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Effects of microplastics on marine organisms and implications to animal, environmental and human health

Luís Gabriel Barboza

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Luís Gabriel Antão Barboza

EFFECTS OF MICROPLASTICS ON MARINE ORGANISMS AND IMPLICATIONS TO ANIMAL, ENVIRONMENTAL AND HUMAN HEALTH

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Author`s declaration

In agreement with the Portuguese law throught the article 4th of the "Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto" of September 12th (GR.08/07/2017), the author states devotion in a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscripts preparation of the published or submitted for publication, corresponding to chapters or sections of the present Thesis.

Publications

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1. Barboza, L.G.A., Cózar, A., Gimenez, B.C.G., Barros, T.L., Kershaw, P.J., Guilhermino, L., 2019. Macroplastics pollution in the marine environment. *In*: Sheppard, C. (Ed.). *World Seas: An Environmental Evaluation*. Vol. III: *Ecological Issues and Environmental Impacts*. 2nd Edition. Academic Press (Elsevier), London, 305-328 pp.

2. Barboza, L.G.A., Frias, J.P.G.L., Booth, A.M., Vieira, L.R., Masura, J., Baker, J., Foster, G., Guilhermino, L., 2019. Microplastics Pollution in the Marine Environment. *In*: Sheppard, C. (Ed.). *World Seas: An Environmental Evaluation. Volume III: Ecological Issues and Environmental Impacts.* 2nd Edition. Academic Press (Elsevier), London, 329-351 pp.

3. Barboza, L.G.A., Vieira, L.R., Branco, V., Figueiredo, N., Carvalho, F., Carvalho, C., Guilhermino, L., 2018. Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, *Dicentrarchus labrax* (Linnaeus, 1758). *Aquatic Toxicology 195*, 49-57 pp.

4. Barboza, L.G.A., Vieira, L.R., Branco, V., Carvalho, C., Guilhermino, L., 2018. Microplastics increase mercury bioconcentration in gills and bioaccumulation in the liver and cause oxidative stress and damage in *Dicentrarchus labrax* juveniles. *Scientific Reports 8*, 15655 p.

5. Barboza, L.G.A., Vieira, L.R., Guilhermino, L., 2018. Single and combined effects of microplastics and mercury on juveniles of the European seabass (*Dicentrarchus labrax*): changes in behavioural responses and reduction of swimming velocity and resistance time. *Environmental Pollution 236*, 1014-1019 pp.

6. Barboza, L.G.A., Vethaak, A.D., Lavorante, B.R.B.O., Lundebye, A., Guilhermino, L., 2018. Marine microplastic debris: an emerging issue for food security, food safety and human health. *Marine Pollution Bulletin 133*, 336-348 pp.

7. Barboza, L.G.A., Lopes, C., Oliveira, P., Raimundo, J., Caetano, M., Vale, C., Guilhermino, L. Microplastic in wild fish from North East Atlantic Ocean in relation to neurotoxicity and other toxic effects, and estimated human exposure to microplastics through fish consumption. (*Submitted to Scientific Reports, under review*).

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Abstract

The presence and accumulation of plastic waste in the marine environment are well known environmental issues. However, the ubiquitous presence and persistence of microplastics (small pieces of plastic less than five millimetres in size) in the marine environment became of particular concern in recent years due to their potential impacts on animal, ecosystem and human health. For this reason, microplastics are now recognized as emerging pollutants of great concern and are considered a priority research topic. In this context, the main goal of this Thesis was to contribute to the advancement of knowledge on the ecotoxicological effects of microplastics on marine organisms and their implications to animal, environmental and human health.

To understand the current knowledge on the marine contamination by plastics and micropastics and its effects, and to identify specific topics deserving further investigation, two reviews of the literature, one on plastics and the other on microplastics, were performed. These reviews correspond to Chapters II and III, respectively, of the present Thesis and are published as two chapters of an international scientific book.

In the first phase of the experimental study (Chapter IV), the short-term effects of microplastics, alone and in mixture with mercury, and the potential influence of microplastics on the bioaccumulation and bioconcentration of mercury were investigated using juveniles of the European seabass (*Dicentrarchus labrax* Linnaeus, 1758) as biological model. Mercury was selected because is an ubiquitous pollutant of particular concern, is very toxic, can be accumulated by organisms and its organic forms, particularly methylmercury, can be biomagnified in trophic webs. Briefly, in a laboratory bioassay, groups of fish were exposed for 96 h to mercury alone (0.010 and 0.016 mg/L), microplastics alone (1-5 μm diameter fluorescent plastic microspheres, polymer of unknown composition, 0.26 and 0.69 mg/L), and mixtures of both pollutants (same concentrations). The actual exposure concentrations of microplastics and mercury were determined during the bioassay. At the end of the exposure period, fish swimming performance, several other biomarkers and the bioaccumulation and bioconcentration of mercury by fish were investigated. The results indicated that microplastics are able to sorb mercury from

the seawater. They also indicated that in the range of concentrations tested microplastics (0.26 and 0.69 mg/L), mercury (0.010 and 0.016 mg/L), and their mixtures caused neurotoxicity, through the inhibition of the activity of the enzyme acetylcholinesterase (AChE) and induction of oxidative stress and lipid damage in the brain, impairment of neuromuscular cholinergic function through the inhibition of the activity of cholinesterase enzymes (ChE) in the dorsal muscle, stress and lipid oxidative damage (gills, liver and muscle), changes in the mechanisms of cellular energy production, reduction of the swimming velocity and of the resistance time when swimming against the water flow. Moreover, toxicological interactions between microplastics and mercury in fish were evidenced by several parameters, and influence of microplastics on the bioconcentration and bioaccumulation of mercury was found. From these results it was concluded that microplastics caused adverse health effects and modulated the toxicity of mercury *in D. labrax* juveniles. In real scenarios, particularly in areas contaminated with microplastics and mercury, juveniles of this species can be considerably affected, which may cause population decline. The results of this study also showed potential risks to predators of *D. labrax* juveniles, human consumers of this species, and the potential of microplastics to cause environmental impacts due to adverse effects on fish populations and their ecological function. The results included in the Chapter IV of the present Thesis are published in the form of three scientific research papers.

In the second phase of the experimental study (Chapter V), the contamination of wild fish from three species (*Dicentrarchus labrax, Trachurus trachurus and Scomber colias*) widely consumed as food by humans in Portugal and other countries by microplastics was investigated. A total of 150 fish (50 animals er species), from the North-East Atlantic Ocean, landed in a Port of the North-West region of Portugal and on sale for human food consumption were studied. In each fish, the gastrointestinal tract, a sample of the dorsal muscle and a sample of gills were analysed for the presence of microplastics which were characterized by type, colour and size. Moreover, the activity of AChE in the brain and ChE in the dorsal muscle, and the levels of lipid peroxidation (LPO) in the brain, gills and dorsal muscle were determined. Based on the total mean of microplastics found in the dorsal muscle (the main edible tissue for humans) and the recommendations of the European Food Safety Authority for fish consumption by different human population groups (three age groups of children; adults and the general

population), and on data from the European Market Observatory for Fisheries and Aquaculture Products and National Marine Fisheries Service regarding human consumption of fish per capita, estimates of microplastics intake through fish consumption by the European population, Portuguese population and in the main importer countries of fish from Portugal were made. A total of 368 microplastics were recovered from the three species studied: 175 from the gastrointestinal tract, 112 from gills and 81 from the dorsal muscle. From the 150 fish analyzed, 49 % contained microplastics, and 32 % had microplastics in the dorsal muscle. The total mean (± standard deviation) of microplastics in the dorsal muscle was 0.054 ± 0.099 items/g of tissue. Fish with microplastics had higher brain AChE activity and increased LPO levels (brain, dorsal muscle and gills) than fish without microplastics. These results indicate neurological alterations and lipid oxidative damage in organs crucial to survival and performance of animals. They also suggest that microplastics may have contributed to these effects, despite the potential contribution of other stressors cannot be excluded. Estimates of microplastic intake through fish consumption by subgroups and the general human population in Europe varied between 112 and 842 microplastic items per year. The estimates of microplastics intake per year/capita for countries in Europe, North America and South America showed that the exposure to microplastics through fish consumption may indeed be considerably higher in countries where fish consumption is high, such as Portugal (3078 microplastic items/year/capita). Considering that fish consumption is only one of the routes of human exposure to microplastics, this study and others in the literature emphasize the need of more research, risk assessment and adoption of measures to minimize human exposure to these particles.

In the last phase of the study (Chapter VI), a literature review focused mainly on food security, food safety and human health issues was made. From this study it was concluded that the presence of microplastics in the marine environment has implications for human food security and safety, and for human health and wellbeing, and that human exposure through multiple routes of exposure (especially food, drinking water and air) increases the concern about the risks associated with long-term exposure microplastics. Several topics deserving future investigation were identified and management actions were recommended. This study is published as a scientific review article.

In the Chapter VII, the main results and findings were integrated and discussed, highlighting the major findings of the Thesis.

Finally, in the Chapter VIII, the contribution of the present Thesis to the advance of knowledge regarding the microplastic paradigm and future work perspectives are presented.

In general, the studies included in the present Thesis indicated that the contamination of the marine environment by plastics and microplastics is a global phenomenon, that microplastics can be uptaken by fish and other organisms and induce diverse types of adverse effects, that microplastics influence the toxicity of other common contaminants in animals exposed to mixtures containing these particles, that fish used for human consumption contain microplastics, and that the environmental contamination by microplastics has implications to animal, environmental and human health. The present thesis also contributed to the advance of knowledge on several specific topics within the paradigm of the marine contamination by microplastics, and identified needs of research, and highlighted the need of risk assessment and management of microplastics alone and in mixture with other contaminants, including in relation to human health.

Keywords: microplastics, mercury, mixtures, biomarkers, behaviour, bioaccumulation, bioconcentration, European sea bass, Atlantic horse mackerel, Atlantic chub mackerel, food security and safety, human health.

Resumo

A presença e a acumulação de resíduos plásticos no ambiente marinho são questões ambientais bem conhecidas. No entanto, a presença e a persistência de microplásticos (partículas de plástico com dimensão inferior a cinco milímetros) em ecossistemas marinhos tornaram-se particularmente preocupantes nos últimos anos devido aos seus potenciais impactos em animais, ecossistemas e riscos para a saúde humana. Por esta razão, os microplásticos são agora reconhecidos como poluentes emergentes de grande preocupação e são considerados um tópico de investigação de elevada prioridade. Neste contexto, o principal objetivo desta Tese foi contribuir para o avanço do conhecimento sobre os efeitos ecotoxicológicos dos microplásticos em organismos marinhos e as suas implicações para a saúde animal, ambiental e humana.

Para analisar e sumarizar o conhecimento atual sobre a contaminação marinha por plásticos e microplásticos, os seus efeitos ecotoxicológicos e identificar tópicos que mereciam uma investigação mais aprofundada, foram realizadas duas revisões da literatura, uma sobre plásticos (Capítulo II) e a outra sobre microplásticos (Capítulo III), as quais estão publicadas na forma de dois capítulos de livro científico internacional.

Na primeira fase do estudo experimental (Capítulo IV), foram investigados os efeitos induzidos a curto prazo por microplásticos, isolados e em mistura com mercúrio, e a sua potencial influência na bioacumulação e bioconcentração do mercúrio, utilizando juvenis do robalo europeu (*Dicentrarchus labrax* Linnaeus, 1758) como modelo biológico. O mercúrio foi selecionado para este estudo porque é um poluente ubíquo de elevada preocupação devido à sua toxicidade, por poder ser acumulado por organismos e porque as suas formas orgânicas, particularmente o metilmercúrio, poderem ser biomagnificadas nas redes tróficas. Resumidamente, num bioensaio laboratorial, grupos de peixes foram expostos durante 96 h a mercúrio (0,010 e 0,016 mg/L), microplásticos (microesferas de plástico com 1 a 5 µm de diâmetro, fluorescentes e de composição desconhecida, 0,26 e 0,69 mg/L) ou a misturas de ambos os poluentes nas mesmas concentrações. Durante o bioensaio foram determinadas as concentrações reais de exposição dos microplásticos e do mercúrio na água. No final do período de exposição, foi avaliado o desempenho de

natação dos animais, determinados vários outros biomarcadores e investigada a bioacumulação e a bioconcentração do mercúrio. Os resultados indicaram que na água do mar o mercúrio pode adsorver aos microplásticos. Indicaram ainda que na gama de concentrações testadas, os microplásticos (0,26 e 0,69 mg/L), o mercúrio (0,010 e 0,016 mg/L) e as suas misturas causaram neurotoxicidade, através da inibição da actividade da enzima acetilcolinesterase (AChE) e da indução de estresse oxidativo e dano lipídico no cérebro, comprometeram a função colinérgica a nível muscular por inibição da actividade das enzimas colinesterases (ChE) do músculo, induziram estresse oxidativo e dano lipídico nas brânquias, fígado e músculo, causaram alterações nos mecanismos de produção de energia celular e reduziram a velocidade de natação e o tempo de resistência a nadar contra o fluxo de água. Foram ainda observadas interações toxicológicas entre os microplásticos e o mercúrio nos peixes e influência dos microplásticos na bioconcentração e bioacumulação do mercúrio. Destas evidências concluiu-se que os microplásticos causaram efeitos tóxicos e modularam a toxicidade do mercúrio em juvenis de *D. labrax.* Em cenários reais, particularmente em áreas poluídas por microplásticos e mercúrio, os juvenis desta espécie podem ser consideravelmente afetados, o que pode levar a uma diminuição da população. Os resultados deste estudo evidenciaram ainda riscos para os predadores de juvenis de *D. labrax* e consumidores humanos desta espécie e o potencial dos microplásticos para causar impactos ambientais devido a efeitos adversos nas populações de peixes e na sua função ecológica. Os resultados incluídos no Capítulo IV da presente Tese encontram-se publicados na forma de três artigos científicos de investigação.

Na segunda fase do estudo experimental (Capítulo V), foi investigada a contaminação de exemplares selvagens de três espécies de peixes (*Dicentrarchus labrax, Trachurus trachurus and Scomber colias*), amplamente consumidas como alimento humano em Portugal e noutros países, por microplásticos. Foram estudados 150 peixes (50 de cada espécie) provenientes do Nordeste do Oceano Atlântico, adquiridos numa lota da região Noroeste de Portugal e destinados a serem vendidos para consumo alimentar humano. Em cada peixe, foram analisados o trato gastrointestinal, uma amostra do músculo dorsal e uma amostra das brânquias relativamente à presença de microplásticos, tendo as partículas sido caracterizadas (tipo de partícula, cor e tamanho). Foram ainda determinados a atividade das enzimas AChE no cérebro e ChE no músculo dorsal e os níveis de peroxidação

lipídica (LPO) no cérebro, nas brânquias e no músculo dorsal. Com base na média total de microplásticos encontrados no músculo dorsal (o principal tecido edível para humanos) dos exemplares analisados e nas recomendações da Agência Europeia de Segurança Alimentar relativamente ao consumo de peixe por diferentes grupos populacionais (três grupos etários de crianças; adultos ou a população em geral), e nos dados do Observatório do Mercado Europeu dos Produtos da Pesca e da Aquicultura e do Serviço Nacional Americano de Pesca Marinha em relação ao consumo de peixe per capita, foi estimada a ingestão de plásticos pela população Europeia, Portuguesa e dos principais países importadores de peixe de Portugal, através do consumo de pescado. Foi encontrado um total de 368 microplásticos em exemplares das três espécies estudadas, os quais estavam presentes no trato gastrointestinal (175 partículas), nas brânquias (112 partículas) e/ou no músculo dorsal (81 partículas). Dos 150 peixes analisados, 49 % continham microplásticos, sendo que 32 % tinham microplásticos no músculo dorsal. A média da concentração de microplásticos no músculo dorsal foi 0.054 ± 0.099 items/g. Os peixes com microplásticos tinham atividade da AChE no cérebro e níveis de LPO no cérebro, músculo dorsal e brânquias superiores aos encontrados nos peixes que não tinham microplásticos. Estes resultados indicam alterações neurológicas e danos oxidativos em órgãos cruciais para a sobrevivência e desempenho dos animais. Sugerem ainda que os microplásticos podem ter contribuído para esses efeitos, embora não se possa excluir a contribuição de outros contaminantes a que os peixes possam ter estado expostos no seu habitat natural. As estimativas de ingestão de microplásticos através do consumo de peixe por subgrupos populacionais e pela população humana europeia em geral variaram entre 112 e 842 items por ano. As estimativas de ingestão de microplásticos por ano per capita para diferentes países, indicaram que a exposição a microplásticos através do consumo de peixe pode ser consideravelmente superior em países onde o consumo de peixe é alto, como em Portugal (3078 MP items/ano/capita). Considerando que os peixes são apenas uma das vias de exposição humana a microplásticos, este estudo e outros da literatura enfatizam a necessidade de mais investigação, avaliação de risco e adoção de medidas que minimizem a exposição humana a microplásticos.

