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Microplastics in fishes from an estuary (Minho River) ending into the NE Atlantic Ocean

Lúcia Guilhermino [a,b,*], Alexandra Martins [a,b], Clara Lopes [b,c], Joana Raimundo [b,c], Luis R. Vieira [a,b], L. Gabriel A. Barboza [a,b], Joana Costa [b], Carlos Antunes [b,d], Miguel Caetano [b,c], Carlos Vale [b]

[a] ICBAS, School of Medicine and Biomedical Sciences, University of Porto, Department of Populations Study, Laboratory of Ecotoxicology and Ecology, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[b] CIIMAR, Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Research Line Global Changes and Ecosystem Services, Avenida General Norton de Matos, 4450-208 Matosinhos, Portugal
[c] IPMA, Portuguese Institute of Sea and Atmosphere, IP Division of Oceanography and Marine Environment (DIVOA), Av. Doutor Alfredo Magalhães Ramalho, 6, 1495-165 Algés, Portugal
[d] Aquamuseu do Rio Minho, Parque do Castelinho, 4920-290 Vila Nova de Cerveira, Portugal

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ABSTRACT

Wild fish (*Cyprinus carpio, Mugil cephalus, Platichthys flesus*) from an estuary of the NE Atlantic coast were investigated for plastic contamination ($N = 128$). From the 1289 particles recovered from fish samples, 883 were plastics. Among these, 84% were fibres and 97% were microplastics. Thirty-six polymers were identified. The number of microplastics (mean ± SD) per individual fish (MP/fish) was 8 ± 6 in *C. carpio*, 10 ± 9 in *M. cephalus* and 2 ± 2 in *P. flesus*. The means of MP/fish per body site were 6 ± 7 in gastrointestinal tract, 0.5 ± 1.1 in gills, 0.3 ± 0.7 in liver and 0.6 ± 1.2 in muscle samples. A few large fibres in liver (≤ 4841 μm) and muscle (≤ 5810 μm) samples were found. The results evidence the existence of high fish contamination by microplastics and reinforce the need of further research on plastic pollution in estuaries.

1. Introduction

The worldwide plastic production and its use tend to increase unless environmental policies are urgently implemented. Despite the measures that have been proposed and in some cases implemented, such as regulations and restrictions (e.g. Marine Strategy Framework Directive - MSFD, Howarth, 2009; Galgani et al., 2013), recycling, and new and ongoing technologies (Hahladakis et al., 2018; Picó and Barcelò, 2019), it is unlikely that the increase of global plastic pollution will revert in the next years (Alimba and Faggio, 2019; Barceló and Picó, 2019). Indeed, the pandemics caused by SARS-CoV-2 likely has been increasing the environmental problem mainly due to the intensive use of plastic materials worldwide, among other reasons (Canning-Clode et al., 2020; Guilhermino et al., 2021). Moreover, available resources have been shifted to face and control the pandemics. Under these circumstances, the development of alternative technologies and the adoption of measures to face plastic pollution have slowed down.

Microplastics (MP) are globally dispersed in different types of ecosystems (Akdogan and Guven, 2019; Yang et al., 2021). They are persistent pollutants that can be long-range transported (Cózar et al., 2017), ingested by organisms and transferred along food chains (Carbery et al., 2018, Savoca et al., 2020; Albano et al., 2021a). Consequently, MP have been accumulated in the environment and biota worldwide (Akdogan and Guven, 2019; Wang et al., 2020; Xu et al., 2020). The plastics and MP present in the environment generally contain other toxic chemicals, such as several types of additives used in the plastic industry and environmental contaminants adsorbed to MP during their permanence into the air, water and soils (Lau and Wong, 2000; Frias et al., 2010; Barboza et al., 2018a). Often, environmental plastics and MP also contain microorganisms (Moore et al., 2020). After plastic and MP ingestion, such chemicals and microorganisms can be transferred from the plastic particles to the animal body and cause adverse effects

* Corresponding author: ICBAS, University of Porto, Deptartment of Populations Study, Laboratory of Ecotoxicology and Ecology, Rua de Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal.
E-mail addresses: lguilher@icbas.up.pt (L. Guilhermino), clara.lopes@ipma.pt (C. Lopes), jraimundo@ipma.pt (J. Raimundo), cantunes@ciimar.up.pt (C. Antunes), mcaetano@ipma.pt (M. Caetano), carlos.vale@ciimar.up.pt (C. Vale).

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(O'Donovan et al., 2018; Lu et al., 2019).

Animals exposed to MP in laboratory conditions have shown several adverse effects, such as histological alterations and lesions in the gastrointestinal tract and gills (Pedà et al., 2016; Collard et al., 2017b; Jabeen et al., 2018), intestinal inflammation (Qiao et al., 2019), neurotoxicity (Oliveira et al., 2013), oxidative stress and damage (Barboza et al., 2018b), immunoregulation (Espinosa et al., 2019), feeding behavior and developmental alterations (Sendra et al., 2020; Albano et al., 2021b), reproductive toxicity (Pacheco et al., 2018; Guilhermino et al., 2021), effects over generations (Martins and Guilhermino, 2018; Wang et al., 2019), among several others (e.g. Barboza et al., 2018a; Limonta et al., 2019; Wang et al., 2020). Nevertheless, no significant adverse effects of MP were also documented, for example in marine zooplankton after acute exposure in the laboratory (Beiras et al., 2018) and in wild fish that did not show oxidative stress or cellular damage in the liver despite having ingested MP (Alomar et al., 1917).

MP present in the environment, including man-made cellulose fibres widely used in the textile industry (Shen et al., 2010; Savoca et al., 2019), show high variety of properties (Andrady, 2017). Such properties change over time influencing the fate of MP in the environment (Andrady, 2017), the sorption of chemicals onto MP and their release (Hahladakis et al., 2018; Tuccori et al., 2019), and the biological effects of MP (Barboza et al., 2018a). Furthermore, MP can be further fragmented into nanoplastics that are considered an emerging additional threat (Peng et al., 2020). Those evidences justify the great concern on the potential impact of MP on biodiversity, ecosystem functioning, and human health.

Coastal areas of anthropogenic impacted regions were identified as areas of MP concentration. Estuaries in particular are highly pertinent to study due to their ecological relevance and services provided to the human society. Plastics enter estuaries mainly through rivers that often contain heavy loads of these materials (Lebreton et al., 2017), urban and industrial sewages, other continental sources, and coastal waters. Studies have documented a high diversity of MP contamination in estuaries (Hitchcock and Mitrovic, 2019; Rodrigues et al., 2019; Robin et al., 2020; Wu et al., 2020), and among estuarine fish communities (Bessa et al., 2018; Pegado et al., 2018; Blettler et al., 2019; Ferreira et al., 2019; Kazour et al., 2020). MP can cause adverse effects on estuarine fish (Oliveira et al., 2013; Luis et al., 2015; Miranda et al., 2019), on their preys, and on predator-prey relationship (Van Colen et al., 2020). MP estuarine pollution raises particular concern regarding its potential impact on wild fish recruitment, population growth and sustainability (Miranda et al., 2019; Kazour et al., 2020).

Most of the studies on wild fish contamination by plastics and MP focus on the ingestion step (e.g. Avio et al., 2017; Bellas et al., 2016; Bour et al., 2018; Garcia-Garin et al., 2019; Naidoo et al., 2016, 2020; Talley et al., 2020). Studies on the distribution of MP in skin and gills (e.g. Abbasi et al., 2018) and internal organs/tissues (e.g. Collard et al., 2017a; Karami et al., 2017; Akhbarizadeh et al., 2018; Barboza et al., 2020) of wild fish are still limited. Further knowledge on partitioning of MP in wild fish internal organs and tissues is necessary to relate exposure and effects, and to assess and manage the risks to fish, environment and human health.

The goal of the present study was to assess to what extent plastics are present in wild populations of fish from the estuary of the Minho River ending into the North East (NE) Atlantic Ocean. To better understand the plastic contamination degree of the specimens sampled, the gastrointestinal tract (GT), and samples of gills, liver and dorsal muscle were checked for plastic presence, and the features of the plastic particles were analysed.

2. Material and methods

2.1. Study area and species

The study area was the estuary of the Minho River, hereafter indicated as Minho estuary, which is located in the North West (NW) coast of the Iberian Peninsula (Fig. 1). It is an international estuary and part of the border between the North Region of Portugal and Galicia, Spain. Its biota and habitat richness led to its inclusion in NATURA 2000 and Long-Term Ecosystem Research network (LTER-Europe, LTER-Portugal). Beside the high ecological and conservational value, the Minho estuary holds a high socio-economic relevance (Guimarães et al., 2012; Ribeiro et al., 2016). It is a shallow-water estuary with about 40 Km long and a salinity gradient ranging from 32 to 34‰ at the mouth to freshwater a few kilometres upstream (Sousa et al., 2008; Vieira et al., 2015; Ribeiro et al., 2016). The influence of the tide and salinity, water temperature, nutrients and other water parameters vary along the year in response to the freshwater discharge of the Minho River, among other factors (Sousa et al., 2008; Guimarães et al., 2012; Vieira et al., 2015). The estuary has considerably natural vulnerability (Ribeiro et al., 2016). Several pressures were identified, including exotic invasive species and several types of anthropogenic activities (Sousa et al., 2008; Ribeiro et al., 2016).

The European flounder (*Platichthys flesus* Linnaeus, 1758), the flathead grey mullet (*Mugil cephalus* Linnaeus, 1758) and the common carp (*Cyprinus carpio* Linnaeus, 1758) were selected for this study mainly because they are long-life species, have wide geographic distribution and are commercially exploited (FishBase, 2021), *P. flesus* is one of the species recommended for monitoring plastic litter in fish (ICES (International Council for the Exploration of the Sea), 2015), and the three species meet the key criteria for the use of bioindicators to monitor marine litter recommended in Fossi et al. (2018).

2.2. Fish sampling and ethical issues

In the present study, 128 fish were analysed. For ethical reasons, 90 fish were obtained from local fishermen shortly after their capture. The other fish were collect by us in a similar way and in the same area of those collected by fishermen. The collection of fish in the wild had authorization from Portuguese Authorities, including the "Instituto Nacional para a Conservação da Natureza e da Floresta" (ICNF), licences 501-505/2018/CAPT, and from the Portuguese National Authority for Animal Health "Direção Geral de Alimentação e Veterinária" (DGAV): 0421/000/000/2017, 014227, 31st May 2017 (PLASTICGLOBAL project). L. Guilhermino and L. R. Vieira are accredited by DGAV as investigator/coordinator (equivalent to FELASA category C) to carry animal experimentation.

All fish were captured in the area defined by the coordinates 42°0'2.62"N, 8°39'53.21"W and 41°52'12.22"N, 8°51'17.69"W (Fig. 1), carps mainly in the upstream and mullets and flounders in mid and low estuary. Carps were collected with traditional trap nets, mullets with trammel nets and flounders with a beam trawl. As changes in estuarine MP pollution over time have been documented (e.g. Rodrigues et al., 2019; Wu et al., 2020), fish were caught in two sampling periods: early March 2018 (winter) and early September 2018 (summer). Typically, the two periods correspond to the start of the ecological recovery of the ecosystem after the extremes of annual abiotic conditions variation. The total number of fish per species in the two sampling periods was 44 *P. flesus*, 43 *M. cephalus* and 41 *C. carpio* (Table 1).

