Understanding Microplastic Contamination in Marine Pelagic Ecosystems

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Resumo

Perante o crescente aumento da produção de plástico a nível global, tem vindo a verificar-se uma maior consciencialização face à poluição por plásticos nos ambientes marinhos. Consequentemente, a investigação nesta área também se desenvolveu, havendo um foco nos microplásticos (MP, <5 mm), atualmente reconhecidos como uma ameaça global emergente. Estas partículas antropogénicas têm origem tanto em fontes terrestres como marítimas. De distribuição generalizada no ambiente marinho, a dimensão reduzida dos microplásticos faz com que estejam acessíveis a uma vasta gama de espécies marinhas, desde o zooplâncton até aos grandes predadores. Vários métodos têm sido desenvolvidos para a amostragem, processamento e análise de microplásticos no ambiente mas ainda não foram estabelecidos procedimentos operacionais padrão o que dificulta a comparação entre estudos. Atualmente já existe alguma legislação que aborda a poluição por plásticos nos oceanos, incluindo os microplásticos, como a Diretiva-Quadro Estratégia Marinha (DQEM). Apesar do aumento da consciencialização e esforços de regulamentação, persistem lacunas significativas de conhecimento, designadamente, como os organismos marinhos internalizam os microplásticos e como estas partículas se distribuem pelos diferentes tecidos. A informação sobre as características e quantidades de microplásticos nos ecossistemas marinhos continua a ser limitada e controversa. Assim, a presente tese teve como objetivo abordar estas lacunas críticas de conhecimento, concentrando-se especificamente no ambiente marinho pelágico do Nordeste do Oceano Atlântico.

O Capítulo 2 apresenta os resultados da integração do Tween-20 nos protocolos tradicionais de digestão com KOH para a otimização de um método de extração de microplásticos de amostras de peixes com uma percentagem elevada de gordura. A incorporação do Tween-20 facilitou o processo de digestão e filtração das amostras, sem afetar significativamente os espectros de FTIR dos polímeros mais encontrados no ambiente marinho. Além disso, o Tween-20 também exibiu um efeito protetor na degradação do policarbonato (PC) e do tereftalato de polietileno (PET), melhorando as suas taxas de recuperação. Esta otimização foi crucial para as fases subsequentes de análise de microplásticos em várias espécies de peixes pelágicos, incluindo algumas com alto teor de gordura.

O Capítulo 3 aborda a acumulação de microplásticos em pequenos peixes pelágicos de elevado valor comercial, incluindo a sardinha, o biqueirão e o carapau. Foram identificados microplásticos no trato gastrointestinal, nas brânquias e no músculo de todas as espécies estudadas. Dos 118 peixes analisados, 92–96% estavam contaminados. Estes resultados destacam a ocorrência generalizada dos microplásticos e as suas potenciais implicações para o consumo humano. Além disso, foi ainda possível estabelecer uma ligação entre a composição dos microplásticos observada nas brânquias de sardinha e biqueirão e na água circundante.

Os resultados do Capítulo 4 mostram que a dieta dos pequenos peixes pelágicos influencia a ingestão de microplásticos. Espécies que consomem presas com dimensões mais pequenas apresentaram concentrações mais baixas de microplásticos nos seus estômagos em comparação com aquelas que se alimentam de presas mesozooplanctónicas maiores. Especificamente, o carapau, que se alimenta de presas maiores (>1000 μm), foi a espécie que demonstrou concentrações mais elevadas de microplásticos nos seus conteúdos estomacais.

O Capítulo 5 explorou a presença de microplásticos nos conteúdos estomacais do peixe-lua pela primeira vez. Foi observado que os espécimes capturados no outono apresentavam um maior registo de fibras em comparação com os capturados na primavera.

De uma forma geral, os microplásticos mais encontrados ao longo desta dissertação foram fibras, partículas azuis e polímeros como o polipropileno, polietileno, acrílicos e polímeros à base de celulose.

De todas as espécies estudadas, o carapau surge como um bioindicador promissor para monitorizar microplásticos nos ecossistemas marinhos pelágicos. Esta seleção teve por base diversos fatores, incluindo a sua distribuição generalizada, histórico, habitat, características tróficas, comportamento alimentar, importância comercial e suscetibilidade à ingestão de microplásticos.

Em conclusão, com esta tese foi possível avançar significativamente na compreensão da contaminação por microplásticos nos ecossistemas marinhos pelágicos, proporcionando uma base para estudos futuros e iniciativas de conservação. Os resultados apresentados destacam a interligação das espécies e defendem uma abordagem holística e abrangente para enfrentar o problema persistente da poluição por microplásticos nos oceanos.

Palavras-chave: Microplásticos, Ecossistema marinho, Peixes pelágicos, Atlântico Nordeste, Bioindicador, trato gastrointestinal, brânquias, músculo, FTIR, Tween-20, monitorização ambiental

Abstract

As global plastic production continues to rise, the awareness of the ubiquity of marine plastics pollution in the marine environments has increased over the past decades. Consequently, the research activity in this field increased as well, recently focusing on microplastics (MP, <5 mm), which are recognized as an emerging global threat. These anthropogenic particles come from both land and sea sources and find their way into the ocean through various pathways. Given their small size and widespread distribution in the marine environment, microplastics are highly accessible to an extensive range of marine species, from zooplankton to top predators.

Efforts have been made to developing methods to analyse microplastics in environmental samples, resulting in various approaches for sampling, processing, and analysis. So far, no standard operating procedures exist, but more and more attempts on harmonization are made. Legal frameworks, such as the European Marine Strategy Framework Directive (MSFD), have been initiated to address marine plastic pollution, including microplastics. Despite increased awareness and regulations, significant knowledge gaps persist, particularly in understanding how marine organisms take up microplastics, the distribution of microplastics in different body tissues, and limited information about microplastic characteristics and quantities in marine ecosystems.

Hence, this thesis aimed to address critical knowledge gaps specifically focusing on the pelagic marine environment in the Northeast Atlantic Ocean. The results presented in Chapter 2 allowed the introduction of a cost-effective and environmentally friendly method that incorporated Tween-20 to enhance the extraction of microplastics from high-fat fish samples. By integrating Tween-20 into traditional KOH digestion protocols, this method effectively prevented the formation of fat layers during digestion, without significantly affecting the FTIR spectra of polymers commonly found in marine environment. Moreover, Tween-20 exhibited a protective effect on the degradation of polycarbonate (PC) and polyethylene terephthalate (PET), enhancing recovery rates. The success of this optimized method was pivotal for subsequent phases analysing microplastics in various marine pelagic fish species, including those with high fat content.

The Chapter 3 of this thesis explored the microplastic accumulation in small pelagic fish of high commercial value, including European sardine, European anchovy, and horse mackerel. The investigation revealed the presence of microplastics in the gastrointestinal tract, gills, and muscle. Microplastics were identified in 92–96% of the 118 individual fish analysed, underscoring their widespread occurrence and potential implications for human consumption. Moreover, the research established a direct link between the composition of microplastics in fish and the surrounding water, particularly evident in the gills of European sardines and anchovies.

Furthermore, the findings from Chapter 4 highlighted that the dietary choices of small pelagic fish had an impact on the accumulation of microplastics. Species that primarily consumed smaller prey showed lower levels of microplastics in their stomachs compared to those that fed on larger mesozooplanktonic prey. Specifically, Atlantic mackerel and horse mackerel, which prefer larger prey (>1000 μm), demonstrated higher concentrations of microplastics in their stomach contents.

Moreover, in Chapter 5, microplastics were identified in the stomach contents of ocean giant sunfish for the first time, revealing seasonal variations. Notably, specimens caught in autumn exhibited a higher registration of fibers compared to specimens caught in spring.

Out of all the species investigated in this thesis, horse mackerel has emerged as a promising bioindicator for monitoring microplastics in pelagic marine ecosystems. This choice considered several factors, including its widespread distribution, background, habitat, trophic characteristics, feeding behaviour, commercial importance, and susceptibility to microplastic ingestion. The predominant microplastics identified exhibited distinctive characteristics, including a fiber-like structure, blue colouration, and a composition of polymer varieties such as polypropylene, polyethylene, acrylic, and cellulose-based polymers.

Overall, this thesis advanced understanding of microplastic contamination in marine pelagic ecosystems, providing a foundation for future research and conservation efforts. The findings emphasize the interconnectedness of species and advocate for a holistic, ecosystem-wide approach to address the pervasive issue of microplastic pollution in oceans.

Keywords: Microplastics, Marine ecosystem, Pelagic fish, Northeast Atlantic Ocean, Bioindicator, Gastrointestinal tract, Gills, Muscle, FTIR, Tween-20, Environmental monitoring

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List of Abbreviations

ABS	ACRYLONITRILE-BUTADIENE-STYRENE
ANCOVA	ANALYSIS OF COVARIANCE
ANOVA	ANALYSIS OF VARIANCE
ATR	ATTENUATED TOTAL REFLECTANCE
BPA	BISPHENOL A
CA	CELLULOSE ACETATE
DDT	DICHLORO-DIPHENYL-TRICHLOROETHANE
DNA	DEOXYRIBONUCLEIC ACID
EC	EUROPEAN COMMISSION
ECHA	EUROPEAN CHEMICALS AGENCY
ECD	ENDOCRINE-DISRUPTING CHEMICAL
EU	EUROPEAN UNION
EVA	ETHYLENE-VINYL ACETATE
FPA	FOCAL PLANE ARRAY
FTIR	FOURIER TRANSFORM INFRARED SPECTROSCOPY
GES	GOOD ENVIRONMENTAL STATUS
GT	GASTROINTESTINAL TRACT
HDPE	HIGH-DENSITY POLYETHYLENE
LDPE	LOW-DENSITY POLYETHYLENE
MP	MICROPLASTIC
MSFD	EUROPEAN MARINE STRATEGY FRAMEWORK DIRECTIVE
NOAA	NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION
OSPAR	CONVENTION FOR THE PROTECTION OF THE MARINE ENVIRONMENT OF THE NORTH-EAST ATLANTIC
PA	POLYAMIDE
PAH	POLYCYCLIC AROMATIC HYDROCARBON
PAN	POLYACRYLONITRILE
PBDE	POLYBROMINATED DIPHENYL ETHERS
PC	POLYCARBONATE
PCB	POLYCHLORINATED BIPHENYL
PE	POLYETHYLENE
PET	POLYETHYLENE TEREPHTHALATE

PMMA	POLY (METHYL METHACRYLATE)
POE	POLYOXYETHYLENE
POP	PERSISTENT ORGANIC POLLUTANT
PP	POLYPROPYLENE
PPE	PERSONAL PROTECTIVE EQUIPMENT
PS	POLYSTYRENE
PTIR	PHOTOTHERMAL-INDUCED RESONANCE
PU	POLYURETHANE
PVA	POLYVINYL ACETATE
PVC	POLYVINYL CHLORIDE
SAN	STYRENE ACRYLONITRILE
SDG	SUSTAINABLE DEVELOPMENT GOAL
SEM	SCANNING ELECTRON MICROSCOPY
S-SNOM	SCATTERING SCANNING NEAR-FIELD OPTICAL MICROSCOPY
SUP	SINGLE-USE PLASTIC
TG-ML	TECHNICAL SUBGROUP ON MARINE LITTER
TL	TOTAL LENGTH
UN	UNITED NATIONS
UNEP	UNITED NATIONS ENVIRONMENT PROGRAMME
UV	ULTRAVIOLET RADIATION
WFD	WASTE FRAMEWORK DIRECTIVE
WIUE	WESTERN IBERIA UPWELLING ECOSYSTEM

Chapter 1

General Introduction

General Introduction

1. Decoding Plastic Definition

Finding a clear definition of what is a plastic material is rather difficult. Derived from the Latin “plasticus” and the Ancient Greek “πλαστικός” (plastikos), the term “plastic” was used long before the first modern plastic was invented to refer to materials that could be easily molded or shaped (Macionis, 2018). They can be cast, pressed, or extruded into a huge range of shapes due to their malleability or plasticity during manufacturing. There are various ways to categorize or classify plastics which facilitate understanding similarities and differences between materials.

Chemically speaking, plastics are synthetic or semi-synthetic organic polymers made from repeating monomer units and can be categorized based on the side chains and backbone molecular building blocks of the polymer (Peters, 2015). Some important groups in these classifications are the acrylics, polyesters, silicones, polyurethanes, and halogenated plastics. Plastics are often divided into two major groups depending on their behaviour when heated: thermoset materials, which cannot be remolded when heated, and thermoplastic materials, which can be reheated and remolded (Peters, 2015).

Other classifications refer to the origin of the raw materials or the ability to degrade under certain conditions. Conventional plastics are derived from petroleum or natural gas, contributing to their low price and high availability. Bioplastics are plastic materials produced from renewable biomass sources, such as vegetable fats and oils, corn starch, straw, woodchips and recycled food waste (Harding et al., 2017). Usually, the terms bio-based and biodegradable are misunderstood or used inappropriately (Lambert and Wagner, 2017). Bioplastics are not necessarily biodegradable and not all petroleum-based plastics are nonbiodegradable. Based on degradation mechanisms, plastics may be considered biodegradable, compostable and oxo-degradable (Abdelmoez et al., 2021). Although these terms are often used interchangeably, they are not synonymous. True biodegradability of plastics results from the ability of the polymeric chain to undergo processes of degradation into simple molecules (i.e., H_2O, CO_2, CH_4, and organic matter) under the action of microorganisms (Iwata, 2015). However, biodegradation may require specific conditions of water and temperature, such as those achieved when composting. Oxo-degradable plastics are composed of petroleum-based polymers that easily degraded into smaller fragments by incorporating pro-oxidants additives that are typically transition metals like nickel, iron, manganese, and cobalt (Abdelmoez et al., 2021). However, these smaller fragments are not easily degradable in the environment, raising concerns about their environmental fate (Abdelmoez et al., 2021). Moreover, international standards for biodegradability present multiple limitations for predicting behaviour under natural environments (e.g., testing under unrealistic scenarios; Patrício Silva, 2021).

2. The Plastic Era: From Invention to Ecological Implications

Plastics have become an integral part of our modern society, but their origins can be traced back to the mid-19th century. The first plastic material was based on nitrocellulose and was produced synthetically by Parkes in 1862 and Hyatt in 1866 (Feldman, 2008). The addition of camphor to nitrocellulose led to the first thermoplastic (a modified natural polymer) known as celluloid, which was utilized as a substitute for ivory in billiard balls (Feldman, 2008). Over time, celluloid gained popularity and found extensive use in applications such as photographic film.

In 1907, Leo Baekeland made a significant breakthrough by inventing the first true thermoset plastic called Bakelite. This revolutionary material was created through the polycondensation process of phenol with formaldehyde. Bakelite proved to be incredibly versatile and was introduced commercially in 1909-1910 (Seymour, 1988). It found widespread use in the manufacturing of various products such as telephones, radios, and electrical insulators. The successful application of Bakelite marked the beginning of the synthetic plastic era and the rising of the plastic industry. Not long after, in 1911, Francis Matthews described the thermal and catalytic styrene polymerization to yield a substance able to replace celluloid, glass and hard rubber filed. Additionally, in 1926, Ostramislenski patented the technique of casting flexible film from a solution containing polyvinyl chloride (PVC) and a plasticizer (Seymour, 1989). It was also discovered that PVC could become flexible, resembling rubber or leather, when heated in the presence of a high-boiling-point liquid. Polypropylene (PP) and polyethylene (PE) were invented in the following decades (Andrady and Neal, 2009), further expanding the range of available synthetic plastics. Carothers was the inventor of the first synthetic fiber, known as nylon 66 and revolutionizing the textile industry (Feldman, 2008). Polystyrene (PS) was first manufactured by BASF in the 1930s. Poly(methyl methacrylate) (PMMA) was produced in 1933, for aircraft glazing and for a variety of applications particularly where transparency and/or good weathering resistance is important (Seymour, 1988). Polyethylene terephthalate (PET), known for its excellent barrier properties, was developed in the 1940s and became popular in beverage bottles and food packaging (Feldman, 2008).

During World War II, the importance of plastics escalated significantly due to the scarcity of natural resources. Nylon 66, used both as a fiber and an engineering plastic, played a crucial role in various military applications such as parachutes, ropes, body armour, helmet liners, and more (Feldman, 2008). PVC found particular use in electrical cable insulation and sheathing (Feldman, 2008). The post-World War II period witnessed remarkable advancements in plastics technology, leading to the continued expansion of the plastic industry.

Plastics have become ubiquitous in our lives, making significant contributions across numerous sectors of society. They have revolutionized food packaging, drug delivery, refuse derived-fuel, protection against transmissible diseases, as well as applications in roads, pavements, and other areas (Kumar et al., 2021). Innovative advancements, such as nano-sized polymers, offer new possibilities for drug delivery against deadly diseases, including cancers (Xiao et al., 2018). Condoms have played a vital role in preventing HIV and other sexually transmitted diseases (Beksinska et al., 2020), while polymers scaffolds are engineered for artificial bone and cartilage implants (Shkarina et al., 2018).

In 2021, the global production of plastics scaled to about 390 million tonnes, demonstrating the success of plastic industry (PlasticsEurope, 2022). The packaging sector emerged as the dominant force in the plastics market, accounting for 44% of the production, while building and construction followed closely at 18%. Additionally, the automotive and transportation sector held a 7% share, as did the electrical and electronics, household, leisure, and sports sectors, while agriculture, farming, and gardening accounted for 4% (PlasticsEurope, 2022). The widespread adoption of plastics can be attributed to their relatively low cost, ease of manufacturing, versatility, and water-resistance. Over time, they have gradually replaced traditional materials such as wood, stone, horn and bone, leather, paper, metal, glass, and ceramics (Andrady and Neal, 2009). In **Table 1** is summarized the main produced polymers in the world and some of their properties.

While plastics have revolutionized modern life, plastic waste, on the other hand, has become the most regularly identified litter type in the ocean and a major global concern. Plastic waste accumulates due to overproduction, inappropriate disposal at landfills, and inadequate recycling management (Thompson et al., 2009). Currently, only 9% of the plastic waste is recycled, while 12% is incinerated, and a staggering 79% is either dumped in the environment or landfilled (Geyer et al., 2017). Disposable products used for a short period known as single-use plastics (SUP) contribute significantly to this issue. The COVID-19 pandemic has increased the consumption of single-use plastic, especially personal protective equipment (PPE) such as face masks, gloves, and gowns, as well as plastic used by households to wrap and take food from supermarkets and restaurants, generating a further release of plastics into the environment and therefore into the oceans (Roberts et al., 2022).

The early 1970s marked the initial reports of plastic pollution in the oceans (Carpenter and Smith, 1972). Subsequent findings, such as the extensive accumulation of plastic waste in the North Pacific Gyre, shed light on the magnitude of this issue (Moore et al., 2001). When plastics leak into the environment, they cause severe problems, such as the blockage of waterways, leading to standing water that serves as a breeding niche (to mosquitoes, pests, vector-borne diseases transmission), becomes a vector for toxic chemicals, and ultimately disturbs the natural cycles (biogeochemical cycle in ecosystems) (Kumar et al., 2021). The effects and long-term implications of plastic pollution on marine ecosystems are a global concern. They encompass harm to wildlife through entanglement or ingestion (Deudero and Alomar, 2015), biomagnification (Mattsson et al., 2017), and the release of chemicals accumulated from contaminated environmental sources and additives used in plastic manufacturing (Teuten et al., 2009; Koelmans et al., 2014). Plastic debris exists in various sizes, categorized as mega (>1 m), macro (25 mm – 1 m), meso (5 – 25 mm), and micro (<5 mm) (GESAMP, 2019). Among these, microplastics stand out due to their small size, enabling extensive interactions with organisms and widespread dispersion in natural systems, with limited potential for recovery.

Table 1 - Types of plastic mainly produced in the world and commonly found in the natural environment: density ($g.cm^{-3}$) and common uses (Yuan et al., 2022; Andrady, 2011).

Type of Plastic	Acronym	Density ($g.cm^{-3}$)	Common uses
Polypropylene	PP	0.83-0.85	Reusable food containers and packaging, bottle caps, drinking straws, laboratory equipment, rope, netting
Low-density polyethylene	LDPE	0.91-0.93	Food wrap film, plastic bags, water pipes, six-pack rings, bottles, netting, drinking straws
High-density polyethylene	HDPE	0.94-0.96	Toy, milk bottles, pipes, plastic bags, detergent and oil bottles, cable insulation
Polyethylene terephthalate	PET	1.38–1.41	Plastic beverage bottles, packaging, processed meat packages, peanut butter/jam jars, pillow and sleeping bag filling, textile fibers
Polystyrene	PS	1.04–1.08	Fast food container, disposable plastic cups and lids, foam (i.e., “Styrofoam”), CD crystal cases, service ware, packaging materials, laboratory ware, electronics
Polyamide	PA	1.13-1.35	Fibers, toothbrush bristles, fishing line, under-the-hood car engine moldings, making films for food packaging
Polyvinyl chloride	PVC	1.37-1.39	Plumbing pipes and fittings, cosmetic containers, electrical conduit, wall cladding, roof sheeting, garden hose, blood bags and tubing
Polycarbonate	PC	1.20-1.22	Construction materials, medical equipment, reusable beverage bottles, CDs, DVDs, street and car lights, sky-lights, baby bottles, roofs of greenhouses, glasses lens, water pipes
Polyurethane	PU	0.40–0.60	Upholstery, sports mats, packaging bags.
Acrylonitrile-butadiene-styrene	ABS	1.02–1.08	Automotive applications, pipes
Styrene acrylonitrile	SAN	1.06–1.10	Cosmetic containers, ballpoint pens, lighters
Cellulose Acetate	CA	1.28-1.31	Textiles, cigarette filters

3. Microplastics as persistent and ubiquitous contaminants in marine waters

The concept of microplastics (MP) was proposed by Thompson et al. (2004) to describe the accumulation of microscopic pieces of plastic in marine sediments and in the water column of European waters. Later, Arthur et al. (2009) introduced an upper size limit, defining microplastics as plastic particles smaller than 5 mm. However, there is still ongoing debate about the appropriate definition of microplastics, with only the upper limit being clearly defined. Various lower size limits have been proposed, commonly falling within the range of 1 to 20 μm (Frias et al., 2019). This variation in definitions presents methodological challenges and makes it difficult to compare studies. Nevertheless, the most widely used definition considers microplastics as particles less than 5 mm in their longest dimensions. It has been adopted by organizations such as the Marine Strategy Framework Directive (MSFD) of the European Union and the National Oceanographic and Atmospheric Administration (NOAA) for monitoring and implementation purposes.

Microplastics are classified into two main categories according to their origin: primary microplastics and secondary microplastics (Cole et al., 2011). Primary microplastics refer to intentionally manufactured microparticles. These primary microplastics include pre-production resin pellets used in the manufacturing of plastic products, industrial abrasives employed for delicate surface treatments and microbeads found in personal care products such as facial cleansers, toothpaste, shower gels, scrubs, peelings, eye shadow, deodorants, blush powders, makeup foundation, mascara, shaving cream, baby products, bubble bath lotions, hair colouring, nail polish, insect repellents, and sunscreens (Auta et al., 2017; Cole et al., 2011; Fendall and Sewell, 2009). Furthermore, microplastics have been increasingly used in medicine as carriers for pharmaceuticals (Auta et al., 2017). Air blasting technology is another application of microplastics, wherein acrylic or polyester microplastic scrubbers are employed to remove rust and paint from machinery, engines, and boat hulls (Auta et al., 2017). Primary microplastics enter the aquatic environment through household sewage discharge, air-blasting technology, or accidental release during transportation and processing (Hale et al., 2020).

Secondary microplastics are formed through the fragmentation and weathering of larger plastics during usage (e.g., textiles, paints, and tires) or after disposal. Weathering is predominantly instigated by exposure to solar ultraviolet radiation and subsequent autocatalytic thermal oxidation, while the fragmentation process likely arises from mechanical forces acting on weathered plastics (Andrady, 2017). The weathering of plastics primarily leads to the accumulation of degradation byproducts, manifesting as yellowing and an augmentation of oxygenated components like aldehydes, carboxylic acids, and ketones (da Costa et al., 2018). These chemical changes introduce carbonyl and hydroxyl functional groups into the polymer structure, serving as indicators to determine the relative levels of surface oxidation and relative weathering of specific polymers (Rodrigues et al., 2018). Additional reactions such as hydrolysis, biodegradation, and complete breakdown of plastics into smaller molecules like H_2O and CO_2 are remarkably gradual processes (Andrady, 2017). The propensity of microplastics to continuously fragment contributes to an anticipated increased number of smaller particle sizes (Hale et al., 2020). In comparison to primary microplastics, the prevalence of secondary microplastics is notably higher within the environment. The process of large plastic items deteriorating into microplastics is particularly active along shorelines due to the synergistic effects of intense ultraviolet radiation from the sun, abundant oxygen, and physical abrasion caused by waves, sediment particles, and wind (Andrady et al., 2022). Conversely, in specific aquatic environments like the deep sea, where ultraviolet radiation exposure is reduced and temperatures are low, the process of weathering tends to be slower.

Land-based sources account for the majority of microplastics found in the marine environment, comprising approximately 80 to 90 percent of the pollution (Andrady, 2011). Rivers serve as pathways for transporting microplastics from inland areas to the ocean (Lebreton et al., 2017). Plastic debris from municipal drainage systems, sewage effluents, and improper management of inland areas can end up in the sea through rivers (Yang et al., 2021). Moreover, atmospheric fallout may be another source of synthetic fibers in the marine environment (Dris et al., 2016). It is assumed that these fibers in the atmosphere come from several sources, including clothes and houses, degradation of macroplastics, and landfills or waste incineration (Dris et al., 2016). Because of their light weights, microplastics can be transported by the wind to the marine environment (Dris et al., 2016). Despite this, ocean-based sources still contribute around 10 to 20 percent of the marine microplastic pollution. Microplastics originate from seaside tourism, commercial fishing, marine vessels, and offshore industries (Yang et al., 2021). Discarded or lost fishing gear, such as plastic monofilament lines and nylon nets, constitutes a significant source of microplastics scattered at various ocean depths (Yang et al., 2021). Furthermore, microplastic waste from shipping and naval vessels, along with plastic waste generated by offshore industries, particularly petrochemicals, infiltrates marine ecosystems and worsens the problem (Yang et al., 2021).

Independent of the source, microplastics constitute the largest portion of plastic pollution in the marine environment, accounting for about 92% of global marine plastic waste (Eriksen et al., 2014). These microplastics display variations in composition, colour, size, shape, density, and other characteristics (**Figure 1**) that along with hydrodynamic conditions and biofouling influence microplastics fate in marine ecosystems (Long et al., 2015; Kowalski et al., 2016; Kaiser et al., 2017). Once released into the ocean, the environmental fate of microplastics primarily depends on the polymer density (**Table 1**) which influences buoyancy, position in the water column and the consequent possible interaction with biota (Wright et al., 2013). Polyethylene (PE) and polypropylene (PP), which have densities lower than water (1.02 g.cm^{-3}), are typically found on the water's surface and in the neustonic environment (Sebille et al., 2020). On the other hand, polymers with higher density, like polyvinyl chloride (PVC) and polyester, tend to sink to the seafloor (Sebille et al., 2020). Additionally, polymer density can change over time due to degradation, biofouling attachment or incorporation into organic aggregates and these changes are likely to impact the transport dynamics of MP in seawater (Laursen et al., 2022; Bagaev et al., 2017; Kaiser et al., 2017). The density of MP can also be altered by degradation, fragmentation, and leaching of additives, impacting their distribution within the water column.