Na última fase do estudo (Capítulo VI), foi efetuada uma revisão da literatura que incidiu particularmente sobre as questões de sustentabilidade alimentar, segurança alimentar e saúde humana. Deste estudo concluiu-se que a presença de

microplásticos no ambiente marinho tem implicações para a sustentabilidade e segurança alimentar e para a saúde e bem-estar humanos. Concluiu-se também que o Homem está exposto a microplásticos por várias vias (especialmente através de alimentos, da água de consumo e do ar), pelo que urge avaliar riscos e potenciais efeitos a longo prazo e adotar medidas de prevenção e mitigação. Foram ainda identificados vários tópicos para investigação futura e recomendadas ações de gestão. Este trabalho está publicado na forma de um artigo científico de revisão.

No Capítulo VII, foram integrados e discutidos de forma mais abrangente os resultados mais importantes e retiradas as conclusões gerais da Tese.

Finalmente, no Capítulo VIII, são apresentados os principais contributos da Tese para o avanço do conhecimento na área e diversas perspetivas de trabalho futuro.

Em suma, os estudos incluídos na presente Tese indicaram que a contaminação do ambiente marinho por plásticos e microplásticos é um fenómeno global, que os microplásticos podem ser absorvidos por peixes e outros organismos e induzir diversos tipos de efeitos adversos, que os microplásticos influenciam a toxicidade de outros contaminantes ambientais em animais expostos a misturas contendo estas partículas, que os peixes utilizados para consumo humano contêm microplásticos e que a contaminação ambiental por microplásticos tem implicações na saúde animal, ambiental e humana. A presente Tese contribuiu ainda para o avanço do conhecimento em diversos tópicos específicos no âmbito do paradigma da contaminação do ambiente marinho por microplásticos, identificou aspetos que requerem mais investigação, evidenciou a necessidade de avaliação e gestão de risco de microplásticos individualmente e em mistura com outros contaminates ambientais, incluindo em relação à saúde humana.

Palavras-chave: microplásticos, mercúrio, misturas, biomarcadores, comportamento, bioacumulação, bioconcentração, robalo, carapau, cavala, sustentabilidade e segurança alimentar, saúde humana.

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♦ Appendix D – Supplementary information Chapter IV, Section 4

Abbreviations list

ALDFG - Abandoned, lost or otherwise discarded fishing gear

ANCOVA - Analysis of Covariance

ANOVA - Analysis of Variance

BAF - Bioconcentration factor

BCF - Bioaccumulation factor

CAT - Catalase enzyme

CBD - Convention on Biological Diversity

CCAMLR - Commission for the Conservation of Antarctic Marine Living Resources

ChE - Cholinesterase enzymes

CMS - Convention on the Conservation of Migratory Species of Wild Animals

DDT - Dichlorodiphenyltrichloroethane

DFG - Derelict fishing gear

DGAV - Portuguese National Authority for Animal Health, "Direção Geral de Agricultura e Veterinária"

EC - European Commission

EEZs - Exclusive economic zones

EFSA - European Food Safety Authority

EU - European Union

EUMOFA - European Market Observatory for Fisheries and Aquaculture Products

FAO - Food and Agriculture Organization of the United Nations

FTIR - Fourier transform infrared spectroscopy

GEF - Global environment facility

GES - Good environmental *status*

GESAMP - Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection

GI - Gills

GPA - Global plan of action

GPML - Global partnership on marine litter

GPx - Glutathione peroxidase enzyme

GR - Glutathione reductase enzyme

GST - Glutathione S-transferases enzymes

GT - Gastrointestinal tract

HDPE - High-density polyethylene

HELCOM - Baltic Marine Environment Protection Commission

Hg - Mercury

Hg-H - High mercury concentration

Hg-L - Low mercury concentration

HIPS - High impact polystyrene

HOCs - Hydrophobic organic chemicals

ICRW - International Convention for the Regulation of Whaling

IDH - Isocitrate dehydrogenase enzyme

IGOs - Intergovernmental Organizations

IMO - International Maritime Organization

IUCN - International Union for Conservation of Nature

IWC - International Whaling Commission

LC50 - Median lethal concentration: the concentration of the tested substance estimated to cause 50% of mortality in the tested population in the specific conditions of the toxicity bioassay

LDH - Lactate dehydrogenase enzyme

LDPE - Low-density polyethylene

LME - Large marine ecosystems

LPO - Lipid peroxidation

MARPOL - International Convention for the Prevention of Pollution from Ships

MEPC - Marine Environment Protection Committee

MPs - Microplastics

MPs-H - High microplastics concentration

MPs-L - Low microplastics concentration

MSFD - European Marine Strategy Framework Directive

MU - Dorsal muscle

NE - North East

NGOs - Non-governmental Organizations

Ni - Nickel

NOAA/USA - National Oceanic and Atmospheric Administration/ United States of America

NW - Northwest

OECD - Organization for Economic Co-operation and Development

OSPAR - Convention for the Protection of the Marine Environment of the North-East Atlantic

PA - Polyamides

PAHs - Polycyclic aromatic hydrocarbons

PAN - Polyacrylonitrile

Pb - Lead

PBDEs - Polybrominated diphenyl ethers

PBTs - Persistent, bioaccumulative, and toxic substances

PC - Polycarbonate

PCBs - Polychlorinated biphenyls

PE - Polyethylene

PES - Polyester

PET - Polyethylene terephthalate

PEVA - Polyethylene-vinyl acetate

POPs - Persistent organic pollutants

PP - Polypropylene

Ppm - Parts per million

PS - Polystyrene

PVAc - Polyvinyl acetate

PVC - Polyvinyl chloride

RASFF - Rapid Alert System for Food and Feed

RFMO/As - Regional Fisheries Management Organizations and Arrangements

ROS - Reactive oxygen species

SD - Standard deviation

SDG - Sustainable goals

SIDS - Small Island Developing States

SOD - Superoxide dismutase enzyme

TBARS - Thiobarbituric acid reactive substances

UN – United Nations

UNCLOS - United Nations Convention on the Law of the Sea (UNCLOS)

UNDP - United Nations Development Programme

UNEP - United Nations Environment Programme

UNESCO - United Nations Educational, Scientific and Cultural Organization

US EPA - United States Environmental Protection Agency

UV - Ultraviolet radiation

w.w. - wet weight

WHO - World Health Organization

Chapter I

General introduction

1.1. Brief introduction to the paradigm of marine environmental contamination by plastics

Plastic pollution is a major challenge of our times and in the last years has gained large attention from scientists, media, general public, and Authorities. It is estimated that each year, about 5 to 10 % of worldwide plastic production will end up at seas and oceans (Jambeck *et al.*, 2015). Derived mainly from land-based sources (~ 80 %), but with contribution of sea-based ones (~ 20 %) (GESAMP, 2016), plastic litter is able to travel great distances across the globe, transported through rivers, draining systems, winds, ocean currents and animals (Barboza *et al.*, 2019a), among other ways. Plastic waste is now so ubiquitous in the natural environment that scientists have even suggested it could serve as geological indicator of the Anthropocene era (Zalasiewicz *et al.*, 2016).

The widespread occurrence of persistent micro-sized plastic debris in the marine environment has been one of the main current concerns (Rochman, 2018). These small plastic bits are called "microplastics" and this designation was introduced in the scientific literature within the last decades, to describe microscopic plastic particles found in the marine environment (Thompson *et al.*, 2004; GESAMP, 2016; Frias and Nash, 2019).

Microplastics may contain very toxic chemicals incorporated during their manufacture, use and/or permanence in the environment and can be ingested by different types of organisms including species widely used in the human diet (Gallo *et al.*, 2018; Barboza *et al.*, 2018a; Smith *et al.*, 2018). Therefore, the increasing quantity of microplastics in the environment poses many potential risks to the wildlife, environmental and human health due to the particles themselves and to the chemicals that they generally contain. Moreover, in the environment, microplastics are often colonized by microbes and other organisms, including pathogenic ones, which may increase the global risk of human and animal diseases via new contamination/infection routes (Wright and Kelly, 2017; Barboza *et al.*, 2018a).

In this framework, microplastics are now considered ubiquitous environmental pollutants of high concern (*e.g.* Rochman, 2018; Frias and Nash, 2019) and regulations to monitor and investigate the problem of these small plastic debris have been implemented (*e.g.* European Marine Strategy Framework Directive – MSFD,

Directive 2008/56/EC). Despite the studies conducted in recent years, several important questions related to the fate of microplastics in organisms and ecosystems, toxicological and ecological effects, and interactions with other contaminants remain open and thus, their impacts on animal, ecosystem and human health are still far of being completely understood (Barboza *et al.*, 2018a; de Sá *et al.*, 2018).

1.2. General and specific objectives of the Thesis

The main goal of this Thesis was to contribute to the advance of knowledge on the ecotoxicological effects of microplastics on marine organisms and their implications to animal, environmental and human health. To reach this main goal, the following specific objectives (SO) were established:

- SO1 – To review the literature regarding the paradigm of the marine contamination by macroplastics;
- SO2 – To review the literature regarding the paradigm of the marine contamination by microplastics;
- SO3 – To investigate the behaviour of microplastics and mercury in the water, the neurotoxicity, oxidative damage and energy-related changes potentially induced by short-term exposure to microplastics alone and in mixture with mercury on juveniles of the European seabass (*D. labrax*), and the possible influence of microplastics on mercury bioaccumulation (brain and muscle);
- SO4 – To investigate the oxidative stress and lipid oxidative damage potentially induced by short-term exposure to microplastics alone and in mixture with mercury in the gills and liver of *D. labrax* juveniles, and the possible influence of microplastics on mercury bioconcentration (gills) and bioaccumulation (liver);
- SO5 – To investigate the short-term effects of microplastics alone and in mixture with mercury, on the swimming performance of *D. labrax* juveniles;
- SO 6 – To investigate the occurrence of microplastics in fish species on sale for human food consumption in relation to fish biomarkers, and to estimate the human intake of microplastics through fish consumption in Europe and selected countries from other regions;

- SO 7 – To provide an overview of marine contaminantion by microplastics and its effects, and the potential risks associated with the presence of microplastics in the marine environment, including in a perspective of human food security, food safety and health.

The European seabass (*Dicentrarchus labrax)*, the Atlantic horse mackerel (*Trachurus trachurus)* and the Atlantic chub mackerel (*Scomber colias*) were selected for the present study mainly because they are highly consumed as food by humans in Portugal and several other countries, and therefore they wild populations have high economic importance (EUMOFA, 2017). Moreover *D. labrax* is an excellent and widely used model in Ecotoxicology (*e.g.* Gravato and Guilhermino, 2009, Almeida *et al.*, 2010, Hernández-Moreno *et al.*, 2011) that can be obtained from aquaculture (avoiding the use of wild specimens in laboratory experiments), and therefore it was selected as test organism for the laboratory bioassays.

1.3. Thesis structure

This Thesis is organized in 9 Chapters and the general Thesis framework is presented in Figure 1.1.

The Chapter I corresponds to the general introduction of the Thesis, and includes a brief introduction to the problem of the marine environmental contamination by plastics, and the general aim, specific objectives and the structure of the Thesis.

Chapter II is a review of the literature regarding the paradigm of the marine contamination by macroplastics, its effects and possible impacts, and compilates agreements and actions to prevent and combat plastics debris in the world`s oceans and seas, thus addressing the SO1.

Chapter III is a review of the literature regarding the paradigm of the marine contamination by microplastics, its global distribution and biological effects, and existing gaps of knowledge, thus addressing the SO2.

In Chapter IV, the short-term effects of microplastics alone and in mixture with mercury were investigated using juveniles of the European seabass (*Dicentrarchus labrax* Linnaeus, 1758) as biological model. The section 4.1 provides a brief

summary of mercury and its toxic effects and the rational for its use in this study. The section 4.2 addressing the SO3, introduces, describes and discuss the behaviour of microplastics and mercury in the water, the effects of the contaminants on brain and muscle of *D. labrax* juveniles, and the potential influence of microplastics on the bioaccumulation of mercury in the brain and muscle. The section 4.3 addressing the SO4, introduces, describes and discuss the effects of contaminants on gills and liver, and the influence of microplastics in the bioconcentration of mercury in gills and in the bioaccumulation of the metal in the liver. The section 4.4 adressing the SO5, introduces, describes and discuss the behavioural effects of microplastics and mercury, namely on fish swimming velocity and time resisting against the water flow.

Given the evidence of microplastic ingestion by fish species used in the human diet available in the literature and the potential risks to human health, this issue was investigated in three species widely consumed as human food in Portugal and other countries (*D. labrax*, *T. trachurus* and *S. colias*). In this way, 50 animals per species were analized regarding their contamination by microplastics (gastrointestinal tract, gills and dorsal muscle). Biomarkers indicative of neurologic alterations and lipid oxidation damage were also determined to investigate potential adverse effects that may be at least partially due to microplastic contamination. Moreover, estimates of microplastics human intake through the consumption of marine wild fish were made. This study is described and discussed in Chapter V that addressed the SO6.

In Chapter VI that addressed SO7, the evidences of seafood contamination by microplastics available in the literature were reviewed, and the potential consequences of microplastic marine contamination for human food security, food safety and health were discussed.

The results of Chapters II to VI are integrated and discussed in Chapter VII, highlighting the major findings of the present Thesis.

In Chapter VIII, the concluding remarks, the contribution of the studies included in the present Thesis to the advance of the knowledge and future work perspectives are provided.

Finally, Chapter IX is the list of references corresponding to text citations.

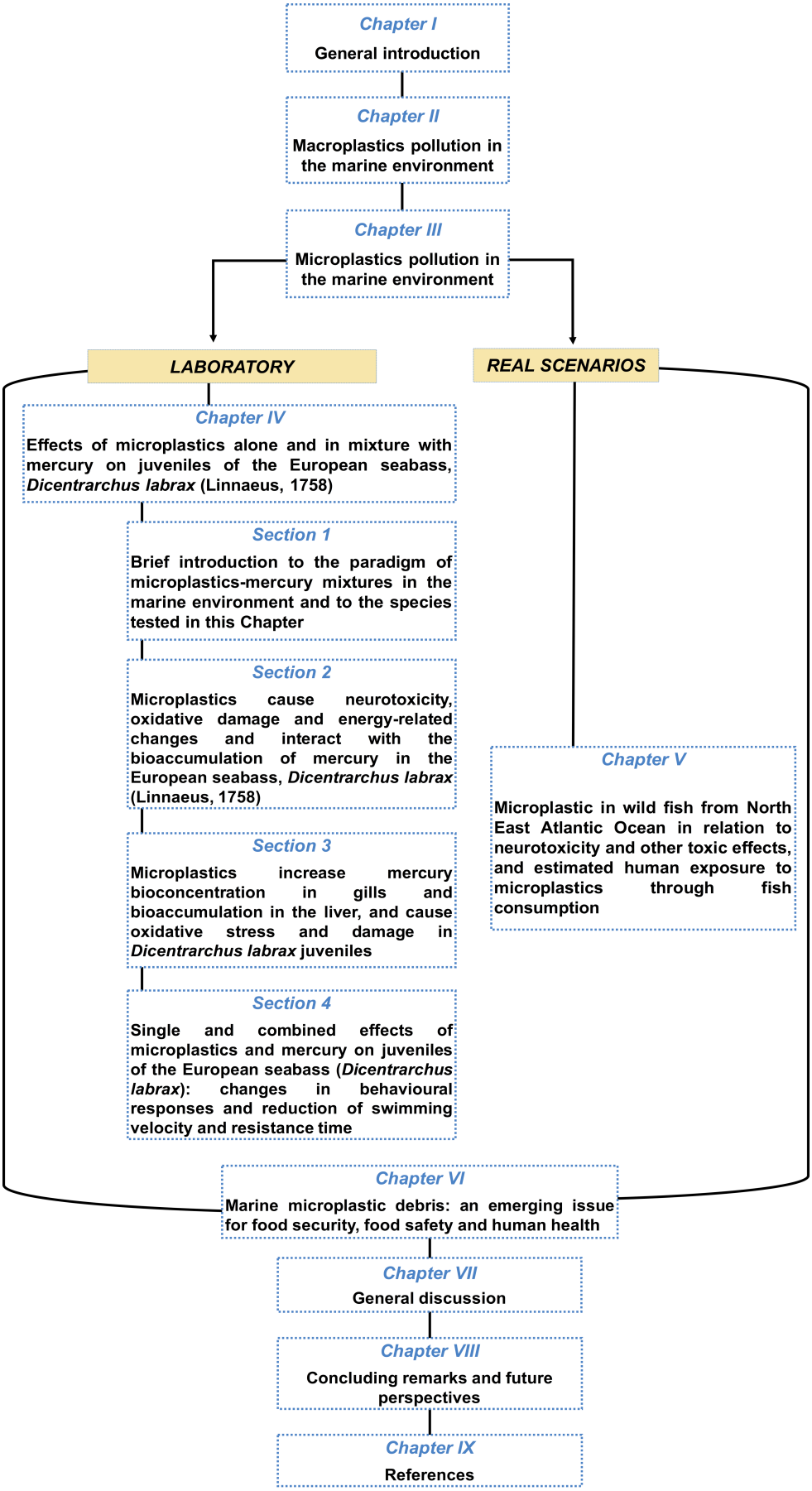

Figure 1.1. Framework of the Thesis.

