedes, 2002). However, despite the potential for MPs to accumulate in stations 4 and 5, this was only verified in st1. Such strong retention of MPs inside the estuary may be greatly attributed to its reported slow flow rate (Biguino, Sousa, & Brito, 2021; I. Cunha, Neuparth, Caeiro, Costa, & Guilhermino, 2007; Vale et al., 1993) and to flocculation (Andersen, Rominikan, Olsen, Skinnebach, & Fruergaard, 2021; Laursen, Fruergaard, & Andersen, 2022). The typical aggregation of suspended particulate matter in the estuary water column (Eisma, 1986; Manning & Dyer, 1999; Meade, 1972) enhanced by the mixture between freshwater and seawater, is suggested to transport MPs from the water surface into the estuarine sediments due to their incorporation in such flocs (Andersen et al., 2021; Laursen et al., 2022). Conversely, the abrupt decrease of MPs abundances at st2 may be eventually explained by the higher hydrodynamism occurring at the mouth of the Sado estuary (the interface with the Atlantic Ocean), preventing MPs entrapment in the sediment. Likewise, MPs at stations 3 to 6, may be under permanent resuspension into the water column, which consequently decrease their availability on the seabed (Näkki, Setälä, & Lehtiniemi, 2019; Shamskhany, Li, Patel, & Karimpour, 2021). An additional explanation is that, eventually, MPs might get buried at the submerged ebb-tide delta (Costas, Rebêlo, Brito, Burbidge, & Prudêncio, Maria Isabel FitzGerald, 2015) located at the estuary mouth south margin, preventing the exportation of higher MPs abundances to the Arrábida coast. However, as sampling in this study was only conducted at the north margin, further studies would be necessary to clarify this possibility.

Nonetheless, if plastic inputs in the ocean continue to increase as estimates predict (Geyer et al., 2017; Isobe, Iwasaki, Uchida, & Tokai, 2019; Jambeck et al., 2015), it is plausible to expect an increase of MPs accumulation in areas with potential for deposition, as described for st4 in particular. Therefore, and considering this station specific location, which is inside the fully protected area of the Arrábida Marine Park, we suggest its integration in a monitoring plan of MPs pollution, along with st1, as a preventive measure. Regarding st5, despite its deposition potential and the extreme proximity to Sesimbra town (even though it consists of a smaller municipality; ca. 52,000 inhabitants; according to INE Statistics Portugal, 2021[10]), MPs accumulation was unexpectedly low. Here we hypothesize that st5 was not close enough to the Sesimbra submarine outfall in order to capture more realistic data about MPs inputs in this area. As mentioned before, this outfall, contrarily to the one inside the estuary

[10] https://www.ine.pt/scripts/db_censos_2021.html

(Setúbal WWTP), is located far from the shore, being exposed to higher hydrodynamic conditions (turbulence) that prevent the deposition of potentially emitted MPs. Nevertheless, the influence of Sesimbra WWTP effluent is not completely inexistent, considering the higher diversity of MPs types in st5, in comparison to st4 and st6 (Figure 3.6E), and the before mentioned absence of significant differences in fibers abundance among the 6 stations.

Despite the absence of a correlation between MPs abundances and sediment grain size, similarly to what was reported by Alomar et al. (2016), Fastelli et al. (2016), Blašković et al. (2017) and Coppock et al. (2021), it should be highlighted that in st1, both the majority of MPs and the dominant grain size fraction belonged to the 0.250-0.500 mm range. Further studies are thus needed to confirm such relationship, which could hence support the use of sediment grain size as a proxy for MPs size characterization inside the estuary. In what concerns grain sorting, and contrarily to what is reported by other studies (Zobkov & Esiukova, 2017), our findings suggest that this sediment feature may contribute to infer and identify areas with potential for MPs accumulation. In fact, sediments at st1 were both the most poorly sorted (i.e., the less calibrated sediments) and a MPs hotspot. Moreover, though only to some extent, the higher accumulation/entrapment of MPs observed in st1 (in comparison to all the other stations) may have possibly resulted from its subtly larger fine grain fraction (silt/clay; Figure 3.9B) and higher organic matter content (significantly higher than st2 and st5). These variables were expected to interfere with MPs accumulation due to the potential of fine grain fraction and organic matter to provide sediment cohesion (Shrestha & Blumberg, 2005) and to enhance particle aggregation (Maes et al. 2017). However, owing to the lack of stronger relations with these sediment characteristics, our data suggests that proximity to MPs sources and the local hydrodynamic conditions (slow flow rate in the estuary and ocean wave turbulence) are the main variables affecting MPs accumulation in sediments at our study area.

Meiofauna abundance and MPs to meiofauna ratios

Despite the unadvised use of grabs as a sampling method for meiofauna studies due to the bow-wave effects that disturb the sediment surface prior to sampling (Somerfield & Warwick, 2013), it should be highlighted that the selection of this method - Petite Ponar grab - was primarily based on MPs as the main target. Also, as such disturbance could be assumed to similarly interfere with the calculation of both MPs and meiofauna abundances, we may consider our MPs to meiofauna ratio patterns robust. As expected, meiofauna abundance was higher in st1 than in the other stations. This could be linked to the higher content of organic matter found inside Sado estuary (Sandulli, De Leonardis, & Vanaverbeke, 2010), where hydrodynamic conditions are weak (inferred by the smaller mean grain size and more poorly sorted sediments). Also, since about 48% of MPs accumulated in the estuary (Figure 3.6F)

overlap the size range of meiofauna organisms (between 63 µm to 500 µm; Giere, 2009), there is a high potential for MPs to be ingested by meiofauna predators in st1, either accidentally or intentionally. In what concerns the MPs to meiofauna ratio, a clear contrast was noticed between st1 and all the other stations, especially with st3. It reinforces the higher exposure of benthic feeders in the estuary, an ecosystem known to provide important habitats and nursery grounds (Beck et al., 2001; Sheaves, Baker, Nagelkerken, & Connolly, 2015) but also known to accumulate high concentrations of pollutants in sediments, as reported for Sado (Carvalho, Rodrigues, Basto, & Vasconcelos, 2009; Nunes et al., 2014; Ribeiro, Ribeiro, & Tiritan, 2016). The conversely lower ratios occurring between st2 and st6, suggesting fewer interactions, is explained by their low abundances of both MPs and meiofauna (at st6 in particular), which is potentially caused by the exposure of these stations to the predominant swell direction (NW; Mota and Pinto, 2014).

Rainfall

Regarding the assessment of temporal patterns in MPs abundance, although there was a slight decrease from October 2018 to February 2019, no significant changes were noticed. In fact, despite the significantly reduced rainfall observed during the two first sampling campaigns, no correlation was detected between rainfall and the monthly abundances of MPs here reported. This is contrary to the patterns reported by Rodrigues et al. (2020) regarding surface water samples collected in the same sampling stations, suggesting that rainfall (or stormwater runoff) mainly interferes with MPs abundances on the sea surface, or has at least causes a more immediate effect at this marine compartment. In fact, when low-density particles (known to ultimately contribute for the pool of MPs found in sediments) enter in the marine environment, they will not sink for several weeks, due to the lack of biofouling (Kaiser et al., 2017; Lobelle & Cunliffe, 2011; Ye & Andrady, 1991). Moreover, the lack of such relation in this study may be also related with an increase of the current velocity at the Sado estuary from late summer to February (Biguino et al., 2021; F. Martins et al., 2002; Vale et al., 1993) preventing a faster settling of the new MPs inputs in the system.

Polymer diversity

As observed in a preceding study focused on the MPs floating at the surface waters from this coastal area, the polymer diversity here determined was also high (11 different polymers), mirroring the multiple activities taking place, both on land (domestic, commercial, industrial and tourism) and at the sea/estuary (fishing, maritime recreational activities and intense traffic to shipyards). In fact, 6 of the 11 polymers here identified (PE, PS, PP, PUR, Copolymer PP/PE and PET) were also detected in the

seawater surface samples, reflecting the widespread of several polymers in the water column. In addition to the potential links already suggested by Rodrigues et al. (2020), here others may be established between the identified polymers and the local sources/activities. For example, the PVAc may have the shipyard as a source, as this polymer is used in shipbuilding (Graca et al., 2017). Others may be released via wastewater as a result of laundry (Polyacrylates; textile fibers; (Mark Anthony Browne et al., 2011; Napper & Thompson, 2016)), use of cosmetic and art/craft products (Polyacrylates and PVC; glitters; Yurtsever, 2019), use of pharmaceutical products for the treatment of hyperkalemia (PS-S; Wong et al., 2020; Rahman and Marathi, 2022) or use of water softening products (PS-S; Saleh, 2009). It should be highlighted that out of the 11 polymers, 7 are denser than seawater (>1.02 g cm^{-3}), namely PET, Polyacrylates, PVAc, PVC, PUR, PA and PS-S. Also, as reported elsewhere about other coastal sediments (Graca et al., 2017; Zheng et al., 2019), the predominant polymer collected in this study was PET, being mostly assigned to fibers and fiber bundles. Besides being widely known to result from textile laundry (Mark Anthony Browne et al., 2011; Napper & Thompson, 2016), they might also result from the degradation of fishing gear and maritime equipment (Cole, 2016; Murray & Cowie, 2011; OSPAR, 2020). The second most abundant polymer in our sediments was PE, which is in accordance with other studies (Coppock et al., 2021; Zheng et al., 2019) and is explained by its multiple applicability, high demand (PlasticsEurope, 2021) and fast discard (mainly packaging of consumer goods and single-use items). As mentioned before, despite these items tendency to float, biofouling processes will ultimately cause them to sink.

Mineralogical content

Lastly, in addition to the different organic matter contents and granulometric parameters found in sediments of each station, they also present distinct mineral composition which strongly mirrors the sediment sources occurring in the nearby shore. We could assume a weak transport of sediments along the shoreline or, at least, a strong influence of local sediment sources in the samples' mineral composition. In fact, the higher proportion of carbonates in st4 and st6 coincided with their proximity to the Arrábida carbonated cliffs, while the higher siliciclastic (quartz) proportions found in the other stations are suggested to come from the adjacent sandy beaches (Figueirinha, st2, Portinho da Arrábida, st3, and Califórnia, st5) or from the Sado river (st1). The reduced transit of sediments between stations (throughout the 6 sampling campaigns) agrees with the reduced exportation of MPs from the estuary to the western marine coast, which contributes to the accumulation of MPs inside the estuary, i.e., in the vicinity of their emission sources.

Conclusion

The temporal fluctuation of MPs abundance in subtidal sediments at the Portuguese west coast, between late summer and winter (Aug18 and Feb19), was not significant and revealed no relationship with rainfall. However, a clear spatial distribution pattern was observed: MPs accumulation was high in the estuary (hotspot; mostly comprised by fragments) and low in all the other five stations (mostly represented by fibers). The high abundance of MPs in the estuary was moderately correlated with its poorly sorted sediments and with the high organic matter content. Also, besides possessing the higher silt/clay fraction, the size range of the majority of MPs inside the estuary was coincident with sediments most abundant grain size fraction: 0.250-0.500 mm.

The residual exportation of MP from the estuary is supported by the distinct mineralogical content of each station, which indicates a reduced transit of sediment along the coast. Conversely, the similar abundance of fibers among the 6 stations is potentially linked to the higher surface area to volume ratio of this type of MPs and to the location a WWTP outfall at each extremity of the study area. The MPs to meiofauna ratio was particularly higher in the estuary (1:15.3) suggesting a higher exposure level faced by biota. As expected, most polymers found in sediment were denser than seawater and may be linked to local activities.

Understanding patterns and identifying environmental factors capable of interfering with MPs accumulation in sediments are critical for the establishment of effective measures that aim to reduce and prevent plastic inputs to the marine environment. In marine protected areas, such information is especially important not only to evaluate their effectiveness in what concerns the protection of species and habitats from MPs pollution (Blašković et al., 2017; Fastelli et al., 2016), but also to adjust their management and/or monitoring plans accordingly. Therefore, continued research and local dissemination at awareness campaigns are necessary to engage citizens and stakeholders to tackle MPs pollution in this Portuguese coastal region.

Author Contributions

DR conducted fieldwork sampling, laboratory procedures (MPs extraction and characterization, sediment characterization), statistical analysis, and wrote the manuscript. JA performed FTIR analysis, collaborated in the discussion and selection of the best method for MPs extraction from sediment samples, provided assistance with laboratory procedures and at reporting FTIR analysis. JPa performed micro - FTIR analysis, assisted in the interpretation of spectra, and at reporting FTIR analysis and results. JPe compiled the rainfall data and gave important contributions to improve the manuscript. PSC provided assistance with sedimentological laboratory procedures and gave important contributions to improve the

manuscript, namely about granulometry analysis, mineralogical content interpretations and XRD technique. FR conducted the XRD technique and assisted with mineralogical content interpretations. PS coordinated the study, discussed results, gave important contributions to the writing and to the English review of the text. MHC reviewed and made important contributions to the text.

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4

IS THE BOGUE *BOOPS BOOPS* (LINNAEUS, 1758) A GOOD INDICATOR OF MICROPLASTICS POLLUTION IN PORTUGUESE COASTAL WATERS?

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Abstract

Understanding the exposure of wild marine fish to microplastics (MPs) and assessing their potential as bioindicators of MPs pollution became an important step for monitoring purposes, being though seldomly validated. Here, further than investigating temporal patterns of MPs ingestion by *Boops boops* (Linnaeus, 1758) caught in coastal waters between Setúbal and Sesimbra, Portugal, we also aimed at comparing them with the described patterns of MPs occurring in the same region and period (October 2018 to January 2019), at both surface water and seabed sediments (this species feeding grounds). Fibers

accounted for 93% of all MPs ingested and both the abundance of MPs per fish gastrointestinal tract (1.80 ± 1.26; mean ± SD) and food intake significantly decreased during this 4 month-period. Such patterns, potentially related to this species reproduction season, have only marginally matched those found in its feeding areas (namely in sediments). Further studies should occur in spring/summer months.

Keywords: pollution; exposure; marine fish; temporal patterns, fibers, gastrointestinal tract

Introduction

Quantifying and categorizing microplastics (hereafter MPs) in the marine environment have been the main goals of countless scientific studies since the beginning of the 21st century. Either to provide baseline data or for monitoring purposes, such estimations were and are critical to understand the exposure levels faced by marine biota and to contribute to human health risk assessment. Moreover, they are also important to support the establishment of preventive measures or to evaluate their efficiency. Simultaneously, the assessment of whether certain species may work as good bioindicators of MPs pollution in specific marine compartments has been increasing (Fossi et al., 2018). In fact, other than informing about the pollution level at the species environment, bioindicators may also reveal disturbance and allow the assessment of detrimental effects occurring in the organisms (Fossi et al., 2018; Rochman et al., 2013). Such research relies on the marine organisms ability to ingest these small sized plastic particles (smaller than 5 mm (Arthur et al., 2009)), which may occur intentionally, when MPs resemble their natural preys (Ory et al., 2017; Shaw & Day, 1994), accidentally, when there is a passive intake of MPs during foraging activities, or by trophic transfer, when predators feed on prey that already carry MPs (Fossi et al., 2018; Nelms et al., 2018; Neves et al., 2015; Romeo et al., 2015; Wright et al., 2013).

Regardless of how MPs are ingested, since they occur in multiple and irregular shapes (with sharp and pointy edges, for example) and possess a strong affinity with chemicals, they may cause either physical harm and/or produce toxicological responses (Rochman et al., 2013; Wright et al., 2013), depending on the organisms contaminant body burden (Albert Aart Koelmans et al., 2016). Such chemicals, adsorbed to plastic surfaces, consist of highly hydrophobic persistent and bioaccumulative organic pollutants available in seawater; and of toxic additives (plasticizers, flame retardants, antimicrobials, dyes or UV-stabilizers) incorporated during plastics manufacture (Teuten et al., 2009). According to Koelmans et al. (2014, 2016), the contribution of MPs ingestion as a contaminant pathway might be residual in comparison to others, such as prey ingestion or dermal uptake, that occur more regularly. Nevertheless, such contribution will depend on the amount and frequency of MPs ingestion, which is reported to vary between species and throughout development. Additionally, it will also depend on the pollution levels occurring at their feeding grounds, namely the concentration of chemicals, MPs

abundance and polymers diversity, as the chemical affinity of MPs will depend on its polymeric composition (Teuten et al., 2009; Lee et al., 2014; Hummel et al., 2021).

Addressing this uncertainty is critical, since an increase of contaminants accumulation in the tissues, which may occur by trophic transfer across the marine food chain (bottom-up cascade effects; bioamplification), may cause detrimental impacts on top predators (Nelms et al., 2018) and eventually on humans (Galloway, 2015; Thompson et al., 2009; Vethaak & Leslie, 2016; Wright & Kelly, 2017). Such human health concerns, especially relevant in Portugal where seafood is a major diet component (FAO, 2022), could be addressed through the regular monitoring of MPs ingestion by marine organisms, such as wild fish, which end up proving to be suitable bioindicators of MPs pollution of their feeding habitats. Since these studies are lacking at Portuguese coastal waters, here we intend to characterize and quantify the MPs ingested by a fish species and to evaluate if they mirror the pool of MPs available in this species feeding habitats. This will be performed by comparing the temporal patterns observed in MPs extracted from fish gastrointestinal tract (GIT), with those detected in MPs extracted from environmental matrices collected at the same study area: surface water (D. Rodrigues et al., 2020) and subtidal sediments (D. Rodrigues et al., 2022).

The selection of the bogue *Boops boops* (Linnaeus, 1758) was not only based on the criteria described by Fossi et al. (2018) and Bray et al. (2019), but also on the findings of several studies reporting this species potential to be used as a bioindicator in the Mediterranean (Garcia-Garin et al., 2019; Nadal, Alomar, & Deudero, 2016; Rios-Fuster, Alomar, Compa, Guijarro, & Deudero, 2019; Sbrana et al., 2020; Tsangaris et al., 2020). Despite the low commercial value of the bogue in Portugal (Docapesca, n.d.; Monteiro et al., 2006), it consists of prey to several commercial fish species (Romeo, Consoli, Castriota, & Andaloro, 2009; Stergiou & Karpouzi, 2002), is used as bait in tuna fishery in Azores (Cruz, Machete, Menezes, Rogan, & Silva, 2018; Cruz, Menezes, Machete, & Silva, 2016) and Madeira (Romero et al., 2021) and is one of the most abundant Sparidae species in Portuguese coastal waters (Gordo, 1995), with considerable reported landings (625 tons in 2018; FAO, 2020). Such features, along with the easy access to samples for monitoring studies, also consist of relevant aspects to evaluate in a bioindicator candidate. Other than that, this benthopelagic fish, mainly marine (Prista, Vasconcelos, Costa, & Cabral, 2003), occurs in inshore waters (up to 300 m in the Atlantic) and may be found in different bottom types, namely sand, mud, rocks, and seagrass beds (Bauchot & Hureau, 1986).