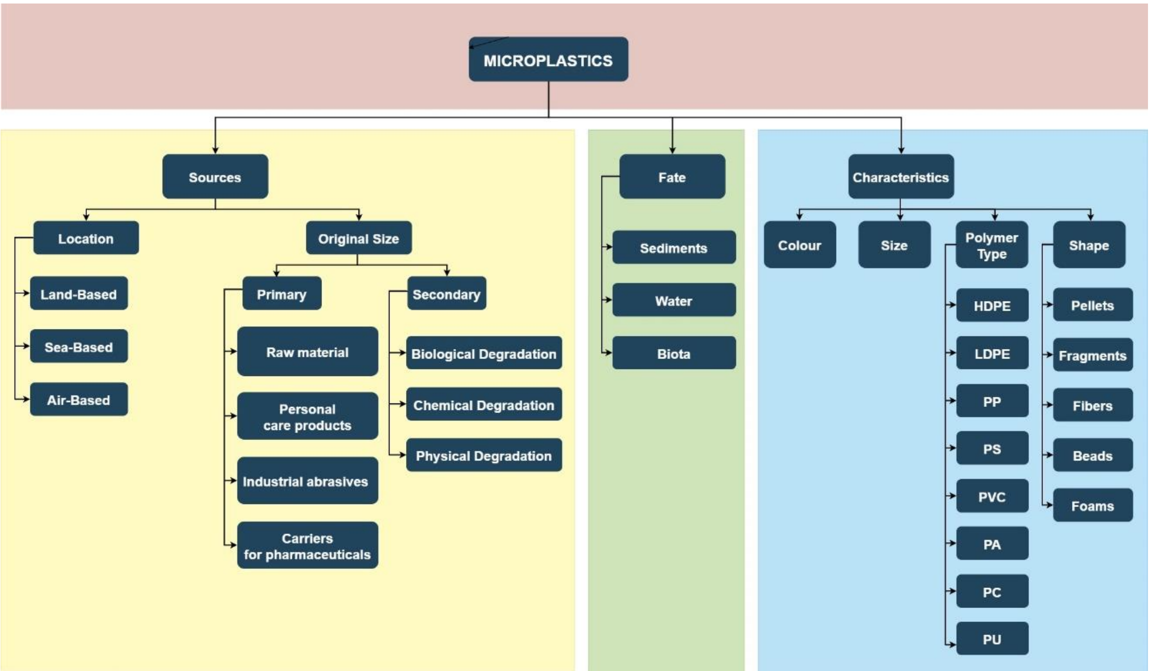

Figure 1 - Key characteristics of microplastics.

The dispersion of microplastics in marine environment is additionally influenced by both anthropogenic and environmental factors. The presence of microplastics is intricately linked to human actions, with coastal regions characterized by dense populations, proximity to industrial sites, and the influx of river discharges (Andrady and Neal, 2009). Once in the ocean, the small size and low density of microplastics contribute to their widespread transport across long distances by ocean currents (Cole et al., 2011). Oceanic transport can move buoyant microplastics to distant shorelines or entrained particles can accumulate in central ocean regions (e.g., Law et al., 2010). Microplastic distribution is further influenced by wind which can promote the vertical movement of particles within the water column (Kukulka et al., 2012). Wind, surface currents and geostrophic circulation drive the dispersal patterns of microplastics at large scales (Law et al., 2010). Conversely, at smaller scales, MP may be confined by turbulent benthic boundary layer currents and thermohaline gradient or transported by underlying currents (Bagaev et al., 2017). Deep currents may also resuspend the sinking microplastics, preventing them from settling to the seafloor and keeping them suspended in the water column (Bagaev et al., 2017). Additionally, a number of oceanic phenomena frequently facilitate sedimentation processes. For example, dense shelf water cascading, which involves the sinking of dense water masses along continental shelves, can transport MP to greater depths (Kane and Clare, 2019). Similarly, severe coastal storms and offshore convection can also play a role to the settling and distribution of MP (Kane and Clare, 2019).

Microplastics are found extensively across marine environments, being widely distributed throughout various ecosystems on a global scale (Auta et al., 2017; Akdogan and Guven, 2019; Adam et al., 2021; Yang et al., 2021). When it comes to seawater, Adam et al. (2021) global overviewed that about 89% of the reported microplastic concentrations range from 10^{-2} to 10^{4} microplastics per cubic meter ($MP.m^{-3}$). In some instances, higher concentrations have been identified in specific regions of the Pacific, Atlantic, and Indian oceans. These three major water bodies also exhibit the widest variability in concentrations. Measurements conducted in the Mediterranean Sea show a common range of concentrations between 10^{-2} and 10 $MP.m^{-3}$. The highest reported value was 129 $MP.m^{-3}$ following a flooding event along the Turkish coast. Examining the Pacific Ocean, concentrations span between 10^{-3} and 10^{5} $MP.m^{-3}$, with 74% of the samples taken in this region containing more than 1 $MP.m^{-3}$, indicating it as the most heavily polluted water body in terms of microplastic presence. The Atlantic Ocean data encompassed samples from diverse areas, including European, African, North American, and South American waters, as well as the North Atlantic subtropical gyre.

Concentrations typically fall within the 10^2 to 10^4 $MP.m^{-3}$ range, with an average concentration of 3.6×10^3 $MP.m^{-3}$. Turning to the Arctic Ocean, many samples showed no microplastics, and approximately 79% of the reported concentrations range between 0.23 and 100 $MP.m^{-3}$. The highest recorded value was 375 $MP.m^{-3}$, which could be attributed to factors like melting sea ice or point sources such as ship wastewater discharge. The data in this paragraph is exclusively sourced from Adam et al. (2021), encompasses estuarine samples as well and is illustrated in **Figure 2**.

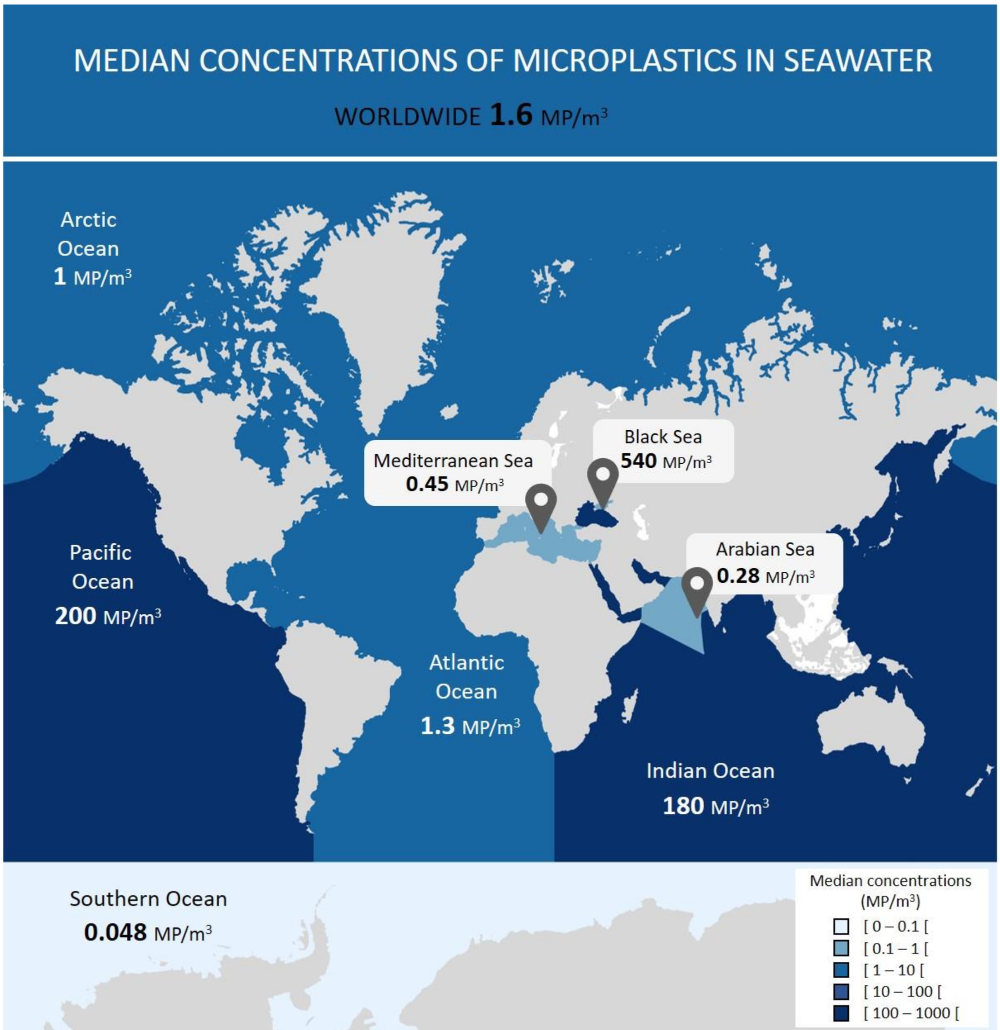

Figure 2 - Global distribution of microplastic concentrations in seawater, based on data from Adam et al. (2021).

4. Adverse effects of microplastics on biota

As oceans and seas become increasingly contaminated with microplastics, marine organisms are exposed to the physical and chemical properties of these small plastic particles (Wright et al., 2013). There are two types of chemicals present in microplastics; (i) additives and raw materials from plastics; (ii) chemicals that are adsorbed from the environment (Campanale et al., 2020). Additives are intentionally included during plastic production to enhance qualities such as colour, transparency, and performance, making plastics more resistant to degradation by factors like ozone, temperature, and light. They include inert or reinforcing fillers, plasticizers, antioxidants, UV stabilizers, lubricants, dyes, and flame retardants (EC, 2012). These additives are usually not chemically bonded to the plastic polymer. Microplastics have a variety of physical and chemical attributes that allow interaction with existing contaminants in the marine environment. Due to their hydrophobic nature, microplastics attract hydrophobic pollutants, including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), dichloro-diphenyl-trichloroethane and its metabolites (DDTs), polybrominated diphenyl ethers (PBDEs), alkylphenols and bisphenol (Agboola and Benson, 2021). Microplastics also demonstrate unique sorption behaviour towards heavy metals, with respect to surface charge and area (Guo and Wang, 2019). In an aquatic environment, pH and ionic strength significantly influence the process of adsorption/desorption (Tang et al., 2020; Zou et al., 2020), as these variables determine the reactivity and charge state of polymer surfaces. The rate of sorption is specific to polymer type and contaminant chemistry, with polystyrene and polyethylene demonstrating higher sorption capacities (Turner and Holmes, 2015; Liu et al., 2018). Surface roughness enhances sorption, especially in older plastics, both in the laboratory and natural conditions (Turner and Holmes, 2015; Liu et al., 2019). Sorption capacity is also influenced by surface area, with smaller microplastics of the same polymer type showing greater sorption capacity due to their increased surface area (Liu et al., 2018).

Microplastics also serve as a substrate for a wide range of microbial communities, leading to the formation of biofilms coined as plastispheres by Zettler et al. (2013). Their study identified microplastics as potential hosts for harmful bacteria such as *Vibrio* spp., which pose significant risks to marine food chains (Zettler et al., 2013). Moreover, Maso et al. (2003) observed the presence of potential harmful dinoflagellates, including *Ostreopsis* sp. and *Coolia* sp., along with resting cysts of unidentified dinoflagellates and both temporary cysts and vegetative cells of *Alexandrium taylori*. This suggests that microplastics may contribute to the proliferation of harmful algal blooms and facilitate the dispersion of marine species (Andrady, 2011). While natural substrates like macroalgae provide habitats for microorganisms, microplastics exhibit a more efficient dispersal mechanism, potentially increasing the distribution of invasive species (Barnes and Milner, 2005).

Marine organisms across many trophic levels interact with microplastics through various pathways, including contact, ingestion, and respiration. One extensively examined interaction is microplastic ingestion (reviewed in Campanale et al., 2020; Lusher et al., 2017). In aquatic environments, organisms may ingest microplastics directly from their surrounding environments due to their inability to differentiate between microplastics and their preys (Wright et al., 2013). Alternatively, they can indirectly ingest microplastics by consuming organisms that are already contaminated with these tiny particles (Wright et al., 2013). The adverse effects of microplastic ingestion have been reported in all the different levels of the aquatic food web, from primary producers to top predators. For example, microplastics retained within the gastrointestinal tracts of fish can result in significant physical abrasions and perforations, leading to reduced nutrient absorption and diminished feeding activity due to a false sense of satiety (Jovanović, 2017; Wright et al., 2013). Furthermore, microplastic intake can induce anatomical and functional alterations in the digestive tracts of fish, leading to dietary and developmental challenges (Huang et al., 2022; Jabeen et al., 2018; Peda et al., 2016). Controlled laboratory experiments have revealed a range of ecotoxicological effects in response to microplastics exposure. For instance, these effects encompass reduced feeding rates, slowed growth, decreased oxygen consumption, and alterations in tunicate development (Paffenhöfer et al., 2020; Messinetti et al., 2018). Mussels and crabs subjected to microplastics have displayed changes in oxidative stress enzymes activity, immune responses, increased DNA damage, and disruptions in amino acid metabolism (Hariharan et al., 2021; Cappello et al., 2021; Horn et al., 2021; Wang et al., 2021; Revel et al., 2019). Zooplankton that consume microplastic particles often suffer from intestinal damage, delayed growth, impaired feeding, and changes in behaviour, reproduction, and the development of their offspring (Botterell et al., 2019).

Notable behavioural changes have been observed in various marine organisms during these experiments. For instance, beach hoppers (*Platorchestia smithi*) exhibited weight loss and decreased jumping heights (Tosetto et al., 2016), zebrafish (*Danio rerio*) displayed altered daily activity rhythms (Limonta et al., 2019), European seabass (*Dicentrarchus labrax*) showed reduced swimming velocity (Barboza et al., 2018), and jacopever (*Sebastes schlegelii*) exhibited prolonged foraging times, reduced food sensitivity, and decreased swimming speeds (Yin et al., 2018). Changes in pulsation frequency and immobility were identified in *Aurelia* sp. jellyfish (Costa et al., 2020). Similarly, reduced swimming capability and diminished predatory prowess were observed in mysid shrimp (*Neomysis japonica*) (Wang et al., 2020).

Furthermore, the toxic effects of microplastics could be enhanced by the adsorbed chemicals. These chemicals linked to microplastics have been documented to induce cellular toxicity (Rochman, 2015) and have adverse impacts on fish populations (Guven et al., 2018). They can also deplete energetic reserves in lugworms and shore crabs (Besseling et al., 2019; Watts et al., 2015; Wright et al., 2013), influence metabolic rates and survival in Asian green mussels (Rist et al., 2016), and affect growth, development, and survival in *Daphnia* sp. under laboratory conditions (Ogonowski et al., 2016). Moreover, after exposure to microplastics, marine copepods, such as *Centropages typicus*, exhibit significant reductions in their algal consumption, ultimately impacting their reproductive capacity and overall survival (Cole et al., 2015).

Microplastics have significant impacts on marine ecosystems, disrupting not only marine organisms but also the overall ecological balance. Notably, biomagnification has been observed in various fish and other species positioned higher in the food chain. For instance, research by Boerger et al. (2010) revealed microplastics in planktivorous fish, which subsequently biomagnified in larger predatory species that preyed on these fish. In the Mediterranean Sea, biomagnification has been documented in bluefin tuna, albacore tuna, and swordfish, as reported by Romeo et al. (2015). Nelms et al. (2019) demonstrated the trophic transfer of microplastics from mackerel (*Scombrus scombrus)* to grey seals (*Halichoerus grypus)*. Biomagnifications from marine species to humans could occur in the same way. However, despite some research efforts in this field, they remain relatively limited. The extensive complexities of biomagnification and trophic transfer cannot be comprehensively understood with the current scope of investigations. Consequently, further research in this domain is imperative.

Finally, while laboratory experiments have revealed numerous adverse effects, the debate surrounding the risks posed by microplastics in real-world environments persists. This debate is powered by disparities between the characteristics of microplastics typically used in toxicology studies and those observed in natural environment (Lenz et al., 2016). Usually, microplastics employed in toxicological studies are smaller, possess more regular shapes, and are in higher concentrations compared to those found in field-collected samples. Consequently, risk assessments based solely on toxicological study results may not be directly applicable. According to existing literature, the available evidence does not conclusively support the assertion that microplastics at environmental concentrations induce adverse effects on individual organisms (Agathokleous et al., 2021). However, some researchers argue that the environmental concentrations of microplastics may be underestimated due to limitations in analytical methods, particularly when quantifying small microplastics (Ivleva, 2021). These undetected microplastics are believed to be more prevalent than their detected counterparts (Conkle et al., 2018), potentially directly impacting the accuracy of risk assessments. Furthermore, the methodology for assessing the risks of microplastic pollution is controversial among scientists (Koelmans et al., 2017). Traditional risk assessment methods developed for conventional pollutants such as heavy metals and organic chemicals may not be directly transferable to microplastics due to differences in their mechanisms of action and forms of existence.

5. Methodologies for monitoring microplastics in marine environment

Methodological choices play a significant role in determining environmental microplastic concentrations. Currently, many different methods are used for sampling, extraction, and analysis of microplastics (**Figure 3**) and there is a lack of procedure standardization although a great effort has been done in this direction in recent years (Frias et al., 2018; Bessa et al., 2019; Gago et al., 2019; GESAMP, 2019). The diversity of methods used in microplastic research difficult direct comparisons between study outcomes.

Plankton nets, which are frequently utilized for seawater microplastic sampling, offer multiple towing options—horizontal, vertical, and oblique (Baini et al., 2018; Collignon et al., 2014). The main advantage of net sampling is that large volumes of water can be sampled relatively quickly (Gago et al., 2019). Their limiting factor is the mesh size that can strongly affect the size spectrum of collected particles. Mesh sizes commonly used are around 333 or 335 μm but they can vary widely from 50 to 3000 μm (Hidalgo-Ruz et al., 2012). These differences can lead to significantly divergent results in terms of microplastic concentrations. For instance, concentrations determined using a 100 μm net were found to be a hundred times higher than those observed with a 333 μm manta trawl (Vermaire et al., 2017). Similarly, the limitation posed by mesh size can result in an underestimation of microplastic counts due to the exclusion of smaller particles. A study conducted by Barrows et al. (2017) revealed significantly higher microplastic concentrations using bulk water methods, indicating both increased counts and a lower size limit when compared to net-based sampling. Bulk water sampling devices such as Niskin bottles, rosettes, and integrating water samplers provide a comprehensive method of collecting microplastics across various size ranges. Yet, their drawback lies in the sampling of relatively smaller water volumes, which might affect the overall representativeness of the findings (Cutroneo et al., 2020).

In the laboratory, most environmental samples require additional processing steps to remove organic matter or separate plastic and non-plastic particles. However, these additional steps can extend the processing time and potentially introduce the risk of particle loss and contamination. Therefore, the standardization and rigorous assessment of techniques for microplastics extraction of complex matrices are imperative.

To isolate plastics from samples, density-based liquids have emerged as a method with varied applications across different sample types (Bessa et al., 2019; Gago et al., 2019; Frias et al., 2018). In essence, this technique involves mixing a known-density saturated salt solution with a sample, allowing settling, and then collecting the overlying material for further analysis. This density extraction method proves particularly effective in separating plastics from environmental samples, especially marine sediments due to their higher density compared to most polymers. The adjustment of solution density allows for the selective collection of heavier polymers, with solutions possessing a specific density exceeding 1.2 $g.cm^{-3}$ being frequently employed (Gago et al., 2019). While sodium chloride (NaCl) is commonly preferred for its cost-effectiveness and environmentally friendly method (Gago et al., 2019), an evaluation of various salt solutions demonstrated that NaCl had the lowest recovery rate of microplastics among the tested options (Quinn et al., 2017). Higher-density solutions like sodium bromide (NaBr) or zinc chloride ($ZnCl_2$) proved more versatile in extracting a broader range of plastic polymers (Quinn et al., 2017).

In many cases, researchers employ digestion to facilitate the isolation of microplastics from matrices with organic matter. Digestion has gained distinction in recent years as the predominant method for extracting microplastics from biota tissues (Bessa et al., 2019). Moreover, digestion can also be applied to sediments and water samples containing particulate organic matter (Gago et al., 2019; Frias et al., 2018). Combining digestion approaches with density separation can further enhance the efficiency of sample extraction. Various digestion methods have been developed, including the use of bases such as sodium hydroxide (NaOH; Karami et al., 2017; Dehaut et al., 2016) or potassium hydroxide (KOH; Thiele et al., 2019; Karami et al., 2017; Dehaut et al., 2016); simple and/or mixtures of acids such as nitric acid (HNO_3), hydrochloric acid (HCl), and perchloric acid ($HClO_4$) (ICES, 2015; Van Cauwenberghe et al., 2015; Claessens et al., 2013); oxidants like hydrogen peroxide (H_2O_2) and peracids (Avio et al., 2015; Nuelle et al., 2014); and enzymatic digestion (von Friesen et al., 2019; Catarino et al., 2017; Cole et al., 2014). However, each digestion method has its own advantages and disadvantages. Acids can lead to substantial destruction of biogenic compounds, ranging between 94–98%, but they can also dissolve polymers (Enders et al., 2016; Claessens et al., 2013). Potassium hydroxide (KOH) at a 10% solution can completely remove organic matter, and various adaptations of this procedure exist, including incubation at room temperature for several weeks or elevated temperatures (40°C or 60°C) (Karami et al., 2017; Dehaut et al., 2016). However, increased temperatures and molarity can lead to discoloration and degradation of certain plastic polymers, such as polycarbonate, cellulose acetate, PET, and PVC (Thiele et al., 2019; Karami et al., 2017; Dehaut et al., 2016). Hydrogen peroxide (H_2O_2) serves as an efficient oxidizer for removing organic material. Nonetheless, the use of a 30% solution of hydrogen peroxide has been associated with polymeric changes, including colour alterations and size reduction (Karami et al., 2017). Sometimes, its application has even led to foam formation and reduced extraction efficiency (Karami et al., 2017). Enzymatic digestion protocols may be preferential due to the biological specificity of enzymes. However, using enzymatic digestion to target specific types of organic matter needs either a comprehensive understanding of the organic matter present in the matrix or a combination of multiple enzymatic digestions for effective results. The use of Proteinase-K as an enzymatic protocol has been most widely applied, but certain biological materials, such as shells, carapaces and wood are not broken down by Proteinase-K (Cole et al., 2014). Given the potential variability in extraction efficiencies, all methods should be tested in laboratory settings both before and during their application, as efficiency outcomes can differ based on the individuals performing the tasks. Therefore, the development of methods that incorporate automated separation and analysis processes, thereby minimizing human errors and the introduction of contaminants, is an urgent necessity.

Various techniques are available for microplastic identification and characterization. Early microplastics studies primarily relied on visual identification. Visual identification involves observing potential microplastics on filters with the naked eye or a microscope. Different microscopes, including fluorescence (Cai et al., 2018), dissection (Sagawa et al., 2018), optical (Bagaev et al., 2018), electron (Leslie et al., 2011), stereo (Zobkov et al., 2019), binocular (de Lucia et al., 2014), inverted (Gorokhova, 2015), and vertical (Setälä et al., 2016) were utilized. Microscopes may be equipped with a camera, and therefore, microplastics can also be photographed and subsequently analysed (Lopes et al., 2020). Three criteria were established for visual microplastics recognition: particles should lack visible cellular or organic structures, fibers should maintain consistent thickness, and particles should display uniform coloration (Hidalgo-Ruz et al., 2012).

Meeting these criteria defines particles as plastic (Hidalgo-Ruz et al., 2012). Melting and hot needle tests can further assess if observed particles are plastic (Enders et al., 2015). However, these tests may compromise particles and are typically used on uncertain particles. For enhanced microplastics identification, artificial colours can be used, with Nile Red being a commonly employed fluorescent dye (Meyers et al., 2022). Nonetheless, visual identification has limitations, notably in terms of observational errors and misclassifications. Even when counting under a microscope, disparities can emerge among different users, especially when dealing with particles smaller than 500 µm, where the risk of misidentification is elevated due to subjectivity (Hidalgo-Ruz et al., 2012).

Currently, the prevalent techniques employed for the chemical characterization analysis of microplastics encompass scanning electron microscope-energy-dispersive X-ray (Wang et al., 2017), Fourier transform infrared spectroscopy (FTIR; Lopes et al., 2020), Raman spectroscopy (Araujo et al., 2018), thermal analysis (Majewsky et al., 2016), pyrolysis gas chromatography mass spectrometry (Py-GC/MS; Hendrickson et al., 2018), and liquid chromatography (Wang et al., 2017). Among these techniques, spectroscopic methods such as FTIR and Raman spectroscopy are particularly popular (Xu et al., 2019). They identify microplastic particles by analysing their unique vibrational spectrum, specific to each polymer type (Xu et al., 2019). Moreover, these spectroscopic methods can be integrated into microscopic setups, enabling chemical imaging. Chemical imaging, which combines digital imaging with spectroscopic measurements, offers several advantages over other analytical methods. Firstly, it is non-destructive and may be non-contact, allowing for multiple results to be collected from various instruments (Xu et al., 2019). Secondly, chemical imaging provides enriched information on spatial and spectral features, facilitating the characterization of morphology and chemical composition. This is especially valuable considering that the analysis of microplastics encompasses multiple dimensions, including the chemical composition, size, shape, colour of individual particles, and the abundance of each polymer type within a sample. Additionally, there is potential to develop an automated pipeline for spectroscopic analysis, which is more efficient, and labour-saving compared to other methods. Raman spectroscopy is a preferred choice for small microplastics due to its higher size resolution (Xu et al., 2019). It can detect particles as small as 1 µm and even smaller (approximately 300 nm) when utilizing µ-Raman imaging (Xu et al., 2019). On the other hand, FTIR has a less precise size resolution, as it can only detect particles down to a size of 10-20 µm (Xu et al., 2019). This limitation arises from the diffraction-limited resolution of infrared spectroscopy, which prevents the analysis of smaller particles. It's worth noting that samples with thicknesses less than approximately 5 µm, such as films or fibers, may struggle to produce sufficient absorbance for interpretable spectra when using FTIR. However, nanoscale IR analysis can be achieved by combining with atomic force microscopy (AFM), specifically using scattering scanning near-field optical microscopy (s-SNOM; Knoll and Keilmann, 2000) and photothermal-induced resonance (PTIR; Lahiri et al., 2013).

Finally, accurate determination of environmental concentrations can only be achieved under strict contamination control procedures, which need to be more widely adopted. Only high-quality data on environmental concentrations of microplastics can be reliably used to determine impacts on ecosystems.

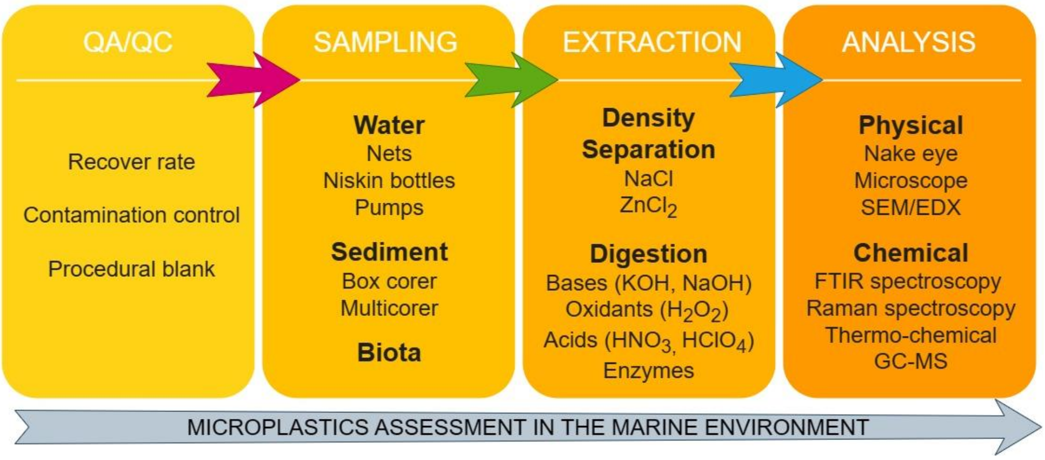

Figure 3 - Flow diagram with the procedure methods for microplastics sampling, extraction and analysis from marine environment.