Chapter II

Macroplastics pollution in the marine environment

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(Book Chapter included in the present Thesis with permission of Elsevier included in the Appendix A, with punctual modifications)

2.1. Introduction

The first synthetic polymers were developed in the middle of the 19th century, marking the beginning of the "Plastic Era," and by the beginning of the 20th century, the manufacture of new plastic types increased rapidly (Law, 2017). The demand for plastic is increasing, with a variety of applications in industries from food packaging, civil construction products, automotive and medical applications, as well as electrical and electronic components, and its worldwide production is estimated to be approximately 322 million tons (Plastics Europe, 2016). There are approximately 50 different basic types of polymers included in 60,000 plastic formulations (Shashoua, 2008), the most common being high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyvinyl chloride (PVC), polystyrene (PS), polypropylene (PP), and polyethylene terephthalate (PET) (Li *et al.*, 2016; Plastics Europe, 2016) (Table 2.1).

Plastics are durable, which allows them to remain for years in the marine environment, where their degradation may take decades (Hammer *et al.*, 2012; Hidalgo-Ruz *et al.*, 2012). According to Jambeck *et al.* (2015), 275 million metric tons of plastic waste were generated in 192 coastal countries in 2010, and 4.8 to 12.7 million metric tons entered into the ocean. Plastic makes up most (60 % – 80 %) of all marine litter found worldwide (Derraik, 2002).

Reports of plastic pollution in the oceans first appeared in the early 1970s (Carpenter and Smith, 1972). Subsequently, the discovery of an extensive area of plastic waste accumulation in the North Pacific Gyre, including abandoned fishing nets, bottles and caps, toothbrushes, containers, boxes, and tiny plastic particles that were fragmented by the action of waves or by photodegradation process (Moore *et al.*, 2001), showed that the problem of plastic in the oceans was on a scale never before admitted (Sobral *et al.*, 2011).

Marine plastic pollution affects many taxa from invertebrates to vertebrates (Deudero and Alomar, 2015). There has been an increase in the number of records about seabirds, marine mammals, turtles, fish, and invertebrates threatened by marine litter throughout the last years (Kühn *et al.*, 2015), and its presence in marine environments is now one of the greatest environmental problems of our time (Law, 2017).

Table 2.1. Types of plastic commonly found in the natural environment: specific gravity (g/cm^3) and common uses (Andrady, 2011; Li *et al.*, 2016).

Type of Plastic	Acronym	Specific gravity (g/cm^3)	Common uses
Polypropylene	PP	0.83-0.85	Dip bottles and ice cream tubs, potato chip bags, microwave dishes, kettles, garden furniture, lunch boxes, blue packing tape
Polyethylene	PE	0.91-0.96	Wide range of inexpensive uses including supermarket bags, plastic bottles
Low-density polyethylene	LPDE	0.91-0.93	Glad wrap, garbage bags, squeeze bottles, clack irrigation tube, black mulch film, garbage bins
High-density polyethylene	HDPE	0.94-0.96	Freezer bags, milk bottles, juice bottles, shampoo bottles, chemical and detergent bottles, rigid agriculture pipe
Polyethylene terephthalate	PET	1.37	Soft drink and water bottles, salad domes, biscuit trays, salad dressing and peanut butter containers
Polystyrene	PS	1.04	CD cases, plastic cutlery, imitation cristal glassware, low cost brittle toys, video cases
High impact polystyrene	HIPS	1.04-1.07	Refrigerator liners, food packaging, vending cups, electronics
Polyamides	PA	1.13-1.35	Fibers, toothbrush bristles, fishing line, under-the-hood car engine moldings, making films for food packaging
Polyester	PES	1.38-1.40	Fibers and textiles
Polyvinyl chloride	PVC	1.37-1.39	Plumbing pipes and fittings, cosmetic containers, electrical conduit, wall cladding, roof sheeting, garden hose, blood bags and tubing
Polycarbonate	PC	1.20-1.22	Compact discs, eyeglasses, riot shields, security windows, traffic lights, lenses, construction materials

2.2. Types of plastic debris

In order to monitor and quantify possible impacts on biota, plastic debris are categorized into different size classes. Although different authorities recommend subtly different size limits (Ryan *et al.*, 2009), plastic debris can broadly be divided into four classes (Barnes *et al.*, 2009; GESAMP, 2016) (Table 2.2). Larger fragments pose the risk of entanglement, ingestion, and suffocation, mostly to birds, fish, and marine mammals living in polluted areas, whereas meso- and micro-debris may be ingested by a wide range of marine organisms and can lead to serious consequences for these species (Gall and Thompson, 2015). The consequences and impacts of mega-, macro-, and meso-plastics are discussed in this Chapter.

Table 2.2. Size categories of marine plastic litter (Barnes *et al.*, 2009; GESAMP, 2016).

Diameter	Source	Examples
MICRO (≤ 5 mm)	Primary and secondary microplastics	Primary: industrial and domestic products; Secondary: textile, fibers, tyre dust
MESO (5–25 mm)	Fragmentation of larger plastic itens	Bottle caps, fragments
MACRO (25–1000mm)	Lost items from maritime activities or from rivers	Plastic bags, food and other packaging, fishing floats, buoys, balloons
MEGA (> 1 m)	Abandoned gear, catastrophic events	Abandoned fishing nets and traps, rope, boat hulls, plastic films from agriculture

2.3. Global distribution of plastic debris

Knowledge of the global distribution of marine plastic debris is severely limited by our capacity of obtaining extensive and comparable data sets. Plastic debris comprises a heterogeneous assemblage, including items of a wide range of shapes, sizes, and chemical composition. The typology of plastic objects entering into the ocean is as wide as the possible uses of the plastic materials. Microbeads from cosmetics, textile fibers, industrial pellets, bags, bottles, toys, ghost fishing nets, and buoys, or fragments from them may be found in the marine ecosystems. Plastic debris spans at least six orders of magnitude in size, from microns to meters (Martí *et al.*, 2017). They are composed of a broad diversity of polymers, which confer varied properties in relation to, for instance, its density (Hidalgo-Ruz *et al.*, 2012). This

makes them susceptible to be transported by surface ocean currents (Lebreton *et al.*, 2012; van Sebille *et al.*, 2012), by bottom currents through the canyons in the continental slope (Galgani *et al.*, 1996), or even by wind flow (Dris *et al.*, 2016), which, together with the wide distribution of land- and sea-based sources, leads to the occurrence of plastic residues everywhere in the world's seas and oceans. Beaches (Browne, *et al.*, 2015a,b), mangroves (Ivar do Sul *et al.*, 2014), coral reefs (Hall *et al.*, 2015), as well as surface and mid waters (Kooi *et al.*, 2016; Law *et al.*, 2010), deep bottoms (Pham *et al.*, 2014), and even sea ice (Obbard *et al.*, 2014) have been reported as reservoirs of plastic debris.

Surface-trawling plankton nets, visual *census* from vessels or on beaches, aerial surveys, bottom-trawling fishing nets, diving, towed video cameras, submersible vehicles, sediment corers, and quadrants, or continuous plankton recorders are some of the most common sampling methods used to quantify plastic debris (JRC, 2013). However, all these techniques only provide local views of a portion of the marine plastic size range, complicating a comprehensive representation of global patterns. Yet the number and spatial coverage of data have widely increased in recent years, and significant progress has been made in the understanding of the global plastic distribution, particularly from the use of surface-trawling plankton nets to measure plastic concentrations on the ocean surface (*e.g.* Cózar *et al.*, 2014; Eriksen *et al.*, 2014; Law *et al.*, 2010, 2014). One of the most relevant findings arising from the surveys with surface-trawling plankton nets is the existence of huge accumulation zones of floating plastic debris (Law *et al.*, 2010), where concentrations are up to four or five orders of magnitude higher than in non accumulation zones (Cózar *et al.*, 2014; Eriksen *et al.*, 2014; van Sebille *et al.*, 2015). These are mainly in the convergence zones of each of the five large subtropical gyres, in the North and South Pacific, North and South Atlantic, and Indian Ocean (Cózar *et al.*, 2014; Eriksen *et al.*, 2014). These accumulation zones are mainly caused by global wind patterns and their effect on ocean surface currents (Fig. 2.1). In each hemisphere, there are steady latitudinal bands of wind flow, easterly winds in the tropics, from 0 to 30 degrees of latitude, and westerly winds in the mid-latitudes, from 30 to 60 degrees. Since the Earth rotates, the resulting surface water currents turn to the right of the wind direction in the Northern Hemisphere, and to the left in the Southern Hemisphere, the Coriolis effect. Therefore, large vortices pump surface

water and floating debris to the centers of the ocean basins, at around 30 degree latitude in each hemisphere.

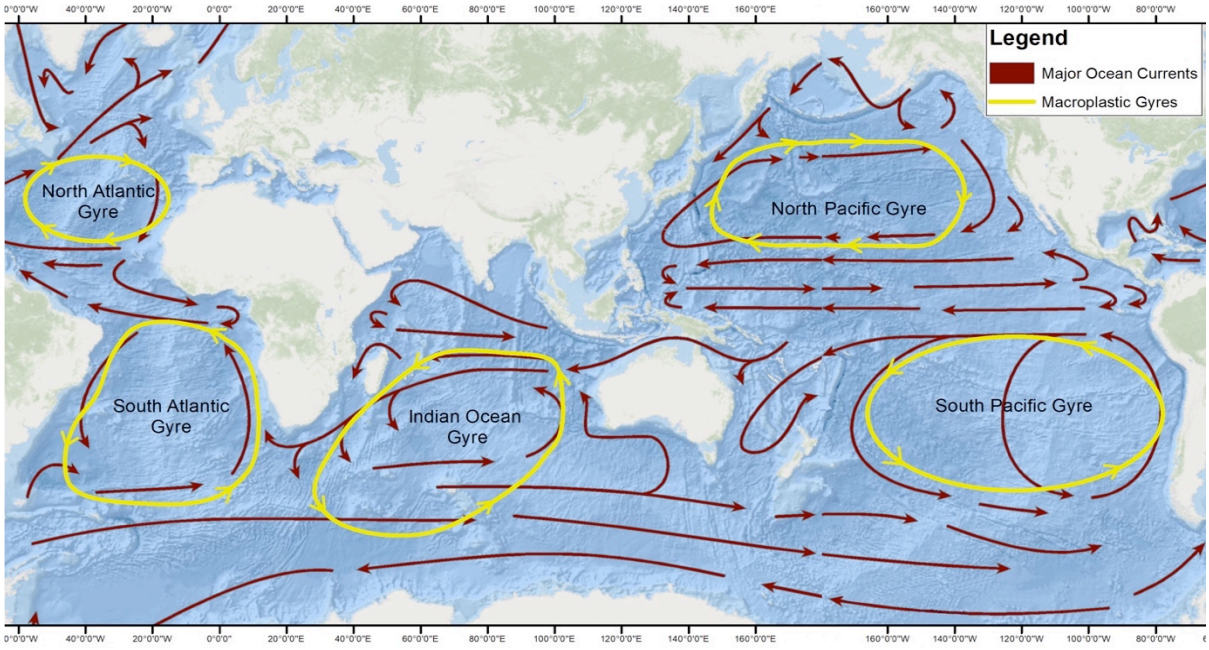

Figure 2.1. The accumulation zones of plastic that form in the five subtropical gyres (kindly designed by Anne Sheppard).

Of the five subtropical gyres, the North Pacific accumulates the largest amount of floating plastic, around one third of the total or higher (Cózar *et al.*, 2014; Eriksen *et al.*, 2014; van Sebille *et al.*, 2015). This observation agrees with the model estimates of plastic waste inputs from land into the ocean, locating also the top four most polluting countries on the Western coast of the Pacific Ocean (Jambeck *et al.*, 2015). Interestingly, the North Atlantic Ocean, also associated with high coastal populations and intense maritime traffic, shows lower plastic concentrations. Subtropical gyres are not closed plastic reservoirs, but they are leaky and some of them, such as the North Atlantic Gyre, release more plastic than others (van Sebille *et al.*, 2012). The leakiness of the accumulations of floating plastic debris at subtropical latitudes leaves room for their dispersion to polar latitudes, a possibility seen in the Arctic Ocean. Hundreds of tons of floating plastic debris are carried from the North Atlantic to the Greenland and Barents seas in the Arctic Ocean by the Thermohaline Circulation (Cózar *et al.*, 2017), the giant convective cell redistributing heat from the warm latitudes to the poles. The formation of deep water by cooling in the Greenland and Barents seas plays a key role in this density-driven global circulation, acting as a large suction pump of surface water that supplies motion to

the Thermohaline Circulation (Kuhlbrodt *et al.*, 2007). Therefore, the North Atlantic branch of the Thermohaline Circulation also collects floating plastic debris from highly populated latitudes and delivers them to the Greenland and Barents seas, where the landmasses, together with the polar ice cap, are a dead end for the surface transport of floating debris (Cózar *et al.*, 2017). The poleward transport of debris adrift in the Northern Hemisphere suggests possible plastic accumulations also in the Southern Ocean, also known as the Antarctic Ocean. Indeed, the Thermohaline Circulation conveys surface waters to the Ross and Weddell seas, in the Pacific and Atlantic sectors of the Southern Ocean, respectively (Kuhlbrodt *et al.*, 2007). On the other hand, the Southern Ocean, surrounded by the vigorous Antarctic Circumpolar Current, differs from the Arctic Ocean in that it has a strong northward Ekman flow that will disperse most floating plastic back toward the subtropical gyres in the Southern Hemisphere. These features should impede the transport of plastic into the Southern Ocean, as observed from satellite-tracked drifting buoys (van Sebille *et al.*, 2012). However, field data are still sparse and the Southern Ocean remains a large gap in our knowledge on global plastic pollution (Waller *et al.*, 2017).

The global map of floating plastic debris is the result of the world distribution of plastic sources and the redistribution due to the main global patterns of ocean circulation, the large wind-driven at low and mid latitudes and the density-driven circulation extending the plastic pollution to the sparsely populated high latitudes. This picture is completed by an additional kind of surface plastic accumulation, the semi-enclosed seas. Global models of ocean surface circulation have identified a wide list of potential accumulation zones in regional seas (Lebreton *et al.*, 2012), though many of these are yet unexplored and, to date, field data have just tested significant accumulations in the Mediterranean (Cózar *et al.*, 2015) and East Asian seas around Japan (Isobe *et al.*, 2015). Both of these regions have high human pressure together with a limited hydrodynamic capacity to transfer the plastics they receive into the open ocean, thus acting as local traps for floating plastic pollution. The plastic concentrations in these accumulation zones are comparable to or even higher than those described for the subtropical gyres (Cózar *et al.*, 2015; Isobe *et al.*, 2015; van der Hal *et al.*, 2017).

