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Accumulation of microplastics in North Atlantic sharks: causes, potential effects, and bioremediation strategies

Roger Busom Casado

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Título: Acumulação de microplásticos em tubarões do Atlântico Norte: causas, potenciais efeitos e estratégias de biorremediação

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In memory of my grandfather,

Jose Casado Pitarque.

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“Agua – Jarabe de Palo”

Resumo

O plástico é um material versátil amplamente utilizado em muitos setores económicos e tem sido produzido em massa desde a década de 1950. No entanto, o plástico é composto por polímeros de difícil degradação que, devido à gestão inadequada atual e outros fatores, resulta na acumulação de grandes quantidades desses polímeros em diferentes ecossistemas da Terra, incluindo nos oceanos. Como o plástico é ubíquo em vários sistemas oceânicos, eventualmente interage com organismos marinhos, levando a eventos de emaranhamento ou ingestão. Muitos estudos têm demonstrado a acumulação de macro- e microplásticos em organismos marinhos. Entre eles, os predadores de topo de vida longa são muito propensos a acumular grandes quantidades de resíduos plásticos devido aos processos de bioacumulação e biomagnificação. Por essa razão, os tubarões carnívoros podem ser considerados bons sentinelas de contaminação por microplásticos, devido à sua alta posição trófica e à diversidade de habitats que podem ocupar (ou seja, desde bentónicos a pelágicos e de ambientes costeiros a oceânicos).

O principal objetivo da presente tese foi avaliar a acumulação de microplásticos e outras partículas antropogênicas na espécie de tubarão bentónico *Scyliorhinus canicula* e compará-la aos dados gerados para o tubarão pelágico *Prionace glauca* da mesma área geográfica, com ambos os estudos seguindo a mesma metodologia. Tal teve como propósito avaliar diferenças na ingestão de partículas antropogénicas entre os dois tubarões, com a hipótese de que o lixo antropogénico não se acumula uniformemente em diferentes zonas marinhas e, portanto, os organismos marinhos podem tender a acumular partículas antropogénicas mais presentes nos seus respetivos ecossistemas. Além disso, também se pretendia avaliar se os resultados do presente estudo estariam alinhados com a literatura existente sobre a acumulação de partículas antropogénicas em outros tubarões e ecossistemas marinhos (ou seja, água do mar e leito oceânico). Por último, este trabalho também visou estudar como parâmetros digestivos, como enzimas e condições de pH ácido, poderiam afetar as partículas antropogénicas retidas no trato gastrointestinal dos tubarões, a fim de entender que mudanças as partículas antropogénicas podem sofrer durante a sua passagem pelo trato digestivo do tubarão. Além disso, o estudo dos efeitos enzimáticos sobre os plásticos também visou avaliar o potencial biotecnológico dessas enzimas digestivas para a degradação de plásticos.

Os resultados mostraram uma incidência de 100% de partículas antropogénicas em ambos os tubarões, com *Scyliorhinus canicula* acumulando menos partículas por indivíduo (7.84 ± 3.49) do que *Prionace glauca* (36.31 ± 23.7). Em relação à forma dos itens ingeridos, os tubarões pata-roxa acumularam até 4 vezes menos fragmentos do que os tubarões-azuis. No entanto, para ambos os tubarões, as fibras foram a forma de partículas mais acumulada. Em relação ao tipo de polímero, *S. canicula* ingeriu principalmente partículas de origem natural (por exemplo, celulose, algodão e outros) e a maioria dos itens ingeridos tinha uma densidade maior do que a da água do mar (flutuação negativa), enquanto *P. glauca* ingeriu principalmente itens de origem sintética (por exemplo, polietileno, polipropileno e outros), e uma grande proporção das partículas acumuladas tinha uma densidade menor que a da água do mar (flutuação positiva). Por último, em relação aos testes digestivos *in vitro*, ao simular algumas das condições do trato gastrointestinal dos tubarões, a enzima pepsina foi capaz de causar perda de peso em filamentos de poliamida (4.64%), em filmes de polietileno de baixa densidade (2.32%) e causou alterações estruturais em fibras de algodão.

Este estudo destaca a importância de monitorizar a ingestão de partículas antropogénicas em predadores de topo para demonstrar ainda mais a vulnerabilidade desses organismos marinhos à acumulação de lixo marinho, o que pode resultar em potenciais impactos para a saúde dos mesmos, ao mesmo tempo em que oferece informações sobre os diferentes níveis de poluição antropogênica em diferentes áreas marinhas (bentônicas versus pelágicas). Além disso, este estudo também destaca a importância de compreender como as partículas ingeridas se podem comportar dentro do trato digestivo dos organismos para futuramente explorar os seus possíveis efeitos.

Palavras-chave: Poluição marinha, partículas antropogénicas, predadores de topo, , ecossistemas marinhos, digestão, degradação enzimática.

Abstract

Plastic is a versatile material used extensively in many economic sectors and has been mass-produced size 1950s. Nevertheless, plastic is composed of hardly degradable polymers which, due to the current inadequate management and other factors, results in the accumulation of large quantities of such polymers in different Earth ecosystems, including oceans. As plastic is ubiquitous across various oceanic systems, it eventually interacts with marine organisms, leading to entanglement or ingestion events. Many studies have demonstrated the accumulation of macro- and microplastics in marine organisms. Among these, long-lived top predators are very prone to accumulate large quantities of plastic debris due to bioaccumulation and biomagnification processes. For that reason, carnivorous sharks can be considered good sentinels of microplastic contamination given their high trophic position and the diversity of habitats they can occupy (i.e., from benthic to pelagic and from coastal to oceanic environments).

The main objective of the present thesis was to evaluate the accumulation of microplastics and other anthropogenic particles in the benthic shark species *Scyliorhinus canicula* and comparing it to the data generated for the pelagic shark *Prionace glauca* from the same geographical area, with both studies following the same methodology. This was done with the purpose of assessing differences in the ingestion of anthropogenic particles between both sharks, hypothesizing that anthropogenic litter does not accumulate uniformly across different marine zones and therefore, marine organisms may tend to accumulate anthropogenic particles more present in their respective ecosystems. Additionally, it was also intended to assess if the findings of the present study would align with existing literature on anthropogenic particle accumulation in other sharks and marine ecosystems (i.e. seawater and seabed). Lastly, this work also aimed to study how digestive parameters, such as enzymes and acidic pH values, could affect the anthropogenic particles retained in shark's gastrointestinal track, to understand which changes can anthropogenic particles undergo during their passage through the shark's digestive tract. Furthermore, the study of enzymatic effects on plastics also aimed to assess the biotechnological potential of these digestive enzymes for plastic degradation.

The results showed a 100% incidence of anthropogenic particles in both sharks, with *Scyliorhinus canicula* accumulating less particles per individual (7.84 ± 3.49) than *Prionace glauca* (36.31 ± 23.7). Regarding the shape of the items ingested, the small-spotted catsharks accumulated up to 4 times less fragments than the blue sharks. However, for both sharks, fibres were the most accumulated shape of particles. In relation to the type of polymer, *S. canicula* ingested mainly particles of natural origin (e.g. cellulose, cotton and others) and most of the items ingested had higher density than seawater (negative buoyancy), while *P. glauca* ingested mostly items of synthetic origin (e.g. polyethylene, polypropylene and others), and a large proportion of the particles accumulated had a lower density than seawater (positive buoyancy). Lastly, concerning the digestive in-vitro tests, when simulating some of the gastrointestinal track conditions of sharks, pepsin enzyme was able to produce weight loss in polyamide filaments (4.64%), in low-density polyethylene films (2.32%) and caused structural alterations in cotton fibres.

This study highlights the importance of monitoring the ingestion of anthropogenic particles in marine top predators to further demonstrate the vulnerability of these organisms to accumulate marine litter, which may result in potential health impacts, while also offering insights into the varying anthropogenic pollution levels in different marine zones (benthic versus pelagic). Additionally, this study also emphasizes the importance of understanding how ingested particles may behave within the digestive tract of organisms to further explore their possible effects.

Keywords: Marine pollution, anthropogenic particles, top predators, marine ecosystems, digestion, enzymatic degradation.

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List of abbreviations

Analysis of variance (ANOVA)

Anthropogenic particles (APs)

Gastrointestinal track (GIT)

Genetically modified organisms (GMO)

Marine litter (ML)

Mediterranean Sea (MED)

Micro-Fourier Transformed Infrared (Micro-FTIR)

Microplastics (MPs)

Non-detected (N.D)

Northeast (NE)

Numerical index (NI)

Occurrence index (OI)

Persistent organic pollutants (POPs)

Poly (butyl acrylate) (PBA)

Polyamide (PA)

Polychlorinated biphenyls (PCBs)

Polycyclic Aromatic Hydrocarbons (PAHs)

Polyethylene (PE)

Polyethylene Hight-Density (PE-HD)

Polyethylene terephthalate (PET)

Polypropylene (PP)

Polystyrene (PS)

Polyurethane (PU)

Polyvinyl acetate (PVA)

Polyvinyl chloride (PVC)

Scanning electron microscopy (SEM)

Ultraviolet (UV)

United Nations Environment Programme (UNEP)

Volatile organic compounds (VOCs)

Chapter 1.

General Introduction

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1.1 Plastic litter in oceanic waters

Plastic is a versatile material used in many economic sectors (Geyer et al., 2017), due to its good qualities and widespread use, the industry of this polymer began to boom in 1950s. Consequently, due to the mass production, its low rate of degradation and inadequate management, plastic waste has been accumulating in natural ecosystems since it started to be produced (Barnes et al., 2009; Kershaw et al., 2011). It is estimated that 146 million tonnes of plastic were globally used for packaging in 2015, which was considered the sector that produce more plastic (35.87 %) being also the product with the shortest shelf life (Geyer et al., 2017). At the beginning of the XXI century, the world was producing 213 million tonnes of plastic per year and, since then, plastics production has permanently increased, having been produced 381 million tonnes of plastic in 2015 (Geyer et al., 2017). Of all plastic produced between 1950 and 2015 globally, approximately 6.3 billion tonnes of plastic waste was generated, from which around 12% was incinerated, only 9% has been recycled and 79% was unprocessed (Geyer et al., 2017; Rhodes, 2018) The unprocessed plastic is commonly stored in landfills, or worse it is released to the environment (Rhodes, 2018).

Land-based plastics contribute approximately with 70-80% of the total amount of plastics in the ocean (Lebreton et al., 2018; Li et al., 2016). Between 1.15 and 2.41 million tonnes of plastic are estimated to be transported from rivers to oceansli annually (Lebreton et al., 2017), and over 1,000 rivers are responsible for 80% of global annual ocean emissions of land-based plastics (Meijer et al., 2021). The remaining 20% of ocean plastic is generated by anthropogenic activities related with marine environments: merchant shipping, ferries and cruise ships; fishing vessels and fish farming; naval vessels, research ships and pleasure crafts; offshore oil and gas platforms (Lebreton et al., 2018; Macfadyen et al., 2009).

Plastic litter have been documented in different oceanic environments: in ocean surface (Suaria et al., 2020; Hansen et al., 2023); ocean sediments (Cannas et al., 2017; Maes et al., 2017; Urbanek et al., 2018); beaches (Cooper & Corcoran, 2010) and estuaries (Corcoran, 2015; Anderson et al., 2018). Due to the omnipresence of plastics in the ocean, marine organisms are constantly exposed to plastic litter. Entanglement and ingestion of microplastics are the most common interactions of plastic litter with marine life. These phenomena have been documented all over the world and affects many marine species

(Goswami et al., 2020; Phuong et al., 2018; Neves et al., 2015; Lopes et al., 2020; Moore et al., 2013; Moore et al., 2009; Matsuoka et al., 2005). Microplastic ingestion occurs after plastic objects are broken down into small particles as a result of abiotic and biotic factors in terrestrial and aquatic environments (Zhang et al., 2021). According to scientific literature, any synthetic solid particle or polymeric matrix, with regular or irregular shape, is commonly referred as 'microplastics' (MPs) when their size is ranging from 1 μm to 5 mm (Van Cauwenberghe et al., 2013; Arthur et al., 2009; Frias & Nash, 2019). The accumulation and dispersion of microplastics in the ocean has led to the emergence of serious marine environmental problems and has been in the public and scientific debate in the recent years.

1.1.1 Accumulation of plastic in ocean surface

Our oceans are dynamic, inter-connected and in continuous movement due to the variation of ocean temperature, salinity, and by the effect of wind. These characteristics allow large oceanic water masses to create currents known as oceanic gyres. There are five main oceanic gyres: North Atlantic gyre, South Atlantic gyre, Indian Ocean gyre, North Pacific gyre, and South Pacific gyre. Most of plastic items have the capability to float and, consequently, they can be transported by the wind and superficial currents, tending to accumulate in the centre of ocean gyres (stable regions without currents) and to create plastic ocean patches (Van Sebille et al., 2012). According to Eriksen et al. (2014), it was estimated that in the year of 2014 there were approximately 268,940 tonnes of plastic waste floating in the ocean. The North Pacific was the area accumulating the most with a total of 96,400 tonnes (35.8%), followed by the Indian Ocean with 59,130 tonnes (22%), the North Atlantic with 56,470 tonnes (21%), the South Pacific with 21,020 tonnes (7.8%), and the South Atlantic with 12,780 tonnes (4.8%). Although it is not considered an oceanic gyre, the Mediterranean Sea also accumulates a high amount of plastic litter 23,150 tonnes (8.6%), mainly due to the high density of the population that lives on its coastlines (Eriksen et al., 2014) and because of the distinguishing semi-enclosed morphology that makes the Mediterranean Sea highly vulnerable to plastic pollution.

From the available studies on plastic debris in ocean surface, plastic lines, ropes and fishing nets represent a 52% of total plastic litter, followed by hard plastic, plastic sheet and film (47%), preproduction plastic pellets (0.5%) and foamed material (0.05%) (

Lebreton et al., 2018). Polyethylene (PE) and polypropylene (PP) are the most common polymer types found in ocean surface (Cózar et al., 2014; Lebreton et al., 2018) due to their low densities.

1.1.2 Accumulation of plastic in marine sediments

There is a generalized tendency to believe that plastic only accumulates in the ocean surface but there are several studies which prove that plastic debris also accumulate in marine sediments in high amounts (Bergmann et al., 2017; Maes et al., 2017; Woodall et al., 2014). Ekman transport and geostrophic currents play a crucial role in the movement of plastic waste and tiny particles across the ocean, enabling to comprehend how these particles can eventually reach the ocean floor (Sangiolo, 2022). Semi-synthetic origin polymers, like rayon and synthetic origin polymers, such as polyvinyl chloride (PVC), acrylic, polyamide (PA), and polyester, which are polymers denser than sea water, can be commonly found in the ocean sediments (Woodall et al., 2014; Courtene-Jones et al., 2020). Furthermore, plastics with positive buoyancy (e.g. PP and PE) can, over a period of weeks to months, become negatively buoyant and sink (Woodall et al., 2014; Lobelle & Cunliffe, 2011). There are several phenomena that can make plastics aggregations denser than water, such as the formation of a biofilm on top of the plastics by algae, bacteria, or other organisms (Morét-Ferguson et al., 2010) or by the adhesion of minerals to the plastics (Corcoran, 2015).

Based on the characterization of microplastic debris in deep sea sediments in North Atlantic Ocean, Mediterranean Sea and South-West Indian Ocean (Woodall et al., 2014; Courtene-Jones et al., 2020), the majority of microplastics found were fibrous in shape (microfibres), commonly measuring between 0.5–3 mm in length and less than 0.1 mm in diameter. Microfibres were typically blue, black, green, red, or transparent and occasionally vibrant colours such as pink, purple, and turquoise. Moreover, polyester and rayon were the most prevalent polymers found in benthic sediments, followed by others such as polyamide, acetate, acrylic, PVC, and polypropylene (Woodall et al., 2014; Browne et al., 2011; Courtene-Jones et al., 2020). Although positive buoyant plastics can be found sedimented as a result of the above-mentioned phenomena, the majority of the polymers accumulated in deep sea sediments have a higher density than seawater.

1.1.3 Environmental processes of plastic degradation and fragmentation

When plastics get into the ocean, they can be degraded/fragmented by abiotic factors such as UV radiation, mechanical stress (waves and currents), heat and chemical interactions (Zhang et al., 2021; Frias et al., 2019), or by biotic factors such as physical fragmentation and biological degradation (Porter et al., 2019; Dawson et al., 2018; Danso et al., 2019), although total degradation/fragmentation of plastics can take hundreds of years. Therefore, some studies are reporting that plastics have been accumulating in oceans since their creation (i.e., in the first half of the XX century) (Kershaw et al., 2011).

Fossil-based plastics are composed of long carbon and hydrogen chains with or without other atoms such as oxygen, nitrogen, or sulphur (Figure 1). These polymers can have different lengths, being made up of monomers, and these different lengths can provide different features to the polymer. Due to their hydrophobicity, crystallinity, and lack of a favourable functional group, fossil-based plastics cannot be easily biodegraded by microorganisms (Wilkes & Aristilde, 2017; Urbanek et al., 2018).

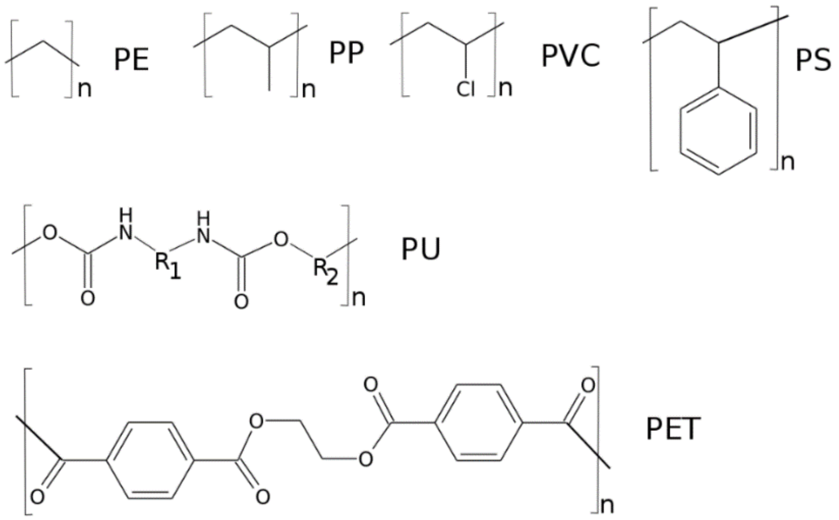

Figure 1. Most common fossil sources plastics: Polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyurethane (PU) and polyethylene terephthalate (PET)

Moreover, the long-chain polymer structure and high molecular weight of fossil-based plastics makes their biodegradation particularly challenging given that plastics cannot easily pass through the cellular membrane of microorganisms (Shah et al., 2008; Urbanek et al., 2018; Wilkes & Aristilde, 2017). Consequently, before biodegradation can occur, long plastic polymers must be depolymerized into smaller monomers. This process can be initiated by the previously mentioned abiotic factors, but the presence of certain bacteria that produce specific extracellular enzymes will help the process (Shah et al., 2008). Once depolymerization is complete, only bacteria with intracellular depolymerases will be able to mineralize the plastic (Gu, 2003; Shah et al., 2008).

Due to the low bioavailability of plastic synthetic polymers, abiotic degradation typically occurs before biotic degradation and can alter the chemical and physical characteristics of plastics. Photodegradation is considered the most important process that initiate plastic degradation (Andrady, 2015; Zhang et al., 2021), with high energy ultraviolet (UV) irradiation UV-B (290–315 nm) and medium energy UV-A (315–400 nm) being the main responsible for the photodegradation (Liu et al., 2019). The impact of photodegradation on plastics is dependent on their unique polymeric structure, leading to variable effects. Additionally, the integrity of plastics can also be impacted by mechanical forces, such as collision and abrasion with rocks and sand caused by wind and waves. Freezing and thawing are also considered mechanical forces (Zhang et al., 2021). Additionally, the presence of pollutants such as nitrogen dioxide (NO_2), ozone (O_3), sulphur dioxide (SO_2), and volatile organic compounds (VOCs) can alter the integrity of plastics directly or indirectly by catalysing the formation of radicals by photochemical reactions, which may also lead to the degradation of plastics (Crawford & Quinn, 2017). Finally, it should be noted that slow thermal oxidation and photo-oxidation can also cause changes in the integrity of plastics, typically occurring in beach environments (Andrady, 2015). It is important to highlight that the processes described above will just affect plastics floating at the sea surface or littered on beaches (Cooper & Corcoran, 2010).

On the other hand, biotic degradation is also involved in plastic fragmentation/degradation, which involves the breakdown of plastics by organisms and can be classified into two categories: physical degradation, in which plastics can be degraded by biting (Cadée, 2002), gnawing (Porter et al., 2019), chewing or by digestive fragmentation (Dawson et al., 2018; Cau et al., 2020) and biological degradation or bioremediation, which occurs through biochemical processes and it is normally carried out by microorganisms (Danso et al., 2019).

Bioremediation is a process that uses mainly microorganisms, plants, or microbial or plant enzymes to detoxify contaminants in the soil and other environments (Gouma et al., 2014). In marine ecosystems, bioremediation of plastic waste is commonly carried out by microorganisms including bacteria and fungi (Caruso, 2015; Shahnawaz et al., 2019). There are many studies evaluating the potential of many microorganisms for biodegradation of plastic waste (Zeenat et al., 2021; Shah et al., 2008; Urbanek et al., 2018; Zhang et al., 2021; Othman et al., 2021). Moreover, biotechnology has enabled major advances to be made in the field of plastic degradation, essentially through the integration of omics technologies (metagenomics, transcriptomics, proteomics, metabolomics): search for microorganisms with potential to degrade plastics (Danso et al., 2019); search for enzymes with potential to degrade plastics (Bollinger et al., 2020; Danso et al., 2019); study of new genes encoding enzymes with potential to degrade plastics (Acinas et al., 2021; Kumari & Chaudhary, 2020) and producing transgenic strains with genes with potential to degrade plastics (genetically modified organisms – GMO) (Kumari & Chaudhary, 2020).

