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Microplastic contamination and its effects in the European Hake (*Merluccius merluccius*) from the North East Atlantic Ocean and aimed at human consumption

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Microplastic contamination and its effects in the European Hake (*Merluccius merluccius*) from the Northeast Atlantic Ocean and aimed at human consumption

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DECLARAÇÃO DE HONRA

Eu, Alexandre Augusto Moutinho Raimundo Alves Aleluia, inscrito no Mestrado em Mestrado em Ciências do Mar - Recursos Marinhos do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto declaro que o conteúdo da presente dissertação é resultado do meu próprio trabalho de investigação e contém contributos que não foram utilizados previamente noutros trabalhos apresentados a esta ou outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico

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Abstract

The widespread contamination of marine ecosystems by microplastics and other debris that constitute marine litter may also pose risks to biodiversity and public health. The objectives of this study were: (ii) to assess the contamination of wild *Merluccius merluccius* specimens from the Northeast Atlantic Ocean by small microplastic particles (< 5 mm) (MPs); (ii) to investigate potential biological effects that may have resulted from MPs contamination; and (iii) to estimate the human dietary exposure through the dorsal muscle of fish from the studied population.

For ethical reasons, 50 fish from commercial fishery and aimed at human consumption were investigated. From each fish, samples were collected to analyse the presence of s, namely the gastrointestinal tract (GT), and samples of the brain, gills, liver and dorsal muscle, hereafter referred as muscle. Tissue samples were also collected for determination of the following biomarkers: acetylcholinesterase (AChE) enzyme activity as a biomarker of neurotoxicity (brain); cholinesterase (ChE) enzyme activity as a biomarker of neuromuscular effects (muscle); lactate dehydrogenase (LDH) enzyme activity as a biomarker of alterations in the energy metabolism (muscle); catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) enzyme activity as biomarkers of oxidative stress (gills and liver); lipid peroxidation (LPO) levels as indicative of oxidative damage in lipids (liver, gills and muscle). Human exposure was estimated based on the concentration of MPs fish muscle and the weekly dose of fish consumption recommended by the European Food Safety Authority.

A total of 221 MPs were extracted from all samples, with sizes ranging from 11 to 4784 µm and a mean (± standard deviation - SD) of 787 ± 993 µm. MPs had the following colours: brown (30%), blue/bluish (25%), black/dark (24%), transparent (8%), grey (6%), red (5%), orange (1%) and green (1%). A considerable proportion (51%) of the MPs had fibre-like shape, but fragments (38%), more regularly shaped particles (6%), and film-like shaped MPs (5%) were also found. From the 50 fish, 96% had at least one MPs in the analysed samples, 50% in GT, 46% in gill, 30% in brain, 50% in liver and 60% in muscle samples. The mean (± SD) number of MPs per fresh weight (gram of tissue analysed) in the different types of samples was: 0.23 ± 0.34 MPs/g in GT samples; 0.24 ± 0.40 MPs/g in gill samples; 2.29 ± 4.22 MPs/g in brain samples; 0.34 ± 0.57 MPs/g in liver samples; and 0.11 ± 0.11 MPs/g in muscle samples. There were significant differences in several biomarkers (AChE, ChE, LDH, CAT, GR, GPx and LPO) between

fish with samples positive and negative for MPs. Relative to fish with samples not having MPs, fish with MPs had lower AChE activity in the brain and ChE activity in the muscle; induction of LDH activity in the muscle; increased activity of CAT, GPx and GR in gills; enhanced GR in the liver; and elevated levels of LPO in the muscle. No other significant differences were found. The estimated human exposure to MPs through the consumption of fish (muscle) from the studied population was 1747 MPs per year.

These results demonstrate the ingestion of different types of MPs by *M. merluccius* specimens from the Northeast Atlantic Ocean, the retention of MPs in gills, and the presence of MPs in the brain, liver and muscle of the fish. Biomarkers indicate neurotoxicity, neuromuscular effects, increased oxidative stress and lipid oxidative damage; and energetic changes possibly associated with MPs contamination. The presence of MPs in the dorsal muscle indicates a possible human exposure through consumption of specimens from the studied population.

Overall, the findings of the present study demonstrated the contamination of wild white hakes (*M. merluccius*) from the Northeast Atlantic Ocean with MPs, suggest relationship with adverse effects in fish, and highlights the risks of human MPs intake through regular of seafood consumption prepared with fish from this species.

Key-words: wild fish, *Merluccius merluccius*, NE Atlantic Ocean, microplastics biomarkers, oxidative stress, human ingestion.

Resumo

A contaminação generalizada dos ecossistemas marinhos por microplásticos e outros detritos que constituem o lixo marinho é um perigo para a biodiversidade marinha, podendo ainda apresentar riscos para a saúde pública. Os objetivos deste estudo foram: (ii) avaliar a contaminação de exemplares selvagens de *Merluccius merluccius* provenientes do Nordeste do Oceano Atlântico por partículas microplásticas de pequenas dimensões (< 5 mm) (MPs); (ii) investigar potencial efeitos biológicos que possa ter resultado da contaminação por MPs; e (iii) estimar a exposição humana através do consumo alimentar do músculo dorsal de peixes da população estudada.

Por razões éticas, foram investigados 50 peixes, provenientes da pesca comercial e destinados a consumo alimentar humano. De cada exemplar, foram recolhidas amostras para análise da presença de MPs, nomeadamente o trato gastrointestinal (GT) e amostras do cérebro, brânquias, fígado e músculo dorsal, doravante referido com músculo. Foram também recolhidas amostras de tecidos para determinação dos seguintes biomarcadores: atividade da enzima acetilcolinesterase (AChE) como biomarcador de neurotoxicidade (cérebro); atividade das enzimas colinesterases (ChE) como biomarcador de efeitos neuromusculares; atividade da enzima lactato desidrogenase (LDH) como biomarcador de alterações de metabolismos energéticos (músculo); atividade das enzimas catalase (CAT), glutationa redutase (GR) e glutationa peroxidase (GPx) como biomarcadores de stress oxidativo (brânquias e fígado); os níveis de peroxidação lipídica (LPO) como indicativo de danos oxidativos em lípidos (fígado, branquias e músculo). O cálculo da exposição humana foi baseado na concentração média de MPs no músculo peixes e na dose semanal de peixe recomendada pela Autoridade Europeia para Segurança Alimentar.

Foram extraídas 221 MPs de todas as amostras analisadas, com dimensões entre 11 e 4784 µm e média (± desvio padrão – DP) de 787 ± 993 µm. As MPs tinham as seguintes cores: castanho (30%), azul/azulado (25%), preto/escuro (24%), transparente (8%), cinzento (6%), vermelho (5%), laranja (1%) e verde (1%). Uma parte considerável (51%) das MPs tinham forma de fibra, mas foram também encontrados fragmentos (38%), forma mais regular (6%), e forma de filme (5%). Dos 50 peixes, 96% tinham pelo menos uma MPs, 30% tinham MPs no cérebro, 50% no GT, 46% nas brânquias, 50% no fígado e 60% no músculo.

A média (± DP) do número de MPs por peso fresco de tecido analisado nos diferentes tipos de amostras foi: 0,23 ± 0,34 MPs/g no GT; 0,24 ± 0,40 MPs/g nas brânquias; 2,29 ± 4,22 MPs/g no cérebro; 0,34 ± 0,57 MPs/g no fígado; e 0.11 ± 0.11 MPs/g no músculo. Houve diferenças significativas em vários biomarcadores (AChE, ChE, LDH, CAT, GR, GPx e LPO) entre peixes com MPs e aqueles onde não foram encontradas MPs. Relativamente aos peixes onde não foram encontradas MPs, os peixes com MPs tinham: menor atividade da AChE no cérebro e das ChE no músculo; indução da atividade LDH no músculo; aumento da atividade das enzimas CAT, GPx, GR nas brânquias e da GR no fígado, e aumento dos níveis de peroxidação lipídica no músculo (LPO). Não foram encontradas diferenças significativas nos restantes biomarcadores. A estimativa de exposição humana a MPs através do consumo de músculo de peixes da população estudada foi 1747 MPs por ano.

Os resultados obtidos demonstram a ingestão de diversos tipos de MPs por exemplares de *M. merluccius* provenientes do Nordeste do Oceano Atlântico, a retenção de MPs nas brânquias e a presença de MPs no cérebro, fígado e músculo dos peixes. Os biomarcadores indicam neurotoxicidade, efeitos neuromusculares, aumento do stress oxidativo, danos oxidativos nos lípidos e alterações energéticas que podem estar associadas à contaminação por MPs. A presença de MPs no músculo dorsal indica uma possivel exposição humana através do consumo de exemplares da população estudada.

No seu conjunto, os resultados deste estudo demostram a contaminação de pescada branca (*M. merluccius)* do Nordeste do Oceano Atlântico por MPs, sugerem relação entre esta contaminação e efeitos adversos nos peixes, e indicam riscos de ingestão de MPs para consumidores humanos através do consumo regular de alimentos preparados com esta espécie.

Palavras-chave: peixe selvagem, Merluccius merluccius, Oceano Atlântico NE, biomarcadores, microplásticos, stress oxidativo, ingestão humana.

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Abbreviations list

AChE – Acetylcholinesterase enzyme

CAT – Catalase enzyme

ChE – Cholinesterase enzymes

EFSA – European Food Safety Authority

Fulton – Fulton's condition index

GPx – Glutathione peroxidase enzyme

GR – Glutathione reductase enzyme

GT – Gastrointestinal tract

H_2O_2 – Hydrogen peroxide

LDH – Lactate dehydrogenase enzyme

LPO – Lipid peroxidation

MPs – Microplastics

MSFD – Marine Strategy Framework Directive

NAD – Nicotinamide adenine dinucleotide

NADH – Reduced nicotinamide adenine dinucleotide

NADPH – Reduced nicotinamide adenine dinucleotide phosphate

NE – Northeast

SD – Standard deviation

TBA – 2-thiobarbituric acid

TBARS – Thiobarbituric acid reactive substances

1. Introduction

Marine pollution is a pressing environmental issue that poses a significant threat to the health of our oceans and the diverse life they support (Mæland,and Staupe-Delgado *et al*., 2020). One of the biggest contributors to this phenomenon and a major concern is none other than the material widely referred to as “plastics” (Letcher., 2020).

Plastics is a general term for synthetic organic polymers formed by polymerisation of subunits named monomers, which are derived from petroleum or natural gas (Cole *et al*., 2011). The development of polymers began with “Parkesine”, which was produced in the 1850s and was the first plastic ever (Geyer, 2020) Half a century later, in 1907, Bakelite was invented and since then production has increased dramatically from 0.5 million tonnes/year in 1960 to about 300 million tonnes in 2013 (Geyer et a., 2017; Avio *et al*., 2017). In 2021, 390.7 million tonnes of plastics were produced worldwide, of which 57.2 million tonnes were produced in Europe (PlasticsEurope, 2022). Recently, municipal waste pollution has skyrocketed as a result of the recent COVID-19 pandepidemic, increasing the threat to the environment and public health on a global scale (Masud *et al*., 2022).