6. Acting Against Plastic Pollution: EU Initiatives and Measures

Plastic pollution has become a worldwide sustainability crisis. Recognizing the global nature of this problem, an increasing number of international agreements and frameworks are laying the foundation for international efforts to address plastic-related issues. As an example, in 2015, all United Nations Member States embraced Agenda 2030, providing a shared standpoint for peace and prosperity for people and the planet. Agenda 2030 includes several targets of relevance to plastics, notably within Sustainable Development Goal (SDG) 12 to ensure sustainable consumption and production patterns, and SDG 14 to conserve and sustainably use the oceans, seas, and marine resources for sustainable development (United Nations, 2015). Additionally, the 2016 United Nations Environment Assembly (UNEA-2) Resolution 2/11 on Marine plastic litter and microplastics invited all countries “in cooperation with industry and other stakeholders, at the national, sub regional, and international levels, to organise and/or participate in annual campaigns for awareness-raising, prevention and environmentally sound clean-up of marine litter" (Watkins and Schweitzer, 2018). The United Nations Regional Seas Programmes (Programmes cover the Mediterranean, North-East Atlantic, the Baltic Sea Area, the Wider Caribbean Region, the Northwest Pacific Region, the Black Sea Area, and the South Pacific) also contain measures designed to reduce and prevent marine litter (Watkins and Schweitzer, 2018). Furthermore, the operational framework of the G20 Action Plan on Marine Litter recognizes the necessity of addressing pollution from both land and sea-based sources (Watkins and Schweitzer, 2018). The measures identified involve promoting the socioeconomic benefits of policies to prevent marine litter, advocating for waste prevention and resource efficiency, and supporting sustainable waste management initiatives.

The legislative framework of the European Union operates on an intergovernmental level but is put into action and enforced at the national level. The dominant piece of legislation governing the marine environment is the Marine Strategy Framework Directive (MSFD). The objective of MSFD is reaching Good Environmental Status (GES) for European seawaters. Descriptor 10 specifically addresses litter, including plastics and microplastics, in the coastal and marine environment and the Technical Subgroup on Marine Litter (TG-ML) has made a significant contribution by identifying the need for a harmonized monitoring approach across all Member States, as well as various short-term research priorities (Galgani et al., 2010). As a result, there has been a surge in research to understand the characteristics, drivers, and processes of litter at sea (Maes et al., 2019). Furthermore, Member States monitor marine litter beyond the MSFD (Gago et al., 2016) at regional seas level within programs under the OSPAR, HELCOM and Barcelona Conventions (Chen, 2015).

Various directives and regulations within the European Union (EU) have a specific focus on polymers and plastic manufacturing. These measures serve to protect the environment and the well-being of EU citizens. In 2008, a significant milestone was reached with the approval of Directive 2008/98/EC (European Parliament, 2008), which established minimum requirements for extended producer responsibility schemes (EPR). These schemes expanded manufacturers' responsibilities across a product's lifecycle, encompassing take-back, recycling, and disposal. Essentially, EPR shifts environmental costs from taxpayers to producers, aligning with the 'polluter pays' principle. Additionally, in 2008, the EU waste framework directive (WFD) (Waste Framework Directive, 2008) and its 2018 amendment (Directive 2018/851) outlined a strategy to reduce waste, limit landfilling, promote innovative waste management and recycling technologies, and encourage sustainable practices throughout society. The goal is to prevent waste and turn it into a resource for a greener, more circular economy.

In 2015, the Directive 2015/720/EU (European Parliament, 2015), required Member States to reduce the use of lightweight plastic carrier bags (those with a thickness below 50 microns). Member States have flexibility in choosing measures, such as setting reduction targets, using economic incentives, or implementing marketing restrictions. These measures must achieve two goals: 1) limit annual consumption to under 90 bags per person by 2020 and 40 by 2026, and 2) prohibit free distribution of these bags at the point of sale unless equally effective alternatives are in place before 2019.

In 2018, the EC presented their European Strategy for Plastics in a Circular Economy (European Commission, 2018), which aimed at laying the foundations to a new plastics economy, where the design and production of plastic products fully respect reuse, repair and recycling needs and more sustainable materials are developed and promoted.

In 2019, the EU adopted Directive 2019/904 (European Parliament, 2019), known as the Single Use Plastic Directive (SUP Directive), which includes several ambitious measures: 1) Bans certain single-use plastic products with alternatives, like cotton buds, cutlery, plates, stirrers, balloon sticks, cups, food containers, and beverage containers made of expanded polystyrene, as well as all oxo-degradable plastic products; 2) Implements reduction measures for plastic food and beverage packaging, along with specific marking and labelling requirements; 3) Introduces EPR schemes, covering the cleanup costs of waste from items like tobacco filters, food containers, beverage cups, lightweight plastic bags, wet wipes, and balloons; 4) Sets targets for plastic bottle collection rates, design standards for bottle caps, and requirements for recycled plastic content in bottles; 5) Establishes monitoring and reporting rules for plastic fishing gear, with plans for an EPR scheme in the future. The European Commission encourages Member States to create a unified standard for circular fishing gear design to promote reuse and recycling.

In 2020, the European Commission presented its Circular Economy Action Plan (European Parliament, 2020a), one of the main pillars of the European Green Deal, which builds upon the 2018 European Strategy for Plastics. The Action Plan focuses on increasing recycled plastics, introducing new requirements for recycled content, and addressing microplastics, bio-based, and biodegradable plastics. As a result, the European Chemicals Agency (ECHA) drafted a restriction proposal in early 2019 to limit intentionally added microplastics. This proposal aims to reduce microplastic releases by

70 to 95%, equivalent to 400,000 to 500,000 tonnes over 20 years. Importantly, it doesn't seek to restrict polymers in general but aims to restrict the market placement of microplastics, introduce labelling for certain uses, and establish reporting requirements to ensure better risk assessment.

In the same year, the European Commission also enacted regulations concerning the export, import, and intra-EU shipment of plastic waste (European Parliament, 2020b). These rules banned the export of plastic waste from the EU to non-OECD countries, except for clean plastic waste intended for recycling. Exporting plastic waste from the EU to OECD countries and imports in the EU will also be more strictly controlled.

Considering the importance of marine litter originating from marine sources, such as abandoned fishing gear, which can have particularly harmful effects on marine animals, the European Commission published a decision on May 31, 2021, establishing models for reporting data and information on fishing gear placed on the market and fishing gear waste collected in Member States (European Commission, 2021). The Port Reception Facilities Directive (European Parliament and Council of the European Union, 2019) aims to effectively address marine litter from ships, including fishing vessels. With this legislation, the delivery of all waste generated on board vessels, all waste caught in passive fishing gear, and end-of-life fishing gear becomes mandatory. The law also provides for the existence of densification units for the disposal of expanded polystyrene, or other suitable systems for the disposal and storage of polystyrene waste/materials. Moreover, several measures to reduce marine litter have also been incorporated into the Fisheries Control Regulation under the Common Fisheries Policy.

Despite these commendable initiatives, challenges remain in aligning the diverse interests of stakeholders, policymakers, industries, and society to effectively combat plastic pollution. Achieving a shared understanding of potential scenarios and consequences is crucial to foster convergence and collective action in the ongoing battle against plastic pollution, safeguarding both the environment and human well-being.

7. Aims of this Thesis

This thesis addresses the pressing issue of marine microplastic pollution and its pervasive presence, which have become a global concern. Despite growing awareness of their ecological impact and the establishment of legal frameworks for their control and monitoring, significant knowledge gaps persist. These gaps encompass the mechanisms of microplastic uptake by marine organisms, the distribution of microplastics within different body tissues, and the limited and contentious information regarding microplastic characteristics and quantities in pelagic marine ecosystems. Therefore, this doctoral thesis aimed to achieve the following specific objectives:

1- Optimization of a methodology for extracting microplastics from the gastrointestinal tracts of high-fat fish species (Chapter 2).

2- Assessment of the distribution of microplastics in the gastrointestinal tract, gills, and muscle of pelagic fish (Chapter 3).

3- Determination of the correlation between microplastic accumulation in pelagic fish and the presence of microplastics in marine water (Chapter 3).

4- Determination of the relationship between microplastic ingestion by pelagic fish and their dietary composition (Chapter 4).

5- Assessment of the seasonal variation of microplastic ingestion by pelagic fish (Chapter 5).

6- Identification of the most suitable fish species as a bioindicator candidate for monitoring microplastics in the pelagic marine environment (Chapter 3-5).

By addressing these objectives, this research enriches our comprehension of microplastic pollution. The acquired information enables the alignment of toxicity studies with real-world scenarios, thus improving the precision of risk assessments. Moreover, the insights gained from this thesis play a pivotal role in informing environmental management policies and in advancing monitoring programs.

8. Thesis publications

The current dissertation is composed by the following published articles in international scientific journals:

Lopes, C., Fernández-González, V., Muniategui-Lorenzo, S., Caetano, M., Raimundo, J. (2022). Improved methodology for microplastic extraction from gastrointestinal tracts of fat fish species. Marine Pollution Bulletin 181, 113911. https://doi.org/10.1016/j.marpolbul.2022.113911 **(Chapter 2)**

Lopes, C., Ambrosino, A.C., Figueiredo, C., Caetano, M., Santos, M.M., Garrido, S., Raimundo, J. (2023). Microplastic distribution in different tissues of small pelagic fish of the Northeast Atlantic Ocean. Science of the Total Environment 901, 166050. https://doi.org/10.1016/j.scitotenv.2023.166050 **(Chapter 3)**

Lopes, C., Raimundo, J., Caetano, M., Garrido, S. (2020). Diet composition and microplastic ingestion by planktivorous fish. Limnology and Oceanography Letters 5, 103-112. https://doi.org/10.1002/lol2.10144 **(Chapter 4)**

Lopes, C., Figueiredo, C., Baptista, M., Caetano, M., Santos, M.M., Raimundo, J. (2023). First evidence of microplastic ingestion in the ocean giant sunfish (Mola mola). Marine Environmental Research 190, 106064. https://doi.org/10.1016/j.marenvres.2023.106064 **(Chapter 5)**

Chapter 2

Improved methodology for microplastic extraction from gastrointestinal tracts of fat fish species

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Improved methodology for microplastic extraction from gastrointestinal tracts of fat fish species

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ABSTRACT

Potassium hydroxide (KOH) digestion protocols are currently applied to separate microplastics from biological samples, allowing efficient digestion with minor degradation of polymers in a time- and cost-effective way. For biota samples with high-fat content, KOH reacts with triglycerides generating an overlying soap layer, making difficult the digestion and solubilization and subsequent microplastics extraction. Here we studied the addition of Tween-20 in different concentrations to evaluate the effect on the soap layer of post-digested samples. Addition of 10 % of Tween-20 presented higher flow rate during filtration, being set as optimal value. Incorporation of Tween-20 in the extraction procedure increased recovery rates of LDPE, PC and PET and appears to have a protective effect on PC and PET degradation. Tween-20 did not interfere in FTIR spectrum of polymers available in the marine environment. Being low-toxic, makes addition of Tween-20 a simple and economical way to optimize KOH digestion protocols for microplastics extraction.

1. Introduction

Microplastics include all small plastic particles ranging from 1 μm to 5 mm in size (Frias and Nash, 2019). Loading into the aquatic environment or resulting from the breakdown of larger plastic debris (Cole et al., 2011), microplastics represent the most abundant and hazardous fraction of marine plastic pollution (Eriksen et al., 2014). Particles' physical properties, hydrodynamic conditions and biofouling influence microplastics fate and their effects in marine ecosystems (Long et al., 2015; Kowalski et al., 2016; Kaiser et al., 2017).

Because of years of cumulative and continuous emissions into the ocean combined with their resistance to degradation, microplastics are now considered ubiquitous in the marine environment which increases the risk of organisms' exposure. Small size fragments and fibers made of low- and high- density polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polyurethane (PUR), polyethylene terephthalate (PET), and polystyrene (PS) are often found in the marine environment, reflecting their global plastic demand and their heterogeneity (PlasticsEurope, 2019). Their uptake has been globally reported and includes primary producers, invertebrates, fish, seabirds, and marine mammals affecting over 220 different species (FAO, 2016). Once ingested, microplastics may cause physical damage, suffocation, gut blockage, and nutritional problems (Watts et al., 2015; Jovanović, 2017). Additives incorporated during plastic manufacturing (e.g., plasticizers, flame retardants, pigments and UV stabilizers) and pollutants adsorbed on their surface may also increase their toxicity (Avio et al., 2015).

The EU Marine Strategy Framework Directive (MSFD), United Nations Environmental Programme (UNEP), and action plans from the Regional Seas Conventions required long-term microplastics monitoring programs to assess their distribution, accumulation trends and impact in the marine environment. To understand the impact of microplastic pollution on marine biota, it is also important to monitor ingestion and subsequent effects. To have reliable results, MSFD's Technical Subgroup on Marine Litter recommends analyzing routinely at least 50 individuals per species (Galgani et al., 2013). Despite that, there is a lack of agreement to establish suitable indicator species, harmonized methodologies and comparable data to optimize monitoring programs (de Sá et al., 2018; Hermsen et al., 2018).

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So far, a wide range of protocols has been established to separate microplastics from biota samples. Protocols should not only consider the degradation efficiency of biological material, but also the level of damage on plastic polymers, costs, potential hazards associated with the reagents, the time consumption and the steps need for extracting microplastics (Bessa, 2019). Some studies extracted microplastic with no (pre)degradation of biological material (Choy and Drazen, 2013), suppressing some steps of sample preparation, but rising analysis time and decreasing separation success due to loss of smaller particles. To minimize the analytical difficulties most established protocols add at least one (pre)treatment to digest organic matter. This may be chemical digestion with simple and/or mixtures of acids (Claessens et al., 2013; ICES, 2015; Cauwenberghe et al., 2015), bases (Foekema et al., 2013; Rochman et al., 2015; Kühn et al., 2017; Karami et al., 2017), enzymatic digestion (Cole et al., 2014; Catarino et al., 2017; von Friesen et al., 2019) and oxidizing agents (Avio et al., 2015; Nuelle et al., 2014).

Acid compounds are highly effective in destroying soft and hard biological tissues, but they also degrade polymers with a low pH tolerance, e.g., polyamide, polystyrene and polycarbonate (Claessens et al., 2013). Enders et al. (2016) reported the complete dissolution of polyamide and polyurethane after applying acidic treatment and some degradation was observed in acrylonitrile butadiene styrene (ABS), polymethyl methacrylate (PMMA) and polyvinyl chloride (PVC), after applying an acid mix of 4:1 (v:v) HNO_3 (69 %) + $HClO_4$ (70 %) for 5 h and subsequent heating in a laboratory oven for 10 min in 80 °C. Avio et al. (2015) showed that nitric acid caused a marked dissolution of both polyethylene and polystyrene tested particles. Such damages could lead to a misidentification of particles properties (e.g., shape, size distribution and color), recommended being reported in monitoring programs (Galgani et al., 2013). Likely, the total loss of some polymers during the sample pretreatment or their break in smaller pieces could systematically under or overestimate microplastic abundance and polymer diversity. Enzymatic digestion showed adequate results in the breakdown of biological material with minor effect on polymer structure, but it is an expensive and time-consuming method increasing potential contamination. Löder et al. (2017) developed a basic enzymatic purification protocol that takes 16 days per sample to accomplish and use many enzymes and reagents that could be not suitable to use in a monitoring program. The alkaline digesting using KOH has been recommended since it was found to effectively digest organic matter with none- to low-degradation of most of the polymers (Dehaut et al., 2016; Kühn et al., 2017). The solution is filtrated to isolate the potential microplastics for subsequent visual and chemical analysis (Hermsen et al., 2018; Lusher and Hernández-Milian, 2018). However, for samples containing elevated lipid content (fat), this method brings additional difficulties for the separation of microplastics from the biological matrices since saponification reaction tends to occur. KOH reacts with triglycerides producing potassium salts of fatty acids. Sodium-based salts form a hard soap while potassium-based salts form soft (liquid suspension) soaps (Konkol and Rasmussen, 2015). The production of the soap layer turns difficult or impossible to perform the filtration step and isolate potential microplastics.

To overcome these limitations, this study aimed to optimize the existing KOH protocol described by Dehaut et al. (2016) by adding a surfactant agent (Tween-20). Recently, López-Rosales et al. (2021) demonstrated that an oxidative method using a surfactant (sodium dodecyl sulfate) plus hydrogen peroxide is a reliable, fast and cheaper alternative than the alkaline (potassium hydroxide) and enzymatic (protease plus lipase) digestions to get rid of the planktonic organic matrix. Protocols were evaluated based on (i) digestion efficiency of different biological matrices; (iii) recovery rate of the nine most common microplastics polymers assessed by their mass loss; and (iv) modifications in post-treated particles FTIR spectra and surface. Tween-20 belongs to the polysorbate family and is composed of a hydrophilic sorbitan polyoxyethylene (POE) head group linked by an ester bond to a hydrophobic fatty acid tail, mainly with lauric acid. Polysorbates are nonionic surfactants widely used in medicinal, food, and cosmetic products formulations as solubilizing and wetting agents, and mostly as emulsifier for stable oil-in-water emulsions, preventing surface adsorption and particle aggregation (as referred in Garidel et al., 2020). They are characterized by their low-toxic, biocompatibility and are economical comparing to other surfactants (Dwivedi et al., 2018). Recently they have been used in the preparation of microplastics homogeneous aqueous dispersion (e.g., Shams et al., 2020; Fernández and Albentosa, 2019).

2. Material and methods

2.1. Microplastic particles

A microplastic reference kit developed during the Baseman project (JPI-Oceans) was used. The reference kit was composed by the nine most frequent polymers found in the environment (Soares et al., 2020), namely high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polycarbonate (PC), polymethyl methacrylate (PMMA) and polyamide-66 (PA66). They were provided by the Universität of Bayreuth (Germany) in powder with an irregular shape and an average size of 300 μm.

2.2. Contamination prevention

To avoid contaminations, the material used was washed with ultrapure water and all procedure was performed inside a clean air laminar flow cabinet (HEPA filter, class ISO5). Samples were kept always in covered glass recipients to minimize airborne microplastics contamination. Team members used a 100 % cotton lab coat throughout the entire process. All the solutions were made using ultrapure water and filtered using a glass fiber filter of 1.2 μm.

Negative controls as procedural blanks were run alongside for each protocol (n = 40) using all reagents and filtration system, according to quality criteria proposed by Hermsen et al. (2018). A total of 4 blue fibers were accounted on three procedural blanks. Since only particles were targeted in this study, fibers were not considered.

2.3. Optimization of Tween-20 concentration

The optimal concentration of Tween-20 was achieved by studying the effect of six different concentrations (0 % v/v, 1 % v/v, 2 % v/v, 4 % v/v, 6 % v/v and 10 % v/v) on the digestion of 25 g of pooled and homogenized livers of thornback ray (*Raja clavata*). Livers were chosen due to the known elevated lipid content (Tufan et al., 2013) and an absence of sediments and bone/chitin materials. The samples were weighed and transferred to a glass recipient where a solution of 10 % KOH (w/v) was added in proportion of three times the volume of samples, prepared by dissolving KOH pellets (≥85 %, Sigma-Aldrich) in ultrapure water. Afterwards, the Tween-20 was added and the samples were digested for 24 h to 60 °C under constant stirring at 130 rpm. The obtained solution was filtered and membranes dried at 40 °C in an oven. Filtration over 8 μm cellulose nitrate membranes was chosen based on lower limits of most common FTIR approaches and to prevent filter clogging. The filtration time of each sample was measured and volumetric flow rate ($mL.min^{-1}$) was determined with Eq. (1):

$$\text{Volumetric flow rate } (mL.min-1) = \frac{V_s}{t_f} \quad (1)$$

where V_s is the volume of the sample filtered (mL) and t_f is the filtration time (min).

2.4. Protocols for microplastic extraction

All samples were treated using a solution of 10 % KOH (w/v, 3× sample volume), for 24 h at 60 °C (Dehaut et al., 2016). Based on the optimized Tween-20 amount (Section 2.3) four protocols were tested to the application of the surfactant:

Protocol 1–10 % KOH (w/v), at 60 °C during 24 h, without stirring.

Protocol 2–10 % KOH (w/v) at 60 °C and 130 rpm during 24 h.

Protocol3–10 % KOH (w/v) + 10 % Tween-20 (v/v) at 60 °C during 24 h, without stirring.

Protocol 4–10 % KOH (w/v) + 10 % Tween-20 (v/v), at 60 °C and 130 rpm, during 24 h

For all protocols, the obtained solution was filtered (8 μm cellulose nitrate membranes) under vacuum and membranes dried at 40 °C in an oven.

2.5. Digestion efficiency

Specimens of thornback ray (*Raja clavata*), chub mackerel (*Scombercolias*), and European sardine (*Sardina pilchardus*) were collected from the western coast of Portugal. In the laboratory, the amount of visceral fat was assessed macroscopically according to the four-stage classification key developed by Furnestin (1943) (1 = no fat; 2 = thin thread of fat; 3 = fat around all the gut; 4 = body cavity full of fat) to ensure consistency of this variable. The fat content of horse mackerel and sardines was between 3 and 4 on the scale of visceral fat, while thornback ray individuals did not show any fat or just thin thread of fat (stages 1 and 2) in their body cavity. This difference allows to mimic different types of biological tissues. Furthermore, the comparison among different species is relevant because their ecological and diet diversity may affect the efficiency of tested protocols (Bianchi et al., 2020). Gastrointestinal tracts (GITs) of these species were pooled into 25 g portions and manually homogenized. For each species, 5 replicated of pooled GITs were treated according to the 4 protocols mentioned and digestion efficiency (%) was calculated according to Eq. (2) (Karami et al., 2017):

$$\text{Digestion Efficiency } (\%) = \frac{\text{Wi} - (\text{Wa} - \text{Wb})}{\text{Wi}} \times 100 \qquad (2)$$

where Wi is the initial weight of biological material, Wa is the weight of dry membrane after filtration and Wb is the weight of dry membrane before filtration.

Reagent controls were run alongside to identify changes on filter membranes. Before and after filtration, filter membranes were rinsed with ultrapure water, dried at 40 °C, and then weighed to assess the effect of reagents on filter weight.

2.6. Effect of KOH and Tween 20 in recovery rates of microplastics

Five milligrams of each polymer (HDPE, LDPE, PET, PP, PS, PVC, PC, PMMA and PA66) were treated alone with digestion protocols described above in independent five replicates. Recovery rates (%) of microplastics were determined through the ratio of the weight of microplastics on the filter membrane before and after each treatment, calculated as follows (Karami et al., 2017):

$$\text{Recovery Rate } (\%) = \frac{\text{Wa} - \text{Wb}}{\text{Wi}} \times 100 \qquad (3)$$

where Wa is the weight of dry membrane after filtration, Wb is the weight of dry membrane before filtration, and Wi is the initial weight of microplastics.

2.7. Effect of KOH and Tween 20 on FTIR spectra

A subsample of each post-treated polymers was examined to evaluate possible modifications in their Fourier Transform Infrared Spectrometry (FTIR) spectra by comparison with pristine polymers spectra acquired before treatments, using a 400 FT-IR/FT-NIR Perkin Elmer Spectrometer (4000–650 cm^{-1}, 4 cm^{-1} nominal resolution, Beer-Norton strong apodization, 50 scans per spectrum, background-, depth-penetration- and baseline-corrected) equipped with a horizontal one-bounce diamond crystal (Miracle ATR, Pike). Spectra were collected in ATR mode (Andrade et al., 2020). Various powder grains of polymer were measured at the same time in the ATR using an ATR powder device, the spectra obtained is an average of the total measurements. It was probe that the reproducibility of the powder measurement is lower than 5 % (RSD). A commercial polymer library (Perkin Elmer) and an ad-hoc polymer library with spectra of weathered polymers were used for the validation and comparison of polymer particles (Andrade et al., 2019; Fernández-González et al., 2021).

2.8. Effect of KOH and Tween 20 on polymer surface by SEM

The same subsample of each post-treated polymer analyzed by FTIR was also analyzed by Scanning Electron Microscopy (SEM) to evaluate possible changes in the surface of the polymers by comparison with virgin plastics. The sample surfaces were coated with a layer of gold and then analyzed in a Scanning electron microscopy, JEOL JSM 6400. Because the surface textures of the powder grains are heterogeneous, visualization was repeated at different surface sites and at different grains.

2.9. Statistical analysis

Statistical analyses were performed using STATISTICA 12 software (Statsoft Inc., Tulsa, OK, USA). Normality and homogeneity of the data were tested using Shapiro-Wilk's test and Levene's test, respectively. The Kruskal-Wallis test and the Mann–Whitney *U* test were used to compare recovery rates of microplastics among protocols for each polymer and among polymers for each protocol. The same tests were performed to compare digestion efficiency of biological material among protocols for each fish species and among fish species for each protocol. Statistical tests were considered significant at p values < 0.05.

3. Results

3.1. Concentration of Tween-20

It was evaluated the effect of Tween-20 at different concentrations in alkaline solution, keeping the KOH concentration constant at 10 %. After 24 h of digestion at 60 °C, all samples were visually inspected for the formation of a viscous soft gel, signifying saponification. Samples treated with Tween-20 (1–10 % of Tween-20) showed a reduction of soft gel at their surface and appeared more homogeneous compared to samples treated without the surfactant (control) (Fig. 1a). Moreover, the median value of volumetric flow rate of samples treated with Tween-20 was significantly higher from the control (all MW p-value <0.05) (Fig. 1b). No significant differences were observed between flow rates of solutions with 1 % and 2 % of Tween-20. With 10 % concentration of Tween-20 the flow rate was significantly higher ($p < 0.05$), take less time (<1 min. per sample) to filter than the other treated samples. Thus, the 10 % concentration of Tween-20 was selected as the optimum value to use in digestion efficiency tests (Protocol 3 and Protocol 4).

3.2. Digestion efficiency

Median values of digestion efficiency (%) for five replicates of each protocol are presented in Table 1 for different species. Mackerel and ray samples showed median values of digestion efficiency superior to 95 % for all protocols, while digestion efficiencies of sardine samples were slightly lower, ranging from 90 % to 95 % (Table 1). Digestion

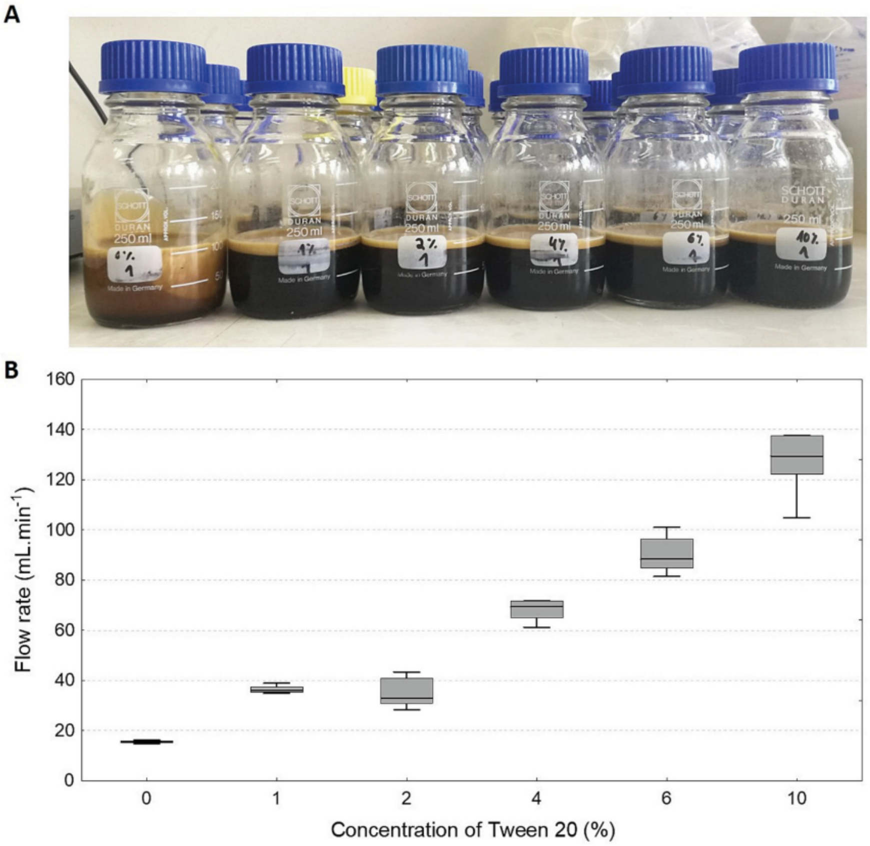

Fig. 1. Study of different concentrations of Tween-20 added to digestion protocol with 10 % potassium hydroxide at 60 °C for 24 h. A- Visual inspection of ray liver samples after being digested with alkaline protocol at different Tween-20 concentrations. B - Median value of volumetric flow rate of ray liver samples filtrated after treated with digestion protocol with different concentrations of Tween-20.

