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MESTRADO EM CIÊNCIAS DO MAR - RECURSOS MARINHOS
AQUACULTURA E PESCAS

Ratio between fish larvae and microplastics in the Douro estuary: temporal and spatial dynamics

Sabrina Rodrigues Magalhães

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Ratio between fish larvae and microplastics in the Douro estuary: temporal and spatial dynamics

Dissertação de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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"How much our climate, food security and economic security and, ultimately, our future on this planet depends on the health of our oceans."

Leonardo DiCaprio

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| Abstract

The quantity of plastic debris in the aquatic environments is increasing at an alarming rate, namely microplastics (MPs) (particles <5 mm) that have reached high densities in several aquatic environments. The durability, floatability of MPs allow them to disperse to other areas distant from their original sources and threatening aquatic life and ecosystems health. The early life stages of fishes are highly vulnerable to environmental factors, including anthropogenic pressures, controlling their abundance and distribution patterns. MPs are particularly dangerous to early life stages of fishes because their ingestion can induce gut blockage, limiting food intake, and expose individuals to contamination through the capacity of MPs to adsorb pollutants.

Over the last years, there are been a notorious increase of studies about MPs, although few have focused on estuarine ecosystems. Moreover, these studies tend to use different methodologies to quantify MPs in water samples, highlighting the need of standardized methodologies. Therefore, this thesis started by adapting a commonly used protocol, the NOAA protocol (National Oceanic and Atmospheric Administration) to quantify MPs in estuarine waters. To achieve this first objective, four types of plastic (Low-density Polyethylene; Polyethylene terephthalate; Polyamide; High-density Polyethylene) were used in artificial samples to test all the steps of the protocol, namely the most important phases, as sieving and drying, organic matter elimination and density separation of MPs. Our results showed that ideal temperature to dry the samples was 90 ˚C; two doses of 20 mL H_2O_2 were effective enough to degrade all the organic matter; and exactly 6 g of NaCl was necessary for the last phase of the protocol. The modifications proposed to the NOAA protocol will allow a better efficiency in the extraction and quantification of MPs, since according to the tests carried out the extraction of MPs was more efficient than 90%, regardless of the type of plastic. Hence, the proposed modifications will allow a better efficiency in MPs extraction and quantification and a less time-consuming protocol.

Investigate the MPs contamination of an urban estuary, the Douro estuary, (NW Portugal) was the focus of the second part of this thesis. Monthly sampling surveys were performed over one year, in nine stations along the horizontal salinity gradient of the Douro estuary. Sub-surface planktonic horizontal trawls were performed to collect fish larvae and MPs. Planktonic samples were sorted, and fish larvae identified

to the highest taxonomic level possible. A total of 1498 fish larvae belonging to 32 taxa were collected, with a mean abundance of 11.66 fish larvae 100 m^{-3}, with a high dominance of very few taxa, namely *Pomatoschistus microps*. MPs abundance was determined using the previously adapted protocol. Different types of MPs were observed in the Douro estuary, namely fibers, soft and hard plastic, colorful and transparent plastic, in a total of 2152 particles, with a mean abundance of 17.06 MPs per 100 m^{-3}. MPs were found in all of the samples, and results showed that in several months the number of MPs surpassed the number of fish larvae, with an average ratio of 1 fish larvae:1.5 MPs. However, there was not a temporal or spatial overlap between fish larvae and MPs, what may indicate that both are mainly influenced by different environmental variables. Such results are concerning, highlighting that a higher availability of MPs may facilitate their ingestion by fish and therefore increase possible impacts in these communities.

| Resumo

A quantidade de detritos plásticos nos ambientes aquáticos está a aumentar a um ritmo alarmante, mais concretamente, os microplásticos (MPs) (partículas <5 mm), que já atingem grandes densidades nos mais variados ecossistemas. Características como a durabilidade e flutuabilidade dos MPs permitem que estes dispersem para locais distantes de suas fontes de origem, ameaçando a vida aquática e o estado ambiental dos ecossistemas. As fases larvares dos peixes são altamente vulneráveis a fatores ambientais, incluindo pressões antropogénicas, que controlam os seus padrões de abundância e distribuição. Os MPs são particularmente perigosos nesta fase inicial de vida dos peixes, pois a sua ingestão pode provocar o bloqueio do intestino, limitando a ingestão de alimentos. Para além disso, podem também expor os organismos à contaminação, devido à capacidade que os MPs têm para adsorver poluentes.

Nos últimos anos, tem-se verificado um aumento notório de estudos sobre os MPs, mas no entanto, ainda são poucos os que focam os ecossistemas estuarinos. Por outro lado, vários trabalhos utilizam diferentes metodologias para quantificar MPs em amostras de água, salientando a necessidade de metodologias padronizadas. Assim, este estudo começou por adaptar um dos mais utilizados protocolos, o protocolo da NOAA para quantificar MPs em águas estuarinas. Para atingir este primeiro objetivo, quatro tipos de plásticos (Polietileno de baixa densidade, Politereftalato de etileno, Poliamida e Polietileno de alta densidade) foram utilizados para testar todas as etapas do protocolo, nomeadamente as três fases mais importantes como a secagem e triagem das amostras, eliminação da matéria orgânica e separação por densidade dos MPs. Segundo os testes efetuados, verificou-se que a temperatura ideal para secar as amostras foi de 90 ˚C; duas doses de 20 ml de H_2O_2 foram suficientes para degradar toda a matéria orgânica; e a quantidade exata de 6 g de NaCl foi necessária para a última fase do protocolo. As modificações propostas ao protocolo NOAA irão permitir uma melhor eficiência na extração e quantificação de MPs, uma vez que segundo os testes efetuados a extração de MPs foi sempre superior a 90%, independentemente do tipo de plástico.

O objetivo da segunda parte desta dissertação foi investigar a contaminação de um estuário urbano, o estuário do Douro (NW Portugal) por MPs, foram realizadas amostragens mensais durante um ciclo anual, em nove locais distribuídos ao longo do gradiente horizontal de salinidade do estuário do Douro. Nestas amostragens

foram efetuados arrastos horizontais subsuperficiais, utilizando a uma rede planctónica para recolher larvas de peixes e MPs. As amostras planctónicas foram triadas e as larvas de peixes identificadas até ao mais alto nível taxonómico, sempre que possível. Um total de 1498 larvas de peixes pertencentes a 32 taxa foram identificadas, com uma abundância média de 11.66 indivíduos 100 m^{-3} e com grande dominância de alguns taxa, nomeadamente *Pomatoschistus microps*. A abundância de MPs foi determinada usando o protocolo previamente adaptado. Diferentes tipos de MPs foram observados no estuário do Douro, nomeadamente fibras, plástico rígido e maleável, colorido e transparente, num total de 2152 partículas, com uma abundância média de 17.06 partículas 100m^{-3}. Foram encontrados MPs em todas as amostras, e os resultados mostraram que em alguns meses o número de MPs foi superior ao número de larvas de peixes, com uma proporção média de 1 larva de peixe:1.5 MPs. Contudo, não foi observado uma sobreposição dos padrões espaciais e temporais dos MPs e estados larvares de peixes, o que pode indicar que estes são influenciados por diferentes variáveis ambientais. Estes resultados são preocupantes, uma vez que a elevada abundância de MPs pode facilitar a sua ingestão, provocando assim impactos negativos nas comunidades de peixes.

Until the moment, the scientific output of this thesis has resulted in:

I) Rodrigues, S., Almeida, C.M.R., Silva, D., Cunha, J., Freitas, V., & Ramos, S. (2019). Microplastics contamination in an urban estuary: abundance and distribution of microplastics and fish larvae in the Douro estuary. *Science of the Total Environment*, 659, 1071-1081.

II) Rodrigues, S., Almeida, C.M.R., Silva, D., Cunha, J., Freitas, V., & Ramos, S. (2018). Microplastics contamination in an urban estuary: interactions between microplastics and ichthyoplankton in the Douro estuary. 11º Congreso Ibérico y 8º Iberoamericano de Contaminación y Toxicología Ambiental

III) Rodrigues, S., Silva, D., Ferreira, M. Almeida, C.M.R., Freitas, V., Antunes, J. & Ramos, S. (2017). Ratio between fish larvae and microplastics in the Douro estuary: temporal and spatial dynamics. IJUP – Investigação Jovem na Universidade do Porto

IV) Rodrigues, S., Silva, D., Almeida, C.M.R., Freitas, V., & Ramos, S. (2017). The ratio between fish larvae and microplastics in the Douro estuary. ABC – Aeicbas Biomedical Congress

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ANOSIM – Analysis of Similarity
ANOVA - One-way analysis of variance
AT- Ambient temperature
MANOVA – Multivariate analysis of variance
MPs - Microplastics (<5mm)
NOAA - National Oceanic and Atmospheric Administration
OM - Organic matter
PA - Polyamide
PE-HD - High-density Polyethylene
PE-LD - Low-density Polyethylene
PET - Polyethylene terephthalate
PRIMER - Plymouth Routines in Multivariate Ecological Research
SIMPER – Similarity Percentages
WFD - Water Framework Directive

Chapter 1

General Introduction

1 | Microplastics

Giving the exponential population increase and industrialization, the demand for manufactured goods has also increased. Part of these products are directly or indirectly discarded into the environment (GESAMP, 2015; 2016; Li et al., 2018), and the quantity of that waste that reaches the aquatic environment is alarmingly growing. Typically, aquatic waste includes paper, glass, metal, and plastics, which one is considered as one of the most problematic, representing 50 to 90% of the marine debris (Eerkes-Medrano et al., 2015; Li et al., 2018). Furthermore, plastic is recognized as the type of debris that most affects the aquatic ecosystems (GESAMP, 2015; 2016; Rezania et al., 2018) (Figure 1). Human plastic consumption has increased at a dramatic rate over the last years, and global plastic production reached a peak of 335 million tonnes in 2016, with Europe being the second largest producer (responsible for 19% of the production) with 60 million tonnes produced only in 2016 (PlasticsEurope, 2017) (Figure 2).

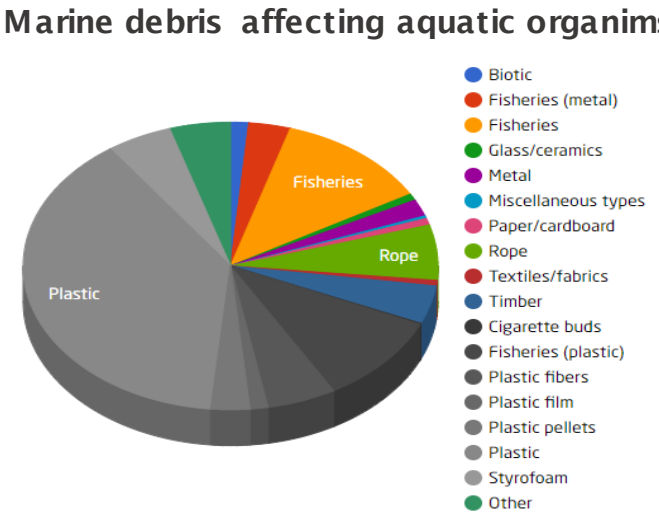

Figure 1 – Proportion and types of marine litter that affect aquatic life (from AWI-LITTERBASE).

Characteristics such as durability, floatability, and resistance increased the interest in plastics that became essential for different products and activities. These same characteristics are also responsible for plastic persistence for very long periods of time and dispersion to other areas distant from their sources (Vermeiren et al, 2016; Krelling et al, 2017). It has been suggested that the temporal and spatial distribution of plastics in aquatic environments are mainly influenced by factors such as the source and type of plastic; proximity to urban areas and high

population density; physical forces; size of the water body and water residence (Eerkes-Medrano et al., 2015). However, there are still major uncertainties about the spatial and temporal distribution patterns of plastics (GESAMP, 2015).

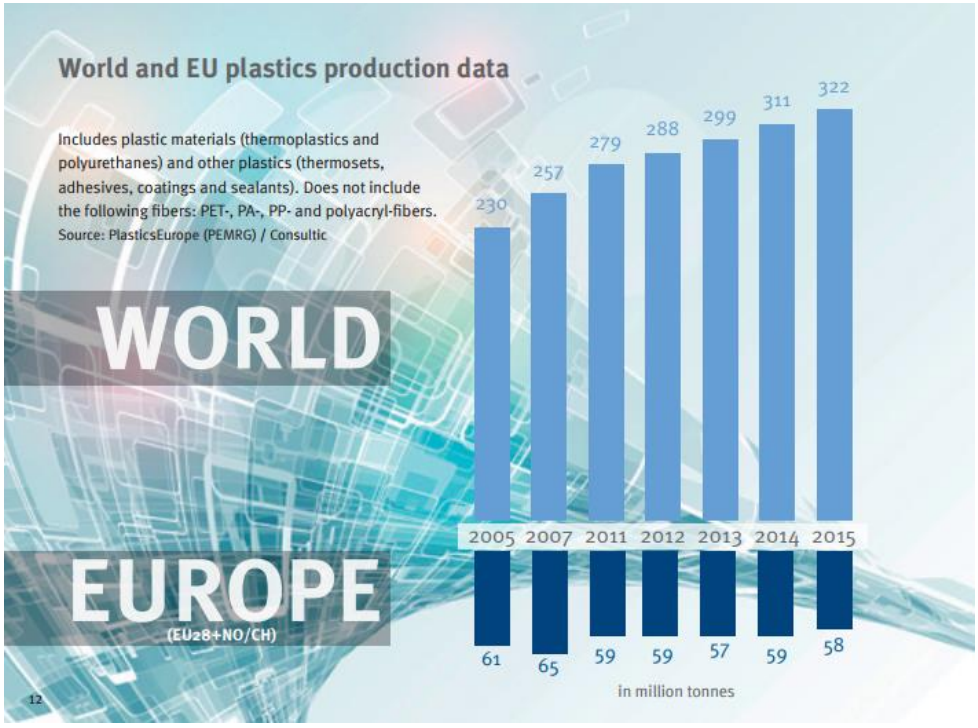

Figure 2 - Plastic production, in million tonnes, in the world and in particular in Europe, in the last decade (2005-2015) (from Plastic Europe, 2016).

Plastic debris are present in aquatic environments in a wide range of sizes, from macroplastics to microplastics. According to the National Oceanic and Atmospheric Administration (NOAA), microplastics (MPs) comprise plastic particles smaller than 5 mm and are subdivided into two categories: I) *primary microplastics*, produced intentionally in that size (e.g. microbeads or plastic production pellets); and II) *secondary microplastics*, as a consequence of the progressive fragmentation of larger plastic debris into smaller particles by photolytic, mechanical and physical degradation processes (Song et al., 2015; Masura et al., 2015; Anderson et al., 2016; Besley et al., 2017).

MPs have already been observed in different aquatic environments, such as oceans, rivers, lakes, beaches, and estuaries. The largest concentrations of microplastic particles are usually found in coastal and urban areas or near industrial sites (Claessens et al., 2011; Desforges et al., 2014). Also, rivers are indicated as one of the contamination pathways of marine MPs, since there are more and more

evidence of MPs found in estuarine environments (Browne et al., 2010; Lima et al, 2014; Gallagher etal., 2016; krelling et al., 2017).

Over the last years, there has been an increasing concern with MPs and many studies have been published (e.g. Li et al., 2018; Rezania et al., 2018; Zhang et al., 2018). These studies greatly increased the scientific knowledge about MPs and revieweing the different methodologies used to quantify MPs, although there is still some inconsistency in sampling protocols highlighting the need for standardized methodologies to sample, extract and quantify MPs (Eerkes-Medrano et al., 2015). Different sampling techniques are reported in the literature such as water pumps, plankton net, manta net, neuston net or bulk samplers at different depth and mesh size, potentially preventing comparison across studies (Li et al., 2018; Zhang et al., 2018). Moreover, different techniques to quantify MPs have been used. For example, MPs can be separated from the sample only by sieving and visual sorting or using laboratory protocols (Zhang et al., 2018). These protocols have many discrepancies and large inconsistency hinders the comparison of results. The lack of precise information about the laboratory procedures as the variety of reagents used to degrade the organic matter of the sample, different techniques used to the MPs separation through flotation can affect the results obtained. All of these discrepancies and gaps highlight the need to establish an efficient and standardized methodology to assess MPs contamination in aquatic ecosystems (Eerkes-Medrano et al., 2015; Li et al., 2018)

2 | Larval stages of fishes

In the life cycle of fishes, the early life stages are particularly vulnerable either to biotic and abiotic factors. The planktonic eggs and larval stages of fish, denominated as ichthyoplankton, play a major role in food webs, forming a link to other species of fishes (Ré & Menezes, 2009). Besides, the success of early life stages is crucial and controls the success of recruitment and ultimately the natural equilibrium of the adult fish stocks (Santos et al., 2017).