Plastic encompasses a wide range of sectors and has found global use due to its low cost, durability, lightness, inertia, resistance and versatility (Avio *et al*., 2017; Geyer, 2020). For example, sectors such as packaging, construction, transportation, electrical and electronic equipment, and agriculture rely on these plastics. In addition, plastics are used in household furniture, leisure and sporting goods, and medical devices and accessories (Geyer *et al*., 2017; 2020). The inherent durability of plastic, results in a resistance to degradation, which in turn leads to an accumulation of waste, especially in oceans and seas (Cole *et al*., 2011; Avio *et al*., 2017). Plastics make up the majority of floating particles and between 60 and 80% of all marine litter, making them the most widespread type of marine litter (Setälä *et al*., 2014). Furthermore, their resistance to degradation ensures that trillions of plastic debris remain in the waters for centuries (Cole *et al*., 2011; Avio *et al*., 2017; Barboza *et al*., 2020).

Despite efforts to reduce the amount of plastic waste entering the marine environment, around 8 million tonnes of litter end up in the oceans (Letcher., 2020). The occurrence of stormwater overflows, sewage discharges and the transport of land-based litter by wind ensure horizontal and vertical distribution of particles in marine ecosystems (Avio *et al*., 2017; Guilhermino *et al*., 2021). Other inputs come from industrial offshore

activities (oil and gas production platforms, aquaculture equipment and operations), fishing equipment (mainly fishing nets) and aquatic activities and services, including tourism efforts (direct marine littering) (Avio *et al*., 2017; Barboza *et al*., 2018a).

The fate of plastics in the aquatic environment is mainly influenced by the density of the polymer, which affects the vertical position in the water column, buoyancy and potential interaction with biota (Avio *et al*., 2017). Larger plastic debris (macroplastics) can have serious impacts on the marine environment as it can disturb, injure and kill its inhabitants through ingestion, suffocation, drowning and entanglement (Cole *et al*., 2011; 2013). For example, the Marine Debris Program of the US National Oceanographic and Atmospheric Administration (NOAA) has identified plastic debris as a growing form of pollution in light of recent research on the various threats plastics pose to the ecosystem (Avio *et al*., 2017). In addition, the Marine Strategy Framework Directive (MSFD) of the European Union implemented regulations to promote the safety of the marine environment (Directive 2008/56/EC).

Microplastics (hereafter referred as MPs) were recently defined as "any synthetic solid particle or polymeric matrix, with regular or irregular shape and with size ranging from 1㎛ to 5㎜, of either primary or secondary manufacturing origin, which are insoluble in water" (Frias and Nash, 2019). Primary MPs are small particles intended for commercial use, such as exfoliants and additional abrasives in cosmetics (Dąbrowska *et al*., 2022). Secondary MPs are small particles produced during the degradation of larger plastic items such as textile fibres and packaging waste (Huber *et al*., 2022). The process of plastic degradation is described as a series of chemical changes that significantly reduce the average molecular weight and mechanical strength of the polymer (Avio *et al*., 2017. The type of polymer, the presence of chemical additives and the temperature of the environment can all affect how quickly it degrades (Avio et al., 2017). Most MPs in the oceans are secondary, while primary ones enter the oceans directly through discharge (Avio et al., 2017). Sewage treatment plants are seen potential sources of MP contamination in continental systems as large quantities of the microplastics are not removed (Browne *et al*., 2011). The untreated domestic sewage itself can contain large amounts of personal care goods, clothing fibres and other microplastics that are transported to marine environments (Mason *et al*., 2016; de Souza Machado *et al*., 2018). An analysis of microplastics from sediment collected from sewage-disposal sites revealed that the polyester and acrylic fibres, commonly found in clothing materials, are present in a proportion that resembles the one observed in environments that receive sewage discharges and sewage effluent (Browne *et al*., 2011).

MPs consist of a variety of polymers that are synthesised and used for domestic and industrial purposes (Shashoua, 2008). Polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyamide (PA) and polyvinyl alcohol (PVA) are the most commonly used plastics in the world and consequently the most abundant polymers in the marine environment (Santana-Viera et al., 2021). There are several morphotypes of MPs, including fibres, fragments, films, foam and pellets (Amelia et al., 2021). Fibres can originate from the abrasion of synthetic fishing gear and ropes; fragments arise from the continuous degradation of larger plastic items, often presenting irregular shapes; films are more slender and irregular particles; pellets are typically associated with plastic manufacturing, and foams are usually related to foamed polystyrene (Cesa et al., 2017; Rosal, 2021). Fibre like microplastics can be released into the environment through the degradation of fibres used in plastic-based textiles and garments, either during production, use or maintenance (Cesa *et al*., 2017; Almroth *et al*., 2018). It is estimated that 0.19 million tonnes of MP fibres from the production and normal use of synthetic textiles are released into the marine environment each year (Henry *et al*., 2019)

Current scientific research on MP shows that these pollutants are ubiquitous and distributed across the globe (Cole *et al*., 2011). Environmental variables such as precipitation, wind and water currents can carry MP to even the most remote locations far from human activities, such as the Arctic and Antarctic regions (Mishra *et al*., 2021; Prakash *et al*., 2020). Many studies have found MP in fish caught in the waters of the Northeast Atlantic, many of which are highly sought after in human diets (Barboza *et al*., 2018b; Chenet *et al*., 2021). Distributed throughout the water column, MP occupies several layers of aquatic ecosystems. Relative position is determined by particle density, and so high-density polymers, such as PVC, tend to be below the currents and are weighted down to the sediments, while low-density polymers, such as PE, tend to be buoyant (Cole et al., 2013). In addition, events such as storms and the settlement of organisms on the surface of the polymer, or even the local flow system, can lead to different positions in the water column, just like their larger counterparts (Avio et al., 2017). Due to their widespread distribution and ease of transport, MP contamination poses an increasing threat to the balance of biodiversity and the equilibrium of marine and freshwater ecosystems (Varg and Svanback, 2022).

Numerous aquatic species come into daily contact with MP through ingestion, digestion, and assimilation of debris, which has been observed both in the laboratory and in the wild (Granek *et al*., 2020). Furthermore, this trend extends to a wide range of taxa at many trophic levels in different environments. Contamination and toxicity have been

observed at lower tropic levels such as plankton (Setälä *et al*., 2014), phytoplankton (Hao *et al*., 2022) or zooplankton (Cole *et al*., 2013), plants (Li *et al*., 2022) and even reach more complex organisms such as invertebrates (Martyniuk *et al*., 2022), fish (Barboza *et al*., 2018b; 10), birds (Deoniziak *et al*., 2022) and mammals (Zantis *et al*., 2021). While there are still gaps in knowledge regarding detailed specific human health effects and respective exposure levels of MP contamination, information is already available on the harm these particles can cause alone, in interaction with other pollutants or even with microbial contamination (Wright and Kelly, 2017).

Due to their small size, MPs are bioavailable for accumulation and can harm organisms (Browne *et al*., 2011). Toxicological and physical effectson wildlife are caused by ingestion, which in turn impedes organism movement as well as hinders food intake, accumulates and blocks digestive tracts, restricts food intake and can even enter the bloodstream (Deoniziak *et al*., 2022). Their large surface area allows them to absorb various pollutants and toxins, whether in the water or in the sediments, which are later absorbed into the food web (Cole et al., 2013; Setaelae et al., 2014). The toxicological effects, compounded by the additive, antagonistic or synergistic effects of leached plasticisers, endogenous additives and dissolved organic carbon, can impair energy homeostasis, cause oxidative stress and cytotoxicity in organisms (Hao et al., 2022; Nessi et al., 2022). Therefore, MP pollution is widely recognised as an urgent global threat of great concern (Barboza *et al*., 2020).

The European hake, *Merluccius merluccius* (Figure 1), is an important demersal fishery resource of critical importance to many Atlantic and Mediterranean coastal countries (Casey & Pereiro, 1995; Cartes et al, 2004; Mancuso et., al 2019). It occurs in the Northeast Atlantic, from the western coast of Norway to the north and the coast of Mauritania to the south. It is also found in the North Sea, Black Sea and Mediterranean Sea (Casey & Pereiro, 1995; Mancuso et., al 2019). It is a bottom-dwelling predatory species that stays near the seabed during the day and moves vertically up the water column at night (Mancuso et., al 2019). Their main food source is fish, including small hake, anchovies, sardines, herring, cod, sardines and gadoid species, as well as squid. In contrast, juveniles feed mainly on crustaceans, especially euphausiids and amphipods (Murua., 2010).

Due to its commercial importance, the European hake is well researched, with studies focusing on MP contamination (Bellas *et al*., 2016 with recent studies showing MPs contamination in its stomach or gastrointestinal tract (Giani *et al*., 2019; Mancuso et., al 2019; Cocci *et al*., 2022). While field research about the toxicological effects of several class of contaminants on this species is available (Solé, *et al*., 2008; Martínez-Morcillo*et al*., 2019), there is still lacking a bridge between microplastic contamination in wild European hakes and potential adverse effects.

Figure 1 - *Merluccius merluccius*.

The goals of the present study were: (i) to assess the contamination of wild specimens of *Merluccius merluccius* from the Portuguese waters of the North East Atlantic Ocean by microplastic debris; (ii) to investigate potential biological effects in relation to microplastic contamination; and (iii) to estimate human intake of MPs through the consumption of *M. merluccius.*

Merluccius merluccius was selected as the subject of this study due to its extensive distribution across both sides of the Atlantic Ocean. Furthermore, this species is an important commercial fish, widely present in fish markets in Europe, with an average consumption of 1.02 kg per capita in a year (EUMOFA, 2021).

2. Material and Methods

2.1. Fish Collection

Fifty specimens of *M. merluccius* from Portuguese coastal waters of the Northeast Atlantic Ocean (NE) were obtained from the commercial fleet shortly after landing in the Port of Matosinhos, between October and December 2021. The fish were obtained in small quantities (5 fish per day) to allow careful sample collection and processing. The intact corps of the fish were transported in thermally insulated boxes under cold conditions to the Laboratory of Ecotoxicology and Ecology of the School of Medicine and Biomedical Sciences (ICBAS) of the University of Porto.