Surface-trawling plankton nets are an easily comparable and workable method to undertake the study of the global distributions. However, comprehensive large-scale assessments for any other marine plastic reservoirs such as seafloor or coasts

are still lacking (Browne *et al.*, 2015a,b). Floating plastic debris captured by these nets are limited to the size window defined by the mesh size (usually hundreds of microns) and by the dimension of the net-mouth aperture (generally tens of centimeters), thereby excluding the smallest and largest sizes of floating plastic debris. More importantly, the floating plastic debris collected by plankton nets accounts for a tiny fraction (< 1 %) of the plastic entering into the ocean (Cózar *et al.*, 2014; van Sebille *et al.*, 2015). Floating plastic debris are efficiently removed from the surface by a combination of multiple processes (*e.g.* ballasting, ingestion), and the seafloor is regarded as the most likely destination (Cózar *et al.*, 2014; Van Cauwenberghe *et al.*, 2013). Moreover, only about half of all produced plastic polymers in seawater are buoyant (Andrady, 2017), pointing to the nearshore bottoms as a major plastic reservoir.

2.4. Threats to wildlife and the environment from plastic debris

Debris of anthropogenic origin, especially plastics, affect marine biota and ecosystems in many ways. In addition to the deeply detrimental impact of the plastic pollution on marine life, there are other underlying costs too, particularly with regards to both marine and coastal activities, and in turn the economic benefits that local communities and nations derive from them. Knowledge of impacts caused by marine litter is the first step in the search for remediation and control measures. However, the direct and indirect effects of oceanic plastic debris on marine organisms, food webs, and assemblage structure remain poorly understood.

2.4.1. Entanglement

For marine fauna in general, the main problematic biological interactions arising from contact with litter are related to entanglement and/or ingestion. Plastic debris account for 92 % of entanglement and ingestion cases, and around 17 % of all species involved are on the IUCN Red List of Threatened Species (Schepis, 2016). Entanglement happens when the loops and openings of any type of debris entangle animal appendages or entrap it, often resulting in death by drowning, suffocation, or strangulation (Laist, 1997; Moore, 2008). If not instantly fatal, entanglement can cause injuries and wounds or impair animal swimming capacity, leading to starvation through reduced feeding efficiency and making it difficult to escape to predators (US EPA, 1992; Allsopp *et al.*, 2006). In addition, the pups of

several marine species may be affected by fishing nets and other plastic artefacts (*e.g.* bands, collars, and straps discarded by vessels) when these are caught around their necks or bodies, tightening and strangling the animal as it grows (Derraik, 2002).

Many different marine species are impacted, including birds, turtles, mammals, fish and crabs (Table 2.3). It is no longer possible to say which sites are most susceptible to this type of incident; since the problem of marine debris has become global, the entanglement can happen anywhere. An emblematic example can be observed in the Henderson Island, the largest of the four islands of the Pitcairn group. This island is a UNESCO World Heritage Site and until recently, owing to its isolation (5000 Km from the nearest human population), was protected against most human activities. During a recent expedition to the site, a team of researchers from the University of Tasmania found the island's beaches covered with plastic waste, accounting for about 670 items per square meter, the highest density ever recorded (Lavers and Bond, 2017). More than this, they also found a huge amount of animals living among plastic, such as cosmetic jars, bottle caps, plastic drums, and fishing nets (Fig. 2.2, A, B). This particular case proves how even the remotest places are not free from plastic pollution and how severe the consequences are for the ecosystem and the local fauna.

A wide variety of marine debris causes entanglement, but derelict fishing gear (DFG), such as nets fragments, lines, lures, rope, six-packs rings, bait boxes, and strapping bands, are the most common sources of this trouble (Woodley, 2002; Allsopp *et al.*, 2009). The impacts of this so-called "ghost-fishing" via DFG are undoubtedly the most serious among all the dangers of entanglement. Ghost-fishing (or ghost catch) refers to lost or abandoned gillnets, trawls, or crab pots/ traps, which continue to capture both target and non target species after the fishing equipment is no longer under the control of a fisherman (Smolowitz, 1978). As the name suggests, the most considerable consequence of this problem is the continuous capture of marine animals that get stuck and die in the fishing gear (NOAA, 2015). Nets can remain active for long periods, so the biological characteristics of organisms found may vary, depending also on the types and sizes of nets, and the nature of the habitat (Kaiser *et al.*, 1996; Browne, *et al.*, 2015a,b).

Table 2.3. Number of marine species worldwide with documented entanglement and ingestion records (adapted from Allsopp, 2006).

Species group	Number of species with entanglement records	Number of species with ingestion records
Seabirds	51	111
Sea turtles	6	6
Marine mammals	32	26
Fish	34	33
Crustaceans	8	0
Total number	131	176

(A) (B)

Figure 2.2. Examples of entanglement on the Henderson Island in the Pitcairn Island group (Overseas Territory of the United Kingdom): (A) one of many hundreds of purple hermit crabs (*Coenobita spinosa*) that now make their homes out of plastic debris washed up on the island. This particular item is an Avon cosmetics jar; and (B) adult female green sea turtle (*Chelonia mydas*) entangled in a ghost net (Photos: kindly provided by Jennifer Lavers).

In recent years, the problem of DFGs has been worsening due to increased fishing operations and the introduction of synthetic equipment with high durability. Although it is very difficult to have a precise global number, estimates suggest that abandoned or lost fishing gear constitutes about 10 % (640,000 tons) of marine waste (Macfadyen *et al.*, 2009). This number is alarming and is obviously responsible for numerous impacts. Entanglement, however, is not the only problem caused by ghost-fishing. It can cause considerable economic loss due to wastage of fishery resources used for consumption. Thus, some solutions have been proposed, such as: (1) guidance for fishermen and inspection of fishing practice; (2) reformulation of fishing

gears in order to reduce waste; and (3) replacement of synthetic material to biodegradable material (Schneider, 2009). To subsidize these actions, research should estimate the mortality of animals killed by ghost-fishing in relation to their population sizes and then predict, probably via statistical modeling, the actual ecological impacts of entanglement (Browne *et al.*, 2015b).

2.4.2. Ingestion

Ingestion of plastic debris represents another major threat to marine animals. According to NOAA (2014), the ingestion effects on wildlife health can be divided into two main categories: physical effects and physiological effects, both intrinsically linked. The physical effects are (1) lacerations and lesions, which happen when sharp debris punctures the lining of the digestive system, leading to ulceration, lesions, infection, and inflammation; (2) blockage, which occurs because sheets and plastic bags are indigested and lodged in the gastric system, exposing organs to an onslaught of digestive fluids and causing a false sense of satiety; and (3) retention, which refers to the long residence of the debris in the digestive tract. All physical effects eventually lead to the physiological effects that can be nutritional, developmental, immunological, and toxicological. Plastic debris are also associated with chemical toxicants responsible for sublethal effects on animal development, as well as reproductive cycle and population dynamics, with long-term consequences (Thompson *et al.*, 2009). Species may also be affected by plastic ingestion, due to transfer and accumulation of pollutants (Nelms *et al.*, 2016).

Plastic ingestion has been reported for a variety of marine organisms. Among these, seabirds and sea turtles are the animals that most eat of the marine waste (Table 2.3). It is estimated that > 90 % of seabirds have traces of plastic in their bowels (Wilcox *et al.*, 2015), mainly because they think it is natural food, such as fish eggs and crustaceans (Azzarello and Vleet, 1987). This sad phenomenon has been often observed in Australia and New Zealand (see the example in Fig. 2.3). More than half of the world's sea turtles have ingested plastic and other human-produced debris (Schuyler *et al.*, 2016). In this case, turtles confuse plastic with jellyfish – their preferred food (Laist, 1987; Schuyler *et al.*, 2014). Debris consumption occurs notably on coasts where they come to spawn. The risk of starvation for seabirds and turtles is very worrying. The animals stop feeding because they feel the indigestible

volume of plastic in the stomach, and owing to this constant satiety they die of starvation (Gregory, 2009).

The debris consumed by marine animals is predominantly plastic, whether of industrial, recreational, or personal origin (NOAA, 2014). Thus, rapid economic growth and increased use of disposable plastic in many parts of the world, where waste collection and management (*e.g.* safe storage and recycling) are inadequate, have contributed drastically to this ingestion problem. Priority measures to minimize the problem involve broader national and international policies to reduce the amount of debris entering the oceans (NOAA, 2014). However, it is also necessary to pay more attention to the nature of debris and to the different types of ecological impacts (Browne, *et al.*, 2015b).

Figure 2.3. Example of ingestion on Lord Howe Island in the Tasman Sea (between Australia and New Zealand): (A) Flesh-footed shearwater (*Ardenna carneipes*), one of the seabirds most impacted by marine plastic debris in the world; and (B) in detail, a stomach of flesh-footed shearwater cut open and plastic revealed. (Photos: kindly provided by Ian Hutton).

2.4.3. Suffocation and general debilitation

While entanglement and ingestion of plastic is considered to be the greatest threat to marine animals (Wilcox *et al.*, 2016), plastic pollution has other adverse effects, such as suffocation, drowning, strangulation, and starvation (Kühn *et al.*, 2015). The entanglement and/or the ingestion of plastic litter, for example, can suffocate marine animals or give an artificial sense of being full, leading to starvation (Nicolau *et al.*, 2016). Death by starvation may occur due to the accumulation of

plastic debris in the animals' gut causing obstruction of the digestive tract. Some species of turtles and marine mammals may be capable of passing plastic through their digestive system and seabirds can regurgitate indigestible contents (Sigler, 2014). However, that debris can still lead to malnutrition and cause internal injuries, perforate or block the digestive tract, and cause ulcers (Pierce *et al.*, 2004; Kühn *et al.*, 2015; Acampora *et al.*, 2017) Moreover, accumulation of plastic within the intestines gives a positive buoyancy to marine animals, modifying their swimming behavior, affecting their buoyancy control, and leading to drowning (Nelms *et al.*, 2016; Stelfox *et al.*, 2016). All these effects may increase the risk of predation, smothering, or even reproductive and developmental disturbances (Oehlmann *et al.*, 2009; Gall and Thompson, 2015).

In addition, marine debris modifies physical parameters of marine environments harming ecologically and commercially important species and altering marine assemblages and the ecosystem services they provide (Green *et al.*, 2015). Such physical changes lead to desiccation of invertebrates, affecting their efficiency during foraging (Aloy *et al.*, 2011; Carson *et al.*, 2011). For instance, alterations in temperature and sediment permeability on turtle nesting beaches may influence hatchling sex ratios and reproductive success (Nelms *et al.*, 2016). The smothering of the seafloor, probably the ultimate sink for marine debris, also causes changes in physical parameters of marine environments (Gregory, 2009). It damages flora and fauna, reduces luminosity, and creates anoxic environments through inhibition of gas exchange between the sediment-water interface (Rochman, 2015). Hence, nutrition of filter feeders is limited due to a decrease in water circulation, and the reduction of luminosity may reduce diatom densities (Kühn *et al.*, 2015). Anoxic conditions reduce primary productivity and organic matter and therefore may alter the infaunal community. The possible main causes are colonization of the plastic by epifauna and migration of mobile species (and obstructing settlement of some species with a planktonic larval stage) (Green *et al.*, 2015). Coral reefs are also widely affected by plastic pollution (Gall and Thompson, 2015). Since the interaction with plastics may shade, suffocate, and kill corals, there is a negative correlation between the level of coral cover and coverage of marine debris (Richards and Beger, 2011).

2.4.4. Transport of invasive species

According to the 1992 Convention on Biological Diversity (CBD), invasive alien

species are defined as any species that settle in places outside their natural range and then proliferate uncontrollably, causing a threat to native species and to ecosystem integrity. The consequences of alien invasions can be irreversible for the ecosystem concerned. Invasion by unwanted and aggressive invasive species can be detrimental to offshore, intertidal, and littoral ecosystems (Thevenon *et al.*, 2014). Despite the influence of shipping (fouling on boats and transport in ballast water), the opening of canals, and aquaculture (Keller *et al.*, 2011), another important vector of non-native species is through drifting debris. Natural floating material (*e.g.* volcanic rock or pumice, macroalgae, seagrasses, seashells, dead wood, tree trunks, and seeds) often serves as a hard surface substrate for the widespread transportation of organisms in the oceans (Barnes, 2002; Aliani and Molcard, 2003; Thiel *et al.*, 2003). Travel of marine species by raft material is a well-known mechanism of long-distance dispersal (Browne, *et al.*, 2015b). Anthropogenic material also carries individuals across waters, and the current substantial introduction of solid wastes into the oceans, dominated by plastic debris (Barnes and Milner, 2005), increases the chances of marine species movement through rafting (Browne, *et al.*, 2015b; Allsopp *et al.*, 2006).

Floating plastic debris is very abundant, moves slowly (*i.e.* is susceptible to move species across biogeographic boundaries while they are still alive), is long-lasting (*i.e.* it can withstand degradation at the sea surface for long time periods), and shows distinct patterns of stranding in relation to natural debris (Barnes, 2002; Barnes and Milner, 2005; Lewis *et al.*, 2005). Such features contribute toward rapid colonization and survival of rafting organisms, so that the threat of invasion from rafting on plastic debris is potentially greater than from natural floating material (Barnes, 2002; Browne, *et al.*, 2015b). For Lewis *et al.* (2005), the major problem is the quantity. That is, species move along the same routes as on natural floating material, but it is quite likely that the number of individuals dispersing across open oceans has increased with the large amount of debris deposited in the marine systems every year (Lewis *et al.*, 2005).

In fact, the considerable amount of synthetic and non biodegradable plastics released to the oceans during the past five decades has created alternative “rafts of ride” for a wide range of marine opportunistic colonizers (Gregory, 2009; Thevenon *et al.*, 2014). A variety of sessile and mobile organisms, including (macro-) algae, invertebrates, fishes, and even iguanas, have been observed floating on marine

waste (Barnes, 2002; Thiel and Gutow, 2005). Species of bryozoans, hydroids, barnacles, molluscs, and polychaete worms are most often found traveling on plastic debris (see Table 2.4) (Barnes, 2002). Although colonized marine litter has been found also in the poles, the processes of marine debris colonization and invasion are clearly more frequent and predominant in tropical regions (Barnes, 2002; Barnes and Fraser, 2003; Barnes and Milner, 2005). However, regardless of the region or environment, such invasion by alien species is more dangerous for endangered biota, for at risk coastal environments, and where the endemism is significant (Gregory, 2009; Thevenon *et al.*, 2014).

Although there are a significant number of studies reporting the invasion by marine life on plastic debris, most of them only compile information about the material amount and the organisms on it (Browne *et al.*, 2015a,b). Thus, the effects of marine plastic debris as a transport vector is shown as one of the less recognized and documented problems. This means that the movement of invasive species on plastic debris in the open ocean has been widely reported in the literature, and the linkages between the presence of alien species on floating material and their arrival, survival, capability of reproduction, and population settlement into novel areas are not established (Browne *et al.*, 2015a,b). In order to fill these gaps, future research in this field should focus on establishing such links to determine the actual ecological impacts caused by rafting on plastic debris by invasive species, especially those that are aggressive aliens.

Table 2.4. Some examples of invasive species on plastic debris or on others synthetic materials, which contain plastic in their composition (adapted from Gregory, 2009).