The handling of fish alive was performed according to the Portuguese and European ethic principles and regulations for the protection of animals used for scientific purposes, except regarding chemical anaesthetics that were not used. Because samples for multiple studies were taken from specimens to maximize their use, including for neurotoxicity research, chemical anaesthetics could not be used as they can interfere with the analyses and results of some biomarkers (Luis et al., 2015). To avoid additional stress during transport to the laboratory and maintain intact the fish corps to prevent internal contamination by external plastic particles in field conditions, fish were sacrificed rapidly after collection by rapid cooling under cold-induced anaesthesia. Rapid cooling induces rapid death with lower levels of stress than some

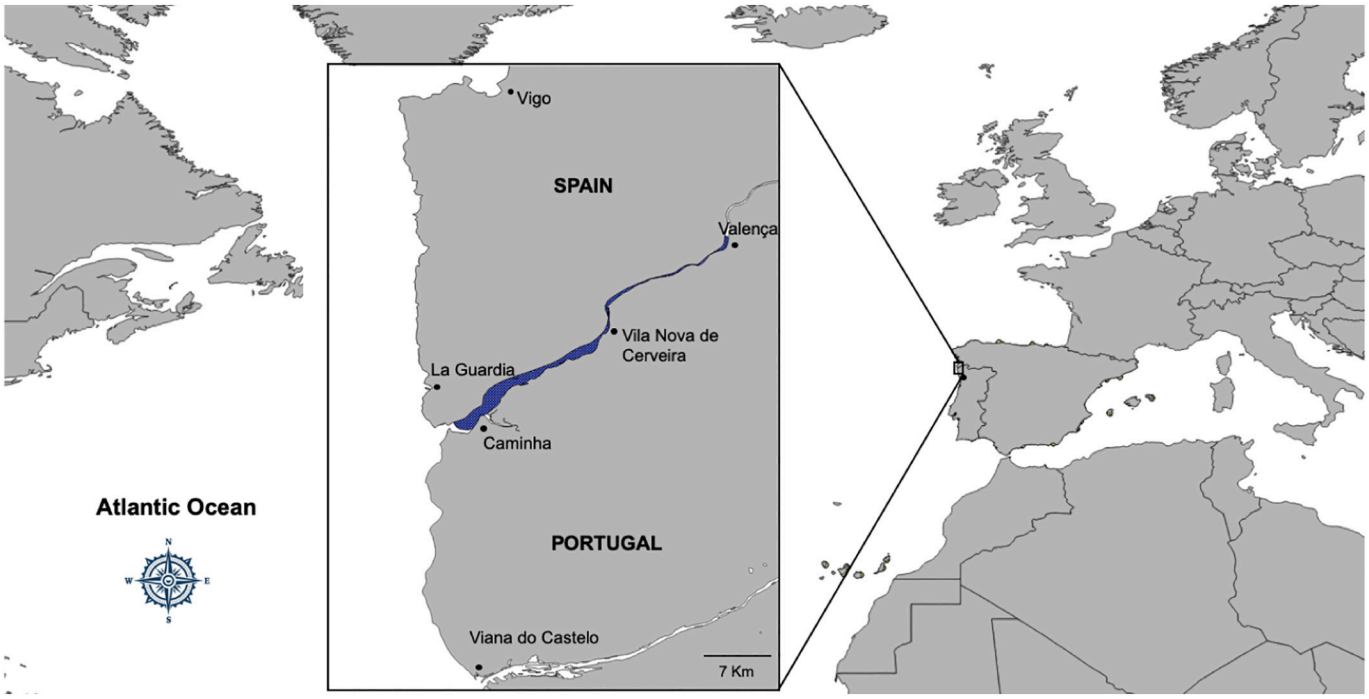

Fig. 1. Localization of the Minho River estuary with the sampling area indicated (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Number of specimens (*Cyprinus carpio*, *Mugil cephalus* and *Platichthys flesus*) analysed per species and body sites in two sampling periods (winter and summer), and mean and standard deviation of fish total length. GT – Gastrointestinal tract. Different letters after the mean indicate significant differences among species (Kruskall-Wallis test, $p \leq 0.05$). * - Indicates significant differences between fish collected in the winter and in the summer (Mann-Whitney test, $p \leq 0.05$).

Parameter	Seasons	Specimens	Specimens per species			Body sites			
		Total	*C. carpio*	*M. cephalus*	*P. flesus*	GT	Gills	Liver	Muscle
Number	Total	128	41	43	44	108	128	128	128
	Winter	60	20	20	29	50	60	60	60
	Summer	68	21	23	24	58	68	68	68
Mean length (cm)	Total	34 ± 12	45 ± 12 a	35 ± 6 b	22 ± 3 c				
	Winter	35 ± 13	46 ± 10	39 ± 5 *	20 ± 3 *				
	Summer	33 ± 12	45 ± 14	31 ± 4	24 ± 1				

chemical-based procedures and has been considered a humane practice to sacrifice fish (Wilson et al., 2009). To prevent contamination of internal organs and tissues, the whole and intact bodies of dead fish were transported to the laboratory in clean thermally isolated boxes containing frozen ice packs within the lowest time possible.

2.3. Sample preparation

Sample preparation included collection and dissection of the biological material, sample weighting, particle recovery from biological samples, particle characterization, and plastic-type identification. In all the steps special care was taken to avoid external and cross-contamination of samples and isolated particles as in Barboza et al. (2020). Briefly, all the procedures were carried out in restricted access and previously cleaned areas, the researchers used 100% cotton laboratory coats and nitrile gloves, all the materials were sterile or were carefully cleaned previously, ultra-pure water was used in all the steps requiring it including washing materials and dissection instruments, samples were covered when not being processed, procedural blanks ran along with the analysis, and contamination in the laboratory was monitored with air-exposed filters (Bessa et al., 2019; Barboza et al., 2020; Capillo et al., 2020). Only 30% of the procedural blanks (reagent blanks) showed a single fibre. Fibres, mainly blue, were found in the 640 control filters accounting for less than an item (in average) per filter. Just the fibres of similar colour and shape to those found in samples were subtracted from each sample to compensate for the atmospheric contamination.

The total length, hereafter indicated as length, and the total body weight (wet weight – ww) of each fish were recorded. From each specimen, the gastrointestinal tract (GT), two brachial arcs (one from each side of the fish), liver and dorsal muscle (hereafter indicated as muscle) were removed and isolated on ice. Afterward materials were weighted individually (Kern 572-33, Kern & Sohn GmbH, Germany) and frozen at −20 °C for later analyses of plastic particles. Table 2 indicates the number of samples for plastic analyses. For gill, liver and muscle samples, the number of samples was equal to the number of fish analysed accounting 128 samples (41 carp samples, 43 mullet samples and 44 flounder samples). The GT of 41 carps, 43 mullets and 24 flounders was analysed accounting 108 GT samples. When plastics were not found in any of the analysed samples of a fish, it was assumed that the fish did not have plastics (Barboza et al., 2020). It should be noted that this may underestimate fish contamination because the parts of the body not analysed may contain plastics. The mean of the sample (whole GT; gill, liver or muscle tissue) mass per species and body site is indicated in the supplementary material (Table S1).

Table 2
Number of samples from *Cyprinus carpio*, *Mugil cephalus* and *Platichthys flesus*, number of plastics (PL) and microplastics (MP) recovered from fish, and percentage of MP, mesoplastics (Meso-PL) and macropastics (Macro-PL). Overall – 3 species, all body sites. GT – Gastrointestinal tract. Gills – Gill samples. Liver – Liver samples. Muscle – Dorsal muscle samples. T – Total, two sampling periods together. W – Winter. S – Summer.

Parameter	Sampling period	Overall	Species			Body sites			
			C. carpio	*M. cephalus*	*P. flesus*	GT	Gills	Liver	Muscle
Number of biological samples	Total	108	41	43	24	108	128	128	128
	W, S	50, 58	20, 21	20, 23	10, 14	50, 58	60,68	60, 68	60, 68
Number of PL	Total	883	326	463	94	697	60	43	83
	W, S	372, 511	184, 142	143, 320	45, 49	285, 412	37, 23	28, 15	22, 61
Number of MP	Total	854	313	448	93	671	59	43	81
	W, S	352, 502	174, 139	133, 315	45, 48	266, 405	36, 23	28, 15	22, 59
MP (%)	Total	97	96	97	99	96	98	100	98
Meso-PL (%)	Total	3	3	2	1	3	2	0	2
Macro-PL (%)	Total	0.7	0.6	0.9	0	0.7	0	0	0

2.4. Plastic extraction, isolation and characterization

The sample preparation towards the recovery of plastics followed the procedures described in Barboza et al. (2020) with punctual adaptations. Briefly, a 10% KOH solution was prepared in ultra-pure water and a volume corresponding to three-fold the biological material was added to each sample. The mixture was digested by incubation at 60 °C for 24 h (GT, liver and muscle samples) or at 40 °C for 72 h (gill samples) in an oven (Drying oven EV50, Raypa, Spain). After digestion, the obtained solution with particulate matter was filtered through glass-microfiber filter membranes 1.2 μm (Munktell & Filtrak GmbH, Germany) using vacuum conditions (pump Millipore WP6122050, Merck KGaA, Darmstadt, Germany). Each filter was put in a glass Petri dish that was closed and dried (24 h at 40 °C in an oven - Binder BD 53, Tuttlingen, Germany). Filters were analysed in a stereomicroscope (Nikon SMZ800, Japan) and images of all the particles were recorded (Nikon DS-Fi1, Japan).

Particles retained on the filters were further analysed and measured according to their longest dimension using image analysis software (ImageJ software, https://imagej.nih.gov/ij/). The potential plastic nature of the particles was inferred by stereomicroscope and microscope observation, image analyses and comparison with particles previously identified as being plastic by Fourier Transformed Infra-Red spectroscopy (FT-IR) analysis. Seventy percent of the particles recovered from fish samples were further FT-IR analysed to investigate their potential plastic nature and polymer type. FT-IR analyses were carried out using a PerkinElmer Spotlight 200i FT-IR Imaging System equipped with a mercury cadmium telluride (MCT) array detector cooled by liquid nitrogen. Spectra were collected in reflectance mode with measurement resolution set at 4 cm^{-1} ranging from 4000 to 600 cm^{-1} with a minimum of 10 scans. The identification of the polymer type was performed by comparison of each spectrum with polymer and additives (coating and paints) libraries made available by PerkinElmer. The spectrum under analysis needed to match the reference spectrum for more than 70% to be accepted.