2.2. Quality assurance and quality control (QA/QC)

To prevent external and cross-contamination of the samples, numerous precautions were taken at each stage of sample collection, dissection, digestion, filtration, MP isolation and visual identification, following the recommendations of previous studies (Karami *et al*., 2017a; Hartmann *et al*., 2019; Barboza *et al*., 2020). Nitrile gloves and clean cotton lab coats were worn throughout the process. The entire process of fish dissection, sample preparation, digestion and filtration took place in a previously cleaned flow cabinet flow-through cabinet to minimise potential contamination with MPs from external sources. All surfaces and materials were thoroughly cleaned with 70% ethanol. Prior to use, filters were carefully examined under a stereo microscope to remove any plastic debris. In addition, all blanks were thoroughly examined to identify any contamination. The colour, shape, and size of the contamination were carefully noted so that any similar debris found in the samples could be excluded.

2.3. Sample preparation

In the laboratory, the total body length was measured with a digital calliper and the total body wet weight of each fish was determined (Kern 572-33, Kern & Sohn GmbH, Germany). A physical examination of the fish was made. Then, each fish was carefuly washed to remove external particles. For MP analyses the following samples were collected: the whole gastrointestinal tract (GT), as well as samples of the liver (approximately 3.686 g), the gills (three branchial arches), the brain (all the tissue except

the cerebellum) and the dorsal muscle (10 g) were collected, weighed, and stored in a freezer at -20° C until further examination. Samples for biomarker analyses were also collected, namely: a small portion of the liver (approximately 0.774 g) one pair of the branchial arches, the cerebellum, and a portion of the dorsal muscle. The wet weight of each sample collected for MP analysis was determined using a precision balance (Kern 572-33, Kern & Sohn GmbH, Germany). Similarly, biomarker samples were weighed using a different another precision balance (HCB 153H, Adam Equipment, United Kingdom). All samples were stored in a cool place, at -20 ºC for MPs and -80ºC for biomarker analysis, until needed.

2.4. Isolation and primary characterization of particles

Particle isolation was performed by tissue digestion followed by filtration as described in Barboza et al. (2020). Briefly, a 10% (w/w) potassium hydroxide solution (> 3 times the volume of the biological sample) was added to tissue samples placed in glass flasks. These flasks were covered with aluminium foil to avoid contamination and incubated at 60ºC (drying oven EV50, Raypa, Spain) for 24 hours. The resulting digested liquid was filtered through glass microfibre philtre membranes (pore size 1.2 µm, VWR International, USA) using a vacuum pump (WP6122050, Merck, Germany). The filters were placed in glass Petri dishes, which were sealed and dried at 40ºC for 24 hours in a drying oven (ED -S 56, BINDER, Germany).

The filter membranes were examined in detail in a stereo microscope (Nikon, SMZ800 stereo microscope, Japan) with an attached camera (Nikon Digital Sight DS-Fi1, Japan). The quantification and categorisation of the particles (by colour, size and shape) followed previous descriptions of plastic debris, and were subsequently photographed. The following types were considered for shape:plastic fragments (irregularly shaped), pellets (spherical or ovoid, more regular shaped), filaments/fibres (thin and elongated particles), plastic films (Hartmann *et al*., 2019; Barboza *et al*., 2020; Rosal, 2021). Regarding colour, the following categories were considered: blue/blueish, black,dark transparent, grey, brown, red, orange and green (Guilhermino *et al*., 2021). The size of the observed MPs analysed (longest dimension for fibres; Feret's diameter for the other shapes) was measured using the ImageJ software available in https://imagej.nih.gov/ij/ and the following size classes were considered: <100 µm; 100-149 µm; 150-499 µm; 500-1499 µm; 1500-3500 µm; 3501 < 5000 µm (Guilhermino *et al.*, 2021). Results of MP contamination were expressed as thenumber of MP per individual

(MP items/individual) and the number of MP per gram of tissue (MP items/g). Presence of MP was expressed in total percentage of fish with at least one MP found and percentage of fish with MP in each organ.

2.5. Biomarker determination

A set of biomarkers were selected based on their crucial roles in essential processes and functions vital to the survival and living organisms. As such, they have been used extensively in studies on microplastic pollution of fish (Alomar *et al*., 2017; Barboza *et al*., 2020; Choi *et al*., 2023 Cohen-Sánchez *et al*., 2023). The biomarkers selected were the following: the Fulton's condition factor (Fulton's K), calculated based on the total length and wet weight of the fish, as a morphometric index that estimates the condition of the organism, which in turn helps to observe potential differences in a population (Lloret *et al*., 2002); the activity of the brain acetylcholinesterase (AChE) and muscle cholinesterase (ChE), as indicative of neurotoxicity (Barboza *et al*. 2018b); the activity of lactate dehydrogenase (LDH) in the muscle as indicative of alterations in the energy metabolism as it is involved in anaerobic pathways (Barboza *et al.*, 2018b); the activity of the enzymes glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) in the liver and gills as crucial oxidative damage prevention (Lima *et al.*, 2007); and the lipid peroxidation (LPO) levels in liver, gills and muscle as indicative of lipid oxidative damage (Barboza *et al.*, 2020).

The samples were defrosted on ice and homogenized (Ystral GmbH d-7801, Germany) on ice in the respective buffer in a ratio of 1 g wet tissue/ 10 ml buffer and centrifuged (centrifuge 5810 R Eppendorf, Germany). The brain samples and muscle samples were homogenized in phosphate buffer (pH = 7.2, 0.1 M) and centrifuged at 3300g for 3 min at 4ºC for AChE and ChE determinations, respectively. LDH muscle samples were homogenized in Tris buffer (pH 7.8, 50 mM) and centrifuged at 3300g for 3min at 4ºC (centrifuge 5810 R Eppendorf, Germany). Muscle, liver, and gill samples were homogenized in phosphate buffer (pH 7.4, 0.1M) to determine the levels of LPO. Then, 4 µL of a 4% solution of butylated hydroxytoluene (BHT) was added to 250 µL of the homogenate. To assess the enzymatic activity of CAT, GPx, and GR, the residual homogenate of the previous step was centrifuged at 10000g for 20 min at 4ºC. After thawing the samples on ice, their protein content was determined using the Bradford method (Bradford, 1976) adapted to a microplate format as described by Guilhermino *et al*. (1996). The quantification was performed using a Biotek PowerWave HT 340

Microplate Reader (USA), with bovine γ-globulin serving as the standard protein for calibration. The protein concentrations of the samples were the adjusted to 0.3 mg/mL for AChE and ChE, and to 0.9 mg/mL for LDH, CAT, GPx and GR. Four replicas of sample, 10 µL in volume, were pipetted to the wells followed by 250 µL of the BioRad Protein Assay solution with agitation for 15 min. After this period, the absorbance was read at 600 nm and 25ºC in a Biotek PowerWave HT 340, Microplate Reader, USA, for all enzymatic activities and LPO levels, except CAT activity which was measured with the aid of a Spectramax® spectrophotometer, Molecular Devices, USA.

AChE and ChE activities were determined by the Ellman's method (Ellman *et al*., 1961) adapted to microplate (Guilhermino *et al*., 1996). Acetylthiocholine was hydrolysed into thiocholine. The absorbance of theproduction rate of thiocholine was read at 412 nm (Biotek PowerWave HT 340, Microplate Reader, USA. The hydrolysis of acetylthiocholine produces thiocholine. The production rate of thiocholine was measured at 412 nm and was expressed in nanomoles per mg of protein (nmol/min/mg protein).

LDH activity was determined according to Vassault et. al (1983) adapted to microplate (Diamantino *et al*., 2001). LDH catalyzes the conversion of pyruvate to lactate and vice-versa, as it also converts NADH to NAD^+ and vice-versa during the reaction. LDH activity was measured by determining the decrease in NADPH levels, using pyruvate as substrate, at 340 nm (Biotek PowerWave HT 340, Microplate Reader, USA).

GPx activity was measured by the method of Flohé and Günzler (1984) adapted to microplate (Lima *et al.*, 2007). GPx catalyzes the oxidation of glutathione (GSH) to glutathione disulfide (GSSG). Its activity is indirectly measured using glutathione reductase, an enzyme that catalyzes the reduction of GSSG to GSH, with the simultaneous oxidation of NADPH to $NADP^+$. GPx activity was measured by observing the decrease in NADPH levels at 340 nm (Biotek PowerWave HT 340, Microplate Reader, USA).

GR activity was measured according to the method of Carlberg and Mannervik (1975) adapted to a microplate (Lima *et al.*, 2007). GR catalyzes the reduction of GSSG to GSH with simultaneous oxidation of NADPH to $NADP^+$. GR activity was measured by monitoring the decrease in NADPH levels at 340 nm (Biotek PowerWave HT 340, Microplate Reader, USA).

CAT activity was determined according to Clairborne (1985). CAT decomposes the hydrogen peroxide (H_2O_2) in molecular oxygen and water. H_2O_2 displays a continuous increase in absorption with a decreased wavelength in the ultraviolet spectrum. This means that the decomposition of H_2O_2 is directly correlated to the decrease in absorbance. As such, CAT activity was measured trough the H_2O_2 decomposition by determining the change in absorbance at 240 nm (Spectramax® spectrophotometer, Molecular Devices, USA) for 1 minute. Activity was determined from the difference in optical density referring to the linear part of the curve.

LPO level in muscle, liver and gills tissues was measurement by quantifying thiobarbituric acid reactive substances (TBARS), according to Ohkawa *et al.* (1979). The amount of TBARS was quantified with absorbance reading at 535 nm (Biotek PowerWave HT 340, Microplate Reader, USA).

The activities of AChE, ChE, GPx, GR; LDH were expressed in nanomoles per mg of protein (nmol/min/mg protein), CAT activity was expressed in micromoles per mg of protein (µmol/min/mg protein) and LPO values were expressed in nanomoles of TBARS per g of tissue (nmol TBARS/g tissue).

2.6. Estimated human risk of MPs intake through the consumption of European hake

The human risk of MP intake (HRI) through the consumption of European hake as food per year, was determined based on the European Food Safety Authority (EFSA) recommendations on fish consumption by age group (i.e., 40 g for 1 year old children; 50 g for children with ages between 2 and 6 years; 200 g for children older than 6 years; 300 g for adults or the general population) (EFSA, 2014), and the total mean of MP present in the dorsal muscle of all fish sampled (i.e. total number of MPs found in muscle tissue / 50 specimens), following the general procedure used by Barboza *et al*., (2020), with punctual differences, using the following formula:

a) HRI = concentration of MPs in the edible tissue x Recommended dose of the edible tissue x Weeks

   Where: estimated human risk of MP intake, expressed as the number of MP items ingested per year (MPs/year); Concentration of MPs in the edible tissue – mean of the number of MP items in the dorsal muscleanalysed (MPs items/g); Recommended dose of the edible tissue – dose of the tissue estimated to be ingested per week; Weeks – number of weeks per year (52).