However, despite *B. boops* considerable vagility (Bray et al., 2019; Harmelin, 1987), it is plausible to consider MPs found in their GIT as particles ingested close to the area where the fishes were caught. According to Grigorakis et al., (2017), MPs found in the fish gut may be assumed as recently ingested items, instead of representing an accumulative retention from several meals. Also, the small gut length of *B. boops*, categorized by Bray et al., (2019), may potentially contribute to a short digestion period. This fish species is considered an omnivore, varying between planktivory (Anato & Ktari, 1983a;

Lopes, Raimundo, Caetano, & Garrido, 2020) and herbivory (Bell & Harmelin-Vivien, 1983; Linde, Palmer, & Gómez-Zurita, 2004; Nadal et al., 2016; Ruitton, Verlaque, & Boudouresque, 2005; Stergiou & Karpouzi, 2002); and is known to ingest both benthic and pelagic preys (Anato & Ktari, 1983a; Bauchot & Hureau, 1986; Bell & Harmelin-Vivien, 1983; Derbal & Kara, 2008; Karpouzi & Stergiou, 2003; Stergiou & Karpouzi, 2002). Considering that *B. boops* feeds on both seawater surface and seabed sediments, we aimed to investigate if the MPs ingested would mirror the patterns of MPs available in this species feeding grounds. Owing to the concurrent collection of samples, from the same study site, a thorough comparison between fish and environmental data was conducted to assess if the bogue consists of a suitable MPs pollution indicator in the North-Eastern Atlantic.

Materials and Methods

Study area and sampling

The collection of zooplankton and subtidal sediments took place in the Sado river estuary and Professor Luiz Saldanha Marine Park (Figure 4.1), located at the Portuguese west coast. The laboratory processing of samples and aliquots, from both matrix types, aiming at MPs extraction and calculation of zooplankton and meiofauna abundances, are described in detail by (D. Rodrigues et al., 2020, 2022). The sampling of environmental matrices was conducted in six campaigns, from August 2018 to February 2019 (late summer to winter) and, whenever possible, fish samples caught by local fishermen with purse seines were concurrently supplied (50 individuals, per month) at Sesimbra fish first sale (Docapesca; Figure 4.1) by a local fish trader. All samples were transported in ice coolers and kept frozen at -20 °C until analysis.

Ultimately, the intended comparison between fish and environmental samples was only viable for 4 campaigns: from Oct18 to Jan19. While in Aug18 the insufficient specimens supplied (only 26 instead of 50) were exclusively used to test and adjust the MPs extraction protocol, at the time of the Feb19 campaign there were no bogue specimens available. Therefore, the data collected in Aug18 and Feb19 from environmental samples were not considered in this study. Also, considering that *B. boops* is a marine species, the MPs extracted from GIT samples were exclusively compared with MPs collected from ocean exposed stations (from st2 to st6, i.e., excluding the estuary; st1). Lastly, an extra set of specimens (50) supplied in March 2019 by the local fish trader when *B. boops* was finally available, was also analyzed with the unique purpose of extending the evaluation of MPs ingestion beyond the time frame considered for comparison (Oct18 to Jan19).

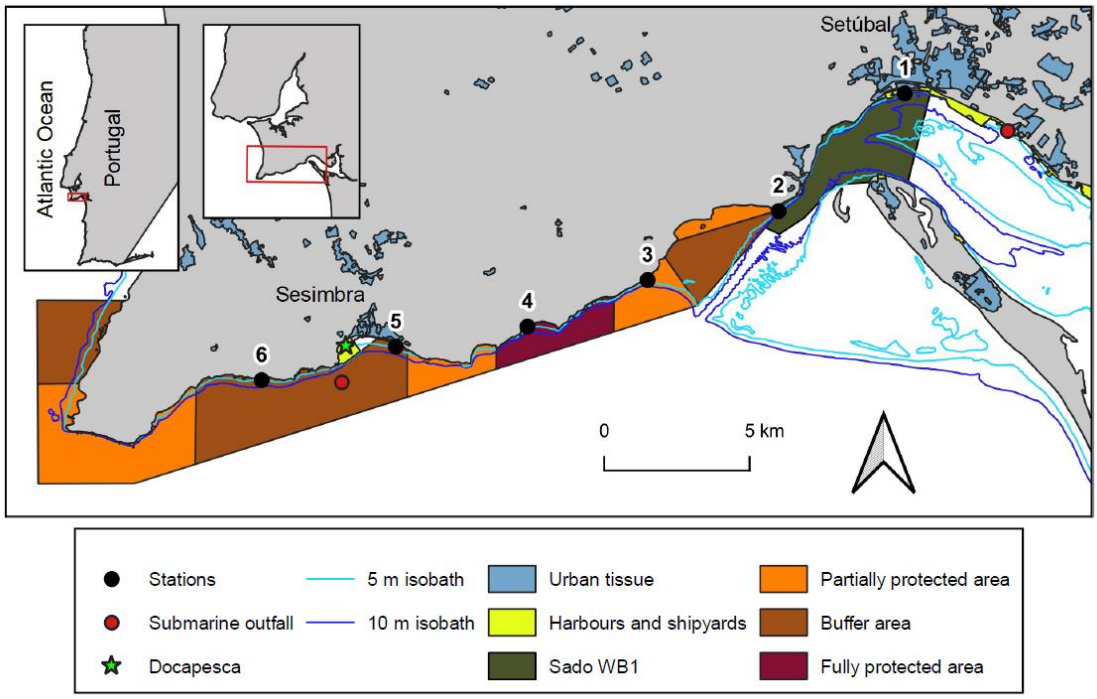

Figure 4.1- Map of the study area with the location of stations where both water and sediment samples were collected (black dots) and with the location of Docapesca (green star) where fish samples were supplied. Map developed in QGIS and adapted from Rodrigues et al. (2020) where the complete information about the map layers, as well as the list with the GPS coordinates of each station, are provided.

In the laboratory, once thawed at room temperature, fish were washed with filtered Milli-Q water (to prevent contamination of airborne fibers), measured (standard length, SL), and weighed (BW), to the nearest 0.1 cm and 0.01 g, respectively. Fish dissection followed, and the gastrointestinal tract (GIT) was removed and weighed (GITW). In addition, from each fish (replicate), a sample of muscle and liver was also collected, labelled and frozen at -20°C in aluminum foil to allow posterior analysis (which were not included in this study). A potassium hydroxide solution (KOH 10%) was used to digest the GIT and its content (Foekema et al., 2013) inside a glass jar covered by a glass Petri dish. The volume of KOH 10% was 3 times the amount of biological material to be digested. After 72 hours of digestion, each sample was sieved through a 38 µm metal mesh and transferred to a 100 ml beaker with distilled water. The beaker content was manually stirred and then, except for GIT deposits and saponified portions, it was filtered out through a vacuum filtration apparatus system onto glass microfiber filters (~1 µm pore size). To increase the extraction potential of MPs in samples, GIT deposits and saponified portions followed a second period of digestion (48 hours), being then submitted to separation by density with a high-density sodium chloride solution (1.2 g cm^{-3}). After stirring, samples were left to settle for 1 hour. Then, the supernatant was filtered, and the remaining deposits were discarded.

Filter analysis followed, allowing the categorization of potential MPs in 4 different types (according to Rodrigues et al. (2020 and 2022); Table 4.1) and 5 size classes (0.038-1, 1-2, 2-3, 3-4, and

4-5 mm). Frequency of MPs occurrence (percentage of fish that had MPs in their GIT) and Fulton condition factor (K; as 100 * (fish weight/fish length^3), Froese (2006)) were calculated. MPs abundance, reported as items per fish, was calculated by considering all fish and, separately, only the fish which contained at least 1 MPs.

Table 4.1 – Definition of each particle type.

Type	**Definition**
Fragment	Hard or soft irregular particle
Film	Thin and malleable, flimsy particle
Fiber	Thin line, equally thick throughout its entire length, frequently curled
Fiber bundle	Several fibers tightly wound together in a knot-like formation

Polymer identification

Particles selected for polymer identification, based on the best expert judgment according to similarity, texture, thickness, and shine, were isolated in covered concave slides. Polymer identification was performed in 9% (17 items) of the total of particles, after discarding fibers considered airborne contamination. Analyses were carried out on a μ-FTIR spectrometer (Perkin Elmer® Spotlight 200i Microscope System), with a microscope aperture of 100 × 100 μm, using a strong Norton-Beer apodization. All spectra were acquired at room temperature under reflectance mode with a resolution of 4 cm^{-1} and 1 cm^{-1} wavenumber intervals, within 4000–500 cm^{-1}. The analysis was performed on the sample surface, sometimes at more than one point, when results were dubious. A background scan was performed before any analysis series. Polymer identification relied on a match over 80% (Pequeno et al., 2021) between the sample and a referenced database (Primpke et al., 2018), and the assignments were confirmed by the analysis of the polymer characteristic bands (Jung et al., 2018; Xiao et al., 2002).

Quality Assurance and Quality Control

To assess airborne contamination in fish samples, control filters (blanks) were exposed to the air throughout lab work (inside Petri dishes, one at the left and one at the right of the working area, per group of 10 fish specimens). Details about preventive measures taken with seawater and sediment samples are described in Rodrigues et al. (2020) and Rodrigues et al. (2022), respectively. All the fibers extracted from samples which were similar to those found in blanks were excluded from the results. Other contamination sources were minimized during laboratory work by using glass and stainless-steel materials. GIT samples were kept covered at all times, a cotton lab coat and nitrile gloves were always

worn and working surfaces were rinsed before use with Milli-Q water and ethanol. Moreover, all prepared solutions and rinsing liquids were filtered before use.

Data analysis

The exploratory data analysis about the most abundant type, size class, color, and polymer, among MPs extracted from fish GIT, have only considered samples collected between Oct18 and Jan19, though data collected from Mar19 has been also depicted in figures. Patterns of MPs ingested by fish were compared with those found in MPs extracted from the environmental matrices collected in the same 4 months. To assess if the abundance of MPs ingested by *B. boops* varied between sampling campaigns (from Oct18 to Jan19), a Kruskal-Wallis test was applied, since parametric assumptions were not met. Post-hoc multiple comparisons were conducted with Dunn's test. Lastly, to understand if fish weight, standard length, GIT weight and Fulton index could explain the temporal patterns found in the abundance of MPs per fish, Kruskal-Wallis tests were conducted and followed by Dunn's test.

The environmental data considered for comparison is part of the dataset collected in the scope of two previous studies focused on distribution patterns of MPs in the water surface (D. Rodrigues et al., 2020) and seabed sediments (D. Rodrigues et al., 2022). Instead of the whole dataset (comprised by 6 monthly campaigns and 6 stations), this study has only considered data from 4 campaigns (between Oct18 and Jan19) and from ocean-exposed stations, i.e., excluding the estuary station, since *B. boops* is a marine species. Thus, new analyses (one-way ANOVA) were conducted with the subset data to assess temporal variations (among 4 campaigns) in MPs abundance (dependent variable) at both environmental matrices (seawater surface and seabed sediments). This parametric test was conducted after transforming the original data as follows: Box-Cox (surface seawater) and log(x+1) (seabed sediments), to meet normality (Shapiro-Wilk test) and homogeneity of variances (Levene test) assumptions. Post-hoc Tukey test was used to identify the sources of significant differences ($p < 0.05$) at the seawater surface. The analysis of variance on zooplankton and meiofauna abundances among campaigns (Oct18 to Jan19) was also assessed by a one-way ANOVA, with log (x+1) transformed data, and followed by Tukey post-hoc test for pairwise comparisons. Since, originally, the assignment of MPs extracted from sediments was made according to granulometric fractions, here they were re-assigned to the size range classes established for MPs extracted from seawater surface and fish, so all items could be comparable.

All the previously mentioned tests were performed with TIBCO Statistica™ 14.0.0 software and the level of significance was set at a p-value ≤ 0.05.

Results

Out of the 251 particles extracted from the 200 specimens (collected from Oct18 to Jan19), 56 (22%) were discarded for being considered airborne contamination fibers, and 11 were excluded due to one of the following µ-FTIR results: non-plastic particles, as cellulose, inconclusive match, or match under 80%. Therefore, the assessment of temporal patterns observed in MPs ingested by the bogue was based on a total of 184 particles (size range 44.48 – 4967.98 µm). From this amount, 93% were fibers (Figure 4.2A) and 58% consisted of particles with less than 1 mm (Figure 4.2B). Among fibers, the predominant color was blue (72%;Figure 4.3). The abundance of MPs per fish in Jan19 was significantly lower than in both Oct18 (Kruskal-Wallis H test, $p < 0.001$; Dunn's $p < 0.001$;Figure 4.4) and Nov18 (Kruskal-Wallis H test, $p < 0.001$; Dunn's $p < 0.05$). Data collected in Mar19 shows an increase in MPs ingestion, particularly on the fragment type (Figure 4.2A).

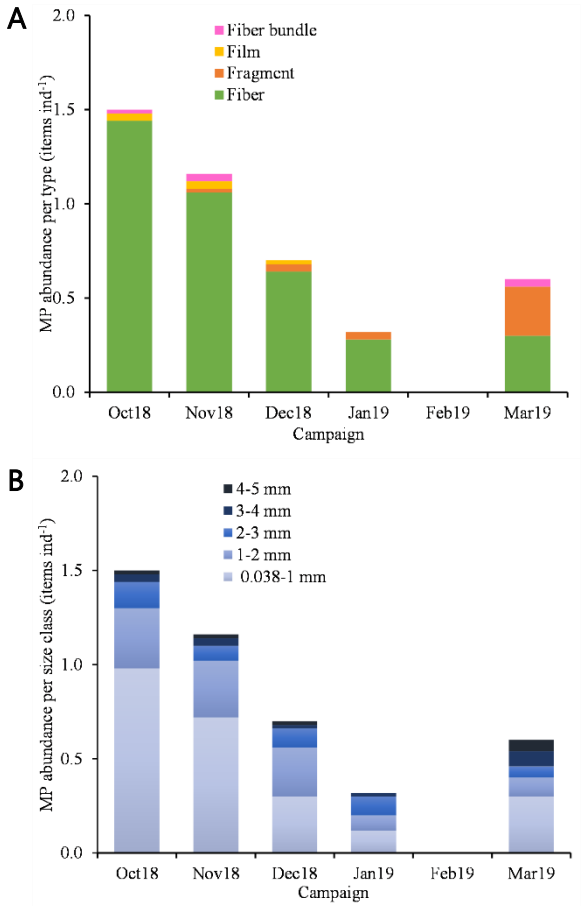

Figure 4.2 - Temporal variation of MPs abundance at fish GIT (mean items ind^{-1}) per particle type (A) and per size class (B).

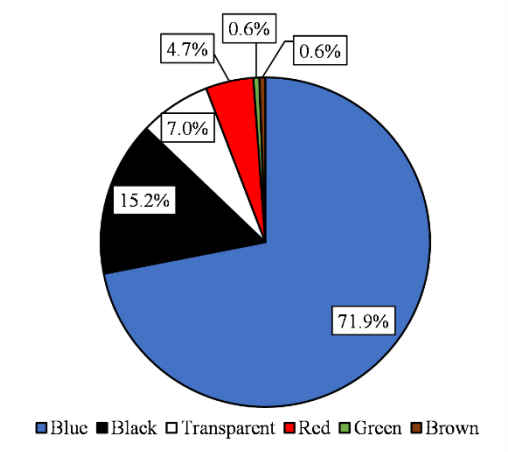

Figure 4.3 – Color composition of fibers ingested by *B. boops* between Oct18 and Jan19.

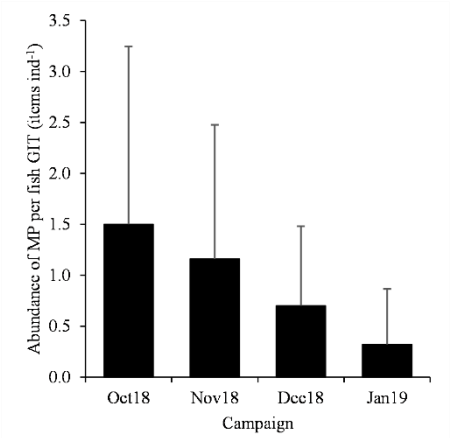

Figure 4.4 – MPs abundance in fish GIT between Oct18 and Jan 19 (mean ± SD; items ind^{-1}).

Among the 17 items analyzed by FTIR, 6 were confirmed as plastic, based on a match over 80% (Pequeno et al., 2021). A total of 4 polymers (Figure 4.5) were identified: Polyethylene Terephthalate (PET; 3 items), Low-Density Polyethylene (LDPE; 1 item), Nylon (1 item), and Polyacrylate (1 item).

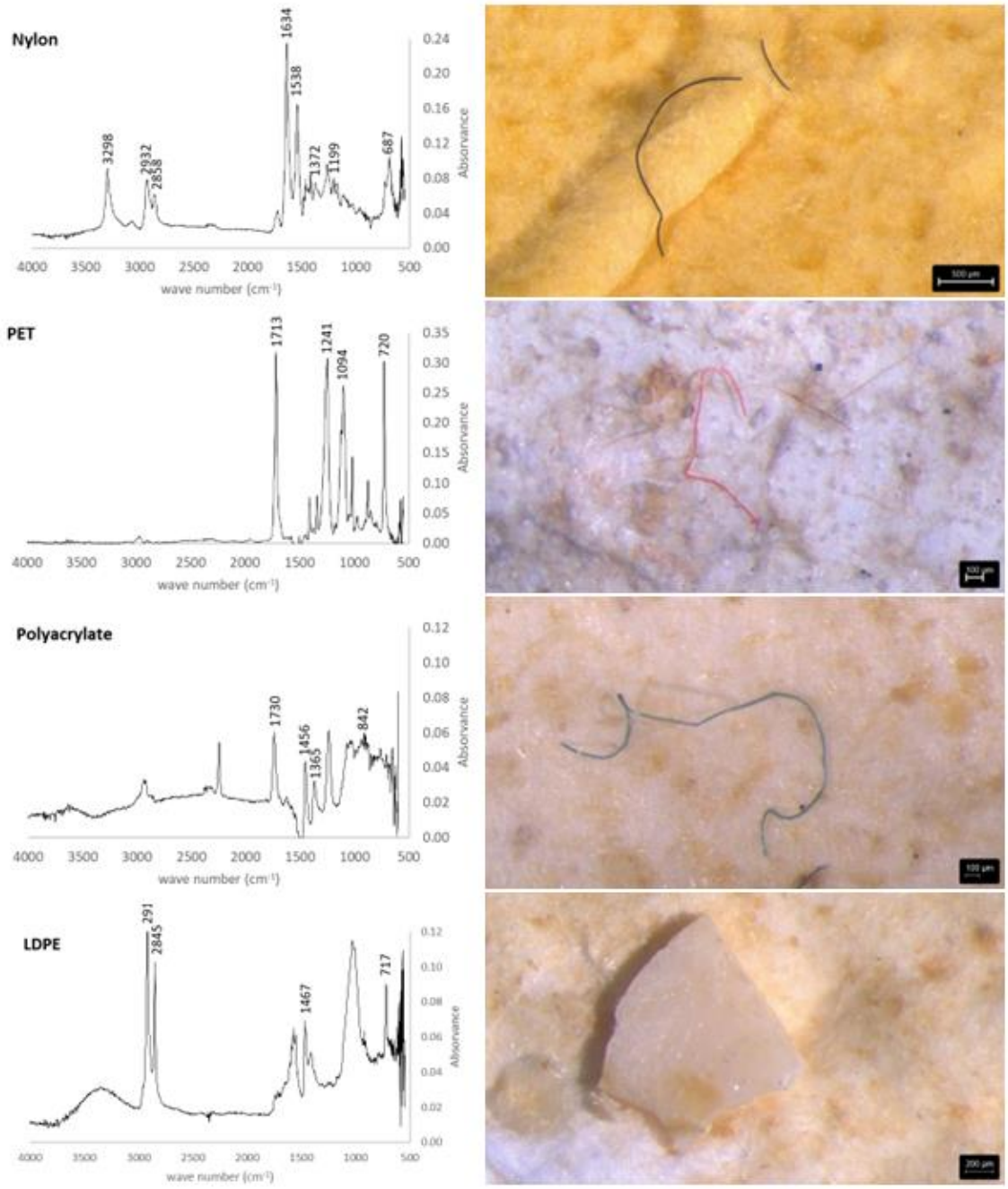

Figure 4.5 - Representative infrared spectra of the 4 identified polymers: Polyethylene Terephthalate (PET), Low-Density Polyethylene (LDPE), Nylon and Polyacrylate. The image assigned to each spectrum corresponds to the MPs analyzed.