As mentioned before, numerous studies have demonstrated the ability of bacteria to degrade plastics (Danso et al., 2019). However, recent research has shown that larger marine organisms can also play a role in the degradation and breakdown of (micro) plastics through the action of digestive enzymes. It has already been reported plastic breakdown in the digestive track of benthic (Cau et al., 2020) and pelagic (Dawson et al., 2018) animals. These findings highlight the need for a more comprehensive examination of the digestive conditions that can play a role in the alteration of plastics integrity and evaluate the additives present in plastic materials, that can be potentially released during the plastic breakdown in the digestive system, and their potential impacts on both marine organisms and ultimately human health.

1.2 Interactions of plastic litter with marine fauna

The accumulation of plastic in the ocean can cause diverse interactions with marine species, including entanglement with plastic structures, ingestion of plastics and microplastics, and the accumulation of contaminants (e.g. persistent organic pollutants (POPs), phthalates, flame retardants and others) released from plastic litter (Bergmann et al., 2015) These phenomena have been documented worldwide and has the potential to induce adverse effects on various species inhabiting different ecological systems.

1.2.1 Entanglement of marine species with plastic litter

One of the most visible impacts of plastic pollution in the ocean is the entanglement of marine species, which occurs when debris entangles with marine animals resulting in a physical interaction (Moore et al., 2009). Entanglement of marine life is documented all over the world (Bergmann et al., 2015) and affects many marine species, from mammals in North Atlantic (Moore et al., 2013), to sea birds in California coast (Moore et al., 2009), octopus in Japan (Matsuoka et al., 2005), crabs in Virginia, USA (Bilkovic et al., 2014) or marine turtles in northern Australia (Wilcox et al., 2015). Most of the entanglements are caused by fishing gears, the called “ghost fishing” which refers to lost or abandoned fishing gear (Breen, 1990) that remain in the sea, entrapping and causing harm to organisms and benthic habitats (Good et al., 2010). Nevertheless, other anthropogenic material such as ropes, balloons, plastic bags, sheets or six-pack drink holders have also been largely described to cause entanglement (Moore et al., 2013; Moore et al., 2009; Rodríguez et al., 2013).

1.2.2 Ingestion of microplastics by marine fauna

Plastic waste has become ubiquitous in marine environments, leading to unintentional ingestion and accumulation of particles in the digestive tracts of marine fauna. Ingestion of plastic by marine organisms is less visible than entanglement (Bergmann et al., 2015), but equally harmful, potentially causing severe health issues (Ryan, 2016). In the worst cases, ingested plastic may completely block or severely damage the gastrointestinal tract, ultimately leading to rapid mortality. Consequently, plastic ingestion has been documented globally and affects a wide range of marine species, including zooplankton (Goswami et al., 2020), bivalves (Phuong et al., 2018), fish (Neves et al., 2015; Lopes et al., 2020), sea birds (Avery-Gomm et al., 2012), sea turtles (Campani et al., 2013), and large marine mammals (De Stephanis et al., 2013; Fossi et al., 2014).

Plastic ingestion can be carried out by three different phenomena: direct ingestion, that includes accidental ingestion of particles through indiscriminate feeding strategies (filter-feeders), active selection due to misidentification of microplastics for food (Nelms et al., 2018) or as a result of secondary ingestion (debris already ingested by prey). Furthermore, there are other factors that can affect plastic ingestion. Among these, the colour of the particles plays a significant role in influencing marine debris consumption. Specific colours may attract predators that mistake them for their prey, thus increasing the likelihood of ingestion (Boerger et al., 2010; Lusher et al., 2013). In seabirds, this phenomenon has been suggested for greater shearwaters (*Puffinus gravis*), red phalaropes (*Phalaropus fulicarius*) (Moser & Lee, 1992) and parakeet auklets (*Aethia psittacula*) (Day et al. 1985). Nevertheless, in sea turtles the issue of plastic colour is more controversial, with some studies indicating that there is no particular preference for plastic ingestion based on colour (Lutz, 1990), while others argue that light-coloured and translucent plastics are more frequently consumed, suggesting similarity to their jellyfish prey (Tourinho et al., 2010; Schuyler et al., 2014).

The age of individuals can also be regarded as a potential factor that influences plastic intake. Young individuals of fulmars (*Fulmarus glacialis*) (Van Franeker et al., 2011), flesh-footed shearwaters (*Puffinus carneipes*) (Hutton et al., 2008), and short-tailed shearwaters (*Puffinus tenuirostris*) (Acampora et al., 2014) tend to accumulate more plastics than adults, which could be explained by parental delivery of food by regurgitation to chicks at the nest (Kühn et al., 2019). By contrast, the mean number of plastic items ingested by planktivorous fish from the North Pacific gyre increased as the size of fish increased (Boerger et al., 2010), which can be explained by the phenomenon of bioaccumulation (Alves et al., 2016; Bernardini et al., 2018). The odour of plastics can be another factor affecting the intake plastic debris in fish (Boerger et al., 2010).

Depending on litter size and on species, marine litter (ML) particles may be excreted or accumulated in the gastrointestinal tract, but may also cause physical and mechanical damage, such as abrasion, inflammation, blockage of feeding appendages or filters, and obstruction of gastrointestinal tract (Cole et al., 2011; Wright et al., 2013). The acknowledgment of the magnitude of the problem has led to various initiatives on different levels – global (G7), regional (OSPAR, UNEP Regional Seas Programme), European (the EU Marine Strategy), and national. However, further evidence is required to support policy actions against marine plastic pollution.

Moreover, the presence of marine plastic debris is often linked to a complex mixture of chemicals, comprising of ingredients found in plastic materials (monomers and additives), by-products from the manufacturing process (such as chemicals created during the combustion of raw petroleum materials), and chemical pollutants present in the ocean that adsorbs on plastic debris, including persistent organic pollutants and heavy metals (Rochman, 2015). In larger organisms, plastics typically tend to exhibit a prolonged residence time within the digestive tract, leading to fragmentation of the objects into smaller sizes through enzymatic or mechanical digestive processes (Bergmann et al., 2015). During the process of fragmentation, plastics can become damaged and potentially more vulnerable to the release of plastic associated chemicals. This presents a major problem, as the chemical contaminants released from microplastics can have ecotoxicological impacts on both marine organisms and humans through their consumption (Yuan et al., 2022). It is documented that all the contaminants cited above have been detected in seabirds (Lavers et al., 2014), fish (Rochman et al., 2013), marine mammals (Fossi et al., 2012), amphipods (Chua et al., 2014) and many others marine organisms (Besseling et al., 2013; Fossi et al., 2014). Therefore, the issue of plastic pollution manifests evident physical and metabolic impacts on marine life and has a global concern.

Therefore, further information is required concerning the levels of microplastic ingestion by marine species and the potential effects of these particles and their contaminants on the organisms, as well as a comprehensive understanding of their impact on the overall health of marine populations and the marine trophic web. The selection of bioindicator or sentinel organisms to monitor MPs ingestion is thus crucial for the assessment of marine plastic pollution and for the establishment of future mitigation strategies.

Accordingly, certain marine organisms have been used as bioindicators of plastic pollution given their capacity to bioaccumulate plastic debris in their digestive systems (Bonanno & Orlando-Bonaca, 2018). Currently, seabirds are the most commonly employed group of species as bioindicators for assessing plastic contamination. They are followed by sea turtles, with a majority of reports focusing on loggerhead sea turtles (*Caretta caretta* Linnaeus, 1758) and green sea turtles (*Chelonia mydas* Linnaeus, 1758). The previous organisms are considered moving bioindicators, and give information about the pollution state of an extesive area. Additionally, mussels, particularly the blue mussel (*Mytilus edulis* Linnaeus, 1758), have also been used to monitor plastic contamination and, in this case, they are considered local bioindicators and give information of a specific area. Nevertheless, there are many other marine species being used as plastic bioindicators, including fish, mammals, polychaetes, bryozoans, holothurians, and also bacterial communities (Bonanno & Orlando-Bonaca, 2018) which can provide information of the pollution state of different ecosystems.

Top predators can be considered as good sentinels of microplastic contamination in marine ecosystems because they are mostly long-lived organisms, and this makes them particularly susceptible to oceanic contamination through bioaccumulation and biomagnification processes. Therefore, they tend to accumulate hight amounts of plastic litter during their lifetime. Studies have demonstrated that top predators can indirectly ingest microplastics from contaminated prey (Farrell & Nelson, 2013) and consequently, these microplastics are transferred through trophic chains (Miller et al., 2020; Nelms et al., 2018).

1.3 Sharks as microplastic sentinels

Sharks can be regarded as reliable bioindicators of microplastic contamination in the marine environment, since many species occupy top predator positions and, as a consequence of their place in trophic chains, they tend to accumulate more plastic compared to species at lower trophic levels (Miller et al., 2020).

There are some studies that report levels of microplastic contamination in different shark species inhabiting different oceanic spots and different ecosystems: *Prionace glauca* of Pacific Ocean (pelagic ecosystem) (Huang et al., 2022); *Scyliorhinus canicula*¸ *Mustelus asterias*, *Scyliorhinus stellaris*, *Squalus acanthias* (Parton et al., 2020) of Atlantic Ocean (benthic ecosystem) and *Lamna nasus* (Maes et al., 2020) of Atlantic Ocean (pelagic ecosystem); *Chiloscyllium punctatum*, *Chiloscyllium hasseltii* of Indian Ocean (benthic ecosystem) and *Scoliodon laticaudus*, *Carcharhinus sorrah*, and *Carcharhinus dussumieri* (Matupang et al., 2023) of Indian Ocean (pelagic ecosystem); *Prionace glauca* (Bernardini et al., 2018) of Mediterranean Sea (pelagic ecosystem), *Galeus melastomus*, *Etmopterus spinax*, *Scyliorhinus canicula* (Valente et al., 2019) and *Galeus melastomus* (Alomar & Deudero, 2017) of the Mediterranean Sea (benthic ecosystem). Although there has been an increasing number of studies reporting microplastic pollution in sharks, polymer detection is still largely lacking.

1.3.1 Small-spotted catshark (*Scyliorhinus canicula*)

1.3.1.1 Biology and conservation status

The Small-spotted catshark *Scyliorhinus canicula* (subclass: *Elasmobranchii*, order: *Carcharhiniformes*, family: *Scyliorhinidae*) has a slim and elongated body, with a short and wide head, a large mouth, and broad gill slits. It possesses large oval-shaped eyes that provide excellent vision in low-light waters. The animal has eight fins: two pectoral fins situated in front of five gill slits, two pelvic fins that are long and joined in males at the inner margin, two dorsal fins located distal to the head, a short anal fin, and an asymmetrical caudal fin behind it (Ferreira, 2019). The back of the animal is yellowish-grey with small brown and black spots, while the belly is entirely cream-colored. Its skin is rough and covered with dermal denticles. The males measure in average around 71 cm and weights between 0.5 and 1 kg and females around 70 cm and can be slightly heavier than males. The maximum length for both male and female *S. canicula* in Atlantic waters has been documented as 100 cm (Compagno, 1984; Quero, 1984), although it is rare to observe specimens measuring more than 80 cm long (Ivory et al., 2005). *Scyliorhinus canicula* are known to have a maximum lifespan of around 10-20 years (Bendiab et al., 2012; Rodríguez-Cabello et al., 2005).

Small-spotted catshark was assessed by the IUCN Red List of Threatened Species in 2020 and is listed as of Least Concern with its populations being considered stable at the global scale (Finucci et al., 2021) It is considered common and one of the most abundant elasmobranchs across its range in the Northeast Atlantic and Mediterranean Sea (Ramírez-Amaro et al., 2020).

1.3.1.2 Distribution in the Atlantic Ocean

Scyliorhinus canicula is a benthic shark found from the shallow sub-littoral to the edge of the continental shelf. This shark typically exhibits a tendency to remain in the same area, with average displacements of 30 km from the area where they inhabit and its largest movements reaching distances of 286 km (Papadopoulo et al., 2023; Rodríguez-Cabello et al., 2004). In the Northeast Atlantic, this species commonly inhabits at > 150 meters deep (Serena et al., 2015) . In the north, it is found at depths of at least 300 m (Ellis et al., 2005), and in more southerly waters and in the Mediterranean Sea it can inhabit greater depths (Serena et al., 2015). This species is distributed through the eastern Atlantic, ranging from Norway and the Shetland Islands to Senegal (possibly along the Ivory Coast), and throughout the Mediterranean and Black Seas (Figure 2).

Figure 2. Geographical distribution of *Scyliorhinus canicula* (Finucci et al., 2021)

1.3.1.3 Diet

According to the work of Martinho et al. (2012), the diet of *Scyliorhinus canicula* captured in the central Atlantic coast of Portugal (Figueira da Foz) at deeps of 70 m, are mostly composed of crustaceans, according to the numerical index (NI) (i.e. the percentage of each prey item in relation to the total number of prey items (number of individuals of a prey category/total number of individuals among all prey categories)×100) and occurrence index (OI) (i.e. the percentage of each prey item in all non-empty stomachs, (number of stomachs containing a prey category/ total number of stomachs containing prey)×100), with values of 49.9% and 66.2%, respectively. The most represented prey species were Pagurus spp. (including *P. bernhardus, P. cuanensis, and Pagurus sp*.) (Martinho et al., 2012). The second and third most abundant prey items, according to OI and NI, were Teleostei (*Sardina pilchardus* and *Scomber scombrus*) and Polychaeta (Martinho et al., 2012). Similar results were reported by (Wieczorek et al., 2018) with individuals captured in west coast of Ireland, being the decapods the group with a higher occurrence (85%), followed by Polychaetes, filter feeders and fish.

1.3.1.4 *Scyliorhinus canicula* as sentinel of microplastics in benthic environments

Microplastic pollution in *Scyliorhinus canicula* have been documented in North East Atlantic Ocean (Parton et al., 2020) and in Mediterranean Sea (Valente et al., 2019). In the study by Parton et al. (2020), 12 individuals were analysed having an occurrence of anthropogenic particles of 66.6% of the total individuals. The analysis just reported occurrence of fibres made of higher density materials, such as cellulose and polyacrylamide. In other study by Valente et al. (2019), 30 individuals were analysed, having an occurrence of anthropogenic particles of 66.7% of the total individuals. The analysis reported an occurrence of 90.7% of fibres, 8% of fragments and 1.3% of films Nevertheless, in this last study, the results for polymer detection were not representative given that just 15 out of 258 particles were analysed by FT-IR spectroscopy.

Based in the literature and taking into account that *Scyliorhinus canicula* is a benthic shark that lives on sandy, coralline, algal, gravel or muddy bottoms, it can be considered a potential sentinel of microplastic pollution for the benthonic environments.

1.3.2 Blue shark (*Prionace glauca*)

1.3.2.1 Biology and conservation status

The blue shark *Prionace glauca* (subclass: *Elasmobranchii*, order: *Carcharhiniformes*, family: *Carcharhinidae*) is a wide-ranging shark, found throughout all oceans in both tropical and temperate waters (Rigby et al., 2019). It has a slender and elongated body with a long and conical snout. It has big eyes which are equipped with a nictitating membrane, a kind of semi-transparent eyelid that runs from top to bottom and protects the eyeballs when fighting with prey (Ferreira, 2021). This species has five gill slits, two dorsal fins, two pectoral fins, two anal fins and a caudal fin that would help to control its buoyancy (Groot, 2010). The pectoral fins are long and slender. It has a white colouring on the ventral part, and a very intense metallic blue on the rest of the body. Its teeth, which are constantly falling out and being replaced, are triangular in shape with serrated edges. The male measures in average between 1.8 and 2.8 m and weights between 27 and 55 kg and females between 2.2 and 3.3 m and weight between 93 and 182 kg. Some specimens, however, were recorded to measure 3.8 m long (Ferreira, 2021). Blue sharks are known to have a maximum lifespan of around 20-30 years (Andrade et al., 2019).

Prionace glauca is listed as Near Threatened at the global scale by the IUCN Red List of Threatened Species in 2018, with a tendency for population decrease (Rigby et al., 2019).

1.3.2.2 Diet

Prionace glauca eats pelagic fish and mesopelagic cephalopods, particularity squid. They can also feed of other invertebrates (mostly pelagic crustaceans), small sharks, cetaceans (possibly as carrion), and seabirds (Hideki & John, 2009).

According to the results of Mendonça (2009) with *Prionace glauca* from the Northeast Atlantic Ocean, *Histioteuthidae* is the most representative cephalopod family in the diet of blue shark with *Histioteuthis arcturi* being the predominant species of this group. This species of cephalopod, *H. arcturid,* is generally found in the mesopelagic zone (twilight zone) between 200 and 1000 m. The study also reports teleost fish in the diet of blue sharks, although less common. The teleost fish identified in the stomachs of blue sharks belonged to the families *Molidae*, specifically the ocean sunfish (*Mola mola*), and *Alepisauridae,* specifically the lancet fish (*Alepisaurus brevirostris*). Finally, mammals were the blue shark's least abundant prey group. *Delphinidae* was the most represented mammal family in the blue shark’s diet followed by *Phocidae*. *Stenella coeruleoalba* (Stripped dolphin) was the most frequent mammal species present in the stomach contents followed by *Tursiops truncatus* (bottlenose dolphin). Seabirds can also be present in the diet of blue shark. However, it is believed that these animals do not have a significant impact on the diet, generally they are only hunted when they are already dead or dying. Nevertheless, in a study conducted by Stevens (1973), it was observed that blue sharks occasionally target healthy individuals.

Blue sharks from the North Atlantic make vertical excursions of hundreds of meters in response to the prey distribution, being more active at night and in the early morning (Hideki & John, 2009).

1.3.2.3 Distribution in the Atlantic Ocean

Prionace glauca is an oceanic epipelagic shark, being mainly distributed from the sea surface to depths of about 350 m, even though deeper dives down to 1,000 m have been recorded (Coelho et al., 2018). It is rarely found inshore, except in the cases where continental shelf is narrow (Hideki & John, 2009). It prefers water temperatures between 12–20ºC.

Blue shark has a wide geographical distribution depending on the age. Immature blue sharks (North Atlantic: females < 182.1 cm Fork Length (FL), males < 197.0 cm FL; South Atlantic: females < 173.8 cm FL, males < 175.5 cm FL based on the ICCAT Shark Working Group report (Anon, 2014), including small juveniles (age ≤ 1) and juveniles of all age classes are typically found in the northeast Atlantic (Gulf of Biscay), central east Atlantic (Azores Islands and west waters of the Azores) and in southwest Atlantic (southern Brazil and Uruguay) (Figure 3).

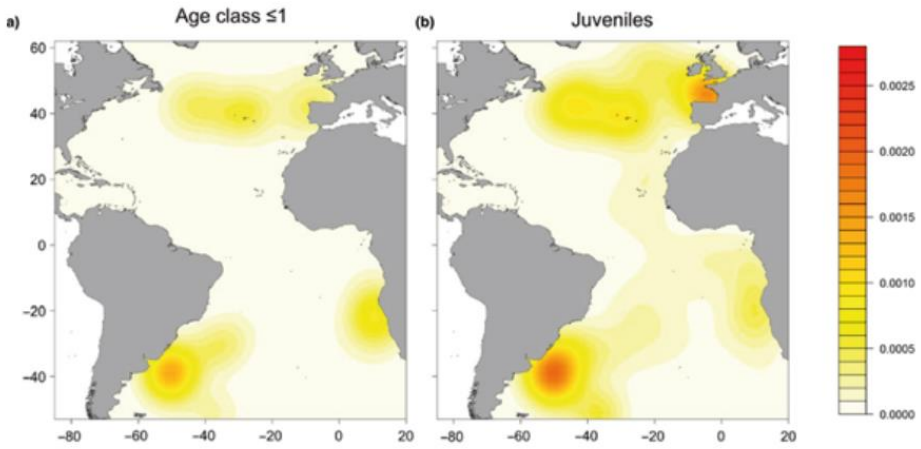

Figure 3. Kernel density distributions for juveniles of blue shark (Redder areas have a higher population density) (from Coelho et al., 2018).

On the other hand, mature blue sharks (North Atlantic: females ≥ 182.1 cm FL, males ≥ 197.0 cm FL; South Atlantic: females ≥ 173.8 cm FL, males ≥ 175.5 cm FL based on the

ICCAT Shark Working Group report (Anon., 2014)), including adult males and adults females, are more abundant in the equatorial and tropical Eastern Atlantic, in the Gulf of Guinea and closer to the Cabo Verde Archipelago (Coelho et al., 2018) (Figure 4).

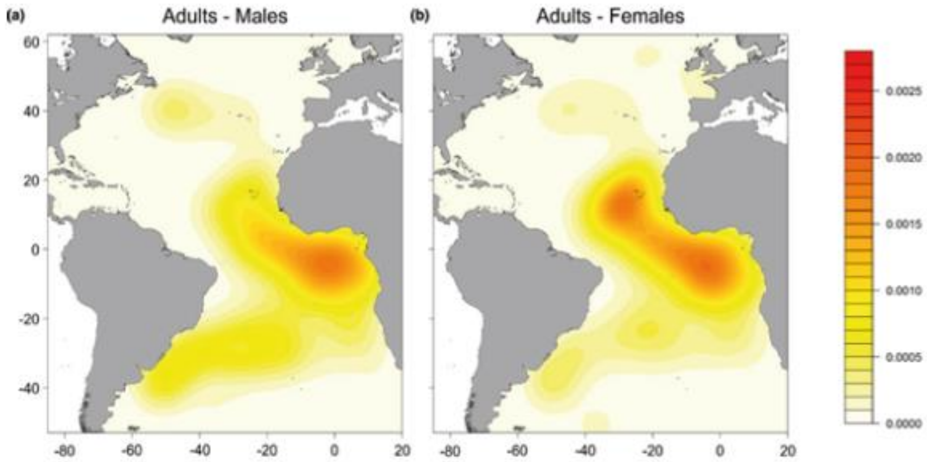

Figure 4. Kernel density distributions for adult males and females of blue shark (Redder areas have a higher population density) (Coelho et al., 2018).