A more accurate estimate was made, in relation to the per capita human consumption offish in Portugal (59.91 kg/year/capita) according to the European Market Observatory for Fisheries and Aquaculture Products (EUMOFA) (EUMOFA, 2021) and the total mean value of MP in the tissue of all fish analysed.

b) HRI = concentration of MPs in the edible tissue x Consumption of fish per year per capita

   Where: estimated human risk of MP intake, expressed as the number of MP items ingested per year per capita (MP items/year/capita); Concentration of MPs in the edible tissue – mean of the number of MP items in the dorsal muscle analysed (MPs items/g); Consumption of fish per year per capita – consumption of fish per year per capita (Kg/year/capita) in Portugal based on EUMOFA data.

2.7. Statistical Analysis

All data analyses were performed using the SPSS statistical package (version 27.0), and the significance level was set at 0.05. The data set of each biomarker was tested for normal distribution and homogeneity of variances using the Shapiro-Wilk test and Levene's test, respectively. For variables that did not achieve normal distribution, appropriate transformations were performed (Zar, 1999). Specifically, liver CAT and GR and gill CAT, GPx and LPO datasets were log transformed and muscle ChE datasets were square root transformed. For data that had a normal distribution or could be transformed to achieve normality, Student's t-test was used to compare fish with MPs to those without MPs in the samples analysed. If a normal distribution could not be achieved, the data sets for each biomarker were subjected to a non-parametric Mann-Whitney U-test.

3. Results

3.1. Microplastics (MP) in *Merluccius merluccius*

The extent of contamination in the blank samples was categorised by shape andcolour, and the control-corrected observations for each sample were then obtained by subtracting the results of the blank samples from the recorded observations. Thus, the MP that had the same shape and colour as those found in the blank samples were neither counted nor considered in the analyses. At least one MP was found in 48 of 50 fish, which means that 96% of the studied fish examined were contaminated. The distribution of MP by tissue was: 15 fish had MP in the brain (30%); 23 fish had MP in the gills (46%);30 fish had MP in the dorsal muscle (60%); 25 fish had MP in the liver (50%) and 25 fishhad MP in the gastrointestinal tract (50%) (Figure. 2).

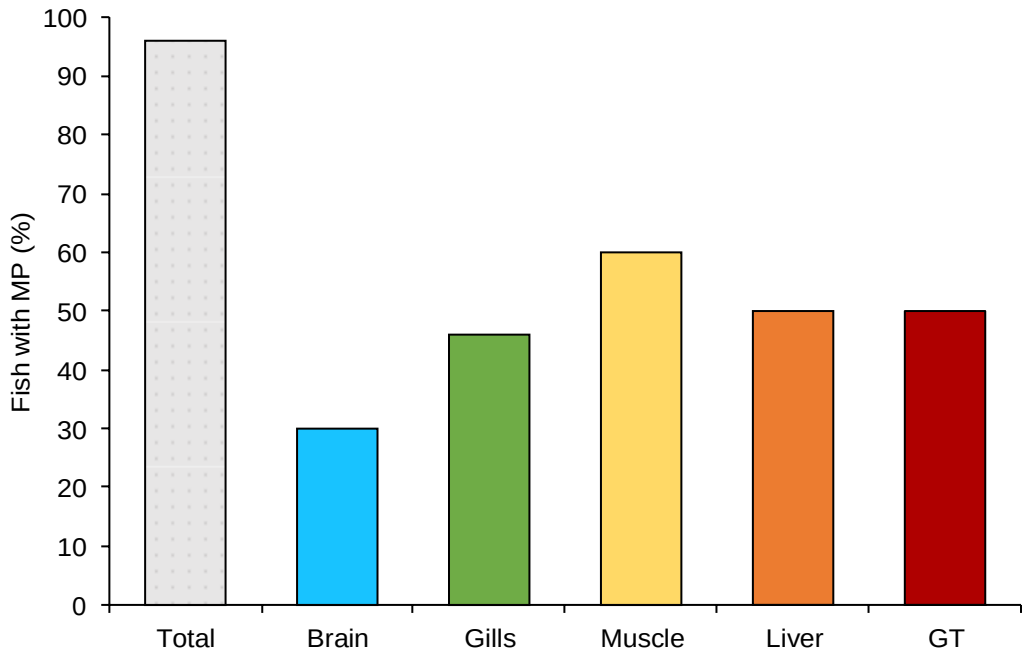


Figure 2 - Percentage of *Merluccius merluccius* (N = 50), with microplastics (MPs) in the brain, gill, dorsal muscle, liver, gastrointestinal tract (GT), or in any these analysed tissues (Total).

A total of 221 MP items were retrieved from the 50 *M. merluccius* specimens: 22 MP were found in the brain (10%), 46 in the gills (21%), 56 in the dorsal muscle (25%), 45 in the liver (20%) and 52 in the gastrointestinal tract (24%). The mean concentration (± standard deviation - SD) of MP was: 0.44 ± 0.79 items/fish (2.29 ± 4.22 items/g) in the brain, 0.92 ± 1.43 items/fish (0.24 ± 0.40 items/g) in the gills, 1.12 ± 1.06 items/fish (1.11 ± 1.1 items/g) in the dorsal muscle, 0.90 ± 1.20 items/fish (0.34 ± 0.57 items/g) in the liver and 1.04 ± 1.37 items/fish (0.23 ± 0.34 items/g) in the gastrointestinal tract.

The shapes of the MP from the *M. merluccius* specimens were fibres, fragments, pellets and films (Figure 3), which are exemplified in Figure 4. From the 221 MP retrieved, 113 were fibre-like shape (51%), followed by 84 fragments (38%), 14 pellet-like shape (6%) and 10 plastic film (5%). Fibres-like shape were the most retrieved debris in the brain (68%), gills (72%), and muscle tissue (75%) and fragments were the most retrieved in the liver (58%) and gastrointestinal tract (58%). Film debris was found in the brain and muscle and no pellet was recovered from the brain.

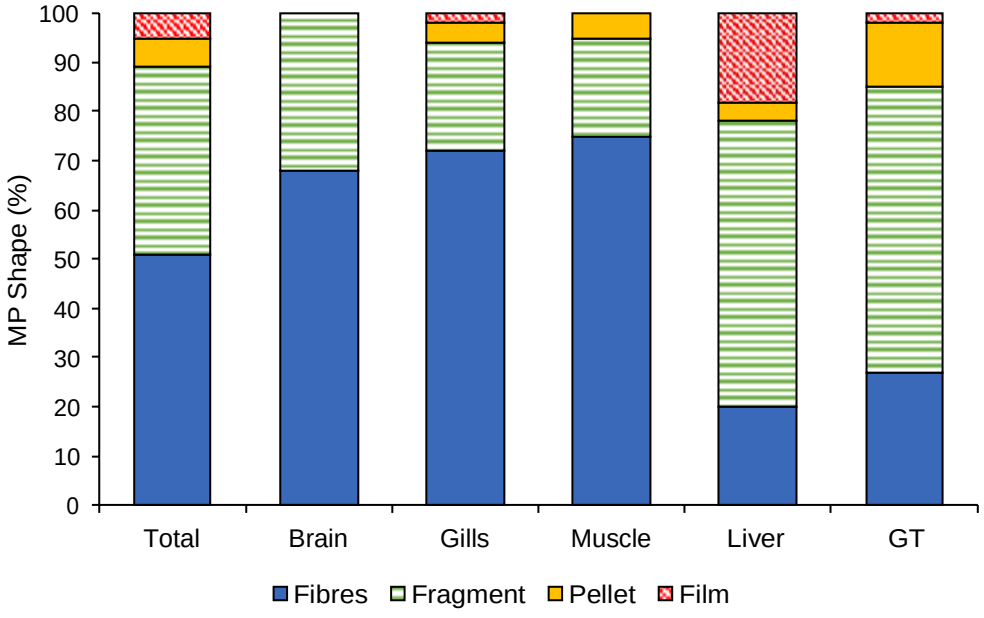


Figure 3 - Percentage of microplastics (MPs) found in *M. merluccius* brain, gills, in the brain, gill, dorsal muscle, liver, gastrointestinal tract (GT), or in any these analysed tissues (Total) categorized by shape.

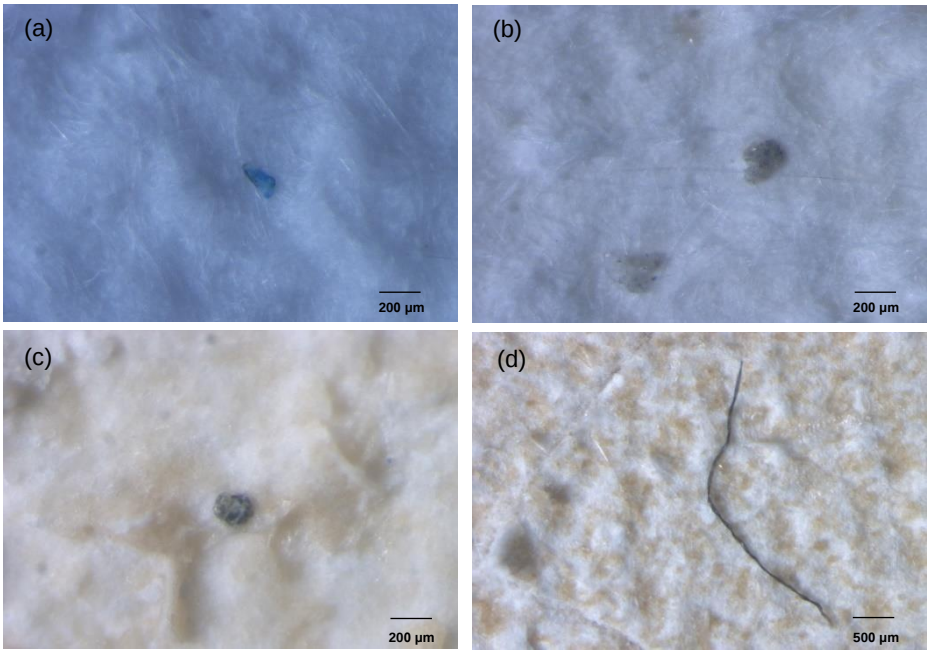


Figure 4 - Examples of microplastics (MPs) retrieved from *M.merluccius* : (a) fragment from the brain; (b) pellet-like particle from the gastrointestinal tract (GT); (c) film from the liver; (d) fibre from the gills.

MP sizes varied from 11 to 4784 µm in the 50 sampled fish. Tissue-specific size the sizeranges of MP were: 43-3356 µm in the brain, 87-4011 µm in the gills, 38-4161 µm in the muscle, 11-3312 µm in the liver and 32-4784 µm in the gastrointestinal tract. The measured MP were divided into six size classes: <100 µm22%), 100-149 µm (9%), 150- 499 µm (27%), 500-1499 µm (22%), 1500-3499 (17%) µm and 3500-4999 µm (3%) (Figure 5).