Who?	Where and how?	Reference
Bryozoan *(Membranipora tuberculata)*	In New Zealand (from Australia) on plastic substrates, including virgin plastic pellets (nibs) and large artefacts	Gregory, 1978
Seeds of three exotic plant species, being one not known on the locality	In a small island near Auckland, New Zealand, at a child's small plastic toy boat stranded	West, 1981
Bryozoan *(Thalamoporella evelinae)*	On Florida shores (from Brazil) through attachment to pelagic plastic artefacts and later stranding on beaches	Winston *et al.,* 1997
Oyster *(Lopha cristagalli)*	In a remote beach of New Zealand, attached to a tangled mass of synthetic rope stranded	Winston *et al*., 1997

Barnacle *(Lepas pectinata)* Isopod *(Idotea metallica)*	In Ligurian Sea waters colonizing plastic artefacts	Aliani and Molcard, 2003
10 species belonging to five Phyla: Annelida, Bryozoa, Cnidaria, Mollusca and Porifera	In Adelaide Island (Antarctic Peninsula) on a plastic strapping band washing ashore	Barnes and Fraser, 2003
Harmful microalgae	Along the Catalan coast (northwestern Mediterranean) on pelagic plastic debris	Masó *et al*., 2003
Anenome (*Diadumene lineata)*	In the lagoon of Pearl and Hermes Reef, Northwestern Hawaiian Islands, on derelict trawl netting	Zabin *et al.,* 2004
Bryozoan *(Galeopsis mimicus)*	At east coast of Canterbury, England (from New Zealand) on a small piece of frayed plastic substrate	Carter and Gregory, 2005

2.4.5. Accumulation of toxic substances

The interaction of plastic marine debris with toxic chemicals in the ocean is a serious threat to marine biodiversity, ecosystem health, and ecosystem services (Yuan *et al.*, 2017). The manufacturing process of plastic polymers uses chemicals derived from non-renewable crude oil, several of which are hazardous and may be released during the production, use, and disposal of the plastic product (Lithner *et al.*, 2011). Monomers, oligomers, bisphenol-A, phthalate plasticizers, and flame retardants are among the plastic related contaminants incorporated during the manufacturing process (Gall and Thompson, 2015). In addition to that, marine plastics usually have high levels of toxic compounds adsorbed from seawater or even from the sediment (Rochman, 2015).

Plastic-associated contaminants include persistent, bioaccumulative, and toxic substances (PBTs), such as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), and dioxins (Engler, 2012). These toxic chemicals usually have very low water solubility and tend to sorb to other hydrophobic compounds, such as sediment, organic matter, and plastic debris, increasing their environmental persistence (Vegter *et al.*, 2014). The contaminants' adsorption by plastic debris is about one hundred times more efficient than by suspended organic matter (Engler, 2012). Plastic debris also accumulates metals from ocean water (Rochman, 2015).

Plastic-associated contaminants transfer from plastic debris to marine animals upon consumption may occur, and then there is a potential for transferring these materials through the food chain (Miranda *et al.*, 2016). For instance, both additive chemicals and chemicals that accumulate in nature desorb from polyvinyl chloride

(PVC) and might be transferred into the tissues of animals (Browne *et al.*, 2013). The biomagnification of harmful chemicals associated with plastics up the food web leads to toxic effects at higher trophic levels even at low ambient concentrations (Engler, 2012). Environmentally persistent and toxic substances accumulate in the sediment, harming benthic communities. Then they are subsequently ingested by detritivores (Richards and Beger, 2011). The digestive process of deposit- and suspension-feeding species may also mobilize hazardous substances from ingested plastic particles (Engler, 2012).

Organisms probably face greater effects if exposed to the mixture of plastic with sorbed chemical contaminants (Rochman, 2015). When bioavailable, those contaminants may cause liver toxicity, development disturbance, endocrine disruption, neurotoxic effects, changes in behaviour, and other adverse effects (Rochman, 2015). For instance, concentrations of PCBs and trace metals in seabirds are positively correlated with the mass of ingested plastic (Rochman, 2015). Sea turtles and seabirds have significant concentrations of those contaminants present in their tissues and eggs (Nelms *et al.*, 2016; Tanaka *et al.*, 2013). In addition, toxins may also be transferred to the offspring via the mother, threatening the successful reproduction of species (Oehlmann *et al.*, 2009).

2.4.6. Disturbance of habitats from mechanical beach cleaning

The input of pollutants in coastal areas with intense urban and tourist activities has made necessary the extensive cleaning of beaches, especially during high seasons (Morton *et al.*, 2015). Many places use mechanical techniques (beach grooming) for cleaning, although manual cleaning is cheaper and more sustainable (Vanhooren *et al.*, 2011). Mechanical techniques usually cause serious threats to habitat integrity because there is no distinction between beach litter and organic material (Poeta *et al.*, 2014). Damage by modifications of the subtidal zone (an important recruitment zone for many sandy beach animals) and the loss of biodiversity, productivity, and critical habitats are common (Mascarenhas, 2015). Further, mechanical beach cleaning is also expensive and short term in nature (Poeta *et al.*, 2014).

Removal of the pioneer vegetation, for example, may cause the increase of sediment transport, lowering beach elevation and reducing the basic ecosystem services provided by beach vegetation (Vanhooren *et al.*, 2011; Poeta *et al.*, 2014;

Kelly, 2016). Thus, the destruction of buried and pioneer species caused by grooming leaves the banks susceptible to erosion (Vanhooren *et al.*, 2011; Attorre *et al.*, 2013). Mechanical cleaning limits the distribution of beach vegetation (Defeo *et al.*, 2009; Kelly, 2014) and is associated with a high silicate, phosphate, and dissolved inorganic nitrogen concentration and turbidity in the adjacent surf zone (Russell *et al.*, 2014). The heavy equipment used may crush mature plants, root systems, seeds, seedlings, and root fragments (Dugan and Hubbard, 2010). Therefore, native plant abundance, species richness, and beach wrack are usually lower on groomed beaches compared to unraked beaches (Nordstrom *et al.*, 2012). In addition to that, the bacterial production is usually higher on uncleaned beaches (Malm *et al.*, 2004).

Beach wrack deposition along coasts provides a nutrient source for beach ecosystems and a microhabitat refuge for resident benthic communities (Mossbauer *et al.*, 2012; O'Brien *et al.*, 2017). Moreover, beach wrack also has an important role in the nutrient flow of beaches. The removal of biological resources such as plants, animals, and organic debris has significant ecological consequences as it is a source of food for many organisms and is essential for pioneer vegetation (Vanhooren *et al.*, 2011). Grooming may significantly reduce richness, abundance, and biomass of important prey for higher trophic levels (Acuña and Jaramillo, 2015). By contrast, taxa with well-developed dispersal abilities can be more prevalent, modifying completely the community structure on groomed beaches (Defeo *et al.*, 2009). After a single and short-term grooming event, meiofauna communities may recover quickly (24h) (Gheskiere *et al.*, 2006). However, recovery of macroinvertebrates may be slow, especially if the grooming was conducted on a daily or weekly basis throughout the year, and it may affect the diversity and population dynamics of sandy beach macroinvertebrates (Defeo *et al.*, 2009; Gilburn, 2012).

Grooming may also negatively affect vertebrates by causing direct mortality of their eggs (Defeo *et al.*, 2009; Lucrezi *et al.*, 2016). There are significant reductions in the density of incubating eggs of shorebirds inhabiting groomed sandy beaches and of beach spawning fish (Martin *et al.*, 2006; Lucrezi *et al.*, 2016). Those effects are maximized when the mechanical cleaning includes removal of beach wrack, such as kelp and debris (Martin *et al.*, 2006). For instance, shorebirds are positively correlated with wrack cover and the biomass of their invertebrate prey (Dugan *et al.*, 2003; Peterson *et al.*, 2006). Moreover, tracks created by grooming can adversely affect the ability of turtle hatchlings to reach the sea (Özdilek *et al.*, 2006).

2.5. Agreements and measures to prevent and combat plastic debris: global action and initiatives

The need to improve governance to reduce the impact of plastic debris has been recognized for several years. Legislation, agreements, measures, actions, and initiatives can all be accommodated within the concept of a governance framework. Such frameworks can apply at global, regional, national, or local scales. They can involve national governments, international bodies, intergovernmental organizations (IGOs), public and private entities, and a wide variety of citizens' groups and non-governmental organizations and initiatives. They exist to promote not only the effective organization of institutions or societal groups but also the desired outcomes. This extends beyond the rather narrow definition of governance being "the exercise of authority, control, management and power of government". A simpler definition of governance is "the ability to get things done without necessarily having the legal competence to command they be done" (E.O. Czempiel in Macfadyen *et al.*, 2009). This requires flexibility in approach, and operating at appropriate spatial scales (Fig. 2.4). A measure introduced at a global scale will only be effective if it meets regional and local needs and circumstances. Conversely, there are many example of measures introduced at a local or national scale being reproduced in other parts of the world. In the present context of reducing the presence and impact of plastic debris in the oceans, governance frameworks may be directed specifically at reducing inputs of plastic from land- or sea-based sources, or applied indirectly as a means to meet some other goals, such as: protecting biodiversity and sensitive habitats; improving human health and well-being; or encouraging economic development (Campbell *et al.*, 2016).

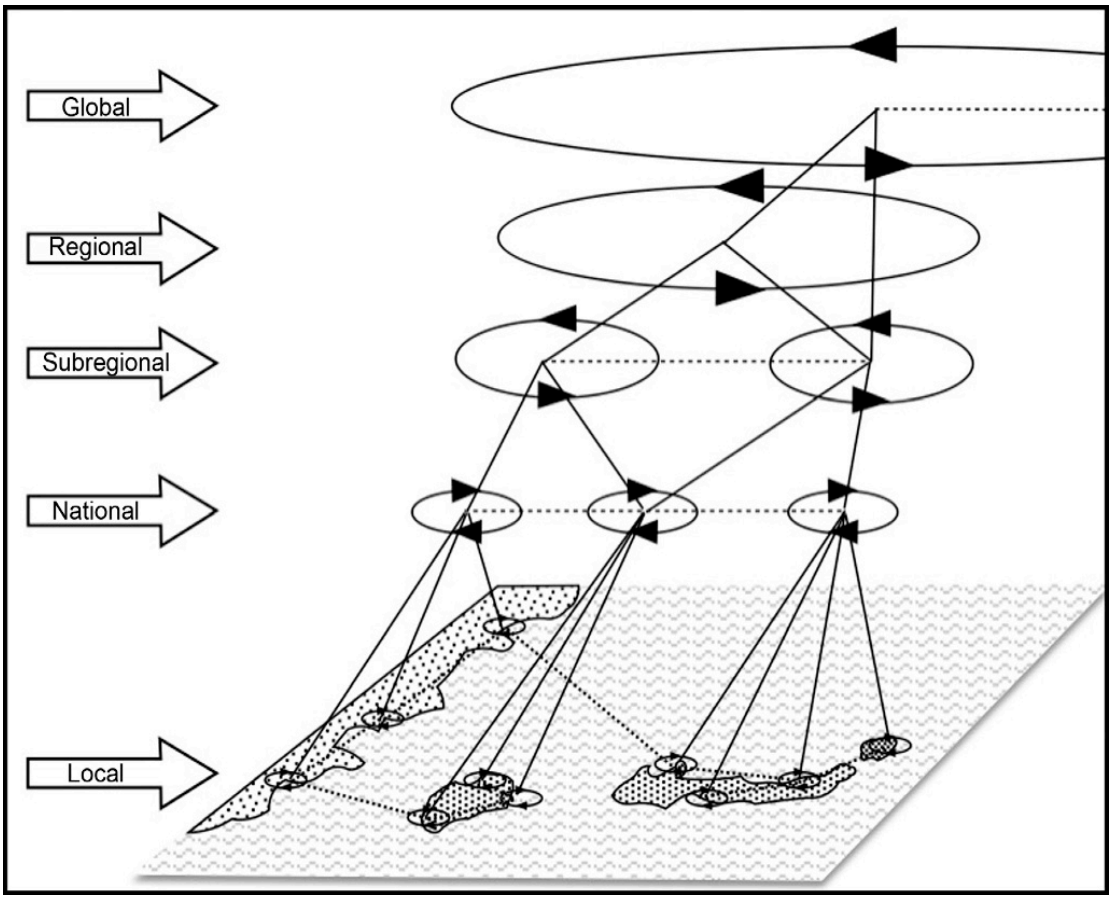

Figure 2.4. Proposed governance framework for connecting local, national, regional, and global scales of governance, showing links (nonbinding or legal) (adapted and redrawn from Fanning et al., 2007).

2.5.1. International Conventions, frameworks, measures and other arrangements

2.5.1.1. Agenda 2030 and the United Nations Sustainable Development Goals

Agenda 2030 (*Transforming our World: the 2030 Agenda for Sustainable Development*) was adopted in September 2015 by the United Nations (UN) General Assembly (UNGA), as an outcome document of the UN summit for the adoption of the post-2015 development agenda. It provides a framework for a very wide range of initiatives aimed at developing a more sustainable future for humankind, and for the sustainable exploitation of natural resources on which we depend. It consists of an action plan comprising 17 UN Sustainable Goals (SDG) and 169 Targets, of which SDG 14 is of key importance:

DG 14 – Conserve and sustainable use the oceans, seas and marine resources for sustainable development.

Target SDG 14.1: Marine pollution: *"By 2025, prevent and significantly reduce marine pollution of all kinds, in particular from land-based activities, including marine debris and nutrient pollution".* Three additional Goals are relevant to the present discussion: SDG 6 – ensure availability and sustainable management of water and sanitation for all; SDG 11 – make cities and human settlements inclusive, safe, resilient, and sustainable; SDG 12 – ensure sustainable consumption and production patterns.

The specific targets and their relevance are summarised in Table 2.5. A major UN conference on the Oceans and SDG14 took place in New York in June 2017[1]. Over 1300 voluntary commitments were made during the five-day conference, by national governments, major institutions, NGOs and individuals, directed at meeting the SDG 14 targets. Of these, 540 (39%) referred to SDG 14.1, with many specifically related to reducing the input and impacts of plastic debris in the oceans.

2.5.1.2. United Nations Convention on the Law of the Sea (UNCLOS)

The UN Convention on the Law of the Sea (UNCLOS) provides the overarching framework for the governance of the oceans. It has 167 parties and entered into force in 1994. There is a General Obligation for states under UNCLOS Part XII Article 192: "*……. to protect and preserve the marine environment'*. Article 192 falls within customary international law, which is binding on all states, whether or not they are parties to UNCLOS. Article 194 further specifies that: '*States shall take, individually or jointly as appropriate, all measures within this Convention that are necessary to prevent, reduce and control pollution of the marine environment from any source*".

Marine litter is included within the definition of pollution adopted by UNCLOS: "*the introduction by man, directly or indirectly, of substances or energy into the marine environment, including estuaries, which results or is likely to result in such deleterious effects as harm to living resources and marine life, hazards to human health, hindrance to marine activities, including fishing and other legitimate uses of the sea, impairment of quality for use of sea water and reduction of amenities*".

[1] https://oceanconference.un.org

As with all legislation, UNCLOS is only effective as far as states are willing and able to introduce and enforce measures to meet the agreed goals. It is apparent that the existence of UNCLOS has not in itself prevented enormous quantities of plastic litter entering the ocean. Each year a meeting of the United Nations Open-ended Informal Consultative Process on Oceans and the Law of the Sea takes place to examine particular topics of interest within the remit of UNCLOS. The 17th meeting of the Consultative Process, in June 2016, took as its main theme: "*Marine debris, plastics and microplastics"*, illustrating that the topic is high on the international policy agenda.

i. United Nations Fish Stocks Agreement

A provision was introduced under UNCLOS in December 1982 concerning "the Conservation and Management of Straddling Fish Stocks and Highly Migratory Fish Stocks". This refers to the need to reduce the impact of fishing gears, gear marking, and the retrieval of abandoned, lost or otherwise discarded fishing gear (ALDFG). This is an important provision, given the disproportionate impact of ALDFG on biodiversity, sensitive habitats, food security, and social well-being (UNEP, 2016). The Review Conference of the Agreement in 2006 recommended that states, individually and collectively through regional fisheries management organizations and arrangements, should, inter alia: *"… enhance efforts to address and mitigate the incidence and impacts of all kinds of derelict gear, establish mechanisms for the regular retrieval of derelict gear and adopt mechanisms to monitor and reduce discards".*

2.5.1.3. International Maritime Organization (IMO)

i) International Convention for the Prevention of Pollution from Ships (MARPOL).

Annex V of the MARPOL convention concerns the discharge of garbage from ships and offshore platforms. A revised version of Annex V came into force on January 1, 2013. This prohibits the discharge of all plastics anywhere in the global ocean including waters within and outside the national jurisdiction. The Marine Environment Protection Committee (MEPC) also adopted the 2012 Guidelines for the development of garbage management plans for ships (resolution MEPC.220(63)).