Estuaries play an important role in the life cycle of fishes, as they provide permanent habitat, nursery grounds, migration routes, refuge and feeding areas for many fish species (Elliot et al., 2007). Several marine species use estuaries as nursery habitats for their early life stages (Ramos et al., 2006a; Ramos et al., 2012; Lima et al., 2015; Amorim et al., 2017). After the marine spawning, larval fish

and/or juveniles migrate into estuaries where they stay until reaching maturation and joining the marine adult stocks (Kimirei et al., 2013; Amorim et al., 2016). The early life stages of fishes are highly controlled by environmental factors, including abiotic factors as salinity, temperature, and biotic factors as food availability and predators (Amorim et al., 2017). These factors play an important role in the density and growth of fish larvae, consequently influencing their temporal and spatial distribution patterns in estuaries (Ramos et al., 2012; 2006b). In addition, ichthyoplankton is also vulnerable to anthropogenic pressures such as climate change and pollution, which can affect their distribution, diversity and possibly increase their mortality (Steer et al., 2017).

Estuaries are traditionally located in areas of high population and intense human activity, and over the past decades have been identified as the most threatened ecosystems (Ramos et al., 2012). The proximity to urban areas and possible anthropogenic disturbances in estuaries might compromise estuarine ecological functions, namely the essential nursery function that may be reduced and compromising several marine fish species that rely on these habitats (Ramos et al., 2012).

Information related to the larval fish community, their patterns, and functioning are essential since abundance and diversity of adult fish strongly depend on the success of the individuals in the early life stages (Ramos et al., 2006b; Primo et al., 2011; Steer et al., 2017). Also, this type of information became increasingly important to understand and categorize the effects of human activities on the environment and perceiving how environmental changes can affect the survival rate of different species of fish (Primo et al., 2011).

3. MPs and Plankton

Microplastics can have a negative ecological and economic impact on ecosystems (GESAMP, 2015). MPs can pose negative effects directly or indirectly to aquatic ecosystems (Eerkes-Medrano et al., 2015), and thus establishing rates of accumulation and monitoring spatial and temporal patterns is fundamental to understand the real impact of MPs. Plankton communities are highly vulnerable to MPs, which can lead to serious physical impacts as blocked digestive tracts, debilitation, direct choking, internal wounds, ulcerating sores, blocked digestive tracts or a false sense of satiation), which can be lethal to planktonic organisms

(Cole et al., 2013; Kang et al., 2015; Eerkes-Medrano et al., 2015; de Sá et al., 2015; Avio et al., 2017; Rezania et al., 2018). MPs ingestion can difficult food digestion, decrease body condition, lead to starvation or even debilitation (Lusher, 2015; Avio et al., 2017). In addition to physical impacts, ingested MPs may act as carriers of chemicals and pollutants. These chemicals can be added to the plastic in their production or sorbed from the environment, such as persistent organic pollutants (POP) (Eerkes-Medrano et al., 2015; Li et al., 2018). Several laboratory studies already showed the potential negative effects and impact of ingestion of MPs, proving that pollutants are transferred from MPs to the organism and their organs (Barboza et al., 2018; Rainieri et al., 2018). Due to these physical and toxicological consequences, MPs may alter the planktonic natural community by decreasing growth rates, alter feeding preferences and innate behaviors, compromising plankton survival and, consequently altering planktonic food webs and the entire ecosystem health (Wright et al., 2013; Lonnstedt et al., 2016). In fact, given that many MPs are buoyant (Barnes et al., 2009; Cole et al., 2011), they will be widely available to planktonic organisms, not only including the larval stages of many commercially important fish species (Boerger et al., 2010) but also their natural preys evidencing MPs potential contamination throughout the food chains. In fact, MPs can be transferred along the food chain affecting food webs and eventually reaching human diets (Browne et al., 2008; Hidalgo-Ruz et al., 2012; Cole et al., 2013; Frias et al., 2014), highlighting the need to investigate relationships between ichthyoplankton and MPs.

4 | Objectives and outline of the thesis

The production and use of plastic are increasing worldwide, and consequently, reaching aquatic environments and threatening wildlife and human wellbeing. MPs are an emergent pollutant, and there are still several knowledge gaps, emphasizing the need for further studies seeking for better methods for MPs quantification, as well as investigating the abundance of MPs in the aquatic ecosystems.

Estuaries are considered the most productive ecosystems, but at the same time are among the most threatened (Ramos et al., 2012). The Douro estuary is an urban estuary, hosting several human activities and still supporting important ecological functions, as the nursery habitat for marine species (e.g. European Flounder, (Vasconcelos et al., 2010). The Douro estuary considered as a highly modified water body by the WFD (2000), is highly impacted by several anthropogenic pressures (Cabral et al., 2012; Ramos et al., 2015). However, there is still no information regarding the contamination by MPs and their possible impact in the planktonic communities. Thus, the overall aim of this study was to investigate the contamination of the Douro estuary by MPs and the ratio between larval stages of fishes and MPs. Also, attending to the different methodologies reported in the literature, this study also aimed to improve a standardized protocol to quantify MPs in estuarine waters. Thus, the specific objectives of this thesis were:

1) adaptation of an existing protocol (NOAA protocol - (Masura et al., 2015) to quantify MPs concentration in estuarine water samples by testing:
    - **I.** the recovery and possible degradation of MPs,
    - **II.** the efficiency of the adapted protocol to eliminate the organic matter of estuarine waters.
2) investigate the dynamics of the ratio between fish larvae and MPs by:
    - **I.** describing the spatial and seasonal patterns of larval fish assemblages in terms of abundance, diversity, and species composition;
    - **II.** evaluating the contamination of the Douro estuarine waters by MPs and analyzing spatial and season patterns of MPs;
    - **III.** study the spatial and season patterns of the ratio between fish larvae and MPs in the Douro estuary.

The present thesis is organized in four chapters, namely:

Chapter 1 includes a general introduction to the thesis theme, as well as the motivation and objectives of the present study.

Chapter 2 describes the optimized procedures and the full protocol to adapt and improve the NOAA (Masura et al., 2015) standardized protocol to determine MPs concentration in estuarine water samples. This chapter includes all the detailed methodology, indicating limitations and testing the efficiency of the protocol using laboratorial assays and 4 different types of polymers.

Chapter 3 focuses on the study of temporal and spatial patterns of larval fish assemblages and MPs in the Douro estuary, and the ratio between fish larvae and MPs.

Finally, Chapter 4 provides the final main considerations of the study and identifies future studies.

The two main studies integrated into thesis (Chapters 2 and 3) were submitted to international peer-reviewed scientific journals, with the following references:

I) Rodrigues, S.M., Almeida, C.M.R., Ramos, S. Adaption of a laboratory protocol to quantity microplastics contamination in estuarine waters. Submitted to Marine Pollution Bulletin

II) Rodrigues, S., Almeida, C.M.R., Silva, D., Cunha, J., Freitas, V., & Ramos, S. (2019). Microplastics contamination in an urban estuary: abundance and distribution of microplastics and fish larvae in the Douro estuary. *Science of the Total Environment*, 659, 1071-1081.

Chapter 2

Adaptation of a laboratory protocol to quantity microplastics contamination in estuarine waters
(submitted to Marine Pollution Bulletin)

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| Abstract

Microplastics have been documented in every aquatic environment and known as an ecological global problem. However, there is still no standardized analysis procedure for their extraction and quantification in estuarine waters. One of the first protocols was the NOAA laboratory methods and, although other efficient methods have been published, there is still no standardized method to estuarine waters. The aim of this work was to assess the effectiveness of the NOAA protocol to quantify microplastics in estuarine water and provide all the details and changes to improve the efficacy of the method. For that, four types of plastic (PE-LD; PET; PA; PE-HD) were used in artificial samples to test all the steps of the protocol. Several criteria were tested, namely: (i) quantities of H_2O_2 used for organic matter degradation; (ii) temperatures of drying samples; and (iii) density separation efficacy. With the proposed modifications, the microplastics extraction were above 90%, regardless the type of plastic, with PE-LD reaching 100% of efficiency. The new adapted protocol that we propose will allow a better efficiency in extraction and quantification of microplastics in samples from estuarine environments.

Keywords: Microplastics; Estuary; water; Contamination; Analytical procedure

1 | Introduction

Plastic production has been progressively increasing and was estimated at 335 million tons in 2016 (PlasticsEurope, 2017). Part of this plastic reaches the aquatic systems, either by being deliberately discarded or unintentionally lost, becoming part of the so-called marine debris (Galgani et al., 2010). These plastic debris eventually disintegrate into smaller particles (< 5 mm) becoming microplastics (MPs) (Thompson et al., 2004). Their small size and increasing accumulation in aquatic habitats (beaches, estuaries, rivers and deep sea) threaten the health of the ecosystems due to their toxic potential, impact on food webs and physical effect on organism's growth and survival rate, hence been considered as emergent pollutants of significant concern (Galgani et al., 2010; Van Cauwenberghe et al., 2013).

Research on the presence and impacts of MPs is growing in importance as it is a worldwide issue. Sampling, separation and quantification of MPs vary widely, being also dependent on the sampling location and sampling depth (Qiu et al., 2016). Analyses made in different locations tend to use different techniques and/or express results in different units, therefore, limiting comparisons. Many studies do not report the exact procedures used or have many discrepancies between separation and sampling steps. This methodological variation and large inconsistency hinder the comparison of data (Masura et al., 2015; Qiu et al., 2016; Andrady, 2017). All of these discrepancies highlight the need for a single efficient and standardized protocol.

One of the procedures typically used to analyze MPs in the marine environment is the NOAA protocol (Masura et al., 2015). This method is composed by three main phases: 1) sieving and drying, 2) organic matter elimination (oxidation step) and 3) density separation (Masura et al., 2015). One of the main advantages of using the NOAA protocol is that it allows the calculation of MPs concentration in a variety of metrics, including per piece, per mass, or per volume, enabling the comparison of results between different studies. However, even this protocol is not consensus. For instance, in the oxidation step, in the NOAA protocol, to eliminate organic matter (OM), the sample is subjected to hot digestion with a 30% H_2O_2 solution. However, other studies refer different concentrations of H_2O_2 (Nuelle et al., 2014; Kang et al., 2015) as being effective in degrading all the organic matter present in

the sample. Moreover, one must assure that this digestion does not destroy the MPs.

In terms of plastic debris, characteristics such as floatability, composition, and density became important, determining if MPs stay buoyant or if they sink, affecting their distribution in water (Anderson et al., 2016). One important phase in NOAA analytical procedure is the MPs separation by flotation in a dense salt solution, usually through addition of sodium chloride. More than half of all manufactured plastics have a density lower than seawater, tending to float and to go with the current. However, not every type of plastic has a density lower than salt water (Song et al., 2015; Besley et al., 2017). Some types of plastic that are also common as marine debris, tend to sink due to their higher density (Desforges et al., 2014; Frias et al., 2014; Andrady, 2017). So, knowing that different types of plastics have different densities, the density separation step of NOAA protocol might not be efficient, which can decrease the protocol's efficiency by underestimating MPs concentration as there is the possibility that denser particles sunk after the addition of the sodium chloride (Qiu et al., 2016). Another concern is the potential contamination during the laboratorial procedures by fibers present in the air (of the laboratory) or in poorly cleaned instruments, what may alter the final results.

Thus, controlling and detecting potential contaminations during the protocol is fundamental to have accurate results (Vermeiren et al., 2016).

The environmental threat posed by MPs is getting increasing attention from the scientific community. Most of the scientific publications that try to decrease inconsistencies between methodologies are usually focused on the sea surface water or beach sediments (Eerkes-Medrano et al., 2015; Li et al., 2018). The characteristics of estuarine waters are very specific and unique, differing greatly from seawater samples, mainly in terms of organic matter content, highlighting the need to establish an efficient and standardized methodology specific to estuarine waters. To overcome this lack in knowledge, in this study we aimed to adapt and improve the NOAA protocol to determine MPs concentration in estuarine water samples, with a descriptive methodology to quantify MPs, reporting the protocol's efficiency and limitations.

2 | Methods

To achieve the objectives of the study, the NOAA protocol (Masura et al., 2015) were tested with laboratory samples containing a known type and concentration of MPs. Experimental assays were designed in order to test: (i) the efficacy of the NOAA protocol in terms of MPs recovery, (ii) the possible degradation or loss of MPs, (iii) the efficacy of organic matter elimination, and also (iv) potential contamination. The samples were subjected to the NOAA protocol, namely the three most important phases, as sieving and drying (*phase 1*), organic matter elimination or oxidation step (*phase 2*), and density separation (*phase 3*). In environmental samples, mainly from estuaries, the amount of organic matter can vary greatly and may influence the time and/or quantity of reagents needed during the protocol. In that way, to test the first and second phases of NOAA protocol, it was also necessary to use field samples, which were collected from the Douro estuary (NW Portugal), to test the time of drying samples (*phase 1*) and influence of the hot digestion in the organic matter (organic matter elimination).

2.1 | MPs preparation

Based on literature, we considered as MPs all type of plastic with less than 5 mm (e.g. (Andrady, 2011; Kershaw, 2015; Masura et al., 2015). The MPs types used in this study were selected from the most common items and types of polymers of marine litter (PlasticsEurope, 2017), namely: *film plastic bags* (Low-density Polyethylene (PE-LD); specific gravity 0.93 g/cm^3), *bottle caps particles* (Polyethylene terephthalate (PET); 1.37 g/cm^3), *fishing line fibers* (polyamide (PA); 1.13 g/cm^3) and *microspheres* (High-density Polyethylene (PE-HD); 1 mm, 1.00 g/cm^3) (Figure 3). Excepting the microspheres, all the remaining types of MPs (e.g. plastics bags, fishing lines) were manually produced and sieved by 5 mm mesh-sieves to discard particles larger than 5 mm. For film plastic bags and bottle caps particles <5 mm were subdivided into 2 size types: 1) between 3 and 5 mm and 2) below 2 mm. Microspheres were commercially acquired (from Cospheric Innovations in Microtechnology) and used as received. An initial mass of 0.5 mg of each type of plastic was defined per sample. For each type of plastic 6 replicates were prepared.

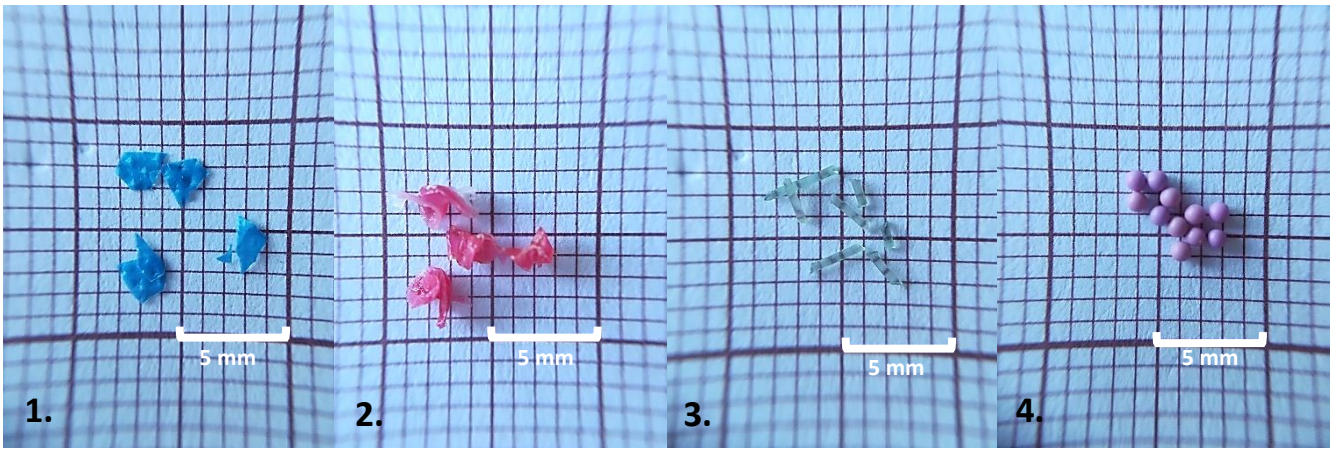

Figure 3- Different types of MPs used: 1.film plastic bags (PE-LD); 2.bottle caps particles (PET); 3.fishing line fibers (PA); 4.microspheres (PE-HD).