Out of the 200 fish, 102 had at least 1 MP in the GIT, corresponding to a frequency of occurrence of 51% (FO%; Figure 4.6). The average of MPs per fish (considering only the fish with MPs; n=102), was 1.80 ± 1.26 (mean ± SD), being the highest registered in Oct18 with 2.27 ± 1.69 MPs per fish, and the lowest in Jan19 with 1.14 ± 0.35 MPs per fish (Table 4.2). Among the fish with MPs (102), 43 had 2 or more MPs in their GIT (22% of the 200 fish), being a maximum of 8 MPs registered in a single specimen collected in the Oct18 campaign. The average of MPs per fish (considering fish without MPs

as well; n=200) was 0.92 ± 1.27 (mean ± SD) and, as observed before, the campaign with the highest MPs abundance was Oct18 (1.50 ± 1.75), whereas Jan19 had the lowest (0.32 ± 0.55).

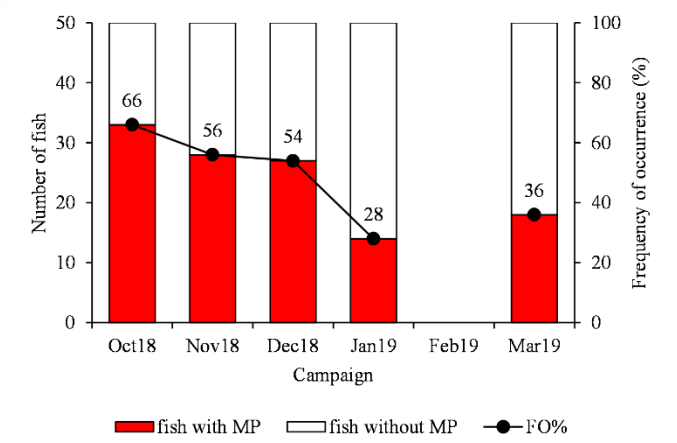

Figure 4.6 – Number of fish with (red bar) and without (white bar) MPs in their GIT, per campaign. Temporal variation of the frequency of occurrence of ingested MPs (black dots).

Table 4.2 – Number of fish (N), number of fish with MPs, frequency of occurrence (FO%), average of MPs per fish considering all the fish collected (MPs/fish (all)), average of MPs per fish considering only the fish with MPs (MPs/fish (with MPs)), number of fish with more than 2 MPs (Fish with ≥ 2 MPs), and maximum MPs per fish, per campaign (Camp).

Camp	N	Fish with MPs	FO%	MPs/fish (all); (average ± sd)	MPs/fish (with MPs); (average ± sd)	Fish with ≥ 2 MPs	Max MPs/fish
Oct18	50	33	66	1.5 (1.75)	2.27 (1.69)	16	8
Nov18	50	28	56	1.16 (1.32)	2.07 (1.1)	19	6
Dec18	50	27	54	0.7 (0.78)	1.3 (0.6)	6	3
Jan19	50	14	28	0.32 (0.55)	1.14 (0.35)	2	2
Total	**200**	**102**	**51**	**0.92 (1.27)**	**1.80 (1.26)**	**43**	
Mar19*	50	18	36	0.6 (1.47)	1.67 (2.05)	4	10

The 200 specimens collected between Oct18 and Jan19, ranged between 72.58 - 297.43 g of body weight, 2.89 - 19.03 g in GIT weight, and 16.1 - 26.2 cm in standard length (mean ± SD are indicated in Table 4.3). Fish collected in Oct18 had significantly higher body weight (Kruskal-Wallis H test, $p < 0.001$; Dunn's $p < 0.001$), GIT weight (Kruskal-Wallis H test, $p < 0.001$; Dunn's $p < 0.001$), standard length (Kruskal-Wallis H test, $p < 0.001$; Dunn's $p < 0.001$) and Fulton condition factor (Kruskal-Wallis H test, $p < 0.05$; Dunn's $p < 0.05$) than fish from the other 3 campaigns. In comparison to Jan19 (the end of the 4-month period), the fish collected in Mar19 show a higher FO and a higher MPs abundance (Figure 4.6 and Table 4.2), and a slightly lower body weight, GIT weight, and Fulton condition factor (Table 4.3).

Table 4.3 – Number of fish (N), average with standard deviation in parenthesis of body weight (BW), GIT weight (GITW), standard length (SL), and Fulton condition factor (K), per campaign.

Campaign	**N**	**BW**	**GITW**	**SL**	**K**
Oct18	50	167.23 (38.96)	9.58 (3.65)	20.91 (1.31)	1.8 (0.16)
Nov18	50	120.81 (13.82)	6.04 (1.4)	19.24 (0.81)	1.7 (0.12)
Dec18	50	111.02 (15.14)	5.34 (1.1)	18.64 (0.9)	1.71 (0.11)
Jan19	50	118.39 (25.31)	5.29 (2.01)	19.05 (1.39)	1.69 (0.13)
Total	**200**	**126.99 (32.73)**	**6.29 (2.72)**	**19.39 (1.42)**	**1.71 (0.14)**
Mar19	50	117.51 (26.48)	5.19 (1.58)	19.1 (1.39)	1.66 (0.11)

Regarding MPs extracted from environmental samples, whereas temporal fluctuations were observed in the abundance of floating MPs, with a significant increased from Oct18 to Jan19 campaign ($F(3,16) = 4.93$, $p < 0.05$, Tukey test, $p < 0.05$); Figure 4.7 A and B), no significant variations were observed in sediments ($F(3,16) = 1.45$, $p = 0.266$; Figure 4.7 C and D). Among the floating MPs, fragments were consistently the predominant type (Figure 4.7A) and the majority of items belonged to the 1-2 and 2-3 mm size classes (Figure 4.7B). The prevalent type of MPs found in these marine coastal sediments were fibers (Figure 4.7C). MPs assigned to the smallest size class (0.063 – 1 mm) were the most abundant in sediments (Figure 4.7D).

Whereas significant fluctuations were observed in zooplankton abundance between Oct18 and Jan19 ($F(3,16) = 20.32$, $p = 0.000$), being the lowest registered in Dec18 (Tukey test, $p < 0.05$; Figure 4.8A), meiofauna abundances remained similar throughout all campaigns ($F(3,16) = 2.98$, $p = 0.063$; Figure 4.8B).

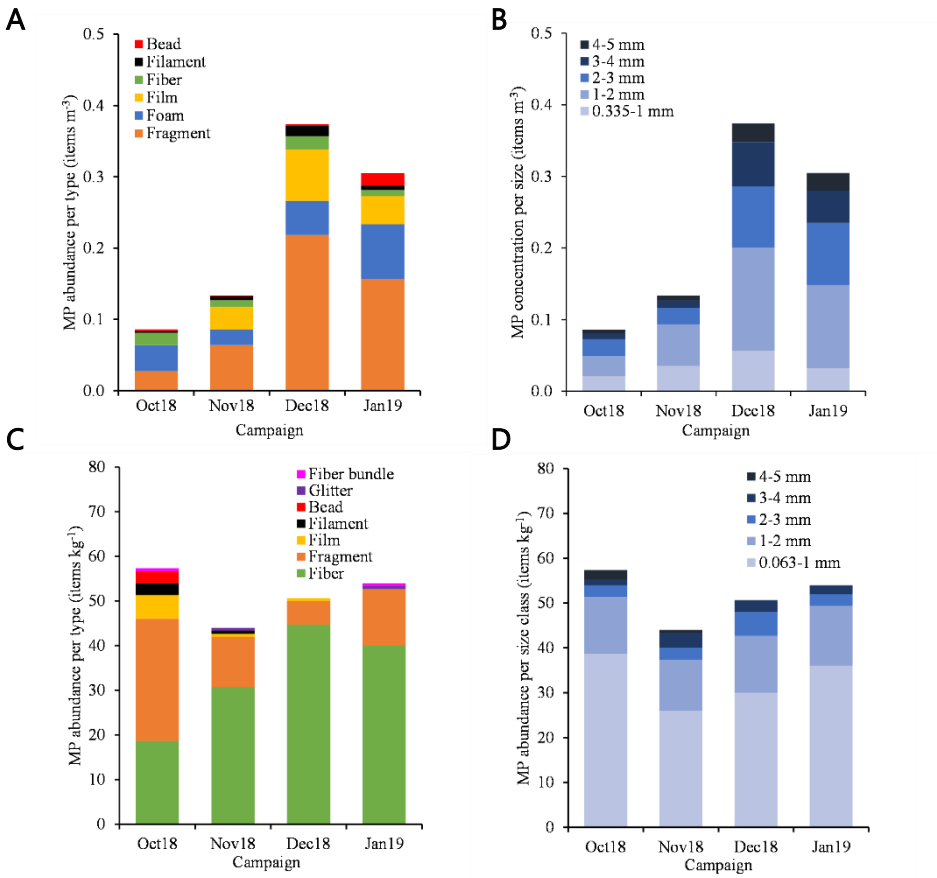

Figure 4.7 – Temporal variation of MPs abundance at seawater surface (mean items m^{-3}) per particle type (A) and per size class (B); at seabed sediments (mean items kg^{-1}) per particle type (C) and per size class (D). Panels A and B adapted from Rodrigues et al. (2020). Panels C and D adapted from Rodrigues et al. (2022).

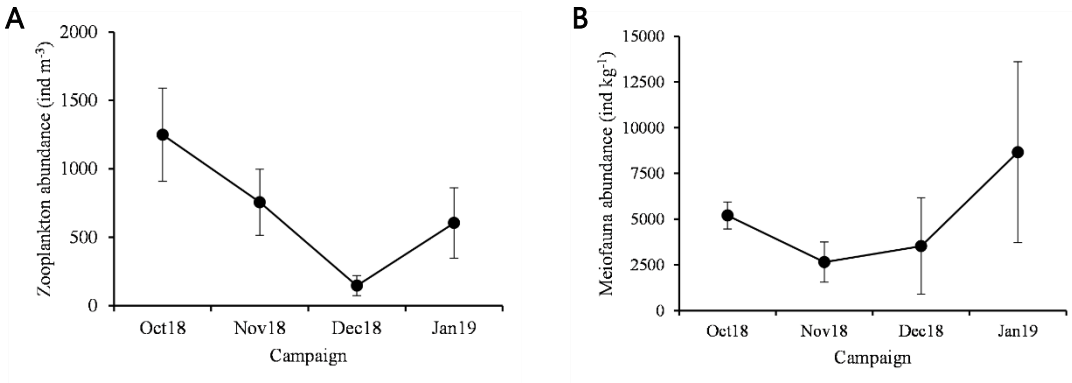

Figure 4.8 – Temporal variation (mean ± SD) of the zooplankton (A) and meiofauna (B) abundances.

Discussion

The temporal patterns observed in the abundance of MPs occurring in *Boops boops* gastrointestinal tracts marginally matched those found in MPs collected concurrently from one of this species feeding grounds: the seabed sediments (D. Rodrigues et al., 2022). The lack of a stronger relation suggests that, during the autumn/winter seasons, the bogue should not be considered a robust bioindicator of MPs pollution in this Portuguese coastal area. Such outcome largely differs from several studies reporting this species potential to be used as a bioindicator of plastic pollution in the Mediterranean (Garcia-Garin et al., 2019; Nadal et al., 2016; Rios-Fuster et al., 2019; Sbrana et al., 2020; Tsangaris et al., 2020), where this species has a high commercial value (Bray et al., 2019) and high reported landings (19 711 tons in 2018, FAO, 2020).

However, it should be highlighted that most of these studies took place during spring. Therefore, the contrasting results could be assumed to be largely related to the sampling periods (different seasons) discrepancy. Regarding the study of Sbrana et al. (2020), despite sharing a similar sampling period with ours (autumn/winter), their findings were based on a comparison between specimens collected from 3 different areas (ranked according to their anthropogenic pressures), while here the samples source was always the same study area, from which environmental data on MPs abundance was in fact available.

Other than the season discrepancy, the distinct levels of MPs pollution occurring in these regions could be also interfering. Probably, *B. boops* occurring in the Mediterranean are exposed to higher abundances of MPs than those in the Atlantic Northeast due to the hydrodynamics of such a semi-enclosed basin (Cózar et al., 2015). While we report a MPs abundance per fish (all fish considered) of 0.92 ± 1.27 MPs per fish, in the study of Sbrana et al. (2020), conducted at the same time frame, it doubles: 1.8 ± 0.2 MPs per fish. Further studies are thus critical to understand if the adequate use of this species as a bioindicator is restricted to the Mediterranean or to specific seasons.

One of the most relevant criteria considered for the selection of *B. boops* in this study was its feeding habits. Since it is known to ingest both pelagic and benthic prey, this species is both exposed to floating and settled MPs. Hence, our aim was to investigate if fluctuations of MPs abundance in wild fish GIT samples would mirror the patterns occurring, at the same period and same study area, in MPs abundance at the seawater surface and/or at the seabed sediments. Yet, patterns did not match. While the ingestion of MPs decreased continuously between Oct18 and Jan19, the inverse pattern occurred in MPs abundance on the seawater surface. On the other hand, although MPs abundances in seabed sediments have also decreased after Oct18, this tendency was weak and not significant.

The absence of evident temporal coincident patterns in our study may be explained by the typical decrease in food intake by fish species when seawater temperature decreases in winter (Miegel, Pain, van Wettere, Howarth, & Stone, 2010; Temming & Herrmann, 2001; Tirsgaard, Svendsen, &

Steffensen, 2015; Volkoff & Rønnestad, 2020). Though it is reported to occur along with a slower evacuation and intestinal transit (Miegel et al., 2010), if food ingestion decreases significantly, the intake of MPs (the passive intake, in particular) will follow. This assumption is supported by the observed significant and continuous decrease of MPs ingestion and GIT weights during the study period, reinforcing the hypothesis that lower water temperatures may alter the propensity and frequency of MPs ingestion, by interfering with fish biological traits.

Since fluctuations in meiofauna abundance were residual, suggesting a similar prey availability in sediments over time, if the decrease in food intake is associated with a lower prey availability, this could suggest a preference from the bogue to feed on zooplankton, which is typically less abundant at that time of the year (M. E. Cunha, 1993). In fact, although our data show a significant increase after Dec18, zooplankton abundances were still low compared with levels known to be usually attained in spring (M. E. Cunha, 1993). Yet, considering the size, type and polymer characteristics of MPs ingested by this omnivore species, which share more similarities with MPs found in sediments, the most plausible explanation is the water temperature decrease.

The particularly low abundance of MPs in fish collected during the 4 months, could be also linked to the allocation of energy from feeding activities into the development of the reproductive system (Özyurt, Mavruk, & Kiyağa, 2012; Vahabnezhad, Kaymaram, Taghavi, Valinassab, & Fatemi, 2016; Villegas-Ríos, Alonso-Fernández, Domínguez-Petit, & Saborido-Rey, 2014; Volkoff & Rønnestad, 2020). Indeed, fish sampling did overlap with *B. boops* reproduction and spawning season, which is known to occur between late winter and spring on the Atlantic North-Eastern coast (Anato & Ktari, 1983b; Gordo, 1995; Monteiro et al., 2006) and, according to our data, the length of all the specimens was above the minimum reproduction length reported for this species (Gordo, 1995; Monteiro et al., 2006). This explanation is supported by the higher Fulton condition factor values observed in our study, with fish collected before or at the beginning of the reproduction season, when compared with the lower values reported by Garcia-Garin et al. (2019), for specimens collected during spring, possibly after spawning. Such seasonal variation in the condition factor was also registered by Dobroslavić et al. (2017).

Overall, despite the weak temporal match, there was a consistent pattern observed in all the 5 sets of specimens analyzed, which should be highlighted. Amongst the different types of MPs known to occur locally in this species feeding grounds (fragment, foam, film, fiber, filament, bead, fiber bundle and glitter), fibers were the main type of MPs ingested by *B. boops*. This could be the result of an active ingestion of fibers, by mistaking them as prey, which would be explained by the use of visual cues while foraging, typically used by planktivorous fish (Roch et al., (2020). Yet, since this species is reported to capture their prey by suction-feeding (Linde et al., 2004; Olmo, M. Fernández, Kostadinova, & Poulin, 2008), which is a non-selective mode (Olmo et al., 2008), the prevalence of fibers could be related

instead to this species capability of distinguishing non-edible particles (which is usually a characteristic attributed to chemosensory forager fish that feeds on benthic fauna (Roch et al., 2020)). Once ingested, fibers may remain unnoticed and follow their transit towards the GIT, while the other types of MPs, if ingested, would be potentially rejected due to a more evident texture and shape.

It was not possible to discriminate if fibers ingestion occurred via trophic transfer since the GIT content was directly digested with KOH 10% without a prior inspection and individualization of prey. Another possible route, also difficult to differentiate, is the potential ingestion of MPs via drinking water, which occurs continuously in marine fish to maintain homeostasis (Fuentes & Eddy, 1997). Contrarily to freshwater fish that carry out the ionic and osmotic regulation in the gills, in seawater fish the gut plays an important role in ionic exchanges (Maetz, 1971). According to Roch et al. (2020), very small particles are thus passively ingested along with water, which agrees with the predominance of fibers found in our fish samples, in particular those belonging to the smaller size class (0.038-1 mm).