Table 1
Median (IQR - interquartile range) values of digestion efficiency (%) of biological materials after treated with different alkaline protocols (n = 5).

	Sardine	Mackerel	Ray
Protocol 1	92.3 (1.11)	95.9 (0.87)	95.2 (0.71)
Protocol 2	91.4 (1.22)	97.1 (1.07)	95.2 (0.63)
Protocol 3	93.1 (0.25)	98.3 (0.19)	95.1 (2.61)
Protocol 4	93.5 (2.95)	98.7 (0.37)	95.8 (0.59)

efficiencies of ray and sardine samples were similar among protocols, while were significantly different for mackerel (KW p-value = 0.002). In the case of mackerel samples, protocols with addition of Tween-20 were more effective than Protocol 1, (MW p = 0.012; MW p = 0.012; respectively), reaching Protocol 4 the best results for this species. These results were also confirmed since it was visible a distinct layer of fat in the top of digested mackerel sample after applying Protocol 1, while solution from Protocol 4 was clearer and did not show a soap layer on the top (Fig. 2). Control experiments showed that used solvents did not cause an increase in the weight of the filter membranes.

3.3. *Effect of KOH and Tween 20 in recovery rates of microplastics*

Median recoveries (%) of all microplastics among treatments ranged between 47.3 % and 103.7 % and are presented in Table 2. In the original protocol (Protocol 1), the recoveries among polymers were statistically different, being observed an increase of the initial weight of PMMA (103.7 %) and recoveries lower than 95 % for LDPE (94.5 %), PC (67.9 %) and PET (47.3 %). Comparing the different treatments, significant weight changes were found for LDPE (KW p = 0.005), PC (KW p = 0.046), and PET (KW p = 0.005). For LDPE, higher recoveries were obtained using Protocol 4 than the original Protocol, while PC showed higher recoveries using Protocol 3 than the original Protocol. For PET, all protocols showed higher recoveries than Protocol 1, being the Protocol 4 the only one that reached a recovery rate of >90 %.

Finally, a Mann–Whitney *U* test was also performed to assess the effect of Tween-20 in recovery rates of microplastics by grouping protocols with (Protocol 3 and Protocol 4) and without (Protocol 1 and Protocol 2) the surfactant. Results shown that Tween-20 improved the recoveries of LDPE (MW p = 0.002), PC (MW p = 0.008) and PET (MW p = 0.005). Stirring at 130 rpm (Protocol 2) have not shown a clear impact on recoveries of the polymers. It improved the recovery of PET but to lower values than Protocol 4.

3.4. *Effect of KOH and Tween 20 on FTIR spectra*

After exposure to the digestion protocols, selected triplicate microplastics of each polymer type were analyzed by FTIR to evaluate potential chemical impacts on their spectra. Exampled spectra of obtained results are provided in Fig. 3. A library search (Section 2.7) was performed for each analyzed particle and all particles were successfully matched to the correct reference spectra. After submitted to Protocol 3 and Protocol 4, HDPE and LDPE spectra presented broad bands approximately at 3600 cm^{-1} and at 1530 cm^{-1}, possibly due to hydrogen bonding related to the presence of water remaining or to the structure of the Tween-20 that contains OH- group. Also, the band around 1600 cm^{-1} increases slightly in all the HDPE samples. PVC and PMMA from Protocol 3 and Protocol 4 also present some weak new

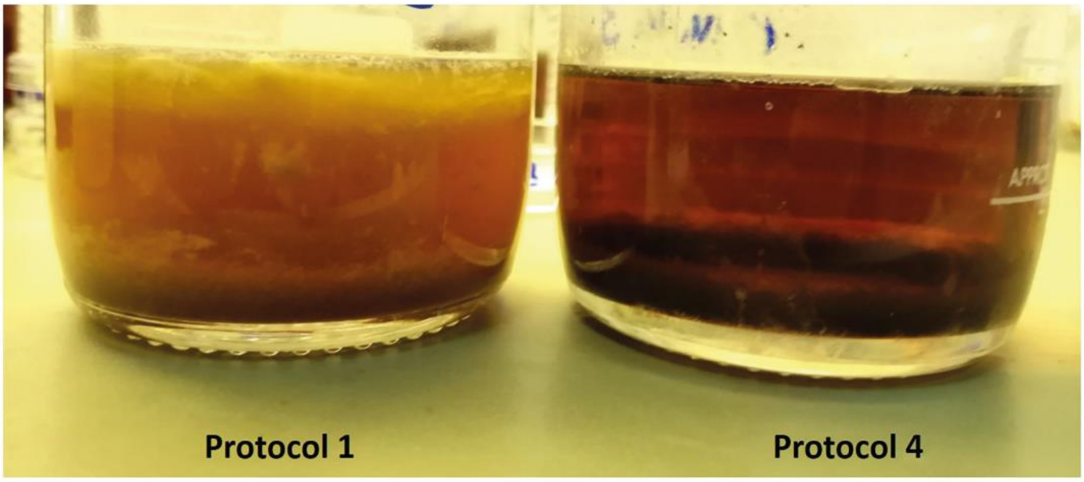

Fig. 2. Visual inspection of mackerel samples after treated with Protocol 1 (left) and Protocol 4 (right).

Table 2
Median (IQR - interquartile range) values of polymer recoveries (%) after treatment with different alkaline protocols (n = 5).

	HDPE	LDPE	Pa66	PC	PET	PMMA	PP	PS	PVC
Protocol 1	98.3 (3.45)	94.5 (1.82)	100.0 (5.77)	67.9 (0.91)	47.3 (10.99)	103.7 (0.99)	97.3 (1.45)	95.3 (5.66)	94.7 (5.36)
Protocol 2	89.3 (5.35)	90.0 (6.25)	100.8 (7.12)	68.5 (9.84)	61.8 (5.05)	100.0 (1.89)	100.0 (1.89)	94.3 (8.19)	99.4 (1.79)
Protocol 3	98.1 (3.70)	95.7 (3.64)	92.9 (9.55)	88.2 (15.88)	68.9 (27.20)	100.9 (1.75)	100.0 (0.86)	100.0 (3.19)	96.2 (7.55)
Protocol 4	100.0 (3.51)	101.8 (2.00)	100.9 (7.55)	86.5 (2.42)	91.2 (1.72)	98.11 (8.24)	100.0 (2.78)	96.1 (5.73)	96.4 (1.37)

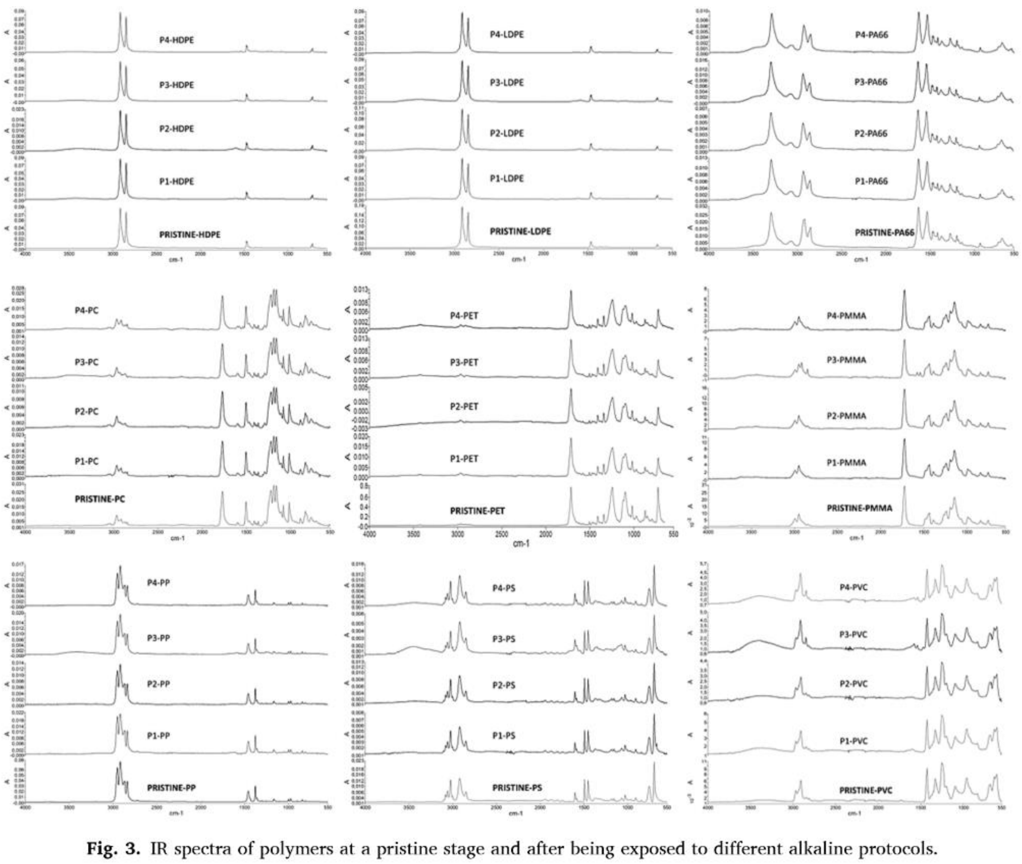

Fig. 3. IR spectra of polymers at a pristine stage and after being exposed to different alkaline protocols.

bands (around 1600–1500 cm^{-1}) in spectra that may be mainly due to the presence of Tween-20. However, it is worth mentioning that these weak bands do not affect the correct identification of polymers.

3.5. *Effect of KOH and Tween 20 on polymer surface by SEM*

Same subsamples of each polymer type used for FTIR analysis were also subjected to SEM analyzes to identify potential modifications on the polymer surface caused by the different protocols. Examples of obtained SEM micrographs are shown in Fig. 4. PET and PC particles from Protocols 1 and 2, were physically degraded, presenting large holes on the surface of the particles. Otherwise, these physical alterations were clearly reduced for protocols using Tween-20. Some minimal physical changes were observed in the surface of PS, HDPE, LDPE, PMMA, PA6,6 and PVC for all treatments compared to their controls, mainly fractures and flaking. Some adhesions can be observed on the surface of some PP and PVC particles, which may be due to reagents used.

4. Discussion

An increasing number of methodologies based on KOH solution has been applied for microplastics extraction due to its simplicity, easy to perform and one of the most economical. However, in biological samples with high-fat content, KOH reacts with triglycerides producing potassium salts of fatty acids. The production of a soap layer, generally on the top of the solution, turns the filtration step too long or even impossible to perform the microplastics separation from the post-digested solution. Dehaut et al. (2016) reported a fatty layer on the filter after digestion of fish tissues with 10 % KOH, 24 h, 60 °C. Also, Enders et al. (2016) using a similar protocol was not able to completely digest cod stomachs and

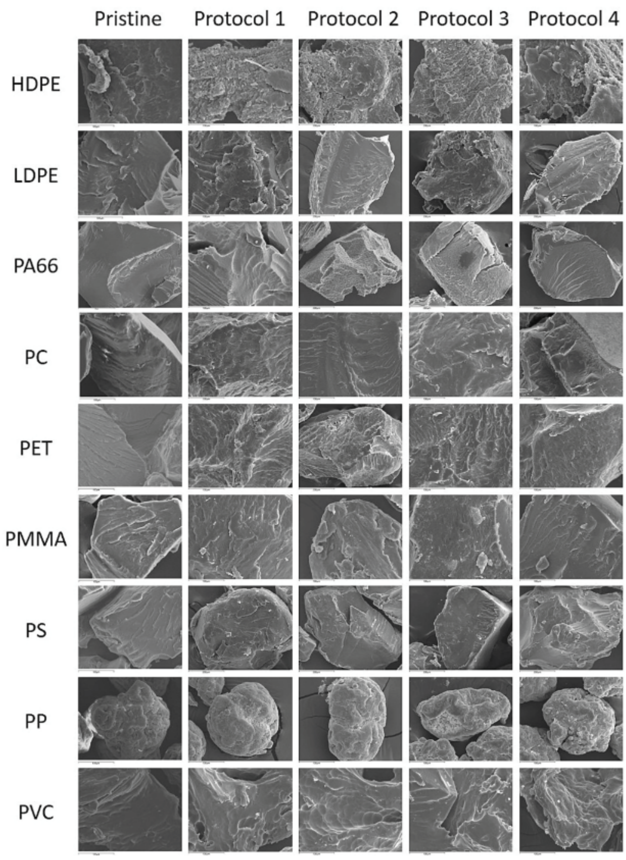

Fig. 4. SEM images of polymers at a pristine stage and after being exposed to different alkaline protocols.

noticed a production of a "Layer of black/brown slime afloat". Here, it was found that adding Tween-20 to the KOH solution before digestion period prevents the formation of fat layers and lead to an increase of volumetric flow rate of post-digested solution during the filtration step. As a surfactant, Tween-20 positioned itself at the interface between alkaline aqueous solution and biological organic phase where it can act to lower surface or interfacial tension, preventing particles agglomeration and decreasing their mean diameter. Tween-20 may also enhance the dispersion of biological material in the alkaline aqueous solution which might let OH- molecules in aqueous phase reach easily to the core of biological material optimizing its breakdown and therefore, increasing the digestion efficiency.

Adults of chub mackerel, European sardines and thornback ray were chosen as representative examples with differences in their diet and fat content to evaluate the digestion efficiency of the protocols studied in this work. According to Karami et al. (2017), a method must be considered suitable when the digestion efficiency is equal to or >95 %. In the case of the thornback ray, similar digestion efficiency was observed in all treatments and all reached the level required. This result was expected since this species has a lower fat content compared with the other two species and feeds mainly on large preys such as Cephalopoda, Osteichthyes and Decapoda:Brachyura (Farias et al., 2006). As a benthic predator, thornback ray can catch its prey from their burrows in the sediment, so is also common to find sediments in their stomach contents. These hard food items such as sediments, bones (calcium phosphate) and chitinous exoskeletons (e.g., the carapaces of crabs and lobsters) are resistant to alkaline hydrolysis and the presence of Tween-20 or agitation do not seem to improve the digestion efficiency of their stomach contents. In case of chub mackerel and sardine, both species are considered fatty species, but only for the case of chub mackerel, there was a clear increase in digestion efficiency of samples treated with Tween-20 when compared to the original protocol. This can be explained by the highest preference of adult sardines for small prey as phytoplankton and microzooplankton (Garrido et al., 2015). Many of these planktonic species have polymeric structures that require longer treatments with an alkaline solution and even some of them such cellulose and chitinous based structures are resistant. For instance, diatoms and dinoflagellates can represent >90 % of sardine dietary carbon contribution during the warmer months (Garrido et al., 2015). The most distinctive characteristic of diatoms is the nanopatterned silica cell wall, and many dinoflagellate species present a theca made of cellulose plates (Durkin et al., 2009). These hard structures represent a higher percentage of small prey constitution than larger prey and they will remain after alkaline digestion, affecting the digestion efficiency of gastrointestinal tracts of individuals who feed preferentially on this type of prey. This is probably why sardines are the only species in this study whose none of the treatments reached 95 % of digestion efficiency.

The recovery rates of polymers are in line with the results of other studies in which KOH was used at 10 % at 60 °C (Thiele et al., 2019; Karami et al., 2017; Kühn et al., 2017; Dehaut et al., 2016). Dehaut et al. (2016) and Thiele et al. (2019) noticed changes in the shape of PET particles and Karami et al. (2017) reported a greater loss of PET mass. In fact, the depolymerization of PET particles in the presence of an alkaline solution has beenstudied (e.g.,Ügdüler et al., 2020; Karayannidis et al., 2002; Wan et al., 2001). Although many of these studies have applied extreme degradation conditions to accelerate the hydrolysis (e.g., high temperatures and pressure), it requiresa 2 KOH: 1 PET reaction stoichiometry repeating unit to produce ethylene glycol and terephthalic potassium salt (Wan et al., 2001). A decrease in the rate of PET degradation was also observed with the increase of particle size, thickness, crystallinity and with the existence of other polymer layers (Ügdüler et al., 2020). In the case of LDPE, most of the studies do not indicate any significant changes. Only Kühn et al. (2017) reported some degradation of one bag labelled as being made of LDPE among several samples representing polyethylene. Likewise, Dehaut et al. (2016) did not report any significant changes for PC.

Since the increase of temperature accelerates alkaline hydrolysis and can cause some microplastic damages, Munno et al. (2018) recommend samples digestion below 60°C and Karami et al. (2017) pointed the 40 °C as optimal temperature to minimize the loss of any microplastics. However, the advantage of Tween-20 in the extraction procedure was found to increase recovery rates of LDPE, PC and PET and a decrease of damage on particles surface without increasing microplastics degradation. One hypothesis is that Tween-20 adsorbed to the surface of polymers and acted as a protective layer during the hydrolysis reaction, making it difficult for hydroxide ions to access unreacted polymers repeating units and, consequently, reducing the efficiency of the hydrolysis reaction. Thus, the "protective" effect of the Tween-20 makes it possible to keep the digestion with KOH 10 % at 60 °C, not compromising the time consumption of microplastics extraction and their degradation. However, the developed method does not include a protocol for cellulose and chitin digestion. Thus, in the case of high gut content of zooplankton and phytoplankton, it is recommended refined the protocol adding a density separation as a last step.

5. Conclusions

A better understanding of factors that influence biological material degradation is useful in optimizing methodologies for microplastics extraction. Results of this study indicate that Tween-20 can be added to traditional digestion protocols using potassium hydroxide with no or minimal interference in the FTIR spectrum of a large fraction of the polymers available in the marine environment. Tween-20 is recognized as environmentally safe and its addition is a simple way to optimize the existing potassium hydroxide protocols. For increasing lipids emulsification, it decreases the formation of the fat layer facilitating the filtration step. Moreover, Tween-20 appears to have a protective effect on degradation of some polymer types of microplastics, increasing their recovery rates.

CRediT authorship contribution statement

C. Lopes conducted the experiments, formal analysis and wrote the original draft. **V. Fernández-González** and **S. Muniategui-Lorenzo** conducted FTIR and SEM analysis. **C. Lopes, M. Caetano** and **J. Raimundo** conceptualized the experimental design. All the authors contributed to manuscript writing and critical revisions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Chapter 3

Microplastic distribution in different tissues of small pelagic fish of the Northeast Atlantic Ocean

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Microplastic distribution in different tissues of small pelagic fish of the Northeast Atlantic Ocean

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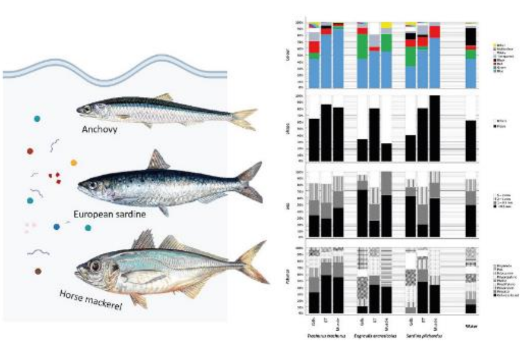
HIGHLIGHTS

- The composition of MP found in the gills of sardines and anchovies closely resembled that of the MP present in water samples.
- Horse mackerel showed the highest MP concentrations in their GT, while anchovy and sardine displayed the lowest MP concentrations in their muscles.
- In horse mackerel and European sardine, there was a weak but significant negative correlation between MP levels in the GT and the Fulton Condition Index.
- Cellulose-based, polyacrylate, and polyester were the main polymers observed in fish tissues.
- The most prevalent MP measured less than 0.5 mm and were blue fibers.

GRAPHICAL ABSTRACT



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ABSTRACT

The accumulation of microplastics (MP) by marine species of ecological and commercial interest represents a major concern, particularly for those present in human diet. This study analysed the accumulation of MP in three species of coastal pelagic fish with high commercial value, European sardine (*Sardina pilchardus*), European anchovy (*Engraulis encrasicolus*) and horse mackerel (*Trachurus trachurus*), collected along the Western coast of the Iberian Peninsula. The gastrointestinal tract (GT), gills and muscle were analysed and a total of 504 particles were observed. MP were found in all target tissues of the studied species. Horse mackerel exhibited significantly higher concentrations of microplastics in GT compared to other tissues. On the other hand, anchovies and sardines had significantly lower microplastic concentrations in their muscle tissue. The accumulation of microplastics in the gills showed a significant difference between species, with anchovy having significantly higher concentrations compared to horse mackerel. Horse mackerel had the highest percentage of individuals with microplastics in their GT (92 %), followed by sardine (75 %) and anchovy (50 %). Horse mackerel was also the species that registered the highest percentage of individuals with particles in the muscle (63 %), followed by

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anchovy (40 %) and finally sardine (39 %). MP in the gills of European sardines and anchovies were similar to those found in water samples. The majority of MP found measured <0.5 mm and were blue fibers. Furthermore, the presence of MP in the GT showed a weak and moderated significant negative correlation with the Fulton Condition Index in horse mackerel and European sardine. Our study confirms the ubiquitous extent of MP contamination in the ocean and provides baseline evidence of MP tissue distribution in three small pelagic fish species with distinct feeding behaviour, while correlating this with the presence of MP in water. Importantly, the results of this study contribute to improve the understanding of biological partitioning of MP in open sea fish species with high commercial relevance, and the potential deleterious effects of our increasingly MP contaminated world.

1. Introduction

Microplastics (MP), ranging in size from 1 to 5000 μm (Frias and Nash, 2019), have been found in a variety of marine environments, including ocean surface, water column, deep sea, and coastal sediments (as reviewed in Cole et al., 2011 and Coyle et al., 2020), from the Arctic (Kanhai et al., 2019) to the Antarctic (Lacerda et al., 2019), demonstrating their global and widespread distribution in ecosystems. MP exhibit a wide range of shapes and morphologies. Among them, microbeads and pellets, deliberately manufactured with sizes in the micron-scale range, are considered primary MP, used in the plastic industry and incorporated into cosmetic and personal care products intentionally (Cole et al., 2011). On the other hand, secondary MP encompass various shapes resulting from photochemical, mechanical, and biological degradation processes within the marine environment (Cole et al., 2011). The most prevalent shapes of MP found in marine environment are fibers originating from synthetic clothing and fishing nets, along with fragments produced by the breakdown of larger plastic items (Cole et al., 2011). MP physical properties, such as density and shape, can influence their vertical distribution in the seawater column and their presence in benthic habitats. Polyethylene (PE) and polypropylene (PP) are positively buoyant and predominate in the upper layers, due to its lower density in comparison with seawater, while polymers having higher density, such as polyvinyl chloride (PVC) and polyester, as well as those subject to biological fouling, will sink to the seafloor (Mountford and Morales Maqueda, 2019).

Marine fish are continuously interacting with MP present in the ecosystems, and many consequences of that contact have been highlighted through laboratory experiments (Wang et al., 2021). Adverse effects on fish are dependent of particle characteristics, their concentrations and exposure routes and include internal obstructions, abrasion, formation of reactive oxygen species, resulting in malnutrition and compromising growth and reproduction (Jeong and Choi, 2019; Jovanović, 2017). In addition to their intrinsic toxicity, MP may be a vector of other toxic compounds for organisms at the base of the food chain, where there is potential for bioaccumulation and biomagnification (Teuten et al., 2009). For instance, endocrine disrupting compounds (ECDs) such as bisphenol A (BPA), nonylphenol, and octylphenol are widely employed as plastic additives and have the potential to induce negative health effects on aquatic organisms, with potential deleterious effects on humans (Pironti et al., 2021).

Most of published works describing the presence of MP in fish focus on gastrointestinal tract. However, recently, the presence of MP in muscles of fish have been reported (Barboza et al., 2020a; Abbasi et al., 2018; Akhbarizadeh et al., 2018). MP with sizes smaller than 150 μm can be absorbed (EFSA Contam Panel (EFSA Panel on Contaminants in the Food Chain), 2016), suggesting that most MP found in fish muscle within this range may result from direct uptake through the abdominal cavity. However, large fibers and fragments, up to 2363 μm and 490 μm respectively, have been observed in the dorsal muscle of fish, indicating that they somehow entered the fish body and reached internal tissues (Barboza et al., 2020a). These evidences raise concerns regarding the ingestion of microplastics by humans through the consumption of contaminated fish muscle and the potential damaging effects on human health (Barboza et al., 2020a). Additionally, these studies have stressed the limitations of using just the gastrointestinal tract for microplastics monitoring purposes, as the gastrointestinal tract is often removed before fish consumption. Furthermore, there are other important exposure routes of microplastics that should not be excluded from monitoring such as gills, as they can help to clarify their environmental prevalence and distribution to other organs. For instance, the primary purpose of the gill rakers is to retain particles from the water flow during breathing, to protect the gill epithelium (Elsheikh, 2013). Likewise, in some species such as *Clupeiformes*, gill rakers are also used as a net to extract food from the water flow and guide it toward the esophagus (Gibson, 1988).

Small and semi-pelagic fish species are economically vital around the world, and account for the majority of fish biomass, particularly in upwelling regions (FAO, 2016). Furthermore, in pelagic food webs, their low and middle trophic positions are critical. These species directly consume plankton, exerting a top-down control over planktonic communities and at the same time a bottom-up control over top predators (Cury et al., 2000). Microplastics have been detected in the gastrointestinal tract of several coastal small pelagic fish species from the Northeast Atlantic (Lopes et al., 2020; Pereira et al., 2020; Collard et al., 2017) and the Mediterranean (Renzi et al., 2019; Compa et al., 2018).

Pelagic fish, including sardines and anchovies, have a planktivorous diet throughout their entire life cycle. In contrast, certain species, like horse mackerel, adopt planktivory during their early developmental stages and transition to piscivory when they reach the adult stage (Garrido et al., 2015). MP accumulation in planktivorous fish is particularly concerning due to the size similarity between microplastics and planktonic species. Fish feeding strategies also play a significant role in the uptake of MP. Filter-feeders, for example, are more likely to passively uptake microplastics from the surrounding water (Collard et al., 2017), while particulate-feeders may actively ingest microplastics by confounding them with prey (Ory et al., 2018). Moreover, differences in the morphology of the feeding apparatus among co-occurring planktivorous fish species can influence their diet composition and the presence of microplastics. For instance, Collard et al. (2017) found that European sardines have the largest filtration area and closest gill rakers compared to Atlantic herring and European anchovy, making sardines more efficient in filtering and more likely to ingest microplastics. Furthermore, sardines were found to ingest a greater quantity of fibers and smaller fragments, in line with the research by Garrido et al. (2007), emphasizing that sardines possess a filtering apparatus morphology that enables them to retain preys as small as 12 μm.

The objective of the current work was to assess and characterize the presence of microplastics in 3 pelagic species from the Northeast Atlantic Ocean: European sardine (*Sardina pilchardus),* European anchovy *(Engraulis encrasicolus)* and horse mackerel (*Trachurus trachurus*), focusing on their accumulation in specific tissues, namely the gastrointestinal tract, gills, and muscle. This work also intended to correlate the concentrations of microplastics found in the studied species with water samples collected in the same area, providing a better understanding of the environmental exposure and potential human risks.

2. Methods

Specimens of pelagic fish from 3 commercial species, the European sardine, horse mackerel, and the European anchovy (*Sardina pilchardus, Trachurus trachurus, Engraulis encrasicolus*) were collected in 5 stations along the northwestern Iberian coast during a research cruise (PELAGO18) carried out between April and May of 2018 on board of RV Noruega. The number of individuals caught at each location ranged between 30 and 52, depending on the availability (Fig. 1, Table 1). Samples taken on board were immediately frozen at −20 °C for posterior laboratory dissection. Water samples ($n = 12$) were also collected obliquely from the 60 m depth to the surface, using bongo nets with a mesh size of 200 μm in 20 min trawls (see Fig. 1).

For each individual fish, total length (cm) and weight (g) were measured and the gastrointestinal tract (GT), gills and a portion of dorsal muscle were removed and weighted individually, for further microplastic analysis. Biota and water samples were processed according to Lopes et al. (2022). A solution of 10 % KOH was added to samples and samples were incubated during 24 h at 60 °C. After alkaline digestion, samples were filtered through 20 μm polycarbonate membrane. Filters were analysed using a stereomicroscope (LEICA S9i) with an integrated camera (IC80 HD) and potential microplastics were photographed and isolated. Particles were sorted by colour (black, blue, transparent, white, red, green, and other) and shape (fibers and others). Size was recorded by measuring particles at their largest cross section using the ImageJ software and they were categorized according to four size classes (≤0.5 mm; 0.5–1 mm; 1–2 mm and 2–5 mm).