Table 2.5. SDG targets of relevance to the reduction of marine plastic debris and its impacts (text in bold indicates key targets).

SDG Target	Description	Relevance
6.3	**By 2030, the proportion of untreated wastewater should be halved**	Reduction in input of land-based plastics
11.6	**By 2030, reduce the adverse per capita environmental impact of cities**, including by paying special attention to air quality and **municipal and other waste management**	Reduction in input of land-based plastics
12.1	Implement the 10-year framework of programmes on sustainable consumption and production, all countries taking action, with developed countries taking the lead, taking into account the development and capabilities of developing countries	Moving towards a circular economy, with decrease in production and 'leakage' of plastics
12.4	**By 2020, achieve the environmentally sound management of chemicals and all wastes throughout their life cycle**, in accordance with agreed international frameworks, and significantly reduce their release to air, water and soil in order to minimize their adverse impacts on human health and the environment	Reduction in input of land-based plastics and associated chemical contaminants
12.5	**By 2030, substantially reduce waste generation through prevention, reduction, recycling and reuse**	Moving towards a circular economy, with decrease in production and 'leakage' of plastics
12 b	Develop and implement tools to monitor sustainable development impacts for sustainable tourism that creates jobs and promotes local culture and products	Encourage improved stewardship of coastal environment, including litter prevention
14.1	**By 2025, prevent and significantly reduce marine pollution of all kinds, in particular from land-based activities, including marine debris** and nutrient pollution	Direct measures to reduce inputs of marine debris to the ocean
14.2	**By 2020, sustainably manage and protect marine and coastal ecosystems** to avoid significant adverse impacts, including by strengthening their resilience, and **take action for their restoration** in order to achieve healthy and productive oceans	Targeted protection of sensitive habits, including removal of ALDFG
14.7	By 2030, increase the economic benefits to Small Island developing States and least developed countries from the sustainable use of marine resources, including through sustainable management of fisheries, aquaculture and tourism	Encourage improved stewardship of coastal environment, including litter prevention
14.a	Increase scientific knowledge, develop research capacity and transfer marine technology, taking into account the Intergovernmental Oceanographic Commission Criteria and Guidelines on the Transfer of Marine Technology, in order to improve ocean health and to enhance the contribution of marine biodiversity to the development of developing countries, in particular small island developing States and least developed countries	Encourage improved stewardship of coastal environment, including litter prevention; facilitate improved monitoring, assessment and implementation of effective prevention and reduction measures
14.c	**Enhance the conservation and sustainable use of oceans and their resources by implementing international law as reflected in UNCLOS, which provides the legal framework for the conservation and sustainable use of oceans and their resources**, as recalled in paragraph 158 of The Future We Want	Strengthen underpinning legal governance framework

ii) London Convention and Protocol

The London Convention (Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter 1972) came into force in 1975. Its objective is to provide effective control of all sources of marine pollution and take all practical steps to prevent pollution by dumping of wastes or other matter at sea. Currently, 87 States are Parties to the Convention. The London Protocol was agreed to in 1996 to modernize and eventually replace the Convention. It came into force in March 2006 and currently has 46 Parties. Plastics cannot be dumped under the terms of the London Convention. However, it is apparent that some plastic material is dumped inadvertently, for example, in dredged harbour sediments (IMO, 2016).

2.5.1.4. Other UN Agencies

i) Food and Agriculture Organization (FAO)

The FAO Code of Conduct for Responsible Fisheries applies globally, is voluntary in scope, and covers all levels of governance. It contains a number of provisions and standards, some of which are related to marine litter prevention or recovery. These include the provision of port reception facilities, storage of garbage on board, and the reduction of ALDFG (Table 2.6).

ii) UN Environment

a. Global Plan of Action (GPA). UN Environment hosts the GPA, the acronym of the global program of action for the Protection of the Marine Environment from Land-based Activities. It represents the only global intergovernmental mechanism directly addressing the connectivity between terrestrial, freshwater, coastal, and marine ecosystems. Marine litter, excess nutrients, and inadequate waste-water treatment have been the recent focus of the GPA activities. These are designed to assist national and/or regional authorities to devise and implement sustained action to prevent, reduce, control, and/or eliminate marine degradation from land-based activities. UN Environment is one of several implementing agencies for projects funded by the Global Environment Facility (GEF).

Table 2.6. FAO Code of Conduct for Responsible Fisheries - provisions related to marine litter.

Provisions under Article 8	
8.4 Fishing activities	
8.4.6	States should cooperate to develop and apply technologies, materials and operational methods that minimize the loss of fishing gear and the ghost fishing effects of lost or abandoned fishing gear.
8.4.8	Research on the environmental and social impacts of fishing gear and, in particular, on the impact of such gear on biodiversity and coastal fishing communities should be promoted.
8.7 Protection of the aquatic environment	
8.7.1	States should introduce and enforce laws and regulations based on the International Convention for the Prevention of Pollution from Ships, 1973, as modified by the Protocol of 1978 relating thereto (MARPOL 73/78).
8.7.2	Owners, charterers and managers of fishing vessels should ensure that their vessels are fitted with appropriate equipment as required by MARPOL 73/78 and should consider fitting a shipboard compactor or incinerator to relevant classes of vessels in order to treat garbage and other shipboard wastes generated during the vessel's normal service.
8.7.3	Owners, charterers and managers of fishing vessels should minimize the taking aboard of potential garbage through proper provisioning practices.
8.7.4	The crew of fishing vessels should be conversant with proper shipboard procedures in order to ensure discharges do not exceed the levels set by MARPOL73/78. Such procedures should, as a minimum, include the disposal of oily waste and the handling and storage of shipboard garbage
8.9 Harbours and landing places for fishing vessels	
8.9.1	States should take into account, inter alia, the following in the design and construction of harbours and landing places: c. waste disposal systems should be introduced, including for the disposal of oil, oily water and fishing gear

b. Honolulu Strategy. The Honolulu strategy was developed through a consultation process, supported by UN Environment and National Oceanic and Atmospheric Administration (NOAA), which took place before, during, and after the Fifth International Marine Debris Conference, held in Honolulu in 2011. It is a framework for a comprehensive global effort to reduce the ecological, human health, and economic impacts of marine debris globally, to complement and support existing arrangements, and to encourage the development of new solutions. It is intended for use as a:

- Planning tool for developing or refining spatially or sector-specific marine debris programs and projects; common frame of reference for collaboration and sharing of best practices and lessons learned; and, monitoring tool to measure

progress across multiple programs and projects.

The further integration of the Honolulu Strategy at a variety of spatial scales, involving many different stakeholders, is being promoted through the Global Partnership on Marine Litter (GPML)[2]. This provides a mechanism for the exchange of information and promotion of best practice, covering land- and sea-based sources of marine litter and reducing the quantities and impacts of litter already in the ocean. UN Environment provides the Secretariat and a Steering Committee provides oversight and monitors progress.

2.5.1.5. Other International Conventions and arrangements

i) The Convention on the Conservation of Migratory Species of Wild Animals

The Convention on the Conservation of Migratory Species of Wild Animals (CMS or the Bonn Convention) was adopted in June 1979. It addresses the conservation of species or populations that cross national jurisdictional boundaries, as well as of their habitats. The Secretariat is provided by UN Environment and is based in Bonn, Germany. The CMS commissioned three reports on marine debris in 2014, which were presented at the 11th Meeting of the Conference of the Parties in Quito Ecuador, November 2014. These covered various aspects of marine debris including impacts on migratory species, commercial shipping best practice, public awareness, and education (CMS, 2014a, 2014b, 2014c). The CMS adopted a resolution in November 2014 (Resolution 11.30) on the "Management of marine debris," based on the recommendations of the report, that referred to:

"a. identifying knowledge gaps in the management of marine debris (paragraphs 5-13)
b. commercial marine vessel Best Practice (paragraphs 14-17)
c. public awareness and education campaigns (paragraphs 18-23)"

ii) The UN Convention on Biological Diversity

The UN CBD came into force in December 1993. It is operated by the Global Environment Facility (GEF) with funding from member states. The GEF has been a financial mechanism for the UN CBD since 1996, and provides financial resources for

[2] https://sustainabledevelopment.un.org/partnership/?progress&id=331

developing countries and countries with economies in transition to implement the Convention. Articles 6 and 8 of the Convention, which contracting parties are required to adopt "…. as far as possible and as appropriate", are particularly relevant to reduce the impact of marine plastic debris (Table 2.7). The Secretariat commissioned a major review of the impacts of marine litter on biodiversity, which was published in 2012 (SCBD, 2012).

Table 2.7. UN Convention on Biological Diversity – measures related to marine litter.

Article 6 General measures for conservation and sustainable use	
a)	Develop national strategies, plans or programmes for the conservation and sustainable use of biological diversity or adapt for this purpose existing strategies, plans or programmes which shall reflect, inter alia, the measures set out in this Convention relevant to the Contracting Party concerned.
b)	Integrate, as far as possible and as appropriate, the conservation and sustainable use of biological diversity into relevant sectorial or cross-sectorial plans, programmes and policies.
Article 8 - *In-situ* conservation	
a)	Establish a system of protected areas or areas where special measures need to be taken to conserve biological diversity;
d)	Promote the protection of ecosystems, natural habitats and the maintenance of viable populations of species in natural surroundings;
e)	Promote environmentally sound and sustainable development in areas adjacent to protected areas with a view to furthering protection of these areas;
f)	Rehabilitate and restore degraded ecosystems and promote the recovery of threatened species, inter alia, through the development and implementation of plans or other management strategies;

iii) International Whaling Commission

The International Whaling Commission (IWC) was set up in 1946, under the auspices of the International Convention for the Regulation of Whaling (ICRW). The Commission has a membership of 88 Contracting Governments. The IWC co-ordinates and funds conservation work on many species of cetacean, in addition to its primary role in regulating whaling and conserving whale stocks as a whole. The IWC began formally to consider marine debris in 2011 following its endorsement of the Honolulu Commitment[3]. It concluded the marine debris, including ALDFG, plastics and microplastics, was a conservation and welfare concern for cetaceans throughout the oceans. In addition to regular work by its Scientific Committee, The IWC has held two expert workshops on marine debris (IWC, 2014), and three on large whale

[3] https://iwc.int/marine-debris

entanglement in all fishing gear, including ALDFG (SC/66a/COMM2). It has established a global network for disentanglement of whales from gear, including a training and support programme for new teams around the world; and increased its efforts to strengthen international collaboration.

iv) Basel, Rotterdam and Stockholm Conventions (BRS)

These three conventions deal with issues concerning hazardous compounds, and work loosely together[4]. The Basel Convention covers the Control of Transboundary Movements of Hazardous Wastes and their disposal. It is of relevance to the present discussion as much of the waste trade involves plastics, and some of these contain relatively high levels of additive chemicals that are in Annex I or II of the Convention. These have known toxicological effects, with serious human health implications. The Convention also requires Parties to: *"ensure that the generation of hazardous wastes and other wastes are minimised".* Illegal trade in hazardous waste, or treatment in poorly managed facilities, can lead to leakage into waterways and hence into the ocean. The Rotterdam Convention covers the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides in International Trade, and forms another important restraint on the unregulated trade in waste. Again, plastics may be included if they contain substances listed within the Convention Annexes. The regulation of Persistent Organic Pollutants (POPs) is covered by the Stockholm Convention. It came into force in 2004 and was established to protect human life and the environment from chemicals that persist in the environment, bioaccumulate in humans and wildlife, have harmful effects, and have the potential for long-range environmental transport. Many POPs are lipophilic and are readily adsorbed by plastics in the environment. These include legacy compounds such as PCBs, as well as more recently introduced compounds. Some durable plastics (*e.g.* PVC) contain significant quantities of additional chemicals, including UV stabilizers and flame retardants, which have known toxicological impacts and are described as either POPs or persistent bioaccumulating and toxic (PBTs) chemicals. These compounds are weakly bound within the plastic matrix and readily leach into the surrounding environment.

[4] http://synergies.pops.int/

v) SIDS Accelerated Modalities of Action Pathway (SAMOA Pathway)

There are three groupings of Small Island Developing States (SIDS): the Caribbean Community, the Pacific Islands Forum and AIMS (Africa, Indian Ocean, Mediterranean and South China Sea). SIDS experience particular pressures and vulnerabilities, including the generation and management of waste (*e.g.* tourism, lack of infrastructure) and the presence of marine plastic debris, often originating from distant waters. The SIDS Accelerated Modalities of Action Pathway (SAMOA Pathway)[5] was adopted in 2014, during the third conference on SIDS, held in Samoa. It addresses priority areas for SIDS, including the promotion of sustainable tourism and protection of the oceans and seas. For dealing with marine pollution the outcome document states:

> "*58. With this in mind, we strongly support action:*
> *(d) To address marine pollution by developing effective partnerships, including through the development and implementation of relevant arrangements,* […] *and, as appropriate, instruments on marine debris and on nutrient, wastewater and other marine pollution, and through the sharing and implementation of best practices"*

2.5.2. Regional Conventions, frameworks, measures and other arrangements

2.5.2.1. The role of regional cooperation

Bringing about changes in how we provide stewardship of the oceans, and promoting the sustainable use of ocean re-sources, requires recognition of both ecological and political boundaries. Some form of regional cooperation is essential, both to ensure that international conventions and agreements are enacted effectively on a regional scale, and to develop and implement additional forms of governance and intervention measures, which are relevant to the specific circumstances of the region and fairly applied to all relevant member states. These arrangements include:

- Regional Seas Conventions and Action Plans
- Regional Fisheries Bodies
- Political and economic organizations

[5] http://www.sids2014.org/index.php?menu=1537

- Leader-driven initiatives
- Large Marine Ecosystem projects

Such forms of regional governance bring many advantages by: (i) taking account of relevant ecological, social, and economic characteristics; (ii) increasing the level of social ambition; (iii) providing flexibility to encourage the participation of the civil society in decision-making; and (iv) encouraging sharing of experience, developing joint processes, and coordinating and harmonizing governance efforts (Wright et al., 2017).

Regional forms of governance are considered essential for delivering the SDG14 targets. The potential for regional implementation of SDG14.1, on marine pollution including marine debris, is considered to be high. However, greater support is considered necessary to overcome recognized gaps and institutional weaknesses (Wright et al., 2017).

2.5.2.2. Regional Seas bodies

There are 18 Regional Seas bodies, covering a significant fraction of the ocean, falling within states' exclusive economic zones (EEZs). The main exceptions include the Atlantic and Pacific coasts of Canada and the United States, and the waters off North-West Africa. Four Regional Seas areas have a significant high seas component (Antarctic, Mediterranean, Pacific and North-East Atlantic), but the majority of waters beyond national jurisdiction are not included. Many Regional Seas bodies have developed Action Plans, covering various aspects of sustainable social and economic development and responding to environmental concerns. Six Regional Seas bodies have established Marine Litter Action Plans and another three are in the process of implementing Action Plans (Table 2.8).

Table 2.8. Regional Seas Conventions and Action Plans.

Region	Convention/Commission/ Coordinating body	Marine litter action plan	Date implemented
Arctic region	Arctic Council	Not yet	-
Antarctic region	CCAMLR; Convention on the Conservation of Antarctic Marine Living Resources	Not yet	-
Baltic Sea	HELCOM; Helsinki Convention	Established	2015
Black Sea	Black Sea Commission	Under development	-

Caribbean Region	Cartagena Convention & Protocols (UNEP)	Established	Approved 2008, revised 2014
Caspian Sea	Tehran Convention	Not yet	-
East Asian Seas	COBSEA; Coordinating Body on the Seas of East Asia (UNEP)	Under development	-
Eastern Africa Region	Nairobi Convention (UNEP)	Not yet	-
Mediterranean	Barcelona Convention (UNEP)	Established as part of Mediterranean Action Plan (UNEP-MAP)	2014
North-East Atlantic	OSPAR Convention	Established	2014
North-East Pacific	Antigua Convention	Not yet	-
North-West Pacific	NOWPAP; Regional Coordination centres: CEARAC (Japan), DINRAC (P.R. China), MERRAC (R. Korea) and POMRAC (Russian Federation)	Established as part of NW Pacific Action Plan	2008
Pacific Region	Noumea Convention; SPREP - Secretariat of the Pacific Environment Programme	Established as part of the Cleaner Pacific 2025 strategy	2015
Red Sea and Gulf of Aden	PERSGA; Regional Organization for the Conservation of the Environment of the Red Sea and Gulf of Aden	Not yet	-
ROPME Sea Area (marine and coastal areas of Bahrain, I.R. Iran, Iraq, Kuwait, Oman, Qatar, Saudi Arabia, and the United Arab Emirates)	Kuwait Convention	Under development	
South Asian Seas	SASAP; South Asia Cooperative Action Plan	Not yet	-
South-East Pacific Region	Lima Convention	Not yet	-
West and Central Africa Region	Abidjan Convention (UNEP)	Not yet	-

Action Plans have been developed taking account of the specific environmental, social and economic context of each region. The strategic framework adopted on the management of marine litter in the Mediterranean contains legally-binding obligations

to take measures to prevent and reduce the impacts of litter from land and sea sources. In the case of the Baltic, the Baltic Marine Environment Protection Commission (HELCOM) has adopted a series of specific recommendations, mostly directed at the shipping and fisheries sectors (Table 2.9).