2.2 | Sieving and drying

The laboratory samples were sieved through a 0.03 mm mesh size (thoroughly rinsed with filtered deionized water), and beakers were rinsed with deionized water several times to transfer all residual solids, ensuring that all MP particles had been properly washed and retained in the mesh. Samples were then transferred to a new clean and weighed (to the nearest 0.1 mg) glass beaker and dried at 90 ˚C until constant weight. Samples were then weighed to confirm the total mass of dried particles.

Since the amount of organic matter can vary in real samples and this fact can possibly increase the time necessary to dry the samples, there was the need to test this NOAA protocol phase with field samples. The field samples, 3 replicates of 3 different stations of the Douro estuary, were sieved with the same procedure and transferred to a clean and weight baker. After, samples were dried at different temperatures: ambient temperature (AT), 60 ˚C, 90 ˚C, and 100 ˚C (Figure 4), to understand if the temperature can decrease the time used in the protocol step. Samples were then weighed (mg) to quantify the total mass of dried matter, including organic and inorganic matter content.

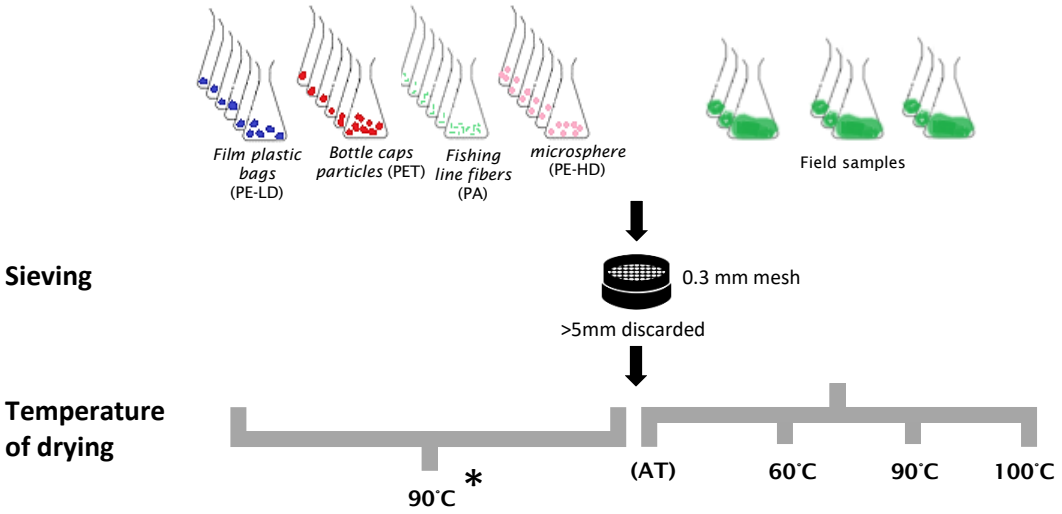

Figure 4 - Experimental draw of different temperatures tested in the first phase of protocol, sieving and drying, in laboratory samples (4 types of MPs) and field samples (3 different stations of the Douro estuary). * - indicates the temperature advise by NOAA.

2.3 | Organic matter elimination

Removing the organic matter in the samples is an important process of the NOAA protocol. H_2O_2 solution is the most common reagent to remove organic matter. In the NOAA protocol, this step is called Wet Peroxide Oxidation, and uses a mixture of a 0.05M Fe(II) solution (7.5 g of $FeSO_4.7H_2O$ (from SIGMA) with 500 mL of water and 3 mL of concentrated sulfuric acid (from SIGMA-ALDRICH) with a 30% H_2O_2 solution (from ACROS ORGANICS), being the one used in our study. In our study, we used the laboratory samples to test if the hot digestion destroys/modify the MPs; and field samples (collected in the Douro estuary) to assess the efficacy of NOAA protocol to eliminate organic matter of samples.

In the laboratory samples, this hot digestion with a H_2O_2 solution was used to assess its effect on the different types of MPs, aiming to test if this digestion destroys/modify the MPs. Therefore, 20 mL of solution was added to each beaker containing the dried MPs particles. The beakers were then, heated until 75 ˚C, as recommended in NOAA. Higher and lower temperatures (95 ˚C and 60 ˚C) were also tested (Figure 5). The NOAA protocol advises the use of 20 mL doses of 30% H_2O_2 solution as many times as needed to ensure that all organic matter, except plastic, is dissolved. In our study, one, two, and three doses of 20 mL of 30% H_2O_2 solution were added to assess if different quantities of H_2O_2 affected characteristics of the

MPs. Samples were heated until they boiled reaching the degradation point. After, the laboratory samples were kept in the hotplate for 30 minutes (as advised by NOAA).

The field samples were subjected to similar procedures of the laboratory samples, using the same reagents in the same quantities. The beakers were heated until 60 ˚C, 75 ˚C or 90 ˚C to analyze if the temperature would help in the degradation of the organic matter. In these samples, one, two, and three doses of 20 mL of H_2O_2 solution were added to ensure that all organic matter was degraded, and test if a higher number of doses was more effective. Samples were heated until they boiled reaching the degradation point.

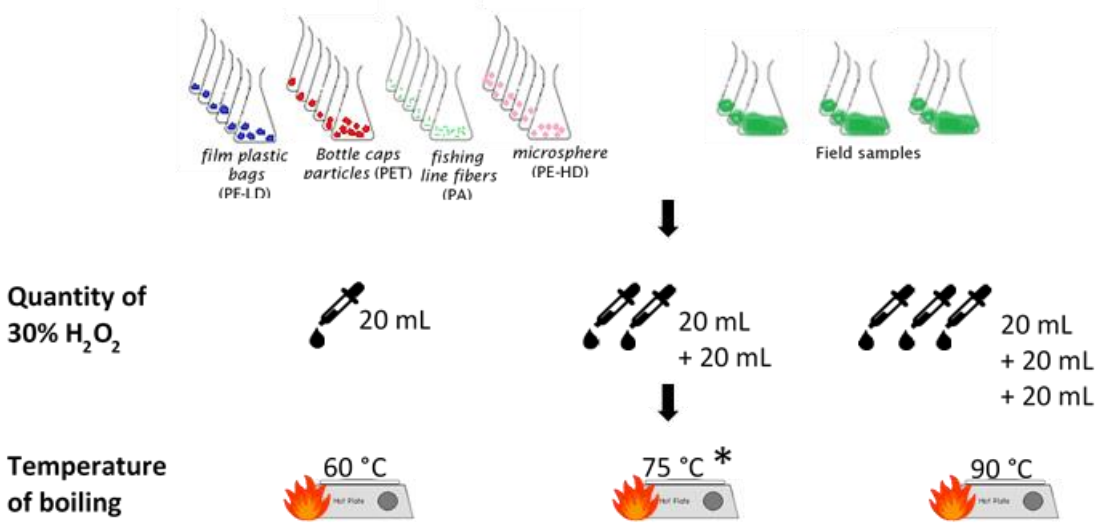

Figure 5 - Experimental draw of the quantity of 30% H_2O_2 and temperature of boiling tested in the second phase of protocol, organic matter elimination, in laboratory samples (4 types of MPs) and field samples (3 different stations of the Douro estuary). * - indicates the temperature advise by NOAA.

After, field samples were dried again at 90 ºC and weighted to quantify the inorganic content and thus estimate the degradation of organic matter content by the protocol.

2.4 | Density separation

Extraction of MPs in NOAA protocol is achieved by density separation. To make all MPs float, high density saturated solutions are normally added to the samples. Following to NOAA protocol, in the laboratory samples, a fully-saturated salt

solution was prepared by dissolving ~6 g of NaCl (from SIGMA-ALDRICH) per 20 mL of sample solution. To improve the dissolution, the mixture is then heated to 75 ˚C and mixed with a stirring magnet for 30 minutes. In the present study, additional periods of 45 minutes and 1 hour of heating and stirring were also tested (Figure 6). Afterward, the solution was transferred to the density separator, cover with aluminum foil and left to precipitate overnight. In the following morning, the floating solids were drained through a 0.03 mm mesh to guarantee that all the plastics particles were collected. The density separator was rinsed several times with deionized water to transfer all the solids to the mesh. The mesh with the MPs was then closed in a petri dish and dried in an oven at 90 ˚C.

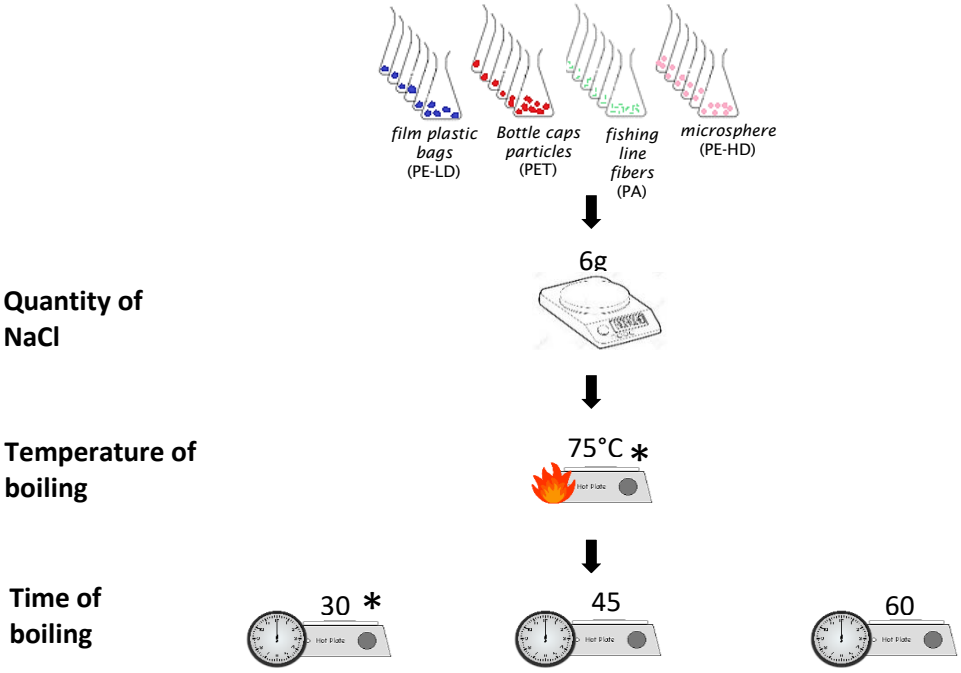

Figure 6 - Experimental draw of the quantity of NaCl, temperature and time of boiling tested in the third phase of protocol, density separation, in laboratory samples (4 types of MPs). * - indicates the temperature advise by NOAA.

Afterward, meshes were weighted and then all the solids were collected, weighted, and visually inspected under a stereomicroscope (Nikon SMZ800) to quantify the original MPs used, and to look for signs of MPs deterioration. Each type of MP was, at the beginning of the experiment, characterized by color and shape to assess if they were altered during the protocol procedures. The percentage of MP recovered was calculated.

2.5 | Contamination

To avoid contaminations during the entire proceeding, all steps were carried out inside a laminar flow cabin to avoid fiber contamination by air. Also, all the equipment used was made of glass and thoroughly rinsed with filtered deionized water before usage. Work surfaces were cleaned with 70% ethanol solution and a lab coat and gloves were worn at all times.

2.6 | Data analysis

One-way ANOVA was used to investigate significant differences between all treatments of the three protocol phases, and between types of plastic. The significance level of all statistical analyses was set at 0.05. Analyses were done with the software IBM SPSS Statistics for Windows, Version 25.0.

3 | Results and discussion

Over the past years, there has been an increasing concern about the impacts of MPs in aquatic ecosystems. Most studies are mainly focused on marine ecosystems, while freshwater and estuarine ecosystems receive very little attention. Currently, there are no unified methods for analysis of MPs in water samples (Blair et al., 2017). Although the NOAA protocol is detailed and often used, not all procedures were found as entirely viable to analyze estuarine water samples. Estuarine samples, unlike marine samples, can have significant amounts of organic matter that can vary with season (Canuel et al., 2015). This poses difficulties when eliminating all the organic matter in some of the samples, ending in a more time-consuming and less effective protocol in the last phase of visual inspection of MPs.

3.1 | Sieving and drying

According to the NOAA protocol, after collection, the water samples are filtered, and the mesh used with all the solid particles is out to dry until constant weight. The majority of studies do not mention the drying temperature or the time used to dry the initial sample, leading to a gap in the protocol and making comparisons between studies difficult (Besley et al., 2017). In our study, four temperatures were tested, based in temperatures used in other similar studies (Jayasiri et al., 2013;

Mohamed Nor et al., 2014; Nuelle et al., 2014; Enders et al., 2015; Lima et al., 2015; Rodrigues et al., 2018), to assess the ideal temperature to dry the water samples (ambient temperature, 60 ˚C, 90 ˚C and 100 ˚C). Our tests showed that when samples were dried at ambient temperature (Jayasiri et al., 2013) it was necessary an average of 48.00 ± 20.78 hours to completely dry the water. Similar, when we used 60 ˚C (Mohamed Nor et al., 2014; Nuelle et al., 2014; Enders et al., 2015; Lima et al., 2015) it was still necessary more than one day (30.66 ± 13.56 hours) until they were completely dried. Thus, these two temperatures led to an even more time-consuming protocol. However, when we used 90 ˚C (Rodrigues et al., 2018) results showed that less time was necessary to completely dry the samples. The number of hours needed to dry the samples at 90 ˚C was significantly lower than dried at 60 ˚C or ambient temperature (ANOVA $F=15.25$ $p \leq 0.01$). PE-HD MPs need 4 hours to dried, PE-LD and PA completely dried after 6 hours, and PET needs 8 hours. In field samples were necessary 10.44 ± 3.09 hours to dry the samples. With temperatures above 100 ˚C, MPs can deform, i.e. altering their initial size and shape. Thus, we consider that 90 ˚C overnight is the temperature and time more appropriate to dry estuarine water samples, in *phase 1*.

3.2 | Organic matter elimination

During the elimination of organic matter phase, it is crucial that the samples reach the boiling point, since only in that point the degradation reaction happens. To induce the boiling point, the temperature adopted by NOAA protocol, 75 ˚C, appeared to be the ideal temperature. Our tests showed that temperatures below 75 ˚C inhibited the reaction, leading to a minimum or none of the organic matter degraded. Above 75 ˚C the solution boiled too violently leading to loss of material. One limitation of the NOAA protocol was found in this step. The NOAA protocol indicates to use doses of 20 mL of 30% H_2O_2 solution as many times as needed to ensure the dissolution of the organic matter present in the sample. However, we observed that one dose of H_2O_2 solution was only effective when the amount of organic matter was minimal (Table 1). For higher amounts of organic matter, a second dose (20 mL + 20 mL) of H_2O_2 solution was necessary, and the majority of the organic matter was only degraded with two doses (20 mL + 20 mL) of H_2O_2. The percentage of organic matter degraded was significantly higher when was added two doses (20 mL + 20 mL) of H_2O_2 (ANOVA $F=80.95$, $p \leq 0.01$) (Table 1), although in some cases there was still organic matter present in solution. However, adding

a third dose of H_2O_2 solution was useless and ineffective, as it resulted in the dilution of the reagents, leading to a weak reaction between reagents and, consequently, none of the remaining organic matter was degraded. Furthermore, a third dose of H_2O_2 leads to changes in size or color in some types of MPs tested (Table 1).

Table 1 – Results of the organic matter elimination testing different quantities of 30% H2O2 in laboratory and field samples. In laboratory samples were analyzed the mean (± standard deviation) percentage of degradation, loss, or alteration of MPs and in field samples the mean (± standard deviation) percentage of organic matter elimination.