However, it is important to note that the movement of sharks can be influenced by various factors, including the migration of their prey, water temperature, reproductive state, sex, and size segregation (Montealegre-Quijano & Vooren, 2010; Coelho et al., 2018). Therefore, due to migrations during their reproductive state, adult specimens are likely to be present along the European coasts. It is documented that individuals living in North Atlantic Ocean breeds in the Mediterranean Sea, the coast of the Iberian Peninsula; and in the Central North Atlantic, closely to Azores Islands (Coelho et al., 2018).

1.3.2.4 *Prionace glauca* as sentinel of pelagic microplastic contamination

Macro-, meso- and microplastics were reported in *Prionace glauca* specimens captured in the Mediterranean Sea (Bernardini et al., 2018), while in the Eastern tropical Pacific Ocean only microplastics were detected in organisms from this species (Huang et al., 2022). In the study from Bernardini et al. (2018), 139 individuals were analysed, but only the individuals with stomach full content (95) were considered. A total of 107 microplastics were found in 24 of 95 specimens processed, having an incidence of 25.26%. The analysis reports a hight occurrence of low density microplastics, such as polyethylene (75.2%) and polypropylene (19.1%) compared with higher density plastics, such as polyester (1.9 %). On the other hand, in the study from Huang et al. (2022), 23 individuals were analysed. A total of 18 microplastics were found in 9 of the 23 individuals analysed (39.1% incidence). The analysis reports a hight occurrence of PET (66.7 %) and PP (27.8 %).

Based in the literature and taking into account that *Prionace glauca* is a pelagic shark that lives in the water column and is a long-lived species, it can be considered a potential sentinel of microplastic pollution, and particularly for the pelagic environments, since it is more commonly found in the ocean surface.

1.4. Objectives of the study and thesis outline

The main objective of this study was to provide a comprehensive understanding of the accumulation of microplastics and other anthropogenic particles (APs) on different marine environments (benthic and pelagic), by using two shark species as sentinels of plastic pollution assessing the accumulation of anthropogenic particles in the benthic shark *Scyliorhinus canicula* and comparing it to the data on the pelagic shark *Prionace glauca*, both inhabiting the North Atlantic Ocean. Furthermore, this work aimed at contributing for a better understanding on how different digestive conditions in the shark stomachs can affect plastics' integrity and contribute for their degradation.

To achieve these goals, the present thesis was organized into 4 chapters, including a general introduction exploring the distribution and accumulation of microplastics across the benthic and pelagic oceanic habitats, characterizing plastic properties, evaluating the impact of abiotic and biotic factors on plastic fragmentation and degradation, and characterizing potentially suitable bioindicator species for monitoring plastic pollution in the ocean. The thesis is further comprised of 2 experimental chapters and a general conclusions and future perspectives' chapter.

The two experimental chapters of this thesis had the following specific objectives:

Chapter 2: "Contrasting Patterns of accumulation of anthropogenic debris in Benthic and Pelagic Shark Environment". The objectives of study were to assess anthropogenic particles' ingestion of the benthic shark *Scyliorhinus canicula* inhabiting the Northeast Atlantic Ocean to understand how biological factors such as the sharks' sex, maturation state, and individual length may influence the abundance and size of the APs ingested. Additionally, this study aimed to compare the findings with the results obtained for the pelagic blue shark (*Prionace glauca*) captured in the same geographical area, in order to assess differences in the anthropogenic particles size, shape, abundance, and polymer type between two shark species inhabiting different marine environments (benthic and pelagic).

Chapter 3: "Simulating the structural alterations of plastic particles retained in the gastrointestinal tract of shark species"**.** The objective of this study was to investigate the capacity of the digestive conditions of sharks (acidic pH and enzymes involved in the digestion) to affect the plastics' integrity, with two main purposes: 1) to better understand how abrasive digestive conditions can affect the plastics retained in sharks' gastrointestinal track; 2) to explore potential biotechnological applications by evaluating enzymes involved in the digestion of shark prey for their potential for plastic degradation, which could be good candidates for environmental remediation purposes.

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Contrasting patterns of accumulation of anthropogenic debris in benthic and pelagic shark environments

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Contrasting patterns of accumulation of anthropogenic debris in benthic and pelagic shark environments

Roger B. Casado[1], Luis M.F. Alves[1], Marco F. L. Lemos[1], Matteo Baini[2], Matteo Galli[2], Cristina Fossi[2], Filipe R. Ceia[3], Filipa Bessa[3], Sara C. Novais[1]

[1]MARE – Marine and Environmental Sciences Centre, ESTM, Politécnico de Leiria, 2520-641 Peniche, Portugal.
[2]Department of Physical Sciences, Earth and Environment, University of Siena, Via P.A. Mattioli 4, 53100 Siena, Italy.
[3]University of Coimbra, MARE – Marine and Environmental Sciences Centre / ARNET – Aquatic Research Network, Department of Life Sciences, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal.

Abstract

Plastic litter has significantly increased in recent decades due to its excessive production and inefficient waste management, which has led to millions of untreated tons of plastic ending up in the ocean. Numerous studies have documented interactions and of macro- and microplastics (MPs) with marine organisms. Among these, long-lived top predators are very prone to accumulate large quantities of anthropogenic debris (plastics and other particles of anthropogenic origin) due to potential bioaccumulation and biomagnification processes. For these reasons, carnivorous sharks can be considered good sentinels of anthropogenic litter contamination given their high trophic position and the diversity of habitats they can occupy (i.e., from benthic to pelagic and from coastal to oceanic environments). The main objective of this study was to assess anthropogenic particles (APs) incidence in the benthic small-spotted catshark (*Scyliorhinus canicula*) and assess how shark length, sex and maturation state can influence the accumulation of such particles. Moreover, this study also compares the findings of the benthic small-spotted catshark (*Scyliorhinus canicula*) with the results obtained in a previous work for the pelagic blue shark (*Prionace glauca)* sampled in the same geographical region (i.e.

Northeast Atlantic) using the same methodology, in order to assess differences in abundance, particles size distribution, shape, colour and polymer type between the particles extracted from two shark species inhabiting different marine environments (benthic and pelagic). Small-spotted catsharks were opportunistically captured aboard commercial fishing vessels (44 individuals) and their stomachs were extracted and analysed for the identification and characterization of potential microplastics and others. The results showed a prevalence of 100% in the frequency of occurrence of anthropogenic particles in the stomachs of *S. canicula*, being fibres (85.5%) and fragments (9.3%) the most comm on shape items accumulated and cellulose (45.5%), cotton (15.9%) and rayon (8.7%) the most common polymer types. The anthropogenic particles accumulation was not influenced by shark length, sex or maturation state. In comparison, small-spotted catsharks accumulated less anthropogenic particles per individual (7.84 ± 3.49), than the pelagic blue sharks (36.31 ± 23.7). Additionally, the proportions of shape, size, and type of polymer of the particles ingested were different in both sharks, with *S. canicula* accumulating a high proportion of fibres (85.5%) and few fragments (9.3%), while *P. glauca* accumulated four times more fragments (36.9%) and less fibres (61.3%). Regarding the polymer type, *S. canicula* accumulated a higher proportion of natural origin polymers (i.e. cellulose, cotton and wool) (65.5%) and less synthetic/semi-synthetic origin polymers (34.5%) compared with *P. glauca,* which predominantly accumulated synthetic origin polymers (i.e. alkyd varnish, polyester and others) (69.4%) and a little proportion of natural origin polymers (7.1%). These results are in accordance with previous studies of anthropogenic litter contamination in marine ecosystems, with fibres being more associated with benthic environments, and fragments with pelagic ones. Our study highlights the high susceptibility of carnivorous sharks to the accumulation by microplastics and other anthropogenic particles, raising concerns on their potential negative effects.

Keywords: Marine pollution, anthropogenic particles, microplastics, top predators.

2.1 Introduction

Plastic production has increased exponentially worldwide in the last decades mostly driven by the increase of single-use plastics (Diggle & Walker, 2022). Consequently, as a result of this overproduction and inadequate plastic litter management, the amount of plastic waste has increased significantly, with approximately 6.3 billion tonnes being estimated to be generated worldwide between 1950 and 2015, from which around 12% was incinerated, only 9% has been recycled while 79% remained unprocessed (Geyer et al., 2017). End-of-life plastic materials are commonly stored in landfills or released directly into natural environments (Rhodes, 2018). The effect of this plastic mismanagement is that millions of disposed tones of plastics can travel along the rivers and can end up in the ocean. It is estimated that between 1.15 and 2.41 million tonnes of plastic are brought from riverine systems into the oceans every year (Lebreton et al., 2017). In addition, considerable amounts of plastics are also released directly into the ocean basins due to anthropogenic sea-based activities such as merchant shipping, ferries and cruise ships, fishing vessels and fish farming, or offshore oil and gas platforms. The United Nations Environment Programme (UNEP) report suggests that abandoned, lost or discarded fishing gear contributes approximately with 10% to total ocean plastics (Nelms et al., 2021; Macfadyen et al., 2009). This over-accumulation had led to serious marine environmental problems over the years, such as entanglement of marine organisms with marine litter (Bergmann et al., 2015; Moore et al., 2013; Moore et al., 2009; Matsuoka et al., 2005; Bilkovic et al., 2014; Wilcox et al., 2015), accumulation of plastic litter in gastrointestinal track (GIT) of marine fauna (Goswami et al., 2020; Phuong et al., 2018; Neves et al., 2015; Lopes et al., 2020; Avery-Gomm et al., 2012; Campani et al., 2013; De Stephanis et al., 2013; Fossi et al., 2014) and release of pollutants carried by plastic items (Gunaalan et al., 2020). It is important to highlight that apart from plastics, which represent around 50-80% of the marine debris (Barnes et al., 2009), there are other anthropogenic particles (APs), such as paint flakes, cellulosic fibres and others, accumulating in oceanic environment which can also affect marine organisms (Rios-Fuster et al., 2019; Collard et al., 2017).

In order to attempt to understand how anthropogenic debris (plastics and other particles of anthropogenic origin) can affect different marine organisms, it is necessary to have an idea of their distribution in the ocean. Marine litter can have distinct behaviours in oceanic environments. Around 60% of plastic debris tend to float and occupy the upper layers in the surface ocean waters because the polymers are less dense than seawater, as is the case with polyethylene (PE) and polypropylene (PP). Nevertheless, the buoyancy effect can be influenced by other factors: increase of buoyancy given by the air contained inside the plastic item (e.g. floating bottles); decrease of buoyancy produced by the presence of biofilms formed by algae, bacteria or other organisms (Morét-Ferguson et al., 2010) or by the adhesion of minerals (Corcoran, 2015) and other items. All these factors can affect the buoyancy of both plastics and other anthropogenic particles. Due to the positive buoyancy of most plastic items, wind and ocean currents can carry considerable amounts of floating plastic pieces across the ocean to finally be accumulated in the centre of oceanic Gyres (Kershaw et al., 2011). Additionally, apart from the studies reporting accumulation of plastics with positive buoyancy in the oceanic gyres, there are other studies reporting accumulation of these plastics in the water column (Gago et al., 2018; Vianello et al., 2013). Therefore, it is expected that pelagic organisms residing in the water column will tend to accumulate these less dense anthropogenic debris inside their organs.

On the other hand, plastics denser than seawater (e.g. polyvinyl chloride (PVC), acrylic, polyester, polyamide (PA) and others) and all other anthropogenic particles with the presence of a biofilm or bonded minerals, which acquire negative buoyancy, will tend to sink. Ekman transport and geostrophic currents play a crucial role in the movement of plastic waste and tiny particles across the ocean, enabling to comprehend how these particles can eventually reach the ocean floor (Sangiolo, 2022). Consequently, a part of anthropogenic litter that reach the ocean will sink and accumulate in marine canyons and sandy sea beds, causing serious problems to benthic populations (Haegerbaeumer et al., 2019; Anani et al., 2022). The available studies estimated that the most prevalent plastics of benthic sediments of North Atlantic Ocean are polyester (>50%), followed by other plastic types (>30%), mainly polyamides and acetate, and lastly acrylic (>10%) (Woodall et al., 2014, Browne et al., 2011). It is also important to note that the majority of detected plastics in those sediments had fibrous shape and were mostly blue, black, green or red fibres (Woodall et al., 2014). Therefore, it is expected that organisms living in ocean sediments will ingest a higher proportion of fibrous anthropogenic particles made of denser polymers.

Due to the effect of abiotic and biotic factors, larger plastic debris slowly fragment into small fragments with various size ranges (Frias et al., 2019). According to GESAMP (2015), plastic debris can be classified according to its size: macroplastics >25 mm; mesoplastics from 5 mm to 25 mm; and microplastics (MPs) <5 mm. Therefore, depending on the size and shape, plastics will have different impacts in marine organisms, from trapping and entanglement to ingestion, bioaccumulation, and potential biomagnification through the marine trophic chains. The incorporation of plastic particles in marine animals can be differentiated depending on the form of ingestion: plastic direct ingestion, that includes accidental consumption of particles through indiscriminate feeding strategies (e.g. filter-feeders) or active selection due to misidentification of microplastics for food (Nelms et al., 2018). Ingestion of microplastic particles have been documented in many different marine animals, including mammals, fishes, turtles, bivalves, crustaceans and others (Ozturk & Altinok, 2020). However, the number of plastic items accumulated in top predators is expected to be significantly higher than in other low-trophic level organisms, as there are evidences of microplastic biomagnification in trophic chains, from preys to top predators (Nelms et al., 2018).

Carnivorous sharks can be considered reliable bioindicators of microplastic contamination given their high trophic position, which make them very prone to biomagnification processes of different pollutants (Lipej et al., 2022). Moreover, because sharks are a diversified group of fish with diverse habitats extending along coastal vs. oceanic and/or benthic vs. pelagic, they can be used to differentiate between the incidence of anthropogenic debris such as plastics in specific environments. Plastic pollution have been recently documented in pelagic (Maes et al., 2020) and benthic sharks (Parton et al., 2020) of North-East Atlantic Ocean, confirming the presence of considerable amounts of microplastics. Nevertheless, it is important to further study the incidence of anthropogenic particles in these organisms to assess the state of contamination in the different shark species inhabiting both ecosystems.

The main objective of this study was to evaluate the ingestion of anthropogenic particles in the benthic shark *Scyliorhinus canicula* inhabiting the Northeast Atlantic Ocean and assess the potential influence of shark length, sex, and maturation state on the accumulation of anthropogenic particles. Moreover, this study also aimed to compare the findings for the benthic small-spotted catshark (*S. canicula*) with the results obtained in a concurrent work for the pelagic blue shark (*Prionace glauca)* using the same methodology (Bessa et al, in preparation), in order to assess if there are differences in abundance, particles size, shape, and polymer type between two shark species inhabiting two different marine environments (benthic and pelagic) in the same geographical region (i.e. Northeast Atlantic).

2.2 Materials and methods

2.2.1 Shark sampling

A total of 44 *Scyliorhinus canicula* specimens were sampled off the coast of Portugal (at 55 km of Peniche coastline) aboard a commercial trawling vessel between late March and late June of 2018 (more details in Marques et al., 2021). The stomachs were extracted and stored at -20ºC until further processing. The sex, total body length mark (cm), and total weight (g) of each individual were recorded. The sex ratio was composed by 55% males and 45% females, and by 43% adults and 57% juveniles. The life stage was defined according to Martinho et al. (2012), with sharks of both sexes being considered adults at the ≥ 50 cm total body length mark. Of the 24 males, 8 were juveniles and 16 were adults and of the 20 females, 11 were juveniles and 9 were adults. The total body length mark ranged between 40.0 cm and 59.0 cm (mean ± SD; 49.93 ± 3.90 cm), and the total weight between 196.6 g and 661.0 g (454.21 ± 110.65 g).

2.2.2 Sample processing

The extraction of anthropogenic particles from the stomachs of sharks was performed following the procedures described in Bessa et al. (2018) and references therein. Each stomach was transferred to individual 500 ml glass jars to which a solution of potassium hydroxide (KOH), 10% concentrated, was added in a 1:3 proportion (m:v). Several studies have identified KOH solution as the most suitable base to digest fish stomach content (Bessa et al., 2018; Kühn et al., 2017; Foekema et al., 2013). A glass lid was used to cover each glass jar to avoid airborne contamination. The digestion took between 4-5 days to occur and was set at room temperature in a closed and controlled lab room. After 1-2 days of digestion, 10 mL of hydrogen peroxide (H_2O_2, 10% concentrated) were added to the less unprocessed samples to accelerate the digestion process and to bleach the sample. Once the stomachs were digested, the content was filtered using a sieve of 63 µm (limit of detection of this study). Afterwards, the content retained in the sieve was vacuum filtered through 1.2 µm Whatman GF/C microfibre filter papers. After filtering, the filters were placed inside individual Petri dishes, dried in an oven (40 ºC) and stored for later visual inspection counting, characterization and identification of anthropogenic particles.

2.2.3 Observation, identification and counting of anthropogenic particles

The counting and the visual characterization of anthropogenic particles was done using a trinocular stereozoom microscope ZEISS STEMI 2000-C (Carl Zeiss Microlmaging Gmbh Göttingen, Germany). The particles suspected to be non-natural origin were sorted, separated, and photographed using AxioCam MRc with Zen 2011 application (blue edition) software. Afterwards, the images of the particles were measured using the software ImageJ (Collins, 2007). Particles were then classified according to the shape into fibres (elongated and thin), fragments, tangled fibres, filaments, and films and, within each shape, according to their colour. All the items were measured (mm) to their largest cross section.

2.2.4 Polymer detection of anthropogenic particles

All the extracted and suspected particles from the sharks' stomachs were used to identity their polymer composition. Visual verification and chemical identification of microplastics was carried out with a micro-Fourier Transformed Infrared (Micro-FTIR) Spectroscope microscope (Nicolet iN10 MX Infrared Imaging Microscope, Thermo Scientific) in reflectance mode. Micro-FTIR spectra of all particles were recorded in the spectral range of 4000 to 650 cm^{-1} with a collection time of 3s and 16 co-scans for each measurement. The spectral resolution was 8 cm^{-1} and the aperture size was a range of 10 × 10 µm to 150 × 150 µm depending on the size of particles. To accurately determine the chemical composition of particles in each sample, a spectra library database was adopted and imported from De Frond et al. (2021). Components with a match of ≥70% were considered acceptable.

2.2.5 Contamination control

All the steps were carried out in an isolated room, in order to avoid airborne contamination that could take place during the entire sample processing steps. All the material used during the sample processing was cleaned with tap water before use, cotton laboratory coats were worn, and plastic material was avoided. To control airborne microplastic contamination, filters in open Petri dishes were placed inside the room every time a session was held. A total of 9 fibres (6 black, 2 transparent and 1 blue) were detected in control filters during all the sample processing sessions. Zero fibres were detected during visual counting and characterization. We assumed a contamination of 2.6% of total particles detected, corresponding to 0,06% per individual shark sample.

2.2.6 Data analysis

Data was organized as '*abundance'* – number of items and '*medium size'* – value of the average size (largest cross section), of the items found in the stomach of each individual. For each group described above ('*abundance'* and '*medium size*') anthropogenic particles detected were classified as total fibres, total fragments, and total APs. To test if the biological factors "sex of the individual" and "maturation stage" could have an influence on '*abundance'* and '*medium size*' of anthropogenic particles (total fibres, total fragments, and total APs), individuals were separated into different groups: sex (24 males and 16 females), maturation state (16 juvenile and 24 adults) and Total (44 individuals). To test the data for normality and homogeneity of variance, Shapiro-Wilk and Levene's Tests were performed, respectively. Given that the assumptions of normality were not met, Mann-Whitney Rank Sum Tests were performed to assess differences between sexes or maturation states in terms of '*abundance'* or '*medium size*' of anthropogenic particles (Total fibres, total fragments, and total APs), and also in terms of the occurrence of total fragments and total fibres. Spearman Rank Order Correlation tests were performed to assess relationships between shark length and the '*abundance'* or '*medium size*' of anthropogenic particles (Total fibres, total fragments, and total APs).

For the comparation of the accumulation data between *Scyliorhinus canicula* and *Prionace glauca,* data on the anthropogenic particles found in 39 stomachs of *P. glauca* sampled in the same geographical area as *S. canicula* were retrieved from Bessa et al. (in preparation). Mann–Whitney tests were performed to assess significant differences between the occurrence and mean size of anthropogenic particles between species, as well as to test differences in mean size of total fragments and total fibres. To test if there were significant differences in the proportion of accumulation of fibres and fragments between the two species, a Pearson's Chi-squared test was performed. Since it was not possible to measure tangled fibres in *Scyliorhinus canicula*, due to its large size which exceeded the limits of the magnifying glass, these were excluded from the comparative analysis of particles' mean size. For all statistical tests, the significance level was as $p < 0.05$. Graphics and figures were made using Adobe illustrator (2023), Excel (2016) and Ggplot2 (Wickham H., 2016), and statistical tests were performed using Sigma Plot 12.0 and R Core Team (2021).