Plastic particles were sorted and quantified by shape in three groups (Barboza et al., 2020): fibres (thin and elongated particles), fragments (particles with an irregular shape), and pellets (spherical or ovoid regular-shaped particles). Plastics were also quantified by colour according the following categories: white/whitish, transparent, blue/blueish, black, grey, brown, red/reddish (including pink and orange), yellow, green, and others (e.g. multi-colour). Regarding size, plastics were classified as microplastics (< 100 - 4999 μm), mesoplastics (5000 - 9999 μm) or macroplastics (≥ 10,000 μm).

2.5. Calculations and statistical analyses

The concentrations of plastics (all plastic particles together) were expressed as the number of plastic items per individual fish (PL/fish) or per tissue wet weight (PL/g). The mean concentrations of particles and the percentages and proportions of fish with plastics, MP, mesoplastics (Meso-PL) and macroplastics (Macro-PL) were calculated in relation to the total number of fish analysed. The overall mean (all species and body sites), the total means per species, and the total mean of plastics, MP, Meso-PL and Macro-PL per species and in the GT were calculated for fish with GT analyses only.

As size may contribute to differences in plastic concentrations within and among fish populations (e.g. Abbasi et al., 2018; McNeish et al., 2018; Pegado et al., 2018), the correlation between fish length and the number of plastic items recovered from fish was investigated. Fish total length was used as indicative of age instead of body weight because is less influenced by environmental factors (Perugini et al., 2014). The correlation between fish length and the number of plastics in each body site was investigated through the analyses of overall data (all fish from the three species per body site), and the data of each species per body site separately. The following correlations were also investigated: sample weight and the number of plastics recovered; sample weight and the number of plastics recovered normalized by sample weight; predominant shape of plastics and each of the predominant polymer types; and predominant colour and each of the predominant polymer types. Comparable analyses were done to investigate the potential correlation between sample weight and plastic items. The Spearman's correlation coefficient was used in all the correlation analyses.

The Fisher exact test was used to compare the proportions of fish with and without plastics, MP, Meso-PL or Macro-PL between pairs of species. It was also used to compare the proportions of fish with and without plastics or MP among fish collected in the winter and in the summer.

Each of the other variables was checked for normal distribution (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test). As for the most part of them, normal distribution and/or homogeneity of variances could not be achieved even after data transformation, nonparametric statistical analyses were used. Plastic, MP or Meso-PL concentrations among the three fish species were compared using the Kruskal-Wallis test followed by a nonparametric Tukey-type test (Zar, 1999). The concentrations of plastics or MP in fish collected in the summer and winter were compared through the Mann-Whitney test. This test was also used to compare Meso-PL or Macro-PL concentrations between two species.

The significance level was 0.05. The nonparametric Tukey-type test was carried out in a Microsoft Excel file. All the other statistical analyses were done using the SPSS statistic package (version 26).

3. Results

The means of fish length per species and seasons are shown in Table 1. Significant length differences among species were found (H_2 = 91.540, $p < 0.001$) and all the species were different. There were also significant differences of length between fish collected in the winter and summer in *M. cephalus* (U = 41.500, p < 0.001) and *P. flesus* (U =

84.500, p < 0.001). Mullets had higher length in the winter than in the summer, whereas the opposite was found for flounders. There were no significant differences between carps collected in different sampling periods (U = 199.500, $p = 0.784$).

3.1. Number, characteristics and types of particles recovered from fish

The total number of particles recovered from the analysed fish samples amounted 1289, from which 69% were plastics and 31% were other types of particles (e.g. sediment particles, organic materials, pigments and other chemicals, including some used in the plastic industry). Table 2 shows the number and size of plastics recovered from fish samples. The number of plastics found in the GT encompassing data from the three species exceeded largely the numbers found in gill, liver and muscle samples. The size of the plastics ranged from 41 to 30,000 μm with MP accounting for 97% of the total number of plastics recovered from fish. The size-ranges of the plastics per body site were: 41–30,000 μm in the GT; 159–5810 μm in gills; 143–4841 μm in the liver; and 63–5810 μm in the muscle.

Thirty-six plastic polymers were identified by FT-IR analysis (Fig. 2a). The six most common polymers were rayon (22%), polyester (17%), polyethylene (10%), polyacrylate (10%), polypropylene (7%) and cellulose acetate (7%). Thirteen polymers were at percentages between 4% and 0.5%, and several others with less than 0.5% each (e.g. cellulose modified, ethylene vinyl acetate copolymer resin, fluorinated ethylene propylene, methacrylate resin, acrylonitrile-butadiene-styrene resin, cellophane, phenolic resin, rubber, among others). The analysis was inconclusive for 0.5% of the total number of particles analysed by FT-IR. Fig. 3 shows the spectra of the six most common polymers found.

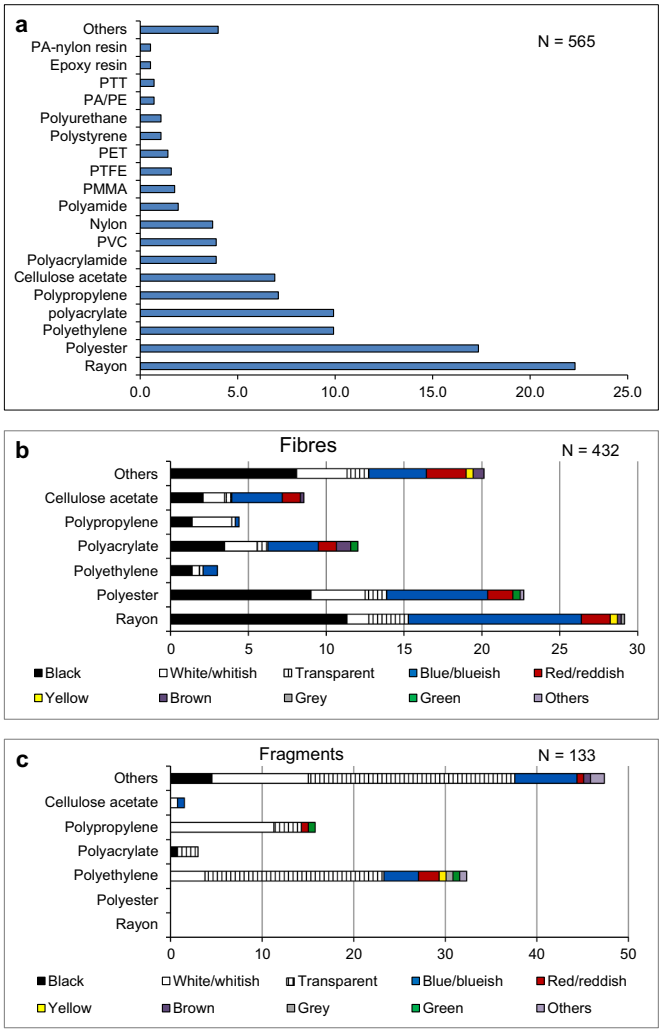

Fig. 2. Percentage of plastic polymers identified by FT-IR analyses among plastic particles recovered from Minho estuary fish (a). Main colour categories of fibres (b) and fragments (c) in relation to the main polymers indentified by FT-IR analyses. PVC – polyvinyl chloride; PMMA - polymethyl methacrylate; PTFE - polytetrafluoroethylene; PET - polyethylene terephthalate; PA/PE - polyamide/polyethylene; PTT – polytrimethylene terephthalate; PA-nylon resin – polyamide/nylon resin.

Among the 883 plastics recovered, 84% were fibres and 16% were fragments (Fig. 4a). A similar pattern was observed in each species, with fibres being 80% in carps, 87% in mullets, and 82% in flounders. Plastics were included in ten colour categories (Fig. 4b): black (45%), blue/blueish (19%), white/whitish (16%), transparent (11%), red/reddish (6%), brown (1%), yellow (0.7%), grey (0.5%), green (0.7%), and others (0.6%). In all the species together, the most common colour categories were black, white/whitish and blue/blueish. The colours of the main polymers differed with the shape, being more diverse in fibres (Fig. 2b) than in fragments (Fig. 2c). When the data of fibres and fragments were analysed together, the following correlations were significant and positive but weak ($r < 0.4$, $p < 0.05$, Table S2): shape vs rayon, shape vs polyester, shape vs polyacrylate, shape vs cellulose acetate, black colour vs rayon, black colour vs polyester, and white/whitish colour vs polypropylene, transparent colour vs polyethylene. Regarding fibres, there were two significant and positive but weak correlations: white/whitish colour vs polypropylene, and blue/blueish colour vs rayon. All the other correlations were not significant ($p > 0.05$) or were negative (Table S2).

The plastics recovered from fish were included in eight size classes (Fig. 4c). The most representative ones were 500-1499 μm, 150-499 μm and 1500-2999 μm, either considering all the fish together or the total number of plastics per species. Fig. 5 shows examples of the plastic particles recovered from fish.

3.2. Variation of the number of plastics with sample weight or fish length

Significant and positive correlations between the number of plastics in the GT and the sample weight were found through the analyses of the data of all species together ($N = 108$, $r = 0.656$, $p < 0.001$), and of *C. carpio* ($N = 41$, $r = 0.601$, $p < 0.001$) and of *M. cephalus* ($N = 43$, $r = 0.322$, $p = 0.035$) separately. All the other correlations between the plastic number and sample weight were not significant ($p > 0.05$) or were negative and weak, including when the number of plastics was standardized per sample weight (Table S3).

Significant and positive correlations between plastic number and fish length were obtained for: number of plastics in all species and body sites versus fish length ($N = 108$; $r = 0.519$, $p < 0.001$); number of plastics in the GT considering data of all species together versus fish length ($N = 108$; normalized by sample weight: $N = 108$, $r = 0.341$, $p < 0.001$; no normalized: $r = 0.526$, $p < 0.001$), in *C. carpio* (no normalized: $N = 41$, $r = 0.601$, $p < 0.001$) and in *M. cephalus* (normalized: $N = 43$, $r = 0.362$, $p = 0.017$). All the other correlations regarding fish length and plastic number were not significant ($p < 0.05$) or were negative and weak (Table S4).