Organic matter digestion		**H_2O_2 solution**		
		20 mL	**20 mL +20 mL**	**20 mL +20 mL +20 mL**
Alteration MPs	Film plastic bags (PE-LD)	Without changes	Without changes	25% ± 0.45 changed in size (fragmentation)
	Bottle caps particles (PET)	Without changes	Without changes	Without changes
	Fishing line fibers (PA)	Without changes	Without changes	100% changed in color (to brownish color)
	Microspheres (PE-HD)	Without changes	Without changes	33% ± 0.51 changed in size (fragmentation)
Organic matter elimination	Field samples	60% ± 0.22	98% ± 0.03	0%

According to the NOAA protocol and several other studies (Free et al., 2014; Tagg et al, 2015; Su et al., 2016), a 30% H_2O_2 solution should be used to dissolve the organic matter. However, other studies have tested other concentrations of H_2O_2 to eliminate organic matter, for example 20% (Kang et al., 2015) or 35% (Nuelle et al., 2014). Also, other studies used other solutions such as a mixture of NaOH and HNO_3 (Castillo et al., 2016), a multienzymatic detergent (Rodrigues et al., 2018) or HCl solution (Desforges et al., 2014). Nuelle et al. (2014) concluded that a H_2O_2 solution was the ideal reagent to dissolve biogenic organic particles when compared with NaOH or HCl solutions. Also, Rodrigues et al. (2018) considered that wet peroxide oxidation was the most effective method in freshwater samples. In our study, 30% H_2O_2 solution (two consequently added doses) also shown to be most adequate to eliminate organic matter of the sample. Results showed that an extra dose of 30% H_2O_2 solution was not a valid option to improve the efficiency of organic matter oxidation and its elimination from samples. However, in some samples, two doses of 30% H_2O_2 solution show not be enough to degrade all the organic matter present (Figure 7). Estuarine samples, unlike marine samples, can

have relevant amounts of organic matter that may vary seasonally according to the estuarine dynamics (Canuel et al., 2015).

Figure 7 – Remains of organic matter and MPs in water estuarine sample, after density separation phase and before drying.

3.3 | Density separation

Addition of NaCl is the most frequently used approach to promote MPs flotation, including in sediment samples (Martins et al, 2011; Jayasiri et al., 2013; Nuelle et al., 2014; Song et al., 2015). According to the NOAA protocol, the exact quantity of NaCl is not important, referring around 6 g. Our tests showed that this quantity is not easily dissolved, and we observed that when the quantity of NaCl was slightly above 6 g (in our study was used 6.3 g), it was not possible to achieve full dissolution of NaCl (Table 2). Thus, when the samples were placed in the density separator, the NaCl precipitated, trapping part of the MPs, and accumulated on the bottom of the system, blocking the flow of the liquid. If the quantity of NaCl added was a little less than 6 g (in our study was used 5.7 g), all the NaCl was dissolved but a percentage of the MPs did not float (Table 2). The percentage of dissolution of NaCl was significantly lower when adding 6.3 g than 5.7 g or 6 g (ANOVA $f=59.50$; $p \leq 0.01$).

In order to improve NaCl dissolution, we tested heating the sample for 30 minutes, 45 minutes and 1 hour. Since increased time did not improve NaCl dissolution, 30 minutes was selected as the time to heat the samples. Therefore, we found that for total NaCl dissolution it was necessary to weight precisely 6 g of NaCl per 20mL of solution and heat the solution for 30 minutes while using a stirring magnet. This

procedure allowed all the microplastics to float and all the NaCl was dissolved. So, this new procedure was added to our proposed protocol.

Table 2 - Results of the density separation testing different quantities of NaCl in laboratory samples. In the laboratory samples was analyzed the mean (± standard deviation.) percentage of dissolution of NaCl and the mean (± standard deviation) percentage of MPs that float in the density * - represents samples where was impossible determine the percentage of MPs that float due the accumulation of NaCl in the bottom of the system trapping part of the MPs and blocking the flow of the liquid.

	Type of MPs	Quantity of NaCl			Type of MPs	Quantity of NaCl	
	PE-LD	5.7	100%		PE-LD	5.7	90% ± 0.05
		6.0	99% ± 0.03			6.0	98% ± 0.08
		6.3	86% ± 0.04			6.3	*
	PET	5.7	100%		PET	5.7	84% ± 0.05
		6.0	99% ± 0.01			6.0	100% ± 0.11
Dissolution		6.3	87% ± 0.05	**Floatability**		6.3	*
of NaCl	PA	5.7	100%	**of MPs**	PA	5.7	87% ± 0.06
		6.0	100% ± 0.01			6.0	93% ± 0.05
		6.3	90% ± 0.06			6.3	*
	PE-HD	5.7	100%		PE-HD	5.7	100%
		6.0	100% ± 0.01			6.0	100% ± 0.02
		6.3	85% 0.04			6.3	*

Another issue of the NOAA protocol that required improvements was the step of rinsing the beaker to transfer the sample to the density separator. The protocol advised to use deionized water to rinse particles from the beaker walls, but this step decreased the density of the separation liquid and resulted in sinking particles that had already been afloat. So, to avoid this, the minimum amount of deionized water was used whenever needed. In other studies, the deionized water was substituted with the same separation liquid for rinsing (Rodrigues et al., 2018).
In relation to the density separator (Figure 8) we also detected some issues, and some components were changed to improve the system efficiency. It was observed that the latex tube at the bottom of the separator degraded very quickly, due to the contact with oxidizing solution, requiring often replacements by a new tube or alternatively the use of a tube made from glass. The NOAA protocol advises on the use of a pinch clamp on the latex tube. However, in this study we opted to use a faucet, becoming easier to control the flow of the solution, discarding it whenever necessary.

Figure 8 – Density separator system used in this study. The system consisted in a glass funnel fitted with a latex tube on the bottom of the stem and a faucet to control de liquid flow.

According to the NOAA protocol, the solution with the microplastics should be transferred to the density separator and left overnight to ensure that all MPs float. After, in the next day, the settled solids (organic matter that wasn't degraded such as wood) are drained from the separator bottom and discarded. Only the floating solids (MPs) are collected with a mesh and posterior drying and weighted. In our study, we did not discard the settled solids, and collected and filtered them through another mesh. This additional step was used to visually evaluate if any MP was trapped in the bottom of the density separator and therefore unable to float, or had a higher density leading to their precipitation.

We noticed that this filtration procedure led to the accumulation of a high amount of NaCl on MPs and on the mesh itself, which could affect the final weight of the MPs. Thus, we found necessary to add a thorough wash of the mesh and particles to eliminate excesses of NaCl. To solve this issue, we created a system with an open flask to immobilize the mesh (Figure 9), allowing to immobilize the filter cloth and wash it several times with running deionized water. The filter cloth was then placed to another flask with deionized water in the bottom and covered with aluminum foil and leave to rest, ensuring that all the remaining NaCl was dissolved in the water. All these steps are detailed in our proposed protocol (please see the supplementary material – section 5).

Figure 9 – System created and used in this work to wash the mesh with the MPs; consists in an open flask and holes in the lid, the mesh is immobilized between the flask and the lid.

3.4 | Method validation

After the established protocol (supplementary material – section 5), including all our proposed adaptations and new steps, samples containing a known type and concentration of MPs (laboratory samples) was processed to evaluate the efficiency of the new protocol for extraction of MPs in terms of quantity and quality of MPs. The majority of MPs were recovered from the top of the density separator, regardless the type of plastic tested. Although the settled solids were always collected and visually inspected, none MPs were found in the bottom of the density separator system or in the settled solids.

The recovery values were all above 90%, (Figure 10). The recovery of the PE-HD (microspheres) was the highest, with 100% of recovery from the top of the density separator system.

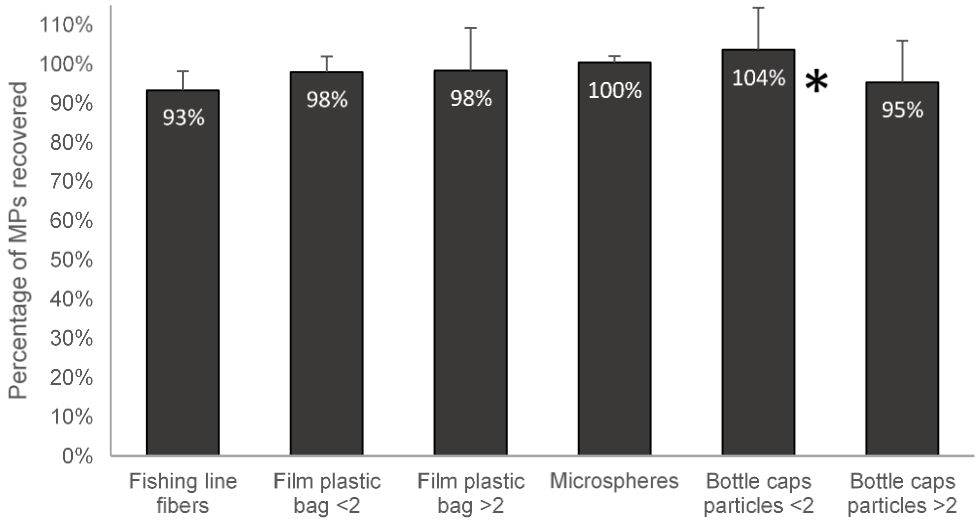

Figure 10 - Percentage of microplastics recovered from the top of the density separator system, for each type of plastic subjected to the protocol. Error bars represent the standard deviation associated (n=6). * - Bottle caps particles <2 had a percentage above 100%, possibly explained by accumulation of NaCl on the rugged parts of this type of MPs.

The lowest recovery percentage was observed for PA (fishing line fibers) with only 93% of recovery, what may be explained by potential lost of this type of plastic with the protocol treatments, namely during the organic matter elimination phase. In fact, after the peroxide oxidation, we noticed that fishing line fibers were visually altered from their transparent color to more brownish colors. Nuelle et al. (2014) used a 7-day exposure assay to determine the impacts of 30% H_2O_2 solution, including in polyamide, and results showed visible changes in this type of plastics, including size (becoming smaller and/or thinner) and color. So, it is important to consider that the NOAA protocol might underestimate the number of this type of fibers, what is quite important since fishing line fibers derived from polyamide are a very common type of plastic in aquatic environments, being reported at high abundances at marine (Kanhai et al., 2017) and lake environments (Su et al., 2016).

In the case of PE-LD (film plastic bag) MPs there were no differences in the percentage of recovery between MPs <2 mm and MPs >2 mm. In the case of PET (bottle caps particles) there was a slight difference in the efficiency of recovery (104% for PET <2 mm and 95% for PET >2 mm) between MPs sizes, but without significant differences between the two size classes (ANOVA $F=1.83$ $p \geq 0.21$).
In the case of PET MPs (bottle caps particles) <2 mm, the efficiency of recovery was slightly above 100%. One of the greatest issues during the use of the NOAA protocol was the accumulation of NaCl around MPs in the separation step, mainly

in the bottle caps particles. It is thus possible that some NaCl precipitated and accumulated on the rugged parts of these particles leading to an increase of the final weight. This fact highlights the need for a thorough washing of the particles after the separation step, that can be more difficult for smaller particles.

Overall, the recovered MPs masses did not show significant differences between types of MPs (ANOVA $F=1.15$ $p \geq 0.36$). These results revealed that no matter the type of MPs, the modified NOAA protocol established in our study had a similar efficiency for high and low-density Polyethylene, Polyethylene terephthalate and polyamide.

4 | Conclusion

According to our results, the adaptations and advice proposed to the NOAA protocol, lead to better results in samples with a high amount of organic matter, such as estuarine water samples. Our study showed an optimum MPs recovery, above 90% for different types of MPs, namely high and low-density Polyethylene, Polyethylene terephthalate and polyamide. The present work allows for a better comprehension of the advantages of NOAA protocol, allowing to be more effective for estuarine waters.

5 | Supplementary material

Protocol to quantify microplastics in estuarine waters detailing all laboratory procedures updated from NOAA (Masura et al., 2015)

1º day

1. Pass the water sample through a filter cloth (mesh <0.3 mm previously rinsed with deionized water and completely dried)
2. Rinse the water containing recipient thoroughly with a squirt bottle filled with deionized water to transfer all residual solids to the filter cloth
3. Rinse the cloth thoroughly with deionized water. Ensure all material has been well washed, drained, and sorted
4. Weigh a clean and dry 500 ml beaker to the nearest 0.1 mg
5. Transfer solids collected in the filter cloth into the beaker using a spatula and minimal rinsing with a squirt bottle containing deionized water
6. Ensure all solids are transferred into the beaker
7. Place beaker drying in oven at 90 ˚C overnight

2º day

1. Weigh the beaker with the dried sample to stable weight, to ensure dryness
2. Turn on the hotplate to 75 ˚C
3. Add 20 mL of aqueous 0.05 M Fe(II) solution to the beaker containing the fraction of collected solids and then 20 mL of 30% H_2O_2 solution
4. Add a stir bar to the beaker and place them in the hotplate inside the fume hood
5. Heat to 75 ˚C for 30 minutes until gas bubbles are observed at the surface. If the reaction is too violent, remove the beaker from the hotplate until boiling subsides. If reaction appears to have the potential to overflow the beaker, add deionized water to slow the reaction.
6. When the reaction stops (no boiling) add another 20 mL of 30% H_2O_2 solution and heat to 75 ˚C letting it react as describe before (step before).
7. Add ca. 6 g of salt (NaCl) per 20 mL of sample to increase the density of the aqueous solution (ca. 5 M NaCl) (around ca. 18 g of NaCl if you add 20 mL Fe(II) solution + 20 mL 30% H_2O_2 solution + 20 mL 30% H_2O_2 solution)
8. Heat mixture to 75 ˚C for 30 minutes or until the salt dissolves
9. Transfer the saturated solution to the density separator
10. Rinse the saturated solution beaker with deionized water to transfer all remaining solids to the density separator. Use the minimum amount of deionized water as possible to avoid diluting the saturated solution
11. Cover loosely with aluminum foil
12. Allow microplastics to float overnight

3º day

1. Visually inspect settled solids for any microplastics. If any is found try to gently hit the separator, making sure all microplastics are floating above
2. Drain settled solids from the separator bottom and discard
3. Weigh a clean and dry filter cloth (mesh <0.3 mm previously rinsed with deionized water and completely dried) to the nearest 0.1 mg
4. Collect floating solids by passing the solution through the filter cloth immobilized in an open flask
5. Rinse the density separator several times with deionized water to transfer all solids to the filter cloth

6. Wash the filter cloth several times with running deionized water and then place the system with the filter cloth in a flask (covered with aluminum foil) with deionized water in the bottom for 5 minutes
7. Place the filter cloth in a petri dish and cover loosely with aluminum foil
8. Allow to dry for 24 hours at 90 ˚C

4º day

1. Weigh the filter cloth with the sample dried to the nearest 0.1 mg
2. Collect all the remain material and microplastics from the filter cloth and weight them
3. Under a dissecting microscope at 40X magnification, use forceps to collect identifiable microplastics from the 0.03 mm mesh and transfer them to a tared vial.
4. Weight only the microplastics

Chapter 3

Microplastic contamination in an urban estuary: Abundance and distribution of microplastics and fish larvae in the Douro estuary

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| Abstract

Estuaries are productive environments used by many fish as nursery grounds. The initial stages of fishes are highly vulnerable to (a)biotic factors, and anthropogenic pressures, influencing fish larvae assemblages along the estuary. Microplastics (MPs) (particles <5 mm) are particularly dangerous to early life stages of fishes because their ingestion can induce gut blockage, limiting food intake, and exposing organisms to contamination due to MPs capacity to absorb pollutants. Present work aimed to investigate the contamination by MPs of an urban impacted estuary (Douro estuary, NW Portugal), and study the temporal and spatial interactions between MPs and larval fish assemblages. Monthly sampling surveys were performed from December 2016 to December 2017, in nine stations along the estuary. Sub-surface planktonic horizontal trawls were performed to collect fish larvae and MPs. Planktonic samples were sorted, and fish larvae identified. MPs abundance was determined using a protocol optimized in our laboratory. A total of 1498 fish larvae belonging to 32 taxa were collected, with a mean abundance of 11.66 fish larvae 100 m^{-3}. During the spring-summer period, it was observed the typical increase in the abundance and diversity of the larval assemblage. Diversity was generally low (H'=0.48) with the high dominance of very few taxa, namely the common goby, *Pomatoschistus microps*. Different types of MPs were found, namely fibers, soft/hard plastic, colorful/transparent plastic, in a total of 2152 particles, with a mean abundance of 17.06 MPs 100 m^{-3}. MPs collected represented approximately 28% of the weight of inorganic matter. Hard MPs and fibers were the most predominant types, representing 83% of the total MPs collected. In some months the number of MPs surpassed the number of fish larvae, with an average ratio of 1 fish larvae:1.5 MPs. Such results are concerning, highlighting that a higher availability of MPs may facilitate their ingestion by fish and therefore increase possible impacts in these communities.