2.3 Results

2.3.1 Incidence of anthropogenic particles

Anthropogenic particles were found in all 44 stomachs of *Scyliorhinus canicula* (frequency of occurrence 100%), with a total of 345 particles being extracted and identified. The average particles per individual was 7.84 ± 3.49 (mean ± SD), ranging from 2 to 18 anthropogenic particles per individual. There were statistical differences between the total occurrence of fibres and fragments (Mann-Whitney Rank Sum Test, U= 56.5 p= <0.001), with individuals having an average of 6.7 ± 3.63 fibres (100% incidence) and 0.73 ± 1.35 fragments (41% incidence).

2.3.2 Characterization of anthropogenic particles

Anthropogenic particles were characterized according to their shape, colour, length, and polymer type. From the total 345 particles found in shark stomachs, 295 had fibrous shape (85.5%), 32 were fragments (9.3%), 10 were filaments (2.9%), 6 were tangled fibres (1.7%) and 2 were films (0.6%).

Colour distribution was different depending on the shape of the particle (Figure 5A). For fibrous shape items, black was the most common colour, followed by blue, red, transparent, and other colours with a smaller percentage (Figure 5A). Regarding fragments, silver was the most common colour, followed by blue and red as the most common colours (Figure 5A). As for the colour of the remaining shaped particles, filaments were all light brown, the tangled fibres were all transparent, and the films were white and blue. Regarding to size classification, fragments had an average size of 0.218 ± 0.166 mm, ranging from 0.018 mm to 0.809 mm, while fibres had an average size of 1.39 ± 0.60 mm, ranging from 0.073 mm to 13.446 mm. Fragments were thus significantly smaller than fibres (Mann-Whitney Rank Sum Test, U= 5 p= <0.001) (Figure 5B).

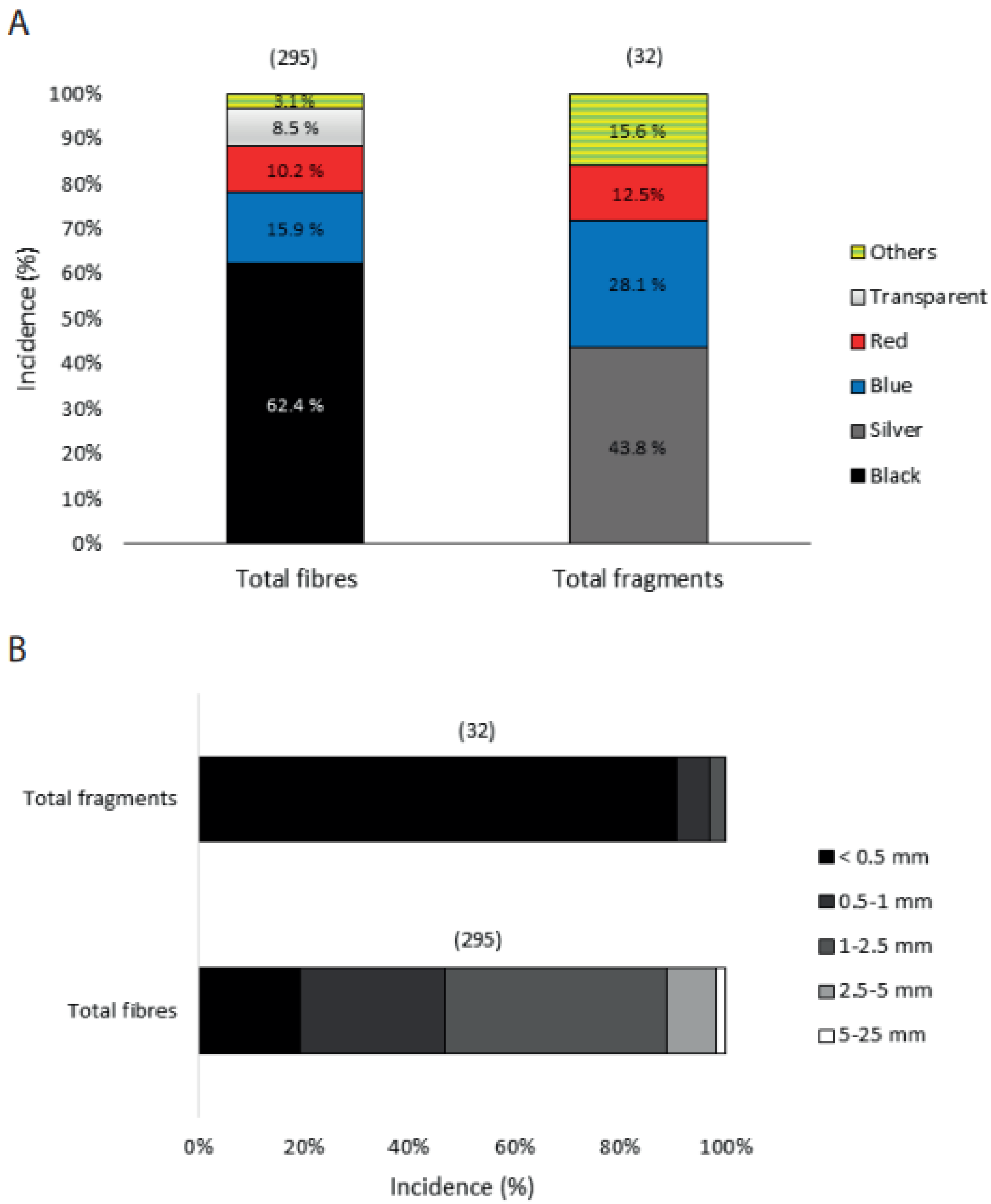

Figure 5: Incidence (%) of anthropogenic particles (fibres and fragments) found in the stomachs of *Scyliorhinus canicula* collected in the Northeast Atlantic (n=44), characterized by (A) colour and (B) size (mm). Other colours include grey, orange, yellow and green. The total number of fibres and fragments are indicated between parentheses.

Polymer identification was carried out for all 345 particles, revealing that 65.5% of the total particles were polymers of natural origin (Figure 6A), with cellulose being the most abundant natural polymer, followed by cotton and wool (Figure 6B). On the other hand, 34.5 % of total particles were categorized as semi-synthetic and synthetic origin polymers (Figure 6A), with rayon being the most abundant semi-synthetic polymer, followed by polyethylene terephthalate (PET) and other synthetic polymers (Figure 6C). Two items (0.6%) were classified as non-detected (N.D) since it was not possible to acquire a particle spectrum from those samples due to the particles thickness and opacity.

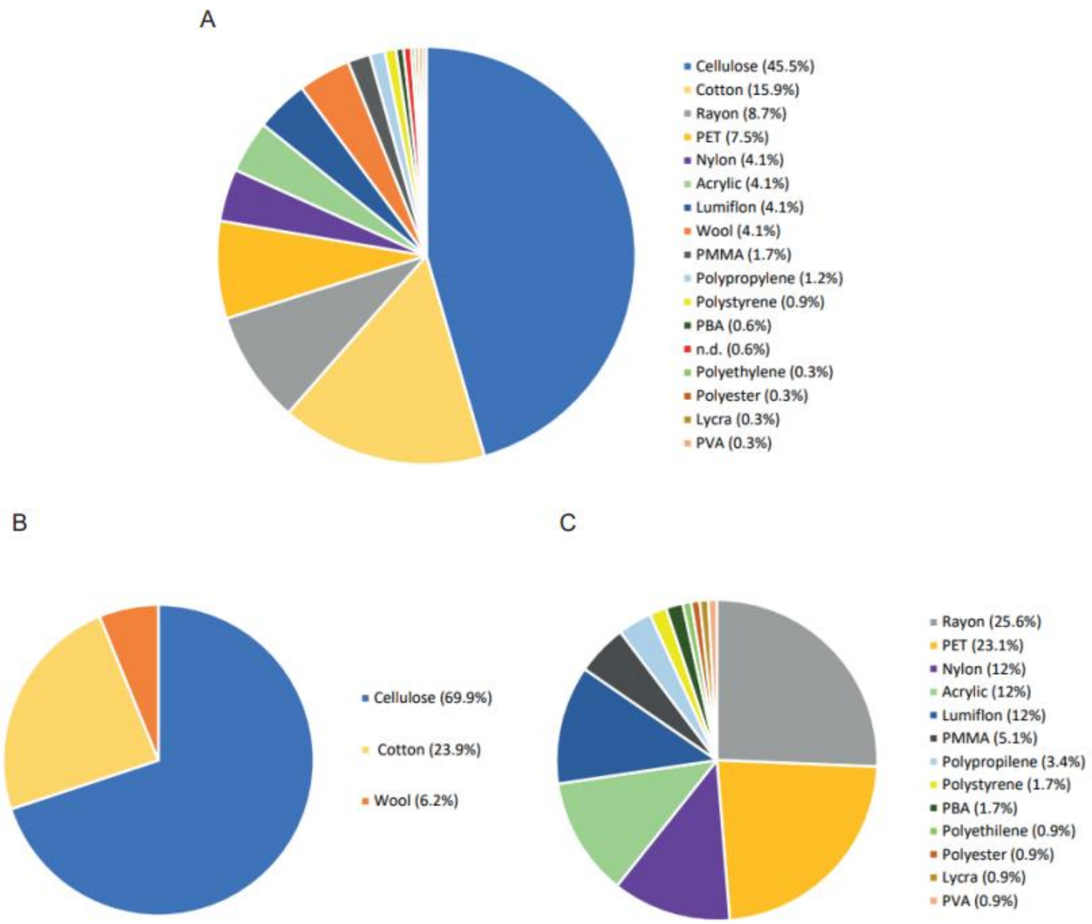

Figure 6: Polymer identification of the anthropogenic particles found in the stomachs of the benthic shark *Scyliorhinus canicula* collected in the Northeast Atlantic (n=44). (A) Total particles (345); (B) Particles of natural origin (226); (C) Semi-synthetic and synthetic particles (117).

Micro-FTIR spectrum and their respective match to some of the most common fragments and fibres polymers accumulated in *Scyliorhinus canicula*, with the correspondent images of the particles, can be seen in Figures 7 and 8.

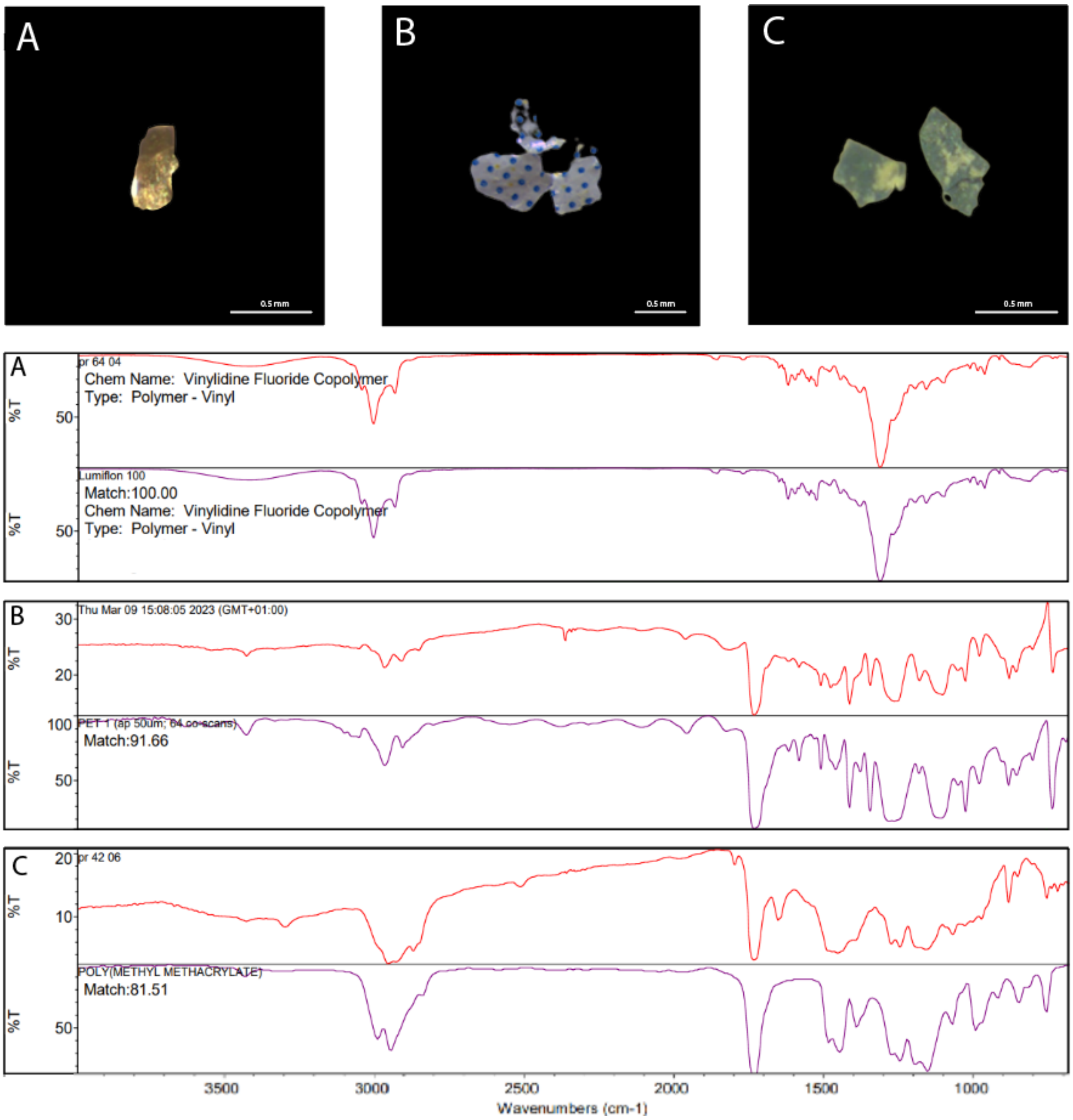

Figure 7: Examples of the most commonly found fragments and respective polymers in *Scyliorhinus canicula's* stomach, characterized by micro-FTIR analysis. Infrared spectra of the particles (red line) and their matches (purple line) of: A) Lumiflon (Match: 100), B) Polyethylene terephthalate (Match 91.66), C) Polymethyl methacrylate (Match 81.51)

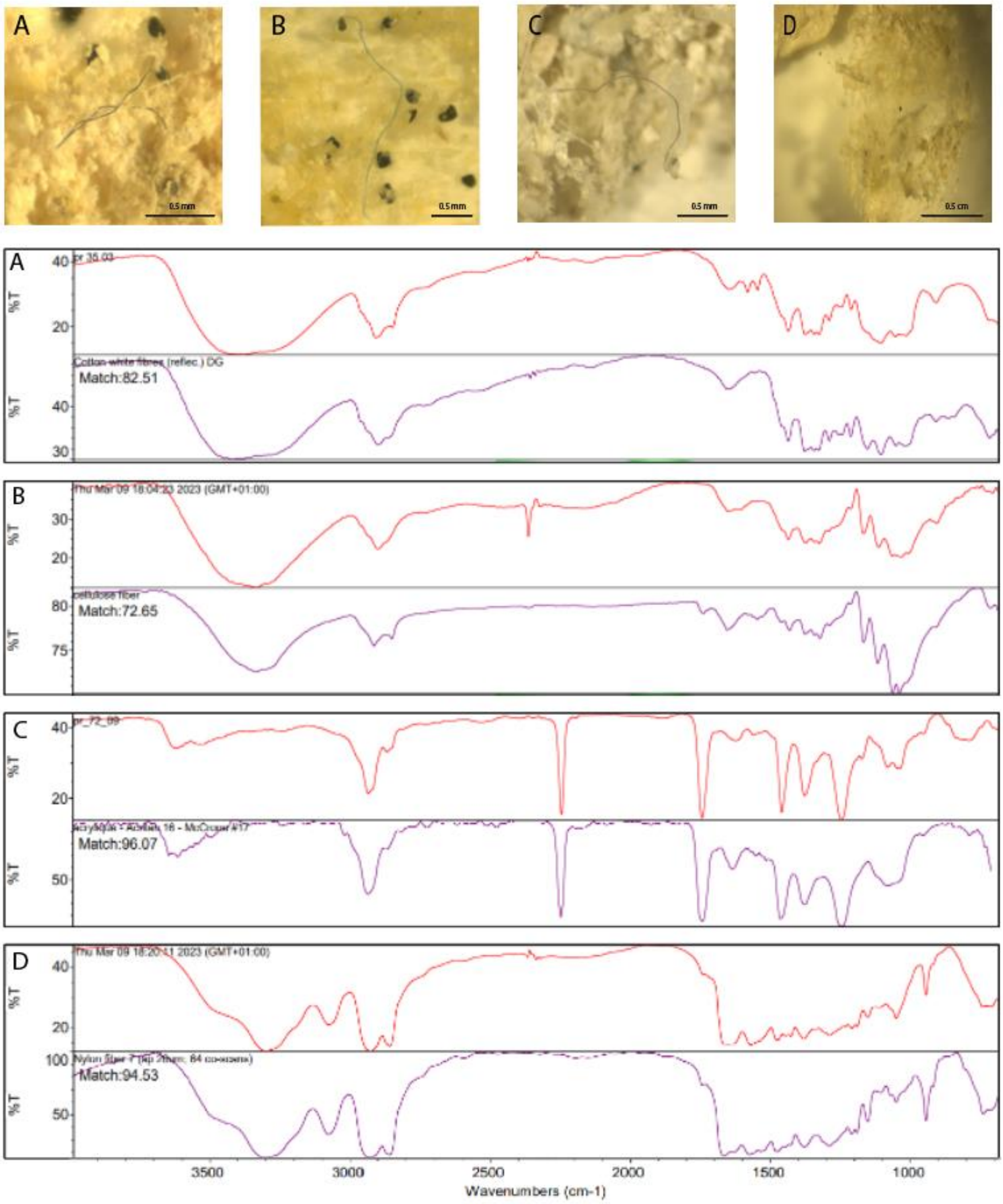

Figure 8: Examples of the most commonly found fibres and respective polymers in *Scyliorhinus canicula's* stomach, characterized by micro-FTIR analysis. Infrared spectra of the particles (red line) and their matches (purple line) of: A) Cotton (Match 82.51), B) Cellulose (Match 72.65), C) Acrylic (Match 96.07), D) Nylon (Match 94.53).

2.3.3 Biological factors affecting anthropogenic particles occurrence

No correlations were found between shark's length and '*abundance*' of either total anthropogenic particles (Spearman's rank correlation, rs = -0.1; p = 0.51), total fibres (Spearman's rank correlation, rs = 0.0033; p = 0.98) or total fragments (Spearman's rank correlation, rs = -0.19; p = 0.22). Likewise, no correlations were found between shark's length and '*medium size*' of total anthropogenic particles (Spearman's rank correlation, rs = -0.049; p = 0.75), total fibres (Spearman's rank correlation, rs = -0.11; p = 0.46) or total fragments (Spearman's rank correlation, rs = 0.034; p = 0.89).

The sex (male or female) or maturation state (adult or juvenile) of the individuals also did not show to have a significant influence in either '*abundance'* or '*medium size*' of the anthropogenic fibres or fragments' accumulation (Table 1).

Table 1: Mean abundance and size of anthropogenic particles (fibres and fragments) accumulated in the stomachs of the studied sub-groups (sex and maturation stage) of *Scyliorhinus canicula*. Mean values are presented alongside standard deviation (SD). Min = minimum value. Max = maximum value. No statistical differences were observed between sexes and maturation stages (Mann-Whitney test, p>0.05).

			Total fibres			**Total fragments**		
	Biological factors	Sample size (n)	Mean ± SD [Min-Max]	U value	P value	Mean ± SD [Min-Max]	U value	P value
Abundance	Male	24	6.3 ± 3.4 [1-14]	222.0	0.678	0.9 ± 1.4 [1-6]	187.5	0.162
	Female	16	7.2 ± 4.0 [2-18]			0.6 ± 1.4 [1-6]		
	Juvenile	16	6.7 ± 4.0 [1-14]	237.0	1.000	0.8 ± 1.4 [1-6]	207.5	0.425
	Adult	24	6.7 ± 3.4 [2-18]			0.6 ± 1.3 [1-6]		
	Total	44	6.7 ± 3.6 [1-18]			0.7 ± 1.4 [1-6]		
Medium size (mm)	Male	24	1.3 ± 0.5 [0.3-2.5]	227.0	0.768	0.2 ± 0.1 [0.1-0.5]	36.0	1.000
	Female	16	1.5 ± 0.7 [0.5-3.2]			0.3 ± 0.2 [0.02-0.7]		
	Juvenile	16	1.4 ± 0.6 [0.3-3.2]	233.0	0.924	0.2 ± 0.2 [0.02-0.5]	29.0	0.331
	Adult	24	1.4 ± 0.6 [0.5-2.9]			0.3 ± 0.2 [0.1-0.7]		
	Total	40	1.4 ± 0.6 [0.3-3.2]			0.2 ± 0.2 [0.02- 0.7]		

2.3.4 *Scyliorhinus canicula* versus *Prionace glauca*: comparison of anthropogenic particles' accumulation between benthic and pelagic environments of North-Atlantic

According to the study of Bessa et al (in preparation), the digestion of *P. glauca* stomachs (n=39) revealed a 100% incidence of anthropogenic particles, similarly to what was observed in the present study with *S. canicula* (n=44). However, the mean occurrence per individual differed significantly for both sharks, with *S. canicula* having a mean occurrence of 7.84 ± 3.49 particles per individual while *P. glauca* had 36.31 ± 23.70 particles per individual (Mann–Whitney test, $U = 124$, $p < 0.001$).