Table 2.9.Recommendations adopted by HELCOM to reduce the impact of marine litter in the Baltic Sea.

Recommendation	Purpose	Implementation date
10/5	Concerning guidelines for the establishment of adequate reception facilities in ports	1989
10/7	Concerning general requirements for reception of wastes	1989
19/14	Concerning a harmonized system of fines in case a ship violates anti-pollution regulations	1998
19/9 (supplemented by 22/1)	Concerning the installation of garbage retention appliances and toilet retention systems and standard connections for sewage on board fishing vessels, working vessels and pleasure craft	1998
28E/10	Application of the No-special-fee system to ship-generated wastes and marine litter caught in fishing nets in the Baltic Sea Area and agreement to raise public awareness on the negative environmental and socio-economic effects of marine litter in the marine environment;	2007
31E/4	Concerning proper handling of waste/landfilling	2010

2.5.2.3. Other regional arrangements

i) Regional Fisheries Management Organizations and Arrangements (RFMO/As)

Regional Fisheries Management Organizations and Arrangements (RFMO/As) have responsibilities to manage either a specific highly migratory species, such as the Atlantic bluefin tuna (*Thunnus thynnus*), or fisheries resources more generally in a particular region. Although the fisheries sector represents a significant source of marine litter, and is impacted by it, there has been a lack of systematic efforts to use most RFMO/As as a mechanism to implement change. In contrast, the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR)[6] has been addressing the issue of marine litter since 1984. The aim has been to monitor and minimize the impact of fisheries in the Convention area, and members have collected

[6] https://www.ccamlr.org/en

data on the incidence and impact of marine litter since 1989. The CCAMLR has introduced mitigation measures to reduce the impact of marine debris on marine life.

ii) Large Marine Ecosystems (LME) Approach

Several systems have been proposed to define distinctive ocean domains on the basis of the physical or biological characteristics, sometimes referred to as eco-hydrodynamic regions. A similar approach was developed by NOAA, in conjunction with the University of Rhode Island, based on the concept of Large Marine Ecosystems (LMEs)[7]. Sixty-four LMEs have been defined. Whatever the justification for this approach, it has become the principal route for targeting funding by the Global Environment Facility (GEF), to encourage eligible countries to take a more ecosystem-based approach to the management of coastal activities. Examples of current LME-based GEF-funded projects include: Agulhas and Somali Currents, Bay of Bengal, Benguela Current, Canary Current, Caribbean (CLME+, with North Brazil Shelf), Guinea Current, Mediterranean (strategic partnership) and Yellow Sea. The LME approach provides a framework for monitoring and assessing LMEs based on 5 modules: productivity, fish and fisheries, pollution and ecosystem health, socioeconomics and governance. United Nations Development Programme (UNDP), Intergovernmental Oceanographic Commission (IOC-UNESCO) and UN Environment act as enabling partners. The relationship between Regional Seas and LME projects is not very clearly defined, and there are advocates for both approaches, with confusion over governance arrangements in some cases (Rochette et al., 2015). There has been criticism that the five-module approach is too restrictive to encompass modern concepts of governance, including an integrated socio-ecological approach to manage resources (McMahon et al., 2009). However, there has been much closer cooperation in the Caribbean and Mediterranean, where the LME and Regional Seas boundaries happen to coincide.

2.5.2.4. Intergovernmental frameworks

i) G7 countries

The Group of 7 (G7) is the group of seven countries[8] with the highest advanced economies, accounting for over 64% of global wealth, according to the International

[7] http://www.st.nmfs.noaa.gov/ecosystems/lme/

[8] Canada, France, Germany, Italy, Japan, United Kingdom, United States of America, plus the EU

Monetary Fund. The G7 adopted a Marine Litter Action Plan, under the presidency of Germany, at the annual summit which took place 7-8 June 2015, at Schloss Elmau.

> "*We acknowledge that marine litter, in particular plastic litter, poses global challenge, directly affecting marine and coastal life and ecosystems and potentially also human health. Accordingly, increased effectiveness and intensity of work is required to combat marine litter striving to initiate a global movement. The G7 commits to priority actions and solutions to combat marine litter as set out in the annex, stressing the need to address land- and sea-based sources, removal actions, as well as education, research and outreach"*
> (Extract from Leaders' Declaration)

The Annex to the Declaration includes details of priority actions to address: land-based sources, sea-based sources, removal and education, research and outreach. Work on implementation of the plan continued under the presidencies of Japan and Italy in 2016 and 2017, respectively.

ii) G20 countries

The Group of 20 (G20)[9] represents about two-thirds of the world's population, 85% of global gross domestic product (GDP) and 80% of global trade. There are several other nations and representatives of major groupings who have guest status or are invited to the annual summits. The G20 has developed a Marine Litter Action Plan, under the German presidency (1 December 2016 – 30 November 2017), partly based on the G7 Marine Litter Action Plan, but designed around the needs and concerns of this wider multi-national community. This was adopted at a meeting in Bremen in May 2017. The Action Plan includes a new voluntary platform, the "Global Network of the Committed" (GNC), to ensure the action plan is implemented. This is open to non-government actors to encourage greater networking and information exchange.

[9] Argentina, Australia, Brazil, Canada, China, France, Germany, India, Indonesia, Italy, Japan, Republic of Korea, Mexico, Russia, Saudi Arabia, South Africa, Turkey, the United Kingdom, the United States and the European Union.

iii) European Union

The European Union of 28 countries[10] occupies an area of over 4 million km^2 with a population of 508 million, the third largest population after China and India. It provides a unique legal framework, allowing the implementation of common legislation and other measures covering many aspects of economic and environmental policy. The principal objective of the Marine Strategy Framework Directive (MSFD) is to achieve Good Environmental Status (GES) in European Seas and ocean waters within national jurisdiction of EU Member States (Baltic Sea, Black Sea, Mediterranean Sea, North Sea, NE Atlantic). The Directive defines GES as: "*the environmental status where these provide ecologically diverse and dynamic oceans and seas which are clean, healthy and productive*". Eleven Descriptors of GES have been agreed, of which Descriptor 10 covers marine litter. The MSFD includes provision for setting indicators and targets for litter reduction, and requires member states to implement measures to meet these.

2.5.2.5. National action plans

A number of countries have developed different forms of response to address the issue of marine litter. For countries within the EU these tend to be aligned with the need to address marine litter under the MSFD. There are additional obligations under international treaties and within Regional Seas Conventions and other agreements. Some countries have gone further and developed and implemented more detailed legislation or guidelines. The most recent and comprehensive national Action Plan has been developed by Indonesia (population ~ 250 million), in which eleven separate ministries have been brought together under the Coordinating Ministry for Maritime Affairs. The plan is based on five pillars, designed to: i) improve behavioural change; ii) reduce land-based leakage; iii) reduce sea-based leakage; iv) reduce plastics production and use; and, v) enhance funding mechanisms, policy reform and law enforcement. The ambitious goal is to reduce marine litter in Indonesian waters by 70% by 2025.

[10] Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, United Kingdom (as of January 2019).

2.6. Final remarks

Plastic litter has been detected worldwide and is now recognized as a real threat on a global scale. Owing to its properties of buoyancy and durability, it floats on the sea surface and can be transported over large distances in the ocean. Hence, plastics of all sizes are found in all ocean regions. The plastic pollution in marine environments can also easily interact with the ocean life and thus poses a threat to wildlife and to environment. However, we still know very little about the consequences of plastic pollution on a global scale and more scientific studies should be conducted. Furthermore, effective governance is vital to bring about significant reductions in the input of plastic and microplastics from land- and sea-based sources, and to reduce the impact of plastic litter in the marine environment. However, legislation in itself is insufficient to bring about the desired outcome. Governance mechanisms need to be inclusive, multi-sectoral, and accepted by stakeholders at all levels. They need to work at a variety of spatial scales, and be flexible enough to be adaptive to changing social, economic, and environmental circumstances. To be fully effective requires support at a political level and capacity building and financial support when appropriate. Despite the major challenge this represents, there are many encouraging signs such as that the need for improved governance that has been recognized, and that governments and other major players are determined to act.

2.7. Acknowledgements

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Chapter III

Microplastics pollution in the marine environment

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(Book Chapter included in the present Thesis with permission of Elsevier included in the Appendix A, with punctual modifications)

3.1. Introduction

The presence and accumulation of plastics and microplastics in the marine environment (including open sea and coastal systems) is of growing concern. Once discarted in the environment, plastic debris disperse and accumulate in marine habitats all over the world (van Sebille *et al.*, 2015; Cózar *et al.*, 2017; Imhof *et al.*, 2017b). Reported concentrations in seawater, in interdital and subtidal sediments, and within marine organisms are highly variable spatially, even within fairly enclosed bodies of water (Eriksen *et al.*, 2014; GESAMP, 2016). Thus, the environmental contamination caused by plastics raises many complex issues and represents an increasing threat to marine organisms and ecosystems.

The presence of small plastic fragments in the open ocean was first documented in the 1970s (Carpenter and Smith, 1972), but the term “microplastics” was first used in 2004 (Thompson *et al.*, 2004) and entered in the scientific and popular lexicon (GESAMP, 2016). Since then, research in this area has increased exponentially, leading to over 150 publications in 2014 (Barboza and Gimenez, 2015), and much more since then.

Microplastics are ubiquitous in the world’s oceans and represent approximately 92.4 % of the global particle counts of plastics (Eriksen *et al.*, 2014). They are present in sediments, throughout the water column, and in the digestive system and tissues of marine organisms (Anderson *et al.*, 2016). Their ability to interact with other environmental contaminants and adsorb them at their surface, their propensity to be ingested by biota and their long residence times in the environment, make them a global concern (Mato *et al.*, 2001; Holmes *et al.*, 2014; Luís *et al.*, 2015; Fonte *et al.*, 2016; GESAMP, 2016; Jabeen *et al.*, 2017). However, despite recent research, many questions still remain open, particularly in ecotoxicology studies (Law and Thompson, 2014).

This section compiles microplastics information regarding: (i) sources, fate, and environmental behaviour; (ii) global distribution in the marine environment; (iii) occurrence in wild marine organisms; and (iv) effects and toxicity in marine species; and aims to further contribute to the international debate on the microplastics global paradigm.

3.2. Microplastics in the marine environment: definition, sources, environmental fate and behaviour

The term “microplastics” was initially applied to plastic particles around 50 µm in size collected on shorelines and in the water column (Thompson *et al.*, 2004). Since then it has become widely used to describe small pieces in the millimetre to sub-millimetre size range (GESAMP, 2016). Other authors (Ryan, 2015; Van Cauwenberghe et al., 2015b) apply the term to particles smaller than 5 mm in diameter or smaller than 500 µm in a more restrictive approach (Fig. 3.1). Because the surface area-volume ratio in microplastics is high, they might also pose a higher risk to marine fauna than macroplastics (Cole et al., 2011).

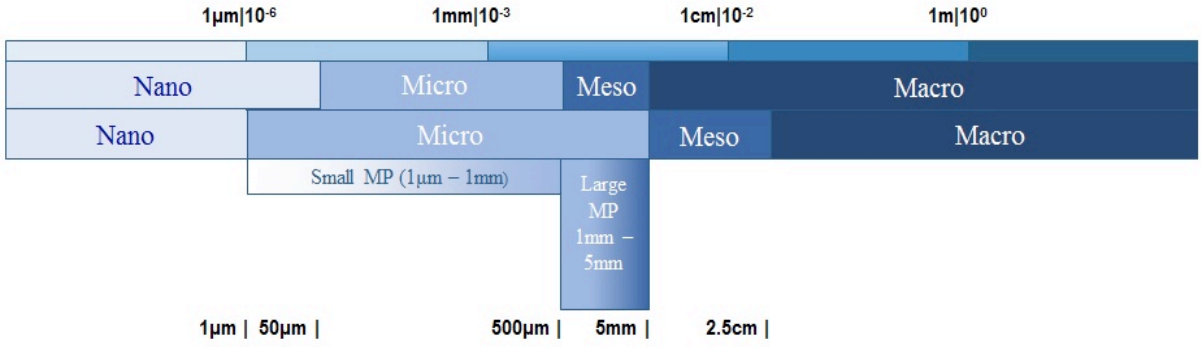

Figure 3.1. Microplastic size limit classifications according to different authors (Adapted from: Ryan, 2015 and Van Cauwenberghe *et al.*, 2015b).

Microplastics can be categorized into primary and secondary according to their source. Most primary microplastics in the environment are generated from industrial and domestic products that contain particles already in the micro or nano size, that is, they are plastics released into the environment in the form of small particles used as raw material in the plastic industry and/or in synthetic textiles, electronic equipment, hygiene and personal care products, such as facial cleaners, bath gels, and toothpastes, among others (Fendall and Sewell, 2009; Cole *et al.*, 2011; GESAMP, 2016). Secondary microplastics result from the fragmentation of larger plastic items into smaller fragments that takes place in the environment under weathering conditions, solar radiation that facilitates oxidative degradation of polymers, salinity and mechanical abrasion such as winds, waves, ocean currents, and even animal bites and other alterations due to biota, and other factors that can break the polymer into ever-smaller fragments (Crawford and Quinn, 2016; Solomon and Palanisami, 2016).

Polymers commonly collected in beach surveys and water surface samples are intrinsically linked to historical worldwide plastic production and usually include polyethylene, polypropylene, polystyrene, nylon, among others (Li, et al., 2016a; 2016b; Andrady, 2017). Thus, microplastics encompass a very heterogeneous set of particles, including fibres (Hidalgo-Ruz *et al.*, 2012). Fibers are one of main environmental microplastic threats as they are widely produced and distributed in water and sediments. Fibers can either result from clothes (Browne *et al.*, 2011) or from degradation of fishing gear (Crawford and Quinn, 2016), among other sources. Examples of different microplastics can be found in Fig. 3.2.

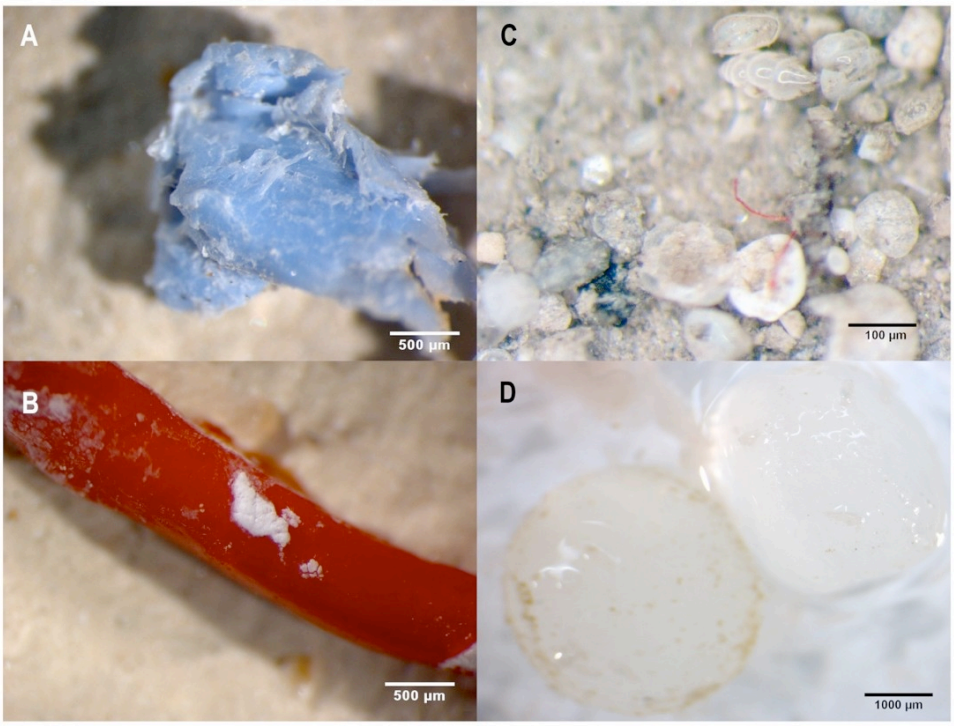

Figure 3.2. Examples of microplastics with different sizes, shapes, and composition. (A) and (B) fragments, (C) fibre, and (D) pellet (Photos: kindly provided by João Frias).