Chemical composition was determined by Fourier Transform Infrared Spectrometry (FTIR), using a PerkinElmer Spotlight 200i FTIR Imaging System equipped with a mercury cadmium telluride (MCT) array detector cooled by liquid nitrogen. Spectra were acquired in reflectance mode. Measurement resolution was set at 4 cm^{-1} ranging from 4000 cm^{-1} to 600 cm^{-1} with a minimum of 10 scans. To confirm the polymer type, all spectra were compared to library databases and then the polymer characteristic bands were compared with spectra assignments. The lower size limit of microplastics in this study was set as 20 μm, corresponding to the mesh size of filters used for filtration process and to particle size limit of the FTIR equipment used in this study.

To account for background contamination, all materials were sterile and thoroughly rinsed with ultrapure water before and between material use and samples were always kept under a clean air laminar flow hood (HEPA filter, class ISO5) or maintained in covered glass recipients. Procedural blanks were run simultaneously with biological and water samples ($n = 76$). During microscopic analyses for microplastics identification, two open Petri dishes were placed with clean filters near the working zone to control airborne fibers contamination ($n = 72$).

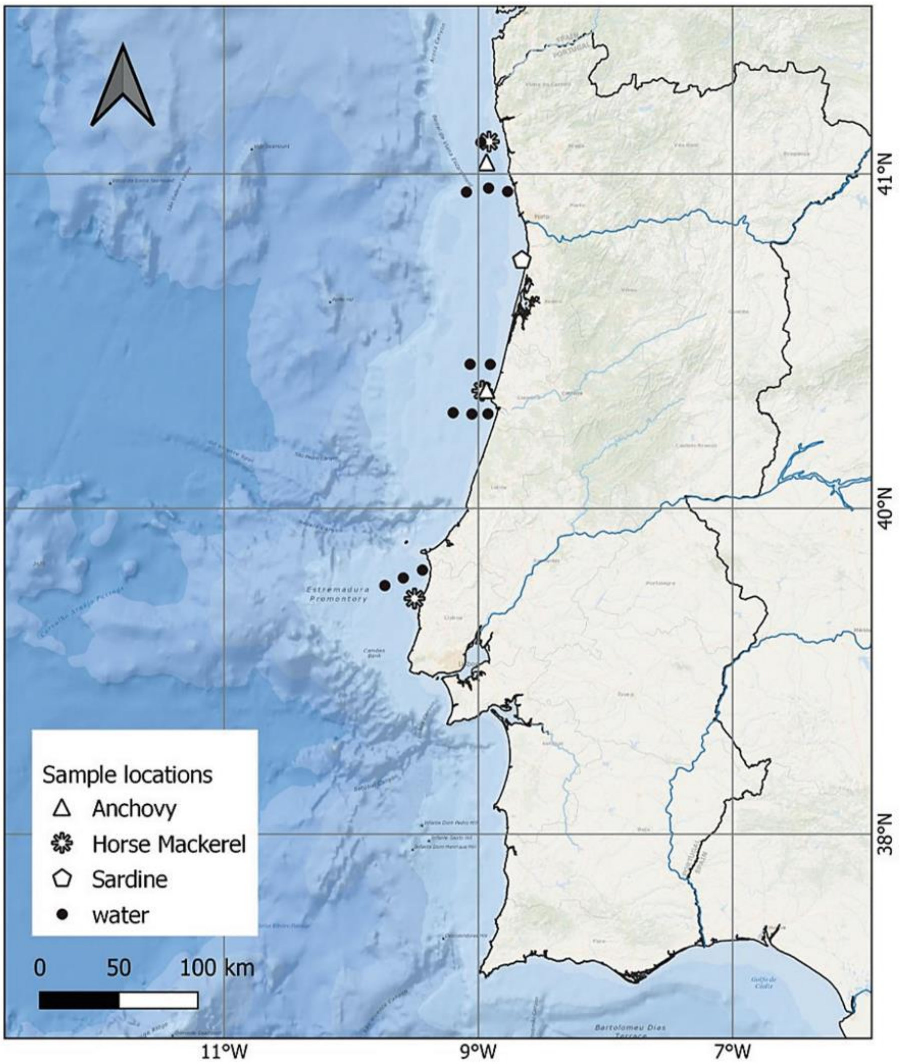

Fig. 1. Sampling sites location of small pelagic fish and water samples caught in the Atlanto-Iberian coast during PELAGO18 survey (May 2018).

Table 1
Biometric parameter fish.

	Trachurus trachurus	*Engraulis encrasicolus*	*Sardina pilchardus*
n Specimens	52	30	36
Mean Total length (cm)	17.2 ± 5.4	11.6 ± 0.5	16.8 ± 1.2
Mean Weight (g)	57.2 ± 51.7	10.2 ± 1.8	41.2 ± 12.9
Fish with MP (%)	96	93	92
Gills			
Speciemens with MP (%)	50	60	61
Median ($MP.g^{-1}$)	0.14	4.93	1.5
Min – Max ($MP.g^{-1}$)	0.00–15.0	0.00–20.0	0.00–28.6
GT			
Specimens with MP (%)	92	50	75
Median ($MP.g^{-1}$)	3.45	0.93	4.8
Min – Max ($MP.g^{-1}$)	0.00–33.3	0.00–25.0	0.00–38.89
Muscle			
Specimens with MP (%)	63	40	39
Median ($MP.g^{-1}$)	0.15	0.00	0.00
Min – Max ($MP.g^{-1}$)	0.00–2.9	0.00–1.09	0.00–0.67

Contamination control measures revealed that 11.1 % of procedural blanks ($n = 8$) and 10.5 % of filters control (n = 8) had caught external sources of microplastic contamination. All particles detected were fibers, 16 in procedural blanks (median = 0.00 fibers/filter) and 20 in filters control (median = 0.00 fibers/filter). To compensate for potential contamination, fibers were excluded from the analysis if they were of similar colour and polymer type to those detected within contamination control measures.

To evaluate the fitness and condition of the fish, the Fulton condition factor (K) was measured (Froese, 2006). It was calculated using Eq. (1) as follows:

$$K = 100 \times \frac{W}{L^3} \quad (1)$$

where W is the weight in grams, and L is the length in centimeters (Fulton, 1904).

Shapiro–Wilk and Levene's test were performed to test normality and homogeneity of variance, respectively. After the invalidation of parametric assumptions, a Kruskal-Wallis test followed by post-hoc multiple comparisons with Dunn's test were used to test for differences of microplastics concentrations between tissues and fish species. Spearman rank correlation test was used to address the relationship between microplastic concentrations in various tissues and the Fulton condition factor (K). A hierarchical cluster analysis was conducted using the group average mode and coupled with similarity profile analysis (SIMPROF) with 999 permutations. This was done on the basis of the Euclidean distance matrix log-transformed/normalized data to inspect the relationship between fish tissues and surrounding water in terms of microplastic composition (colour, shape, size, and polymer type). Additionally, SIMPER analysis was performed to evaluate the contribution of each microplastic characteristic to the average dissimilarity between groups. Analyses were performed using STATISTICA and PRIMER 7 + PERMANOVA considering a 5 % significance level.

3. Results

3.1. Microplastics in fish

In this study, 118 fish specimens of European sardine, horse mackerel, and European anchovy from the Portuguese coast were examined, and a total of 504 microplastics (MP) were recovered. MP were found in all species and tissues studied (Table 1 and Fig. 2).

Horse mackerel had the highest percentage of individuals with microplastics (MP) in their gastrointestinal tract (GT), at 92 %. The median concentration of microplastics in horse mackerel's GT was 3.45 $MP.g^{-1}$, with a maximum of 33.3 $MP.g^{-1}$. Sardines followed with 75 % of individuals having microplastics in their GT, with a median concentration of 4.8 $MP.g^{-1}$ and a maximum of 38.89 $MP.g^{-1}$. Anchovies had 50 % of individuals with microplastics in their GT, with a median concentration of 0.93 $MP.g^{-1}$ and a maximum of 25 $MP.g^{-1}$.

In terms of microplastic presence in the gills, 50 % of horse mackerel specimens had at least one microplastic, with a median concentration of 0.14 $MP.g^{-1}$ and a maximum of 15 $MP.g^{-1}$. Sardines and anchovies showed slightly higher values, with 61 % and 60 % of individuals, respectively. Sardines had a median concentration of microplastics in the gills of 1.5 $MP.g^{-1}$ and a maximum of 28.6 $MP.g^{-1}$, while anchovies presented the highest median concentration of 4.93 $MP.g^{-1}$ and a maximum of 20 $MP.g^{-1}$.

Regarding the muscle, horse mackerel had the highest percentage of individuals with microplastics (63 %), with a median concentration of 0.15 $MP.g^{-1}$ and a maximum of 2.9 $MP.g^{-1}$. Anchovies followed with 40 %, showing a median concentration of 0 $MP.g^{-1}$ and a maximum of 1.09 $MP.g^{-1}$. Sardines had 39 %, with a median concentration of 0 $MP.g^{-1}$ and a maximum of 0.67 $MP.g^{-1}$.

The relationship between MP concentrations in the GT and Fulton Condition factor exhibited a low, statistically significant negative correlation for horse mackerel ($rho = -0.281, p = 0.043, N = 52$; Fig. 3) and a moderate, statistically significant negative correlation for European sardine ($rho = -0.513$, $p = 0.001$, $N = 36$; Fig. 3).

3.2. Tissue-specific variation in microplastic concentrations

Microplastic concentration differed significantly between tissues (*KW* test: $H = 71.896$, $p = 0.000$), with GT presenting the highest concentration, followed by the gills and, finally, the muscle. For horse mackerel, the GT had significantly higher concentration of microplastics than the gills ($p = 0.000$) and the muscle ($p = 0.000$). In the case of anchovies and sardines, the muscle contained significantly lower concentration of microplastics than the other tissues studied ($p < 0.05$).

3.3. Species-specific variation in microplastic concentrations

The accumulation of microplastics was not significantly different between species in the GT (*KW* test: $H = 2.089$, $p = 0.352$) and muscle (*KW* test: $H = 5.5142$, $p = 0.064$). Only the accumulation of microplastics in the gills showed a significant difference between species (*KW* test: $H = 9.3788$, $p = 0.009$), with anchovy having significantly higher concentrations than horse mackerel ($p = 0.014$).

3.4. Microplastics in water samples

Among the 12 water samples analysed, a total of 218 microplastics were recovered, with microplastics being present in all the samples. The median, minimum and maximum of the concentration of microplastics in water was 0.806 $MP.m^{-3}$, 0.228 $MP.m^{-3}$ and 1.856 $MP.m^{-3}$, respectively.

3.5. Microplastics characterization

Different types of microplastics were found in fish tissues and water samples, in terms of shape, colour, size and polymer type (Figs. 4 and 5). Fibers were the most common type of particle collected. Blue (44 %) and black (27 %) were the most common microplastic colors in the water. Blue and other colors such as green and red were also prevalent in different fish tissues, being black microplastics rarely found in any of the

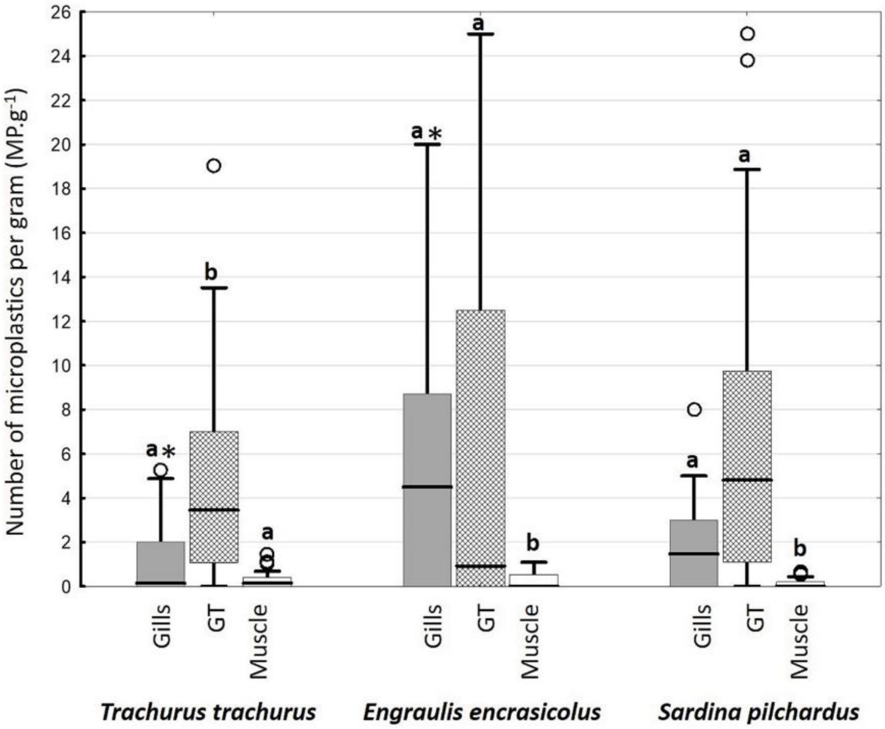

Fig. 2. Number of microplastics per gram ($MP.g^{-1}$) found in tissues of small pelagic fish caught in the Atlanto-Iberian coast during PELAGO18 survey (May 2018). Different letters indicate significant differences in microplastic concentrations among tissues for each species ($p < 0.05$). The asterisk (*) indicates significant differences in microplastic concentrations among species for the gills ($p < 0.05$).

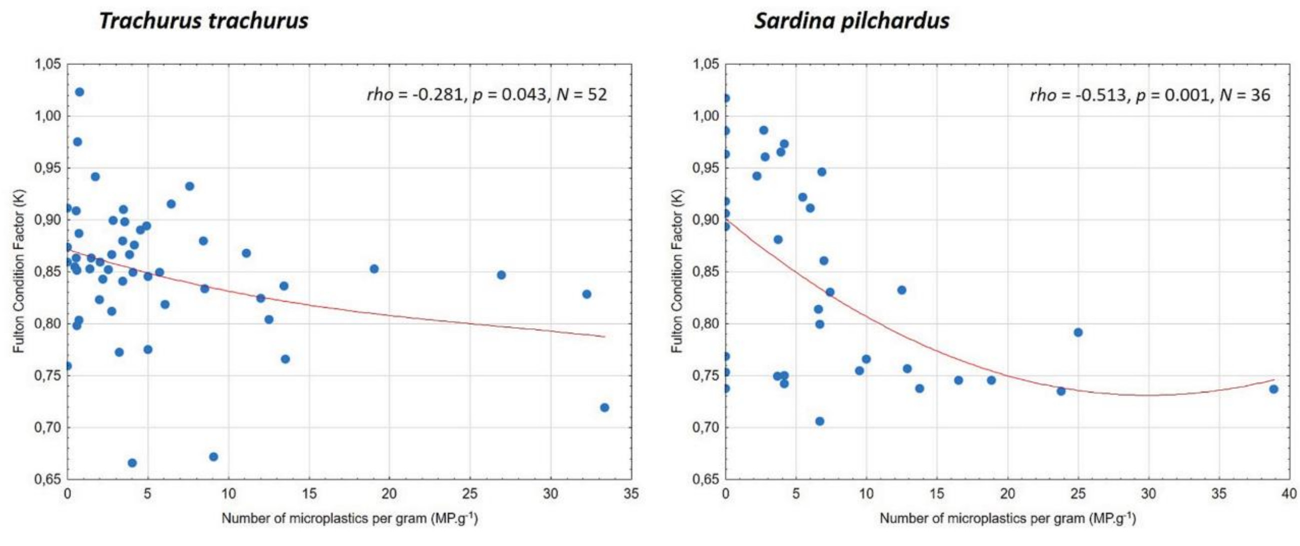

Fig. 3. Spearman Rank Correlation between Microplastic Concentrations and Fulton Condition Factor in the Gastrointestinal Tract of horse mackerel (*Trachurus trachurus*) and European sardine (*Sardina pilchardus*) from the Atlanto-Iberian coast during the PELAGO18 survey (May 2018).

fish tissues studied.

The percentage of fibers found in the digestive tract was very similar across the three species studied, ranging from 81 % to 87 % and was higher than the percentage found in water (62 %). In the case of muscle, the percentage of fibers varied greatly between fish species, accounting for 27 % of the particles found in anchovy, 65 % in horse mackerel, and the total particles found in European sardine's muscle. The percentage of fibers found in the gills of anchovies and sardines was lower than that found in water, 34 % and 40 %, respectively, whereas horse mackerel accounted for 82 % of the particles.

In general, microplastics below 0.5 mm were the most common in both the analysed species and water samples, while those between 5 and 2 mm are the least common. The percentage of microplastics below 0.5 mm in the gills of European sardine and anchovy reached high levels, with 62 % and 72 %, respectively, while horse mackerel had only 33 %. The same was true for muscles, where this size class accounted for 59 % of microplastics in sardines and 64 % in anchovies, but only 44 % in horse mackerel. Similar percentages of microplastics of all size classes

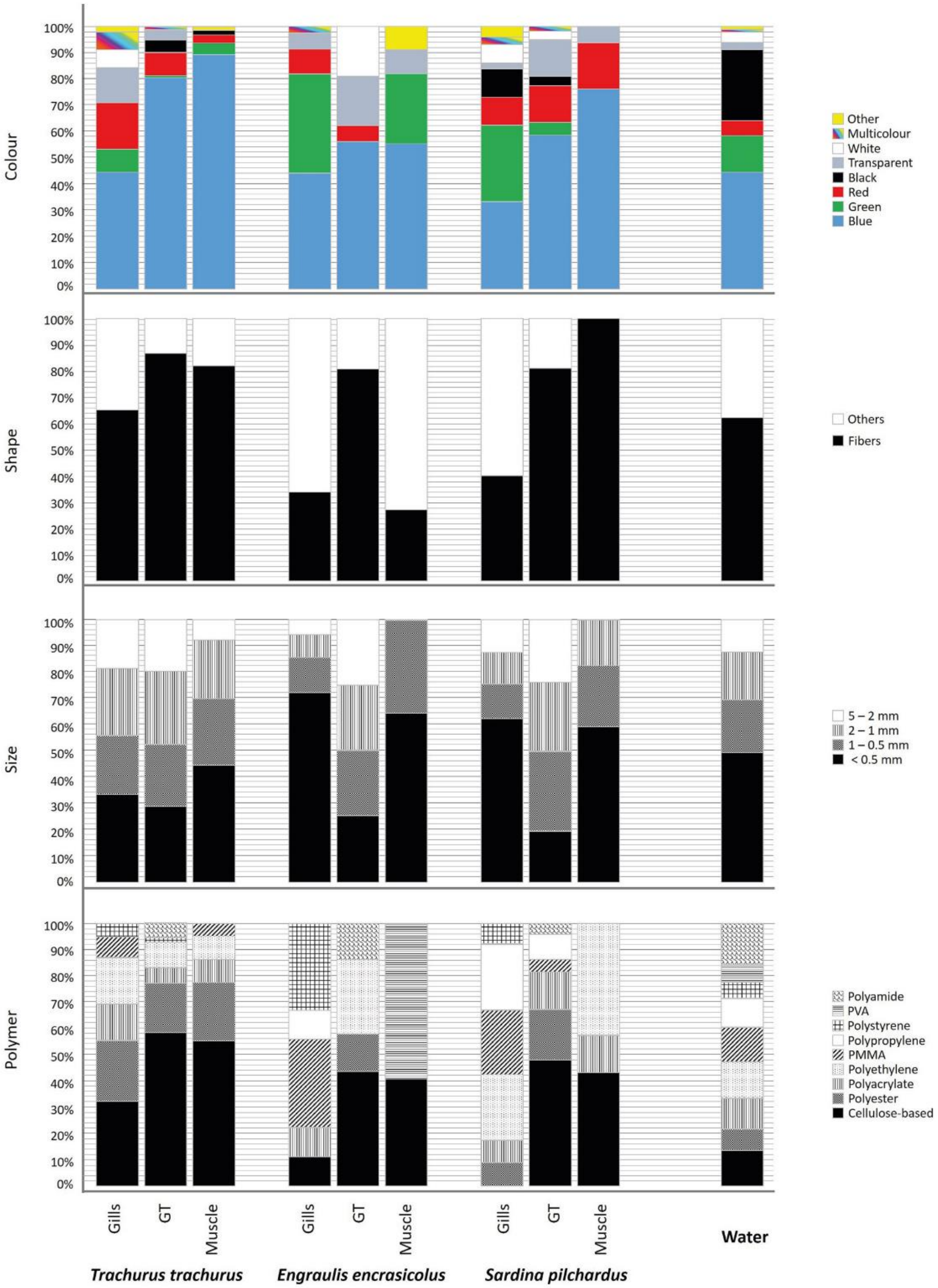

Fig. 4. Relative abundance of microplastic composition (colour, shape, size and polymer type) found in tissues of small pelagic fish and water samples from the Atlanto-Iberian coast during PELAGO18 survey (May 2018).

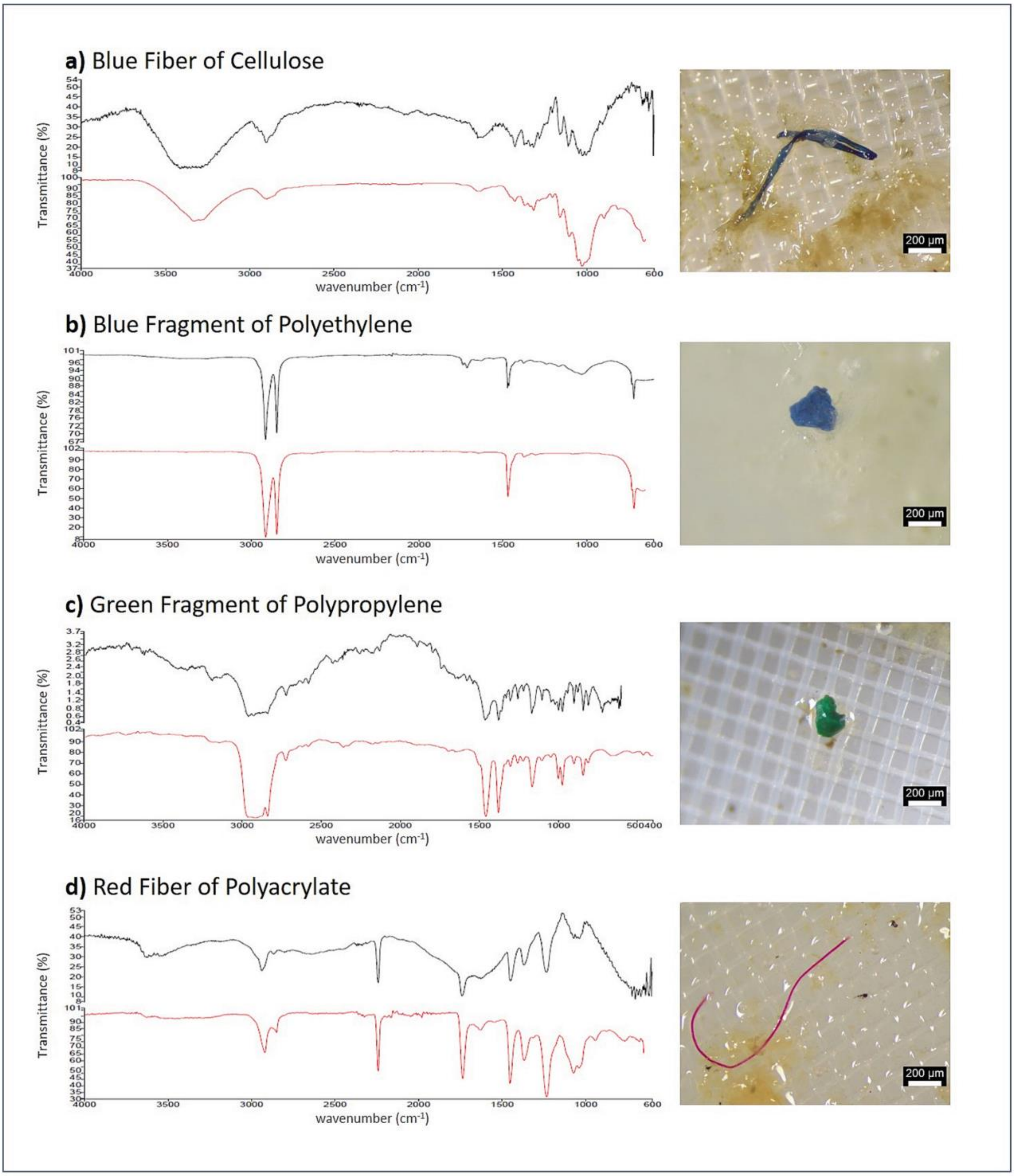

Fig. 5. Examples of microplastics found in tissues of small pelagic fish and water samples from the Atlanto-Iberian coast during PELAGO18 survey (May 2018) and their respective FTIR spectra. The black spectrum is the FTIR measurement of microplastic sample, while the red spectrum is the reference spectrum from the PerkinElmer library.

were found in the gastrointestinal tracts of all species studied.

In total, nine different polymers were identified in water samples, including polyamide (PA, 16 %), polyethylene (PE, 15 %), polymethyl methacrylate (PMMA, 13 %), cellulose-based (13 %), polyacrylate (12 %), polypropylene (PP, 11 %), polyester including polyethylene terephthalate (PET, 8 %), polystyrene (PS, 6 %), and polyvinyl alcohol (PVA, 5 %). PVA, on the other hand, was not found in any fish tissue, PP was not found in any horse mackerel tissue, and PA was only found in fish gastrointestinal tracts. Polymers based on cellulose, polyacrylate, and polyester were the most abundant in fish tissues, and the majority of them are fiber-related.

3.6. Grouping and differentiation pelagic fish tissues and water samples

The SIMPROF test identified three groups (A-C) of statistically distinct ($R = 0.79$; $p < 0.05$) samples (Fig. 6). The anchovy and sardine gills, as well as the water samples, were grouped in group B. The group A contained only anchovy muscle samples, whereas group C contained the greatest number of samples. SIMPER analyses revealed that the differences between groups B and C were primarily due to a higher percentage of PP, PMMA, PS, black, green, and non-fibers related microplastics and a lower proportion of cellulose-based polymer, polyethylene, and polyester in group B than in group C.

4. Discussion

The present study describes the distribution and prevalence of microplastics in three distinct tissues (gills, muscle and gastrointestinal tract) of three coastal pelagic fish species (*Sardina pilchardus, Engraulis encrasicolus* and *Trachurus trachurus)*, characterized by different feeding behaviors, collected off the coast of Portugal, in the Northeast Atlantic. In contrast to previous research that primarily focused on gastrointestinal tract analysis, our study emphasizes the significance of examining other tissues, particularly edible ones such as the muscle. Microplastics were detected in 92–96 % of the 118 individual fish analysed, and they were found in all species and tissues studied. Our findings reveal a widespread occurrence of plastic particles in water samples and in tissues of the studied species, highlighting the increasing plastic pollution in our oceans. These results align with the prevalence of microplastics described in various aquatic environments, including wild fish, bivalves, and polychaetes (Pequeno et al., 2021; Lopes et al., 2020; Fernández and Albentosa, 2019), and emphasize the well-known escalating issue of plastic pollution in our oceans.

This study surpassed conventional research that solely focuses on fish samples by incorporating an analysis of microplastic composition in water samples. This additional step aimed to investigate whether the microplastics found inside the fish were directly related to the environment or selectively ingested. Interestingly, we observed that the microplastics composition detected in the gills of European sardines and anchovies closely resembled the microplastics identified in the water samples. In fact, European sardines and anchovies have the ability to switch between particulate-feeding and filter-feeding, depending on factors such as light intensity, size, and availability of prey (Garrido and van der Lingen, 2014). Fish species that are able to filter-feeding engulf bulky amounts of water, comprising both planktonic organisms and plastic particles, that enters the body through the mouth and is then transferred out through the gills. By this, both prey and microplastics are entrapped in structures, like the gill rakers, and are then moved to the esophagus (Ningrum et al., 2019). This feeding behaviour makes these species more susceptible to accumulating microplastics in their gills without distinguishing between prey and plastic particles. Consequently, this non-selective feeding mechanism could explain the similarity in microplastic composition between the water and gills of European sardines and anchovies. Another explanation could be related to the behaviour of horse mackerel, which is often found in mixed shoals with other small pelagic fish in the Iberian area but tends to distribute and feed at deeper levels than other species (Lopes et al., 2020). As a consequence of this behaviour, horse mackerel is more prone to accumulate other types of microplastics, including those denser than seawater, which might not be commonly found in the pelagic environment.