Regarding the shape of the particles, fibres and fragments were the most common shapes accumulated by both sharks (Figure 9A, B). However, its proportion was significantly different between the two species (Pearson's Chi-squared test, X=73.87, $p < 2.2e\text{-}16$): fibres represented 85.5% and 61.3% of the total anthropogenic particles accumulated in *S. canicula* and *P. glauca*, respectively, while fragments accounted for 9.6% and 36.9% of the total anthropogenic particles accumulated in *S. canicula* and *P. glauca*, respectively (Figure 9A, B). Tangled fibres were found in similar proportions in both species, but filaments and films were only observed in *S. canicula* (Figure 9A, B).

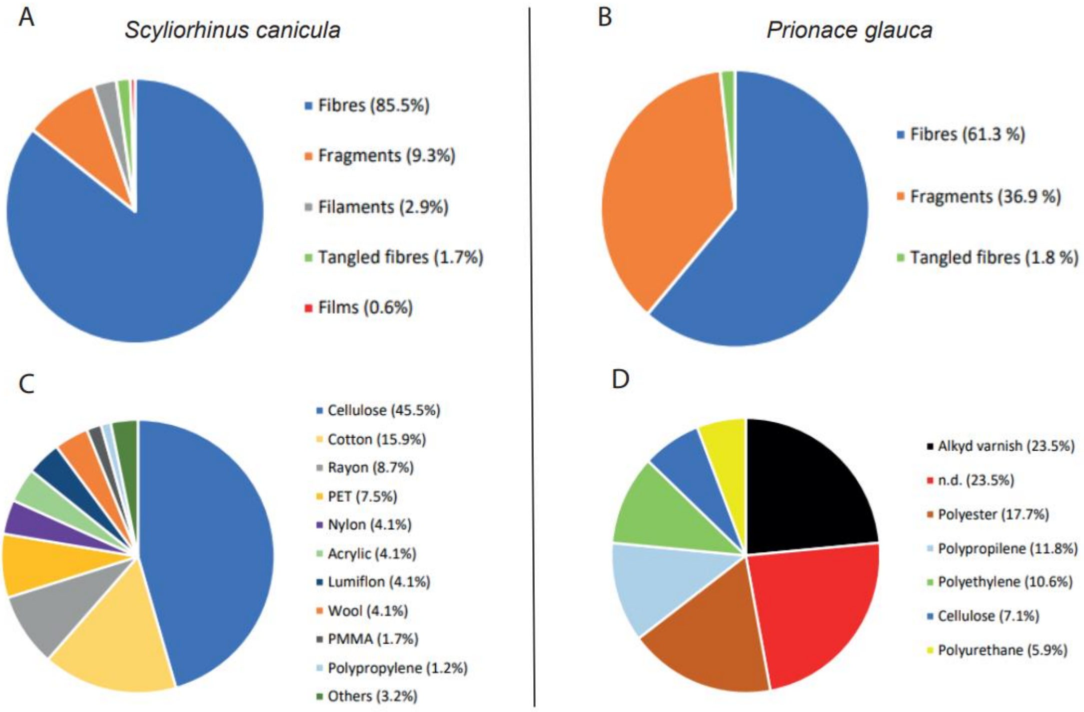

Figure 9: Proportion of shape (A, B) and polymer type (C, D) of anthropogenic particles accumulated in the stomachs of *Scyliorhinus canicula* (A, C) and *Prionace glauca* (B, D) sampled in the North-Atlantic. n.d. = non detected; "Others" = Polystyrene (0.9%), Poly (butyl acrylate) (PBA) (0.6%), n.d. (0.6%), Polyethylene (0.3%), Polyester (0.3%), Lycra (0.3%) and Polyvinyl acetate (PVA) (0.3%).

In relation to the type of polymers, both sharks accumulated polymers of different origins. *Scyliorhinus canicula* accumulated mostly natural and semi-synthetic origin polymers, representing 74.2% of total particles (Figure 9C), while for *Prionace glauca* semi-synthetic origin polymers represented only 7.1% of the total particles accumulated and no natural origin polymers were found (Figure 9D). In relation to the polymers of synthetic origin, they represented 25.8% of total polymers accumulated by *Scyliorhinus canicula* (Figure 9C) while in *Prionace glauca* represented 69.4% of total particles accumulated (Figure 9D). It is important to highlight that 0.6% and 23.5% of total particles could not be identified in *S. canicula* and *P. glauca*, respectively (Figure 9C, D).

Overall, the mean sizes of total particles accumulated in both sharks showed to be significantly different (Mann–Whitney test, $U = 497$, $p = 0.001$). *S. canicula* accumulated in average larger particles (1.35 ± 0.69 mm) than *P. glauca* (0.80 ± 0.41 mm). However, no significant differences between species were present when analysing the mean size of fibres and fragments separately (Figure 10). In the case of accumulated fibres, it can be observed bigger sizes in *S. canicula* (1.39 ± 0.60 mm) than in *P. glauca* (1.14 ± 0.34 nm), but without significant differences (Figure 10A), while for fragments an opposite trend is only marginally seen with slight smaller sizes in *S. canicula* (0.218 ± 0.166 mm) than in *P. glauca* (0.236 ± 0.153), and again without significant differences (Figure 10B).

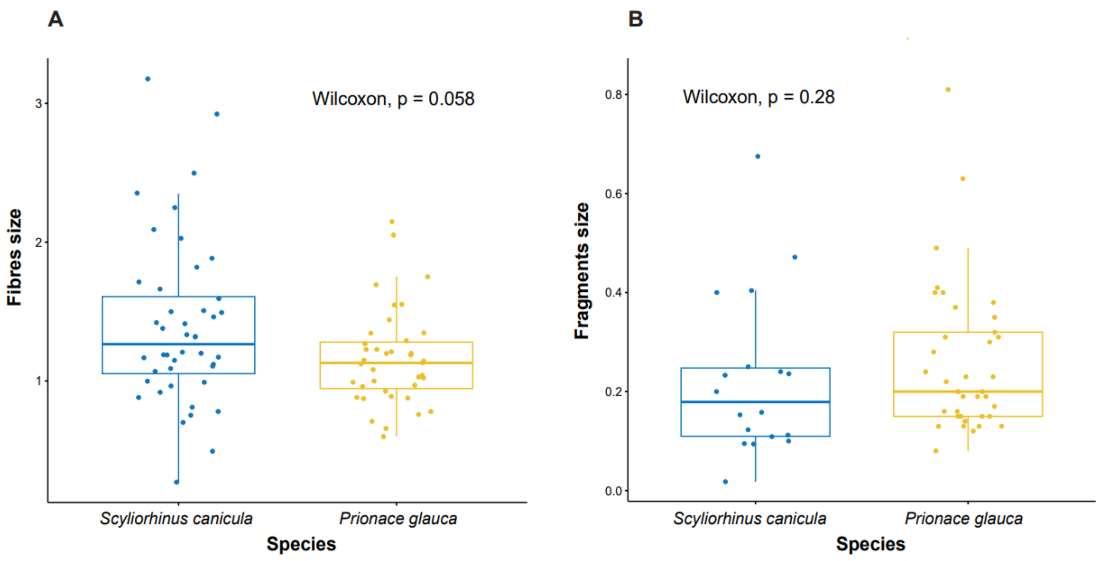

Figure 10: Mean size of fibres (mm) (A) and fragments (B) accumulated in the stomachs of *Scyliorhinus canicula* and *Prionace glauca* sampled in the North-Atlantic. No significant differences were seen for the mean sizes of fibres (Wilcoxon, p = 0.058) and fragments (Wilcoxon, p= 0.28) between species.

2.4. Discussion

2.4.1 Anthropogenic particles ingestion in *Scyliorhinus canicula*

The results of the present study with the benthic shark *Scyliorhinus canicula* showed an overall higher occurrence and mean number of anthropogenic particles ingestion in this shark in comparison to other fish species analysed through the years along the Portuguese coast (Table 2). This reinforces the generalized idea that top predators like sharks can be more susceptible to accumulate higher amounts of anthropogenic particles than other fish, since they can be more affected by bioaccumulation and biomagnification processes

(Nelms et al., 2018; Bernardini et al., 2018; Alves et al., 2016), due to long lifespan and their position in the trophic chain (Lipej et al., 2022). Nevertheless, Neves et al. (2015) documented a lower occurrence and lower mean numbers of anthropogenic particles per individual in *Scyliorhinus canicula* in comparison with the present study (Table 2). Although the sampling region and the study year was different between both works [Neves et al. (2015) conducted their catches during the summer of 2013, along the north, central (i.e. Sesimbra), and south coasts of Portugal, while the present study focused on the Peniche coastline in the central coast], leading to potentially different contamination sources and anthropogenic particles concentration, it has to be taken into account that Neves et al. (2015) did not perform KOH digestion and the particles were identified directly in the stomach. This, along with the fact that the captures could have been made in less polluted places in that study, could justify that the lower occurrence and mean number of anthropogenic particles.

Fibres were the most common shape of anthropogenic particles found in the present study followed by fragments. Similar results were reported for the same species sampled in other regions worldwide (Mancuso et al., 2022; Valente et al., 2019; Parton et al., 2020), suggesting that fibres are much more common to accumulate in gastrointestinal track than fragments for this type of bottom-dwelling sharks. Fibres are described to be one of the most common anthropogenic particles found in benthic ecosystems (Woodall et al., 2014; Courtene-Jones et al., 2020; Fagiano et al., 2023), and consequently marine organisms inhabiting these areas are thus more susceptible to ingest fibrous particles than fragments. In relation to the colour of the items accumulated in the present sharks, black was the predominant colour for fibres, while silver was dominant for fragments. The remaining fragments and fibres exhibited a similar distribution of colours, with blue being the second most common colour, followed by red. This is in accordance of what has been found in other studies where black, blue, and red were the most common colours of anthropogenic particles found in *S. canicula* (Mancuso et al., 2022; Parton et al., 2020). However, silver-coloured fragments were not previously detected in such high abundances in this species. Such fragments were identified as having Lumiflon in their composition thus suggesting that the area where the individuals were captured in this study may have been contaminated with this type of polymer used for ship coating. The size of the anthropogenic particles was in accordance with what was found previously for this species with the great majority of particles having <5 mm (Parton et al., 2020; Mancuso et al., 2022).

Lastly, in relation to the polymer type, natural origin polymers (e.g. cellulose, cotton and wool) were the most common polymers accumulated in the *S. canicula* of the present study, which was also observed by Parton et al. (2020) for the same species where fibres of cellulose were the most common material found, followed by other synthetic polymers. Natural origin fibres should not be disregarded in this type of assessments, because although they are considered biodegradable materials, they can also adsorb toxins (PAHs, PCBs and others) with potentially harmful effects on marine organisms (Lithner et al., 2011; Yuan et al., 2022). The high degree of accumulation of natural fibres in marine ecosystems is thus an issue of high concern (Détrée et al., 2023). Some studies have demonstrated that the presence of natural fibres in ocean basins exceeds largely (91.8%) the synthetic ones (8.2%), with the cellulosic (e.g. cotton) being the most common (79.5%), followed by animal fibres (12.3%) (e.g. wool) (Suaria et al., 2020).

Apart from characterizing the ingested items, this research also aimed to assess if the accumulation of anthropogenic particles in *S. canicula* individuals could be influenced by biological factors such as shark size, maturation state, and sex. Results showed that abundance and size of particles accumulated in the stomachs were not related with the shark's size, maturation state or sex. Similar studies showed no relationships between sex of the sharks and abundance of accumulated particles (Huang et al., 2022; Parton et al., 2020; Mancuso et al., 2022). Anthropogenic particle ingestion of small items (<5 mm) can be considered a random phenomenon (Nelms et al., 2018), given that such small sizes cannot be detected by most organisms, and especially vertebrates like sharks. However, associations between shark length and abundance of anthropogenic particles in their systems can be expected given that larger individuals are supposedly older and, consequently, more exposed to the intake of anthropogenic particles due to bioaccumulation processes (Alves et al., 2016; Bernardini et al., 2018). Although this was not corroborated during the present study, Parton and co-authors (2020) were able to find a correlation between abundance of ingested particles and shark length for the same shark species.

Table 2: Anthropogenic particles incidence in different fish sampled in Portuguese waters. Values are given in percentage (%) of occurrence and number of anthropogenic particles per fish (mean ± standard deviation).

Species	Sampling Period	Area	Number of speciments	Sieve pore size	Anthropogenic particles occurrence (%)	Mean anthropogenic particles per fish (±SD)	Reference
Platichthys flesus	2014	Mondego estuary	40	na	13	0.18 ± 0.55	(Bessa et al., 2018)
Diplodus vulgaris	2014	Mondego estuary	40	na	73	3.14 ± 3.25	(Bessa et al., 2018)
Dicentrarchus labrax	2014	Mondego estuary	40	na	23	0.30 ± 0.61	(Bessa et al., 2018)
Boops boops	2014	Portuguese West and South coast	19	20 µm	60	0.25	(Lopes et al., 2020)
. *Engraulis encrasicolus*	2014	Portuguese West and South coast	131	20 µm	79	0.48	(Lopes et al., 2020)
Sardina pilchardus	2014	Portuguese West and South coast	76	20 µm	58	0.16	(Lopes et al., 2020)
Scomber colias	2014	Portuguese West and South coast	58	20 µm	64	0.23	(Lopes et al., 2020)
Scomber scombrus	2014	Portuguese West coast	19	20 µm	100	1	(Lopes et al., 2020)
Trachurus trachurus	2014	Portuguese West coast	24	20 µm	100	1.75	(Lopes et al., 2020)
Boops boops	2013	Portuguese coast	32	na	9	0.09 ± 0.3	(Neves et al., 2015)
Sardina pilchardus	2013	Portuguese coast	12	na	0	0	(Neves et al., 2015)
Scomber japonicus	2013	Portuguese coast	35	na	31	0.57 ± 1.04	(Neves et al., 2015)
Scomber scombrus	2013	Portuguese coast	13	na	31	0.46 ± 0.78	(Neves et al., 2015)
Trachurus picturatus	2013	Portuguese coast	29	na	3	0.03 ± 0.18	(Neves et al., 2015)
Trachurus trachurus	2013	Portuguese coast	44	na	7	0.07 ± 0.25	(Neves et al., 2015)
Trigla lyra	2013	Portuguese coast	31	na	19	0.26 ± 0.57	(Neves et al., 2015)
Scyliorhinus canicula	2013	Portuguese coast	17	na	12	0.12 ± 0.33	(Neves et al., 2015)
Scyliorhinus canicula	**2018**	**Peniche coastline**	**44**	**63 µm**	**100**	**7.84 ± 3.49**	**This study**

na = not available; SD = Standard deviation

2.4.2 Comparison of anthropogenic particles' accumulation between benthic and pelagic environments of North-Atlantic using sharks as bioindicators

This study also aimed to compare the anthropogenic particles ingestion of two different shark species inhabiting different ecosystems (i.e., benthic and pelagic), sampled in Northeast Atlantic Ocean. The results showed a significantly higher number of anthropogenic particles per individual accumulated in *P. glauca* in comparison with *S. canicula*. The migratory nature of the pelagic blue shark, traveling long distances during its lifetime (Hideki & John, 2009; Coelho et al., 2018), compared with *S. canicula,* which tends to remain in the same area throughout its life (Papadopoulo et al., 2023; Rodríguez-Cabello et al., 2004), can, in part, explain these results due to the potential exposure to more polluted marine areas in the case of *P. glauca*. Moreover, and possibly the most determining factor that can explain the differences observed between both sharks, is the fact that the mean concentrations of plastics floating on the surface are higher than those on the seafloor (Eriksen et al., 2014; Bernardini et al., 2018).

Regarding to the shape of the anthropogenic particles ingested by both sharks, significant differences were found in the proportion of fibres and fragments. *Prionace glauca* accumulated a higher proportion of fragments, up to 4 times more, than *Scyliorhinus canicula*. Other studies conducted on pelagic sharks in various oceanic regions also indicated a greater prevalence of fragments and films in these organisms when compared to benthic sharks (Table 3). The exception is the study by Huang et al. (2022) in which pelagic sharks sampled presented a proportion of fibres and fragments more similar to those described in benthic sharks (Table 3). However, this study relied on the analysis of only 18 microplastics collected from 23 individuals and thus the limited number of accumulated items was insufficient to draw reliable conclusions. Although *P. glauca* had a higher proportion of fragments compared to *S. canicula*, both sharks exhibited a high ingestion of fibres, being the predominant type of anthropogenic particles accumulated. This is in accordance with what was described by Fagiano et al. (2023), in a study of the presence of microplastics in sea surface water, seafloor sediments and beach sediments of a coastal Mediterranean marine protected area. Their results report fibres as the most prevalent anthropogenic particles identified in all analysed habitats (benthic, pelagic and beach sediments). Although fibres are ubiquitous in all environments, they seem to be accumulated in greater proportion in benthic environments. This aligns with the results provided by Woodall et al. (2014) and Courtene-Jones et al. (2020), where the presence of microplastics in deep-sea sediments of Atlantic Ocean, Mediterranean Sea and Indian Ocean were studied. In both studies, fibres were the anthropogenic particles more abundant in benthic ecosystems, and in much higher proportions than fragments. These results are in accordance with the findings of the present study for *S. canicula* and for the most studies using benthic sharks (Table 3). On the other hand, concerning the increased ingestion of fragments by pelagic sharks, the results align with the study of Maes et al., (2017), describing the microplastic contamination in sediments of the Southern North Sea and at the sea surface of Northwest Europe, and in which the fragments were the dominant item in sea surface. These results are also in accordance with the findings for the most studies using pelagic sharks (Table 3). Nevertheless, there is a need for more studies assessing the proportions of fragments and fibres present on the ocean surface because microfibres have been largely ignored by traditional methods used to sample floating microplastics at sea. These methods use 300–500 µm mesh nets that are too coarse to sample most textile fibres (Ryan et al., 2020). Moreover, the potential for sample contamination by environmental fibres further complicates the issue. Consequently, in the majority of surface studies, only fragments and other shapes are sampled, without considering the inclusion of fibres.

In terms of the size of anthropogenic particles accumulated in both sharks, significant differences were found in the mean size of total particles ingested, with *Scyliorhinus canicula* accumulating overall significant larger particles than *Prionace glauca*. Nevertheless, it must be taken into account that *S. canicula* exhibited a higher accumulation of fibres than *P. glauca* and, given that the fibres characterised in both shark species tend to be considerably bigger than fragments, the values associated with fibres may be increasing the mean size of total particles in *S. canicula*. Most of the particles found in the sharks´ stomachs were comprised in the categories mesoplastics and microplastics, with no macroplastics being detected in *S. Canicula*, and only one macroplastic detected in *P. glauca*. (Bessa et al., in preparation). The absence of macroplastics can be explained by the fact that some elasmobranchs may exhibit stomach eversion as it was demonstrated in a free-swimming Caribbean reef shark (*Carcharhinus* *perezi* Poey, 1876) in its natural habitat (Brunnschweiler et al., 2005). Gastric eversion serves to egest small indigestible food particles, sloughed mucosa, and mucus (Sims et al., 2000), which would not be egested by gastric compression or vomiting (Andrews & Young, 1993). Matching outcomes are described in the sharks analysed in the different studies presented in Table 3, being most of the anthropogenic particles accumulated in the benthic and pelagic sharks classified as micro- and mesoplastics.

Lastly, concerning polymer types of anthropogenic particles ingested, the benthic shark *S. canicula* exhibited a high accumulation of natural origin polymers and generally, most of the particles ingested had a higher density than seawater (at a Salinity = 35 ppt and Temperature = 15 °C; $\rho = 1.026\ g \cdot cm^{-3}$), while the pelagic shark *P. glauca* predominantly accumulated particles of synthetic origin, and the vast majority of accumulated particulates tended to be less dense than seawater. Coinciding results are described in marine ecosystems by the study of Gago et al., (2018), which summarize information on microfibres in seawater and sediments from available scientific information (100 original publications were retrieved, dating back to 1960 until 2017), being polypropylene and polyethylene (i.e., polymers less dense than seawater) the polymers more commonly found in sea surface, while rayon and polyamide (i.e. polymers denser than seawater) were more prevalent in sea sediments. Polyester has been documented in both environments (Gago et al., 2018). Similar outcomes are reported in the studies carried out in pelagic sharks (Table 3), which accumulated mostly polymers of synthetic origin (less dense than seawater, with the exception of polyester), while for benthic sharks (Table 3), a greater presence of materials of natural origin was documented, although they occurred at a lower rate than what was found in the present study. Additionally, anthropogenic particles with higher density than seawater were also detected, but again, in smaller quantities than in the current study.

Table 3: Incidence, size, shape, and polymer type of anthropogenic particles found in benthic and pelagic sharks sampled in different geographical regions of the world.