However, independently of the via of MPs ingestion, the prevalence of fibers in fish collected in this study also suggests a preference to feed on benthic prey during autumn/winter: firstly, fibers were the prevalent type of MPs found in sediments of the study area, while on seawater their abundance was residual; second, this type of MPs was consistently the most abundant in all the ocean exposed stations (st2 to st6; see Figure 4.1) where *B. boops*, as a oceanodromous species (Bray et al., 2019), is expected to occur and feed (rather than the estuary sediments where fragments predominate (D. Rodrigues et al., 2022)); third, the density of most polymers identified among MPs extracted from fish GIT was higher than seawater, suggesting that fish ingested MPs from the sediment while foraging. A possible additional coincident pattern to take into account is that the majority of MPs found in both fish and sediments belong to the smallest size class (< 1mm). Lastly, the prevalence of fibers in the gut of the bogue, a benthopelagic fish, is in agreement with the pattern usually observed in benthic fish species (Neves et al., 2015).

Regarding fish collected in Mar19 (early Spring), our findings suggest an increase in MPs ingestion, even though fish GIT weight and Fulton condition factor remain low, and a higher representation of fragments among them. Hypothetically, this may indicate a less fine-tuned selection of prey after the reproduction season; a higher amount of MPs available in the environment; or a change in the prevailing type of MPs occurring in sediments. Moreover, it could also consist of a change in feeding habits, with foraging activities occurring more frequently at the sea surface (where fragments predominate). This eventual change of habits, though noticed from a single sampling moment (Mar19), reinforces the need for further studies to be conducted in this region during spring/summer months because the higher foraging intensity expected to occur (Villegas-Ríos et al., 2014), does not imply a higher ingestion of MPs. In fact, according to a previous study conducted in several locations of the Portuguese coast during spring (Lopes et al., 2020), the ingestion of MPs by the bogue is also low.

Nevertheless, if sediments are in fact the main source of MPs ingested by the bogue, this suggests a higher potential for the bioaccumulation of organic and inorganic pollutants which are known to occur in higher concentrations in marine sediments (Bellanova et al., 2022; Castro & Vale, 1995; Lacorte et al., 2003; Vieira et al., 2021) and to associate to MPs (Bakir et al., 2014b). Thus, monitoring the ingestion of MPs is crucial to inform when (season) and where (MPs hotspots) the accumulation of plastic associated chemicals is enhanced in the animal tissues (including edible tissue). This is a question of concern to be addressed, as the Marine Strategy Framework Directive criteria D10C3 (Commission Decision 2017/848) states that the Member States should establish thresholds and ensure that "The amount of litter and micro-litter ingested by marine animals is at a level that does not adversely affect the health of the species concerned".

Lastly, the collection of MPs from seawater surface in further studies, during spring and summer, would be also important to estimate the exposure of this species during larval development, when feeding on copepod nauplii (Sánchez-Velasco & Norbis, 1997). Since the bogue uses coastal waters as nursery areas (Monteiro et al., 2006) and larvae are usually found in surface layers (Sánchez-Velasco & Norbis, 1997), their exposure to MPs could be substantial at Arrábida Marine Park, considering the observed retention effect of floating MPs in the sampling stations sheltered by Arrábida mountain chain (D. Rodrigues et al., 2020) which were potentially exported from the Sado estuary.

Conclusion

Our findings suggest that during the autumn/winter seasons the bogue should not be considered a reliable bioindicator of MPs pollution in Portuguese coastal waters, though the patterns observed among the MPs ingested by fish have partially matched those described for MPs accumulated in local sediments. Further studies are essential to clarify if this ecologically important species might be considered a good indicator as suggested for the Mediterranean Sea, namely during the spring/summer months.

Author contributions

Diana Rodrigues: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft, review & editing. João Pequeno: Methodology, Writing - review & editing, Funding acquisition. Joana Pais: Methodology, Writing - review & editing. Joana Antunes: Methodology, Writing - review & editing. Paula Sobral: Conceptualization, Supervision, Resources, Writing - review & editing, Funding acquisition. Maria Helena Costa: Resources, Writing - review & editing, Funding acquisition.

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5

ASSESSING THE EFFECTS OF NONYLPHENOL AND MICROPLASTICS IN *SPARUS AURATA* L., 1758 LARVAE: PREY INGESTION AND DERMAL UPTAKE PATHWAYS

Manuscript #4 – In preparation

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Abstract

Understanding the risk posed on biota upon microplastics (MPs) ingestion has driven the scientific community to address this topic of concern in the last years. However, as experimental trials have been frequently testing concentrations that exceed environmentally relevant ones, the detrimental effects reported in those toxicological studies cannot be considered representative of what occurs in nature. Moreover, considering the several contaminant pathways taking place *in situ*, laboratorial assays should include combined exposures in their experimental design, besides testing single stressors. Here we aimed to identify effects resulting from MPs ingestion (of polyethylene terephthalate fibers and ultra-high molecular weight polyethylene fragments) in fish larvae which were simultaneously exposed to an organic compound (nonylphenol; NP), both by trophic transfer through prey and directly through dermal uptake, at environmental realistic concentrations. This was achieved by exposing larvae of *Sparus aurata*, for 6 days, to 5 treatments: one combining MPs an NP, and four control groups (absolute, solvent, NP, and MPs). Although no significant effects have been observed in mortality and biomarkers response, fish larvae simultaneously exposed to NP and MPs presented the lowest levels of antioxidant enzymes (catalase and glutathione S-transferase) and the highest levels of vitellogenin. Conversely, growth was significantly affected in larvae singly exposed to waterborne nonylphenol, which may suggest that MPs ingested contribute to reduce the NP body burden, thus attributing a detoxification role to MPs. Yet, since the measured concentration of NP in aquaria was considerably lower than the nominal concentration (5 µg/l), further studies are necessary to understand if the activity of such biomarkers would occur differently at a higher NP concentration and during a longer exposure.

Keywords: growth, mortality, biomarkers, Artemia, fibers, trophic transfer

Introduction

The occurrence of microplastics (MPs) in the marine environment was firstly reported more than five decades ago (E. J. Carpenter & Smith, 1972; Edward J. Carpenter et al., 1972). Their ubiquity, diversity, and potential to be ingested has been demonstrated over the years. Owing to their small size (smaller than 5mm; Arthur et al. (2009)), MPs end up being ingested either intentionally, when MPs resemble natural preys (Ory et al., 2017; Shaw & Day, 1994), or accidentally, when there is a passive intake of MPs during foraging activities (Besseling et al., 2015; Desforges, Galbraith, & Ross, 2015; Van Cauwenberghe & Janssen, 2014). Moreover, their intake may also occur indirectly (by trophic transfer), when predators feed on prey that already carry MPs (Farrell & Nelson, 2013; Fossi et al., 2018; Nelms et al., 2018; Neves et al., 2015; Romeo et al., 2015; Wright et al., 2013).

Understanding the risk posed on biota upon ingestion become a research goal promptly pursued by the scientific community. Such concern was enhanced with the perception of plastics strong association with organic contaminants (Andrady, 2011; Bakir et al., 2012, 2014a; Teuten et al., 2009). Indeed, in addition to physical tissue damage (Corinaldesi et al., 2021; Espinosa et al., 2019; Hsieh et al., 2021; Pedà et al., 2016; von Moos et al., 2012), MPs intake could also induce toxicological responses (Mark Anthony Browne et al., 2013; Oliveira et al., 2013; Rochman et al., 2013; Rochman, Kurobe, et al., 2014; J. Wang et al., 2019). Yet, since most laboratorial trials have been frequently conducted under extreme or unrealistic environmental conditions, uncertainties remain about the impacts of MPs in biota (Law & Thompson, 2014). For example, if the concentration of MPs provided in diets of laboratorial studies is not environmental relevant, the consequent detrimental effects will not be representative of what is occurring to marine wildlife (Cole, 2016; Albert A. Koelmans et al., 2014; Neves et al., 2015).

Furthermore, as contaminants desorption from plastic largely depend on gut retention time and on the gradient of the chemical concentration between the plastic and the organism contaminant burden (Albert A Koelmans, 2015; Albert A Koelmans et al., 2014), it is essential that laboratorial trials simultaneously consider several contamination pathways. However, until recently, experimental studies have focused on testing MP ingestion as an exclusive route of persistent organic pollutants (POPs) bioaccumulation. According to Gouin et al. (2011) and Koelmans et al. (2013, 2014a, 2016), the ingestion of MPs should have a negligible impact, or even play a detoxification role (i.e., attenuate bioaccumulation in tissues (Albert A Koelmans, 2015)), if the chemical fugacity direction of the organic pollutants occur from the organism tissues to the MPs, which will ultimately be egested (Gouin et al., 2011).

It should also be highlighted that, so far, experimental data specifically resulting from assays with fish are scarce (de Sá et al., 2015; P. Ferreira, Fonte, Soares, Carvalho, & Guilhermino, 2016; Rochman, Kurobe, et al., 2014), especially those concerning larvae of marine fish species (Katzenberger & K.Thorpe, 2015; Mazurais et al., 2015). This is largely insufficient considering the extreme vulnerability of larval stages to environmental stressors (Houde, 1987). As fish larvae survival largely influences fish recruitment success and population fluctuations (Houde, 1987), possible economic (and social) implications for fisheries may arise if MPs ingestion have detrimental effects during these stages. Moreover, experimental studies have been predominantly focused on the direct ingestion of MPs and rarely test ingestion through trophic transfer (Athey et al., 2020; Batel et al., 2016; Bour, Sturve, Höjesjö, & Carney Almroth, 2020; Katzenberger & K.Thorpe, 2015; Tosetto, Williamson, & Brown, 2017b).

Considering the above, our purpose was to address the highlighted gaps by assessing the effects of MPs ingestion (both directly and/or by trophic transfer) on fish larvae, which were also exposed to chemical pollutants in seawater by dermal uptake and through the ingestion of natural prey.

Since MPs occur in the marine environment as a cocktail of different types, polymers and sizes, this study tested the effects of 2 distinct MPs: polyethylene terephthalate (PET) fibers and ultra-high

molecular weight polyethylene (UHMWPE) fragments. While PET fibers are among the most abundant MPs in seawater (Mark Anthony Browne et al., 2011), though only investigated by a few experimental studies (Jemec, Horvat, Kunej, Bele, & Kržan, 2016; Watts, Urbina, Corr, Lewis, & Galloway, 2015), the UHMWPE fragments were selected due to its wide application: in orthopedics (Kurtz, 2004), marine structures[11], automotive sector (Minak, Brugo, & Fragassa, 2019) and military field (Y. Wang & Hou, 2022); but also due to their previous use in studies focused on sorption of organic pollutants (Bakir et al., 2012; Teuten et al., 2007). Both PET and PE are abundant polymers in the Portuguese coast (e.g., Rodrigues et al., 2020, 2022).

Nonylphenol, the chemical compound selected for the exposure, occurs in the marine environment, mainly close to the effluents of wastewater treatment plants (Ahel, Giger, & Schaffner, 1994; Petrovic et al., 2002). This pollutant is the major product of degradation of nonylphenol ethoxylates, which are widely used as surfactants (Soares, Guieysse, Jefferson, Cartmell, & Lester, 2008) namely in household laundry detergents (U.S. Environmental Protection Agency, 2010); and it is often used as a plastic additive (Hermabessiere et al., 2017; Mato et al., 2001; Teuten et al., 2009) from which it may leach (Guenther et al., 2002; Hamlin, Marciano, & Downs, 2015; Soto, Justicia, Wray, & Sonnenschein, 1991). This persistent contaminant, known to bioaccumulate in fish (D.-H. Lee, Jo, Eom, Yum, & Rhee, 2018; Rice et al., 2003), is a xenoestrogen (reported to cause endocrine disruption), as it can mimic the 17β-estradiol (E2) hormone (White, Jobling, Hoare, Sumpter, & Parker, 1994) and induce vitellogenin (VTG) (Ackermann, Schwaiger, Negele, & Fent, 2002; Lavado, Thibaut, Raldúa, Martín, & Porte, 2004). Moreover, besides a decreasing tendency observed in the detoxification activity of glutathione S-transferase (GST) in the presence of NP (Teles, Gravato, Pacheco, & Santos, 2004), which affects the biotransformation of this xenobiotic, NP has been also suggested to cause oxidative stress in fish by decreasing the activity of antioxidant enzymes such as catalase (CAT) and GST, thus compromising the elimination of reactive oxygen species ROS (D.-H. Lee et al., 2018; Shirdel, Kalbassi, Esmaeilbeigi, & Tinoush, 2020).

Here we aimed to determine the effects of an environmental realistic exposure to MPs and NP, in the growth and biomarkers response (CAT, GST and VTG) of early life stages of the gilthead seabream, *Sparus aurata*. Larvae of this species are frequently found at the Portuguese coast, namely in the Arrábida marine park (R. A. D. Borges, 2006), a nursery area where the authors have developed previous research about microplastics and fish larval ecology. Ultimately, this study will contribute to understand the impacts of anthropogenic contamination in commercial fish.

[11] https://jhmenge.com/manufacturers/polymer-industries/

Materials and Methods

Experimental design and chemical exposure

The experimental design of this assay consisted of five treatment groups (Table 5.1). To understand the effects of MPs ingestion in fish larvae facing other ecotoxicological risks, as exposure to a pollutant (nonylphenol; NP) through dermal uptake and prey ingestion - the NP&MPs group – four control groups of fish larvae were assured. These consisted of absolute (CT), solvent (CS), NP (CNP; without MPs), and MPs (CMPs; without NP) controls.

Table 5.1 - Rearing conditions (artificial seawater, ASW; solvent, sv; nonylphenol, NP) and diet exposure (either natural, Nat, or microplastics, MPs) of both Artemia (AT) and fish larvae (Algae, Alg; Fibers, FB; Fragments, FG), per treatment (T).

T	Rearing conditions	Artemia diet		Fish Larvae diet	
		Nat	MPs	Nat	MPs
CT	ASW	Alg	-	AT	-
CS	ASW + sv	Alg	-	AT-sv	-
CNP	ASW + sv + NP	Alg	-	AT-sv-NP	-
CMPs	ASW	Alg	FB	AT & AT◆FB	FB & FG
NP&MPs	ASW + sv + NP	Alg	FB	AT-sv-NP & AT-sv-NP◆FB	FB & FG

Nonylphenol (CAS Number 84852-15-3; analytical standard PESTANAL®; Sigma-Aldrich[12]) was dissolved in distilled water and 0.01% of dimethyl sulfoxide (DMSO; CAS Number 67-68-5; Sigma-Aldrich[13]) (Ackermann et al., 2002; Saravanan, Nam, Eom, Lee, & Rhee, 2019). According to Blackburn and Waldock (1995) and Blackburn et al. (1999) the aimed NP concentration of 5 µg/l in the aquarium is environmentally relevant. Such concentration (nominal) was achieved by adding 1.0 ml of the NP stock solution (10 mg/l; prepared from an intermediate solution of 100 mg/l) to the aquaria (CNP and NP&MPs treatments) every day, after water renewals. Similarly, 1.0 ml of DMSO stock solution (also at 0.01%) was added to the CS aquaria group. Both NP and DMSO stock solutions were stored at 4 ºC, covered with aluminum foil.

[12] https://www.sigmaaldrich.com/PT/en/product/sial/46018
[13] https://www.sigmaaldrich.com/PT/en/product/sigald/472301

The exposure assay was conducted in early June 2021. Approximately 500 larvae (*S. aurata*), at the age of 28 days post hatch (dph), were provided by EPPO, an Aquaculture Research Station (IPMA – Olhão, Portugal), and transported to ISPA - Instituto Universitário fish facilities. Fish larvae were randomly distributed in 15 aquaria (H 18 x L 13 x W 13 cm), at a density of 30 individuals per aquarium, with 3 replicate aquaria per treatment. The aquaria were placed inside a water bath (H 0.3 x L 1.73 x W 0.41 cm), permanently refrigerated to maintain water temperature throughout the experiment (22.4 ± 0.1 ºC). Each aquarium was filled with 2 L of artificial seawater (ASW, 35 ppt), 75% being daily renewed. All aquaria were continuously aerated with a glass Pasteur pipette. The aquaria setup was installed in a small room compartment (H 2 x L 2.25 x W 1.7 m; made of MDF white board) built inside the assay room, where the personnel access was restricted, to minimize airborne contamination (MPs in indoor air), with a 12:12 photoperiod. The 15 aquaria were randomly distributed to avoid the influence of both light and temperature conditions inside the small room.

After one day of acclimation, larvae were exposed to the five treatments, for six days. Larvae from all treatments were fed with Artemia (prey) at a density of 4 instar II/ml per day (based on Fernández et al. (2008) and Galvão dos Santos (2013)). The daily amount of *nauplii* (~8000) was added to each aquarium in 4 meals (~2000 *nauplii* per meal) (Table 5.2). Food residues and fecal pellets were daily removed (siphoned) before water renewals. Dead larvae were also removed and registered daily. The harvest of Artemia was conducted inside a small compartment of the assay room, to guarantee constant light and temperature. Considering that, in the natural environment, both prey and predator occur in the same water body and are thus exposed to the same contaminants, both Artemia and fish larvae, of each treatment, were reared in similar ASW conditions.

Table 5.2 – Time and age (hours post-hatch; hph) of Artemia *nauplii* of each meal provided to fish larvae.

Meal	Time	Age of Artemia *nauplii*
1st	10:00 am	15 hph
2nd	12:30 pm	~18 hph
3rd	03:30 pm	~21 hph
4th	06:30 pm	~24 hph

Microplastics: selection criteria and preparation

The MPs selected for the dietary exposure were polyethylene terephthalate (PET) fibers and ultra-high molecular weight polyethylene (UHMWPE) fragments. Other than aiming to expose fish larvae to MPs of different sizes in simultaneous, we intended to ensure that only one of the two MPs would be small enough to be ingested by Artemia *nauplii* at the instar II stage (i.e., smaller than 50 µm; Ferreira, 2009), facilitating the interpretation of results.

PET fibers (diameter 14 µm x length 50 µm) resulted from a multi-filament yarn sectioning (Goodfellow; ref. ES305710; density: 1.39 gcm^{-3}) by following the method published by Cole, 2016; whereas the UHMWPE fragments (125-250 µm) resulted from sieving the supplied powder, firstly with a 250 µm sieve and then with a 125 µm sieve (Goodfellow; ref. ET306010; density: 0.94 gcm^{-3}). Both types of MPs followed the dyeing protocol of Karakolis et al. (2019) (Figure 5.1) to allow a fluorescence-based detection during samples observation under a Leica DMLB Fluorescence microscope with objective HC PL FLUOTAR 40x/0.80 PH2. This was assured with the Jacquard iDye Poly PINK (JID I 456). Further information on the dyeing protocol and stock preparation for both types of MP is provided in the *supplementary materials*.