Among the studied species, only the accumulation of microplastics in

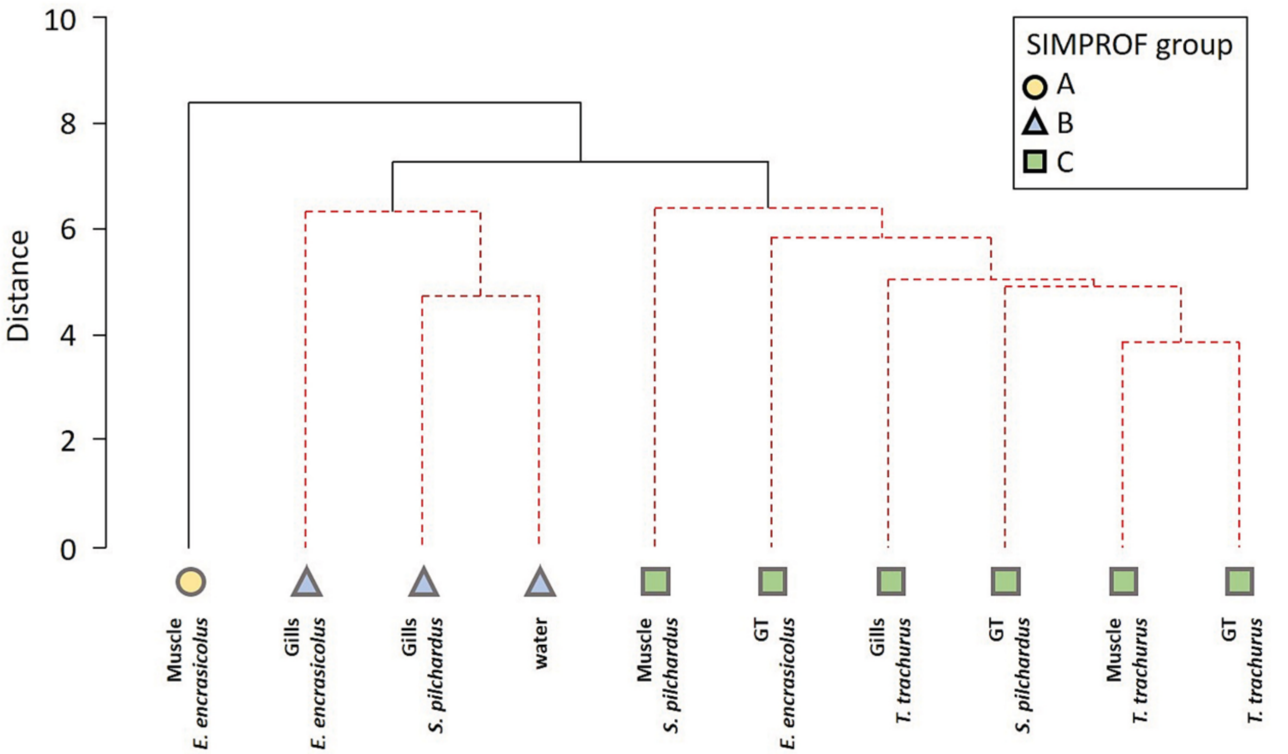

Fig. 6. Dendrogram showing the hierarchical cluster analysis with SIMPROF test on similarity of fish tissues and surrounding water based on their microplastics composition (colour, shape, size, and polymer type). Black lines indicate samples that significantly differ in their microplastic composition at 5 % level (SIMPROF test). Red dashed lines represent samples that do not significantly differ in their microplastic composition.

the gills exhibited a significant difference, with anchovy displaying notably higher concentrations compared to horse mackerel. For instance, Garrido et al. (2015) found that anchovy from Iberian waters had significantly higher feeding intensity than sardines and horse mackerel, which could explain the higher MP concentration in anchovy gills compared to horse mackerel. Additionally, these variations can be attributed to several factors, including environmental microplastic contamination and the structures of the gill rakers (Abbasi et al., 2018; Lopes et al., 2020; Collard et al., 2017).

Concerning the distribution of MP in the different tissues studied, horse mackerel displayed higher MP concentrations in the GT, whereas anchovy and sardine exhibited significantly lower concentrations in the muscle compared to the other tissues. Some authors have reported no differences in microplastic occurrence between gills and GT of fish (Lin et al., 2020). Our study aligns with these findings for sardine and anchovy, which are filter-feeding species, but it differs for horse mackerel, a particulate-feeding species. This discrepancy for horse mackerel may be attributed to the fact that microplastics in their gills are more influenced by the surrounding environment, while those in the GT are influenced not only by the microplastics present in the surrounding environment but also by the microplastics in their available food sources. While the size of microplastics overlaps with the important prey size class for all studied species, there are variations in the types of prey consumed. For instance, sardines consume a significant amount of prey in the size class <500 μm, followed by anchovy. In contrast, horse mackerel does not significantly consume prey in this size class (Garrido et al., 2015). Sardine primarily consumes small plankton (phytoplankton, crustacean eggs, and nauplii) which can represent as high as 90 % of its dietary carbon. On the other hand, anchovy and horse mackerel mainly feed on calanoid copepods and decapods, with crustacean eggs and nauplii also playing a crucial role in anchovy's diet (Fonseca et al., 2022; Garrido et al., 2015). Additionally, Lopes et al. (2020) reported that microplastic accumulation in small pelagic fish from the same area as our study was more closely associated with the size of prey consumed by the fish rather than the specific type of prey. They found that horse mackerel, which depends on larger mesozooplankton prey, accumulated more microplastics in their stomachs than species that rely on smaller planktonic prey, such as sardines. However, in our study, we did not find any significant differences in microplastic concentrations in the GT among the different fish species.

Fibers were the prevailing microplastic shape recorded for all species. This was expected, as fibers have been described as one of the most frequent microplastics in the ocean (Cole et al., 2011), representing the major constituents of synthetic clothing and fishing nets. Considering the same sampling area, fibers have been appointed as the most common microplastics in fish (Neves et al., 2015; Bessa et al., 2018), agreeing with our results. Although this percentage is consistent with the described prevalence of fibers in various fish species (Pequeno et al., 2021; Murphy et al., 2017), Barboza et al. (2020a) described that horse mackerel from the North coast of Portugal showed a higher percentage of fragments (76 %) than fibers (22 %), in the gastrointestinal tract. Nevertheless, this distinct pattern could be linked to diverse pollution sources and illustrates the importance of monitoring and collecting environmental data, such as water samples to correlate data with MP availability. Here, the majority of microplastics found in water were fibers (62 %) and so were most microplastic shape found in organisms. Interestingly, the studied species presented much higher percentages of fibers in their tissues, ranging from 81 to 87 %, than that present in water, showcasing the great bioaccumulation potential of these microplastic form.

According to Filgueiras et al. (2020) polyethylene, polyester, polypropylene, polyamide and acrylics are the most common types of polymers present in the ocean as these are used for fishing and may also be discharged on wastewater. In this study the most common polymer types present in fish were cellulose based, polyacrylate and polyesters, all fiber related. Although our findings are consistent with others describing microplastic types in fish in the Northeast Atlantic, PP was not quantified in any of the horse mackerel tissues despite it being a widely distributed polymer in water (Erni-Cassola et al., 2019) and also found in surrounding water. Another interesting result regards PVA comprising 5 % of particles found in water samples, but being absent from fish tissues. The lack of bioaccumulation of this polymer observed here also stresses the need for further investigation.

In the present study the most common microplastic size class was <0.5 mm. This result is in line with microplastic size assessment in other fish species (Atamanalp et al., 2021). This incidence of smaller microplastics was expected as smaller sized microplastics contribute to unintentional ingestion of this particles by fish (Wang et al., 2021). Furthermore, several oceanic phenomena contribute to the breakdown of plastic, such as mechanical action, biodegradation and photochlorination (Isobe et al., 2015), which increases the availability of smaller pieces. Globally, larger microplastics are more common in the GT and gills, in comparison to muscle as bigger particles enter the digestive tract freely, encountering little to no obstruction. Considering the results of the present study, the percentage of MP < 0.5 mm observed in the analysed species gills were comparable to the ones in the muscle. Collard et al. (2017) argued that particles may cross the gut barrier by either intracellular or paracellular endocytosis. As the resemblance between gills and muscle microplastics percentage did not occur for bigger particles, we suggest that this mechanism may be behind this percentage similarity, for the three studied species, being this passage facilitated for smaller microplastics.

According to the colour analyses, our results showed that the blue colour was the most dominant both in the water samples and the studied fish species. Other studies have described blue as the dominant microplastic colour present in fish (Neves et al., 2015; Bessa et al., 2018; Barboza et al., 2020a). In fact, blue particles in sea water are very common and may be directly or indirectly bioaccumulated (Barboza et al., 2020a), while black and red are other frequent colors (Lin et al., 2020). Although microplastic colour may be transformed by UV radiation, weathering, and microbial degradation (Zhang et al., 2021), previous studies have described the attractiveness of marine organisms toward the blue colour (Ory et al., 2018). In the present study we sampled adult specimens, and as Bellas et al. (2016) described that young fish prefer black microplastics, as they closely mimic their food, if we had sampled fish of different age classes the colour results may have differed. Hence, we suggest that future studies focus on the accumulation of microplastics throughout ontogeny. Interestingly, 27 % of microplastics found in water were black, however a comparable percentage was not found in the studied species tissues. This suggests a selectivity ability, to some degree, by the studied species.

The concentrations of microplastics found in water samples were much lower than the mean predicted no-effect concentration (PNEC) value of 3.84×10^6 part.m^{-3} determined for microplastics in the marine water column (Adam et al., 2020), indicating that there is no immediate risk. Overall, PNEC values for microplastics are important for understanding the potential ecological risks associated with microplastic pollution and for developing policies and regulations to mitigate these risks. However, there are significant discrepancies between the polymer types, shapes, and sizes of particles detected in the ocean and those used in hazard studies, which seriously compromises the usefulness of the hazard data set for risk assessment. For example, the shapes of microplastics tested in bioassays are mainly spheres, while fibers were the main shapes found in our study. Additionally, the average diameters of microplastics used in toxicity studies are smaller than the lower size limit of microplastics set in this study. Therefore, although risks are highly unlikely, they cannot be excluded.

Lastly, we noted a statistically significant negative correlation between the presence of microplastics in the GT and the Fulton condition factor for horse mackerel and sardine. However, the strength of this correlation was weak, indicating that it is insufficient to draw robust predictions or establish a direct cause-and-effect relationship. These results highlight the need for further investigation to better understand the complex interaction between microplastic exposure and fish condition, taking into account potential non-linear patterns and other factors that may confound the results. Regarding microplastic toxicity, its prevalence may prompt histological alterations and oxidative stress (Lu et al., 2016), while freeing incorporated or adsorbed potential contaminants (Ashton et al., 2010). This shows the potential negative impacts of microplastic is fish tissues, while denoting that plastic ingestion and/or retention affects fish body condition. For example, microplastic in gills may provoke physical damage and obstruct gas exchange with implications for breathing (Jabeen et al., 2018). Regarding the GT, internal abrasion and obstruction may occur, and malnutrition has been described as a toxic consequence of microplastic bioaccumulation (Jeong and Choi, 2019; Jovanović, 2017). Moreover, various field studies have provided evidence of a connection between microplastics and the presence of harmful substances in fish tissues, such as bisphenol A (BPA), polybrominated diphenyl ethers (PBDEs), and lower chlorinated polychlorinated biphenyls (PCB) congeners (Barboza et al., 2020b; Gassel and Rochman, 2019; Rochman et al., 2014a) and some laboratory investigations have explored the ecotoxicological effects of microplastics in combination with these associated chemicals on fish. For instance, Chen et al. (2017) discovered that the simultaneous exposure of nanoplastics and BPA led to increased neurotoxic effects in adult zebrafish. Similarly, when Japanese medaka fish were exposed to a mixture of plastic and sorbed PAHs, PCBs, and PBDEs congeners, early warning signs of endocrine disruption (Rochman et al., 2014b) and hepatitic stress (Rochman et al., 2013) were induced. Despite microplastics seemingly making a negligible contribution to overall contaminant exposure compared to intake through food and water (Bakir et al., 2016), it is crucial to emphasize the necessity for comprehensive studies evaluating the effects of chemicals migrating from plastics and microplastics on fish. Overall, these consequences may impact fish growth and reproduction, with profound impacts on biological food chains, sustainability, ecosystems functioning and global health and economy.

5. Conclusion

Our study confirms the ubiquitous extent of microplastic contamination in the ocean. Data described here is pivotal for the assessment of microplastic bioaccumulation, particularly in edible tissues as the muscle, and consequent human consumption of contaminated fish. Hence, we provide baseline evidence of microplastic tissue distribution in three small pelagic fish species, presenting distinct feeding behaviors. Microplastics were present in every studied tissue and species with body condition being impacted by increasing loads of microplastic for *T. trachurus* and *S. pilchardus*. As the studied species are present in human diet, and present a key ecologically and economically role, we advise the scientific community to come together into unveiling the potential implication of microplastic intake for human health. Portugal is the third country in the world with the biggest consumption of fish per capita, reaching a value as high as 61.5 Kg per capita per year (Guillen et al., 2019). Therefore, further research is needed for a better understanding of the microplastic distribution in biological matrices, human diet and the integrated potential deleterious effects of our increasingly microplastic contaminated world.

CRediT authorship contribution statement

C. Lopes: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **A. C. Ambrosino:** Methodology, Validation, Writing – review & editing. **C. Figueiredo:** Methodology, Validation, Writing – review & editing. **M. Caetano:** Validation, Writing – review & editing **M. M. Santos:** Validation, Writing – review & editing. **S. Garrido**: Validation, Writing – review & editing, Funding acquisition. **J. Raimundo:** Conceptualization, Validation, Investigation, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Chapter 4

Microplastic ingestion and diet composition of planktivorous fish

LIMNOLOGY AND OCEANOGRAPHY
Letters

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SPECIAL ISSUE-LETTER

Microplastic ingestion and diet composition of planktivorous fish

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Scientific Significance Statement

Microplastics (MP) pollution in marine ecosystems is a worldwide problem. Factors that influence their ingestion by different fish species are still not well understood. To our knowledge, this is the first work coupling MP accumulation and a full taxonomic description of planktivorous fish diet. We found that MP accumulation was more associated with prey size consumed by fish than with prey type, and species depending on larger mesozooplankton prey accumulated more MP than those dependent on smaller planktonic prey. We identify horse mackerel as a suitable bioindicator for MP monitoring in the pelagic Iberian ecosystem.

Abstract

Planktivorous pelagic fish are susceptible to accumulating microplastics (MP), which have the same size range as their prey and accumulate in their feeding and spawning grounds. We analyzed stomach contents of pelagic fish (European sardine, horse mackerel, anchovy, chub mackerel, Atlantic mackerel, and bogue) from Atlanto-Iberian waters to investigate the relationship between MP ingestion, their diet composition and select a potential bioindicator. We found significant differences between diet of the studied fish species in terms of prey type and size. MP ingestion was significantly related to diet composition. Species with diets that include smaller prey (European sardine, chub mackerel, and bogue) had lower MP concentration in the stomachs than fish depending on larger mesozooplanktonic prey. Horse mackerel had the highest proportion of larger prey ($> 1000\ \mu$m) and the highest MP abundance in the stomachs, and thus are a suitable bioindicator for MP monitoring in the pelagic Iberian ecosystem.

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Author Contribution Statement: C. Lopes and J. Raimundo conducted MP analysis. S. Garrido conducted prey analysis. C. Lopes and S. Garrido conducted data analysis. All the authors contributed to experimental design, manuscript writing and critical revisions.

Data Availability Statement: Data are available in the Dryad repository at https://doi.org/10.5061/dryad.0246352.

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Edited by: Dr Elise Granek, Portland State University, Dr Susanne Brander, Oregon State University, and Dr Erika Holland, California State University, Long Beach.

Over 92% of ocean plastic pollution is composed of particles smaller than 5 mm, known as microplastics (MP) (Eriksen et al. 2014). MP enter directly into the aquatic environment (e.g., microbeads used in cosmetics and microfibers from textiles) or result from the breakdown of larger plastic debris (Cole et al. 2011). Being dispersed by currents and wind, MP are globally distributed in different marine environments (Woodall et al. 2014; Waller et al. 2017; Antunes et al. 2018).

MP are difficult to detect and can interact with a wide range of organisms from all trophic levels with unknown effects on biota and human health (Barboza et al. 2018). MP were detected in several marine fish, including commercial value ones (e.g., Neves et al. 2015; Bellas et al. 2016; Bessa et al. 2018). Once ingested, MP may cause physical damage, suffocation, gut blockage, and nutritional depletion (Jovanović 2017). Additionally, additives incorporated during plastic manufacturing and pollutants adsorbed on their surface may increase their toxicity (Avio et al. 2015).

Laboratory observations suggest that MP uptake by fish is influenced by particle properties (shape, size, color, and density) and by trophic ecology of the species (e.g., feeding behavior, diet composition, and habitat) (Ory et al. 2018). MP trophic transfer may occur indirectly by consumption of contaminated prey (Chagnon et al. 2018), or directly through water or sediment (Besseling et al. 2013; Ory et al. 2018). Experimental work is crucial to identify potential risks and pathways but does not provide an accurate representation of natural conditions (Phuong et al. 2016).

Small- and semi-pelagic fish species have high economic importance worldwide and represent the bulk of fish biomass, particularly in upwelling regions (FAO 2016). Their low and middle trophic position is crucial in pelagic food webs, having an important effect on lower trophic levels dominated by plankton organisms and, at the same time, controlling predatory fish (Cury et al. 2000). Pelagic fish are planktivorous during entire their life cycle (such as sardines and anchovies) or at least during the initial stages of development, incorporating piscivory at adult stage (such as horse mackerel and chub mackerel) (Garrido et al. 2015). Planktivorous fish are particularly susceptible to accumulating MP with similar size, shape, and color to their prey, particularly planktonic organisms (Wright et al. 2013). Differences in feeding strategies may affect the MP consumption. For instance, filter-feeders are more susceptible to passively uptake MP from surrounding water (Collard et al. 2017), while particulate-feeders may actively consume MP by confounding them with their prey (de Sá et al. 2015; Ory et al. 2018).

The most abundant coastal pelagic fish species in the Western Iberia Upwelling Ecosystem (WIUE) are European sardine, horse mackerel, anchovy, and chub mackerel, all of which mostly planktivorous, at least during the larval and juvenile stages (Garrido et al. 2008, 2015; Garrido and Murta 2011). There are known differences in the feeding behavior and diet composition of these species (Garrido and van der Lingen 2014; Bachiller and Irigoien 2015; Garrido et al. 2015), but the implications on MP accumulation are unknown.

Our three objectives are: (1) investigate the presence of MP in stomach contents of six of the most common pelagic fish species in the WIUE: European sardine (*Sardina pilchardus*), horse mackerel (*Trachurus trachurus*), anchovy (*Engraulis encrasicolus*), chub mackerel (*Scomber colias*), Atlantic mackerel (*Scomber scombrus*), and bogue (*Boops boops*); (2) investigate the relationship between MP ingestion and interspecific differences of diet composition and feeding behavior, as well as habitat; and (3) assess the most susceptible species and the best candidate as bioindicator for MP monitoring in the pelagic marine environment. To our knowledge, this is the first work coupling MP accumulation and a full taxonomic description of planktivorous fish diet.

Methods

We collected a total of 327 pelagic fish from six commercial species (European sardine, horse mackerel, anchovy, chub mackerel, Atlantic mackerel, and bogue) in 16 stations along the west and south Iberian coast (Fig. 1; Table 1) during a research cruise (PELAGO14) carried out from April 3rd to May 12th in 2014. Western and southern parts of Iberian coast were analyzed separately due to known differences in oceanography and productivity (Mason et al. 2005) that were shown to affect diet composition of planktivorous fish populations (Garrido et al. 2008). We used a 20 mm mesh size pelagic trawl at depths between 16 and 50 m. Total length (cm) and weight (g) of fish were determined onboard and stomachs removed and frozen for posterior laboratory analyses (Table 1).

We conducted microscopic analysis of food items in pools of 2–10 stomachs from the same fish species and length class ($\pm$ 1 cm), and collected in the same trawl haul (Table 1) (Garrido et al. 2015). After identifying prey items, we processed samples to extract potential MP, using a solution of 10% KOH, prepared in ultrapure water, for 24 h at 60°C (Dehaut et al. 2016) and filtered through 20 μm polycarbonate membrane. All potential MP were visualized and photographed using a stereomicroscope (LEICA S9i) with an integrated camera (IC80 HD). We categorized particles by color (black, blue, transparent, white, red, green, and other) and size using the ImageJ software. We measured particles at their largest cross section and categorized them according to five size classes ($\leq$ 0.5 mm, 0.5–1 mm, 1–2 mm, 2–3 mm, and > 3 mm). We distinguished fibers from other types of MP shapes.

To account for background contamination, we washed all material with ultrapure water and samples were always kept under a clean air laminar flow hood (HEPA filter, class ISO5) or maintained in covered glass recipients. Procedural blanks were performed simultaneously with real samples. During microscopic analyses for prey and MP identification, we placed two open Petri dishes with filters near to the working zone in order to control airborne fiber contamination.

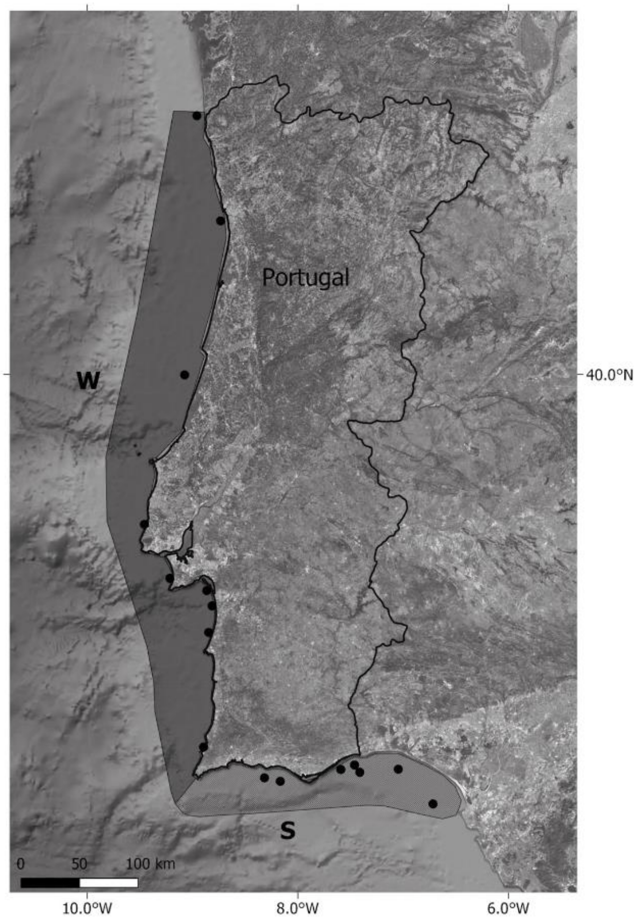

Fig. 1. Sampling sites location of small pelagic fish caught in the Atlanto-Iberian upwelling ecosystem during PELAGO14 survey (May 2014) in two different areas: Western Iberia (W) and Southern Iberia (S).

We selected a subset of 38 potential MP in order to identify their chemical composition by Fourier transform infrared spectrometry (FTIR) using a PerkinElmer Spotlight 200i FTIR Imaging System equipped with a mercury cadmium telluride (MCT) array detector cooled by liquid nitrogen. We collected spectra in attenuated total reflection mode using a germanium crystal. Measurement resolution was set at 4 cm^{-1} ranging from 4000 to 600 cm^{-1} with a minimum of four scans. To confirm the polymer type, we compared all spectra to library databases and then compare analysis of the polymer characteristic bands with spectra assignments. Only polymers matching reference spectra for more than 70% were accepted.

We performed all statistical analysis using the open source software R version 3.5.0 (R Development Core Team, 2011; www.r-project.org). Stomach content weight was compared between species using a nonparametric Kruskal-Wallis test, to investigate if the potential difference in the number of MP found in the stomachs per species was related to stomach fullness. We used analysis of covariance to test for differences of MP ingestion between fish species and areas. Since Atlantic mackerel and horse mackerel were not represented in all areas, the study of the combined effect of species and area on MP accumulation were restricted to European sardine, anchovy, bogue, and chub mackerel. Statistical tests were considered significant at p values < 0.05.

Differences in the diet composition between species and areas were investigated using Adonis (permutation multivariate ANOVA; Anderson 2001) in "vegan," using the Bray-Curtis distance and 999 permutations (Oksanen et al. 2010). We analyzed data dispersions of the prey communities among groups using the Betadisper test (permutational analysis of multivariate dispersions) in "vegan" and performed multiple comparisons using the "pairwise.adonis" function (Martinez Arbizu 2019) to identify the differences between each species pair.

We applied the principal component analysis (PCA, using the "prcomp" from the package "stats" in R) to inspect the relationship between prey type or prey size in the diet and MP accumulation. Prey size classes 0–200, 200–500, 500–1000, 1000–2000, and > 2000 μm were used in the analysis. The variables were scaled before the analysis to have unit variance. In

Table 1. Fish species, sample size (*n*), area, size class (cm), total number of MP, occurrence (%) of pool of stomachs with MP, and MP per fish in the stomach contents of *B. boops*, *E. encrasicolus*, *Sardina pilchardus*, *Scomber colias*, *Scomber scombrus*, and *T. trachurus* from the Portuguese coast.

Species	Sample size (n)	Area	Size class (cm)	Total number of MP	Occurrence (%)	MP per fish: Median	MP per fish: Interquartile range
B. boops	19	W, S	20–24	9	60	0.25	0.00–0.75
E. encrasicolus	131	W, S	12–17	77	79	0.48	0.10–0.90
Sardina pilchardus	76	W, S	14–22	22	58	0.16	0.00–0.53
Scomber colias	58	W, S	19–27	27	64	0.23	0.00–0.67
Scomber scombrus	19	W	22–36	17	100	1.00	0.29–2.50
T. trachurus	24	W	13–21	41	100	1.75	1.20–2.00

order to identify the significant principal components to explain the variance in the data, the Broken Stick criterion was followed, using the "evplot" function (package "lmom").

Results

We found MP in stomach contents of all analyzed fish species, identifying a total of 193 potential MP (Supporting Information Fig. S1). No MP were observed in procedural blanks. Fibers were the primarily MP found in all fish species representing more than 80% of particles, except for horse mackerel (76%) and Atlantic mackerel (65%) that also accumulated other forms of MP (Fig. 2A). The most prevalent MP colors were blue (44%) and black (29%) (Fig. 2B). MP size ranged from 21 μm to 5 mm (except one fiber at 20 mm), being the most common size class < 500 μm (34%) (Fig. 2C).