Species	Sampling Period	Geographical Area	Body part	Number of specimens	Sieve pore size, µm	Plastic occurrence (%)	Size range, mm (%)	Plastic shape (%)	Polymer type (%)	Reference
Pelagic sharks										
Lamna nasus	2014	Celtic Sea (NE Atlantic)	Spiral valve	10	100 µm	100	<5 mm (100%)	Fragments (65.9%) Fibres (32.9%) Pellets (0.9%) Films (0.5%)	Weathered synthetic (na) Polymeric material (na)	(Maes et al., 2020)
Prionace glauca	1999-2015	Western Ligurian Sea (MED)	Stomach	139	1000 µm	25.26	>25 mm (20%) 5-25 mm (>50%) <5 mm (25.71%)	Films (72.4%) Fragments (18.1%) Fibres (5.7%) Others (3.8%)	Polyethylene (75.2%) Polypropylene (19.1%) Polyester (1.9%)	(Bernardini et al., 2018)
	2019	**North-East Atlantic Ocean**	**Stomach**	**39**	**63 µm**	**100**	**5-25mm (0.8%)** **<5mm (99.2%)**	**Fibres (61.3%)** **Fragments (36.9%)** **Tangled fibres (1.8%)**	**Alkyd varnish (23.5%)** **Polyester (17.7%)** **Polypropylene (11.8%)** **Others (47 %)**	**(Bessa et al., 2023)**
	2019-2020	Eastern tropical Pacific Ocean	Pylorus	23	2.7 µm	39.1	0.046 - 3.2 mm	Fibres (83.3%) Fragments (16.7%)	Polyester (66.7%) Polypropylene (27.8%)	(Huang et al., 2022)
Benthic sharks										
Etmopterus spinax	2018	Tyrrhenian Sea (MED)	Stomach and intestine	34	100 µm	61.8	[1 mm-5 mm] (7.5%) [330 µm-1 mm] (5%) [100 µm-330 µm] (87.5%)	Fibres (77.5%) Fragments (12.5%) Films (10%)	Polyester (na) Polypropylene (na) Polyethilene (na)	(Valente et al., 2019)
Galeus melastomus	na	Western Mediterranean	Stomach	125	na	16.80	<5mm (100%)	Fibres (86.36%) Fragments (12.12%) Films (1.51%)	Cellophane (33.33%) Polyester (27.27%) Polypropylene (12.12%) Others (27.28 %)	(Alomar et al., 2017)

	2018	Tyrrhenian Sea (MED)	Stomach and intestine	32	100 µm	78.1	[1 mm-5 mm] (4.2%) [330 µm-1 mm] (7%) [100 µm-330 µm] (88.8%)	Fibres (85.3%) Fragments (9.1%) Films (2.8%) Spheres (2.8%)	Polyester (na) Polypropylene (na) Polyethylene (na)	(Valente et al., 2019)
Mustelus asterias	na	North-East Atlantic and Celtic Sea	Stomach and intestine	12	na	75	0.3 - 14.4 mm	Fibres (100%)	Syntethic cellulose (na) Polyester (na) Cellophane (na) Olefin polypropylene (na)	(Parton et al., 2020)
Scyliorhinus canicula	na	North-East Atlantic and Celtic Sea	Stomach and intestine	12	na	66.6	0.3 - 14.4 mm	Fibres (100%)	Syntethic cellulose (na) Polyacrilamida (na)	(Parton et al., 2020)
	2018	Tyrrhenian Sea (MED)	Stomach and intestine	30	100 µm	66.7	[1 mm-5 mm] (2.7%) [330 µm-1 mm] (5.3%) [100 µm-330 µm] (92%)	Fibres (90.7%) Fragments (8%) Films (1.3%)	Polyester (na) Polypropylene (na) Polyethylene (na)	(Valente et al., 2019)
	2018	**North-East Atlantic Ocean**	**Stomach**	**44**	**63 µm**	**100**	**5-25 mm (2.4%) <5 mm (97.6%)**	**Fibres (85.5%) Fragments (9.3%) Filaments (2.9%) Others (2.3%)**	**Cellulose (45.5%) Cotton (15.9%) Rayon (8.7%) Others (29.9%)**	**This study**
Scyliorhinus stellaris	na	North-East Atlantic and Celtic Sea	Stomach and intestine	10	na	70	0.3 - 14.4 mm	Fibres (100%)	Polyester (na) Olefin polypropylene (na)	(Parton et al., 2020)
Squalus acanthias	na	North-East Atlantic and Celtic Sea	Stomach and intestine	12	na	58	0.3 - 14.4 mm	Fibres (100%)	Polyester (na) Polyacrylamida (na)	(Parton et al., 2020)

na = not available; MED = Mediterranean Sea; NE= North-East

2.5. Conclusion

It is still unclear which biological or environmental factors may influence the vulnerability of some animal species for ingesting larger quantities of anthropogenic particles. However, there are studies that prove that long lived organisms and top predators are more vulnerable to accumulate higher quantities of anthropogenic litter due to bioaccumulation and biomagnification phenomena. The present study showed that *Scyliorhinus canicula*, being a carnivorous shark, accumulated more anthropogenic particles than other fish (i.e., benthonic and pelagic) sampled in the Portuguese coast. Additionally, it was seen that this accumulation was not influenced by the sharks' sex, maturation state or length in this study. Lastly, this study also demonstrated differences in the shape, size and polymer type of the anthropogenic particles ingested by the benthic shark analysed in the present study and the pelagic shark from another study performed in the same geographical area. Moreover, results were compared with other sharks inhabiting different ecosystems and it was possible to confirm this same pattern of differences in the accumulated particles between benthic and pelagic sharks around the globe. This type of comparative studies is important to continue monitoring the accumulation of anthropogenic particles in the ocean, which can help in the adoption of different courses of action needed to tackle the problem in each specific region. This study has also contributed with important information to further demonstrate the vulnerability of top predators, in this case sharks, to the ingestion of anthropogenic particles. However, further studies are needed to better understand what the real impacts of this accumulation may be for the sharks and their respective environmental compartments and ecosystems.

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Chapter 3.

Simulating the structural alterations of plastic particles retained in the gastrointestinal tract of shark species

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Simulating the structural alterations of plastic particles retained in the gastrointestinal tract of shark species

Roger Casado[1], Marco F. L. Lemos[1], Filipe R. Ceia[2], Filipa Bessa[2], Sara C. Novais[1]

[1]MARE – Marine and Environmental Sciences Centre & ARNET—Aquatic Research Network, ESTM, Politécnico de Leiria, 2520-641 Peniche, Portugal.
[2]University of Coimbra, MARE – Marine and Environmental Sciences Centre / ARNET – Aquatic Research Network, Department of Life Sciences, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal.

Abstract

Long-lived top predators are known to be very susceptible to accumulate large quantities of plastic debris due to bioaccumulation and biomagnification processes, however, it is still unclear how deterioration of plastic particles occurs within the GIT and the factors that can influence this process (residence time, ageing and abrasive factors). The objective of the present study was to evaluate the effects of several digestive substances (e.g., enzymes and acids) described in shark stomachs on various plastic samples. Additionally, this study also aimed to assess the potential of digestive enzymes for plastic degradation for biotechnological purposes. To achieve these objectives, this research started by exploring the conditions present in the stomach of sharks, including pH values and enzymes involved in the digestion, as well as the chemical structure of plastics. Based on this information, three sequential trials were conducted using 6 different types of plastics samples: polypropylene, polyethylene terephthalate, high-density polyethylene (in the form of fragments); low-density polyethylene (in film form); polyester and cotton (as fibres), the latter as a positive control. These plastic samples were exposed to different conditions of pH and pepsin enzyme concentrations, from 7 to 18 days. Results showed that adding pepsin enzymes induced weight loss in plastic films and filaments. The maximum total weight loss detected was 4.64 % for polyamide filaments and 2.32% for low-density polyethylene films. Moreover, for fibrous samples, adding pepsin enzyme

resulted in structural alterations in cotton fibres. There are several studies evaluating how digestion process of different organisms (including humans) can have effects on the morphology and properties of plastics but there is still a lack of information of the effect of each variable (i.e., pH, enzymes and others). This study pretends to evaluate two digestive parameters (pH and enzymes) described in sharks and test the effects on different plastic samples.

Keywords: Marine pollution, top predators, microplastics, digestion, enzymatic degradation.

3.1. Introduction

The production of plastic has grown exponentially over the past 50 years, as it is a cheap and versatile material used in various economic sectors such as packaging, construction, building, and textiles (Geyer et al., 2017). However, due to its low rate of degradation in natural settings and inadequate management, plastic waste has been accumulating in natural ecosystems since then (Barnes et al., 2009; Kershaw et al., 2011).

The accumulation of plastic debris in the ocean is a result of a significant portion of plastic waste originating from land-based sources that typically ends up in rivers (Meijer et al., 2021) and ultimately in the oceans. This happens due to the flow of rainwater that carries away all types of litter in its path, combined with the disposal of wastewater from neighbouring towns and factories. During their way to the ocean and upon reaching it, plastics can potentially become fragmented into smaller pieces. Depending on the size of the plastic items, they can be classified as macroplastics (>5 mm), microplastics (MPs) (<5 mm), or nanoplastics (<100 nm) (GESAMP, 2015). Abiotic factors such as sunlight, mechanical forces, and chemical interactions will affect the integrity of plastic litter (Zhang et al., 2021). In addition, biotic factors such as animal biting (Cadée, 2002), gnawing (Porter et al., 2019), digestive fragmentation (Dawson et al., 2018; Cau et al., 2020; Krasucka et al., 2022), and microbiological degradation (Zeenat et al., 2021) can also contribute to plastic fragmentation in the marine ecosystems.

The fragmentation of plastic litter can promote the ingestion of plastic particles by marine organisms, by two different ways: direct ingestion, which can occur accidentally through indiscriminate feeding strategies such as filter-feeding, or through active selection due to misidentification of microplastics as food (Nelms et al., 2018). As a result of the ingestion, plastic items have been found to accumulate in the gastrointestinal tracts of many species in different marine ecosystems. Many studies have reported this phenomenon from different trophic levels and habitats (Nelms et al., 2016; Lusher et al., 2013; Wilcox et al., 2015). For example, in pelagic sharks, plastic accumulation has been documented in *Prionace glauca* (Huang et al., 2022; Bernardini et al., 2018) and in *Lamna nasus* (Maes et al., 2020). Moreover, plastic accumulation has also been documented in benthic sharks like *Scyliorhinus canicula* (Valente et al., 2019; Parton et al., 2020), *Mustelus asterias* (Parton et al., 2020), in *Galeus melastomus* (Alomar et al., 2017; Valente et al., 2019) and others. Plastic items are known to remain in the gastrointestinal track (GIT) of marine species for unknown periods of time and during this period of residence, digestive enzymes can influence the integrity of the plastic items (Cau et al., 2020; Dawson et al., 2018; Krasucka et al., 2022). These enzymes are able to fragment plastics over time (Cau et al., 2020) and alter their properties, such as morphological characteristics, functional groups, degree of crystallinity and adsorption capacity (Krasucka et al., 2022).

The main objective of this study was to explore the digestive conditions of long-lived top predators, focusing in sharks, and investigate the capacity of those abrasive agents (i.e. acidic pH and digestive enzymes) to potentially affect plastics' integrity. Sharks can be considered good sentinels of microplastic accumulation, given that this group includes several long-lived top predators very prone to accumulate large quantities of plastic debris due to bioaccumulation and biomagnification processes. Additionally, sharks can be an interesting source of diverse digestive enzymes, given that they are active predators that hunt a wide variety of prey, from small fish and crustaceans to turtles and marine mammals (Papastamatiou et al., 2007). Therefore, it is expected that they will have strong digestive conditions to deteriorate very diverse organic matter, which leads to hypothesise that these enzymes could have impacts in plastic morphology.

In sum, this study was performed with two main purposes: 1) to better understand how sharks' digestive process could impact plastics retained in gastrointestinal track by assessing if the acidic conditions and enzymes involved in the digestion are able to induce morphological changes (fragmentation or deterioration) and loss weight in plastic samples; 2) to explore potential biotechnological applications of sharks' digestive enzymes for plastic degradation, by evaluating if these can induce degradation (morphological changes or loss weight) in different plastic samples, to determine if they could be good candidates for environmental remediation purposes.

3.2 Materials and methods

3.2.1 Plastic samples and assay conditions

The plastic samples were obtained from commercial food and water serving, cleaning products and fishing equipment, based on the most common plastic polymers found washed ashore in natural environments. The plastic fragments were cut into rounds of approximately 1 cm x 1 cm, plastic filaments were cut manually to obtain items of ~ 2 cm (maximum length) and fibres of ~ 0.6 cm (maximum length) were removed manually with steel forceps. Plastic items were categorised as meso- and macroplastics and were classified based on the polymer type. The polymer of each plastic was confirmed by its commercial product label and included: Polypropylene (PP) (dustpan), polyamide (PA) (nylon thread), polyethylene terephthalate (PET) (water bottle), high-density polyethylene (HD-PE) (detergent bottle), low-density polyethylene (LD-PE) (plastic packaging) and polyester fibres (kitchen cloth). Cotton fibres (jumper) were used as positive control. Plastic fragments and filaments were washed with distilled water to remove any scraps of material, and dried.

The assay conditions, including pH values and enzymes, were selected based on Papastamatiou et al. (2007), which describes the stomach parameters in free-swimming blacktip reef sharks, *Carcharhinus melanopterus*. The assays were conducted using glass vials to prevent any enzyme reaction with plasticware, and covered with a hermetic cover, in order to prevent solution evaporation, and to minimize the risk of contamination.

3.2.2 Plastic characterization

The structural alterations of the plastic samples were detected via a trinocular stereozoom microscope ZEISS STEMI 2000-C (Carl Zeiss Microlmaging Gmbh Göttingen, Germany). Afterwards, the items were photographed using AxioCam MRc with Zen 2011 application software (blue edition) and edited using the Adobe Illustrator 2023. Moreover, plastic fragments and filaments were weighted before and after enzymatic treatment using a Sartorius CPA225D Competence Analytical Balance (Göttingen, Germany), in order to calculate loss weight after the enzymatic assays. The fragments and filaments were cleaned and dried at a temperature of 50°C for 30 minutes before being weighed, before and after applying the enzymatic treatment.

3.2.3 Preliminary in vitro digestion assay

Peptidase pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, USA) was used for the digestive trials. The selection of this enzyme was based on the fact that peptidases had been previously documented in shark stomachs (Papastamatiou et al., 2007; Guerard & Le Gal, 1987) and also thanks to its great solubilization properties in aqueous medium.

The assay was performed using several pepsin concentrations [0, 0.25, 1 and 2 mg/mL] and different plastic fragments (PE-HD, PP and PET) and filaments (PA) of diverse polymer types. The range of concentrations used in this initial test was determined based on the average concentration of pepsin documented in the human stomach (Liu et al., 2015; Krasucka et al., 2022). Pepsin was solubilized in k-phosphate buffer (0.1 M) at the optimal pH of the enzyme (pH 4.5). Afterwards, 1 mL of the pepsin solution was introduced in each of the 12 mL glass vessels, each containing one selected plastic sample. The assay was conducted at room temperature (20ºC), under maintained shaking at 70 rpm (Stuart SSL4 Rocker, See-saw, Lab Scale) for a period of 7 days.

3.2.4 Additional in vitro digestion assay

An additional in vitro digestion assay was performed using the previous pepsin concentrations [0, 0.25, 1, 2 mg/mL] for cotton and polyester fibres. The objective was to determine the optimal degradation concentration of the pepsin enzyme. For this purpose, only visual degradation (structural alterations) was monitored, using a trinocular stereozoom microscope ZEISS STEMI 2000-C (Carl Zeiss Microlmaging Gmbh Göttingen, Germany). The trial was conducted at the same pH (4.5) and molarity (0.1 M), however, the temperature of the assay was increased to 37 ºC in order to work at the optimal temperature of the enzyme, and the shaking was augmented to 300 rpm to increase the enzyme-substrate contact surface. The duration of the assay was extended to 14 days, under maintained shaking (300 rpm) at 37 ºC (Stuart SI500 Incubator, Orbital Shaker). A daily visual check was performed in order to assess the progress of the digestive test and a replacement of medium was done, to prevent enzyme inactivation, 7 days after the start of treatment.

In order to contrast the effect of temperature rising, a complementary assay was done at 20ºC, with cotton and polyester fibres using all the pepsin concentrations of the preliminary in vitro digestion assay [0.25, 0.5, 1, 2 mg/mL].

3.2.5 Final in vitro digestion assay

Peptidase enzyme was used at a concentration of 0.25 mg/mL, while temperature and shaking were maintained at 37 ºC and 300 rpm, respectively (Stuart SI500 Incubator, Orbital Shaker). Molarity of the k-phosphate buffer for the pepsin solution was maintained at 0.1 M. Additionally, two different pH conditions were tested: pH 4.5 (optimal TºC of peptidase pepsin from porcine gastric mucosa) and pH 1.7 (stomach pH value) (Krasucka et al., 2022; Papastamatiou et al., 2007). The experiment was performed during 18 days, with a medium renewal at day 9.

For the purpose of this study two fibrous materials were used (i.e. polyester and cotton fibres, the latter used as positive control), one plastic filament (i.e. PA filament) and one film (i.e. PE-LD film). Three replicates were made for each treatment.

3.2.6 Data analysis

The different digestive treatments of the final assay were named as '*Treatment A*', the one containing k-phosphate buffer (0.1 M; pH = 4.5); '*Treatment B*', containing k-phosphate buffer (0.1 M; pH = 1.7); '*Treatment C*', containing k-phosphate buffer (0.1

M; pH = 1.7) with pepsin enzyme [0.25 mg/mL] and '*Treatment D*', containing k-phosphate buffer (0.1 M; pH = 4.5) with pepsin enzyme [0.25 mg/mL].

To test the data for normality and homogeneity of variance, Shapiro-Wilk and Levene's Tests were performed, respectively. One Way ANOVA tests were performed to assess significant differences between the different treatments (A, B, C and D) of each plastic. To evaluate differences between the degradation rates of PA and PE-LD, a Mann-Whitney Test was done. For all statistical tests, the significance level was set to 95% confidence interval ($p < 0.05$).

3.3 Results

3.3.1 Preliminary in vitro digestion assay

No signs of degradation (visual and weight lost) were detected after 7 days of treatment, for polypropylene (PP) and polyethylene terephthalate (PET) (Table 4). However, polyethylene high-density (PE-HD) showed a marginal weight-loss in the treatments with higher enzyme concentrations (1 and 2 mg/mL) and polyamide samples showed the higher degradation, with 0.36% of total weight loss, in the treatment without the enzyme, followed by pepsin concentration 0.25 mg/mL, which induced 0.33% of total weight loss (Table 4). No morphological alterations were observed in any of the analysed samples.

Table 4. Weight loss (expressed both in mg and % of the total weight) for each type of plastic, using solutions with 5 different concentrations of pepsin (0, 0.25, 0.5, 1, 2 mg/mL).

	Peptidase [mg/mL]	**0**	**0.25**	**0.5**	**1**	**2**
Polyamide (PA)	Weight loss (mg)	0.05	0.03	0.03	0.01	0.01
	Total weight loss (%)	0.36	0.33	0.28	0.10	0.05
Polyethylene (PE-HD)	Weight loss (mg)	0	0	0	0.03	0.01
	Total weight loss (%)	0	0	0	0.08	0.02
Polypropylene (PP)	Weight loss (mg)	0	0	0	0	0
	Total weight loss (%)	0	0	0	0	0
Polyethylene terephthalate (PET)	Weight loss (mg)	0	0	0	0	0
	Total weight loss (%)	0	0	0	0	0

3.3.2 Additional in vitro digestion assay

No signs of visual degradation were detected after 14 days of treatment for polyester fibres (Figure 11). However, for cotton fibres, potential visual effects of degradation were observed at 0.25 mg/mL and 0.5 mg/mL of pepsin concentration (Figure 12). Degradation effects were more pronounced at a concentration of 0.25 mg/mL. As a result, this concentration was chosen for the final in vitro digestion assay.

No visual degradation effects were observed in the complementary test conducted at 20ºC for any of the concentrations and fibrous samples.

Figure 11. Trinocular stereozoom microscope images of visual effects of pepsin enzyme degradation on polyester fibres at 0, 0.25, and 0.5 mg/mL. Bottom images show the effects of the enzyme after 14 days of treatment.

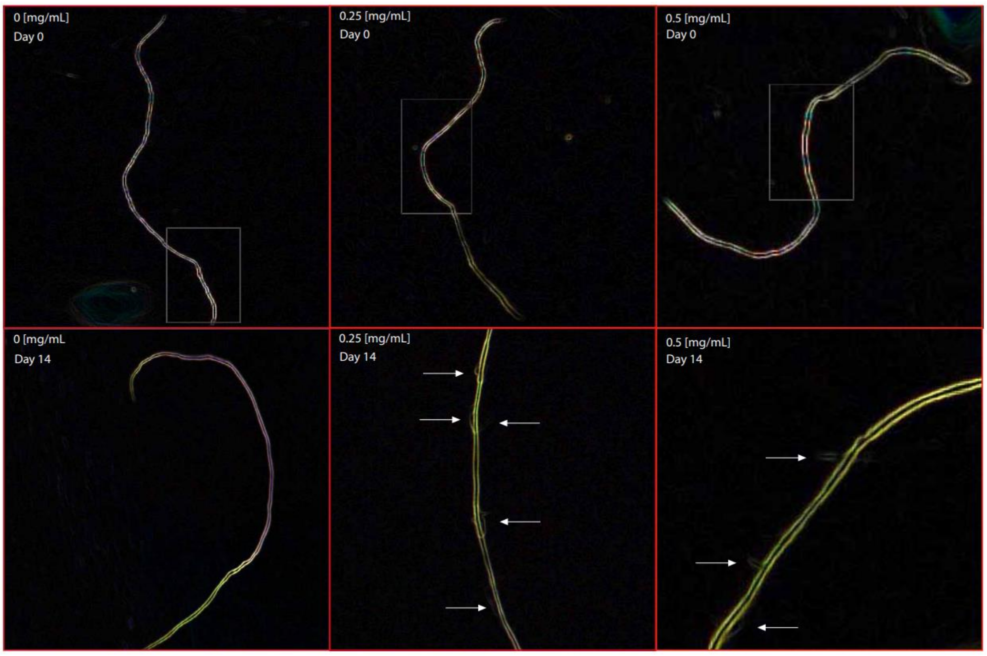

Figure 12. Trinocular stereozoom microscope images of visual effects of pepsin enzyme degradation of cotton fibres at 0, 0.25, and 0.5 mg/mL. Bottom images show the effects of the enzyme after 14 days of treatment. White arrows indicate the degradation points.

3.3.3 Final in vitro digestion assay

Filaments (PA) and films (PE-LD) showed weight loss (Table 4), although no visible degradation was detected for both samples.