The MPs were included in seven colour categories: blue/bluish (25%), black/dark(24%), brown (30%), transparent (8%), grey (6%), red (5%), orange (1%) and green (1%). Blue was most common in the brain (50%) and muscle (54%), black was the predominant colour in the gastrointestinal tract (35%) and brown particles were most commonly found in both the gills (50%) and liver (36%) (Figure 6).

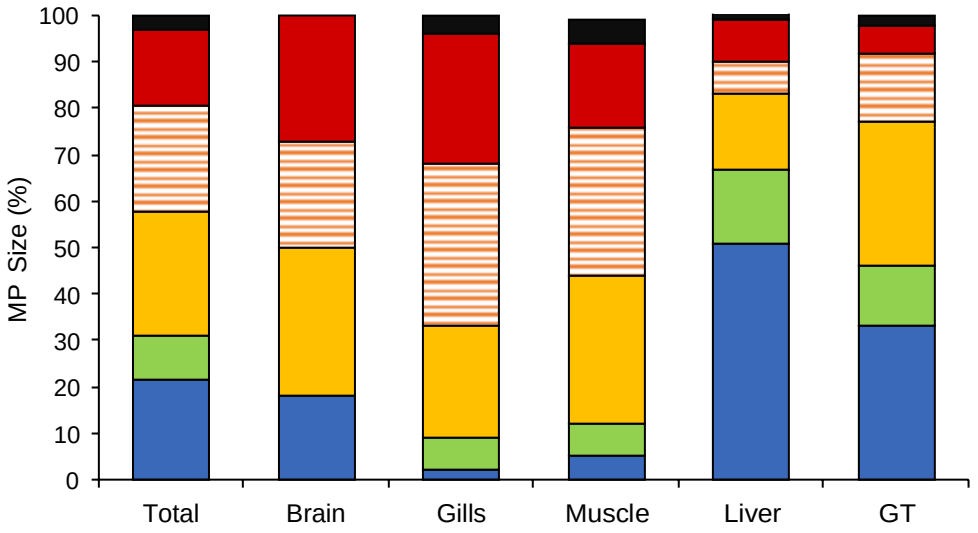


Figure 5 - Percentage of microplastics (MPs) found in *M. merluccius* brain, gills, in the brain, gill, dorsal muscle, liver, gastrointestinal tract (GT), or in any these analysed tissues(Total) categorized by size class.

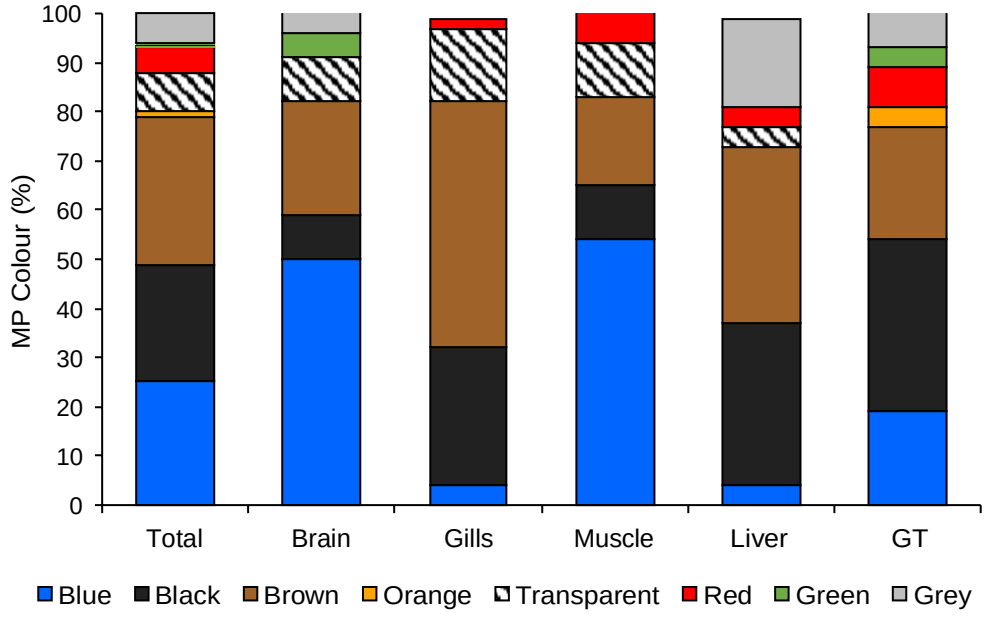


Figure 6 - Percentage of microplastics (MPs) found in *M. merluccius* brain, gills, in the brain, gill, dorsal muscle, liver, gastrointestinal tract (GT), or in any these analysed tissues (Total) categorized by colour.

3.2. Biomarkers associated to MPs contamination.

No significant differences in total length, total weight, and Fulton's condition between fish with and without MP in the brain, muscle, liver, and gills ($p > 0.05$) were found (Table 1).

Table 1 - Mean ± standard deviation (SD) of body weight, total body length, and Fulton condition index (Fulton) in groups of fish with (with MP) and without (No MP) microplastics in the brain, muscle, liver, and gills. N = number of individuals per group. * Indicates significant differences between groups of fish with or without MPs (Student's *t* test, $p \leq 0.05$).

Tissue	Parameter	Level	N	Mean±SD	*t* test
Brain	Total weight (g)	No_Brain	35	24 ± 41	$t_{(48)} = 0.980$; p = 0.332
		MP_Brain	15	223 ± 29	
	Total length (cm)	No_Brain	35	31 ± 2	$t_{(48)=} 0.968$; p= 0.338
		MP_Brain	15	31 ± 1	
	Fulton condition	No_Brain	35	0.8 ± 0.1	$t_{(48)=} 0.951$; p = 0.860
		MP_Brain	15	0.8 ± 0.1	
Muscle	Total weight (g)	No_Muscle	20	226 ± 32	$t_{(48)} = -0.767$; p = 0.447
		MP_Muscle	30	235 ± 41	
	Total length (cm)	No_Muscle	20	31 ± 1	$t_{(48)} = - 0.424$; p = 0.673
		MP_Muscle	30	31 ± 2	
	Fulton condition	No_Muscle	20	0.8 ± 0.1	$t_{(48)} = - 0.770$; p = 0.445
		MP_Muscle	30	0.8 ± 0.05	

Liver	Total weight (g)	No_Liver	25	230 ± 321	$t_{(48)}$ = - 0.244; p = 0.808
		S-MP_Liver	25	233 ± 43	
	Total length (cm)	No_Liver	25	31 ± 1	$t_{(48)}$ = - 0.445; p = 0.658
		S-MP_Liver	25	31 ± 2	
	Fulton condition	No_Liver	20	0.8 ± 0.05	$t_{(48)}$ = 0.562; p = 0.577
		S-MP_Liver	30	0.8 ± 0.1	
Gills	Total weight (g)	No_Gills	27	234 ± 38	$t_{(48)}$ = 0.617; p = ,540
		S-MP_Gills	23	228 ± 38	
	Total length (cm)	No_Gills	27	31 ± 2	$t_{(48)}$ = 0.395; p = 0.695
		S-MP_Gills	23	31 ± 1	
	Fulton condition	No_Gills	25	0.8 ± 0.1	$t_{(48)}$ = 0.671; p = 0.505
		S-MP_Gills	25	0.8 ± 0.1	

Regarding the biomarkers analysed, significant differences in the activity of AChE between fish with and without MPs in the brain were found ($t_{(48)}$ = 2.223, p = 0.031) (Figure 7-a). The mean (±SD) of AChE activity was lower (46 ± 16 nmol/min/mg protein) in fish with MPs in the brain than in fish where MPs were not found in the analysed samples (58 ± 18 nmol/min/mg protein). Significant differences were found for muscle ChE activity ($t_{(48)}$ = 2.202, p= 0.032) (Figure 7, (b)), with a lower mean (±SD) in fish with MPs in the tissue (22 ± 13 nmol/min/mg protein) than fish without MPs (29 ± 11 nmol/min/mg protein).

Muscle LDH activity was significantly different ($t_{(48)}$ = -7.176, p <0.001) (Figure 7, (c)) between the two groups with a higher mean (±SD) in fish with MPs (105 ± 31 nmol/min/mg protein) compared to fish without MPs (57 ± 14 nmol/min/mg protein).

LPO levels were significantly higher (U = 36.000, p < 0.001) (Figure 7, (d)) in the muscle of fish with MPs (46 ± 5 nmol TBARS/g tissue) than muscle of fish without MPs (65 ± 20 nmol TBARS/g tissue).

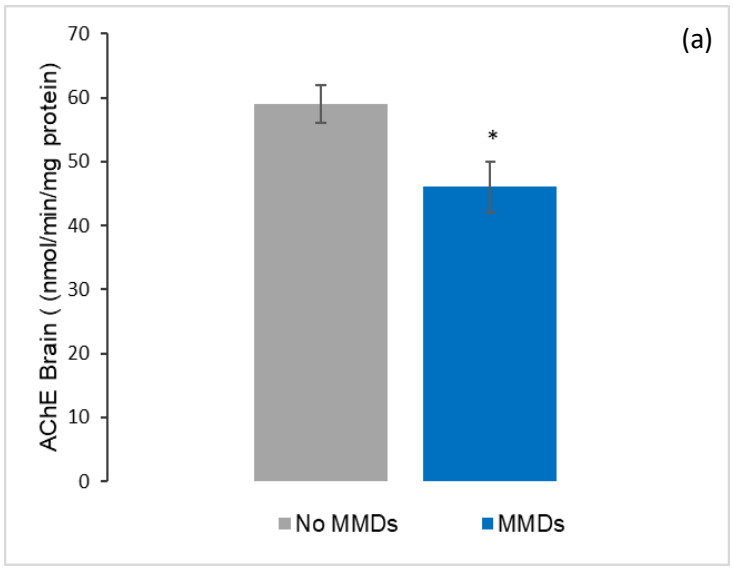
Gill CAT activity was significantly higher ($t_{(48)}$ = - 3.818, p < 0.001) (Figure 7, (e)) in fish with MPs (184 ± 38 µmol/min/mg protein) than fish without MPs in the tissue (149 ± 31 µmol/min/mg protein).