Keywords: Microplastic; Fish larvae; Estuarine water; Temporal and spatial patterns

1 | Introduction

Estuaries are transitional ecosystems between the ocean and rivers, recognized as ecologically important habitats (McLusky & Elliott, 2004). Traditionally hosting important uses to cities and tourism, they are also important for industrial and agricultural activities (Raz-Guzman & Huidobro, 2002). Estuaries play an essential role as habitat, not only for estuarine species but also for marine species, offering protection and food resources, temporary shelter or even functioning as migration routes (e.g. Berletta-Bergan et al., 2002a; 2002b; McLusky & Elliott, 2004; Correa-Herrera et al., 2017; Santos et al., 2017). Nonetheless, estuaries worldwide are exposed to numerous anthropogenic perturbations (Elliott & Whitfield, 2011), including disposing of large amounts of debris, pollution, and contaminated discharges. These increasing perturbations possibly compromise the ecological function and quality of estuarine environments (Whitfield and Elliott, 2002; Zhao et al., 2015; Santos et al., 2017).

Many fish species use estuaries as nursery areas, ensuring food availability, enhancing the growth, and maximizing the survivor rate of early life stages of fishes, namely ichthyoplankton (North and Houde, 2003; Amorim et al., 2017). In their early life stages, fish species are highly vulnerable, especially to environmental conditions (e.g. salinity, temperature, and turbidity). Variations on these conditions influence their distribution, abundance and diversity patterns in estuaries (Hoffmeyer et al., 2009; Ooi & Chong, 2011; Ramos et al., 2012). Furthermore, fish larvae are also vulnerable to a growing number of anthropogenic pressures, as overfishing, environmental stress, pollution and also climate change (Lima et al., 2014; Correa-Herrera et al., 2017), which could ultimately lead to changes in fish community structure (Fonseca et al., 2013; Santos et al., 2017). Hence, the larval fish assemblages of an estuary change continually in time and space, according to reproductive seasons of the species, the environmental fluctuations and possible anthropogenic stressors (Garcia et al., 2003; Ficke et al., 2007; Santos et al., 2017).

In the last decades, plastic production increased drastically along with their accumulation and contamination in the environment (Lima et al., 2014; Correa-Herrera et al., 2017). Characteristics such as durability, buoyancy, and resistance allow plastic being extremely durable and long-lasting. With the influence of wind,

rain, and land runoff, plastics can disperse reaching almost any habitat, including the estuaries (Browne et al., 2010; Frias et al., 2014; Lima et al., 2014) and, over time, plastics can fragment into smaller particles, becoming microplastics (MPs <5 mm). When ingested, microplastics pose a high harmful risk to biota, affecting marine organism physically and chemically (Frias et al., 2010; 2014; Eerkes-Medrano et al., 2015; Rezania et al., 2018). Due to their size, MPs can be easily mistaken for food, and their ingestion may cause injuries such as internal abrasions and blockages (Eerkes-Medrano et al., 2015; Rezania et al., 2018). Also, MPs have potential capacity to adsorb metals or persistent organic pollutants (POP) from the environment and when ingested may eventually increase the risk of toxic effects on the organism (Fendall & Sewell, 2009; Cole et al., 2011; Frias et al., 2010; 2014). Beside this type of interaction between fish larvae and MPs, predominantly studied, other types of interaction can occur. Competition for light, space and nutrients or other density-related factors are other examples of interactions that can affect the distribution and abundance of several groups of plankton (Murphy et al., 1988; Miner et al., 1993; Roy, 2008; Hansen et al., 2017). MPs are an emerging concern and our knowledge about their impact on the aquatic life and habitats is still very limited, highlighting the need to understand the dynamic and proportion between MPs and biota.

In this context, the present study focused on investigate the ratio between fish larvae and MPs by: (1) describing the spatial and seasonal patterns of larval fish assemblages in terms of abundance, diversity, and species composition in an urban estuary (Douro estuary); (2) evaluating the contamination of the Douro estuarine waters by MPs, (3) assessing whether MPs vary seasonally and spatially along the salinity gradient of the Douro estuary.

2 | Methods

2.1 | Study area

The Douro river extends along 930 km in the Iberian Peninsula, draining into the Atlantic Ocean near Porto city, in the northwest coast of Portugal (41.14° N, 8.66° W) (Azevedo et al., 2008). The Douro estuary is a narrow mesotidal and semi-diurnal estuary, with an average depth of 8 m and extending 21.6 km upstream of the river mouth (Azevedo et al., 2014) (Figure 11). It is vertically stratified (salt-wedged estuary) and its hydrodynamics is highly influenced by the river discharge

regime, controlled by the Crestuma-Lever dam (Bordalo & Vieira, 2005; Azevedo et al., 2010; Azevedo et al., 2014). The estuary includes three geomorphological zones; the lower estuary - characterized by a partially obstruction by a sand bar in the mouth of the estuary and with an average width of 333 m; the middle estuary - heavily urbanized and 271 m wide; and the upper estuary - with an average width of 645 m (Azevedo et al., 2008; 2014) (Figure 11).

2.2 | Sampling method

Monthly sampling surveys were conducted from December 2016 to December 2017 in the Douro estuary. Nine sampling stations (1 to 9) were selected along the initial 17 km of the estuary, covering the horizontal salinity gradient of the estuary (Figure 11). Sampling stations 1, 2 and 3, were located in the lower estuary, at 0.5, 1.5 and 2.5 km from the river mouth, respectively.

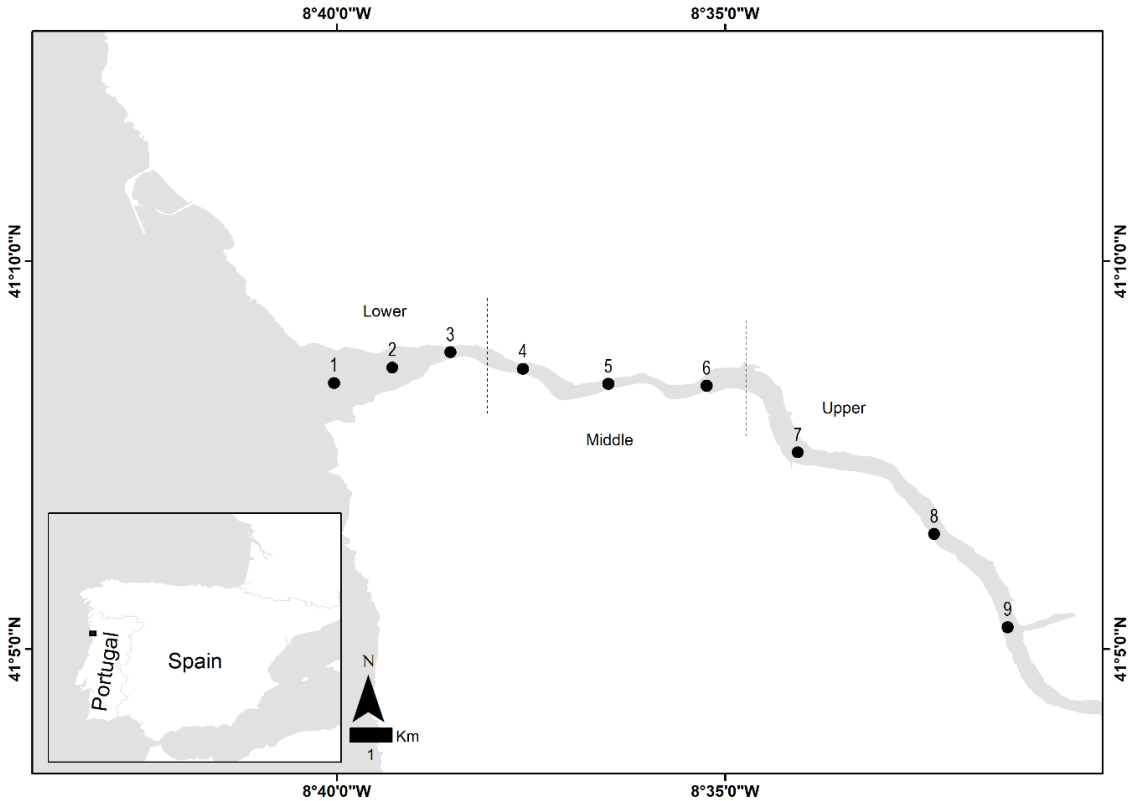

Figure 11 - Douro estuary and location of the nine sampling stations. (A) Lower estuary, with stations 1, 2, and 3; (B) Middle estuary, with stations 4, 5 and 6; (C) Upper estuary, with stations 7, 8, and 9.

Stations 4, 5 and 6 were located in the middle estuary, at 3.5, 5.5 and 7.5 km from the river mouth, respectively. The last three stations, 7, 8, and 9, were located in the upper estuary, at 10.5, 14.5 and 17 km from the river mouth, respectively.

At each sampling station, daylight planktonic samples were collected with a conical 1 m diameter, 4 m long and 500 µm mesh size net (Figure 12.A). Subsurface (1-2 m depth) planktonic circular tows were performed at a constant velocity of ca. 1 ms^{-1} for five minutes, and during the slack phase of spring tides and daylight (i.e. two hours before high tide). The volume of the water filtered was determined by a flowmeter attached to the net (Hydro-Bios). Samples were immediately fixed with 70% ethanol and preserved until laboratory procedures. At each sampling station, vertical profiles of physical-chemical water parameters as salinity (PSU), water temperature (˚C), dissolved oxygen concentration (mg/L) and saturation (%), pH and turbidity (NTU) were performed with a multiparameter probe (YSI EXO1 Sonde) (Figure 12.B). River flow data were obtained from Crestuma-Lever dam.

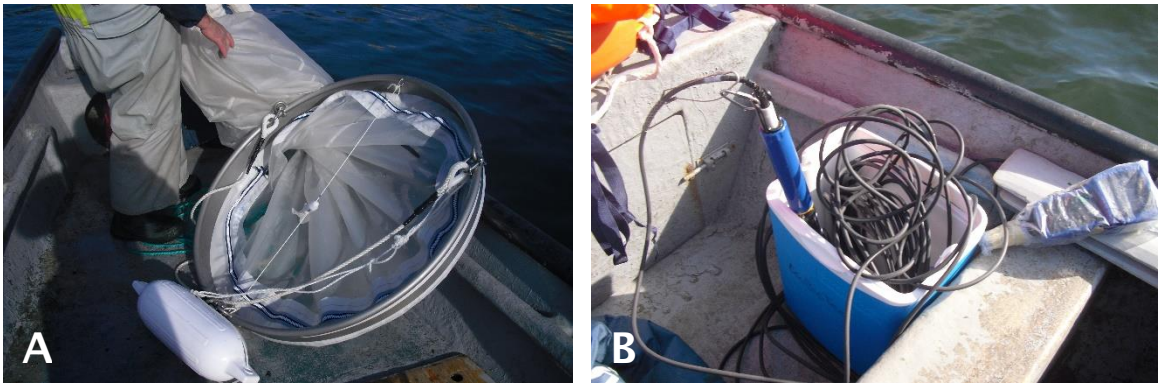

Figure 12 - Planktonic 1 m diameter and 500 µm mesh net used to collect planktonic samples (A); multiparameter probe used to perform vertical profiles of physical-chemical water parameters (B).

2.3 | Sampling processing

To prevent airborne contamination in the laboratory, the following measures were taken: (1) lab coats and gloves were worn during sample processing; (2) all the containers used during sample processing were covered and cleaned using distilled water before reuse; and (3) a petri dish with distilled water was exposed to the environment near the stereomicroscope and inspected for MPs in the end of all the sorting processes (including fish larvae and MPs).

2.3.1 | Larval fish

In the laboratory, the collected samples were sorted under a stereomicroscope (Nikon SMZ800) and fish larvae collected were preserved in 70% ethanol. Individuals were identified to the highest possible taxonomic level, including to species level whenever possible, using specialized literature (Russel, 1996; Ré, 1999; Munk and Nielsen, 2005; Ré and Meneses, 2009; Rodriguez et al., 2017). The number of individuals per taxa were counted from the entire sample and then standardized to the number of fish larvae 100 m^3 of filtered water. After sorting for fish larvae, the remaining material of the sample was placed into a glass container and preserved in 70% of alcohol for latter MPs determination.

2.3.2 | MPs

The remaining sample, previously sorted for fish larvae, was submitted to a protocol for MPs extraction and quantification. MPs quantification was done adapting and optimizing the NOAA protocol (Masura et al., 2015), for estuarine waters (please see further details in chapter 2 – supplementary material). Briefly, samples were first sieved through a 0.03 mm mesh size, washed with deionized water and dried at 90°C. Dried samples were then subjected to 30% H_2O_2 to degrade all the organic matter. The remaining material was then subjected to a density separation with NaCl, allowing for the collection of MPs. All the MPs collected were visually inspected and identified under the stereomicroscope. MPs were weighed, counted and classified according to hardness and color, and their abundance was standardized to the number of MPs 100 m^{-3} of filtered water. The percentage of MPs present in the inorganic part of the sample was obtained by calculating the difference between the mass of MPs at the end of the protocol and the remaining dry matter after the digestion step.

2.4 | Data analysis

Data was analyzed per season (three-month groups) as follows: winter 2016 (W16) comprised December 2016, January and February 2017; spring 2017 (Sp17) comprised March, April and May 2017; summer 2017 (S17) June, July, and August 2017; autumn (A17) September, October and November 2017. The winter 2017 (W17) only comprised a month, December 2017.

In order to ascertain the effect of seasons and estuarine areas on the abundance and diversity of the larval fish assemblage and MPs abundance, a two-way analysis of variance (ANOVA) was used, with seasons and areas as fixed factors. In order to analyze the effect of season and area on the sub-surface water physical-chemical parameters (average 1-2 m depth), a type II multivariate analysis of variance (MANOVA) was performed, with season and area as fixed factors. Whenever necessary, variables were log transformed [log 10 (x+1)] for biological variables such as larval fish, Shannon-Wiener index (H') and Pielou's evenness index (J'); Ln(x) for MPs abundance and physical-chemical variables], in order to stabilize the variance and to fit data to a normal distribution, fulfilling the ANOVA assumptions of homogeneous variance and normally distributed data (Zar, 1996). Homogeneity of variance was tested with Cochran test. Post-hoc analyses were performed with Fisher LSD. ANOVA and MANOVA analyses were performed with TIBCO Statistica™ 13.3 software. The correlations between physical-chemical water parameters and larval fish and MPs abundance were analyzed through Pearson correlation coefficient. A significance level of 0.05 was considered for all analyses.

Each larval fish species was assigned to an ecological guild accordingly to its estuarine use pattern, following Franco et al. (2008), namely: estuarine species (ES), marine migrants (MM; spawn at sea and regularly enter estuaries in large numbers, including marine species using estuaries as nursery grounds), marine stragglers (MS; spawn at sea and enter estuaries accidentally in low numbers), freshwater species (F) and catadromous species (CA). The diversity of the larval fish assemblage was expressed by Shannon-Wiener index (H') (Shannon & Wiever, 1963), and equitability was measured by Pielou's evenness index (J') (Pielou, 1966). Two-way analysis of similarity (ANOSIM) (Clarke & Warwick, 1994) was used to determine the significance of spatial or temporal trends in the structure of the larval fish assemblage, considering $p < 0.05$ (i.e. significance level) and R statistic > 0.5 (i.e. the strength of the factors on the samples). Similarity percentages (SIMPER) (Clarke, 1993) were used to identify the percentage contribution of each taxon to the average dissimilarity between samples of the various seasons and areas pairwise combinations. ANOSIM and SIMPER were based on a Bray–Curtis rank similarity matrix, calculated using the fourth root transformed data. Only species with more than 0.1% of the total catch were included in the analysis avoiding any undue effect of rare species. Multivariate analyses were performed with PRIMER

software (Plymouth Routines Multivariate Ecological Research) (Clarke & Warwick, 1994).

3 | Results

3.1 | Larval fish assemblages

In total, 1498 fish larvae were collected at the Douro estuary, including 11 families distributed through 32 taxa, whereby 23 could be identified to species, 2 to genus and 7 to family (Table 3). A total of 1.5% of the total catch were unidentified larvae, mainly representing yolk-sac stages or damaged larvae. At the species level, *Pomatoschistus microps* reached 37.75% of the total fish larvae collected during this study, representing the most abundant taxon (Table 3), followed by Clupeidae n.i. (29.04%), *Sardina pilchardus* (10.21%), *Pomatoschistus minutus* (5.35%) and *Solea senegalensis* (2.85%). These top five species comprised 85.20% of the total larval fish abundance (Table 3).