3.3. General fish contamination by plastics per species and body site

The overall (all species and body sites) and total (per species or body site) percentages of fish having at least one plastic, MP, Meso-PL or Macro-PL are shown in Table 3. The overall percentage of fish with plastics and MP (94%) was considerable higher than the percentage of fish with Meso-PL and Macro-PL. Carps and mullets had higher percentages of fish with plastics, MP and Meso-PL than flounders. Macro-PL were only found in carps and mullets with no significant differences in the percentage of fish with this type of particles between the two species. Considering the data of all the species together per body site, the total percentage of fish with plastics or MP in the GT was 89%, and the

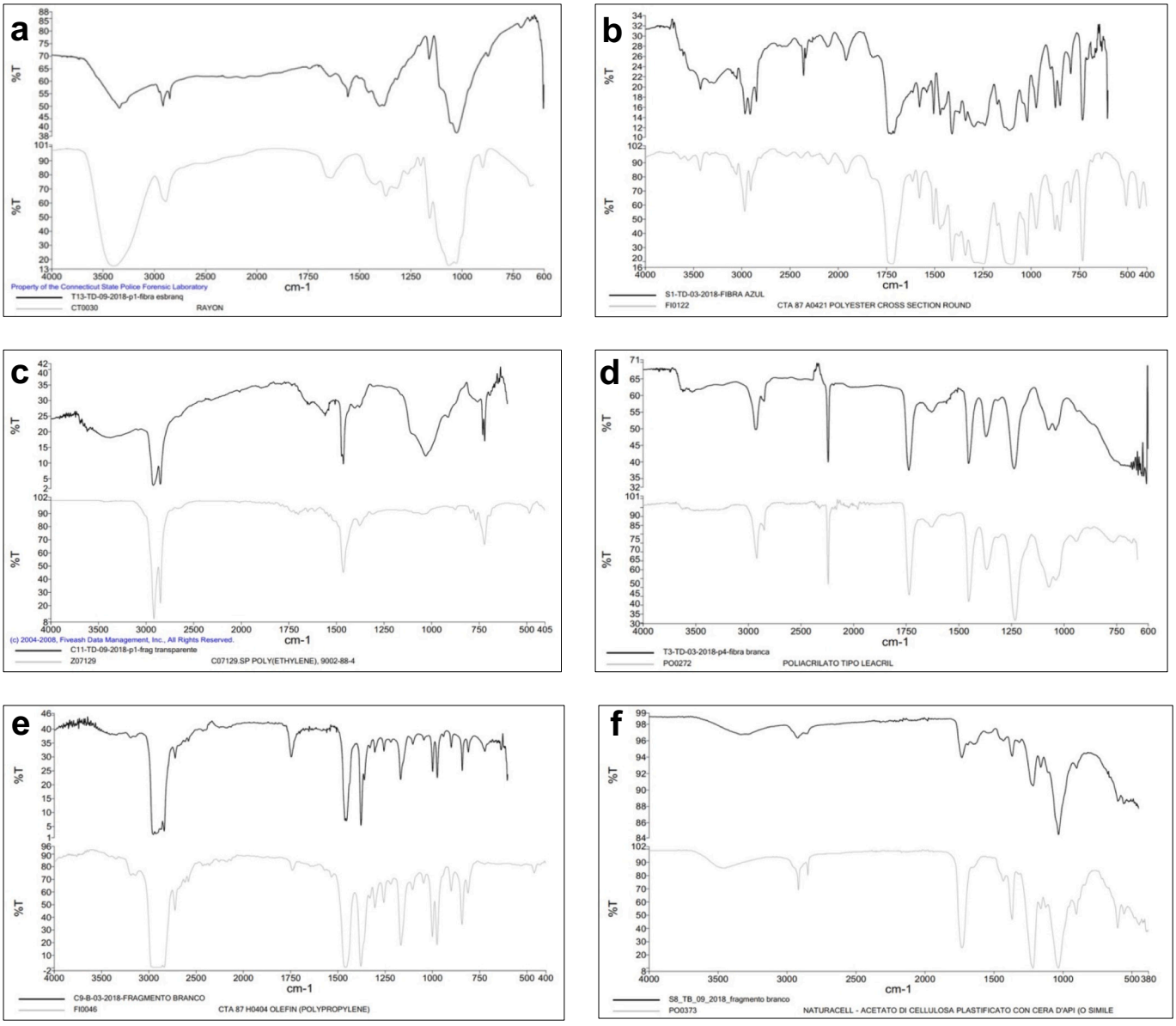

Fig. 3. Representative spectra of the plastic polymers most commonly found in Minho estuary fish: rayon (a), polyester (b), polyethylene (c), polyacrylate (d), polypropylene (e) and cellulose acetate (f).

corresponding values in gills, liver and muscle were much lower.

The overall and total concentrations of plastics, MP, Meso-PL and Macro-PL in fish are shown in Table 4 and the detailed results of statistical analyses are indicated in Table S5. There were significant differences in the total concentration of plastics and MP among species, with carps and mullets having higher concentrations than flounders. In all the species, the concentration of MP was considerably higher than those of Meso-PL and Macro-PL. There were no significant differences in the concentrations of Meso-PL among the three species nor in the Macro-PL concentrations between carps and mullets. Among body sites, the GT had the highest total number of plastics and MP per fish, whereas the liver had the highest concentration when expressed per sample weight.

3.4. General fish contamination by plastics in different sampling periods

The percentage of fish with plastics or MP in the winter and summer per species and body sites are indicated in Table 3. Significant differences were only found in the liver, with more fish having plastics and MP in the winter than in the summer.

The overall concentrations of plastics and MP normalized per sample weight, and the total concentration of MP in the liver (either expressed per fish or per sample weight) were higher in the winter than in the summer (Table 4 and Table S6). The number of fish with Meso-PL or Macro-PL and the mean concentrations were very low, therefore comparisons between sampling periods were not relevant.

3.5. Partitioning of plastics in fish body sites in distinct species

The percentages of fish with plastics or MP in the GT per species and the corresponding concentrations are indicated in Table 5. MP were found in the GT of all species, whereas Meso-PL and Macro-PL were not found in the GT of flounders. Mullets and carps had higher percentage of fish with plastics and MP in the GT than flounders, and the percentage of fish with Macro-PL in the GT was higher in mullets than in carps. Regarding concentrations, carps and mullets had higher number of plastics and MP per fish in the GT than flounders (Table 5). However, when the concentrations of plastics and MP in the GT were expressed per sample weight, there were no significant differences among species

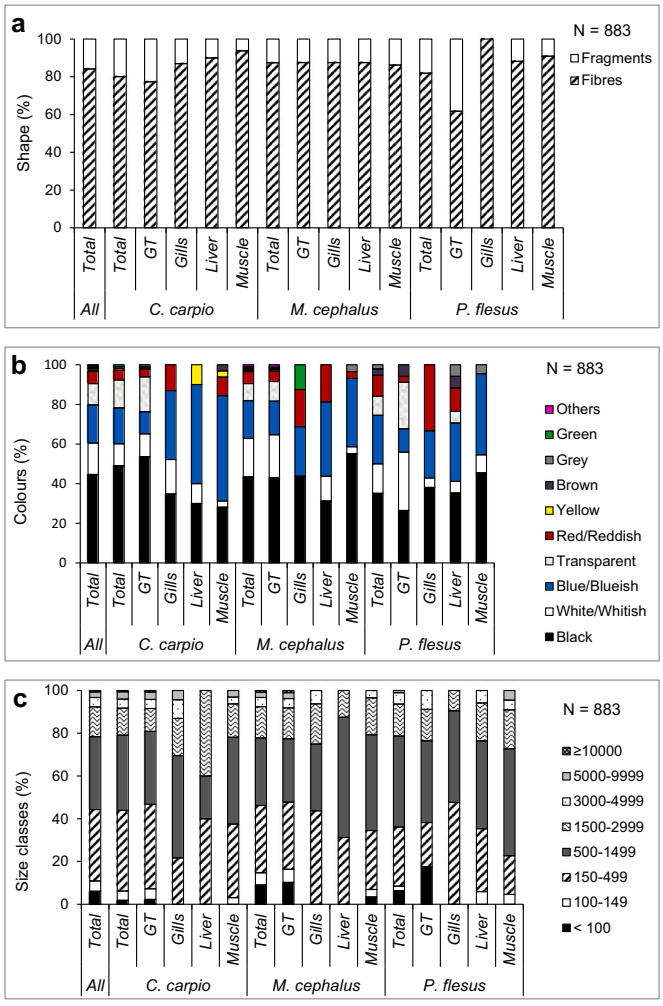

Fig. 4. Shape, colours and size of the plastic particles recovered from fish. (a) - Percentage of fibres and fragments in all the fish (All), per species in all body locations (Total), gastrointestinal tract (GT), gills (Gills), and dorsal muscle (Muscle). (b) – Percentage of plastic items per colour category. (c) – Percentage of plastic items per size class. Number of plastic items recovered: 883 (all fish); 326 in *C. carpio* (261 in the GT, 23 in gills, 10 in the liver, 32 in the muscle); 463 in *M. cephalus* (402 in the GT, 16 in gills, 16 in the liver, 29 in the muscle); 94 in *P. flesus* (34 in the GT, 21 in gills, 17 in the liver, 22 in the muscle).

(Tables 5 and S5). No significant differences in Meso-PL or in Macro-PL concentrations in the GT between carps and mullets were found. In all the species, most of the plastics isolated from the GT were fibres (Fig. 4a). The class sizes and colour categories of the plastics from the GT of the three species are shown in Fig. 4. In the GT, the larger plastics were fibres with size up to 30 mm, and the highest number of plastics found in the GT of a single fish (mullet) was 41 (Table 5).

In all the species, the percentages of fish with plastics and MP in gills were lower than in the GT (Table 5), as well as the number of plastics and MP in gills per fish. However, the pattern changed when the concentrations were expressed per sample weight. There were no significant differences in the percentage of fish with plastics or MP in gills among species, and similar results were found for the concentrations of these particles in gills (Tables 5 and S5). Meso-PL were found in gills of a reduced percentage of carps at low concentrations but not in gills of mullets or flounders. Macro-PL were not found in gills. In all species, fibres were the most common shape of the plastics recovered from gills (Fig. 4a), and there were some differences in colour categories (Fig. 4b) and size classes (Fig. 4c) among species. In gills, the larger plastics were fibres with size up to 5810 μm, and the highest number of plastics recovered from the gills of a single fish (carp) was 9 (Table 5).

All the plastics found in liver samples were MP. In all the species, the percentage of fish with MP in the liver was much lower than the corresponding value in the GT, was close to the values found in gills, and there were no significant differences in the percentage of fish with MP in gills among species (Table 5). Regarding hepatic concentrations, the number of MP per fish was considerably lower in the liver than in GT but the trend changed when the concentrations were expressed per sample weight. There were no significant differences in the hepatic concentrations of MP among species (Tables 5 and S5). In all the species, the MP recovered from fish liver were mainly fibres (Fig. 4a) with the colour categories and size-classes shown in Fig. 4. In the liver, the larger MP were fibres with size up to 4841 μm, and the highest number of MP recovered from the liver of a single fish (carp) was 4 (Table 5).

The percentages of fish with plastics or MP in the muscle were much lower than the corresponding values in the GT, were higher than in gills and liver, and there were no significant differences among species (Table 5). Regarding concentrations, the number of plastics and MP per fish in the muscle were much lower than in the GT, and were close to those recorded in gills and liver (Table 5). When expressed per sample weight, the concentrations in all body sites were close. There were no significant differences in the muscle concentrations of plastics among species (Tables 5 and S5). In all the species, fibres were the most common shape of the plastics recovered from muscle samples (Fig. 4a). The colour categories and size classes of the plastics isolated from the muscle per species are shown in Fig. 4. The highest number of plastics recovered from the muscle of a single fish (carp) was 8, fibres were larger than fragments, and the largest fibre had 5810 μm long (Table 5).

4. Discussion

4.1. High variety of plastic particles in Minho estuary fish

The 36 plastic polymers, ten colour categories, and eight size classes of the plastic particles recovered from the Minho estuary fish illustrate their diversity. However, the large majority of the plastics was MP (97%) with the shape of fibres (84%). Diversity was mainly related to colour, size categories and polymer type.