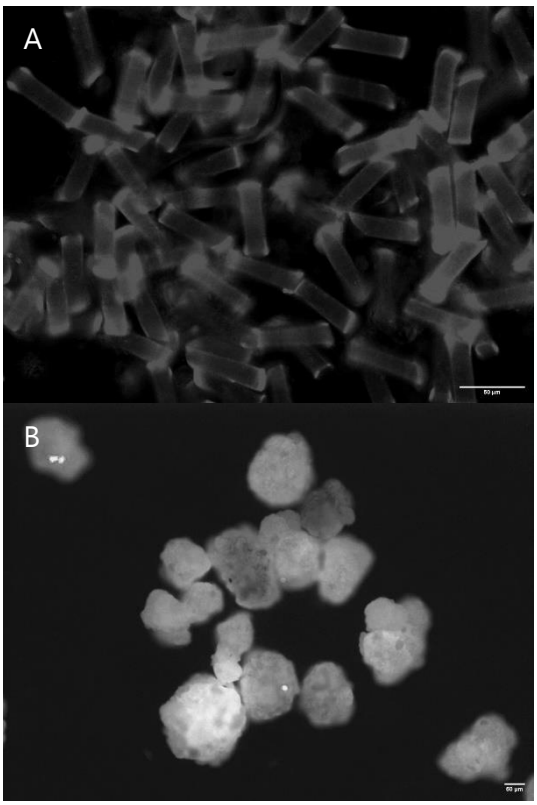

Figure 5.1 – (A) PET fibers (diameter 14 µm x length 50 µm) and (B) UHMWPE fragments (125-250 µm)

Fish larvae diet preparation and microplastics to Artemia nauplii ratios

Since *S. aurata* is a diurnal species in spring and summer (Velázquez, Zamora, & Martinez, 2004), we could infer that, in the wild, the exposure to MPs (through ingestion) is limited to 12 hours per day. Also, to mirror the natural pelagic environment where this species feeds, we intended to provide both prey and MPs at a realistic ratio. Consequently, to estimate the total amount of MPs that would pass by the feeding area of the larvae during the 12h period, we calculated the volume (V) of water passing by the individuals at that same period. This was estimated according to the work of Müller et al. (2020) (V

= Base Area × Height (h) = 0.13 m*0.13 m*h; h = 0.13 ms^{-1}*60 seconds*60 minutes*12 hours; 94910 m^3), considering both the medium current velocity at Sado estuary (0.13 ms^{-1}; Biguino et al., 2021) and the dimensions of each aquarium.

Environmentally realistic abundances of MPs and prey were considered for the estimation of their ratio, which was then simulated in the diets provided in this assay. In the wild, the main prey of gilthead seabream larvae, as most marine fish larvae, are copepods (Mona, Rizk, Fiky, & Elawany, 2019; Sommerfeld & Holzman, 2019). They are usually among the most abundant groups of zooplankton (O'Brian, Wiebe, & Falkenhaug, 2013) and are known to co-occur with MPs in superficial waters (e.g. Rodrigues et al., 2020). Therefore, to estimate the ratio between copepods and MPs, occurring in spring (when *S. aurata* larval stage occurs), we assumed an abundance of 3500 copepods per cubic meter as reported for Cascais bay, Portugal (O'Brian et al., 2013). Yet, due to the lack of data about MPs within the small range that can be ingested by fish larvae and their prey, for Portuguese waters, we considered the abundance data reported for UK coastal waters (J. Li, Green, Reynolds, Shi, & Rotchell, 2018). According to that study, from the 6.7 MPs/l, about 90% were fibers and 10% fragments; also, 30% of the items were smaller than 250 µm. Altogether (i.e., considering the volume of water calculated before (94910 m^3), we estimated an abundance of 1.8 fibers/l and 0.2 fragments/l. As a result, this dietary experiment was based on the following ratios: 0.5 fibers to 1 copepod and 0.1 fragments to 1 copepod. The replacement of copepods by Artemia in the laboratory assay was related with the well-known, simple and fast cultivation of the later (FAO, 1996; P. M. P. Ferreira, 2009).

It was crucial that the 28 dph fish larvae were able to feed on Artemia AF instar II (size: 600-650 µm), which consists of the *nauplii* stage when exogenous feeding begins. This would not only enhance *nauplii* capability to ingest fibers and consequently transfer them to larvae (during the 4 meals) but would also guarantee enough yolk reserves which were essential to provide the nutritional value required for fish larvae at this development phase. Due to all the highlighted reasons, the daily stock of Artemia *nauplii* provided to fish larvae was fresh. The protocol consisted of starting cysts incubation (1.5 g; Artemia AF; 300.000 npg; ZEBCARE[14]) every day at 07:00 pm, in 1 L of clean ASW, continuously aerated with a glass Pasteur pipette for 24h. The incubation took place in a borosilicate glass pear-shaped separatory funnel, with a glass stopcock, at 27°C and 35 ppt salinity.

After hatching, the aeration was stopped, and a light focus helped to concentrate *nauplii* on the funnel bottom. *Nauplii* would then be slowly poured into a 120 µm sieve (supplier: Aquasabi[15]) and gently transferred to a 2 L glass beaker containing ASW (26°C). The volume was topped up to 1.8 L and the content was continuously aerated with a glass Pasteur pipette. At this point, three subsamples of

[14] https://webshop.zebcare.nl/product/micro-artemia-af-300000-naupllii-per-gram-500-gram-package/
[15] https://www.aquasabi.com/Hobby-Artemia-Sieve

1.0 ml each were collected (the aeration pipette was removed instantaneously for this purpose) and *nauplii* were counted under a stereomicroscope using a Bogorov counting chamber, to calculate density.

Subsequently, 5 aliquots of 300 ml were collected from the Artemia supply, by stirring and pouring the content into 500 ml glass jars. Each Artemia group was assigned to one of the 5 treatments, and the ASW conditions adjusted accordingly: two groups were reared in ASW contaminated with NP (CNP and NP&MPs), the CS group was developed in ASW with DMSO and the other two groups (CT and CMPs) in clean ASW (Figure 5.1). The five *nauplii* jars were placed inside a large water bath aquarium (H 0.4 x L 0.58 x W 0.4 cm), with a heater that allowed to maintain temperature constant, at 25.7 ± 0.2 ^{o}C (mean ± SD). Lastly, all the Artemia groups were continuously aerated with a glass Pasteur pipette and enriched with Nannochloropsis microalgae (supplier: Necton[16]; 4 drops per jar (~$6.2x10^{9}$ cells); measured with a medicine dropper) until the last fish meal (Table 5.2).

To ensure that fish larvae from CMPs and NP&MPs treatments would ingest MPs by trophic transfer, the preparation of every meal (of all treatments) began one hour in advance. It consisted of transferring 10,000 *nauplii* of the enriched Artemia to a new 400 ml glass jar with aeration, where ASW of the respective treatment (stored at 26^{o}C) was added until a volume of 200 ml was achieved. This procedure was run with the five treatments, but only *nauplii* from treatments CMPs and NP&MPs were exposed to PET fibers. This was ensured by injecting 4.0 ml (5000 fibers; collected from the fiber stock solution after 1 min vortex) in the Artemia jar. Then, at the end of this 1-hour period, *nauplii* aliquots were sieved into a 120 µm mesh and gently washed back to a 100 ml beaker with clean ASW. This sieving step would ensure that only *nauplii* would be provided to fish larvae - and not free fibers. The volume in the beaker was toped up to 80 ml, to guarantee the collection of three meals (one per replicate) after hand stirring the beaker. Meals were provided into the respective aquaria by injecting 2000 Artemia in 16 ml.

The frequency of *nauplii* with fibers in their GIT, after the one-hour exposure, was later assessed, not only in CMPs and NP&MPs treatments, but in the others as well, to discard the possibility of contamination between treatments. Artemia leftovers from each meal stock were always discarded, except in the last day (for sampling purposes). During this assay all the generated waste was firstly poured to an 80 L container with activated charcoal and then sieved through a 38 µm sieve before being discarded.

Along with Artemia, every meal in CMPs and NP&MPs treatments were also comprised of free fibers (~1000) and free fragments (~200), which were added to the aquaria at the established ratios. While 0.8 ml from the fiber stock solution was injected in each aquarium after a 1 min vortex, fragments were sprinkled into the water surface (~0.00025g stored in aluminum foil).

[16] https://phytobloom.com/nannochloropsis-2/

Preventive measures for quality assurance

To prevent cross contamination between treatment groups, all the supplies used in each treatment (aquaria, *nauplii* jars, aeration pipettes, hose for water renewals, *nauplii* sieves, feeding syringes and beakers, etc.) were labelled with a different color; also, a new micropipette tip was used in each aquarium to inject free fibers. The 15 aquaria were disinfected before the experiment: firstly, soaked in a tank with sodium hypochlorite (ClNaO; CAS: 7681-52-9; 1.7 ml/l) and secondly, in a tank with sodium thiosulfate pentahydrate (Na2S2O3·5H2O; CAS: 10102-17-7; 0.8 g/l).

Sampling

Sampling was performed at day 6. Since NP may adsorb to organisms and tank walls, we aimed to measure the actual concentration of NP in the ASW of each aquarium. Sampling was conducted in one replicate (aquarium) per treatment, throughout 7 time points after the water being renewed at the 6th day (0h, 1h, 3h, 6h, 9h, 12h and 22h). Each sample (4 ml) was immediately syringe filtered (supplier: GVS; 0.2 µm; Ø 13 mm; Regenerated cellulose) to 10 ml amber glass vials (PTFE septa) and stored at -20°C.

To quantify the incidence and number of MPs ingested by fish larvae, and perform morphometric measurements, replicates were collected 15 min after the fourth meal (9 fish larvae per treatment). Regarding the collection of Artemia samples to allow the estimation of fibers incidence, it consisted of storing the leftovers of the first (15 hph) and fourth meal (24 hph); each sample consisted of 4000 *nauplii*. Both fish larvae and Artemia collected for this purpose were euthanized with an overdose of the anesthetic MS222 (1000 ppm), then fixated in Davidson for 24 hours and finally stored in amber glass vials with 70% isopropanol (following Gonçalves et al., 2018).

Additionally, also after the fourth meal, four fish larvae per aquarium were collected, euthanized by rapid cooling, and immediately frozen at -80°C in amber glass vials to assess biomarkers responses, namely, to quantify the catalase (CAT) and glutathione *S*-transferase (GST) activities and vitellogenin concentrations (VTG).

Samples analysis

NP extraction and quantification

Sample preparation

The sample clean-up and analytes pre-concentration was carried out with a 20 mm x 0.5 mm (length × film thickness) polydimethylsiloxane (PDMS) coated twister stir bar (SBSE) (50 µL PDMS volume) supplied by Gerstel GmbH (Mülheim, Germany) (Pinto, Vale, Sontag, & Noronha, 2013). This extract was used to quantify NP from water samples. Each sample was transferred into a 100 ml Erlenmeyer and diluted with 50 ml of MQ water. A stir bar was introduced in the Erlenmeyer and placed in a magnetic stirring plate at room temperature, at 400 rpm, overnight (~24h). Then, with the support of a magnetic rod and forceps, the stir bar was removed and dried with a lint-free tissue. Afterward, it was inserted (with forceps) in a previously labeled 10 ml graduated cylinder, which already contained 5 ml of hexane/acetone (9:1). Then, the NP desorption was performed, for one hour, at 400 rpm. Subsequently, whereas the swollen stir bar was removed with a magnetic rod and left to dry in filter paper, the sample was gradually transferred into a previously labelled 1 ml conic vial, where the solvent was left to evaporate under a gentle nitrogen stream. Then the resulting extract was dissolved in 50 µl of hexane/acetone (9:1), stirred in the vortex and transferred into a vial with insert closed with a crimp PTFE septa cap. Samples were stored at -20ºC and later analyzed through gas chromatography–mass spectrometry (GC-MS), by injecting 2 µl of sample into the GC-MS system. A standard curve was built by preparing 0 to 1000 µg l^{-1} of standard NP (Sigma-Aldrich, USA) in hexane, to quantify NP in samples. Quality control of the results was performed by running blanks, spiked samples and samples in triplicate.

GC-MS analysis

NP was separated on a silica capillary column (30 m × 0.25 mm i.d.; df: 0.25 µm) covered with 5% phenyl and 95% dimethylpolysiloxane (HP-5MS, Agilent-J&W Scientific) with a helium flow rate of 1 mL/min. For mass spectrometry (MS) detection of the ion source, the transfer line and detector temperature were maintained at 230°C, 150°C and 290°C, respectively. MS spectra were obtained by Electronic Impact (EI) at 70 eV using Agilent ChemStation Software. MS detector operated under selected ion monitoring acquisition (SIM) mode. The GC injection parameters were: 2 µL of solution injected in a pulsed splitless divided mode (solvent delay 5 min); injector temperature 280 °C. GC temperature: 120 °C, 20 °C/min at 315 °C isothermal 4.25 minutes. The mass spectrometric detector (MSD)

was operating in full scan acquisition mode and SIM mode. The Limit of Detection (LOD) and Limit of Quantification (LOQ) of Nonylphenol were calculated based on the calibration curve method (Wenzl, Haedrich, Schaechtele, Robouch, & Stroka, 2016).

Fish larvae morphometric measurements

Morphometric measurements to the nearest 0.01 mm included: standard length, total length, pre-anal length, head length, head depth, eye diameter, body depth at the anus and postanal length (described in Solomon et al. (2017)). Fish larvae were observed under a stereoscopic microscope (Leica Wild MZ8) equipped with a camera (MOTICAM 10+) and measured with the Motic Images Plus 3.0 software.

Quantification of MPs ingested by fish larvae and Artemia

After conducting the morphometric measurements of a larvae, its gastrointestinal tract (GIT) was sectioned with a scalpel, placed in a previously identified slide and smashed with a coverslip. Each GIT was immediately observed in a Leica DMLB Fluorescence microscope equipped with a HC PL FLUOTAR 40x/0,80 PH2 objective, for MPs quantification. Such quantification at the microscope was also conducted in Artemia (with 15 hph and 24 hph), being 3 replicates of 50 *nauplii* observed in each treatment. The number of *nauplii* with at least one fiber was registered per replicate, being the mean of the sample subsequently calculated.

Biomarkers analysis in fish larvae

Samples of pooled fish larvae (n=4 per replicate) were homogenized in 600 µl of cold buffer (pH 7.4) containing 0.1 M Na_2HPO_4, 0.15 M KCl, dH_2O, 1 mM EDTA, 1 mM DTT and glycerol 87% and then centrifuged at 12,000 × *g* , for 20 min and 4°C (protocol adapted from Martins et al. (2015)). Then, supernatants were collected and centrifuged at 100,000 ×*g*, for 60 min and stored at -80ºC until further biochemical analysis (GST and CAT). The microsomal pellet was resuspended in buffer pH 7.4 containing 0.1 M Na_2HPO_4, 0.15 M KCl, dH_2O, 1 mM EDTA, 1 mM DTT and glycerol 87% and stored at -80 °C for subsequent VTG analysis.

Total protein content of each sample was determined through the Bradford method (Bradford, 1976) and later used for biomarkers normalization. A Bovine Serum Albumin (BSA) solution was used as a standard and protein concentration in each sample was calculated from a calibration curve (0–16 µg/ml). The absorbance was read at 595 nm on a microplate reader (EZ Read, Biochrom Ltd).

CAT activity

The assay was performed in a 96 well microplate essentially as described by Matos et al. (2020). First, 100 µL of Assay Buffer, 30 µL of methanol and 20 µL of formaldehyde standards (prepared from a 4.25 mM formaldehyde stock solution) were added per standard well. Second, to each sample well, 100 µL of Assay Buffer, 30 µL of methanol and 20 µL of sample were added. These two steps were performed in triplicate (technical replicates). The reaction was initiated by adding 20 µl of Hydrogen Peroxide (30%) to all wells. Afterwards, the microplate was incubated for 20 minutes on a shaker at room temperature. Then, 30 µL of Potassium Hydroxide (10M) was added to each well to terminate the reaction, followed by adding 30 µL of Purpald (chromogen) to each well. Next, the microplate was covered and incubated for 10 minutes on a shaker, at room temperature. Subsequently, 10 µL of potassium periodate was added to each well, being the microplate covered and incubated again, for 5 minutes, on a shaker at room temperature. Finally, the absorbance was read at 540 nm on a microplate reader (EZ Read, Biochrom Ltd), and the calibration curve (range: 0 – 75 µM) was attained owing to the formaldehyde standards.

GST activity

GST activity was determined according to Habig et al. (1974) and optimized for 96-well microplates, following Matos et al. (2020). In brief: a reaction mixture solution (10 ml) was firstly prepared by adding 9.8 ml of PBS (Phosphate-buffered saline), 0.1 ml GSH (200 mM) and 0.1 ml CDNB (1-Chloro-2,4-dinitrobenzene; 100 mM; used as substrate). Then, 20 µl of sample and 180 ml of the mixture solution were added to the wells of a 96 well microplate. The absorbance was read at 340 nm every minute for 6 minutes on a microplate reader (EZ Read, Biochrom Ltd).

VTG

Expression of vitellogenin was evaluated by Western Blot in the soluble fraction (40 µg of protein) by SDS–PAGE on a 4–12% Bis–Tris gel with MES running buffer under reducing conditions (140 V for 1 h). Electrophoresis was followed by dry transfer (20 v for 7 min) to a nitrocellulose membrane with the iBlot™ 2 Dry Blotting System (Invitrogen) and blocked for 1 h at room temperature with a 5% milk solution. Primary antibody incubation proceeded overnight at 4 ºC, followed by washing with PBS and incubation with the appropriate secondary antibody. Blot density was assessed through geneQUANT software system (Clever Scientific, UK) then the protein expression levels were normalized

for total protein loading on the gel which was assessed by Ponceau S staining (Branco et al., 2014). The primary antibody was a rabbit anti-sole vitellogenin polyclonal antibody (Agrisera; AS06 127; dilution 1:2000) and the secondary was a mouse anti-rabbit IgG-HRP antibody (sc-2357, Sta. Cruz; dilution 1:2000).

Statistical analysis

Data was analyzed through non-parametric tests whenever parametric assumptions (normality by Shapiro-Wilk test and homogeneity of variances by Levene test) were not met. A one-way ANOVA analyzed differences in fish larvae mortality among treatments. In order to detect variance in fish larvae total length among treatments, a one-way ANOVA, followed by Tukey post-hoc test for pairwise comparisons, was also conducted. Fibers abundance per fish larvae, among CMPs and NP&MPs treatments, was evaluated with a Mann-Whitney U test. The frequency of fibers occurrence in Artemia, from CMPs and NP&MPs treatments, was calculated and compared in a t-test. Eventual differences between treatments regarding the activity of CAT and GST (log transformed) in fish larvae, were investigated with a one-way NOVA. Treatment effect was also analyzed in the activity of VTG with a Kruskal-Wallis test (fold-change to control). All the previously mentioned tests were performed with TIBCO Statistica™ 14.0.0 software and the level of significance was set at a p-value ≤ 0.05.

Results

Nonylphenol was detected in the ASW of five treatments (Table 5.3; Figure 5.2). While in CT and CS treatments, the concentration variance is residual, an increasing tendency is observed in CMPs. The measured concentration of NP in ASW of CNP and NP&MPs treatments tend to decrease over time, the average being considerably smaller than the aimed target concentration (5 µg/l). The highest concentration (0.34 ± 0.17 µg/l; mean ± SD) was registered in the CNP aquarium.

Table 5.3 – Measured (mean ± SD) and nominal concentration (µg/l) of nonylphenol in the ASW of aquarium, per treatment.