During the study, the larval fish density was on average 11.66 ± 17.36 fish larvae 100 m^{-3}, varying between 0.48 and 108.60 fish larvae 100 m^{-3}. The larval fish density varied significantly between seasons ($F=13.19$, $p<0.01$) and estuarine areas ($F=4.00$, $p\leq0.02$). In general, larval fish assemblages show a higher density in the lower estuary (14.90 ± 1.86 fish larvae 100 m^{-3}) and during spring 2017 (16.28 ± 13.85 fish larvae 100 m^{-3}) (Figure 13.A). The larval fish density exhibited a similar seasonal pattern in the lower and in the middle estuarine areas, with higher densities during spring and autumn and significantly lower densities (Fisher's LSD test, $p<0.01$) in the upper area during winter 2016 and spring 2017 (Figure 13.A). In fact, no fish larvae were collected in the upper area during the winter 2016.

Table 3 – Larval fish species collected in the Douro estuary between December 2016 and December 2017 and their density throughout the year. Ecological guilds (Eco G): ES - estuarine species; MM - marine migrants; MS – marine stragglers, according to Franco et al., 2008. (W16: winter 2016; Sp17: spring 2017; S17: summer 2017; A17: autumn 2017; W17: winter 2017).

				Lower					*Middle*					*Upper*				
Family	Taxon	% of the total catch	Habitat	W16	Sp17	S17	A17	W17	W16	Sp17	S17	A17	W17	W16	Sp17	S17	A17	W17
Gobiidae	*Pomatoschistus microps*	37.75%	ES	0.27	1.69	7.30	7.95	2.33	0.08	3.19	4.76	7.14	0.35	0.00	1.26	6.51	10.37	17.36
Clupeidae	Clupeidae n.i.	29.04%		2.68	14.20	0.00	3.47	0.14	0.16	12.74	1.50	2.49	1.40	0.00	0.17	5.87	0.16	0.18
Clupeidae	*Sardina pilchardus*	10.21%	MM	3.00	2.73	0.00	1.41	0.26	1.48	2.66	0.00	0.09	0.18	0.00	0.00	3.96	0.00	0.00
Gobiidae	*Pomatoschistus minutus*	5.35%	ES	0.00	0.12	1.47	2.88	0.00	0.00	0.34	0.82	0.68	0.00	0.00	0.39	0.60	0.81	0.00
Gobiidae	Pomatoschistus spp.	5.35%		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00
Soleidae	*Solea senegalensis*	2.85%	MM	0.00	0.00	1.80	0.97	0.25	0.00	0.00	0.19	1.02	0.00	0.00	0.00	0.18	0.08	0.00
Gobiidae	Gobiidae n.i.	2.10%		0.00	0.14	0.13	0.17	0.85	0.00	0.00	0.19	0.10	0.33	0.00	0.14	0.09	1.86	0.00
Engraulidae	*Engraulis encrasicolus*	1.70%	MM	0.00	1.00	0.00	0.00	0.00	0.00	1.52	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Atherinidae	*Atherina presbyter*	1.40%	MM	0.00	0.00	0.11	0.00	0.00	0.00	0.00	2.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Soleidae	*Solea solea*	1.40%	MM	0.00	0.00	0.00	1.86	0.00	0.00	0.00	0.00	0.28	0.00	0.00	0.00	0.00	0.00	0.00
Gobiidae	*Parablennius gattorugine*	1.40%	MS	0.09	1.17	0.38	0.00	0.00	0.00	0.28	0.10	0.00	0.00	0.00	0.11	0.00	0.00	0.00
Gadidae	Gadidae n.i.	0.70%		0.00	0.63	0.00	0.00	0.00	0.00	0.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Blenniidae	*Lipophrys pholis*	0.60%	MS	0.11	0.08	0.11	0.35	0.00	0.08	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.08	0.00
Syngnathidae	Syngnathus spp.	0.50%		0.00	0.39	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.12	0.00
Gobiidae	*Gobius* niger	0.50%	ES	0.00	0.48	0.00	0.00	0.00	0.00	0.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gobiidae	*Gobius cruentatus*	0.40%	MS	0.00	0.00	0.00	0.00	0.00	0.00	0.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Labridae	*Labrus Bergylta*	0.40%	MS	0.00	0.00	0.19	0.17	0.00	0.00	0.00	0.10	0.08	0.00	0.00	0.00	0.00	0.00	0.00
Soleidae	Soleidae n.i.	0.30%		0.00	0.00	0.17	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mullidae	*Mullus surmuletus*	0.30%	MM	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00
Labridae	Labridae n.i.	0.20%		0.00	0.00	0.34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gadidae	*Trisopterus minutus*	0.20%	MS	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00
Blenniidae	*Parablennius pilicornis*	0.20%	M	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gobiidae	*Pomatoschistus pictus*	0.10%	MS	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Blenniidae	*Blennius occelaris*	0.10%	MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.10	0.00	0.00	0.00	0.00	0.00	0.00
Soleidae	*Pegusa lascaris*	0.10%	MM	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gadidae	*Trisopterus luscus*	0.10%	MM	0.00	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Labridae	*Centrolabrus exoletus*	0.10%	MS	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Soleidae	*Buglossidium luteum*	0.10%	MS	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Atherinidae	Atherinidae n.i.	0.10%		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Blenniidae	Blenniidae n.i.	0.10%		0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cottidae	*Taurulus bubalis*	0.10%	MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00

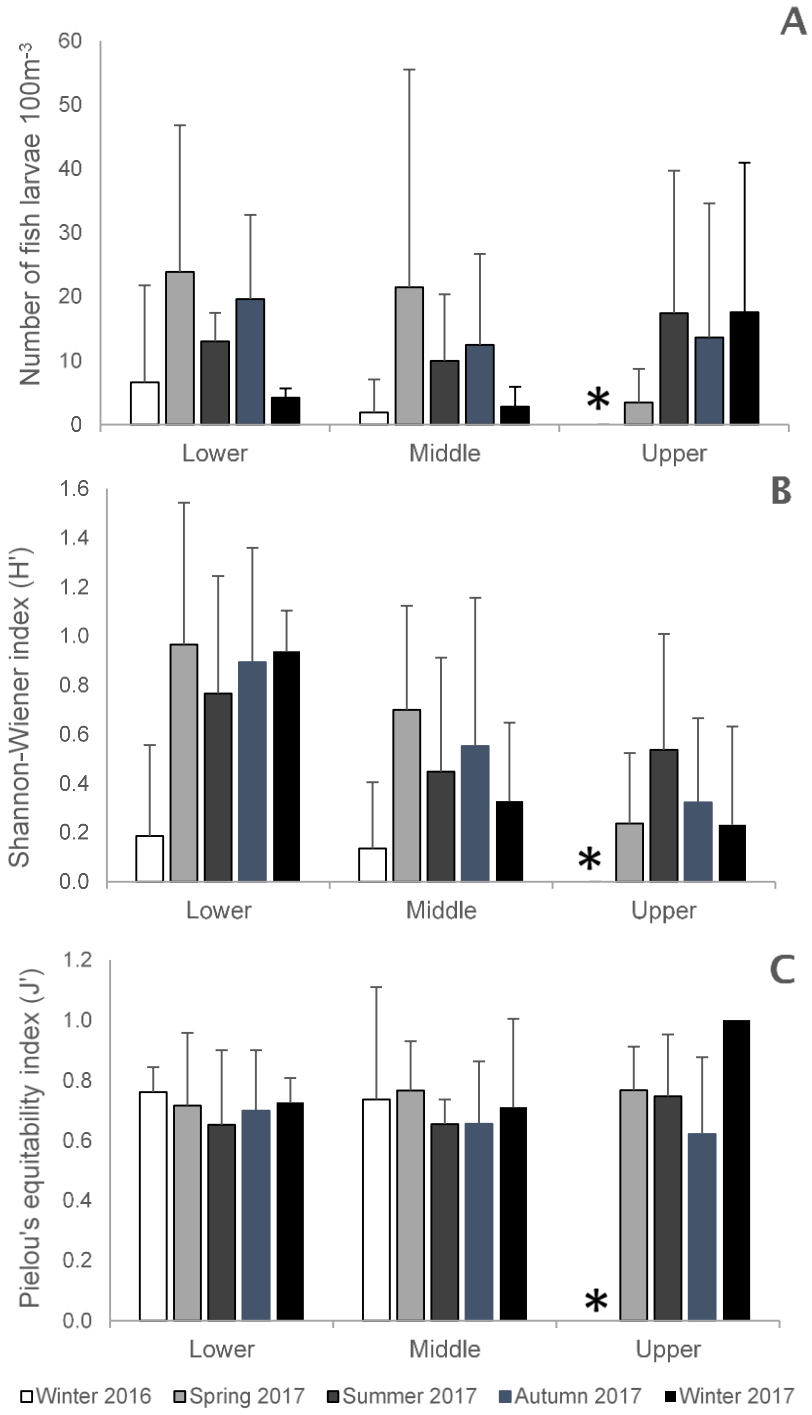

Figure 13 - Temporal and spatial variation of: (A) number of fish larvae 100 m^{-3} (mean ± standard deviation) (B) Shannon Wiener index and (C) Pielou's equitability index of the larval fish assemblage collected in the three areas of the Douro estuary. * - indicates that during winter 2016 in the upper estuarine area there were no fish larvae in the planktonic samples.

The number of taxa per sample ranged between 0 and 15, corresponding to values of 0 and 1.85 for the Shannon Wiener diversity index (H'), and 0.22 to 1 for the Pielou's evenness index (J'). H' varied significantly between seasons ($F=7.13$ $p<0.01$) and areas ($F=11.06$ $p<0.001$). In general, H' was significantly ($F=11.06$ $p<0.01$) higher in the lower estuary ($H'=0.72 \pm 0.54$) and tended to decrease with increase distance from the river mouth (Figure 13.B). Overall, the H' showed significantly higher values in the lower area in spring 2017 (Fisher's LSD test, $p<0.01$) in comparison with other seasons in the middle and upper areas (Figure 13.B). The equitability of the larval fish assemblages did not vary significantly between seasons ($F=1.08$ $p\geq0.36$) or areas ($F=0.01$ $p\geq0.93$) (Figure 13.C). However, the highest equitability value was observed in winter 2017 in the upper estuarine area ($J'=1$) (Figure 13.C).

According to the ANOSIM results, the structure of the larval fish assemblage varied significantly between seasons ($p<0.01$), although, seasonal groups were not clearly separated (global $R=0.32$). The larval fish structure of winter 2016 was significantly different from summer 2017 ($R=0.61$ $p<0.01$). *P. microps* was responsible for 44.56% of the dissimilarity between these two seasons (Table 4), associated with the relatively low abundances of *P. microps* during winter 2016 in the three estuarine areas (Table 3).

Table 3 - Results of one-way ANOSIM (R values and significance levels) and SIMPER analysis on the abundance of the top twenty-two most abundant species from seasonal groups (W16: winter 2016; Sp17: spring 2017; S17: summer 2017; A17: autumn 2017; W17: winter 2017). * - indicates the seasons with statistical differences.

	ANOSIM		*SIMPER*		
Groups	R	*P*	Mean dissimilarity (%)	Discriminating species	% contribution
W16 vs Sp17	0.162	0.001	90.59	Unidentified Clupeidae	26.95
Sp17 vs S17	0.362	0.001	84.42	*P. microps*	27.89
S17 vs A17	0.268	0.002	69.95	*P. microps*	26.86
A17 vs W17	0.130	0.115	69.37	*P. microps*	32.08
W16 vs W17	0.299	0.007	93.67	*P. microps*	44.23
W16 vs S17	**0.614**	**0.001***	**96.17**	***P. microps***	**44.56**
Sp17 vs a17	0.121	0.022	78.64	*P. microps*	25.71
Sp17vs w17	0.164	0.061	81.67	*P. microps*	35.09
W16 vs A17	0.221	0.001	92.78	*P. microps*	40.69
S17 vs W17	0.089	0.218	66.54	*P. microps*	25.04

The larval fish assemblage structure did not vary significantly along the estuary ($R=0.076$, $p\geq0.003$).

3.2 | MPs spatial and seasonal patterns

A total of 2152 MPs particles were collected during the study period. MPs were found in all planktonic samples collected, with a mean density of 17.06 ± 16.50 MPs 100 m^{-3}, and an average weight of 16.000 ± 0.002 mg. In average, MPs represented approximately 27.76 ± 23.09% of the inorganic matter weight present in the planktonic samples.

MPs abundance varied significantly between seasons ($F=5.88$, $p<0.01$) and estuarine areas ($F=3.98$, $p\leq0.02$). The highest average values of MPs density were observed in winter 2016 (21.97 ± 8.82 MPs 100 m^{-3} – average of all seasons) and spring 2017 (23.98 ± 10.61 MPs 100 m^{-3} – average of all seasons) and in the middle area of the estuary (22.20 ± 20.28 MPs 100 m^{-3}) (Figure 14). Significantly lower abundance was observed in the upper estuarine area in summer 2017 and winter 2017 relative to spring 2017 in every estuarine area ($p<0.01$) (Figure 14).

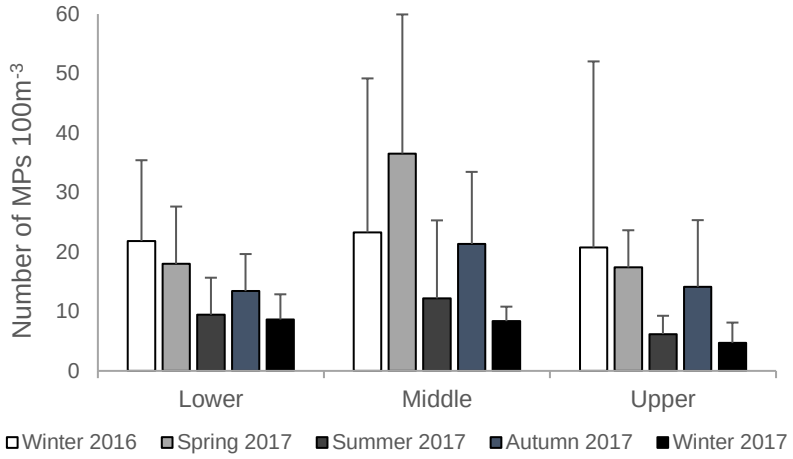

Figure 14 - Temporal and spatial variation of average density of MPs 100 m^{-3} in the three areas of the Douro estuary.

Forty-eight percent of the MPs collected were hard colorful particles, 35% fibers, 13% soft colorful particles, 3% soft transparent particles and 1% hard transparent particles (Figure 15). Hard colorful particles and fibers were the predominant types of MPs, representing 83% of the total MPs collected and with mean abundances of 8.07 ± 10.63 MPs 100 m^{-3} and 5.98 ± 9.75 MPs 100 m^{-3}, respectively. Also, these two types of MPs had the highest percentages of occurrence, namely 72% for hard colorful MPs and 54% for fibers of the total MPs collected.