We selected randomly a subset of 38 potential MP (20% of total particles) between all species in order to verify their polymeric identity. Within these particles, 23 were fibers and 15 belong to other shape categories (fragments, films, and filaments). FTIR analyses revealed that the predominant polymeric form was polypropylene with an overall frequency of 21%, followed by polyethylene (16%), cellulose (16%), rayon (13%), styrene/acrylic copolymer (11%), polyacrylate (8%),

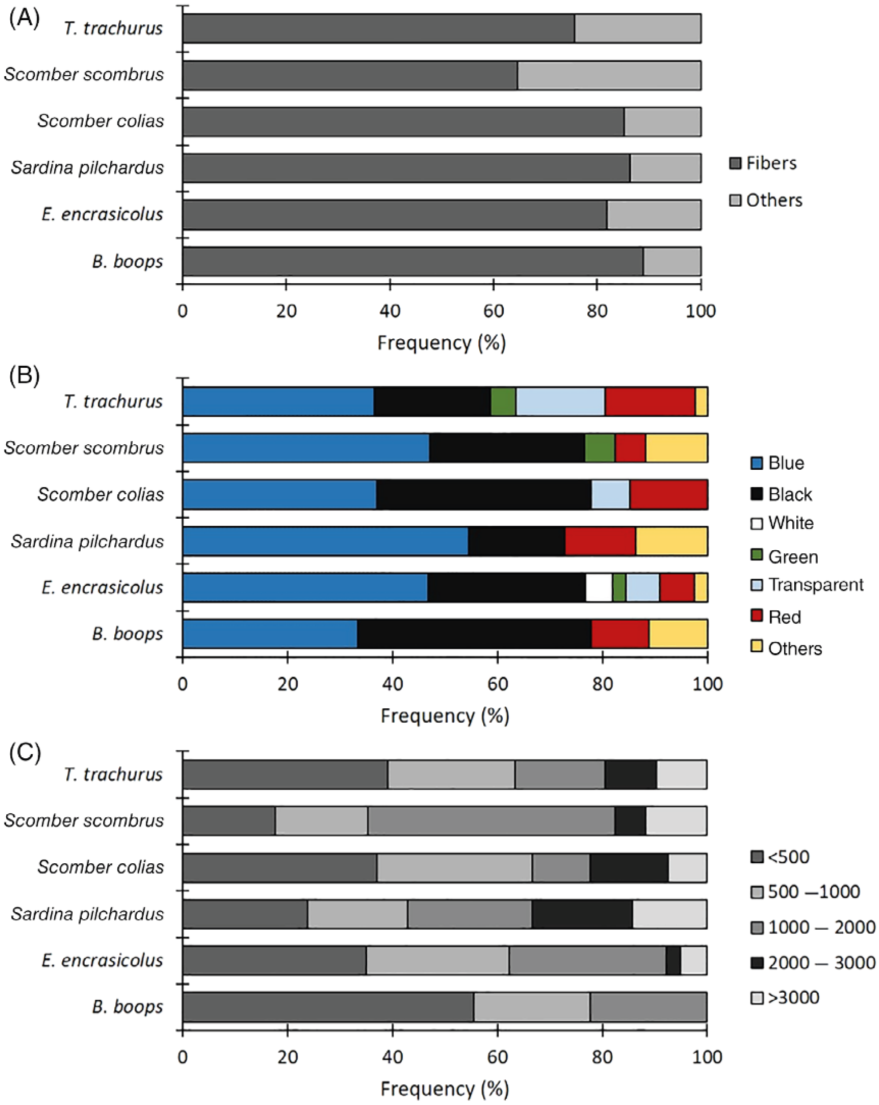

Fig. 2. Microplastics frequency (%) found in *B. boops*, *E. encrasicolus*, *Sardina pilchardus*, *Scomber colias*, *Scomber scombrus*, and *T. trachurus* caught in the Atlanto-Iberian ecosystem characterized by shape (**A**), color (**B**), and size class (**C**).

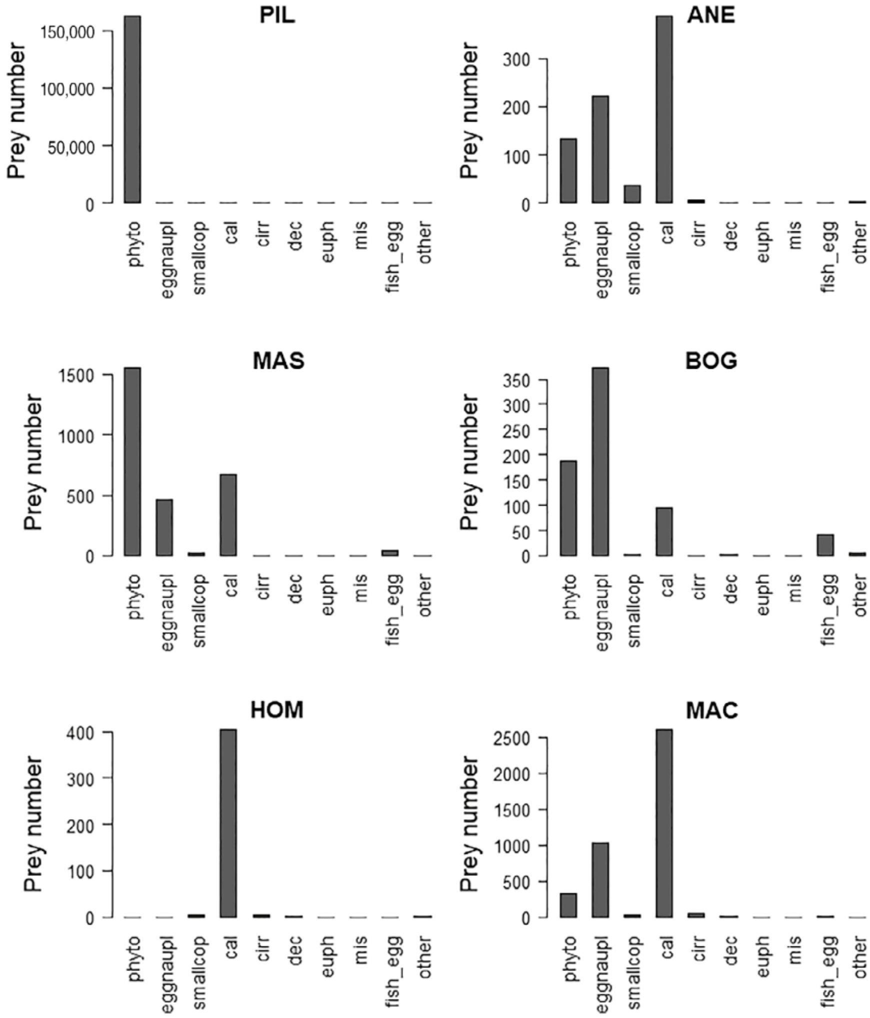

Fig. 3. Number of most important prey types found in the stomachs of *Sardina pilchardus* (pil), *E. encrasicolus* (ane), *Scomber colias* (mas), *B. boops* (bog), *T. trachurus* (hom), and *Scomber scombrus* (mac) collected in the Portuguese coast during the PELAGO14 survey.

nylon-6 (4%), polyethylene terephthalate (4%), and polymeric epoxy plasticizer (4%). A total of 13% of all particles analyzed did not reach the pre-established threshold of 70% match with any of the materials within the FTIR spectra libraries, being excluded from analysis.

No statistical differences were found between the number of ingested MP and the sampling area (F = 0.066, p = 0.798). We found significant differences between the number of MP accumulated among species (F = 6.022, p = 0.001). Particularly, horse mackerel presented higher number of MP per stomach than European sardine, anchovy, and chub mackerel (Table 1, Fig. 4B). The number of fibers and particles accumulated was also similar between the two areas (F = 0.434, p = 0.51 and F = 1.245, p = 0.28, respectively) and significantly different between species (F = 3.586, p = 0.022 and F = 6.022, p = 0.001, respectively), being higher for horse mackerel and anchovy comparing to European sardines and chub mackerel.

Stomach content weight was similar between all species (Chi-squared = 10.40, p = 0.065). All pelagic species analyzed in this study were planktivorous but the diet composition was different between them (Fig. 3, Supporting Information Table S1). Calanoid copepods were a major prey group identified for all studied species, namely *Calanus helgolandicus*. European sardine, chub mackerel, and bogue consumed smaller prey items, including phytoplankton (mostly dinoflagellates). This contrasts with Atlantic mackerel and horse mackerel, for which the contribution of small prey such as phytoplankton was lower. Anchovy diet was intermediate between these two groups,

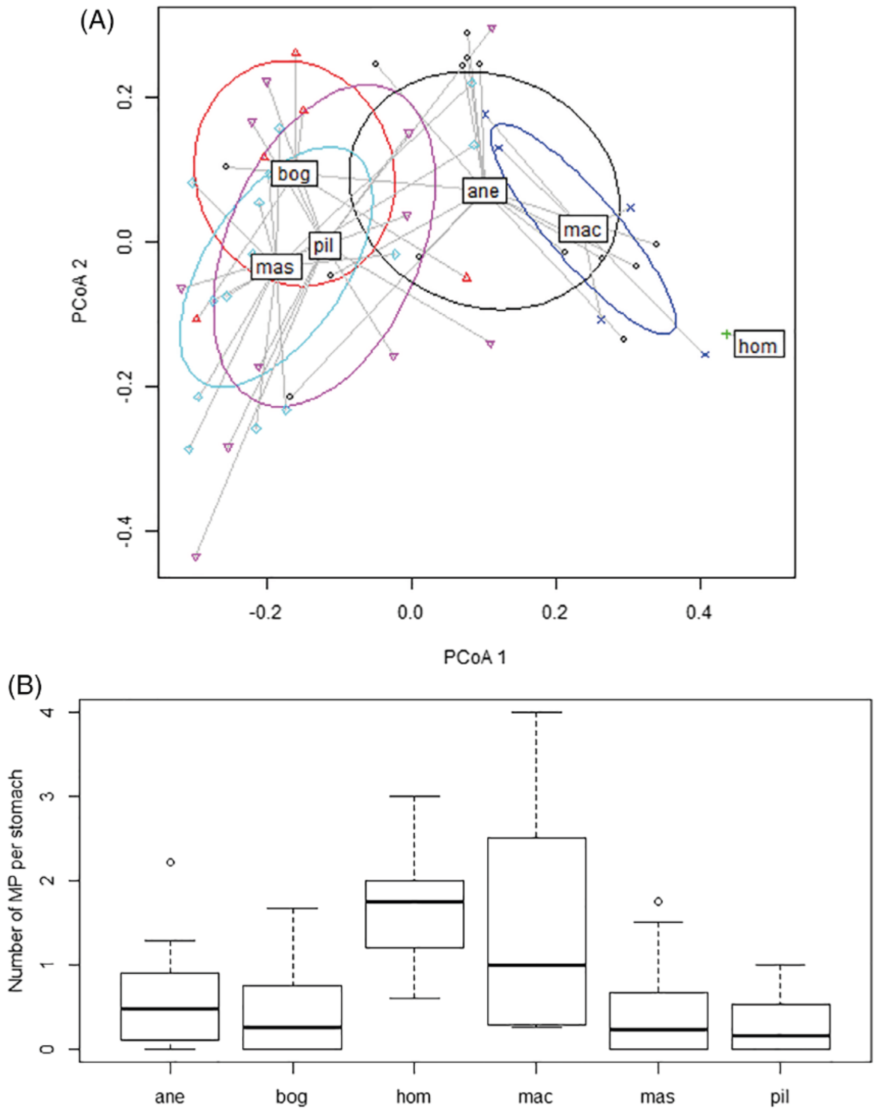

Fig. 4. Biplot of distances to the group centroid of multivariate dispersion analysis of prey composition (**A**) and boxplot of number of MP (**B**) by fish species (*Sardina pilchardus* [pil], *E. encrasicolus* [ane], *Scomber colias* [mas], *B. boops* [bog], *T. trachurus* [hom], and *Scomber scombrus* [mac]) collected in the Portuguese coast during the PELAGO14 survey. PcoA1 and PcoA2 in pannel A refer to principal coordinates of the multivariate dispersion.

ingesting small microplankton and large mesozooplankton, such as decapods and euphausiids. Permutational multivariate analysis of variance (PERMANOVA) revealed that diet of pelagic fish tested here did not vary significantly between areas (Adonis test $F = 2.49$, $p = 0.07$) but varied significantly between species (Adonis test $F = 7.86$ $R^2 = 0.44$, $p < 0.01$) in combination with the Betadisper test ($F = 5.46$, $p = 0.001$) (Fig. 4A). Multiple comparisons revealed significant differences of diet between horse mackerel and several species namely anchovy ($F = 15.19$, *p*-adjusted = 0.01), chub mackerel ($F = 36.22$, *p*-adjusted = 0.015), and European sardine ($F = 18.15$, *p*-adjusted = 0.01). Anchovy diet was significantly different from chub mackerel ($F = 8.25$, *p*-adjusted = 0.01). Diet of Atlantic mackerel was different from that of European sardine and chub mackerel ($F = 10.56$, *p*-adjusted = 0.01 and $F = 5.96$, *p*-adjusted = 0.06, respectively).

The first principal components (PCs) of the analysis of MP and prey type in stomachs accounted for a small percentage of the variance. The Broken stick model identified seven significant PCs explaining 77% of the variance. PCA results of MP accumulation and prey size identified three significant components for prey size explaining 83% of variance. Small prey vs. large prey defined the first component and MP accumulation was mainly associated with prey > 1000 μm. The second and third components showed preferential association to prey of size class 1000–2000 μm. Species whose diet depends mostly on prey of that size range accumulated a higher percentage of MP, particularly horse mackerel and Atlantic mackerel, followed by anchovy (Supporting Information Fig. S2).

Discussion

Most MP found in this study were fibers and the predominant color was blue, similarly to other studies (Neves et al. 2015; Bessa et al. 2018; Herrera et al. 2019). Polypropylene and polyethylene were the main polymer forms found, agreeing with previous studies carried out at the Portuguese coastal area (Frias et al. 2014; Neves et al. 2015; Antunes et al. 2018). These polymers were the most produced by plastic industry being frequently found in different marine environments around the world (GESAMP 2015). Most colored fibers were made of cellulose (cotton), semisynthetic cellulose (rayon), and polyethylene terephthalate (polyester), from textiles and entering marine environment via wastewater treatment (Browne et al. 2011; Rochman et al. 2015). Nylon-6 fibers are widely used in fishing industry, while styrene/acrylic copolymer and polyacrylate are waterproof polymers frequently used in paints and coating products for nautical activities (GESAMP 2015). MP ranged between 21 and 5000 μm, overlapping prey size range of pelagic fish studied. However, the methodology used does not allow detection of particles < 20 μm, which would be preferentially retained by European sardine and chub mackerel. Moreover, small particles may easily pass through the digestive tract and be rapidly eliminated with faeces (Karakolis et al. 2018).

Atlantic mackerel and horse mackerel ingested more MP of various colors and shapes that the other pelagics. This can be related to their feeding behavior, selecting larger prey and generally found deeper in the water column than the other investigated species. Some studies point to demersal fish as being more prone to accumulating MP than pelagic fish (e.g., Bellas et al. 2016; Bessa et al. 2018), this is probably associated with plastic/MP sink and accumulation in the bottom sediments (Woodall et al. 2014). Other studies found opposite results or no relation between MP ingestion and fish distribution in water column (e.g., Anastasopoulou et al. 2018). Most fish were collected from mixed shoals, pointing that feeding behavior is also important for differential ingestion of MP.

Fish species depending on smaller prey, such as European sardines and chub mackerel, had the lowest concentration of MP in stomachs. These species are the most efficient in retaining small particles (Garrido et al. 2007, 2015). European sardines can alternate their feeding behavior between particulate-feeding and filter-feeding, depending on the light intensity, size, and available prey concentration (Garrido and van der Lingen 2014). Laboratory studies suggest that particles > 890 μm (the size of most of the MP found in this work) elicit a particulate-feeding mode, which is a visual and selective feeding mode (Garrido et al. 2007). Chub mackerel presented the highest diet similarity with European sardine. Particularly, both ingest high numbers of pelagic fish eggs, suggesting that they mostly feed in the upper water column, according to Zwolinski et al. (2006). These two species are distributed upper in the water column than Atlantic mackerel and horse mackerel off the Atlanto-Iberian waters. Since MP tend to accumulate in the neuston layer as buoyant pelagic fish eggs, one could expect higher accumulation of MP for these species.

Horse mackerel, a particulate-feeding species, accumulated a high number of MP but mostly < 1000 μm (63%), while the modal prey size class was 1000–2000 μm. Although this species is frequently found in mixed shoals with other small pelagics off the Iberia, it is known to distribute and feed deeper than other small pelagics in this area (V. Marques pers. comm.). On the contrary, Atlantic mackerel was one of the species that accumulated the highest concentration of MP with similar size range to its prey (1000–2000 μm), suggesting that MP might be confounded with prey. However, the data presented here do not allow us to determine if there is selection of a given MP size, for which the MP availability in the water would have to be known.

MP abundance found in the coastal pelagic fish species caught off Atlanto-Iberian was in agreement with concentrations found in digestive tract of other pelagic fish species from the Portuguese coastal and estuarine waters (Neves et al. 2015; Bessa et al. 2018) and from western Spanish Mediterranean coast (Compa et al. 2018). Conversely, Herrera et al. (2019) reported high numbers of MP per stomach for chub mackerel in the Canary Islands coast while Neves et al. (2015) found lower numbers of MP in stomachs of horse mackerel and Atlantic mackerel. Differences among studies can be related with environmental and ecological factors, given that vertical migration and trophic ecology of populations of the same fish species are known to vary seasonally (Garrido et al. 2008) and spatially (Costalago et al. 2015).

In conclusion, MP ingestion was significantly influenced by diet of planktivorous fish, with species feeding on smaller prey (such as European sardine, chub mackerel, and bogue) accumulating less MP in their stomachs when compared to species feeding on larger mesozooplankton organisms. Species that ingested more MP were Atlantic mackerel and horse mackerel, having a greater susceptibility suffering adverse effects posed by MP when compared to other species from this study.

The fish species studied in this work were assessed considering the quality criteria defined by Fossi et al. (2018) to select appropriated sentinel species for monitoring marine litter ingestion (background, habitat and trophic information, feeding behavior, spatial distribution, commercial importance, and sensitivity to litter ingestion). Our study evidences horse mackerel as a suitable bioindicator since is widely distributed in the WIUE and has high abundance in the pelagic environment and a widespread distribution in the Mediterranean Sea and eastern Atlantic Ocean (Abaunza et al. 2008 and references within). The commercial interest of horse mackerel has attracted a considerable number of studies providing information on its biology and ecology (e.g., Abaunza et al. 2008), including its feeding ecology (e.g., Garrido and Murta 2011). European horse mackerel stocks are annually assessed by ICES in Atlanto-European waters; therefore, the species is included in regular monitoring programs, with specimens routinely collected and analyzed from scientific surveys and fishery samples. This allows the access to a large number of specimens, which is required for a bioindicator. Most of the species studied here would fulfill the requirements identified in Fossi et al. (2018); however, horse mackerel ingested the largest number of MP.

Additional studies are needed, incorporating other regions, and focusing on the differential accumulation of MP throughout ontogeny, particularly for those species that change their diet dramatically as they grow, such as horse mackerel, chub mackerel and Atlantic mackerel, becoming piscivory as adults, contrary to anchovy and European sardine that remain planktivorous throughout the life cycle.

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Chapter 5

First evidence of microplastic ingestion in the ocean giant sunfish (*Mola mola*)

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First evidence of microplastic ingestion in the ocean giant sunfish (*Mola mola*)

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ABSTRACT

Mola mola is the largest teleost inhabiting our ocean and the presence of microplastics (MP) in this flagship species was, before this study, never described. Thus, this investigation focused on analysing MP ingestion in 53 ocean giant sunfish in the Northeast Atlantic Ocean. A total of 116 MP were found in 79% of the specimens, with a median of 1 MP.ind^{-1}, ranging from 0 to 11 MP.ind^{-1}. Seasonal differences were observed, with more fibers registered in specimens caught in autumn. Among the different size classes observed, the smallest category (<300 μm) was the most frequent (43%). Blue (43%) was the most prevalent color, followed by green (29%) and black (10%). The majority of fragments were styrene acrylic copolymer (53%), while most fibers were rayon (78%). These findings emphasize that the ocean sunfish population crossing the southern waters of Portugal is exposed to microplastic pollution and highlight the need for effective management policies to address plastic pollution in marine ecosystems.

1. Introduction

The field of microplastics research has experienced significant growth, driven by the recognition of their ecological impact. Microplastics, ranging in size from 1 to 5000 μm, have become a major concern (Frias and Nash, 2019). The industry produces microplastics as microbeads for cosmetics, cleaning products, and industrial abrasives, as well as pellets (Cole et al., 2011). Additionally, larger plastics can undergo physical and chemical degradation through factors such as wave action and UV-light, resulting in the formation of microplastics (Cole et al., 2011). Given their small size and widespread distribution in the environment, microplastics are highly accessible to an extensive range of marine species, from zooplankton to top predators, either through direct ingestion or indirectly through the consumption of contaminated prey (Santos et al., 2021; Nelms et al., 2018; Wright et al., 2013). The diverse range of organisms that consume microplastics, particularly at lower trophic levels, facilitates the continued spread of plastic across food webs via trophic transfer (Santos et al., 2021; Wright et al., 2013).

The ocean sunfish *Mola mola* (Linnaeus, 1758) is a widely distributed species, spanning from 70 °N (Norway and North America) to 55 °S (South America and New Zealand; Kaschner et al., 2016). It exhibits seasonal latitudinal migrations, moving north in late winter-spring and south in late summer-autumn in the northern Atlantic hemisphere (e.g., Sousa et al., 2016a; Thys et al., 2015). *M. mola* is generally considered to have a low commercial value on a global scale (Fulling et al., 2007), and targeted fisheries are predominantly limited to Asian waters (Kang et al., 2015). However, it is important to note that high rates of bycatch are reported globally, and significant localized population declines have been observed. These factors have led to the recent classification of *M. mola* as a "vulnerable" species by the International Union for Conservation of Nature (IUCN; Liu et al., 2015).

In the waters off southern Portugal (SW Europe), *M. mola* exhibits two peaks in abundance annually, during spring and autumn (Baptista et al., 2018), aligning with the reported seasonal latitudinal movements of ocean sunfish in this region (Sousa et al., 2016a). The majority of specimens found in this location are juveniles, ranging in total length from 32 to 120 cm (TL; Baptista et al., 2018; Sousa et al., 2016a; Kang et al., 2015). Consequently, the southern waters of Portugal have been

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recognized as an important area for the development of juvenile *M. mola* (Sousa et al., 2016a).

Smaller ocean sunfish (≤150 cm TL) exhibit migratory movements in close proximity to the coast (Thys et al., 2015). Their range typically does not extend beyond 500 km from the shoreline, with most individuals remaining within 300 km of the coast. Furthermore, juvenile *M. mola* exhibit a generalist diet, consuming a combination of inshore pelagic and benthic prey, including teleosts, crustaceans, bivalves, cephalopods, gastropods, and gelatinous zooplankton (Sousa et al., 2016b). Their vertical and horizontal migratory movements, combined with the fact that *M. mola* occupy higher trophic levels makes them particularly susceptible to accumulating microplastics. Moreover, previous studies have documented the presence of microplastics in the waters (Frias et al., 2014), seabed sediments (Frias et al., 2016), and beaches (Antunes et al., 2018) along the Portuguese coast. Particularly, the North, Center, and Lisbon regions have been identified as the most contaminated areas. It is worth noting that a significant proportion of these microplastics consist of fibers originating from textiles and fishing gear, likely associated with the high population densities and fishing activities in these regions. Considering these findings, it is highly probable that *M. mola* populations crossing Portuguese coastal waters are exposed to microplastic pollution.

In this sense, with the goal of expanding existing knowledge on this ocean giant and taking advantage of its presence off the coast of southern Portugal, the current study sought to assess the presence of microplastics in the stomach of these individuals for the first time.

2. Material and methods

Fifty-three specimens of the sunfish, *Mola mola*, were collected in the SW Europe coastal waters in April–May (spring) and September–November (autumn). These specimens were by-caught in a set-net targeting tuna (Tunipex) located off Olhão, southern Portugal (Fig. 1). Each specimen was measured (32.2–104.6 cm, total length-TL), and gender determined. All individuals were juveniles (i.e., immature; Kang et al., 2015). Stomachs were collected and kept frozen (−20 °C) to be analysed in the laboratory.

Microplastics extraction was performed according to Lopes et al. (2022). The weight (g) of each sample was measured, and a 10% KOH (w/v) + 10% Tween-20 (v/v) solution was added. The samples were incubated at 60 °C for 24 h before being filtered through a 20 μm polycarbonate membrane. Afterwards, filters were placed in Petri dishes and oven-dried at 40 °C before analysis. All potential microplastics were visualised and photographed using a stereomicroscope LEICA S9i (Leica Microsystems GmbH, Wetzlar, Germany) with an accoupled IC80 HD camera. Particles were categorized by color (black, blue, transparent, white, red, green, and other) and measured at their largest cross section using the ImageJ software and categorized according to the following size classes: ≤300 μm; 300–500 μm; 500–1000 μm; 1000–2000 μm; 2000–5000 μm; >5000 μm. In addition, fibers were distinguished from other shapes of microplastics.

All potential microplastics were analysed by Fourier Transform Infrared Spectroscopy (FTIR) using a PerkinElmer Spotlight 200i, equipped with a mercury cadmium telluride array detector (MCT) cooled by liquid nitrogen. Spectra were collected in μ-ATR mode, 4000–600 cm^{-1}, with a resolution of 4 cm^{-1} and a minimum of 4 scans. To confirm the polymer type, all spectra were compared to library databases and then compare analysis of the polymer characteristic bands with spectra assignments. Only polymers matching reference spectra for more than 65% were accepted.

The lower size limit of microplastics in this study was set as 20 μm, corresponding to the mesh size of filters used during the filtration process and to particle size limit of FTIR equipment used in this study.

All materials were sterile or thoroughly rinsed with ultrapure water before and between material use and samples were always kept under a clean air laminar flow hood (HEPA filter, class ISO5) or maintained in covered glass recipients. Negative controls as procedural blanks (n = 15) were run alongside real samples using all reagents and filtration system, according to quality criteria proposed by Hermsen et al. (2018). During microscopic analyses for microplastics identification, two open Petri dishes were placed with clean filters near the working zone and checked after every sample in order to control airborne fibers contamination. Contamination control measures revealed that three of procedural blanks had caught external sources of microplastic contamination. All particles detected were fibers, three black and one blue fiber. To compensate for potential contamination, fibers were excluded from the analysis if they were of similar color and polymer type to those detected within contamination control measures.

All statistical analyses were conducted in Statistica 12 (StatSoft) software. Normality and heteroscedasticity testing was conducted using Shapiro-Wilk and Levene's tests, respectively. After the invalidation of parametric assumptions, the non-parametric Mann-Whitney test was used to compare number of microplastics and year, season, and gender. Spearman rank correlation was used to account for the relationship

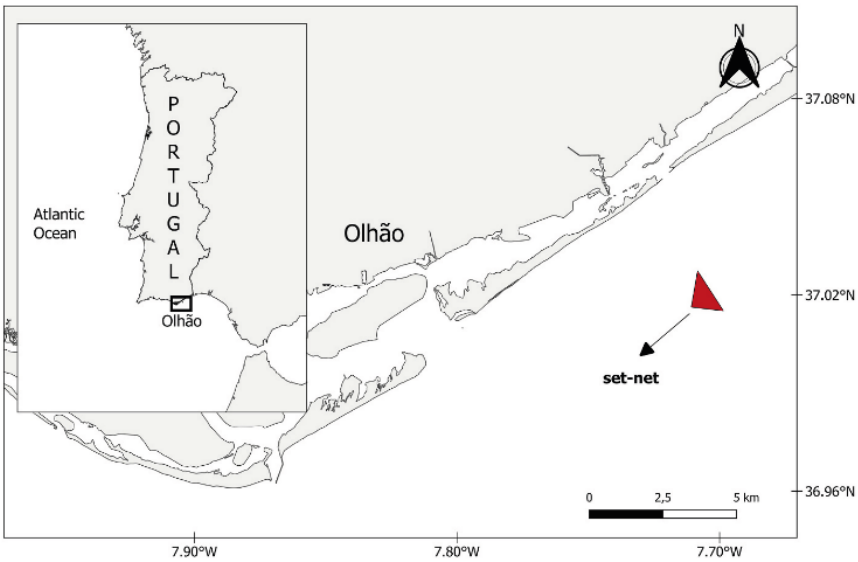

Fig. 1. Location of the sampling site of *Mola mola* off Olhão, in the southern waters of Portugal.

between the number of microplastics and Fulton condition factor (k). A significance level of $\alpha = 0.05$ was considered for all analysis.

3. Results

A total of 116 microplastics were recovered from 79% specimens of *M. mola*, 42 out of the 53 sampled, with a median of 1 $MP.ind^{-1}$, ranging from 0 to 11 $MP.ind^{-1}$. These microplastics make up 94% of the total plastic particles found. Examples of microplastics found are shown in Fig. 2.