The size range of the plastics found in Minho estuary fish (0.041–30 mm) is among the wider ranges previously documented in plastics recovered from fish, such as 0.130–14.3 mm in fish from the English Channel (Lusher et al., 2013) and 0.180-50,000 mm in fish from the North Sea and the Baltic Sea (Rummel et al., 2016). It is also higher than the size range of the plastics recovered from fish collected in the Mondego River estuary (≤1–5 mm, Bessa et al., 2018) also located in the Portuguese coast but in this study only MP were investigated. The size range among the plastics from Minho estuary fish can be even wider as very small MP may have not been detected or have being lost during sample processing, as previously suggested (Roch et al., 2019). The number of polymer types identified in the plastics recovered from Minho estuary fish exceeded the 26 polymer types observed in plastics in several species from coastal and fresh waters in China (Jabeen et al., 2017), which is already a very high number relatively to other studies.

The predominance of MP being mainly fibres, some of the polymers (e.g. polypropylene, polyethylene and polyamide) and colours (e.g. transparent, black, blue, red) found in Minho estuary fish are in line with the characteristics reported for MP found in sediment samples from the continental shelf adjacent to the Minho River estuary and from nearby Rías Baixas, Galicia, Spain (Carretero et al., 2021). Predominance of fibres and diversity of size, polymers and colours were also documented in MP isolated from fish of the Mondego estuary (Bessa et al., 2018). High MP diversity and abundance of fibres were also found in water from the Douro River estuary, a close estuary in the NW Portuguese coast (Rodrigues et al., 2019). Fibres with size and polymer diversity were also documented in fish from NE Atlantic Portuguese waters (Neves et al., 2015; Barboza et al., 2020; Lopes et al., 2020), in water and

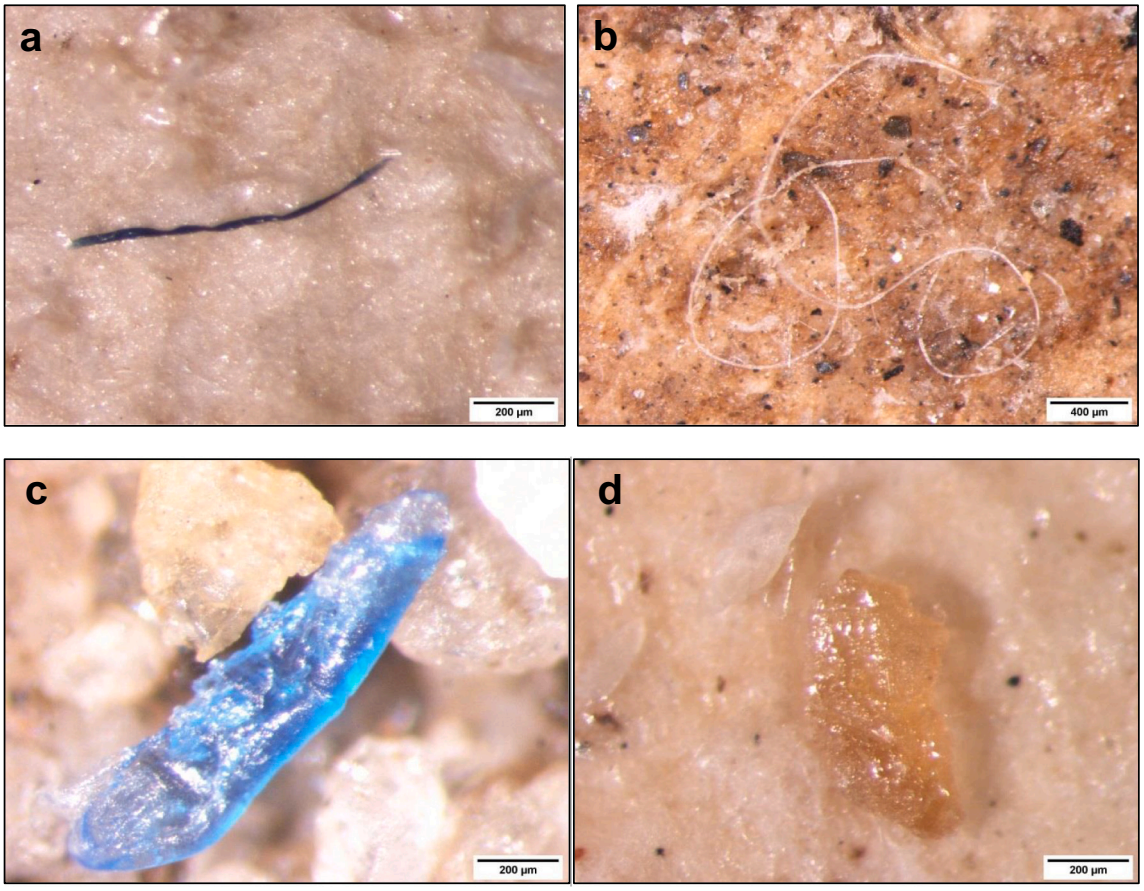

Fig. 5. Examples of microplastics recovered from fish: (a) fibre from *Cyprinus carpio* liver; (b) fibre from *C. carpio* gastrointestinal tract; (c) fragment recovered from *Mugil cephalus* gastrointestinal tract; d) fragment from *C. carpio* muscle.

Table 3
Percentage of fish with plastics (PL), microplastics (MP), mesoplastics (Meso-PL) and macroplastics (Macro-PL) per species and body sites. Overall – all the species together. GT – gastrointestinal tract. Gills – gill samples. Liver – liver samples. Muscle – dorsal muscle samples. Total – fish collected in the winter and summer together. Winter – fish collected in the winter. Summer – fish collected in the summer. NF – not found. Different letters indicate significant differences in the proportion of fish with and without PL or MP between pairs of species (Fisher exact test, $p \leq 0.05$). * indicates significant differences in the proportion of fish with and without PL or MP between the winter and the summer (Fisher exact test, $p \leq 0.05$).

Fish with plastics (%)	Sampling period	Overall	Species			Body sites			
			C. carpio	*M. cephalus*	*P. flesus*	GT	Gills	Liver	Muscle
PL	Total	94	98 a	100 a	79 b	89	27	23	35
	W, S	94, 95	100, 95	100, 100	70, 86	90, 88	32, 22	32, 15*	30, 40
MP	Total	94	98 a	100 a	79 b	89	26	23	34
	W, S	94, 95	100, 95	100, 100	70, 86	90, 88	30, 22	32, 15*	30, 8
Meso-PL	Total	17	24 a	16 a	4 b	15	1	NF	2
Macro-PL (%)	Total	6	7	9	NF	6	NF	NF	NF

fish from other areas of the Atlantic Ocean (e.g. Lusher et al., 2013, 2014; Murphy et al., 2017; Wieczorek et al., 2018) and from other marine ecosystems all over the world (e.g. Avio et al., 2017; Bråte et al., 2016; Baalkhuyur et al., 2018; Bour et al., 2018; Halstead et al., 2018; Zhang et al., 2020). This distribution pattern illustrates the global contamination of the marine environment by a high diversity of plastic particles, with MP and fibres being particularly abundant.

4.2. Potential influence of sample weight, fish length and sampling period

Lack of significant correlations or negative ones between plastic number and weight of gill, liver and muscle samples indicate that the mass of the analysed tissue did not influence significantly the number of plastic items recovered.

The results of the correlation analyses between GT weight and plastic number, and between fish length and plastic number in the GT, which were not significant or were weak when the number of particles was normalized by sample weight, point to the contribution of fish size and natural food ingestion rates to the total amount of plastics recovered from the GT, as documented in other studies (Halstead et al., 2018). The results of the correlation analyses highlight the importance of using the number of plastics standardized per sample weight to compare groups of animals minimizing the influence of factors such as size and food ingestion rates.

Previous studies relating fish length and plastic or MP concentration in the GT or stomach point to various relationships: lack of correlation (Pazos et al., 2017; McNeish et al., 2018), significant correlation, either positive (Peters and Bratton, 2016; McNeish et al., 2018; Pegado et al.,

Table 4
Mean and standard deviation of the number of plastics, microplastics, mesoplastics and macroplastics per fish (PL/fish, MP/fish, Meso-PL/fish and Macro-PL/fish, respectively) and per sample weight (PL/g, MP/g, Meso-PL/g and Macro-PL/g, respectively). Overall – 3 species, all body sites. GT – Gastrointestinal tract. Gills – Gill samples. Liver – Liver samples. Muscle – Dorsal muscle samples. T – Total, two sampling periods together. W – Winter. S – Summer. Different letters after the mean indicate significant differences among species (Kruskall-Wallis test, $p \leq 0.05$). * - Indicates significant differences between fish collected in the winter and in the summer (Mann-Whitney test, $p \leq 0.05$). NF – not found. The overall means and the total means of plastic particles in the GT and per species, and the corresponding means of mass of tissue were calculated for fish with GT analyses only.

Mean concentration	Overall	Total per species			Total (all species) per body site			
		C. carpio	*M. cephalus*	*P. flesus*	GT	Gills	Liver	Muscle
PL/fish T	8 ± 8	8 ± 6 a	11 ± 9 a	2 ± 2 b	6 ± 7	0.5 ± 1.0	0.3 ± 0.7	0.6 ± 1.2
PL/fish W	7 ± 5	9 ± 6	7 ± 4 *	2 ± 2	6 ± 5	0.6 ± 1.4	0.5 ± 0.8 *	0.4 ± 0.6
PL/fish S	9 ± 9	7 ± 6	14 ± 11	3 ± 2	7 ± 9	0.3 ± 0.7	0.2 ± 0.6	0.9 ± 1.5
PL/g T	0.22 ± 0.17	0.3 ± 0.2 a	0.2 ± 0.2 a	0.1 ± 0.1 b	0.3 ± 0.3	0.6 ± 1.5	0.7 ± 2.0	0.1 ± 0.2
PL/g W	0.24 ± 0.13 *	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.2	0.3 ± 0.3	0.4 ± 1.0	1.1 ± 2.5 *	0.1 ± 0.2
PL/g S	0.20 ± 0.19	0.3 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	0.3 ± 0.3	0.7 ± 1.8	0.3 ± 1.3	0.1 ± 0.2
MP/fish T	8 ± 7	8 ± 6 a	10 ± 9 a	2 ± 2 b	6 ± 7	0.5 ± 1.1	0.3 ± 0.7	0.6 ± 1.2
MP/fish W	7 ± 5	9 ± 6	7 ± 4 *	2 ± 2	5 ± 5	0.6 ± 1.4	0.5 ± 0.8 *	0.4 ± 0.6
MP/fish S	8 ± 9	7 ± 5	14 ± 11	3 ± 2	7 ± 9	0.3 ± 0.7	0.2 ± 0.6	0.9 ± 1.0
MP/g T	0.22 ± 0.17	0.2 ± 0.2 a	0.2 ± 0.2 a	0.1 ± 0.1 b	0.3 ± 0.3	0.6 ± 1.5	0.7 ± 2.0	0.1 ± 0.2
MP/g W	0.23 ± 0.13 *	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.2	0.3 ± 0.2	0.4 ± 1.0	1.1 ± 2.5 *	0.1 ± 0.2
MP/g S	0.20 ± 0.19	0.3 ± 0.2	0.2 ± 0.2	0.09 ± 0.09	0.3 ± 0.3	0.7 ± 1.7	0.3 ± 1.3	0.1 ± 0.2
Meso-PL/fish T	0.2 ± 0.5	0.3 ± 0.5	0.3 ± 0.7	0.04 ± 0.20	0.2 ± 0.5	0.01 ± 0.09	NF	0.02 ± 0.12
Meso-PL/g T	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.02	0.001 ± 0.004	0.01 ± 0.02	0.001 ± 0.016	NF	0.001 ± 0.012
Macro-PL/fish T	0.1 ± 0.2	0.05 ± 0.22	0.09 ± 0.29	NF	0.1 ± 0.2	NF	NF	NF
Macro-PL/g T	0.002 ± 0.008	0.002 ± 0.007	0.003 ± 0.010	NF	0.003 ± 0.014	NF	NF	NF