Treatment	Measured	Nominal
CT	0.24 (0.04)	0
CS	0.20 (0.03)	0
CNP	0.34 (0.17)	5
CMPs	0.25 (0.12)	0
NP&MPs	0.24 (0.14)	5

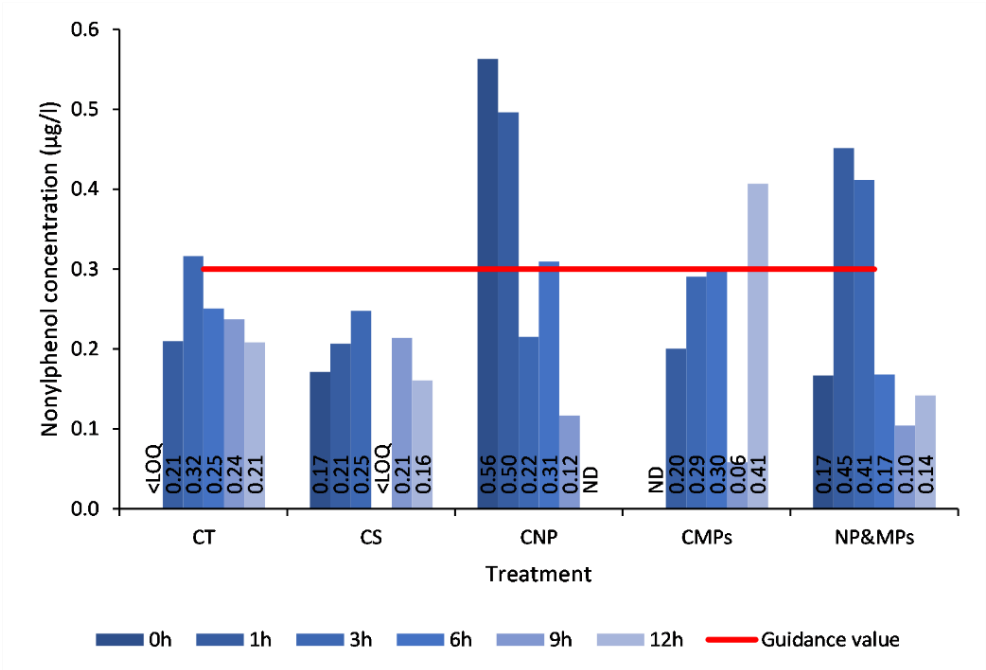

Figure 5.2 – NP measured concentration (µg/l) at 0h, 1h, 3h, 6h, 9h and 12h after ASW renovation in each treatment, in the last day of the assay.

No significant differences were detected between treatments regarding mortality during the experiment ($F(4,10) = 1.94$, $p = 0.18$). However, significant differences in fish larvae total length, namely between the CNP and CS groups ($F(4,40) = 3.07$, $p = 0.03$, $p < 0.05$) were observed, being fish larvae smaller in the CNP group (Figure 5.3).

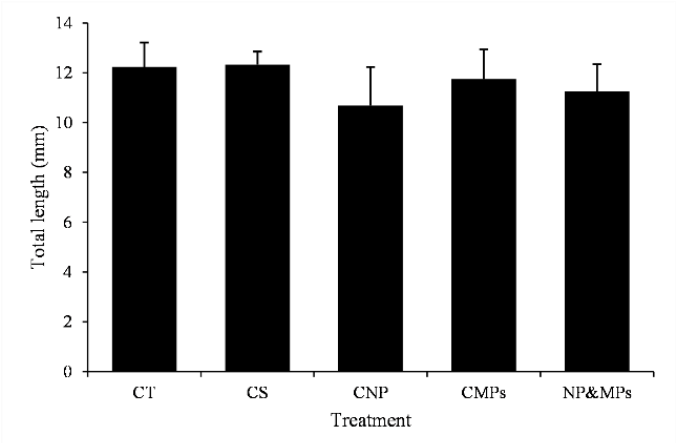

Figure 5.3 - Fish larvae total length (mean ± SD) per treatment, at the end of the 6-day trial

No fibers nor fragments were detected in fish larvae belonging to CT, CS and CNP groups, as expected. Conversely, all replicates from CMPs and NP&MPs treatments (9 larvae per treatment, collected after the 4th meal) had fibers in their GIT (Figure 5.4), ranging from 3 to 32 fibers in a single individual (Figure 5.5). Yet, no significant differences were found between both treatments (U = 30.0,

p = .594). There was no visual nor clear confirmation about fragments intake by fish larvae, despite the identification of fluorescent areas in a few replicates (stains with a poorly defined silhouette).

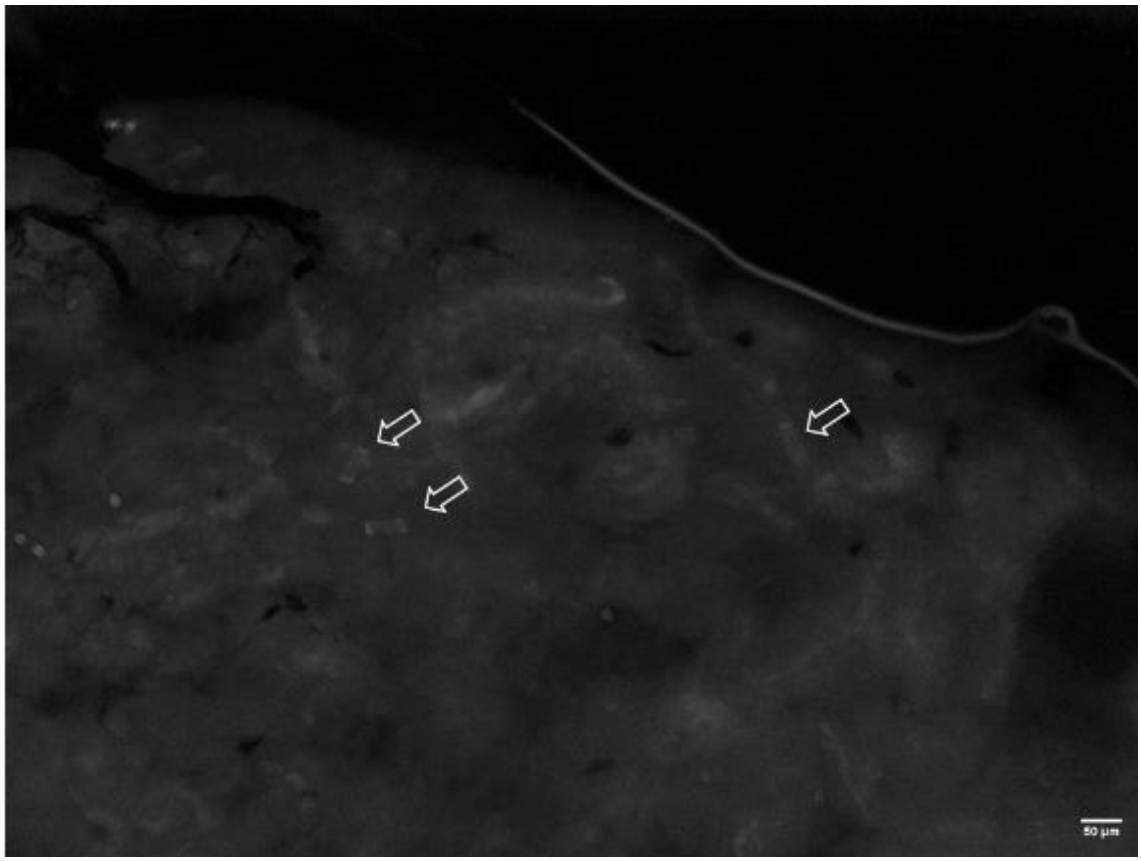

Figure 5.4 – Fibers detected in the GIT content of *S. aurata* larvae reared in the CMPs group.

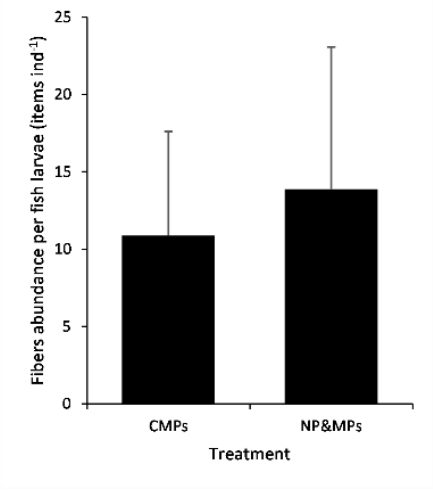

Figure 5.5 – Fibers abundance per fish larvae (items ind^{-1}) collected from the CMPs and NP&MPs treatments (mean ± SD) after the fourth meal, in the last day of the assay

Fibers were not detected in Artemia belonging to CT, CS and CNP group, as expected. Regarding the Artemia collected from CMPs and NP&MPs treatments, while the frequency of fibers in *nauplii* collected at the first meal was residual (Figure 5.6A), it ranged from 12 to 18% in *nauplii* collected after the fourth meal (Figure 5.6B; Figure 5.7). No differences were observed ($t(4) = -1.64$, $p = 0.18$) between CMPs and NP&MPs treatments, in the percentage of Artemia containing fibers in GIT in the last meal of the day (the 4th).

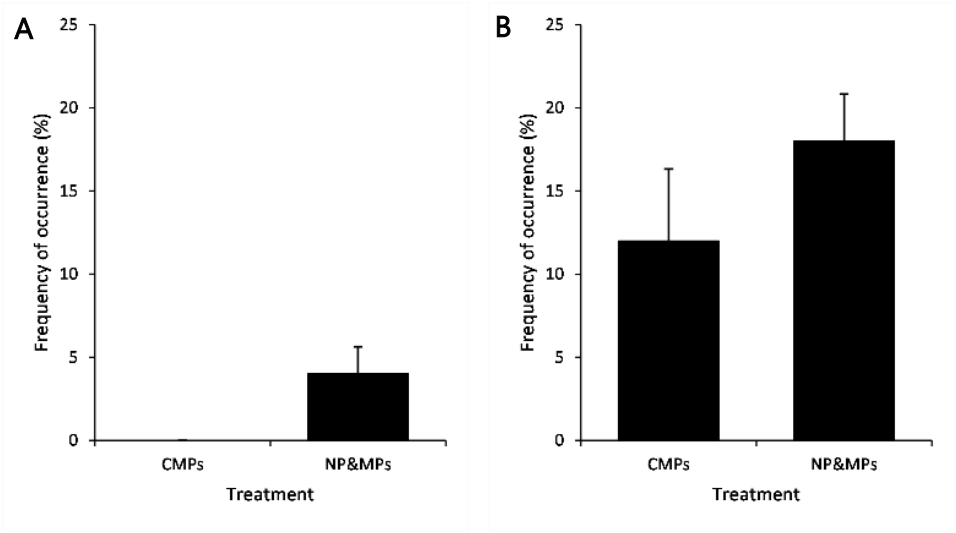

Figure 5.6 – Frequency of occurrence of fibers in Artemia from the CMPs and NP&MPs treatments (mean ± SD) at the first (A) and fourth (B) meals of the last day of the assay

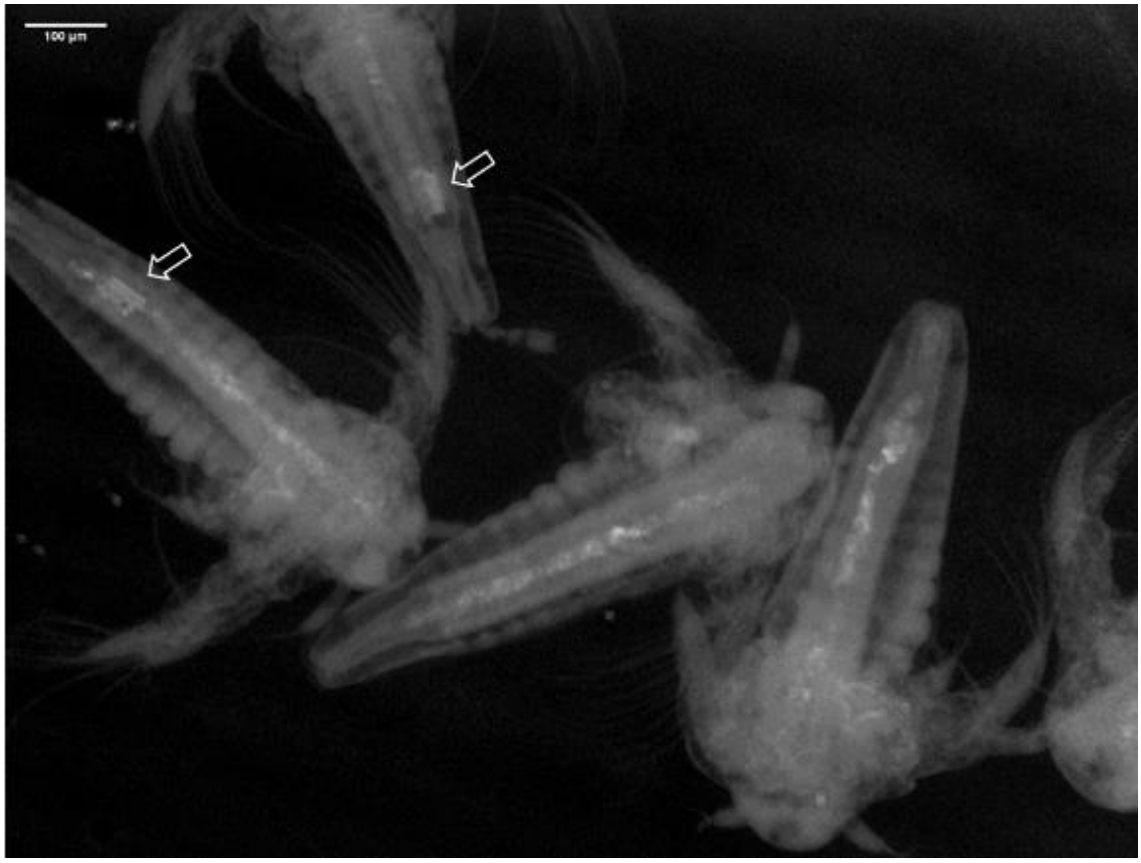

Figure 5.7 – Fibers detected in Artemia nauplii exposed to the CMPs group

No significant differences were found between treatments regarding the activity of both CAT ($F(4,10) = 1.76$, $p = 0.21$; Figure 5.8A) and GST ($F(4,10) = 1.64$, $p = 0.24$; Figure 5.8B). In comparison to fish exposed to the absolute and solvent control groups, the average levels of CAT and GST activities were lower in CNP, CMPs and NP&MPs treatments. Yet, while CAT activity was similarly low in these 3 groups, the lowest GST activity was observed in NP&MPs (0.021 ± 0.004 µmol/min/mg total protein).

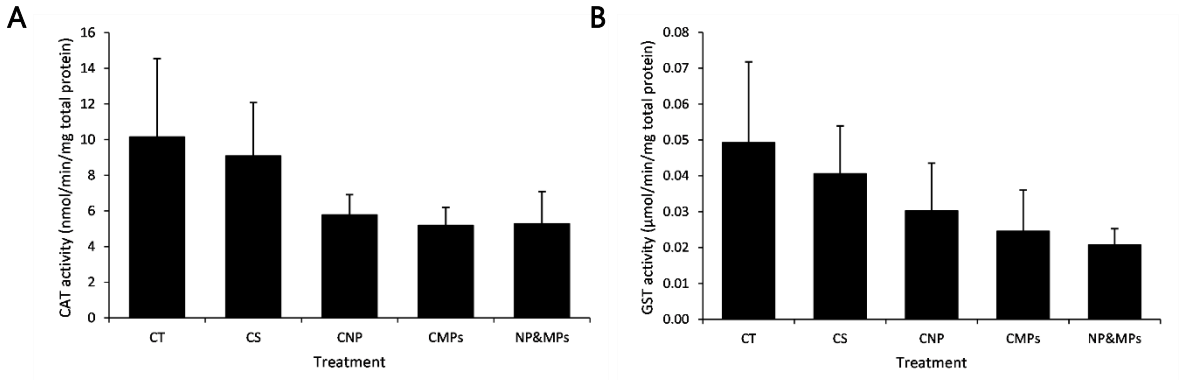

Figure 5.8 – CAT (A) and GST (B) activities (mean ± SD) determined in *S. aurata* larvae exposed to the five treatments for six days.

Regarding the expression of VTG, no significant differences were found between treatments ($H(4) = 4.20$, $p = .38$). However, the higher levels were observed in fish larvae reared in the NP&MPs group (Figure 5.9).

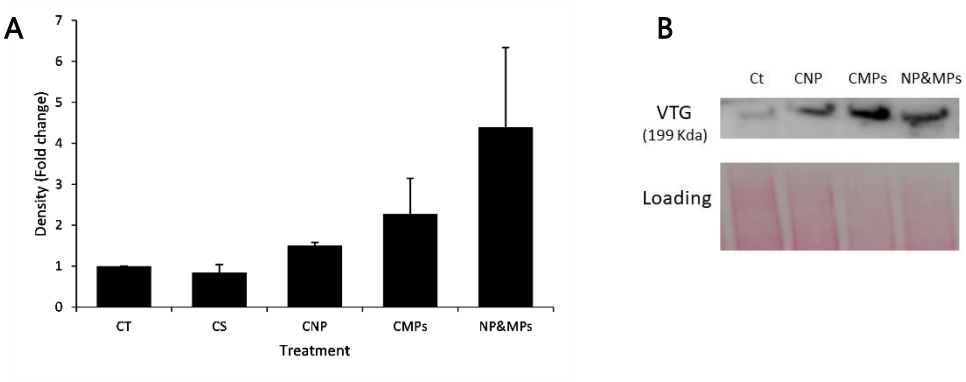

Figure 5.9 - Expression of VTG by Western blot in *S. aurata* larvae exposed to the five treatments for six days.

Discussion

According to our results, *S. aurata* larvae are not significantly affected when exposed to MPs and nonylphenol in simultaneous, at environmentally relevant conditions.

PET fibers were the only type of microplastic clearly detected in fish larvae GIT (from CMPs and NP&MPs groups). Although the exposure to UHMWPE fragments was 5-fold smaller than the exposure to fibers, the cause for their absence in GIT contents, or difficult detection, remains unclear. Besides their reduced availability in each meal (in comparison with PET fibers), it could be related with ingestion avoidance. Another possibility could be a combination of a dye loss during GIT transit associated with their foamy texture, which eventually allows them to better blend in within the GIT content. Such difficult detection in the Fluorescence microscope could be overcome in further studies by following a potassium hydroxide (KOH) digestion to extract ingested items, as performed in a previous study to test UHMWPE ingestion by fish (Jovanović et al., 2018), where MPs were considerably smaller and not fluorescent. Regardless of the cause, we have assumed that fish larvae from groups exposed to MPs (CMPs and NP&MPs) have only ingested fibers. Moreover, based on the lack of significant differences in the abundance of fibers found in larvae GIT from these two groups, it suggests no influence of nonylphenol in feeding behavior.