Polyamide showed the higher total weight loss (4.64%) with Treatment C. However, it is important to note that this polymer also showed a significant value of total weight loss (3%) in the control treatment. Weight loss was also seen in Treatment B (3.13%), and Treatment D *(*4.44%) (Table 5). No statistical differences were found between treatments (One Way ANOVA, p= 0.052).

Polyethylene showed the higher total weight loss (2.32%) with Treatment D. The other treatments induced very little effects on the weight loss of PE-LD (Table 2). No statistical differences were found between treatments (One Way ANOVA, p= 0.065).

Polyamide samples showed an overall higher rate of weight loss when compared to polyethylene samples (Mann-Whitney Rank Sum Test, U= 0 p= <0.001).

Table 5. Weight loss (expressed both in mg and % of the total weight) for each type of plastic, using 4 different treatments: Treatment A [k-phosphate buffer (0.1 M; pH = 4.5)]; treatment B [k-phosphate buffer (0.1 M; pH = 1.7)]; treatment C [k-phosphate buffer (0.1 M; pH = 1.7) + pepsin (0.25 mg/mL)]; treatment D [k-phosphate buffer (0.1 M; pH = 4.5) + pepsin (0.25 mg/mL)]

	Treatments	**A**	**B**	**C**	**D**
Polyamide (PA)	Weight loss (mg) ± SD	0.46 ± 0.11	0.48 ± 0.22	0.89 ± 0.34	0.95 ± 0.17
	Total weight loss (%)	3.00	3.13	4.64	4.44
Polyethylene (PE-LD)	Weight loss (mg) ± SD	0.02 ± 0.04	0.03 ± 0.02	0.05 ± 0.01	0.11 ± 0.06
	Total weight loss (%)	0.42	0.77	0.99	2.32

For fibrous samples, no visual degradation was detected in polyester fibres (Figure 13). Nevertheless, cotton fibres showed visual degradation in two replicates of Treatment C and in two replicates of Treatment D (Figure 14). The most significant visual degradation was observed in replicate 1 of Treatment D. In this treatment, degradation was possible to detect after 9 days and continued to increase after 18 days of treatment (Figure 14).

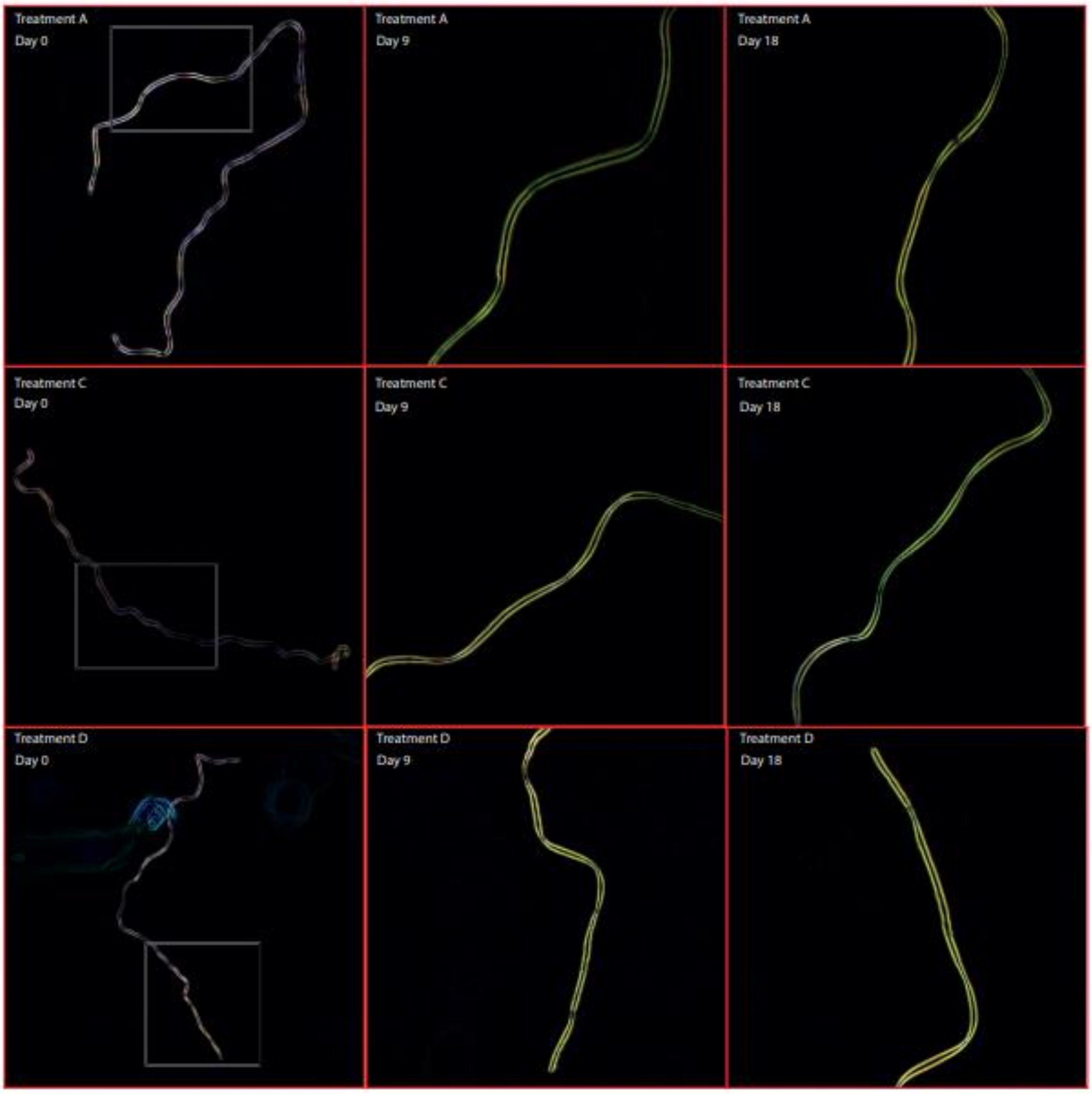

Figure 13. Trinocular stereozoom microscope images of visual deterioration in polyester fibres for treatment A - [k-phosphate buffer (0.1 M; pH = 4.5)], treatment C - [k-phosphate buffer (0.1 M; pH = 1.7) + pepsin (0.25 mg/mL)], and treatment D - [k-phosphate buffer (0.1 M; pH = 4.5) + pepsin (0.25 mg/mL)] for 9 and 18 days.

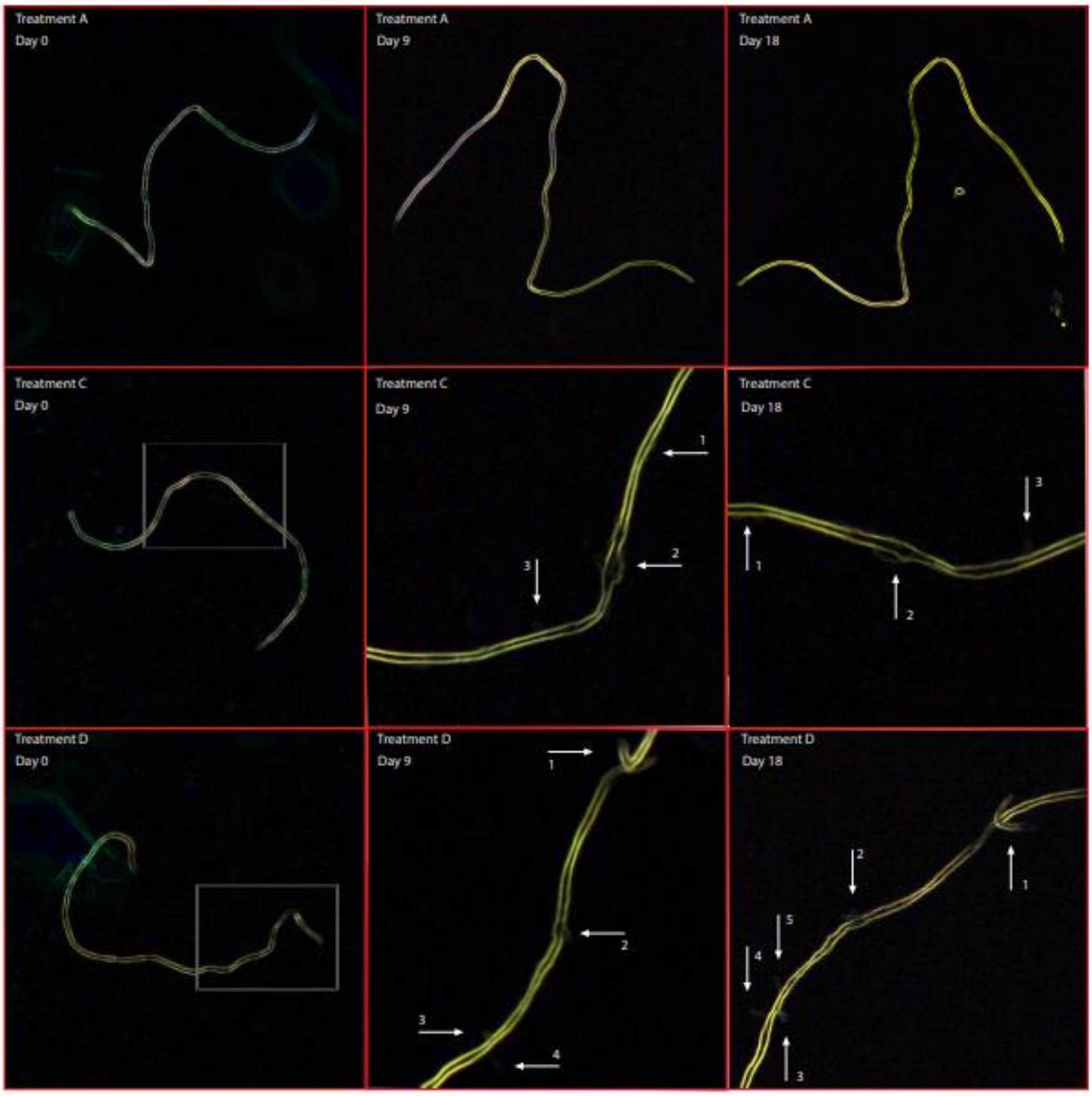

Figure 14. Trinocular stereozoom microscope images of visual deterioration in cotton fibres for Treatment A - [k-phosphate buffer (0.1 M; pH = 4.5)], treatment C - [k-phosphate buffer (0.1 M; pH = 1.7) + pepsin (0.25 mg/mL)], and treatment D - [k-phosphate buffer (0.1 M; pH = 4.5) + pepsin (0.25 mg/mL)] for 9 and 18 days. White arrows indicate the points of degradation, and the numbers represent the same degradation point at day 9 and 18.

3.4 Discussion

The gastrointestinal track of sharks needs to have abrasive conditions to process the food bolus, including: acidic solutions that can range from pH values of 1.5 to 5.5 (Papastamatiou et al., 2007) and digestive enzymes, such as chitinases and peptidases (Guerard & Le Gal, 1987; Papastamatiou et al., 2007). Based on this knowledge, this study intended to verify if these digestive enzymes and acidic conditions would function as non-selective agents, indifferently affecting both the food bolus and the plastic waste ingested by organisms. To achieve this objective, the experimental conditions (temperature, shaking and duration of the trial) were optimised in order to be able to work with the enzyme under optimal conditions. This is the first time that a study intends to simulate in-vitro digestive conditions of marine top-predators (sharks) to better understand how digestive parameters (acidity and enzymes) could affect the structure of different plastic samples.

3.4.1 Preliminary in vitro digestion assays

The preliminary in vitro digestion assay with fragments and filaments aimed to evaluate the effect of different enzyme concentrations for a wide range of plastic polymers, including PE-HD, PP, PET, and PA. These plastics had varying characteristics, such as differences in density, molecular structure, and melting point and some of them have shown to suffer structural alterations when exposed *in vitro* to human digestive conditions (Krasucka et al., 2022). The initial assays were carried out at room temperature (20ºC) and pepsin enzyme was selected for the trials because of: (1) its good solubility in aqueous solutions; (2) the fact that it is documented in shark stomachs (Papastamatiou et al., 2007; Guerard and Le Gal, 1987); (3) the fact that it is recognized to induce hydrolysis of the peptide bonds found in proteins (CO-NH) (Polzonetti et al., 2010), which possess similar chemical properties to those that connect polyamide monomers; (4) its degradation effects were shown on PE-LD and polystyrene (PS) in an in-vitro assay where it was combined with other digestive enzymes (Krasucka et al., 2022). No remarkable results were obtained in this first trial, as no weight loss and structural changes were detected for PP and PET samples and only a negligible weight loss was detected in PE-HD and PA samples. Based on these results, it was hypothesised that the conditions used could be suboptimal for the correct functioning of the enzyme and the temperature for next assays was modified from 20ºC to 37ºC, which was the optimal pepsin temperature (commercial enzyme from porcine pancreas).

The additional in vitro digestion assay with fibres pretended, on the one hand, to test whether an increase in temperature (from 20ºC to 37ºC) would improve the activity of the enzyme and also to identify the optimal concentration at which the pepsin enzyme operates. For this purpose, experiments were carried out to monitor the structural alterations of fibrous samples (cotton and polyester fibres) when exposed to different concentrations of the enzyme. Visual tests were conducted to detect the morphological alterations; however, weight loss could not be controlled in the fibrous samples due to their small size. The effects of temperature rising were contrasted performing a test at room temperature (20ºC) with all enzyme concentrations (0.25, 0.5, 1, 2 mg/mL). No structural alterations were detected in the test performed at 20 ºC for any concentration and for both fibrous polymers. However, experiments conducted at 37°C resulted in significant structural alterations in the treatments with enzyme concentrations of 0.25 and 0.5 mg/mL. Surprisingly, the lowest concentration (0.25 mg/mL) produced better results. This phenomena has been previously observed in the works by Zhuikov et al. (2017) and Yamashita et al. (2003), where different enzymes showed lower degradation rates at higher enzyme concentration. Authors hypothesised that hight enzyme concentrations could lead to lower degradations because the enzyme molecules compete with each other for access to the catalytic centre, hindering their overall effectiveness (Zhuikov et al., 2017; Yamashita et al., 2003).

3.4.2 Final in vitro digestion assay

For this final trial, 4 different plastic polymers were selected: polyester (fibres), cotton (fibres), PA (filaments) and PE (films). The last two polymers (PA and PE) were chosen because they showed to be susceptible to suffer structural alterations by pepsin enzyme in the previous tests. PE low density was used instead of PE hight density (previously used in preliminary tests), because previous studies have shown that exposure to light and oxygen can induce the PE low density to a loss of strength and a loss of tear resistance which can result in an increase of susceptibility to be degraded by pepsin enzyme (Sam et al., 2014).

The highest rates of weight loss for PA samples were observed in the presence of the pepsin enzyme (4.64% and 4.44%), both in treatment C (with enzyme; pH = 1.7) and treatment D (with enzyme; pH = 4.5), respectively. This was an expected result since pepsin target links are peptide bonds of proteins (Polzonetti et al., 2010), which have a chemical structure almost identical to the amide bonds of the polyamide polymer. No significant differences were detected by the effect of pH (from 4.5 to 1.7). Nevertheless, it is important to highlight that significant weight loss was observed in all the treatments of PA samples, including the negative control (without enzyme; pH = 4.5), which suggests that factors, such as temperature (37 ºC), lightly acidic pH (4.5) and shaking (300 rpm), may also affect weight loss in this type of polymer. On the other hand, for PE-LD samples, the higher loss weight was observed in the presence of the enzyme (2.32%) in treatment D (with enzyme; pH = 4.5). In contrast to the PA results, a decrease in pH (from 4.5 to 1.7) seems to negatively affect the capacity of the enzyme to degrade the polymer. Moreover, the loss weight for treatments without enzyme (A and B), was lower than for polyamide, which may suggest that PE-LD is more resistant than PA to factors such as temperature (37 ºC), acidic pH (4.5 and 1.7) and shaking (300 rpm). In sum, filaments and films showed higher weight losses in the PA and PE-LD samples with the presence of pepsin enzyme, although no visual structural alterations were detected, and no statistical differences were seen between the different treatments in each plastic type (although the p-values were very close to 0.05). For future experiments, an increase in the number of replicates would be advisable in order to increase the statistical power of the study for weight loss analysis.

In relation to the cotton and polyester fibres, degradation was only visible in cotton, with polyester not exhibiting any visible signs of morphological alterations. However, this does not necessarily imply that there was no weight loss or any other alteration on its properties not addressed in the present study. The absence of visible changes simply indicates that no apparent structural alterations were detected that could suggest potential degradation. For cotton fibres, structural alterations were detected in the treatments with the presence of pepsin enzyme (treatments C and D). In relation to the pH effect, the results obtained do not allow to observe clear visual differences between both tested pH (1.7, 4.5) combined with the enzymes (i.e treatments C and D). As additional information, it is important to note that cotton fibres were employed as a positive control and thus used to confirm the enzyme's activity under the test conditions. However, although used as positive control, it is important to consider these fibres as potential harmful microfibres. Even if cotton is considered a biodegradable material (240 days for 75% biodegradation), the literature shows that it can contain toxins (PAHs, PCBs and others) with potentially harmful effects on marine organisms (Détrée et al., 2023). The high degree of accumulation of natural origin fibres (e.g. cotton) in marine ecosystems is thus an issue of high concern (Détrée et al., 2023). Studies demonstrate that the presence of natural fibres in ocean basins (91.8%) exceeds largely the synthetic ones (8.2%), and within the group of natural fibres, the most commonly found were the cellulosic (79.5%) (e.g. cotton), followed by animal fibres (12.3%) (e.g. wool) (Suaria et al., 2020).

Although this is a preliminary study, this research has potential applications in both the biotechnology field, where it can be used to explore enzymes capable of degrading plastic, and ecology, for assessing the effects of anthropogenic particles accumulating in the gastrointestinal tracts of marine organisms. For future experiments, it would be valuable to carry out analyses of the physico-chemical alterations of the plastic polymers. For instance, a Fourier-transform infrared spectroscopy (FTIR) analysis would be useful to compare the spectra of the plastics before and after treatment, in order to detect specific variations in the absorbance peaks (Sait et al., 2021; Krasucka et al., 2022). Improving imaging using electronic microscopy instead of a trinocular stereozoom microscope, such as scanning electron microscopy (SEM), would also highly improve the assessment of degradation results (Sait et al., 2021; Krasucka et al., 2022). Moreover, it would be also important to evaluate the effects of other factors such as peristaltic movements, residence times and GIT temperature, apart from exploring other ‘natural’ and synthetic particles. As a future proposal, it would also be interesting to analyse and characterize shark stomach content, as it will contain all the enzymes involved in digestion as well as specific values of the parameters necessary for the optimal functioning of the enzymes, adapting what was described for the human digestive model (Krasucka et al., 2022) to the shark GIT conditions.

3.5 Conclusion

There is limited knowledge regarding the effects that digestive conditions can have on ingested anthropogenic particles. However, there are several studies made for different organisms (including humans) that prove that digestion conditions can produce alterations on the morphology and properties of plastics. The present study demonstrates that the pepsin enzyme documented in the shark's gastrointestinal tract can cause morphological alterations in cotton fibres and lead to weight loss in polyamide and low-density polyethylene samples. This study is a first approach to evaluate how anthropogenic particles (e.g. fibres, films and others) of different polymers types and commonly accumulated in marine ecosystems behave when they are ingested by marine organisms and exposed to digestive agents, which can drive future studies enabling a better understanding of the impacts that these digestive processes could have on sharks' health, as well as future biotechnological investigations for plastic degradation.

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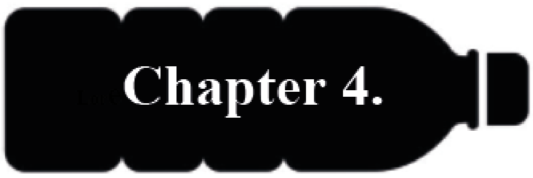

Concluding remarks and future perspectives

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Concluding remarks and future perspectives

Plastic waste has increased markedly in the last few decades due to the exponential increase in plastic production, the lack of efficient waste management, and other factors. Consequently, every year, millions of tons of plastic end up in the ocean. Plastic debris can exhibit distinct behaviours in marine environments, sinking and accumulating in marine sediments or remaining in the water column potentially affecting organisms in both benthic and pelagic zones.

The present study reports the ingestion of microplastics and other anthropogenic particles in the benthic shark *Scyliorhinus canicula* and compares it to the data generated for the pelagic shark *Prionace glauca* from the same geographical area in the Northeast Atlantic and following the same methodology. Results revealed that *S. canicula* individuals accumulated a lower number of particles, with differing shapes, sizes, and polymer types when compared to *P. glauca*. Specifically, *S. canicula* accumulated a higher proportion of fibres and most of the items were made of polymers of negative buoyancy whereas, the pelagic shark *P. glauca* predominantly accumulated a higher proportion of fragments, smaller-sized particles, and mainly items made of positive buoyancy polymers. It is important to highlight that the findings of the present study align with studies available in the literature from other regions, conducted on sharks in various habitats (i.e., benthic and pelagic), as well as with research done to monitor the accumulation of anthropogenic particles in seawater and the seabed. Thus, this study underscores the variation in marine litter distribution between benthic and pelagic zones, with a higher proportion of fragments being found on the surface, primarily composed of polymers with lower density than seawater, like polyethylene and polypropylene, while benthic ecosystems tend to accumulate a higher proportion of fibres, which are usually made of polymers denser than seawater, such as cellulose, rayon, and nylon. Consequently, sharks tend to ingest the predominant anthropogenic particles available in their respective habitats, suggesting an absence of mechanisms to avoid them.