Table 5
Percentage of fish (*Cyprinus carpio*, *Mugil cephalus* and *Platichthys flesus*) with plastics (PL) microplastics (MP), mesoplastics (Meso-PL) and macroplastics (Macro-PL) in the gastrointestinal tract (GT), gills (gills), liver (Liver) and dorsal muscle (Musc) samples, mean (± standard deviation) of the number of these particles per fish (PL/fish, MP/fish, Meso-PL/fish and Macro-PL/fish, respectively) and per sample weight (PL/g, MP/g, Meso-PL/g and Macro-PL/g, respectively), and some characteristics of the particles in each species and body site. Highest PL – highest number of PL per fish. Min – minimal; Max – maximal. Frag – fragments. SD standard deviation. Different letters after the mean indicate significant differences among species (Kruskall Wallis, $p \leq 0.05$). Different letters after the percentages indicate significant differences between pairs of species (Fisher exact test, $p \leq 0.05$). # - only one; $ - mid point (only 2).

Parameter	*Cyprinus carpio*				*Mugil cephalus*				*Platichthys flesus*			
	GT	Gills	Liver	Musc	GT	Gills	Liver	Musc	GT	Gills	Liver	Musc
Fish with PL (%)	93 a	27	20	32	100 a	23	26	40	63 b	30	23	34
Fish with MP(%)	93 a	24	20	32	100 a	23	26	40	63 b	30	23	32
Fish with Meso-PL (%)	22	2	NF	2	16	NF	NF	NF	NF	NF	NF	2
Fish with Macro-PL (%)	7 a	NF	NF	NF	9 b	NF	NF	NF	NF	NF	NF	NF
PL/fish	6 ± 6 a	0.6 ± 1.5	0.2 ± 0.5	0.8 ± 1.6	9 ± 9 a	0.4 ± 0.8	0.4 ± 0.8	0.7 ± 1.0	1 ± 1 b	0.5 ± 0.8	0.4 ± 0.8	0.5 ± 0.9
PL/g	0.3 ± 0.3	0.6 ± 1.4	0.5 ± 1.7	0.1 ± 0.2	0.3 ± 0.2	0.3 ± 0.7	0.4 ± 0.8	0.1 ± 0.2	0.2 ± 0.2	0.8 ± 2.1	1 ± 3	0.1 ± 0.2
MP/fish	6 ± 6 a	0.5 ± 1.5	0.2 ± 0.5	0.8 ± 1.6	9 ± 9 a	0.4 ± 0.8	0.4 ± 0.8	0.7 ± 1.0	1 ± 1 b	0.5 ± 0.8	0.4 ± 0.8	0.5 ± 0.9
MP/g	0.3 ± 0.3	0.6 ± 1.4	0.5 ± 1.7	0.1 ± 0.2	0.3 ± 0.2	0.3 ± 0.7	0.4 ± 0.8	0.1 ± 0.2	0.2 ± 0.2	1 ± 2	1 ± 3	0.1 ± 0.2
Meso-PL/fish	0.2 ± 0.4	0.02 ± 0.16	NF	0.02 ± 0.16	0.3 ± 0.7	NF	NF	NF	NF	NF	NF	0.02 ± 0.15
Meso-PL/g	0.01 ± 0.02	0.02 ± 0.16	NF	0.003 ± 0.022	0.01 ± 0.02	NF	NF	NF	NF	NF	NF	0.001 ± 0.005
Macro-PL/fish	0.05 ± 0.22	NF	NF	NF	0.09 ± 0.29	NF	NF	NF	NF	NF	NF	NF
Macro-PL/g	0.003 ± 0.014	NF	NF	NF	0.005 ± 0.017	NF	NF	NF	NF	NF	NF	NF
Highest PL number	20	9	2	8	41	4	4	4	4	3	3	4
Min PL size (μm)	41	206	381	413	95	159	175	63	95	206	143	127
Fibres max size (μm)	13,900	5810	2032	5206	30,000	3175	2175	3206	4254	2063	4841	5810
Fibres mean size (μm)	1313	147	1074	1152	1326	1175	110	1164	1195	835	1272	1363
± SD (μm)	± 184	± 1430	± 747	± 1125	± 2619	± 964	± 504	± 959	± 1259	± 665	± 1226	± 1399
Frag max size (μm)	3429	4333	381	683	1016	254	397	492	2651	NF	190	714
Frag mean size (μm)	604	1889	381 #	540 $	499	206 $	270	770	770	NF	167 $	540 $
± SD (μm)	± 634	± 2136			± 436		± 189	± 838	± 838			

2018) or negative (Bessa et al., 2018), sometimes in the same study. In addition to fish size and food ingestion rates, other factors may contribute to distinct results of correlation analysis, such as differences in the bioavailability of MP among ecosystems and specific habitats of fish, in abiotic factors and other characteristics of the habitat influencing the behavior and uptake of MP by fish, in ecological traits of the species or life-cycle phase analysed, morphology of the filtering apparatus and biological features other than size and filtering rates (Peters and Bratton,

2016; McGoran et al., 2017; Pazos et al., 2017; Bessa et al., 2018; McNeish et al., 2018; Pegado et al., 2018), in procedures (e.g. analysis of whole GT or its content), among other factors.

The higher overall concentration of plastics expressed per sample weight, and the total hepatic concentration in Minho estuary fish collected in the winter than in the summer indicates increased fish contamination in the former season. As suggested for other estuaries (e.g. Rodrigues et al., 2019), likely a considerable part of the plastics found in the Minho estuary comes from upstream areas. In estuaries, MP abundance is linked to hydrological processes (Wu et al., 2020). As result of more frequent and intense rain in the region, the water flow of Minho River and its tributaries is higher and stronger in the winter than in the summer, resulting in increased water volume and decreased residence time in the estuary facilitating the elimination of some contaminants into the sea (Ribeiro et al., 2016). However, in estuaries with considerable natural vulnerability, such as the Minho estuary mainly due to its relatively small area and water volume, this positive effect may be decreased and overcome by the influence other factors also occurring in the winter, such as increased inputs of seawater caused by storms and high water dynamics at the sea, more variability in the Minho river flow due to strong dam discharges decreasing the efficacy of contaminant elimination, higher entry of contaminants dragged from upstream, and from soil lixiviation in adjacent areas (Ribeiro et al., 2016). Moreover, strong water flow can also cause the release of contaminants from the sediment into the water column, including some MP (Wu et al., 2020). This process that may be particularly important in shallow estuaries such as the Minho estuary increases the concentration of contaminants in the water column and their bioavailability (Ribeiro et al., 2016), especially to benthic and benthopelagic species. High hydrodynamics and changes in estuarine water properties (e.g. decreased salinity, lower temperature) may prevent the aggregation and sedimentation of some MP particles, maintaining them more time in the water column and increasing their bioavailability. These processes may have contributed to the higher contamination of Minho estuary fish in the winter than in the summer. Higher MP contamination when rain is more frequent and abundant causing increased river flow have been also documented in the water (Rodrigues et al., 2019) and fish (Ferreira et al., 2019) from other estuaries.

4.3. High contamination of fish by plastics, potential sources and implications

The overall percentage of fish with MP (94%) and the overall mean concentration (8 ± 7 MP/fish) found in the present study are among the highest ones documented in fish at worldwide level (Table 6). They indicate very high bioavailability of MP in the Minho estuary and very high contamination of the local populations of carps, mullets and flounders by this pollutant. These findings raise high concern on the potential adverse effects of MP pollution on the biota of this estuary that includes species with high conservational interest, such as the European eel *Anguilla anguilla*, among others (Costa-Dias et al., 2010; Sousa et al., 2008). Because several species inhabiting the Minho estuary or spending sometime in it are commercially important (Costa-Dias et al., 2010; Ribeiro et al., 2016) and consumed as food by humans, their contamination by MP raises also concern regarding food safety.

Rivers are important vehicles of plastic input into estuaries, coastal seas and oceans (Lebreton et al., 2017). Only relatively small cities and villages are located in the proximity of the Minho estuary and, hence, the Minho River, its tributaries such as the Louro River that is considerably polluted (Santos et al., 2013; Ribeiro et al., 2016), as well as local urban and industrial effluents are likely the major sources of plastics to the Minho estuary. Urbanization, tourism, fluvial transport, and recreational activities that have been increasing in the region may also contribute, as well as direct inputs of plastic objects by persons, lost fishing gear and other materials, and seawater entering the estuary during flooding, especially during spring tides and storms. These sources have been related with plastic pollution and biota contamination in other estuaries and coastal areas of the Iberian Peninsula (Antunes et al., 2018; Bessa et al., 2018; Rodrigues et al., 2019; Carretero et al., 2021; Vital et al., 2021) and other regions (Peters and Bratton, 2016; Lebreton et al., 2017; Garcia-Garin et al., 2019; Robin et al., 2020; Talley et al., 2020). MP are also present in agroecosystems (Ng et al., 2018). Therefore, agriculture fields near the margins of the Minho River estuary should not be excluded as another potential contributor. Plastic transfer from the net to the fish during fish capture may also have contributed, as previously suggested (Lusher et al., 2013).

The Minho River estuary ends in the Atlantic Ocean and, hence, many of the MP present in the estuary likely reach the coastal zone. This input may contribute to the presence of MP in sediments in front to the estuarine mouth which abundance decreases towards offshore (Carretero et al., 2021). *M. cephalus*, *P. flesus* and other species developing in the estuary and/or in the Minho River and its tributaries, such as *Anguilla anguilla* and *Petromyzon marinus* (Costa-Dias et al., 2010; Dias et al., 2019, 2020), migrate to the sea. Such species may carry river and estuarine MP to the Atlantic Ocean and contribute to the contamination of its food webs with these particles, a hypothesis that deserves further investigation.

4.4. Plastic ingestion by different species

In all the species, the GT was the main contributor to the total number of plastic particles found in fish. In *C. carpio* and *M. cephalus*, it was also the body site where the larger plastics, and the highest diversity of plastic colours and sizes were found.