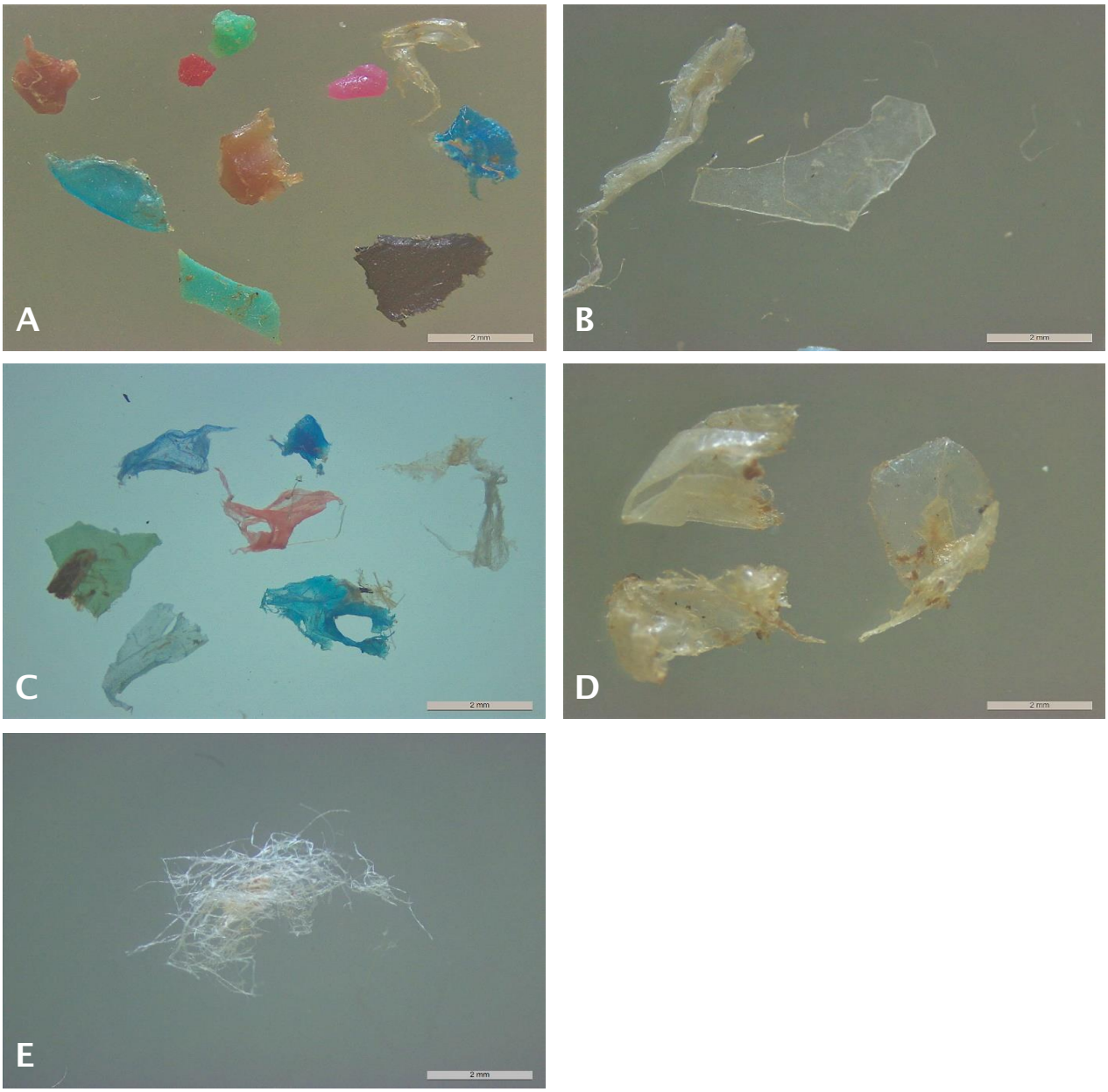

Figure 15 – Examples of microplastics collected in the Douro estuary: (A) hard colorful MPs; (B) hard transparent MPs; (C) soft colorful MPs; (D) soft transparent MPs and (E) fibers. Images captured with a digital camera coupled to a stereomicroscope.

Furthermore, hard colorful particles were the predominant MP type in all seasons and estuarine areas, except for Spring 2017, where fibers were the most abundant type (Figure 16.A). On the other hand, soft particles, either colorful or transparent, and hard transparent particles were the less abundant types of MPs. Hard and soft transparent particles were not observed in the winter 2017 samples (Figure 16.A).

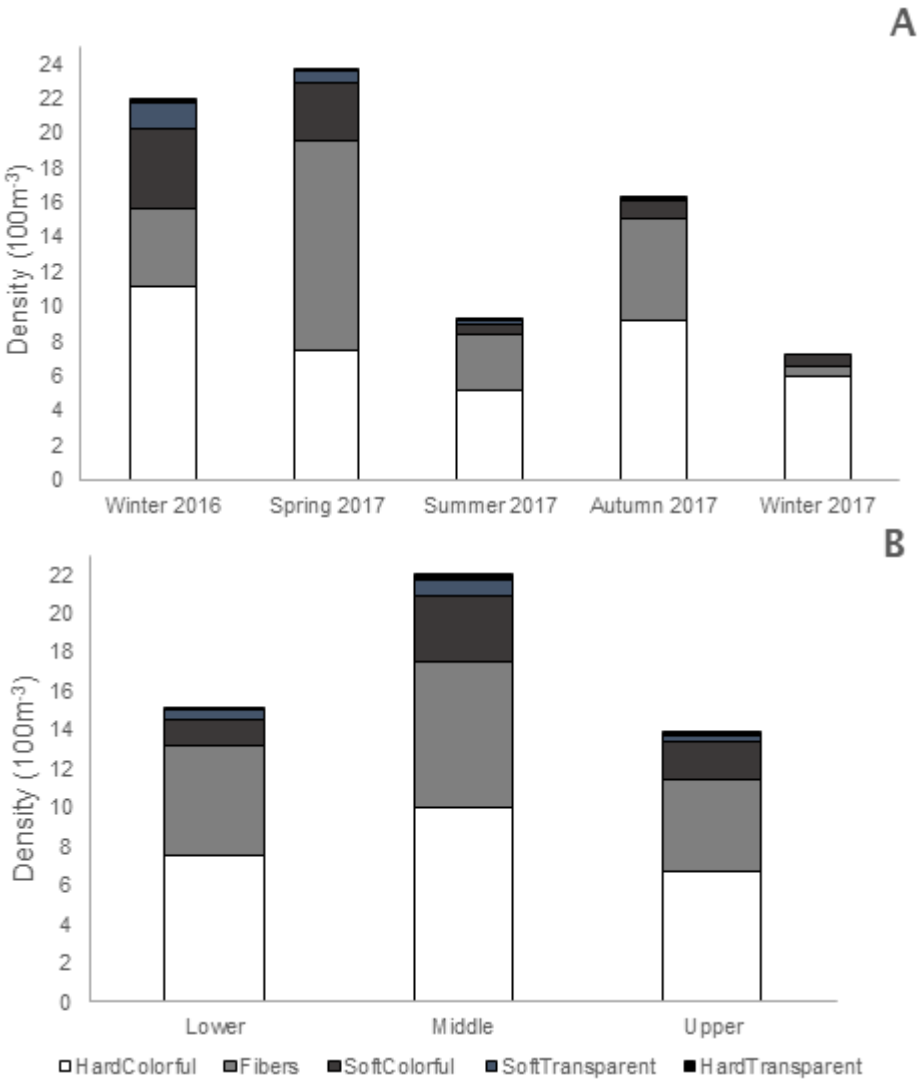

Figure 16 (A) Seasonal distribution of each type of MPs collected in the Douro estuary; (B) Spatial distribution of each type of MPs collected in Douro estuary.

3.3 | Fish larvae versus. MPs

From a total of 107 planktonic samples collected, all of them contained MPs and only 87 samples contained fish larvae. MPs were, on average, more abundant than fish larvae (17.06 ± 16.50 MPs 100 m^{-3} VS 11.66 ± 17.36 fish larvae 100 m^{-3}). Overall, the average ratio obtained in the Douro estuary was 1 fish larvae:1.5 MPs.

The temporal variation of the fish larvae:MPs ratio, showed that MPs were more abundant during most of the seasons, reaching a maximum of 1 fish larvae:4.4 MPs during the winter 2016 (Figure 17.A). Only in the months of summer 2017 fish

larvae surpassed the abundance of MPs, with an average ratio of 1 fish larvae:0.7 MPs (Figure 17.A).

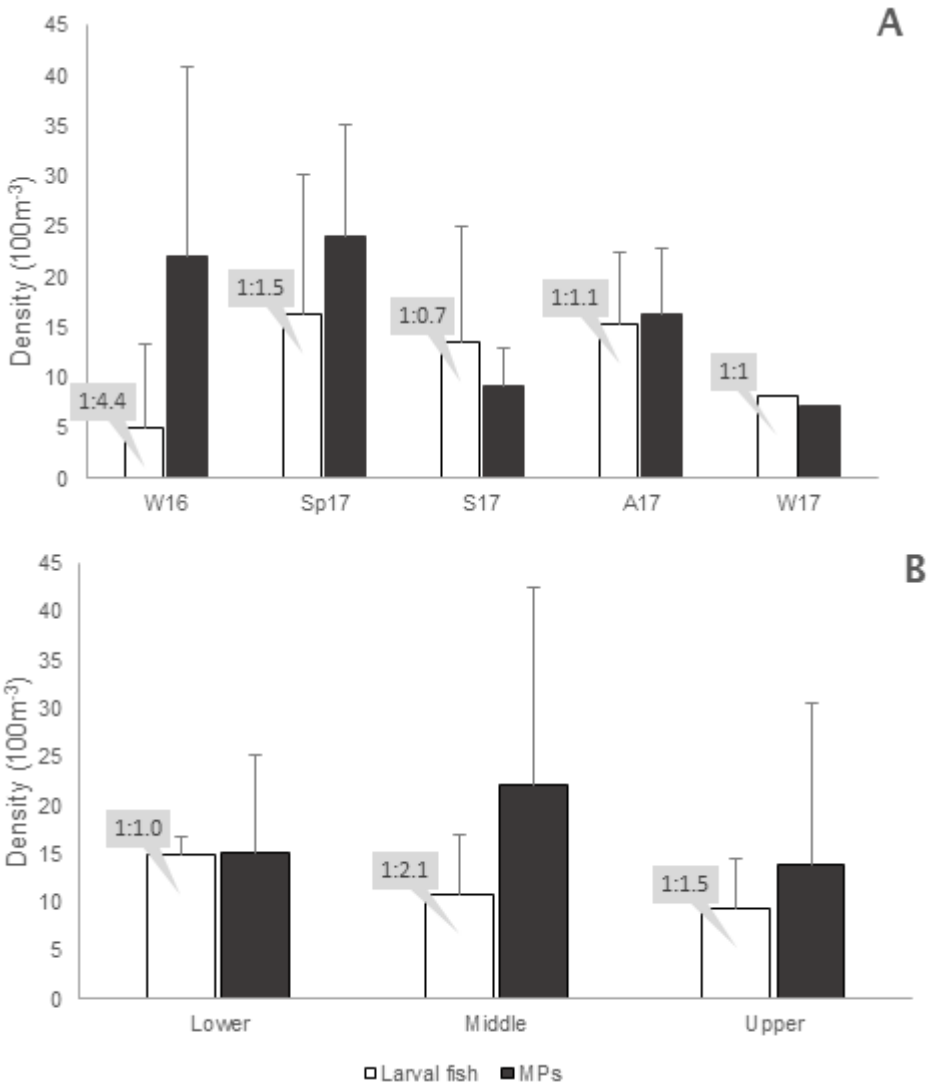

Figure 17 - Larval fish and MPs average abundance (error bars represent standard deviation) and the respective ratio (Larvae fish : MPs) in each season (A) and in each area of the Douro estuary (B). (W16: winter 2016; Sp17: spring 2017; S17: summer 2017; A17: autumn 2017; W17: winter 2017.

In terms of estuarine areas, a similar scenario was observed, with MPs tending to be more abundant than fish larvae, mainly in the middle and upper estuarine areas (Figure 17.B). In the middle estuary, where MPs were more abundant, the abundance of MPs doubled that of the fish larvae (1.0 fish larvae:2.1 MPs).

3.4 | Environmental variables

The physical-chemical properties of the sub-surface water layer of the Douro estuary varied significantly between seasons (Wilks' lambda= 0.33, $p<0.01$) and along the three estuarine sections (Wilks's lambda= 0.08, $p<0.01$). In general, the upper estuary exhibited a higher seasonal fluctuation of the environmental variables, mainly in terms of water temperature, oxygen concentration and oxygen saturation (Figure 18). Water temperature exhibited a typical seasonal pattern in the three estuarine areas, increasing from winter until summer-autumn (Figure 18). This pattern was more evident in the upper estuary, where water temperature was significantly higher (Fisher's LSD test, $p<0.01$).

Despite the seasonal variations within each estuarine area, salinity decreased significantly from the lower to the upper estuary (Fisher's LSD test, $p<0.01$). Overall, the Douro sub-surface water layer was oxygenated (Figure 18), although oxygen saturation and concentration significantly (Fisher's LSD test, $p<0.01$) decreased to 87.23 ± 8.80 % and 7.81 ± 1.45 mgL^{-1}, respectively during autumn 2017. Turbidity and pH only showed significant differences among seasons (turbidity: $F=4.19$, $p<0.01$; pH: $F=8.3$, $p<0.01$).

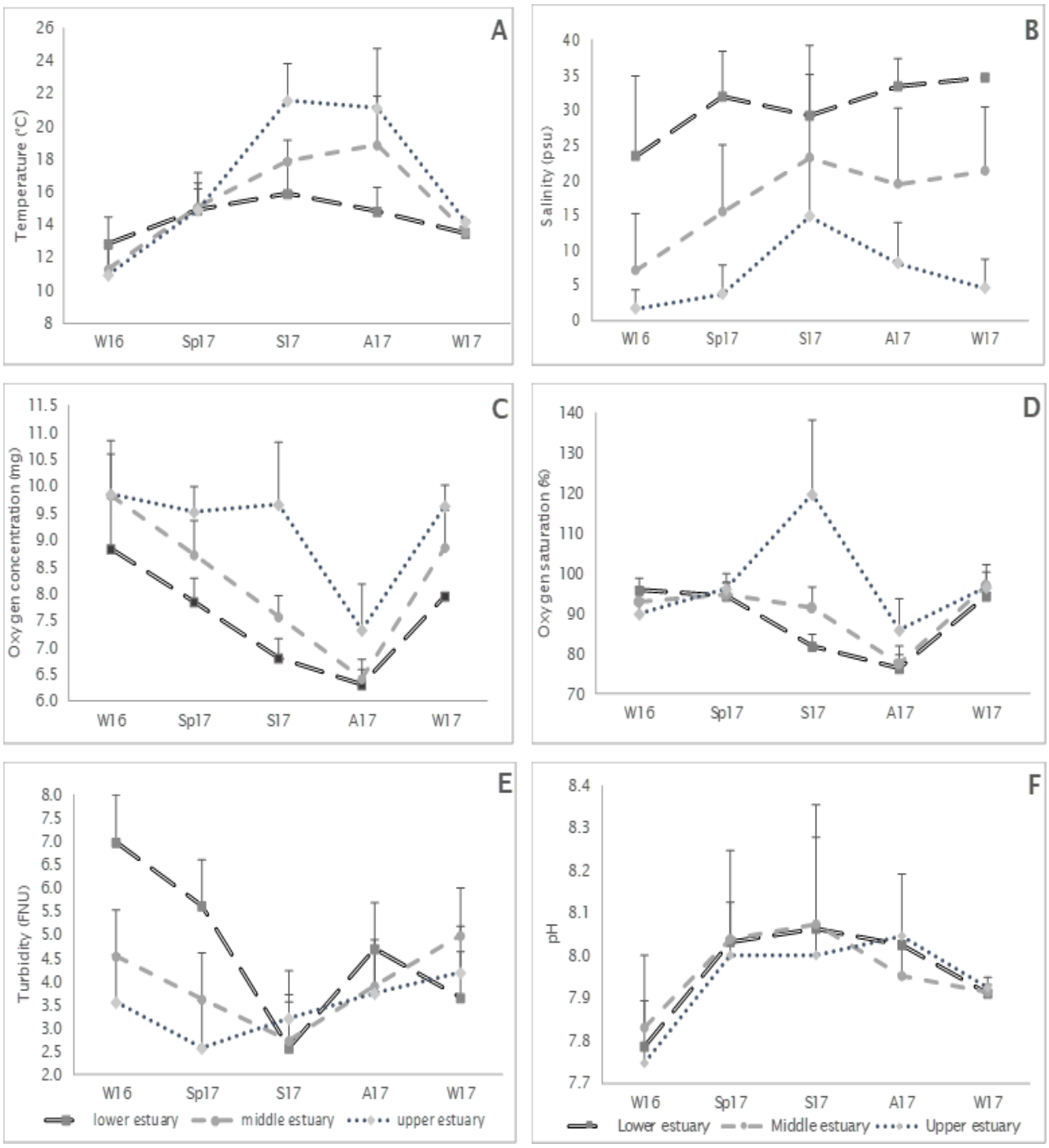

Figure 18 - Seasonal mean values for (A) temperature, (B) salinity, (C) oxygen concentration, (D) oxygen saturation, (E) turbidity and (F) pH in the three areas of the Douro estuary (error bars represent standard deviation). (W16: winter 2016; Sp17: spring 2017; A17: autumn 2017; W17: winter 2017).