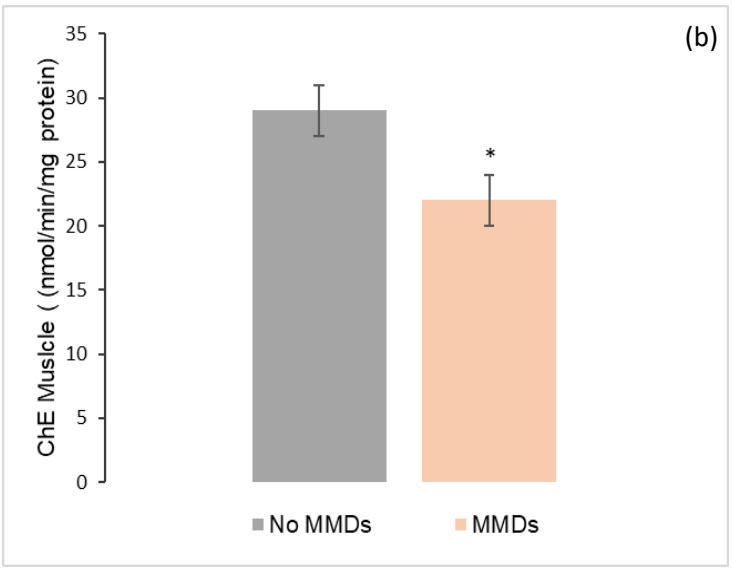
The GPx activity in the gills was significantly different between the studied groups ($t_{(48)}$ = -3.591, p = 0.001) (Figure 7, (f)), with a higher mean (±SD) in fish with MPs (18 ± 10 nmol/min/mg protein) than fish without MPs (11 ± 7 nmol/min/mg protein). GR activity in the gills had significant differences (U = 51.000, p < 0.001) (Figure 7, (g)) between fish with (10 ± 3 nmol/min/mg protein) and without MPs (8 ± 3 nmol/min/mg protein).

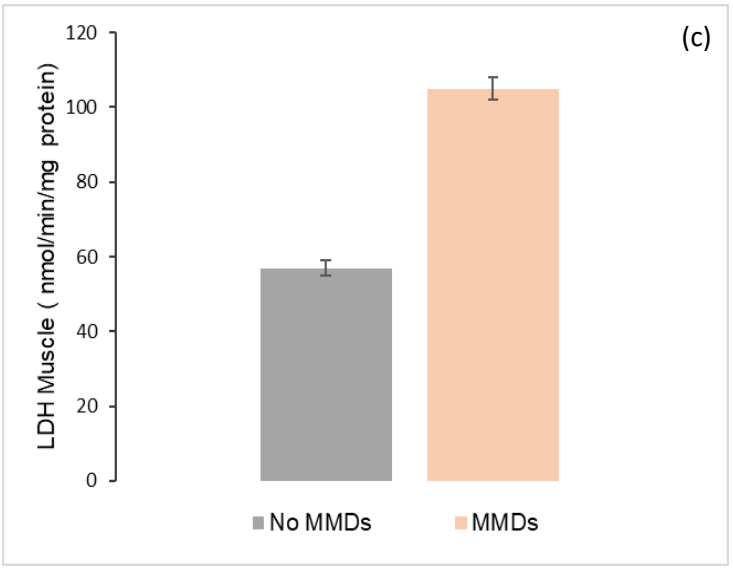
No significant differences were observed in LPO values ($t_{(48)}$ = 0.872, p < 0.388) (Figure 7, (h)) in the gills of fish with MPs (175 ± 62 nmol TBARS/g tissue) and without (185 ± 51 nmol TBARS/g tissue).

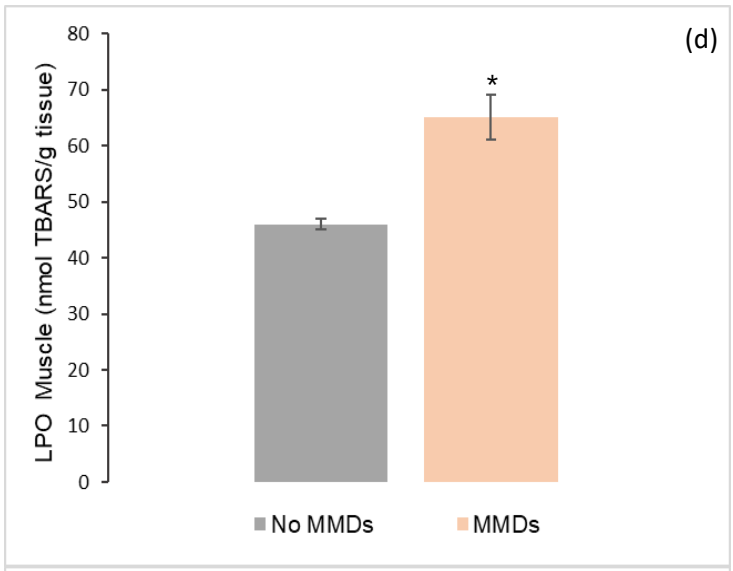
Liver GR ($t_{(48)}$ =-4.918, p < 0.001) (Figure7, (k)) activity had significant differences between fish with (15 ± 8 nmol/min/mg protein) and without MPs (7 ± 4 nmol/min/mg protein).

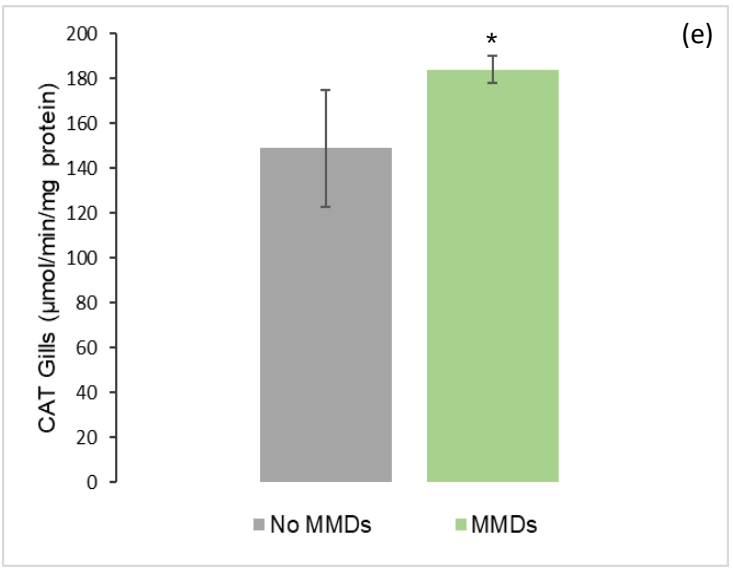
No significant differences were observed in CAT ($t_{(48)}$ = 0.168, p = 0.868) (Figure 7, (i)), GPx (U = 273.000, p = 0.443) (Figure 7 (j)) and LPO (U = 215.500 p = 0.060) (Figure 7, (l)) values in the liver of fish with (134 ± 64 µmol/min/mg protein; 20 ± 10 nmol/min/mg protein and 163 ± 59 TBARS/g tissue protein) MPs and without (159 ± 104 µmol/min/mgprotein; 21 ± 8 nmol/min/mg protein and 142 ± 67 TBARS/g tissue protein).