In fact, the ingestion of fibers was not associated with any significant effect during this 6-day assay. This is in accordance with findings from a study which also tested PET fibers (Bunge et al., 2022), even at an abundance level which exceeded realistic pollution levels. Conversely, the growth of larvae only exposed to nonylphenol was significantly affected, being inferred by their smaller average standard length. The confrontation of such outcome with the average larval length from the NP&MPs group,

indicates that the potential accumulation of nonylphenol in fish larvae tissues by dermal uptake may be continuously reduced due to this contaminant adherence to virgin MPs during their transit inside the GIT, playing thus a detoxification role as suggested by Koelmans (2015).

Regarding biomarkers responses, despite the absence of significant differences to report after this short-term trial (6 days), larvae exposed to MPs, NP or to both in simultaneous, showed to be coping with stress factors, when comparing to larvae reared in both absolute and solvent controls. Such signal, even being weak, suggests that both MPs and NP can interfere with *S. aurata* larvae biochemical processes at environmentally relevant concentrations. This was particularly clear in the NP&MPs group, owing to the higher expression of VTG. The induction of VTG in fish exposed to low NP concentration has been reported before and are usually indicative of either a short-term or a recent exposure (Ackermann et al., 2002).

Other than a slower growth noticed in the CNP group, the absence of adverse effects in this study must be carefully interpreted, as such outcome may have resulted from several constraints. Firstly, the detection of NP in the exposure media of fish larvae reared in CT, CS and CMPs treatments suggests the presence of this contaminant in storage water (sump) used for water renewals, as noticed by Gautam, Chaube, & Joy (2015), and implies that the 5 treatments were equally subject to this negligible contamination. In fact, the NP concentrations measured in both CT and CS aquaria were always lower than the guidance value (300ng/l) established for this compound in the European Union, in what concerns water for human consumption (COMMISSION IMPLEMENTING DECISION (EU) 2022/679). Other explanation for the detection of NP in such control groups could be the detergent used for all 15 aquaria washing, conducted prior to the beginning of this essay.

Yet, owing to the ASW collection at multiple time points per treatment, it was possible to perceive the respective NP kinetics tendencies. While in CT and CS treatments, the concentration variance was residual, in CMPs, there was an increasing tendency over time which might be linked to the leaching of NP from virgin MPs (Hamlin et al., 2015; Staniszewska, Graca, & Nehring, 2016). Conversely, the measured concentration of NP in ASW of both CNP and NP&MPs treatments decreased over time, probably due to the adsorption of NP to organisms and aquarium walls (Nimrod & Benson, 1998) and, in the case of the NP&MPs treatment, due to the possibility of adsorbing to MPs as well (Beiras et al., 2019). Such potential fugacity direction of NP from the ASW to MPs in NP&MPs treatment - contrary to the one apparently observed in CMPs treatment - suggests that the pre-existent content of NP in the MPs used in this assay is small.

With all this in consideration, we assume that the unexpected extra source of NP in the 5 treatments, was negligible and does not prevent the validity of further interpretations.

It should be pointed out that the average concentration of NP in both CNP and NP&MPs treatments was considerably smaller than 5 µg/l (the aimed target concentration). Such high discrepancy

between nominal and measured concentrations (also found in previous studies, e.g. Nimrod and Benson (1998)), besides being related with the nonylphenol low solubility in water, may be also associated with the solvent selected to prepare the NP solutions (DMSO), with the capability of NP (a semi-volatile organic compound) to perform water/air exchange and to become associated with aerosols (Soares et al., 2008), or with the fast adsorption of NP to surfaces inside the aquarium, including fish larvae. Thus, in the absence of data about NP accumulation in fish larvae replicates, which could clarify this hypothesis, and also considering the environmental quality standards (EQS) regarding this pollutant, which establishes that the annual average should not exceed 0.3 µg/l in coastal and territorial waters (DIRECTIVE 2008/105/EC), we may conclude that fish larvae exposure to NP during the 6-day assay was comparable to the NP concentration reported during spring in the outer Sado estuary (239.9 ± 13.8 ng/l (mean ± SE); Rocha et al., 2013), a less polluted environment compared to what was initially planned to simulate in our experiment.

The lack of significant differences in the analyzed biomarkers, between the CT and CS control groups and the other 3 treatments, indicate that no toxicological effects occurred under the environmental realistic conditions tested. Nevertheless, it must be highlighted that, despite the lack of significant differences, fish larvae simultaneously exposed to NP and MPs presented the lowest levels of both CAT and GST. According to other researchers also testing low NP concentrations (Shirdel et al., 2020; Wu, Xu, Shen, Qiu, & Yang, 2011), such reduced activity of the antioxidant enzymes may consist of an inhibition attributed to the presence of NP, consequently causing an accumulation of reactive oxygen species (ROS). Further studies are thus necessary to understand if such biomarkers would have responded differently after a longer exposure or to a NP concentration closer to 5 µg/l. Nevertheless, the smaller fish larvae in CNP group after the 6-days exposure to NP suggest a disruption on *S. aurata* growth, as observed by Sun et al. (2017), though at a much higher concentration (200 µg/l).

An additional explanation for the lack of adverse effects in fish larvae from CMPs and NP&MPs could be the reduced ability of Artemia with 15hph (consisting of the 1st meal of the day) to ingest fibers. Further studies should thus consider exposing more developed *nauplii* to the fibers, to increase the intake of MPs by trophic transfer to fish larvae. This adjustment would however require a compensation of the lower nutritional level of older *nauplii*, by adding an extra food item to fish larvae diet (e.g., rotifers).

It is also worth referring that, although this experiment was intended to be conducted under environmental realistic conditions, the use of virgin MPs ended up to merely represent recent inputs of these particles in the marine ecosystem. Consequently, it does not reflect the majority of MPs in the ocean which are already covered by a biofilm and that exhibit signs of weathering (Vroom et al., 2017). Biofouling, which provides an attractive odor to MPs, has been reported to enhance ingestion (Savoca et al., 2017), and this should be taken into account in further studies.

In fact, there were ~33 fibers available for ingestion per fish larvae, in each meal, in both treatments considering MPs exposure; if we focus on fish larvae from the NP&MPs treatment, the observed average was about 14 fibers per individual GIT in one meal (i.e., ca 50% of the pool of fibers available per individual). Therefore, assuming that our ratios between MPs and Artemia were environmentally relevant, biofouling could have increased MPs intake and potentiated the observation of detrimental effects. On the other hand, we cannot exclude the possibility of the *S. aurata* being able to avoid the ingestion of MPs or to reject them (spit) immediately after ingestion, even that environmentally representative shapes and polymers have been selected for the experiment.

This study reveals that fish larvae are residually affected by MPs exposure and ingestion at environmental realistic conditions and reinforces the importance of combining different exposure pathways when aiming at understanding the impacts of MP ingestion by wild fish.

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Supplementary material

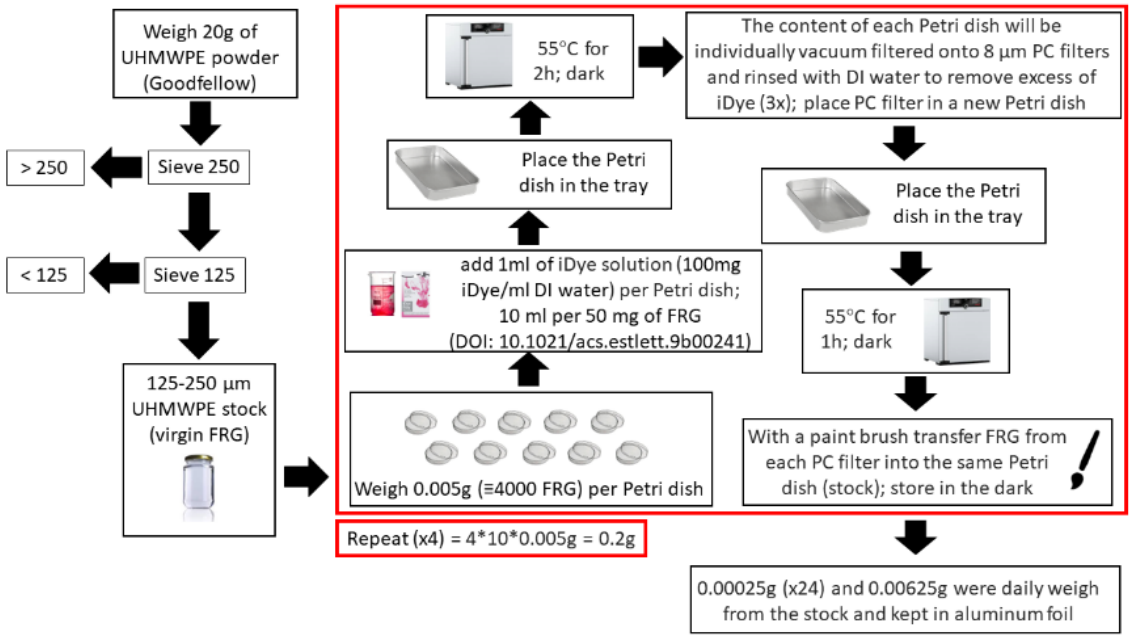

Figure S1 – Fragments preparation

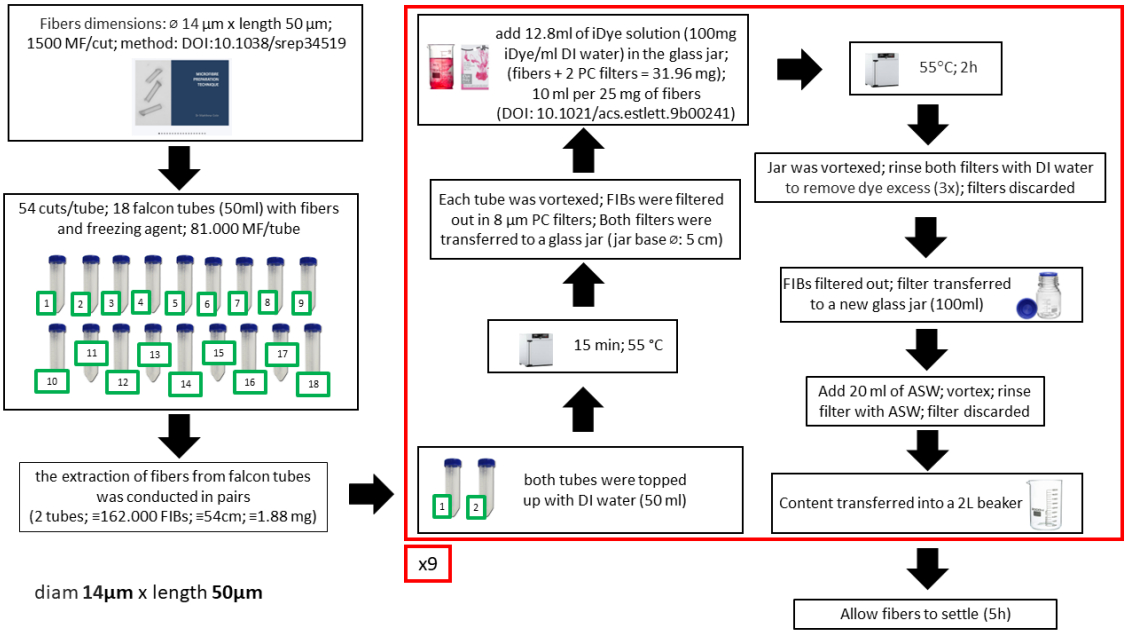

Figure S2 – Fibers preparation

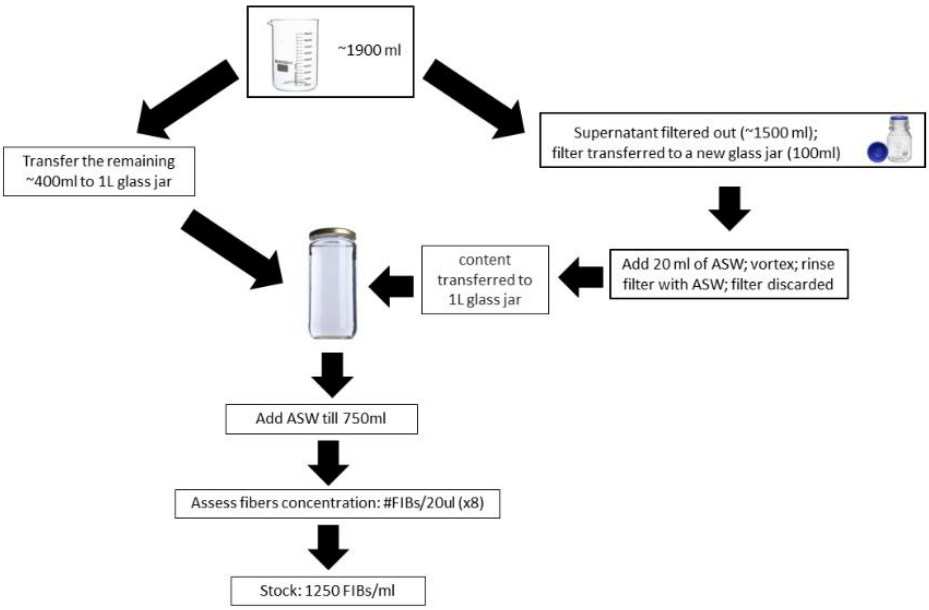

Figure S3 – Fibers preparation (continuation)

6

SCIENCE OUTREACH

Science communication is known to benefit both scientists and society. While for scientists, finding a simple way to explain their research consists of a challenge which improves their communication skills, for the general audience it is suggested to promote interest, curiosity and trust in science (Clark et al., 2016; Woitowich, Hunt, Muhammad, & Garbarino, 2022).

Another critical aspect about science communication is its potential to increase public engagement onto environmental and health problems. This powerful tool has been increasingly used to inspire actions that seek to tackle unsustainable fishing practices, unsustainable water consumption, plastic pollution or the COVID-19 pandemic.

Thus, considering both the important role of science outreach and all the knowledge aimed to acquire in the scope of this thesis, it was my intention from the beginning to combine both and to fulfil the following objective along the way: to raise awareness about marine environment pollution by microplastics and to disseminate main findings of my research to the non-specialist public. Such objective was accomplished in multiple initiatives, which may be separated in 2 groups.

The first group includes the initiatives which occurred during the first years of my PhD workplan, when research was ongoing and final results were still pending. At this early stage, the purpose was only to share the general knowledge about plastic pollution, with different audiences, mainly located in the Lisbon metropolitan area. The language, images and examples shared were adjusted according to the age group of the target audience, to increase comprehension. The titles and context of each initiative are listed below:

- 25 October 2018 - Oral communication: “Lixo Marinho e microplásticos”. Context: “Jornadas Solidárias - Are you awake? | 2.ª Edição”. Universidade Europeia, Campus de Santos. (Link)
- 2 March 2019 – Oral communication: “Microplásticos no mar da Arrábida | Ameaça no ambiente e no prato”. Context: “Sábados no Museu”. Museu Oceanográfico, Portinho da Arrábida. (Figure 6.1)

Figure 6.1 – Flyer made to disseminate the talk given in Museu Oceanográfico, Portinho da Arrábida, in 2019

- 13 March 2019 - Oral communication: "No mar Português também há microplásticos para todos os gostos!" at Escola Básica 2,3 de Álvaro Velho, Barreiro (middle school). Context: OSOS European projects – Open Schools for Open Societies[17], coordinated by Pavilhão do Conhecimento - Centro Ciência Viva. Link
- 14 March 2019 - Oral communication: "Poluição marinha por microplásticos" for 3rd year students from the Bachelor in Biology at ISPA. Context: curricular unit "Curso de campo em biologia marinha" Link
- 12 July 2019 – Oral communication: "Marine Plastic Pollution". Context: Ocean Alive Summer Course, Instituto Politécnico de Setúbal. Link
- 15 October 2019 – National Geographic | Meio Ambiente. "Diana Rodrigues Estuda a Poluição por Microplásticos no Sado e Mar da Arrábida" Link
- 15 June 2020 – Episódio 876 do programa "90 segundos de Ciência". Link
- 17 May 2021 – (virtual) Oral communication: "Poluição marinha por microplásticos" for 3rd year students from the Bachelor in Biology at ISPA. Context: curricular unit "Curso de campo em biologia marinha"
- 31 January 2022 – Antena 2 - Programa Fundos e Novos Mundos Link

[17] https://www.cienciaviva.pt/projectos/osos/

I was also challenged to develop a hands-on workshop to integrate the Kids Dive[18] program. This ocean literacy program, comprised of several educational activities, has the purpose of enhancing kids and teens interest about the ocean and to encourage them to act as ocean ambassadors. Participants become aware about the importance of biodiversity conservation, curious about the underwater world by experiencing scuba diving and are informed about the threats posed to the marine environment. The workshop "Desplastificar o Mar" (Deplastify the sea) was thus designed to meet the last purpose, being already put into practice multiple times since March 2019, mostly in Continental Portugal, but also in Madeira Island, and internationally (at Jessheim Videregående Skole, Norway). The workshop starts with an interactive theoretical part and is followed by 3 dynamic group activities (Link 1; Link 2) in order to provide a full experience. The first activity consists of confronting participants expectations with reality, regarding the fate of plastics used in daily life when they reach seawater (floating/sinking); the second aims to challenge participants to make a 3D ad with plastic items (toys, fishing gear and single use items), with the purpose of raising other kids' awareness about the topic; and in the third, they are invited to choose a well-known song, transform its lyrics according to the concepts learned, and finally to sing it to others. The success of such activities was witnessed in first-hand, by watching the effort and enthusiasm put in each task by participants. It was also noticed through testimonials written by them and respective professors (e.g. Link), by hearing about posterior initiatives conducted at their schools; and, through the increasing demand from other schools to enroll this program that is still ongoing.

The second group of initiatives were later developed, when main findings of my PhD work were ready to be shared. At this point, I shifted my target audience towards the citizens of Setúbal and Sesimbra municipalities. This shift aimed to potentiate the change of habits and encourage actions, since local inhabitants would be familiarized with the study area (both the Sado river estuary and the Arrábida marine park) either by living/working nearby or by relying/depending on this coastal area for subsistence (fisheries and tourism).

These one-hour sessions, either virtually or in-person, consisted of interactive presentations to promote participation. In each municipality (Setúbal city and Sesimbra village) two sessions were conducted: one at a secondary school (to both middle and high school students) and one to local stakeholders. Though a virtual flyer (Figure 6.2) was sent to each stakeholder, as an invitation and to explain the scope of these sessions, the local press and social media of each municipality have also collaborated in the publicizing of these sessions.

[18] http://www.kidsdive.pt/

The dates and audience of each talk (always entitled “Microplásticos na Água, Fundo e Peixes do mar da Arrábida”) were the following:

- 9 February 2022 – to middle and high school students from Escola Secundária du Bocage, Setúbal Link
- 10 February 2022 – (virtual) – with stakeholders from Setúbal municipality (Link 1, Link 2 and Link 3)
- 17 February 2022 – to middle and high school students from Agrupamento de Escolas de Sampaio, Sesimbra (Link 1 and Link 2)
- 25 February 2022 – to citizens from Sesimbra, in the Museu Marítimo de Sesimbra, Fortaleza de Santiago (Link 1; Link 2; Link 3)
- 6 June 2022 – (virtual) to middle school students from Escola Secundária du Bocage, Setúbal

An educational kit about microplastic pollution was prepared to complement the theoretical part of such sessions and to generate interactive moments for better understanding. This kit included: 1) samples of primary and secondary microplastics, 2) single-use items (take away boxes, cutlery, straws, cups), 3) a sample of bigger plastic pieces (> 5 mm) collected in my sampling campaigns, 4) glitter makeup, 5) sample of fibers accumulated in the filter of a dryer machine, 6) estuarine and marine sediment samples, 7) the LIFE magazine August 1, 1955 (volume 39, number 5), and 8) different types of microplastics stored in glass slides to be observed through a pocket microscope (Figure 6.3).