Plastics present in the GT of the studied specimens may have been ingested directly from the water and/or through contaminated prey, as proposed in other studies (de Sá et al., 2015; Jovanović, 2017; Barboza et al., 2020; Talley et al., 2020). The higher percentage of specimens with plastics in the GT than in gills, and the differences on shape, size and colours point to selective ingestion of some plastics by fish and/or plastic intake through contaminated prey. All the Meso-PL and Macro-PL recovered from the GT of carps and mullets were fibres, suggesting possible ingestion of large fibres because they look like prey.

Percentages of specimens with plastics and means of plastic number per fish higher in carps and mullets than in flounders suggest differences in the likelihood of ingesting these particles. Presumably, food ingestion rates by specimens with different sizes contributed to those differences, as previously discussed and also suggested in other studies (e.g. Halstead et al., 2018). Nevertheless, differences in the morphology of the plastics ingested suggest that other factors were likely also involved.

C. carpio and *M. cephalus* are benthopelagic species that move actively through the water column, are omnivorous and have a high diversity of prey that include benthic, pelagic and plankton species (FishBase, 2021). In shallow waters, such as in the Minho River estuary, carps and mullets are likely exposed and uptake plastics through contaminated plankton, pelagic, and benthic prey, and directly from the water when moving through and across the water column. High mobility, diversity of prey, and spending considerable time in the water column may increase the exposure of fish to plastics and the likelihood of their ingestion (Rummel et al., 2016; Karami et al., 2017; Bessa et al., 2018). *P. flesus* is a demersal fish, spending the most part of the time on the top layer of the sediment or buried in it, is mainly carnivorous, preys mostly on benthic organisms (FishBase, 2021), and ingests sediments when feeding (Rummel et al., 2016; McGoran et al., 2017). Hence, the flounders analysed were likely mainly exposed to plastics present either in bottom water and sediment, and in contaminated benthic prey. Therefore, ecological traits may also have contributed to the higher percentage of fish with plastics, number of plastics per fish, and diversity of plastics in carps and mullets than in flounders. Other factors that may have also played a role are the distribution of the species along the estuary, their habitat and prey preferences, and morphological and physiological distinct features, as suggested in other studies (Rummel

Table 6
Microplastic (MP) and/or plastic (PL) contamination of fish from different regions across the world (illustrative examples from the literature). All the fish are from wild populations except if other indication is provided. N – number of fish; n – number of plastic particles. Fish with MP (%) - percentage of fish with MP, if plastics are other than MPs this will be indicated after the mean of the particles; MP/fish – mean of MP number per fish; MP/g – mean of MP number per weight of sample (g); PL/fish – mean of the total number of plastics (MP and PL) per fish; PL/g – mean of the total number of plastics (MP and PL) per weight of sample (g); L-PL/fish – mean of the number of plastics larger than 5 mm quantified separately; L-PL/g – mean of the number of plastics larger than 5 mm per weight of sample (g) quantified separately; All – all types of samples analysed; SD – standard deviation; GT – gastrointestinal tract.

Fish origin	Fish species	N	n	Body site	Fish with MP (%)	MP, PL or L-PL concentration Mean ± SD	Reference
Portugal, Atlantic coast,	3 species	120	157	GT	38	1.67 ± 0.27 MP/fish	Bessa et al., 2018
Mondego River estuary	*Platichthys flesus*	40		GT	13	0.18 ± 0.55 MP/fish	
Portugal, NE Atlantic Ocean	26 species	263	73	GT	32.7	0.27 ± 0.63 MP/fish	Neves et al., 2015
Portugal, NE Atlantic Ocean	3 species	150	368	All	49	1.0 ± 1.9–1.3 ± 2.5 MP/fish	Barboza et al., 2020
				GT	35	1.2 ± 2.0 MP/fish	
				Gills	54	0.7 ± 1.2 MP/fish	
				Muscle	32	0.054 ± 0.099 (MP/g)	
Portugal, NE Atlantic Ocean	6 species	327	193	Stomach	60-100	0.25–1.75 MP/fish (median)	Lopes et al., 2020
Spain, NE Atlantic Ocean	*Scyliorhinus canicula*	48		Stomach	4.2–20.8	1.0–1.2 ± 0.45 MP/fish	Bellas et al., 2016
United Kingdom, English Channel	10 species	504	351	GT	36	1.90 ± 0.1 MP/fish	Lusher et al., 2013
Northwest Atlantic Ocean	7 species	233	452	GT	73 (74-100)	1.8 (1.15 - 2.36) MP/fish	Wieczorek et al., 2018
Argentine, La Prata River estuary	11 species	87	1679	GT[a]	100	18.5 ± 18.9 MP/fish (fibres only)	Pazos et al., 2017
Brazil, Amazonas River estuary	46 species	189	228	GT	30	1.2 ± 5 MP/fish	Pegado et al., 2018
China, Yangtze estuary, East China Sea, South China Sea & Taihu Lake	27 species	486	2557	GT[#]	95.7-100	1.5–7.2 MP/fish, 1.5–17.2 MP/g 0.2–3.0 L-PL/fish; 0.1–3.9 L-PL/g	Jabeen et al., 2017
	Cyprinus carpio	18				2.5 ± 1.3 MP/fish; 0.5 ± 0.3 MP/g 0.5 ± 1.0 L-PL/fish; 0.1 ± 0.2 L-PL/g	
	Mugil cephalus	18				3.7 ± 1.0 MP/fish; 0.5 ± 0.2 MP/g 1.6 ± 0.5 L-PL/fish; 0.2 ± 0.1 L-PL/g	
Australia, Sydney Harbour	*Mugil cephalus*	45	76	GT	64	2.5 MP/fish	Halstead et al., 2018
South Africa, Durban Harbour	*Mugil cephalus*	70	260	GT	73	3.8 ± 4.7 PL/fish	Naidoo et al., 2016
Hong Kong, Eastern coast	*Mugil cephalus*	60	129	GT	60	4.3 ± 14.5 MP/fish	Cheung et al., 2018
Indonesia, Jacarta (estuary)	9 species	174	2063	GT	97.13	12.21 ± 9.76 MP/fish	Hastuti et al., 2019
	Mugil cephalus	27			100	10.07 ± 6.4 MP/fish	
France, English Channel, French coast estuaries	*Platichthys flesus* (juvenile)	86	149	GT	36.4-91.7	Feral: 2.04 ± 1.93 MP/fish Caged: 1.67 ± 1.43 MP/fish	Kazour et al., 2020
United Kingdom, Thames River estuary	*Platichthys flesus*	66	75	GT	71 - 90	0.33 ± 0.49–0.85 ± 1.17 MP/fish (fibres only)	McGoran et al., 2017
United Kingdom, Scotland, coastal & offshore waters	*Platichthys flesus*	47	48	GT	51	0.8 ± 0.94 (PL/fish)	Murphy et al., 2017
Mediterranean Sea	*Engraulis encrasicolus*	10	9	Liver	80	0.8 ± 0.4 MP/fish[a]	Collard et al., 2017a
China	13 species	95	83	GT	22-100	0.3 ± 0.7–5.3 ± 2.4 MP/fish	Su et al., 2019
Hangzhou Bay & Yangtze estuary				Gills	22-78	0.3 ± 0.5–2.6 ± 1.6 MP/fish	
						0.1 ± 0.1–5.4 ± 3.9 MP/g	
				Liver		considered free of MPs	
				Muscle		considered free of MPs	
Iran, Persian Gulf Northeast	4 species	71		Muscle		0.57 ± 0.17 to 1.85 ± 0.46 MP/g	Akhbarizadeh et al., 2018
Iran, Persian Gulf & Musa estuary	4 species	44	734	All		12–21.8 MP/fish	Abbasi et al., 2018
				GT		1.5 - 2.8 MP/fish	
			180	Gills		8–27 MP number	
			86	Liver		4–24 MP number	
			132	Muscle		11 - 21 MP number	
Canada, Ontario, Lake Simcoe	7 species	69		All	99	17.0 ± 15.5 particles/fish (most MP)	McIlwraith et al., 2021
				GT		7.6 ± 7.5 particles/fish (mainly MP)	
				Fillet		8.2 ± 11.4 particles/fish (mainly MP)	
				Liver		1.5 ± 2.0 particles/fish (mainly MP)	
China, Haizhou Bay	6 species	124	514	GT		4.16 ± 0.44 MP/fish	Feng et al., 2019
(farmed fish)			746	Gills		6.08 ± 0.67 MP/fish	

[a] Calculated from the article data considering livers of fish with and without MP.

et al., 2016; Bessa et al., 2018; Barboza et al., 2020).

Higher plastic ingestion by carps and mullets than by flounders is in agreement with some studies in the literature where higher percentage of fish with plastics and higher plastic concentrations in the GT of fish spending more time in the water column than in the GT of demersal ones was documented (Rummel et al., 2016; Bessa et al., 2018). However, no significant differences between pelagic and demersal fish (Neves et al., 2015; Lusher et al., 2013; Phillips and Bonner, 2015) and higher MP content in demersal fish than in pelagic and/or benthopelagic ones (Jabeen et al., 2017; Akhbarizadeh et al., 2018) were also found. These distinct findings may be explained by differences in the availability of plastics and their distribution in the water column and top layer of the sediment among ecosystems and habitats, in environmental conditions and their variation that may influence the availability and distribution of plastics, in other ecological traits and biological features of the analysed species, in local anthropogenic pressures, in methods and procedures, among other factors (Bessa et al., 2018; Pegado et al., 2018).

As shown in Table 6, the mean number of MP per fish in the GT of carps from the Minho estuary (6 ± 6 MP/fish, ± SD) is higher than documented in *C. carpio* specimens from Chinese waters (Jabeen et al., 2017). Likely fish size differences contributed to these findings as the concentration of MP normalized per sample weight (0.3 ± 0.3 MP/g) is lower in the present study than in Jabeen et al. (2017). Chinese specimens also had higher GT concentration of plastics with more than 5 mm than Minho estuary carps. As in our study, fibres were the predominant type of plastics found in Chinese carps and a considerable variety of sizes and colours were found (Jabeen et al., 2017).

The percentage of mullets from the Minho estuary with MP in the GT (100%) and their mean (± SD) GT concentration (9 ± 9 MP/fish) are among the highest values documented in *M. cephalus* from other ecosystems (Table 6). The highest number of plastics recovered from a single fish in the present study (41) is higher than the corresponding number found in mullets (39) from Indonesia (Hastuti et al., 2019), which also had considerable MP contamination. Most of the plastics isolated from the GT of Minho estuary mullets were fibres with a high diversity of colours and size, in agreement with the characteristics of plastics found in *M. cephalus* from Australia (Halstead et al., 2018), China (Jabeen et al., 2017; Cheung et al., 2018), Indonesia (Hastuti et al., 2019) and South Africa (Naidoo et al., 2016).