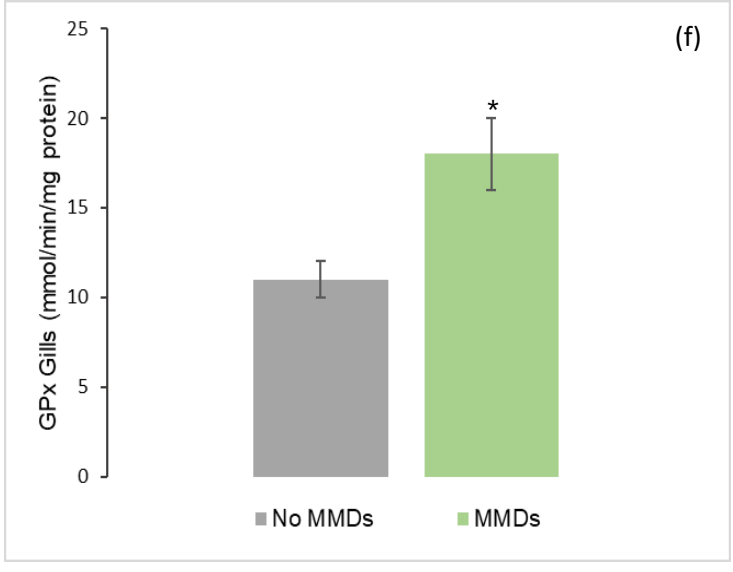

(a)
AChE Brain ((nmol/min/mg protein)
70
60
50
40
30
20
10
0
*
No MMDs
MMDs

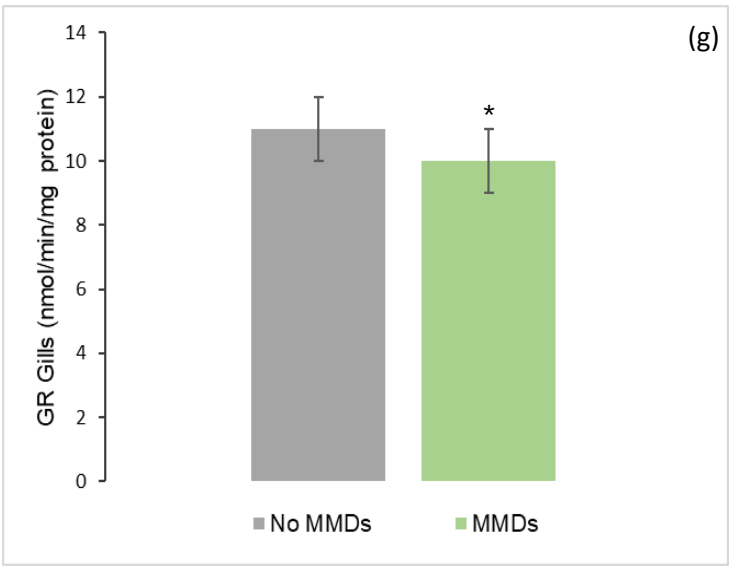

(b)
ChE Muslcle ((nmol/min/mg protein)
35
30
25
20
15
10
5
0
*
No MMDs
MMDs

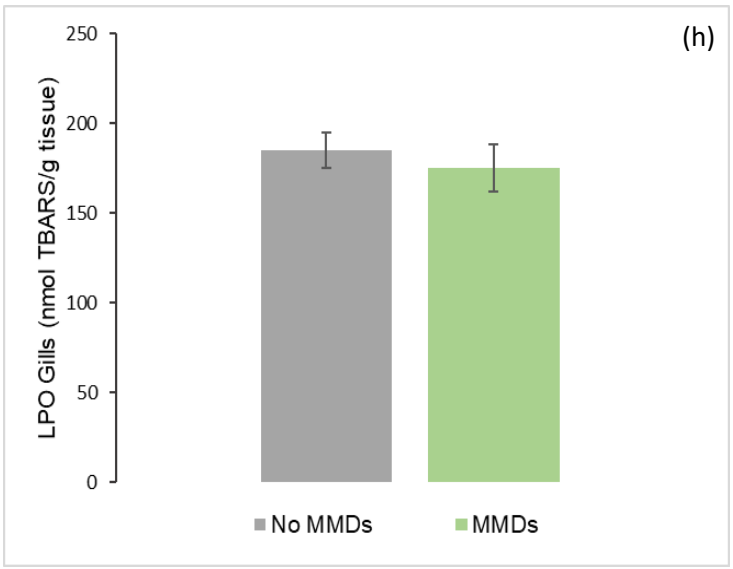

(c)
LDH Muscle (nmol/min/mg protein)
120
100
80
60
40
20
0
No MMDs
MMDs

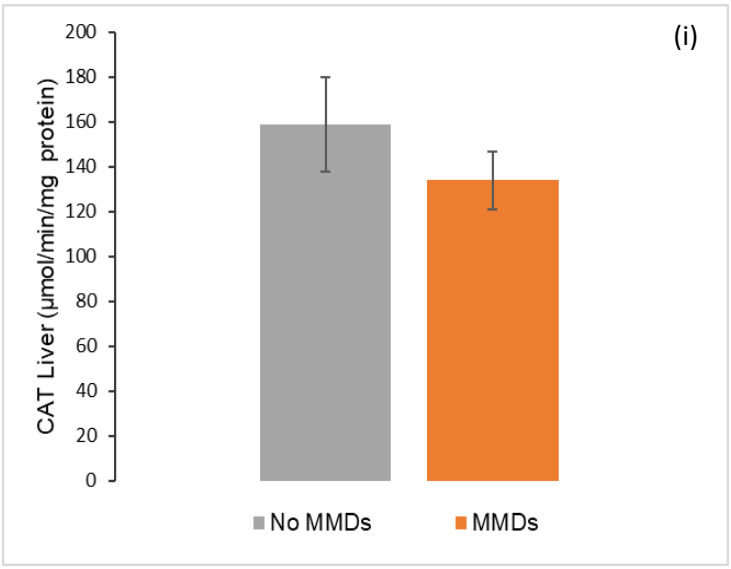

(d)
LPO Muscle (nmol TBARS/g tissue)
80
70
60
50
40
30
20
10
0
*
No MMDs
MMDs

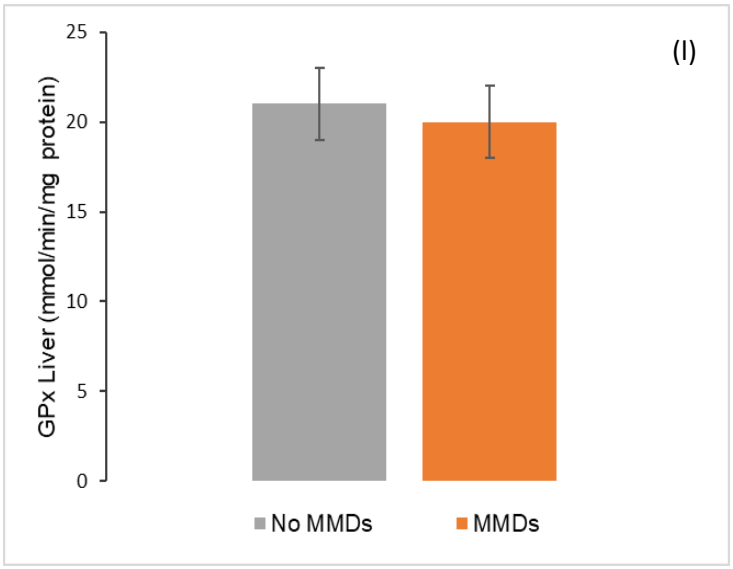

(e)
CAT Gills (µmol/min/mg protein)
200
180
160
140
120
100
80
60
40
20
0
*
No MMDs
MMDs

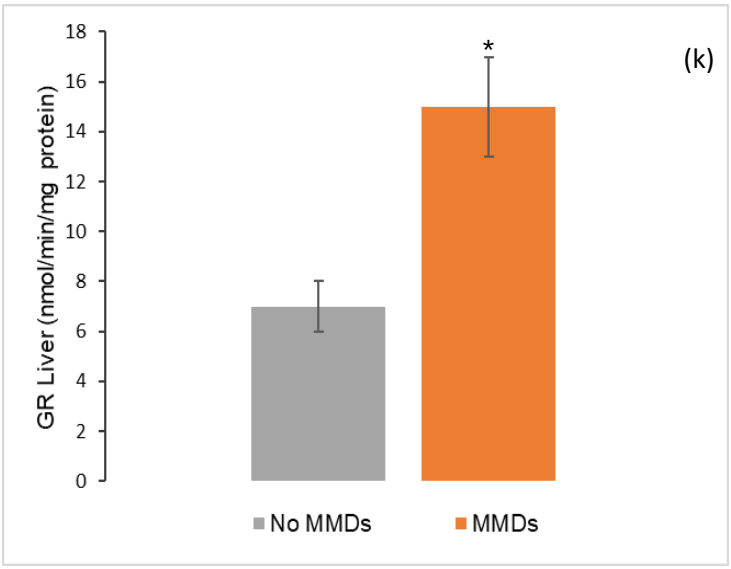

(f)
GPx Gills (mmol/min/mg protein)
25
20
15
10
5
0
*
No MMDs
MMDs


(g)
GR Gills (nmol/min/mg protein)
14
12
10
8
6
4
2
0
*
No MMDs
MMDs


(h)
LPO Gills (nmol TBARS/g tissue)
250
200
150
100
50
0
No MMDs
MMDs


(i)
CAT Liver (µmol/min/mg protein)
200
180
160
140
120
100
80
60
40
20
0
No MMDs
MMDs


(l)
GPx Liver (mmol/min/mg protein)
25
20
15
10
5
0
No MMDs
MMDs


(k)
GR Liver (nmol/min/mg protein)
18
16
14
12
10
8
6
4
2
0
*
No MMDs
MMDs

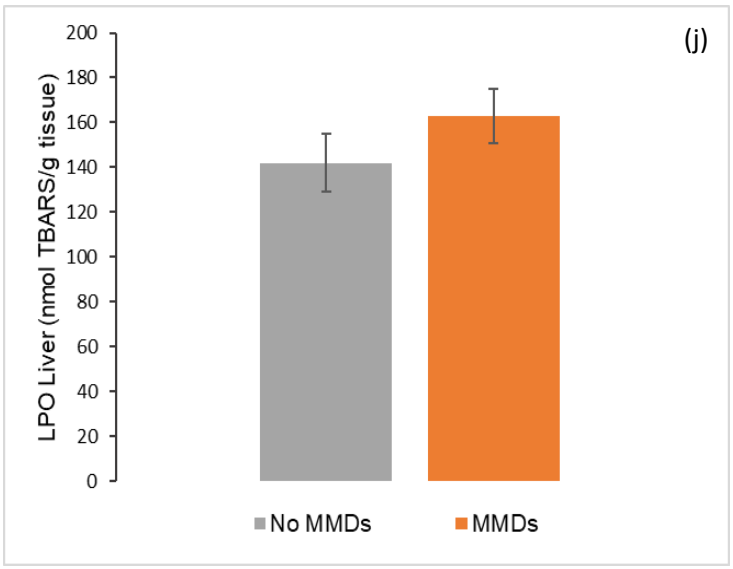

(j)
LPO Liver (nmol TBARS/g tissue)
200
180
160
140
120
100
80
60
40
20
0
No MMDs
MMDs

Figure 7 - Mean ± standard deviation (SD) of: (a) brain acetylcholinesterase activity (AChE-B); (b) muscle cholinesterase activity (ChE-M), (c) lactate dehydrogenase activity (LDH-M) and d) muscle lipid peroxidation levels (LPO-M); (e) gill catalase activity (CAT- G), (f) glutathione peroxidase activity (GPx-G), (g) glutathione reductase activity (GR-G) and (h) lipid peroxidation levels (LPO-G); (i) liver catalase activity (CAT-G), (j) glutathione peroxidase activity (GPx-G), (l) glutathione reductase activity (GR-G) and (l) lipid peroxidation levels (LPO-G) in groups of fish with (with MP) and without (No MP) microplastics in the respective organ, (e.g.:AChe activity in the brain between fish with and without MP). Enzymatic activities are expressed in nmol/min/mg protein. LPO levels are expressed in nmol TBARS/mg protein. *Indicates significant differences between groups of fish with or without MPs (Student's *t* test, $p \leq 0.05$ or non-parametric Kruskall-Wallis test, $p \leq 0.05$).

3.3. Human risk of MPs intake through the consumption of European hake

Table 2. Estimated human intake of MPs from *Merluccius merluccius* consumption based on microplastics (MPs) found in the dorsal muscle of the fish and on EFSA recommendation for fish consumption per week by children of different age groups and adults or the general population.

	Children			Adults or the general population
	1 year	2-6y	>6y	≥ 18y
g/fish muscle/ week	40 g	50 g	200 g	300 g
MP itens/ week	4	6	22	34
g/fish muscle/ year	2080 g	2600 g	10400 g	15600 g
MP itens/ year	233	291	1165	1747

The estimates of the human risk of MPs intake through the consumption of European hake from the Northeast Atlantic Ocean expressed per year were 233 MPs/year for 1 year old children, 291 MPs/year for 2 to 6 year old children, 1165 MPs/year for children above 6 and 1747 MPs/year for adults or the general population (Table 2). The estimates of consumption per capita in Portugal are indicated in Table 3.

Table 3. Estimated human intake of microplastics from *Merluccius merluccius* consumption based on microplastics (MP) found in the dorsal muscle of the fish and on the consumption of fish per capita in Portugal.

Fish consumption	Portugal
Consumption per capita (Kg/year/capita)	59.91 kg
g/fish muscle/ week/ capita	1152 g
MP itens/ week/capita	129
g/fish muscle/ year/ capita	59910 g
MP itens/ year/capita	6710

4. Discussion

4.1 MP in *Merluccius merluccius*

The presence of MPs in the brain, gills, dorsal muscle, liver and gastrointestinal tract (GT) of *M. merluccius* specimens is indicative of the extensive contamination of the waters of the NE Atlantic by MPs. Contamination of the NE Atlantic by MPs is well documented and has been detected in organisms (Barboza et al., 2020; Maaghloud et al., 2020), sediments (Van Cauwenberghe et al., 2013; Courtene-Jones; Lechthaler et al., 2020) and water (Vega-Moreno et al., 2021). Many other aquatic ecosystems such as estuaries like the Minho River in Portugal, NE Atlantic Ocean (Barboza et al., 2020) and seas like the Mediterranean Sea (Mancuso et al., 2019) have also shown signs of MP contamination. This is most likely due to discharges and effluents contaminated with plastic waste from places with high population density, such as many cities in Europe (Browne et al., 2011, de Souza Machado et al., 2018). Many of these bodies of water, that have been previously reported as polluted with this type of waste, are the natural habitat of the *Merluccius merluccius*. These fish can then be contaminated by ingesting polluted organisms or by eating plastic particles (de Sá et al., 2015; Ory et al., 2017), by water filtration (Karlsson et al., 2017) and possibly by fishing nets (Egessa et., 2020).