Figure 6.2 - Flyer made to disseminate the talks given in Setúbal and Sesimbra, in 2022

Figure 6.3 – A student is observing microplastics stored in glass slides through a pocket microscope

To measure the educational progress at such awareness sessions, a quick exercise was conducted in some school classes. It consisted of inviting all participants, at the beginning of the session, to write down at a post-it, a maximum of 3 words/concepts from their knowledge about microplastic pollution. They were told that the post-it should be left anonymous and that I would collect them in the end. However, only by the end of the session, participants became aware that I would invite them to repeat the exercise: to write down 3 new words/concepts about the topic, at the same post-it. This data enabled to compare the word clouds generated (1 from the beginning and 1 from the end; Figure 6.4) in what concerns number and diversity of concepts.

Finally, a rollup (where main findings of my PhD are displayed) was prepared (Figure 6.5) to continue dissemination about what is actually happening in this region. The purpose is to expose the rollup, for 1 or 2 weeks, in the facilities of each stakeholder and to invite/challenge them to share on social media an action taken to prevent plastic pollution in the scope of their activity/business. By sharing good practices, stakeholders will empower consumers and will stimulate others to follow such sustainable initiatives in their own activities/businesses. The final goal is to provide an opportunity for the local stakeholders to share with the community how compromised they are with the sustainability of their activities regarding plastic pollution. The rollup initiative will only begin with the publication of scientific outputs, which may then be accessed through the QR code placed at the bottom of the rollup.

All the science communication mentioned in the second group of initiatives was conducted in the scope of the project funded by National Geographic Society entitled “Reducing microplastics pollution by combining scientific data and local awareness campaigns” (early career grant EC-397R-18).

To conclude this chapter, I must add that owing to my participation in all these different initiatives, I became aware that just as important as the “what” we want to share, is the “how” we choose to share. Also, though all the time dedicated to this purpose has implied a significant effort, it was gratifying to testify the comprehension from elements at the audience, through facial expressions, questions asked, and comments made. I am proud to have contributed for a more aware society, even knowing that it represents “a drop in the ocean”. The lack of time and funding is often reported as what prevents the scientists to participate more in science outreach activities (Woitowich et al., 2022). Although there is undoubtedly a lot to do regarding those limitations, we should step up and follow Bodmer (1985) message: “Learn to communicate with the public, be willing to do so and consider it your duty to do so”.

Figure 6.4 - Word clouds generated from words written by kids, teens, and university students, before and after the educational sessions

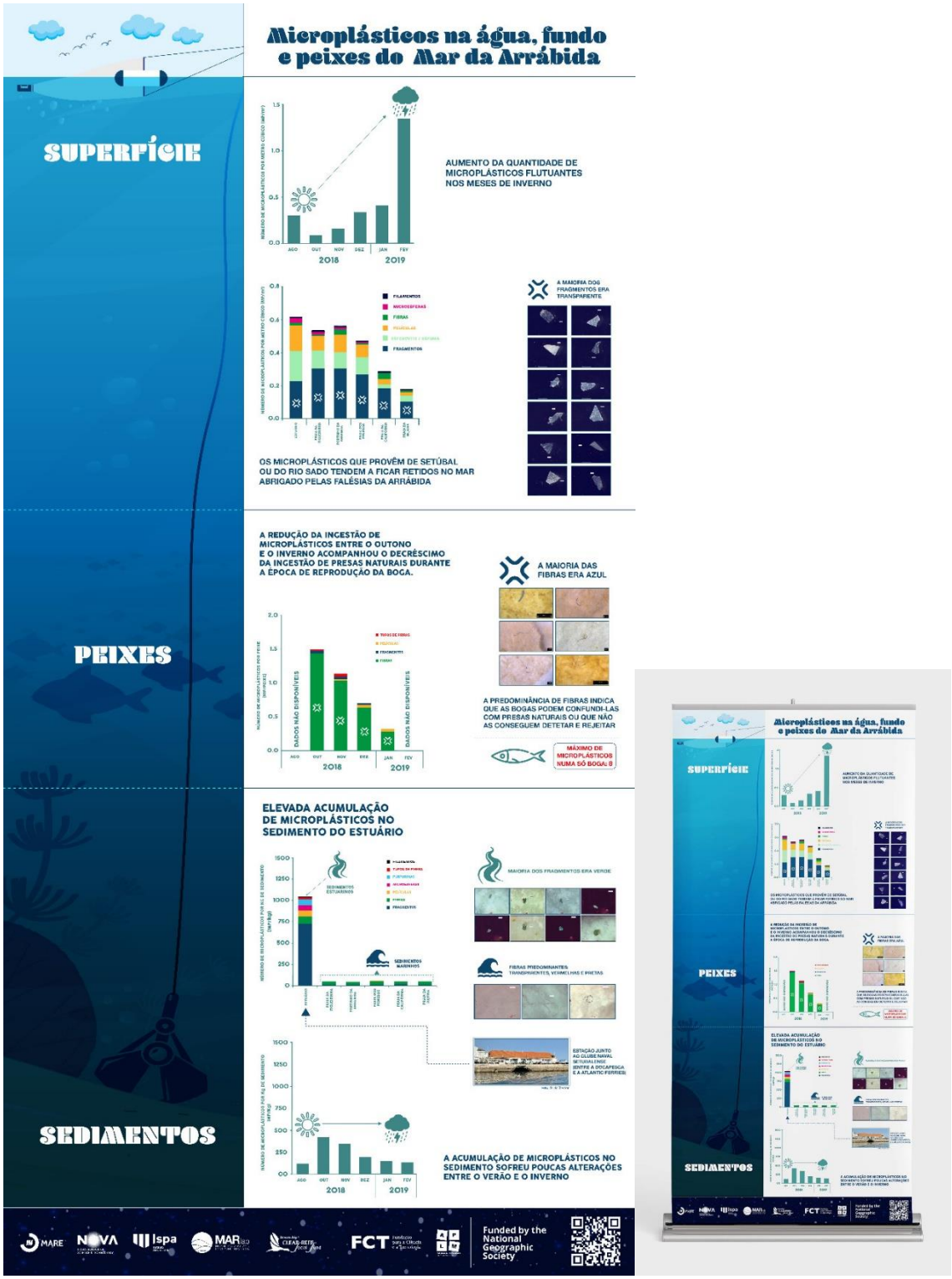

Figure 6.5 – A rollup which displays the main findings of this PhD. To be exposed in the local stakeholders facilities

7

FINAL CONSIDERATIONS

Achieving a blue economy is a critical goal in the European Union (EU). Economic activities, either marine-based or marine-related, both traditional and innovative, have been encouraged to develop their practices in a way that ensures a successful long-term business without putting the health of the ocean at risk (European Commission, 2021, 2022). Inevitably, while all these sectors (e.g., shipping, fisheries, ports, shipyards, aquaculture, and coastal tourism) adapt to these regulations, they end up causing diverse impacts in the marine environment on which they depend on.

Plastics pollution, in particular the widespread of MPs in the marine environment, has been recognized as one of the major problems of the XXI century. Though aimed to be combated, with businesses increasingly aware and focused on improving their practices, the zero emissions target is still far from being accomplished. However, while such effort is undoubtedly important, the major sources of plastics entering the oceans are located on land. As recognized by Jambeck et al. (2015), to overcome or at least reduce such constant inputs, from littering and inadequate disposal, it would require an enhanced management of plastic waste.

However, in addition to the responsibility of each nation to follow general governmental guidelines, either in the EU or beyond its territory, it is as critical that such concern echoes further into a regional level. The identification of the local particularities (e.g., potential sources and hydrodynamics) contributing for MPs pollution in a specific vulnerable area will enhance the success of preventive measures established accordingly. Scientific research has thus a determinant role and this thesis aimed to address this purpose.

Two of the studies here compiled (chapter 2. (D. Rodrigues et al., 2020) and chapter 3. (D. Rodrigues et al., 2022)) provided a first insight about MPs availability in the coastal region of Setúbal and Sesimbra, Portugal. Though consisting of short-term investigations, both shared the same 6 monthly campaigns which ended up covering the (late) summer, autumn and winter seasons. While findings from both studies suggest possible links between some of the identified polymers and potential local sources,

the highest concern is centered on the extreme accumulation level found in sediments collected in the estuarine station, which is certainly attributed to the untreated sewage and stormwater discharges occurring nearby.

While the intensification of urban runoff and river discharges, typical of the winter season due to rainfall, ended up fueling the abundance of MPs at the surface, this was not observed in sediments. The absence of such seasonal influence in the accumulation of MPs in the seabed may be linked to the increase of the Sado river current velocity in winter, delaying the sinking process of MPs. Despite such dissimilarities regarding temporal distribution patterns, both studies indicate a higher accumulation in the station closer to Setúbal city and suggest that the pool of MPs occurring in the remaining stations result from the weak, but continuous, exportation from the Sado estuary. In fact, the expected gradual seaward decrease in MPs abundance, at this south faced coastal area, was not observed. Instead, while in seawater surface there is a nearshore retention effect potentially occurring due to the shelter provided by Arrábida mountain chain against the prevailing north and north-west winds, in sediments there is an abrupt decrease of MPs abundance at the ocean exposed stations.

The categorization of MPs found in both matrices suggests a consistent predominance of fragments at the most polluted station: inside the estuary. However, while at the water surface, both foam and film types were also quite represented, being a pattern also observed in the remaining stations; at the estuarine sediments, the fragment type largely prevailed in contrast to the other types, and, in marine sediments, fibers were the most abundant type of MPs. Whereas the preponderance of secondary MPs (fragments, foams and films) at the surface may be attributed to the breakdown of larger objects, as a potential source; the predominance of fragments in estuarine sediments, i.e., close to their potential sources, was majorly linked to their lower surface area to volume ratio in comparison to fibers, that allows a faster sinking process.

Another aspect to be noted, regarding MPs vertical distribution inside the estuary, concerns to their size. Although both studies do not share the same exact size classes, their comparison suggest that, at the surface, MPs are bigger, being the 1–2 mm the predominant size class, while in sediments most MPs belong to the 0.250–0.500 mm size class. Although such size difference might be strongly related to the sampling methods applied, the smaller MPs in sediments could be also explained by granulometric characteristics of sediments and by the fragmentation process occurring during the settlement of particles. Further sampling taking place in our study area should consider the collection of surface samples with a smaller mesh size to guarantee the quantification of smaller MPs which remained underestimated.

At the end, this baseline data also intends to be use as a reference dataset in both future monitoring and experimental studies. For example, the MP:neuston, MP:ichthyoplankton and MP:meiofauna ratios provided in chapter 2. (D. Rodrigues et al., 2020) and chapter 3. (D. Rodrigues et al., 2022) may be useful in further ecotoxicological research when aiming at selecting environmental realistic exposure

conditions. Moreover, the analysis of MPs composition and distribution patterns in subtidal compartments has also enabled the evaluation of the use of a fish species as a bioindicator of MPs pollution in this coastal area (chapter 4.).

In fact, the concurrent collection of fish specimens and environmental samples (water and sediments) enabled a proper comparison which has been rarely performed. Though the selected species, *Boops boops* (Linnaeus, 1758), has been frequently suggested as a potential bioindicator in the Mediterranean, our study shows a weak match between MPs ingested and those available in this species feeding grounds (most similarities with sediments). Such match mainly relies in the predominance of fibers among the MPs ingested and, on the polymers identified, which were denser than seawater. The lack of a stronger coincident tendency could be explained by the observed decrease in food intake along the sampling campaigns, which consequently relates to this fish species reproduction season. Therefore, in order to properly conclude about this species suitability as bioindicator in Portuguese waters, further studies should be conducted during the spring/summer months.

Lastly, this thesis last aim was to contribute to the understanding of the effects of MPs ingestion (directly and by trophic transfer) in fish larvae, a critical and fragile life stage, which also faces other ecotoxicological risks, as being exposed to pollutants (as nonylphenol; NP) through dermal uptake and prey ingestion. Although the analysis of water samples from the aquaria indicated a considerably smaller concentration of NP in water than the nominal one (5 µg/l), thus approaching levels which are not considered as toxic, our findings suggest that under a realistic exposure, namely to more than one type of MPs, to different polymers, to an environmentally relevant MP:prey ratio and to a relevant concentration of NP in water, there are no significant effects occurring in mortality and biomarkers response to report. Nevertheless, fish larvae simultaneously exposed to NP and MPs presented the lowest levels of both antioxidant and detoxification enzymes (catalase and glutathione S-transferase) and the highest of vitellogenin. It should be also noticed that growth was only affected in larvae exposed to water borne nonylphenol, suggesting that the exposure to MPs may contribute to remove NP from their tissues and consequently play a detoxification role. Further studies are thus necessary to understand if the activity of such biomarkers would occur differently if the exposure was longer or if the measured concentration of NP was in fact closer to 5 µg/l.

To conclude, besides the immediate contribution of this thesis for the scientific community working on microplastics pollution, I believe that it consists of a critical foundation for further studies that aim to monitor such important study area. Moreover, findings here reported mirror the importance of sharing the knowledge with all sectors of society, which I intended to assure in all the opportunities which crossed my PhD path. Investing in environmental education is critical and should be faced as a solution for microplastics pollution.

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APPENDICES

A

LIST OF THESIS OUTPUTS

A.1 Scientific articles

A.1.1 Published

Rodrigues, D., Antunes, J., Otero, V., Sobral, P. & Costa, M. H. (2020). Distribution Patterns of Microplastics in Seawater Surface at a Portuguese Estuary and Marine Park. *Frontiers in Environmental Science, 8*(December), 1-15. doi: 10.3389/fenvs.2020.582217

Rodrigues, D., Antunes, J., Pais, J., Pequeno, J., Caetano, P. S., Rocha, F., Sobral, P. & Costa, M. H. (2022). Distribution patterns of microplastics in subtidal sediments from the Sado river estuary and the Arrábida marine park, Portugal. *Frontiers in Environmental Science, 10*(September), 1-21. doi: 10.3389/fenvs.2022.998513

A.1.2 Submitted

Rodrigues, D., Pequeno, J., Pais, J., Antunes, J., Sobral, P. & Costa, M. H. (2022). Is the bogue *Boops boops* (Linnaeus, 1758) a good indicator of microplastics pollution in Portuguese coastal waters? *Marine Pollution Bulletin*. Submitted on September 24, 2022

A.1.3 In preparation

Rodrigues, D., Lopes, A. R., Matos, B., Bramatti, I., Diniz, M., Noronha J. P., Costa, M. H., Sobral, P. & Faria A. M. (2022) Assessing the effects of nonylphenol and microplastics in *Sparus aurata* L., 1758 larvae: prey ingestion and dermal uptake pathways.

A.2 Oral communications

A.2.1 International conferences

Diana Rodrigues, João Pequeno, Joana Antunes, Paula Sobral, Maria Helena Costa (2021) "Are microplastics detected in the bogue *Boops boops* (L.) reflecting those available in its coastal feeding grounds?". Oral communication. ECSA 58 - EMECS 13 Estuaries and coastal seas in the Anthropocene – Structure, functions, services, and management, Online Live and On-demand, 6-9 September 2021 Link

Diana Rodrigues, Joana Antunes, Vanessa Otero, Paulo Sá Caetano, Paula Sobral, Maria Helena Costa (2020) "Distribution of microplastics in subtidal sediments at the Arrábida coast, Portugal". Oral communication. Micro 2020 International Conference "Fate and Impacts of microplastics: knowledge and responsibilities" held virtually, from Lanzarote and each local node, 23-27 November 2020. Link (Session 27.3_Me. Chaired by Juan Baztan, Crozon; page 759)

Diana Rodrigues, Paula Sobral, Ana Margarida Faria, Maria Helena Costa (2019) "Temporal and spatial distribution of microplastic in subtidal sediments and seawater surface at a Portuguese coastal area". Oral communication. 15th European Ecological Federation (EEF) Congress and 18th National SPECO Meeting, FCUL, Lisboa

A.2.2 National conference

Diana Rodrigues, Paula Sobral, Ana Margarida Faria, Maria Helena Costa (2019) "Distribuição temporal e espacial de microplásticos em sedimentos subtidais e águas de superfície numa área costeira portuguesa". Oral communication. 2ª Conferência Portuguesa sobre Lixo Marinho e Microplásticos, Casa da Baía, Setúbal, 19-20 September 2019

A.3 Poster

Diana Rodrigues, Paula Sobral, Ana Margarida Faria, Maria Helena Costa (2019) "Microplastics and associated contaminants in Portuguese coastal waters: impacts of plastic ingestion on marine fish". Poster. NOVA SCIENCE DAY 2019, Reitoria da UNL, Lisboa, September 18, 2019 Link

A.4 Media Outreach

31 January 2022 – Antena 2 - Programa Fundos e Novos Mundos Link
15 June 2020 – Episódio 876 do programa "90 segundos de Ciência". Link
15 October 2019 – National Geographic | Meio Ambiente. Diana Rodrigues Estuda a Poluição por Microplásticos no Sado e Mar da Arrábida. Link

A.5 Students mentoring

28 February 2020 to 27 January 2021 – Master student: **Dalila Leonor** - Integrated Master in Environmental Engineering (FCT NOVA). Master thesis

6 January 2020 to 29 June 2020 – Bachelor student: **Filipe Borges** - Bachelor in Biology (Universidade de Aveiro). Internship

21 January to 21 February 2020 – Master student: **Marta Pereira** - Integrated Master in Environmental Engineering (FCT NOVA). Internship

8 April to 9 July 2019 – Master student: **Alua Dyussenbayeva** - (International Master in Applied Ecology - IMAE). This student did not deliver the thesis, therefore did not completed her master's degree.

B

FUNDING

13 July 2018 - **Clear Reef Social Fund**. Project entitled “Microplastics and associated contaminants in Portuguese coastal waters: impacts of their ingestion by adult marine commercial fish”. Link

25 April 2018 - Early Career grant – funded by **National Geographic Society**. Project entitled "Reducing microplastics pollution by combining scientific data and local awareness campaigns."

1 January 2018 - Ph.D. Grant (SFRH/BD/130652/2017) – funded by **Fundação para a Ciência e a Tecnologia**.

2022

DIANA DUARTE RODRIGUES

MICROPLASTICS IN A PORTUGUESE COASTAL AREA: DISTRIBUTION PATTERNS ON SURFACE WATERS AND SEDIMENTS, INGESTION BY WILD MARINE FISH, AND RELATIVE CONTRIBUTION AS A CONTAMINATION PATHWAY