The percentage of flounders from the Minho estuary that ingested MP (63%) and their mean per fish (1 ± 1 MP/fish) are in the range of values documented for the species in other ecosystems (Table 6). As in the present study, predominance of fibres over other MP shapes and several common colours (e.g. black, white, blue, red) were previously documented in flounders from the Mondego River estuary, Portugal (Bessa et al., 2018), Thames River estuary, United Kingdom (McGoran et al., 2017), and estuaries from the French coast of the English Channel (Kazour et al., 2020).

Higher percentages of fish from the three analysed species with plastics in the GT and greater means of plastic number per fish in the GT than in the gills, liver and muscle indicate that most of the plastics ingested were eliminated from the body. The absence of the three upper size classes among the MP recovered from the liver, and of the upper size class among the plastics collected from the muscle also supports the hypothesis. Likely, plastics were eliminated with faeces, as documented in previous studies (Jabeen et al., 2018). Nevertheless, while staying in the GT of fish, plastics can cause false food satiation (Boerger et al., 2010) potentially leading to decreased individual fitness (Miranda et al., 2019), GT obstruction, and several types of alterations along the GT walls, including lesions (Pedà et al., 2016; Jovanović, 2017; Jabeen et al., 2018; Espinosa et al., 2019).

4.5. Retention of plastics in gills of distinct species

Plastics are retained in fish gills during respiration through water filtration (Barboza et al., 2020). Therefore, the number of colour categories and size classes of the plastics recovered from gill samples, and the lack of significant differences in the percentage of fish with plastics in gills and in the mean concentrations of these particles in these organs among species reinforce the hypothesis of Minho water contamination by a high diversity of plastic particles. Plastic retention in gills may cause local physical damage, facilitate infections, and decrease the efficiency of respiration and other important functions of these organs, as suggested for fish exposed to MP in laboratory conditions (Collard et al., 2017b; Jabeen et al., 2018; Lu et al., 2018).

The percentages of fish with MP in gills (23 - 30%) and the mean (± SD) concentrations determined (0.4 ± 0.8 to 1 ± 1 MP/fish; 0.3 ± 0.7 to 1 ± 2 MP/g) are in the range documented in the literature (Table 6). In all the investigated species, predominance of fibres over fragments and lower size of plastics in gills than in the GT were found, in agreement with findings in other fish species (Su et al., 2019). In all the species analysed, the number of plastics per fish was higher in the GT than in gills. Opposite findings were also documented in farmed fish (Feng et al., 2019). The properties of plastic particles influence the likelihood of being retained in gills (Lu et al., 2018), and morphological and physiological differences among species influence the filtration process (Collard et al., 2017b). Such factors may contribute to differences among studies.

Fibres up to 5810 μm and fragments up to 4333 μm were retained in gills of the studied specimens. The overall size range of plastics from gills of Minho estuary fish (159–5810 μm) partially overlaps with those documented in other studies, such as: < 100 to >1000 μm (Abbasi et al., 2018); 20 - 5000 μm (Su et al., 2019); < 100 to 1501-3000 μm (Barboza et al., 2020).

4.6. Plastics in liver and dorsal muscle

MP in the liver and MP and Meso-PL in the dorsal muscle of the three species of fish analysed suggest that they were able to reach internal organs and tissues, where they were accumulated or at least retained for some time. Fibres larger than fragments in these tissues suggest that fibres may be more prone to be internalized than other plastic shapes but may be just because they are more uptaken by fish and likely more abundant in the water, as previously suggested (Akhbarizadeh et al., 2018). Because fibres were far more abundant than fragments among the plastics recovered from fish, further studies are needed to test these hypotheses.

Fibres with size up to 4841 μm in the liver and up to 5810 μm in the muscle of Minho estuary fish were found, which is not common. Previous studies documented smaller MP in the liver of wild fish from different locations, such as <100 to 250 μm (Abbasi et al., 2018) and from 124 to 439 μm (Collard et al., 2017a), and in the muscle, such as <100 to >1000 μm and < 100 to 3000 μm (Barboza et al., 2020). Fibres larger than 5000 μm were documented in the dorsal muscle of three wild fish species (Akhbarizadeh et al., 2018). A recent study with wild fish also documented particles suspected of being plastics with size ranging from 71 μm to more than 5000 μm in the liver, and from 12 μm to more than 5000 μm in fillets (McIlwraith et al., 2021).

The smaller MP found in the liver and muscle of Minho estuary fish likely resulted from their translocation in the GT, and possibly also in gills, as suggested in other studies (Karami et al., 2017; Barboza et al., 2020). Laboratory experiments with fish exposed to small MP through the water (Avio et al., 2015; Lu et al., 2016) or diet (Jabeen et al., 2018) demonstrated the presence of the tested particles in the liver. However, understanding how larger plastic particles are able to reach internal organs and tissues is a more complex issue and the mechanisms and/or conditions involved are still unrevealed (Barboza et al., 2020). Some authors (e.g. EFSA Panel on Contaminants in the Food Chain (CONTAM), 2016) consider the absorption of MP larger than 150 μm and their passage from the GT into the lymphatic and circulatory systems unlike. Other authors (e.g. Lusher et al., 2017) consider that MP larger than 0.5 mm do not cross the intact gut wall. Among the processes allowing MP to

reach internal organs and tissues that have been proposed and discussed (e.g. Collard et al., 2017a; Jabeen et al., 2017; Karami et al., 2017; Akhbarizadeh et al., 2018; Paul-Pont et al., 2018), perhaps the most likely one for relatively large MP and small Meso-PL is the passage through lesions in gastrointestinal walls, skin and/or gills, as suggested before (Akhbarizadeh et al., 2018). MP, including fibres and irregular particles, are able to cause inflammation, lesions and several other alterations in the GT and gills of fish (Pedà et al., 2016; Collard et al., 2017b; Jabeen et al., 2018). Fish from the Minho estuary had high concentrations of plastic particles in the GT and in gills and they were likely exposed to plastic pollution for a long period of time in the natural habitat. Therefore, over time, plastics may have caused lesions in protective barriers of the GT and gills allowing the internalization of relative large plastic particles. Such potential damage was not investigated as it was out of the scope of our study.

The percentages of Minho estuary fish with MP in the liver (20 - 26%) and plastics in the muscle (32 - 40%), and the corresponding mean concentrations in the liver (0.2 ± 0.5 to 0.4 ± 0.8 MP/fish; 0.4 ± 0.8 to 1 ± 3 MP/g) and muscle (0.5 ± 0.9 to 0.8 ± 1.6 PL/fish; 0.1 ± 0.2 PL/g in all species) are in the range of corresponding values available in the literature (Table 6). The contamination of dorsal muscle tissue with plastic particles found in the present study and in others with wild fish (e.g. Karami et al., 2017; Abbasi et al., 2018; Akhbarizadeh et al., 2018; Barboza et al., 2020; McIlwraith et al., 2021) raises concern in relation to human exposure to MP through seafood and potential adverse effects on human health and wellbeing. Thus, more studies are needed, particularly on the presence of plastic particles in internal organs and tissues and processes involved, and on contamination-effects relationship in wild species.

5. Summary and final remarks

A total of 883 plastic particles were recovered from 128 fish (*C. carpio*, *M. cephalus* and *P. flesus*) collected in the Minho River estuary. Plastic size ranged from 41 to 30,000 μm and exhibited various colours, such as black, blue/blueish, white/whitish, transparent and red. Most of the plastics were MP (97%) with the shape of fibres (84%). Thirty-six polymer types were identified in the plastics analysed, being the most abundant rayon, polyester, polyethylene, polyacrylate, polypropylene and cellulose acetate. These findings indicate high availability of MP fibres with diverse colours and sizes to the biota.

The plastic pollution in the Minho estuary is also reflected by the 94% overall percentage of fish with plastics (79 to 100% per species) and the overall mean (± SD) concentration of 8 ± 8 PL/fish (2 ± 2 to 11 ± 9 PL/fish per species) which are among the highest values reported in the literature. Eighty-nine percent of fish had plastics in the GT and 27% in gills, and the overall means (± SD) were 6 ± 7 and 0.5 ± 1.0 PL/fish, respectively. These results indicate very high ingestion and gill contamination by plastics, especially MP and fibers, meaning the presence of these small plastic fibres in the water, some of them being retained in fish gills during respiration, and likely also in fish prey. The species with higher number of plastics was *M. cephalus* and *P. flesus* had the lowest one. Fish contamination was higher in the winter than in the summer highlighting the importance of sampling in different periods of the year.

MP were found in liver of the three species, with 20 to 26% of the analysed specimens having fibres up to 4841 μm in this organ. The overall hepatic mean (± SD) of particles was 0.3 ± 0.7 MP/fish (0.7 ± 2.0 MP/g), and the mean (± SD) per species ranged from 0.2 ± 0.5 to 0.4 ± 0.8 MP/fish. MP were also found in the dorsal muscle of 34% of the fish analysed with an overall mean (± SD) of 0.6 ± 1.2 MP/fish (0.1 ± 0.2 MP/g), and mean (± SD) per species ranging from 0.5 ± 0.9 to 0.8 ± 1.6 MP/fish. Meso-PL were also found in 2% of carps and 2% of flounders, namely fibres up to 5810 μm, with an overall mean (± SD) concentration of 0.02 ± 0.12 MP/fish. These findings raise concern in relation to potential adverse effects of internal MP contamination on fish health and highlight the need of more studies on fish internal concentrations of plastic particles.

The high MP contamination of fish from an estuary of great conservation value and relatively low impacted by human activities reinforce the high dispersion of plastics in coastal ecosystems and their availability to biota. Conclusions from this work are in line with others studies stressing the urgent need of further research on the contamination of estuaries and their biota by plastics and on the potential resulting biological and ecological effects. Ecosystem services might be at risk and, in the case of fish and other organisms consumed by humans, food safety should be assessed.

CRediT authorship contribution statement

L. Guilhermino: Conceptualization, Methodology; Investigation – Planning; Data analysis; Writing – Original draft preparation, Review & Editing; Data curation; Visualization – Graphical abstract and Figures 2 – 4; Resources; Project administration; Funding acquisition.

A. Martins: Investigation – Plastic extraction and primary characterization; Visualization - pictures of plastic particles; Writing - Review & Editing.

C. Lopes: Investigation – Identification of plastic polymers by FTIR analysis, Visualization – spectra of plastic polymers; Writing - Review & Editing.

J. Raimundo: Methodology – FTIR analysis, Investigation – Identification of plastic polymers by FTIR analysis, Visualization – spectra of plastic polymers; Writing - Review & Editing.

L. R. Vieira: Methodology - Field work; Investigation – Fish collection and sample collection; Visualization - Fig. 1; Writing - Review & Editing.

L. G. Barboza: Investigation – Sample collection and preparation, plastic extraction and primary characterization; Writing - Review & Editing.

J. Costa: Investigation – Sample collection; Writing - Review & Editing.

C. Antunes: Methodology – Field work; Investigation – Fish collection; Writing - Review & Editing.

M. Caetano: Investigation – Identification of plastic polymers by FTIR analysis; Resources; Project administration; Funding acquisition; Writing - Review & Editing.

C. Vale: Conceptualization, Writing – Original draft preparation; Methodology – planning; Funding acquisition; Writing – Original draft preparation, Review & Editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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