An assay during this study also demonstrated the potential of the pepsin enzyme to induce weight loss when in contact with polyamide and low-density polyethylene items and to cause structural alterations in cotton fibres. This enzyme was documented in the gastrointestinal tract of sharks and in other organisms, including humans. Therefore, this study contributed with some preliminary evidence that the plastics and other anthropogenic particles (i.e., cotton) ingested by marine organisms can potentially undergo changes (i.e., structural alterations and changes in properties) when exposed to abrasive agents in their gastrointestinal tracts. Within this preliminary test, it can therefore be proposed that the pepsin enzyme can be a potential candidate as catalyst for bioremediation processes in plastic degradation.

Overall, these results contributed to demonstrate the ubiquity of anthropogenic particles in different oceanic ecosystems and their potential to accumulate in the organisms such as sharks, particularly those higher up in the food chain and with longer lifespans. Additionally, it showed that anthropogenic particles (even made from natural sources such as celluloses and cotton), are not inert items. For synthetic particles, it was demonstrated that both plastics with a higher tendency to be found in marine sediments (e.g., polyamide filaments) and those that are more common in the water column (e.g., low-density polyethylene films) can undergo morphological changes during their passage through the gastrointestinal tract of marine organisms.

The reasons why sharks are more prone to accumulate larger quantities of APs than other organisms are not precisely known, and it is also unclear why some sharks' species accumulate more plastic than others. As future research perspectives, comprehensive and continuous monitoring programs should be established to track plastic accumulation in shark populations across different habitats and regions (i.e., nursery areas and the geographic distribution of juveniles and adults) and determine the extent to which their accumulation in top predators results from biomagnification (i.e., where plastics pass through prey), or from bioaccumulation (i.e., where plastic is ingested from the organism's environment).

On the other hand, there is a considerable knowledge about the physical and mechanical effects that plastics can have on marine organisms. However, there is still a lack of comprehensive studies on the effects that the additives added to plastics (phthalates, flame retardants, pigments, and others) can have on the organisms once ingested, particularly the effects at a metabolic level. Therefore, it would be also important to explore the pathways of incorporation of these pollutants in top predators such as sharks, trying to determine if these pollutants are present in seawater and are incorporated by biomagnification and bioaccumulation processes or if they are released from plastics during their passage through gastrointestinal track of marine organisms. Additionally, research efforts should focus on developing early-warning techniques, such as biochemical or molecular biomarkers, to assess the long-term effects of plastic ingestion on sharks.

Moreover, it would be interesting to further optimise the plastic degradation processes based on enzyme peptidase, as well as to explore the effects of other digestive enzymes, in order to further evaluate their biotechnological potential for plastic bioremediation processes.

Ultimately, safeguarding the future of sharks requires a holistic and coordinated effort that combines scientific research, conservation measures, but also public engagement.

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Chapter 5.

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APPENDICES

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EFB2022 Virtual conference (Barcelona, Spain)

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Microplastics biodegradation in aquaculture water

Roger B. Casado, Alexandra Cruz
MARE – ESTM, Polytechnic of Leiria, 2520-614 Peniche, Portugal (alexandra.cruz@ipleiria.pt)

Introduction

Plastic production has increased exponentially worldwide in the last decades, mostly driven by plastic packaging, building and construction and textiles. Consequently the amount of plastic waste has increased markedly. Approximately 6.3 billion tonnes of plastic waste was generated between 1950 and 2015, from which around 12% was incinerated, only 9% has been recycled and 79% was unprocessed. The unprocessed plastic is commonly stored in landfills, or worse it is released to the natural environment (Rhodes, 2018).

Objectives

- Isolate and characterise microplastic contamination from an aquaculture tank.
- Try to breakdown the microplastics using two immobilized lipases, in order to increase their bioavailability for further bacterial degradation in a RAS aquaculture system.
- Use commercial plastics as model systems.

Methodology

Detection of microplastic contamination in aquaculture tank

Collect 12 L of aquaculture water tank.
⇩
Make a prefiltration with a metallic filter of 50 µm.
⇩
Remove the microplastics attached in the metallic filter with the help of water and decant them to a crystal glass.
⇩
Make a last filtration with a celulose filter (use vacuum bomb)
⇩
Use the magnifying glass to identify microplastics.

Enzymatic plastic degradation

PET (Plastic type 1) x6
PE- LD (Plastic type 4) x6
PE- HD (Plastic type 2) x6
Fibers from aquaculture tank (Unknow plastic type) x6
⇩
Macroplastics (PET; PE-LD and PE-HD) lose weight was taken into account and for microfibers the alteration of size. Both types of plastic were characterised (colour and shape) before the enzymatic assay.
⇩

Enzymatic assay
Lipozyme immobilized from *Rhizomucor miehei*
One plate for plastic type (Triplicates of different shape)
⇩
Assay conditions
- 10 mL phosphate buffer (Plate)
- 0,02 g of immobilized enzyme (Plate)
- Incubate at 30°C, with movement, for 7 days.

Enzymatic assay
Lipozyme immobilized from *Thermomyces lanuginosus*
One plate for plastic type (Triplicates of different shape)
⇩
Assay conditions
- 10 mL phosphate buffer (Plate)
- 0,02 g of immobilized enzyme (Plate)
- Incubate at 30°C, with movement, for 7 days.

Results

Enzymatic degradation tests (Plastic fragments)

Lipozyme immobilized from *Thermomyces lanuginosus*+ Phosphate buffer (PH= 7) -> Weight loss (grams)

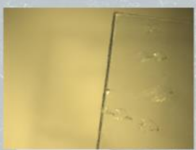
SHAPE	COLOUR	% degradation
Square	blue	0,61
Triangle	blue	1,18
Rectangle	blue	0,00
Square	Transparent	2,07
Triangle	Transparent	1,79
Rectangle	Transparent	10,51
Square	Transparent	2,41
Triangle	Transparent	1,49
Rectangle	Transparent	0,98

Lipozyme immobilized from *Rhizomucor miehei* + Phosphate buffer (PH= 7) -> Weight loss (grams)

SHAPE	COLOUR	% degradation
Square	blue	1,30
Triangle	blue	0,23
Rectangle	blue	0,59
Square	Transparent	2,08
Triangle	Transparent	1,77
Rectangle	Transparent	2,61
Square	Transparent	0,81
Triangle	Transparent	0,29
Rectangle	Transparent	0,00

Control (-) test Phosphate buffer (PH= 7) -> Weight loss (grams)

SHAPE	COLOUR	% degradation
Triangle	Blue	-0,2
Square	transparent	0
Square	transparent	0,1



PET pretreatment of *Rhizomucor miehei* lipase

PET post-treatment of *Rhizomucor miehei* lipase

Enzymatic degradation tests (Fibers)

Lipozyme immobilized from *Rhizomucor miehei*+ Phosphate buffer (PH= 7) -> Size loss (mm)

SAMPLE ID	SHAPE	COLOUR	AMPLIATION	PreT_25/03 LENGTH (mm)	PosT_31/03 LENGTH (mm)
MM_Black	fiber	black	2	7,098	4,319 + 1,917
MM_Blue	fiber	blue	2	3,148	-
MM_Red	fiber	red	2,5	1,831	1,833

Lipozyme immobilized from *Thermomyces lanuginosus* + Phosphate buffer (PH= 7) -> Size loss (mm)

SAMPLE ID	SHAPE	COLOUR	AMPLIATION	PreT_25/03 LENGTH (mm)	PosT_31/03 LENGTH (mm)
TL_Black	fiber	black	2,5	4,552	4,447
TL_Blue	fiber	blue	2,5	1,781	1,781
TL_Red	fiber	red	2	5,79	2,744 + 0,548

Black fiber post-treatment of *Rhizomucor miehei* lipase

Black fiber pretreatment of *Rhizomucor miehei* lipase

Black fiber post-treatment of *Rhizomucor miehei* lipase

Conclusions

- The results obtained are very promising, with degradation rates quite high for some types of plastics.
- Positive results were also obtained in for the enzymatic degradation of fibers.
- Future trials are needed to find the optimal conditions of pH, enzyme concentration and temperature to optimise plastic degradation with lipase enzymes.

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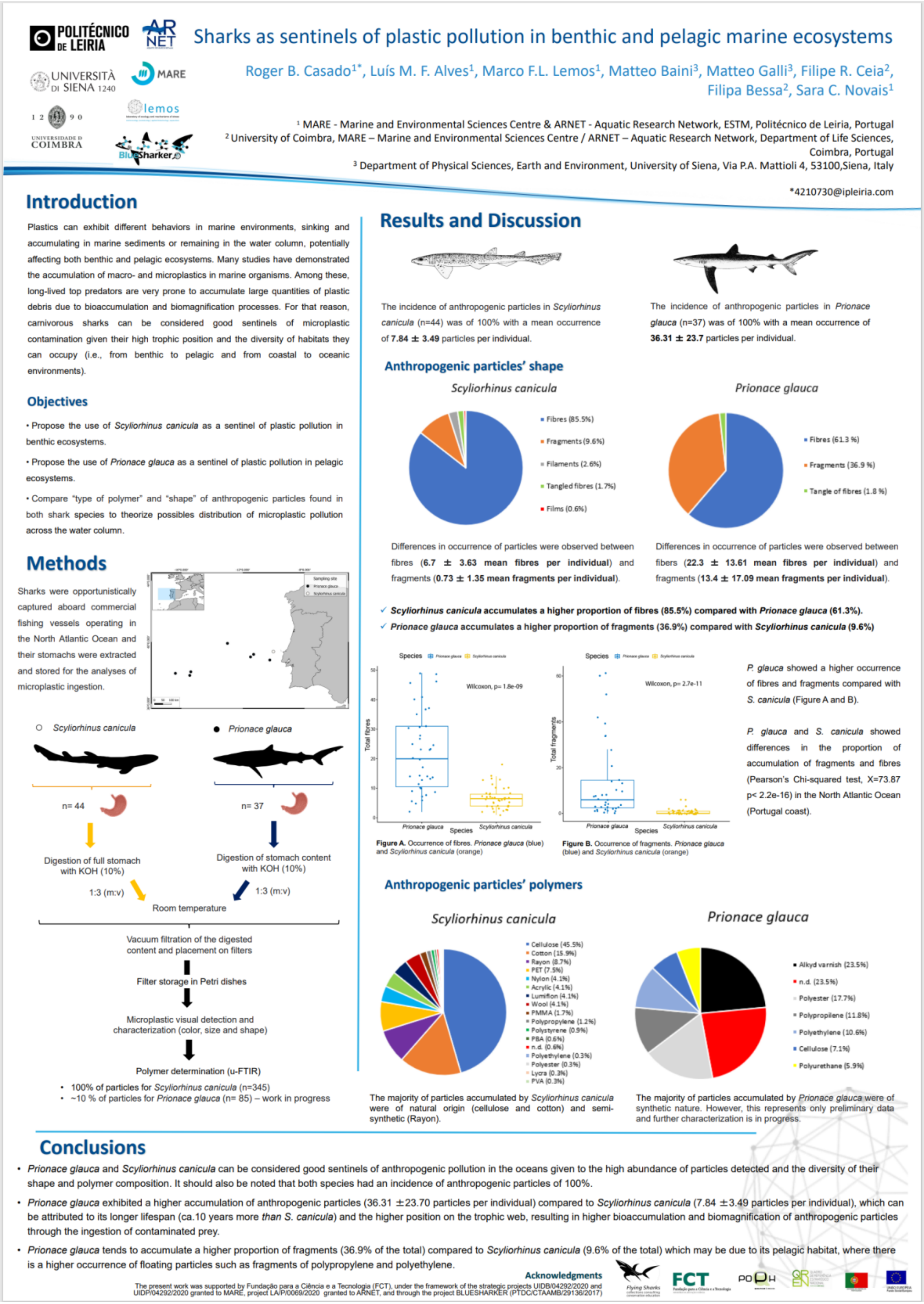
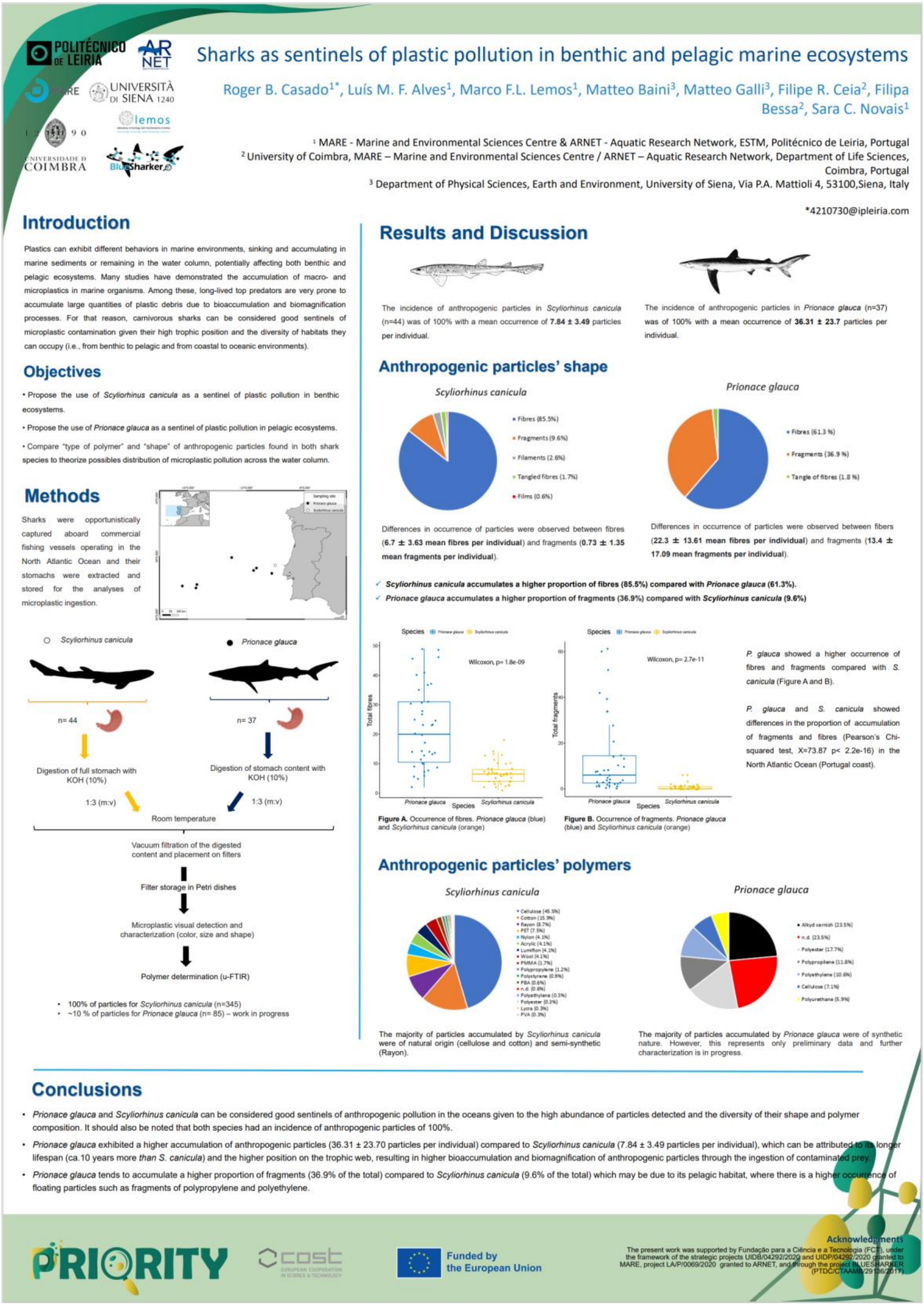
SETAC Europe 33rd Annual Meeting (Dublin, Ireland)



PRIORITY Training School (Jena, Germany)



Sharks as sentinels of plastic pollution in benthic and pelagic marine ecosystems

Roger B. Casado[1*], Luís M. F. Alves[1], Marco F.L. Lemos[1], Matteo Baini[3], Matteo Galli[3], Filipe R. Ceia[2], Filipa Bessa[2], Sara C. Novais[1]

[1] MARE - Marine and Environmental Sciences Centre & ARNET - Aquatic Research Network, ESTM, Politécnico de Leiria, Portugal
[2] University of Coimbra, MARE – Marine and Environmental Sciences Centre / ARNET – Aquatic Research Network, Department of Life Sciences, Coimbra, Portugal
[3] Department of Physical Sciences, Earth and Environment, University of Siena, Via P.A. Mattioli 4, 53100,Siena, Italy

*4210730@ipleiria.com

Introduction

Plastics can exhibit different behaviors in marine environments, sinking and accumulating in marine sediments or remaining in the water column, potentially affecting both benthic and pelagic ecosystems. Many studies have demonstrated the accumulation of macro- and microplastics in marine organisms. Among these, long-lived top predators are very prone to accumulate large quantities of plastic debris due to bioaccumulation and biomagnification processes. For that reason, carnivorous sharks can be considered good sentinels of microplastic contamination given their high trophic position and the diversity of habitats they can occupy (i.e., from benthic to pelagic and from coastal to oceanic environments).

Objectives

- Propose the use of *Scyliorhinus canicula* as a sentinel of plastic pollution in benthic ecosystems.
- Propose the use of *Prionace glauca* as a sentinel of plastic pollution in pelagic ecosystems.
- Compare "type of polymer" and "shape" of anthropogenic particles found in both shark species to theorize possibles distribution of microplastic pollution across the water column.

Methods

Sharks were opportunistically captured aboard commercial fishing vessels operating in the North Atlantic Ocean and their stomachs were extracted and stored for the analyses of microplastic ingestion.

○ *Scyliorhinus canicula* ● *Prionace glauca*

n= 44 n= 37

Digestion of full stomach with KOH (10%) — 1:3 (m:v)
Digestion of stomach content with KOH (10%) — 1:3 (m:v)
Room temperature
Vacuum filtration of the digested content and placement on filters
Filter storage in Petri dishes
Microplastic visual detection and characterization (color, size and shape)
Polymer determination (u-FTIR)

- 100% of particles for *Scyliorhinus canicula* (n=345)
- ~10 % of particles for *Prionace glauca* (n= 85) – work in progress

Results and Discussion

The incidence of anthropogenic particles in *Scyliorhinus canicula* (n=44) was of 100% with a mean occurrence of **7.84 ± 3.49** particles per individual.

The incidence of anthropogenic particles in *Prionace glauca* (n=37) was of 100% with a mean occurrence of **36.31 ± 23.7** particles per individual.

Anthropogenic particles' shape

Scyliorhinus canicula: Fibres (85.5%), Fragments (9.6%), Filaments (2.6%), Tangled fibres (1.7%), Films (0.6%)

Prionace glauca: Fibres (61.3 %), Fragments (36.9 %), Tangle of fibres (1.8 %)

Differences in occurrence of particles were observed between fibres (**6.7 ± 3.63 mean fibres per individual**) and fragments (**0.73 ± 1.35 mean fragments per individual**).

Differences in occurrence of particles were observed between fibers (**22.3 ± 13.61 mean fibres per individual**) and fragments (**13.4 ± 17.09 mean fragments per individual**).

✓ ***Scyliorhinus canicula* accumulates a higher proportion of fibres (85.5%) compared with *Prionace glauca* (61.3%).**
✓ ***Prionace glauca* accumulates a higher proportion of fragments (36.9%) compared with *Scyliorhinus canicula* (9.6%)**

Wilcoxon, p= 1.8e-09

Figure A. Occurrence of fibres. *Prionace glauca* (blue) and *Scyliorhinus canicula* (orange)

Wilcoxon, p= 2.7e-11

Figure B. Occurrence of fragments. *Prionace glauca* (blue) and *Scyliorhinus canicula* (orange)

P. glauca showed a higher occurrence of fibres and fragments compared with *S. canicula* (Figure A and B).

P. glauca and *S. canicula* showed differences in the proportion of accumulation of fragments and fibres (Pearson's Chi-squared test, X=73.87 p< 2.2e-16) in the North Atlantic Ocean (Portugal coast).

Anthropogenic particles' polymers

Scyliorhinus canicula: Cellulose (45.5%), Cotton (15.9%), Rayon (8.7%), PET (7.5%), Nylon (4.1%), Acrylic (4.1%), Lumiflon (4.1%), Wool (4.1%), PMMA (1.7%), Polypropylene (1.2%), Polystyrene (0.9%), PBA (0.6%), n.d. (0.6%), Polyethylene (0.3%), Polyester (0.3%), Lycra (0.3%), PVA (0.3%)

Prionace glauca: Alkyd varnish (23.5%), n.d. (23.5%), Polyester (17.7%), Polypropilene (11.8%), Polyethylene (10.6%), Cellulose (7.1%), Polyurethane (5.9%)

The majority of particles accumulated by *Scyliorhinus canicula* were of natural origin (cellulose and cotton) and semi-synthetic (Rayon).

The majority of particles accumulated by *Prionace glauca* were of synthetic nature. However, this represents only preliminary data and further characterization is in progress.

Conclusions

- *Prionace glauca* and *Scyliorhinus canicula* can be considered good sentinels of anthropogenic pollution in the oceans given to the high abundance of particles detected and the diversity of their shape and polymer composition. It should also be noted that both species had an incidence of anthropogenic particles of 100%.
- *Prionace glauca* exhibited a higher accumulation of anthropogenic particles (36.31 ± 23.70 particles per individual) compared to *Scyliorhinus canicula* (7.84 ± 3.49 particles per individual), which can be attributed to its longer lifespan (ca.10 years more *than S. canicula*) and the higher position on the trophic web, resulting in higher bioaccumulation and biomagnification of anthropogenic particles through the ingestion of contaminated prey.
- *Prionace glauca* tends to accumulate a higher proportion of fragments (36.9% of the total) compared to *Scyliorhinus canicula* (9.6% of the total) which may be due to its pelagic habitat, where there is a higher occurrence of floating particles such as fragments of polypropylene and polyethylene.

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