Gender appears to significantly affect microplastics ingestion (MW-U = 193.5; $p = 0.008$), with females presenting enhanced number of microparticles in comparison to males. Fragments were the type of microplastic that contributed to the difference in microplastic concentrations between gender (MW-U = 209; $p = 0.019$). The number of males and females sampled in this study are equal, meaning that the comparison among gender is not biased.

Seasonal differences in the number of fibers were registered (MW-U = 164.5; $p = 0.029$), where fibers were found in highest number in the stomachs of specimens caught in the autumn. The size classes of

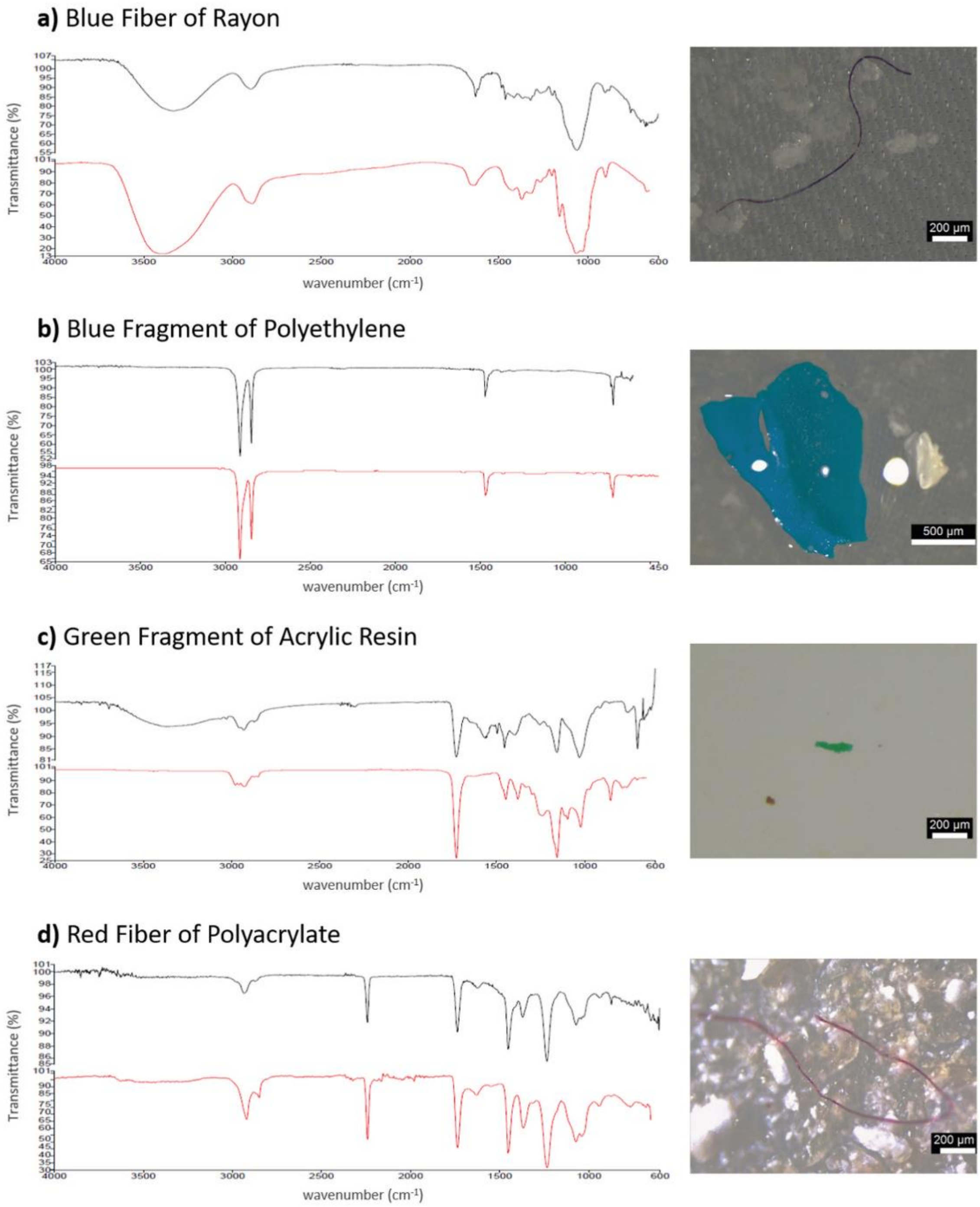

Fig. 2. Examples of microplastics extracted from stomach contents of *Mola mola* and their respective FTIR spectra. The black spectrum is the FTIR measurement of microplastic sample, while the red spectrum is the reference spectrum from the PerkinElmer library.

specimens, separated according to changes in feeding habits (Sousa et al., 2016), did not affect significantly ($p > 0.05$) the number or type of particles observed in the stomach contents of *M. mola* and no significant correlation was found between the number of microplastics in the stomachs and Fulton condition factor ($p > 0.05$).

Notable, fibers and fragments were similarly identified (52%:48%) (Fig. 3). Plastic particles size ranged from 32 μm to 16,000 μm, median size of 570 μm, being the smallest size class particles the most representative ones (<300 μm, 43%), representing a total of 80% of the fragments observed (Fig. 3). For fibers, the most representative size classes were between 500 μm and 2000 μm (68%). Mesoplastic particles (>5000 μm) were recovered, representing 10% of all the fibers counted.

Microparticles had different colors being blue (43%) the most representative followed by green (29%), black (10%), transparent (9%), red and white (4%) and other colors (1%). Fragment ranged across all color categories, with green being the most prevalent (59%), followed by blue (21%). Blue (63%) and black (18%) were the most common fiber categories, with no green or other colors observed (Figs. 2 and 3).

The largest proportion of fragments was identified as styrene acrylic copolymer (53%), followed by polyethylene (35%) and polyacrylate (12%). Most of the fibers were identified as rayon (78%), followed by polyacrylate (11%) and PVC (11%) (Fig. 3).

4. Discussion

The Portuguese coast, like many other areas globally, experiences a significant distribution and occurrence of plastic. Numerous studies have confirmed the abundant presence of plastic debris in this region (e.g., Antunes et al., 2018; Neves et al., 2015; Oliveira et al., 2015). Therefore, it was not surprising to find microplastics in the stomach contents of *Mola mola*. Among the 53 specimens of *M. mola* that we analysed, we discovered 116 microplastics in 79% of them, with a median concentration of 1 MP.ind^{-1}. In our search of the literature, we only found a single report describing a plastic debris in *Mola mola* from western Mediterranean Sea. This report described a single blue mesoplastic in the digestive system of one stranded specimen in a Valencian beach. For this reason, our study provides the first evidence of microplastic occurrence in *Mola mola* and discussion of our results can only be made with other fish species. In a related study, Lopes et al. (2020) sampled 327 pelagic fish from six commercial species (European sardine, horse mackerel, anchovy, chub mackerel, Atlantic mackerel, and bogue) in the west and south Portuguese coast and noted great occurrence of microplastics in the gastrointestinal tract of these species ranging between 58% and 100%, with concentrations varying from 0.16 to 1.75 MP.ind^{-1}. These findings are aligned with the results obtained in our study.

The stomachs of specimens sampled in the autumn contained higher number of fibers compared with those caught in the spring. One possible explanation for this observation is the seasonal latitudinal movements of *Mola mola*. During late winter and spring, *M. mola* individuals in waters off southern Portugal may migrate northward along the west coast of the Iberian Peninsula, and then switch to a southward movement during the summer-autumn period (Sousa et al., 2016a). Consequently, fish inhabiting the southern waters of Portugal in autumn may have spent part of the summer in the western waters of the Iberian Peninsula. The utilization of different spatial areas during these seasonal movements may influence the composition of microplastics ingested by the fish. In fact, the western coast of Portugal, characterized by high population densities, industry, and fishing activities, is the more contaminated by plastics and microplastics, mainly comprised of consumer products and fishing gear (Prata et al., 2020; Frias et al., 2014). On the other hand, Frias et al. (2014) discussed that the southern part of Portugal, being a highly touristic region with various beach clean-up actions in place, exhibits lower levels of these pollutants in the environment. Additionally, the authors suggest that the lower concentration of microplastics found in this southernmost part of Portugal could be attributed to the hauling effect caused by currents from the Northeast Atlantic Ocean towards the Mediterranean Sea, although this hypothesis requires further testing. However, there may be another explanation for the differences found in fiber concentrations between seasons. It has been also reported that smaller sunfish tend to stay closer to the tagging region compared to larger individuals, indicating a clear relationship between size and residency (Sousa et al., 2016a). In this context, the results may also be influenced by the availability of microplastics in the aquatic environment, which can be affected by factors such as river flows and extreme events like storms (Galgani et al., 2013). Along these lines, it is possible that the observed patterns are related to increased precipitation in the autumn, leading to higher river flows and storm events that can ultimately transport microplastics back to coastal environments (Mascarenhas et al., 2008; Kataoka and Hinata, 2015). Additionally, the increased frequency of discharges from wastewater treatment plants during this season could also contribute to the higher availability of microplastics (Ziajahromi et al., 2017).

We observed that gender appeared to influence microplastic distribution, with fragments being more frequent in females than males, which was not expected. This statistical difference found in our data is not biased as an equal number of females and males were sampled. The outcome obtained contradicts the biological and ecological traits of *Mola mola*, as there are no discernible differences in feeding habits, habitat, or food selection between males and females. Additionally, all the

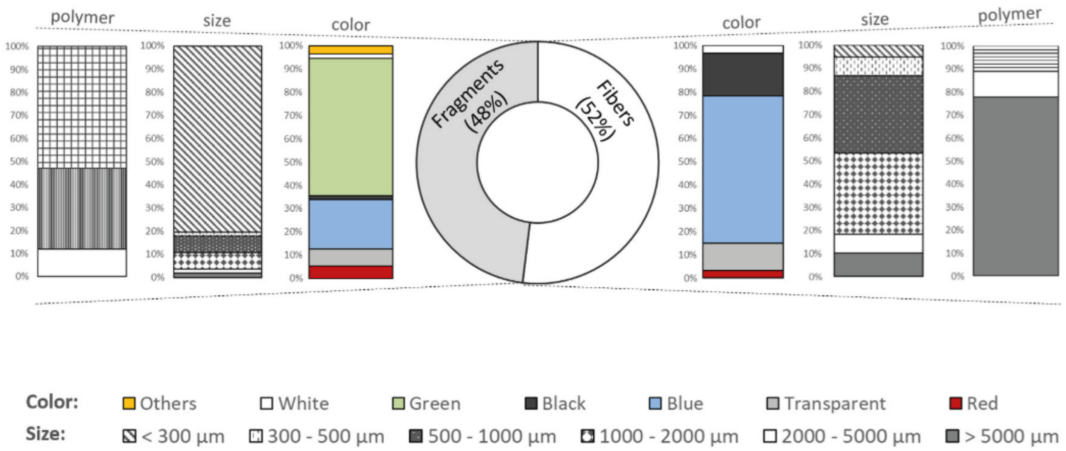

Fig. 3. Relative abundance of microplastic shapes extracted from stomach contents of *Mola mola*, alongside their color, size and polymer composition.

specimens examined were immature, indicating that sex-related behavioral distinctions are unlikely to be present. Although our sample size of 53 individuals aligns with the recommendation of the MSFD's Technical Subgroup on Marine Litter (Galgani et al., 2013), we suggest that conducting more comprehensive studies with larger sampling efforts could help eliminate the possibility of observing reproductive-related differences.

Here, size classes did not affect the number and type of plastic and no correlation between microplastics occurrence and Fulton was observed. The gastrointestinal tract is recognized to be a primary site of microplastic accumulation in various fish species. Concerning this aspect, it is possible for small microplastics to be excreted through feces, as indicated by Karakolis et al. (2018). Given that the majority of microplastics found in this work are of small sizes (<0.3 mm, 43%), this process could take place. While it was not the objective of this study, the possibility of small microplastics translocating through the intestinal epithelium into the circulatory system cannot be excluded and their presence have been already reported in muscles and liver of fish, with several adverse effects to fish wellbeing (e.g., Barboza et al., 2020). Moreover, it is crucial further investigating other significant routes of microplastic exposure, such as the gills and skin (Handy et al., 2008). Examining these routes can provide valuable insights into the environmental prevalence and distribution of microplastics to other organs.

In the present study, blue was the most common microplastic color found in *Mola mola* stomachs (53%), followed by green (29%) and black (10%). Given that *Mola mola* is a visual predator and as color is key on prey perception this fish could ingest a greater number of blue microplastics by confusing them with prey (Wright et al., 2013). However, previous studies have reported blue microparticles as one of the predominant colors found in fish (Neves et al., 2015; Lopes et al., 2020), as well as in the seawater and sediments of the Northeast Atlantic Ocean (Lusher et al., 2014; Woodall et al., 2014). Consequently, the elevated presence of blue microplastics in the stomachs of *Mola mola* could be attributed to their greater abundance in the fish's habitat.

In our study, we observed a similar ratio between fibers and fragments. This finding is interesting as it diverges from the predominant occurrence of microplastic shape described in literature. Typically, fibers are identified as the most common shape of microplastics found in fish species (e.g., Bessa et al., 2018). We hypothesize that the equal proportion of fibers and fragments observed in our study could be attributed to the feeding behavior of juvenile *Mola mola*, which involves consuming a mixture of inshore pelagic and benthic prey (Sousa et al., 2016b). Concurring, Neves et al. (2015) described that benthic species accumulate a higher number of fibers, while pelagic fish accumulate higher content of fragments.

The majority of fibers present in *Mola mola* stomachs were composed of rayon (78%). This aligns with the findings of Frias et al. (2016), who reported that between 60% and 87% of microplastic fibers in sediment samples from the south coast of Portugal were composed of rayon, a semisynthetic cellulose-based polymer. The types of polymers found in this study weight up to the previously found in fish from the Portuguese coast and estuaries (Lopes et al., 2020; Bessa et al., 2018) and follow the described availability of microplastic polymer types on the marine environment worldwide (e.g., polyethylene, rayon; Browne et al., 2011). Regarding fragments, the majority (53%) were composed of styrene acrylic copolymers. These polymers are emulsions formulated for the manufacturing of decorative paints due to their outstanding pigment binding properties. These are frequently applied as waterproof paints and coating products for nautical practices and vessels (GESAMP, 2015; Lopes et al., 2020). These polymers are subject to weathering (Hale et al., 2020), hence contributing to the availability of these fragments in the aquatic environment. Furthermore, fragmentation of these particles is shaped by temperature, salinity, currents, winds, and radiation (Cole et al., 2011). These results are likely related to the fact that the *Mola mola* sampling occurred in Algarve, south coast of Portugal, which is very well composed of beaches with great fishing activities and contains several fishing ports (Antunes et al., 2018). The Algarve coast also serves as a major shipping corridor for vessels entering and exiting the Mediterranean Sea (Sá et al., 2016).

Microplastic data on megafauna is lacking and *Mola mola* is a charismatic species that should serve as a flagship species for marine restoration and conservation strategies. Flagship species are a vehicle for awareness communication and this study serves as a starting point for marine plastic pollution actions with this key species.

5. Conclusion

Overall findings indicate that the ocean sunfish population that crosses the southern waters of Portugal are exposed to microplastic pollution and vulnerable to its ingestion. This work serves as an important, yet concerning, example of the ongoing impacts of our anthropogenic activities. A mixture of good practices within the industry and the fishing sectors are required to mitigate marine anthropogenic litter build up and consequent weathering into smaller particles. The prospective hazards of microplastics ingestion in the flagship species *Mola mola* should as well be investigated in upcoming research. Finally, our results support the need for effective management policies focus on plastic pollution of the marine ecosystem.

Credit author statement

C. Lopes: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **C. Figueiredo:** Methodology, Validation, Writing – review & editing. **M. Baptista:** Conceptualization, Methodology, Writing - Review & Editing. **M. Caetano:** Validation, Writing – review & editing, Funding acquisition. **M. Santos:** Validation, Writing – review & editing. **J. Raimundo:** Conceptualization, Validation, Investigation, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Chapter 6

General Conclusion

General Conclusion

1. Final Remarks

The objective of this thesis was to contribute to a deeper understanding of the existing levels of microplastics in marine pelagic ecosystems. The approach involved method development, concentration assessments, and characterization of microplastics in both biological and environmental matrices. The primary research findings, in alignment with the specific PhD objectives, are as follows:

- *Tween-20 is a cost-effective and environmentally friendly method for optimizing microplastic extraction from high-fat biological samples.*

The inclusion of Tween-20 in traditional digestion protocols using KOH proved to be a crucial solution to improve the efficiency of microplastic extraction from high-fat biological samples (Chapter 2). In high-fat biological samples, the reaction between KOH and triglycerides typically forms a soap layer, hindering microplastic extraction. However, it was demonstrated that the addition of Tween-20 effectively prevents the formation of fat layers during digestion, facilitating the filtration step. Importantly, the study found that Tween-20 could be added to KOH digestion protocols without causing significant interference in the FTIR spectrum of a substantial portion of the polymers commonly found in marine environments. Additionally, Tween-20 exhibited a protective effect on the degradation of certain polymers (PC and PET), leading to increased recovery rates. This protective action empowers the maintenance of the KOH 10% digestion at 60°C, ensuring efficient microplastic extraction.

The successful development of an optimized microplastic extraction methodology has several significant implications. Firstly, this optimized method holds promise for applications beyond gastrointestinal tracts, extending to other tissues with high fat content, such as the liver. Secondly, the incorporation of Tween-20 as a surfactant in the extraction protocol introduces a cost-effective and environmentally safe approach to microplastic analysis. Lastly, the success of this method was pivotal for the subsequent phases of this research, which involved the analysis of microplastics in various marine fish species, including those with high fat content.

- *Small pelagic fish with commercial value from Northeast Atlantic Ocean have microplastics in the gastrointestinal tract, gills and muscle*

Chapter 3 revealed a concerning reality regarding the widespread presence of microplastics in the gastrointestinal tracts, gills, and muscles of small pelagic fish species from the Northeast Atlantic Ocean. Microplastics were detected in 92–96% of the 118 individual fish analysed, and they were found in all species and tissues studied, highlighting the prevalence of these particles in this marine ecosystem. By examining multiple tissues, it offered a more holistic perspective on how microplastics are distributed and accumulated within these organisms, which could have potential ramifications for human consumption. Furthermore, the study underscored the need for additional research on the partitioning of microplastics in the internal organs and tissues of wild fish. Such investigations are crucial for establishing connections between exposure and effects, and for evaluating and mitigating risks to fish, the environment, and human health.

- *The composition of microplastics found in the gills of sardines and anchovies closely resembled the ones present in water samples.*

In Chapter 3, the research went beyond traditional research that only examines fish samples, by including an analysis of the composition of microplastics in water samples. This provided concrete evidence of a direct connection between the microplastic composition in fish, particularly sardines and anchovies, and the microplastics present in the surrounding water.

- *Small pelagic fish feeding on smaller preys had lower microplastics concentration in the stomachs than fish depending on larger mesozooplanktonic preys (>1000 µm).*

Chapter 4 revealed significant differences in microplastic accumulation in small pelagic fish based on their dietary preferences. Smaller prey-consuming species, such as European sardines, chub mackerel, and bogue, displayed lower levels of microplastics accumulation compared to their counterparts feeding on larger mesozooplanktonic preys. Atlantic mackerel and horse mackerel, with their preference for bigger preys *(>1000 µm)*, exhibited higher microplastics abundances, highlighting the ecological implications of dietary choices in microplastic ingestion.

- *More fibers were registered in Ocean Giant Sunfish specimens caught in autumn than those caught in the spring*

Chapter 5 marked a groundbreaking discovery, revealing the presence of microplastics in ocean giant sunfish (*Mola mola*) for the first time, serving as a critical addition to the limited data on megafauna and microplastics. The research unravelled seasonal variations in microplastic composition within ocean giant sunfish specimens, with higher concentrations of fibers observed in specimens captured during autumn compared to spring. While this phenomenon may be associated with the seasonal latitudinal movements of ocean giant sunfish, it could also be influenced by factors like river flows, storm events, and wastewater discharges. This finding emphasizes the complexity of microplastic dynamics in marine ecosystems, reflecting the intricate interplay of environmental variables. Moreover, these results highlight the importance of considering seasonal factors when using *Mola mola* or similar species as bioindicators. One unexpected observation was the gender-related distinctions in microplastic distribution among *Mola mola*, with females exhibiting a higher prevalence of fragments. This discovery highlights the need for further research to elucidate the origins and underlying factors of this gender-based difference.

- *The most prevalent microplastic found in marine pelagic ecosystems was characterized by its size, which was under 500 µm, its fiber-like structure, a blue colour, and primarily consisted of polymer varieties such as polypropylene, polyethylene, acrylic, and cellulose-based polymers*

This research extensively characterized the most common microplastics found in marine pelagic ecosystems, including small pelagic fish, giant ocean sunfish and water samples. It consistently found that fiber-shaped microplastics were the predominant type, comprising 62% in water samples and 52-87% in fish tissues. These fibers likely originate from sources such as synthetic clothing and fishing nets, with a high potential for bioaccumulation in marine organisms. The prevalent colour observed was blue, highlighting its importance in marine pelagic ecosystems. Furthermore, the most prevalent polymer types included polypropylene, polyethylene, acrylic, and cellulose-based polymers, reflecting the diverse sources of microplastics in the marine environment. Smaller microplastics (<500 µm) were prevalent across all fish species and water samples, emphasizing their likelihood of ingestion by marine organisms and potential ecological implications. These findings also align with the methodological limitation in detecting particles smaller than 20 µm, highlighting the pressing need for improved research methods to access the presence of smaller microplastics and nanoplastics more effectively in marine ecosystems.

These results support the documented prevalence of microplastics in wild fish along the coast of Portugal and the Northeast Atlantic, underscoring the escalating issue of plastic pollution in oceans. Similar outcomes have been observed in other studies based on the shape, colour, size, and polymer type of microplastics (Guilhermino et al., 2021; Pequeno et al., 2021; Barboza et al., 2020; Bessa et al., 2018; Neves et al., 2015). However, Barboza et al. (2020) reported a higher percentage of fragments (76%) than fibers (22%) in the gastrointestinal tract of horse mackerel from the North coast of Portugal. Analysis of the gastrointestinal tract of fish captured off the coast of Portugal also revealed contamination with microplastics, with rates of 19.8% for all continental Portugal (Neves et al., 2015), 38% in the Mondego estuary (Bessa et al., 2018), 97% in the Minho River (Guilhermino et al., 2021), and 35% in the North of Portugal (Barboza et al., 2020). Discrepancies in results from different locations may be linked to varying sources of pollution and waste management strategies, necessitating ongoing monitoring. Species-specific factors such as feeding zones, habitats, migratory routes, and the potential confusion of certain plastics with prey could contribute to these differences.

Regarding water concentrations, the recorded values ranged from 0.228 MP.m^{-3} to 1.856 MP.m^{-3}, surpassing earlier reports for the Portuguese coast, which varied between 0.002 and 0.036 MP.m^{-3} (Frias et al., 2014). Nevertheless, these concentrations aligned with findings in other locations, including the Douro estuary (0.17 MP.m^{-3}; Rodrigues et al., 2019), values reported by Bessa et al. (2018) for the Mondego estuary (1.53 MP.m^{-3}), and data from Professor Luiz Saldanha Marine Park on the west coast of Portugal (0.45 MP.m^{-3}; Rodrigues et al., 2020). Despite similarities in the physical and chemical characteristics of microplastics, drawing comprehensive conclusions is limited by the restricted number of studied areas and differences in sampling methodologies, including variations in the size of mesh nets.

All this environmental data draws attention to the disparities between hazard assessment and monitoring assessment datasets. By aligning toxicological research with realistic exposure conditions and utilizing microplastics representative of those found in the environment, we can gain a better understanding of how the size and type of microplastics impact the ecosystems and human health. This thesis provides a solid foundation for future hazard and risk assessment studies. Additionally, comprehending the prevalent types, shapes, and colours of microplastics in marine pelagic ecosystems enables the development of focused strategies for mitigating plastic pollution and safeguarding the environment.

- *Horse mackerel was identified as the most suitable fish species as a bioindicator for monitoring microplastics*

This research covered a broad range of marine fish species, from the ocean's megafauna, represented by the ocean giant sunfish, to smaller pelagic fish species such as European sardine, horse mackerel, anchovy, chub mackerel, Atlantic mackerel, and bogue. This comprehensive approach allowed to assess the potential of these species as bioindicators for monitoring microplastics in marine pelagic ecosystems, particularly in areas governed by the Marine Strategy Framework Directive (MSFD). It was applied established criteria outlined by Fossi et al. (2018), considering factors such as background, habitat, trophic characteristics, feeding behaviour, spatial distribution, commercial significance, and susceptibility to litter ingestion. Ocean sunfish, despite its unique ecological importance, is less suitable as a bioindicator in MSFD regions due to its limited distribution, low economic value, and vulnerable status. In contrast, all the small pelagic fish species examined in this study have commercial significance. Among them, species like anchovy, Atlantic horse mackerel, and Atlantic mackerel stand out as promising candidates, given their widespread distribution across multiple MSFD-regulated regions. Notably, horse mackerel emerges as an outstanding candidate with a high incidence of individuals containing microplastics in their gastrointestinal tracts and muscle. Its propensity for microplastic ingestion indicates a close interaction with these pollutants, making it a valuable indicator of microplastic exposure. To ensure the consistency and reliability of microplastic ingestion monitoring, it is advisable to choose individuals with lengths that are characteristic of the same life stage. This method reduces potential variations in microplastic ingestion linked to alterations in feeding behaviours at various life stages. These findings hold substantial relevance for policymakers and environmental agencies endeavouring to establish robust monitoring programs and protect the integrity of marine ecosystems.

2. Future Perspectives

In the continuation of the studies conducted in the scope of this thesis, key areas for future research and action have been identified:

Method Development:

- Development of methods for smaller microplastic and nanoplastic sampling, extraction, and detection in marine ecosystems to enhance contamination precision.
- Creation of automated approaches for sampling, extraction, and analysis of microplastics to reduce analysis time and operator error.
- Standardization of methodologies for the sampling, extraction, and analysis of microplastics to facilitate comparisons across different studies.

Toxicological Research (Lab experiments):

- Alignment of toxicological research with realistic exposure conditions using environmentally relevant concentrations and representative microplastics.
- Investigation of the impact of additives present in the composition of plastics on the toxicity of microplastics.
- Investigation of synergistic effects of microplastics in the context of climate change.

Field Research:

- Investigation of the presence of smaller microplastics/nanoplastics in other tissues and organs of fish to gain a comprehensive understanding of their distribution within marine organisms.
- Investigation of potential gender-based, seasonal, and life stage differences in the composition and concentration of microplastics in marine organisms.
- Examination of the potential biomagnification of microplastics through the marine food web.
- Investigation of the principal monomers, additives, and sorbed contaminants present in microplastics.
- Characterization of the presence of microplastics in seafloors and the identification of species most susceptible to this type of contamination.

Human Health:

- Investigation of human health implications of consuming fish with high microplastic levels.

Long-Term Monitoring:

- Establishment of long-term monitoring programs for bioindicator species like horse mackerel, anchovy, and Atlantic mackerel to track trends in microplastic contamination.

Mitigation Strategies:

- Development of targeted strategies for mitigating plastic pollution based on prevalent microplastic polymer types, shapes, and colours in marine pelagic ecosystems.
- Provision of evidence-based policy and management recommendations at regional and international levels to address microplastic pollution challenges.

3. Conclusion

This thesis has significantly advanced our understanding of microplastic contamination in marine ecosystems, offering valuable insights into extraction methods, tissue distribution, dietary correlations, and the presence of microplastics in marine pelagic species. The research conducted contributes to the growing body of knowledge needed to address the challenges posed by microplastic pollution and provides a foundation for future research and conservation initiatives aimed at preserving our marine environments and safeguarding human health. The similarities in microplastic prevalence and composition between ocean giant sunfish and small pelagic fish underscore the widespread nature of microplastic pollution in marine ecosystems, transcending size, and trophic position. This suggests that addressing the impacts of microplastic pollution needs an holistic, ecosystem-wide approach that acknowledges the interconnectedness of species and the common threats they face. By tackling microplastic pollution at its source and implementing effective mitigation measures, we can work towards safeguarding the health and integrity of our oceans for all marine life, from the majestic giant ocean sunfish to the smallest pelagic fish.

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