MP presence in fish tissue has been widely reported (Collard *et al*., 2017; Karami *et al.*, 2017b; Abbasi *et al*., 2018; Atamanalp *et al*., 2021), however values varied between different species, locations and methods were used. In the present study, of thetotal fish analysed (n=50), 48 (96%) were contaminated with MP, meaning they had at least one particle in at least one of the sampled tissues.According to the literature, this is not the first observation of contamination of wild hake documented in both the Mediterranean (Giani et al., 2019; Mancuso et al., 2019) and the Atlantic (NE) (Bellas et al., 2016), however the present values are higher than those reported in the literature (between 16% and 48%). This discrepancy can be attributed to the fact that in these studies only samples of the gastrointestinal tract were examined to detect MP contamination by ingestion, whereas in this study 4 tissues were examined for possible contamination.

With more than a single tissue, it is possible to describe a more complex picture of the pathways of MP contamination. As fish were exposed to SM-P in the wild, many ingested these particles. Accumulation in the gastrointestinal tract, can obstruct the tract and cause a false sense of satiety, leading to starvation and even death (Sun *et al*., 2019). Many particles are usually eliminated through excretion, but some suffer enough fragmentation or have adsorbed chemicals that they can overcome physical barriers and even enter the bloodstream (Dawson *et al*., 2018), making their way to various tissues.

The appearance of MP in the muscle indicates the internalisation of particles. According to previous studies (Barboza *et al*., 2020), these particles may have been absorbed in the digestive system or even in the gills. Skin sores, and small cuts are also a possible pathway of MP contamination (Abbasi *et al*., 2018).

The presence of MP particles in the gills of *M.merluccius* were most likely trapped during water filtration. This process is influenced by variables such as microplastic size, morphology, and filtering efficiency (Barboza *et al*., 2020). Accumulation of MPs from water passing through the gills may affect the fitness of the fish and its ability to survive, as MPs can cause structural damage to various organs and tissues, including the gills filaments (Jabeen *et al*., 2018).

Data from the brain showed an increase in total contaminated samples (30%) compared to previous data (7.7% in Atamanalp *et al*. 2021). MP particles in the liver also show evidence of internalization, as they can reach internal organs and amass for unknown periods of time (Collard *et al*., 2017). Plastic translocation lacks extensive knowledge, specifically about the ability to move between organs and tissues. This information would greatly improve microplastic understanding and expand the objects of research, as more and more tissues would be considered for MP contamination assessment.

Of the total MP particles found in *M. merluccius* (221), the predominant colour was brown (31%), followed by blue (25%) and black (24%) (Figure 2). Previous studies have shown that the majority of reported plastic waste is either blue (Guilhermino et al., 2021) or black (Atamanalp et al., 2021). This difference could be due to the fact that European hake is a groundfish that moves vertically at night to catch and eat prey (Casey & Pereiro, 1995). Nocturnal feeding habits and habitat arrangement may reduce accidental ingestion and blue particle ingestion as there is less likelihood of environmental confusion. Diet may also play an important role in the outcome of MP data. *M. merluccius* varies its food preferences depending on depth, from small

crustaceans such as euphausiids to small fish such as Myctophidae. Their natural colour and small size can easily be mistaken for small brownish particles, leading to accidental ingestion and incorporation into the liver. Passive uptake via the gills may also play an active role in MP as most brown coloured particles were found in the gills (50%) and liver (36%). The brain and muscles were predominantly blue in colour (50% and 54%, respectively), and the gastrointestinal tract contained predominantly black debris (35%), which is consistent with the literature (Giani et al, 2019; Mancuso et., al 2019).

The predominant forms of the total MP *M. merluccius* specimens found were fibres (51%) (Graph 3). As reported in several studies, fibres are the most common debris in the Atlantic (54% reported in Barboza et al, 2020) and in *M. merluccius* (81% reported in Giani et al, 2019). High contamination levels in the gills indicate passive uptake of MP resulting from an abundance of fibres in the water, a consequence of urban plastic runoff into the sea. The fragment values indicate increased uptake of these mouldings by ingestion in the gastrointestinal tract and accumulation in the liver, an important organ for the breakdown of toxic products.

The size of the debris particles found ranged from 32 to 4784 µm. As they are below the threshold of 5 mm, all MP are classified as microplastics. These dimensions are consistent with similar studies (Barboza *et al*.,2020; Mancuso *et al.,* 2019; Neves *et al.*, 2015). The gastrointestinal tract shows the greatest variability of all tissues studied. As these MP originate from food ingestion, there is no strict limit to particle size in the mouth of fish. Passive ingestion can also allow for a wide range of particle sizes, depending on the particle contamination in the water (Kılıç et al.,2022). The smaller size ranges found in the brain and gills can be explained by the path they take to their ultimate accumulation.

4.2 *Merluccius merluccius* biomarkers

Specimens with MPs in the brain had significantly lower brain AChE activity than specimens in which no MPs were found. Inhibition of AChE has already been found under laboratory conditions in several exposed species, such as *D. labrax*, (Barboza *et al*, 2018), *Danio rerio* (Santos *et al*., 2022) *Paramisgurnus dabryanus* (Wang *et al*., 2022) and *Cyprinus carpio* (Chen *et al*., 2022). It may cause a diversity of effects, including growth inhibition, behaviour alteration and inactivity which can lead to lower survivability and overall fitness of the fish (Chen *et al*., 2022; Santos *et al*., 2022).

ChE levels decreased significantly in fish with MP, compared to fish without S-MP. This was reported in previous studies (Martínez-Morcillo *et al*.,2019). This decrease, similar to the brain AChE, is a sign of oxidative stress derived from MP toxicity (Gambardella *et al*., 2017).

LDH activity increased significantly, between fish with MP and fish without MP. LDH values for *M. merluccius* have been reported in previous studies (Saavedra *et al*., 2016). The increase in LDH values is a result of cellular hypoxia, derived from MP interaction, that produces an anaerobic condition causing damage to the cells (Saavedra *et al*., 2016).

Muscle LPO levels increased significantly, between fish with MP and fish without MP. High LPO levels are a sign of lipid peroxidation damage and can be caused by MP debris itself or by-products of other contaminants adsorbed in the microplastic surface (Barboza *et al*., 2018b). In other studies, with *M. merluccius* samples, LPO values varied between tissues (Ognjanović *et al*., 2008).

Gill CAT values increased significantly in fish with MP, when in comparison with fish with no MP, effect that has already been recorded (Solé *et al*.,2010). The results show antioxidative response from CAT enzyme, in the gills, while in the liver there was no increase.

GPx and Gr work as an antioxidant defence system, which in turn means that their activity is a sign of oxidative stress. Gill GPx activity had a significant increase in activity in fish with MP, compared to fish without MP contamination. Gill GR activity had a significant increase in activity in fish with MP, compared to fish without MP contamination. Gr activity was significantly higher in the liver of fish with microplastics

compared to those without MP. Additionally, the low levels observed in the gills and liver may describe a small, countermeasure to the oxidative stress (Xia *et al*., 2020; Banaei *et al*., 2022).

All the data mentioned so far suggest that the contaminated fish suffered biological effects such as neurotoxicity in the brain, oxidative stress in the muscle, gills and liver, and oxidative damage, but only in the muscle. The effects observed in the liver and gills differed in severity, as oxidative stress was more pronounced in the liver due to increased activity of all three antioxidant enzymes, while only GR activity was observed in the gills These results highlight the importance of further studies on wild fish populations exposed to contamination by MP. Various toxicity effects may play a role in the survival and fitness of many wild organisms, all of which are important for the balance of the ecosystem.

4.3 Human risk of MP intake through the consumption of European hake

According to the EFSA's fish consumption recommendations (EFSA, 2014), an adult ingesting 300 g of *M. merluccius* per week will result in an intake of approximately 34 MP items/week and 1747 MP items/year, equivalent to 0.112 items/g/week and 5.824 items/g/year. These estimated values are higher than the values previous estimated from the consumption of fish species and/or other seafood where the number of items per week was 16 and the number of items per year was 842 MP, equivalent to 0.054 MP items/g/week and 2.8 MP items/g/year (Barboza *et al*., 2020). As such when measured per capita, the consumption of MPs by Portuguese consumers (6710 MPs items/year/capita) may be much greater.

The estimates of human risk of MPsintake through the consumption of *European hake* from the Northeast Atlantic also vary according to age. Due to their high intake of fish muscle tissue, adults are likely to consume high amounts of MPs. Children, however, are less likely to be exposed in the same way as adults as their fish consumption habits are lower. However, there is a noticeable jump in fish consumption for children aged 6 and lower, compared to children older than 6.

As many other fish species, crustaceans, cephalopod molluscs and shrimps have been reported to have been contaminated with MP (Alfaro-Núñez *et al*.,2021), it is crucial to continue tracking these organisms to generate current estimates of intake, especially as MPs contamination is predicted to increase in the future.

5. Conclusions

This study collects and provides evidence of contamination of commercialy important fish species (*Merluccius merluccius*) from Portuguese coastal waters of the north-eastern (NE) Atlantic Ocean available for human consumption with microplastics. Most fish sampled had at least one particle in one of the tissues examined. A total of 221 particles were retrieved from 50 wild European hakes, from brain, liver, gill, muscle, and GT tissue. The majority of the particles were fibre and fragment shaped, were coloured blue, black and brown, and their most common sizes were between 150-499 µm and 500-1499 µm.

Biomarker assessment showed that fish with MP found exhibited neurotoxicity, oxidative stress, and lipid oxidation damage in various tissues resulting potential threat to organism, that are likely to get contaminated MP. Furthermore, the discovery of microplastics in the edible tissues (dorsal muscle) of wild fish brings to light the possibility of human contamination by microplastics. Highly commercial fish such as the *Merluccius merluccius* can be a source of these contaminants and given the fish-eating habits and the recommended intake of fish in Europe and especially in Portugal, adults and children above of 6 are likely to ingest large quantities of microplastic items.

Merluccius merluccius is a species of fish that has previously been reported to be contaminated with MPs, but the numbers appear to be increasing. As the human population continues to rise, feeding the masses depends more and more on what the sea has to offer. In addition, the growing consumer market generates ever-increasing amounts of waste, which can then end up in various bodies of water. As this cycle continues, it is important to identify the pathways of MP plastic pollution, especially in food sources. Available data shows pollution in NE Atlantic waters as well as in native fish that are often caught and sold for consumption. This work highlights the risk of microplastic pollution in wild fish in coastal countries with a developed fishing industry and market, such as Portugal.

6. References

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