The river flow ranged from 0 and 1337 m^3/s, and in average, gradually decreased from winter 2016 until autumn 2017 (Figure 19).

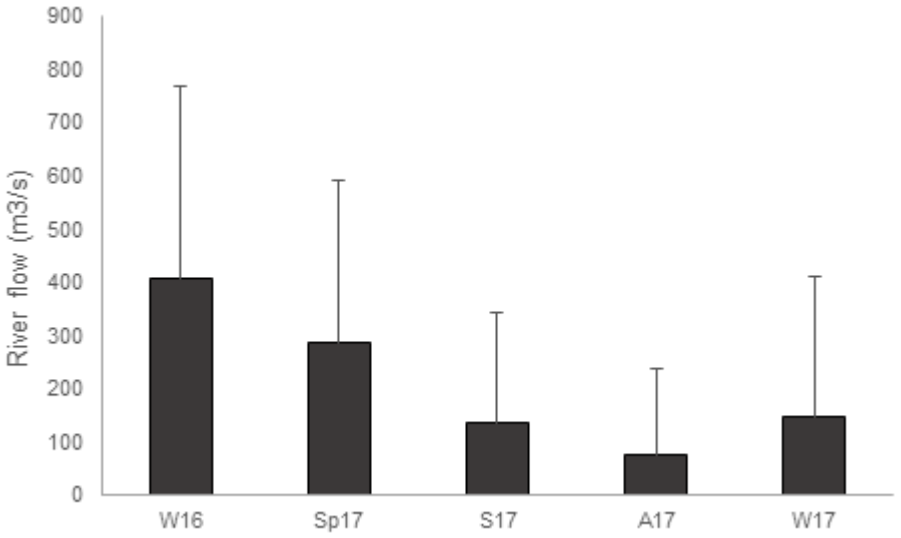

Figure 19- Mean river discharge (m3/s) in the Crestuma-Lever dam, during the study period (error bars represent standard deviation). (W16: winter 2016; Sp17: spring 2017; A17: autumn 2017; W17: winter 2017).

According to Pearson correlation results, larval fish abundance was positively correlated with water temperature and salinity and negatively with oxygen saturation (Table 5). MPs were significantly correlated with river flow. Although MPs were significantly correlated with pH and turbidity, the coefficient of Pearson correlation was lower than 0.4 (Table 5).

Table 4 - Correlation between physico-chemical parameters with larval fish and MPs abundance. * - indicates significant correlations.

	Larval fish density	*MPs density*
Temperature	**0.44***	0.01
Salinity	**0.56***	0.11
Oxygen saturation	-0.22*	0.03
Oxygen concentration	**-0.57***	-0.03
pH	0.39	0.21
Turbidity	-0.03	0.21
River flow	-0.29	**0.51***

4 | Discussion

4.1 | MPs contamination in the Douro estuary

Microplastics contamination of the aquatic environment is becoming a major global concern, and so the number of studies focusing on MPs contamination has increased, namely in rivers and estuaries (e.g. Eerkes-Medrano et al., 2015; Horton et al., 2017). The present study, the first to investigate MPs contamination in the Douro estuary, provides basic information on the MPs contamination of an urban estuary. This study showed that MPs were present in all planktonic samples with an average density of 17.06 ± 16.50 MPs 100 m^{-3}. These values were in the same order of magnitude as those reported by Lima et al. (2014; 2015) for a tropical estuary in Brazil. However, different levels of contamination have been reported among areas, e.g. increasingly higher contamination values have been reported for several other estuaries in China (e.g. Zhao et al., 2014; 2015), but relatively lower values have been found for other temperate estuaries such as the Tamar (UK) (2.8 MPs 100 m^{-3}) (Sadri & Thompson, 2014). Comparisons on the average concentration of MPs in different estuaries should, however, be made with caution and tempered by the different sampling and quantification methods. In fact, the different sampling techniques reported in the literature, e.g. water pumps (Zhao et al., 2014), plankton net (Lima et al., 2014), and manta net (Sadri et al., 2014), highlight the need for a standardized methodology to quantify MPs in aquatic environments (Rocha-Santos & Duarte, 2015).

Along the Douro estuary, MPs tended to concentrate in the middle estuary, an urban area with several touristic activities. The proximity to urban centers has been suggested as one of the most important contributors for MPs pollution (Free et al., 2014; Wagner et al., 2014), based on the assumption that MPs derive from the human careless discharge of plastic debris into aquatic environments. Yet, all the MPs collected in this study were possibly secondary MPs (resulting from degradation of larger plastic debris (Masura et al., 2015) suggesting that they have been in the water for a long time, which might not be compatible with the Douro estuary hydrodynamics. The fact that MPs concentrate in the middle estuary needs to be further investigated in order to ascertain if it is associated with (i) the fact that MPs tend to deposit in areas where the movements of the water are slower (Browne et al., 2010), or (ii) that there are other sources of MPs contamination in the middle estuary, such as effluents of wastewater treatments.

The highest number of MPs was observed during winter 2016 and spring 2017, coinciding with higher river flow values. Similar results were obtained in the Goiana estuary (Brazil), where maximum MPs concentration were observed during the rainy season, the period with higher freshwater inflow (Lima et al., 2015). The positive correlation between river flow and MPs concentration may indicate that MPs collected in the Douro estuary mainly originated from upstream sources. The presence of a large dam in the upstream limit of the Douro estuary, which controls the freshwater inflow of the estuary, may lead to a high retention of MPs in the dam reservoir (Zhang et al., 2015), that can be exported to the estuary, explaining the increasing MPs concentration in the estuary with the increasing river flow. Nevertheless, this hypothesis needs to be investigated in further studies in order to understand why MPs concentrates in the middle estuary.

Hard colorful particles and fibers were the most well-represented MPs types in the Douro estuary and a similar scenario was found in other studies (Zhao et al., 2015; Gallagher et al., 2016). Fibers are the most common MPs found in ingestion investigations and can pose a risk to aquatic organisms being tangled and create agglomerates, preventing food ingestion (Avio et al., 2015; Botterell et al., 2019). Fibers are one of the most common MPs types reported by other studies, however, many of those studies did not apply any type of laboratory procedure (e.g. H_20_2 digestion) or used FTIR to ensure degradation of organic fibers . Hence, a possible explanation for such high fiber values is that those studies included not only the plastic fibers but also the cotton fibers derived from clothes. In the present work, organic matter in all the samples was firstly degraded to ensure that all the fibers counted were plastic fibers. These methodological differences highlight the need to continue seeking standardized methods to quantify MPs in aquatic environments.

4.2 | MPs interactions with larval fish assemblages

In the last decades, the amount of studies investigating the impact of MPs on planktonic communities has increased (Cole et al., 2011; Eerkes-Medrano et al., 2015), although few have focused on the planktonic stages of fishes. This study is the first assessment in the Douro estuary, and results showed that MPs exceeded larval fish density, with an average ratio of 1 fish larvae:1.5 MPs. A similar study, in the Goiana estuary (Brazil), found an inverse ratio, with MPs representing almost

half of the total fish larvae density (Lima et al., 2014). Lechner et al. (2014) also observed higher quantity of MPs in comparison with fish larvae in the Austrian Danube. But, in Ballona Creek and San Gabriel River (USA), Moore et al (2005) also found that MPs concentration exceed the zooplankton density.

In the Douro estuary, MPs and fish larvae had different temporal and spatial distribution patterns. Despite the higher MPs concentration, there was not a temporal or a spatial overlap with the peaks of fish larvae. These different temporal and spatial patterns may indicate that MPs and fish larvae are influenced by different environmental variables, as shown by the Pearson correlations. In fact, results showed that water temperature and salinity were positively correlated with larval fish density, whereas MPs were only correlated with river flow. This desynchronized pattern may also indicate that the potential negative impact of MPs to fish larvae might have been minimized, because when fish larvae density peaked it coincided with the lower MPs concentration in the Douro estuary.

Despite the desynchronized pattern and seasonality between fish larvae and MPs, MPs were found everywhere along the Douro estuary and coexisted with ichthyoplankton during the entire year. The temporal and spatial pattern of the Douro estuarine larval fish assemblage exhibited a similar pattern with other Portuguese estuaries. The winter period was characterized by a strong decrease of larval fish density (Ramos et al., 2006a), and the highest diversity of fish larvae occurred in the lower estuary, near the ocean (Faria et al., 2006). The number of taxa observed in this study was similar to other Portuguese estuaries such as Mondego (31 – Primo et al., 2011) and Guadiana (22 - Faria et al., 2006), although lower than in the Lima estuary (50 – Ramos et al., 2006b). Overall, the larval fish assemblages were dominated by few highly abundant species, including resident species (*P. microps*) and marine migrants (Clupeidae), similar to other temperate estuaries (Faria et al., 2006; Ramos et al., 2006a; Primo et al., 2011).

One of the negative impacts of MPs is the possibility of ingestion by living organisms. MPs can be ingested by fish larvae, zooplankton or small fish, directly affecting the survival of those organisms, or indirectly entering in the food chain and transferred to higher trophic levels (Possatto et al., 2011). Depending on the size of the organism, ingested MPs can be easily expelled or be retained in the gastrointestinal tract. This last case can cause a variety of problems in the animal,

such as: (i) cause a false sense of satiety (Eerkes-Medrano et al., 2015); (ii) block organs by an obstruction; both cases indirectly preventing food ingestion (Derraik, 2002). A study performed in early juveniles *P. microps* showed that MPs were ingested, even when natural prey was present, and the fish predatory performance and efficiency was significantly reduced when MPs were mixed with the prey (de Sá et al., 2015). Taking into consideration that *P. microps* was the most abundant taxa in the Douro estuary, it is expected that the larval stages of this resident species might be negatively impacted by MPs ingestion. However, this scenario should be investigated in future studies. The lower trophic level organisms are more susceptible to ingest MPs due to their limited ability to differentiate between plastic and food (Moore, 2008; Cole et al., 2011). Hence the urgent need to understand the effects that MPs contamination can have on the organisms and on the overall ecosystem.

Due to the numerous functions and services provided by estuaries, namely their role as habitat, refugee and nursery grounds for a variety of marine species, it is of great importance to evaluate their environmental status and ecological health. Estuarine MPs pollution is a complex process as there are many different sources and types of MPs possible of reaching estuaries. Furthermore, MPs can affect several species and ecological functions in a variety of ways, posing new environmental threats to estuarine communities. Globally, considerable progress has been made in characterizing the presence and the potential effects of MPs in the aquatic environment, however, our actual knowledge of MPs pollution in Portuguese waters, and especially in estuaries, is still relatively limited and represent opportunities for further research.

5 | Conclusions

In the Douro estuary, the density of MPs surpassed the density of fish larvae in most of the seasons and estuarine areas, with an average ratio of 1 fish larvae:1.5 MPs. MPs were found in all the planktonic samples, being available to planktonic organisms during the whole year. However, there was not a temporal or spatial overlap of the peak of densities between MPs and fish larvae, what may indicate that both are mainly influenced by different environmental variables. Five types of MPs were identified, whereby hard colorful particles and fibers were the predominant ones. The present study contributed to increasing our scientific

understanding of MPs contamination in the Douro estuary and also raised some questions that represent opportunities for further research, namely gaps in the sources and patterns, as well as on the effects of MPs in the aquatic environment and planktonic organisms.

Chapter 4

Final considerations

Over the last years, there has been an exponential increase of studies focusing on MPs contamination in aquatic environments, with several of them suggesting a contamination worldwide. The presence of MPs in estuaries has initially being investigated focusing its role as a pathway of MPs from land to the ocean, although there are evidence that estuaries accumulate large quantities of MPs, due to their proximity to urban areas (Eerkes-Medrano et al., 2015).

For an accurate MPs environmental quantifications, allowing comparisons between locations, is essential to have standardized sampling and quantification methodologies. There have been several studies attempting to decrease inconsistencies and proposing standardized methodologies (e.g NOAA methods), although these studies are usually focused on the marine water or beach sediments. Estuarine waters are very specific and differ from marine or freshwater, for example in terms of organic matter content (Canuel et al.,2015). Such specific characterizes can compromise the efficiency of protocols develop for marine or freshwater samples. In this context, we tested the NOAA protocol to quantify MPs from water samples (Chapter 2) before starting our monthly monitoring program (Chapter 3). This was done with the intent of knowing that the data collected accurately represented the contamination of our study area, the Douro estuary. Thus, several laboratory tests were realized to test the efficiency of the NOAA protocol for estuarine waters, and with the final objective of proposing recommendations/adaptations to improve the protocol. Results showed that the ideal temperature to dry the estuarine water samples was 90 ˚C, the temperature advised by NOAA, since lower temperatures lead to an even more time-consuming protocol; and on the other hand, with temperatures higher than 90 ˚C some types of MPs, as fibers were deformed. The organic matter elimination is essential and one of the most important steps, mainly for estuarine samples that may content high amount of organic matter. This was one of the steps of the NOAA protocol that was optimized during the study. According to our results, two doses of 20 mL were the ideal quantity of 30% H_2O_2 solution to use for degradation of organic matter, efficient for low or high quantities of organic matter. Also, in this step, we made some precautions regarding the appropriate time and temperature of solution in the hotplate, and we tested the negative effects of using higher quantities of 30% H_2O_2 in shape/color of different types of MPs tested. For the density separation of MPs, the NOAA protocol showed some inconsistencies and limitations, and we proposed several modifications to improve it. For example,

NaCl showed to be the ideal to promote MPs flotation, however, add precisely 6 g of NaCl per 20 mL is fundamental to achieve full dissolution. Besides, details such as the time and temperature to dissolve the NaCl, the use of a stirring magnet and the system created to immobilize the mesh (allowing a properly wash to eliminate the excesses of NaCl), revealed to be more efficient and less time-consuming methodology and, ultimately, avoid the overestimation of MPs. With all the adaptations proposed, the MPs recovery values were all above 90%, with PE-HD achieving the 100% of recovery. All the adaptations proposed by our protocol showed similar effects and efficiency for all the MPs types tested. Thus, this study provided a fully detailed protocol to quantify MPs in estuarine water samples, suitable for being used in future studies and promoting harmonization of methodologies to investigate MPs contamination.

This study is the first to investigate MPs contamination in the Douro estuary, contributing to baseline data for future studies and increasing our scientific knowledge of MPs contamination in estuarine ecosystems. The results obtained showed that MPs were present in all planktonic samples and with an overall ratio of 1 fish larvae:1.5 MPs. It was also found that the spatial and temporal patterns of fish larvae and MPs did not match, since MPs were more abundant in the middle estuary and during winter and spring seasons, while larval fish assemblages tended to be more abundant and diverse in the lower estuary, mainly during summer-autumn period. This desynchronized temporal and spatial pattern might have minimized the potential negative impact of MPs to fish larvae. However, the larval stages of resident species, namely the most abundant taxa, the common goby *Pomatochistus microps*, were exposed to MPs during the entire year. Considering that this species can ingest MPs (de Sá et al., 2015), MPs could have a negative influence on the *P. microps* population of the Douro estuary and this aspect should be investigated in future studies. Since, the lower trophic level organism is in the same range of size of MPs, this increase the urgency to understand the effects that MPs can have on these organisms. Hard colorful particles and fibers were the most abundant in the Douro estuary and a similar scenario was found in other studies. Although, these two MPs types possible affect the organism in different ways, both pose a risk to the aquatic organisms and can lead to death and/or decrease of larval fish communities (Avio et al., 2015; Botterell et al., 2019). Likewise, is necessary to investigate the type of plastic of their origin and understanding the

specific effects that they pose to the most representative fish species of the Douro estuary.

This study emphasized the need for further studies in order to investigate the sources of MPs contamination of the Douro estuary and understand why MPs tended to concentrate in the middle estuary. MPs were more abundant than the early life stages of fishes highlighting the need to understand the potential effects of MPs for planktonic organisms in the Douro estuary. Also, results suggested that MPs and fish larvae were influenced by different environmental variables and future studies are needed to further investigate this feature. Indeed, a better understanding of the factors that can affect the transport and spatial and temporal patterns of MPs in aquatic habitats will help to design effective measures to prevent MPs release into the environment.

Nowadays, plastic waste is so ubiquitous in the natural environment that we need to rethink plastic, take action and raise awareness on the issue of plastic pollution. The results obtained in this thesis can be used as a baseline for other studies in estuarine environments and contributed to increase the scientific knowledge of MPs problematic, and can help us to preserve and improve the ecosystem health of our planet.

Chapter 5

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