Derived mainly from land-based sources (~ 80 %), and also from sea-based sources (~ 20 %) (GESAMP, 2016) (Fig. 3.3), plastic litter is able to travel great distances across the globe due to characteristics such as light weight, floatability, shape, and colour (Maximenko *et al.*, 2012; Andrady, 2017). In the marine environment, microplastics formation from larger plastic debris is influenced by a combination of environmental factors and the properties of the polymer (Anderson *et al.*, 2016; GESAMP, 2016; Andrady, 2017). Table 3.1 summarizes the material characteristics of the plastics that influence their environmental behaviour.

Table 3.1. Characteristics of polymers that constitute microplastics (Andrady, 2017).

Characteristic	Influence on behavior of microplastics	Comments
Density	Buoyancy in seawater determines where in the water column the microplastic is likely to initially reside in	Density ranges of classes of plastics are generally known but can be modified by fillers as well as by surface foulants
Partial crystallinity	The degree of crystallinity determines the ease of oxidative degradation and fragmentation during weathering	General ranges of values are available for different plastics but these can change based on sample history
Oxidation resistance or weatherebility	Chemical structures determine how easily oxidizable the plastic will be in the environment. Fragmentation is a consequence of extensive oxidative degradation	Ease of oxidation suggested by the chemical structure may be very different in compounded plastics that incorporate stabilizers and additives
Biodegradability	Determines the rate of mineralization and potential partial removal of plastics from the water column or sediment	Common plastics are generally bio-inert. Exceptions do exist in synthetic plastics as well as biopolymers
Residual monomer	Toxicity of leaching residual monomers in microplatics to marine organisms that ingest plastics	Both residual monomer levels in common plastics as well as their toxicities are reliably know
Transport	Bioavailability of residual monomers, additives and POPs sorbed by the microplastics depends on their leaching rates in the environment	These properties are known for virgin resins but can change because degree of crystallinity can be varied by sample history or additives
Additives	Concentration and toxicity of additives in microplastics may contribute to the adverse impacts on ingesting species	Chemistry, levels of use in plastics and toxicities, are generally known. But these levels for endocrine disruptors are not reliably known
Surface properties	Rate of fouling of floating debris determines rates of weathering and sinking of microplastics	Surface properties and fouling rates for common plastics are known

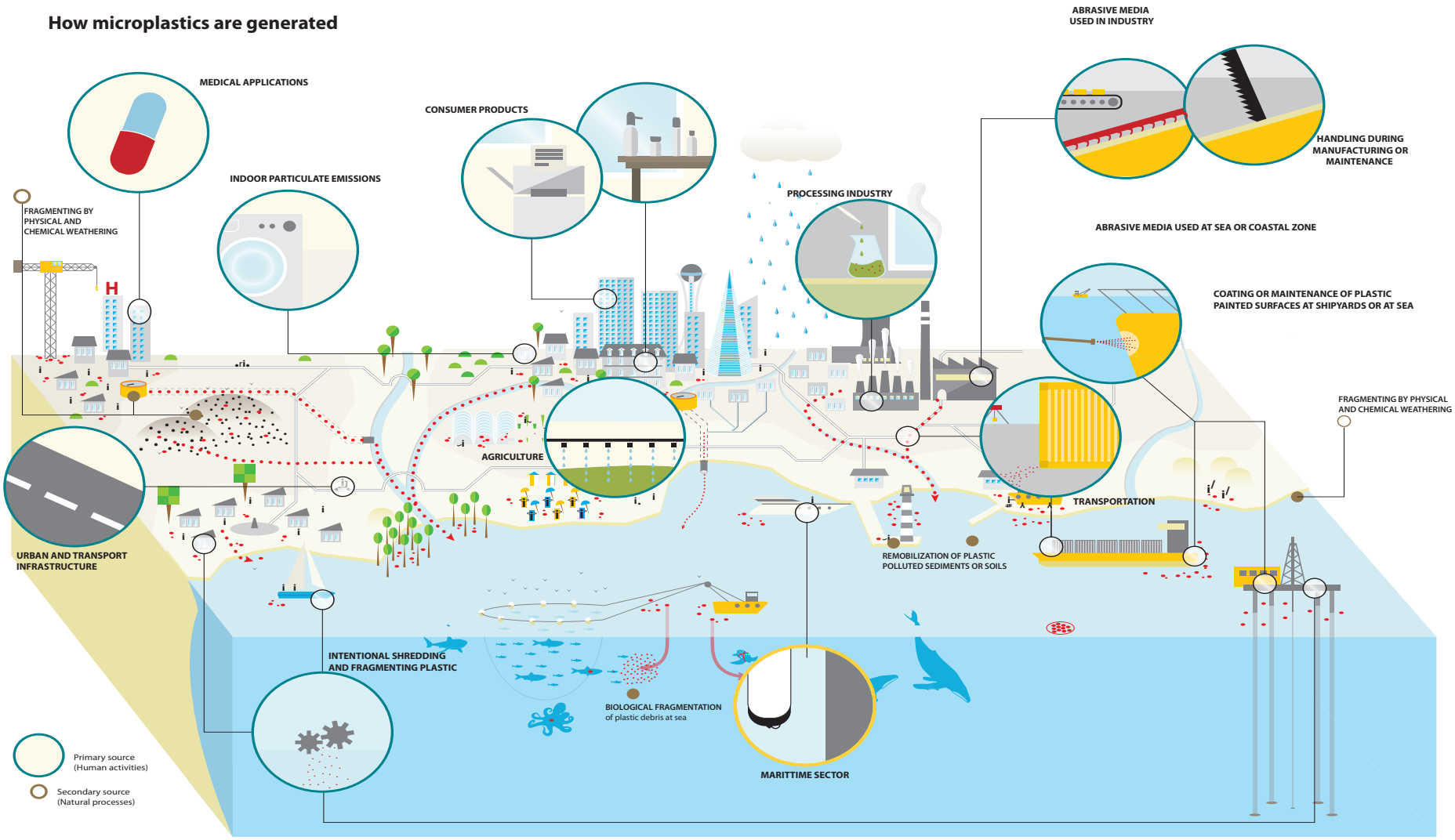

Figure 3.3. How microplastics are generated (kindly provided by GRID-Arendal, Maphoto/Riccardo Pravettoni).

The density of the plastic material is one factor that will determine its buoyancy and position in the water column, and thereby influence the possibility for interaction with different organisms (Anderson *et al.*, 2016; Andrady, 2017) (Fig. 3.4). Several factors may influence buoyancy including its biofouling, that is, the colonization by organisms on the polymer after it enters the sea (Andrady, 2011; Wright *et al.*, 2013b) and de-fouling in the water column by foraging organisms that are a potential pathway for microplastic particles to return to the sea-air interface (Wright et al., 2013a; 2013b). Alternatively, high-density plastics together with fouled microplastics can sink to the sediment (Wright *et al.*, 2013b; GESAMP, 2016). Storms and turbulence conditions can then cause their resuspension and further redistribution in the water column (Anderson *et al.*, 2016) (Fig. 3.4).

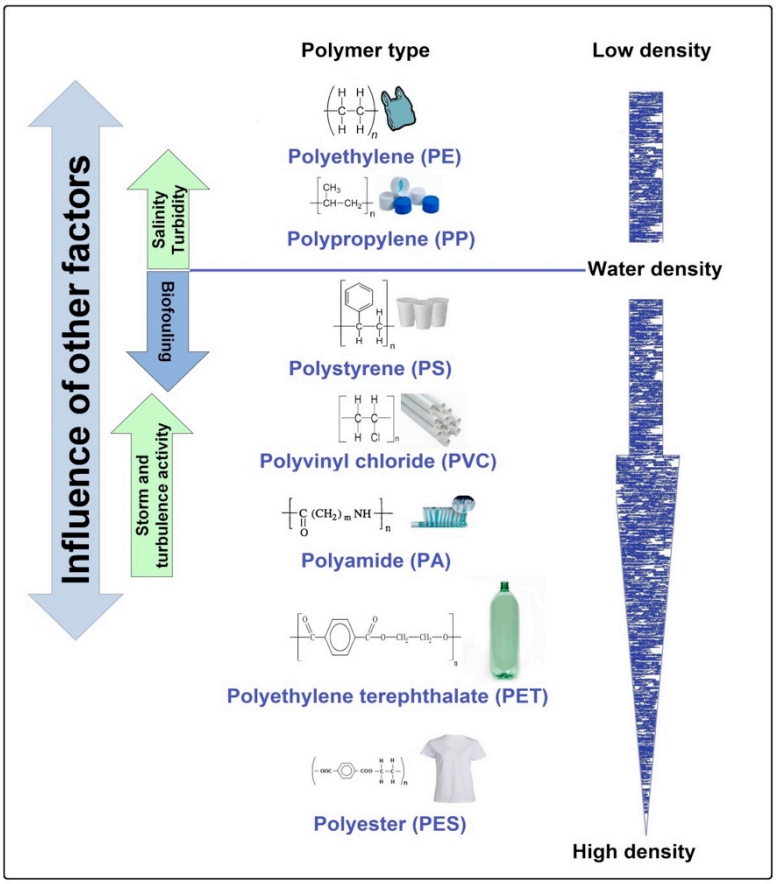

Figure 3.4. Densities, structures, and expected distributions of different plastic polymers in the water column (Adapted from Anderson *et al.*, 2016).

Low- and high-density microplastics are ingested by many marine species (Ivar Do Sul and Costa, 2014) (Fig. 3.5). Such microplastics can contain other adsorbed hazardous chemicals (*e.g.* metals, PCB, PBDEs, and PAHs) which are incorporated during their manufacture, industrial use, and/or presence in the environment (Holmes *et al.*, 2014; Rochman *et al.*, 2014a; Bakir *et al.*, 2016). They may act as a vector of potentially harmful microorganisms, including pathogenic taxa (Keswani *et al.*, 2016; Zettler *et al.*, 2013) and may cause impairment of key functions that normally sustain health and biodiversity (Koelmans *et al.*, 2016).

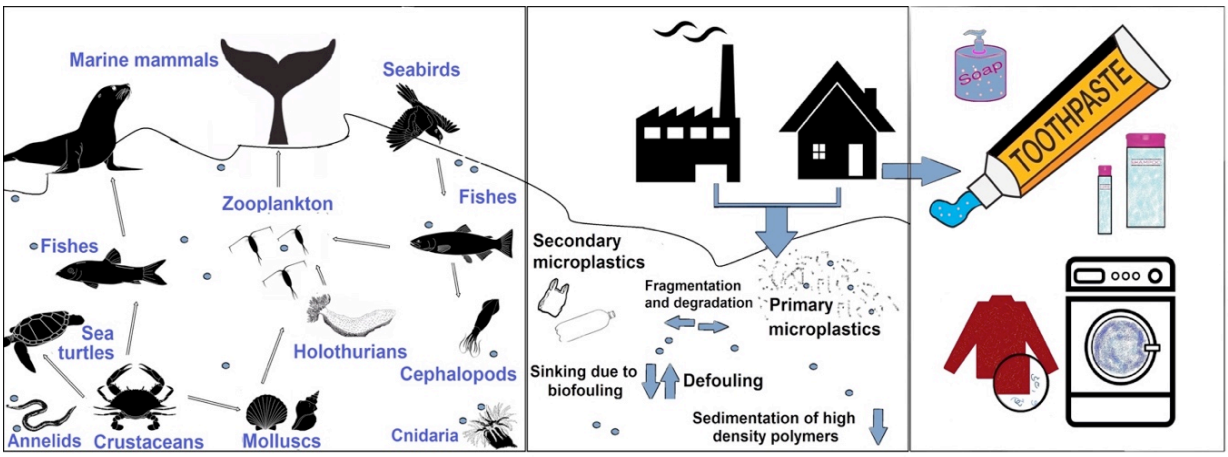

Figure 3.5. Potential pathways of microplastics transportation and its biological interactions. (Adapted from Wright *et al.*, 2013b and Ivar Do Sul and Costa, 2014).

3.3. Global distribution in the marine environment

3.3.1. Accumulations on beaches and coastal areas

Marine litter is directly linked to human behaviour, for which consumption and discard rates play relevant roles (Vaz *et al.*, 2009). Beaches and coastal areas commonly have high population densities, proximity to industrial facilities, and river inputs (Antunes *et al.*, 2013), all of which lead to microplastics accumulation (Andrady and Neal, 2009). Thus, plastics are ubiquitous and present in every coastal area (Hidalgo-Ruz and Thiel, 2013; Lusher, 2015), including estuaries, deltas and coastal lagoons. Not surprisingly, plastics and microplastics are more abundant in densely populated areas, ranging from tiny fibers (microns) to fragments (few millimetres).

One difficulty when comparing microplastics densities or concentrations in different areas is the usage of different quantitative units (*e.g.* Lusher, 2015). Plastics and microplastics are present in almost 40 % – 98 % of collected samples, mainly fibers and fragments. Densities are highly variable depending on the region, sampling season, and weather conditions (Ivar Do Sul and Costa, 2014; Lusher, 2015), among other factors such as sampling equipment and approaches.

3.3.2. Surface and water column floating debris

The Joint Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP) estimates that 95 % of marine debris floating in the ocean is plastic. It is also estimated that 5.25 trillion plastic fragments, weighing perhaps 35-270 thousand tons, are currently floating in ocean and seas (Cózar *et al.*, 2014; Eriksen *et al.*, 2014). Stranded plastics suffer degradation and the resulting secondary microplastics can be transported by ocean currents and accumulate in gyres (Maximenko *et al.*, 2012; Cózar *et al.*, 2014; Eriksen *et al.*, 2014; Ryan, 2014; Woodall *et al.*, 2014). Gyres are vortex areas, where lightweight materials congregate and so far five plastic accumulation areas have been identified: two in the Atlantic Ocean (North and South), two in the Pacific Ocean (North and South), and one in the Indic Ocean (Lebreton *et al.*, 2012; Maximenko *et al.*, 2012). In the North Pacific Subtropical Gyre, one of the most studied, there is a maximum density of 32.76 particles m^{-3} (~ 250 mg m^{-3}) (Goldstein *et al.*, 2012).

3.3.3. Deposition and accumulation of litter in the seafloor

Few studies have focused on benthic marine litter accumulation on the seabed due to difficulties in collecting samples that usually involve the use of expensive technology. Authors have described the likelihood of denser debris sinking, due to their chemical properties (*e.g.* PVC) or due to weight of attached biofouling (Fazey and Ryan, 2016).

3.3.4. Records of plastic pollution in polar regions

Not even remote regions such as the Artic and Antarctica are free from plastic pollution. There were no direct studies on microplastics on Polar Regions before 2014 (Lusher, 2015). Floating marine litter and microplastics trapped in ice cores

have been reported (Obbard *et al.*, 2014; Zarfl and Matthies, 2010) showing that microplastics can reach remote regions where human presence is limited. In the Arctic Ocean, it is estimated that the total load of floating plastic is from 100 to 1200 tons, where 400 tons are composed of 300 billion plastic items (midrange estimate) (Cózar *et al.*, 2017). From these, about 90% are fragments and ~ 7 % are fishing line.

In the Antarctic, microplastic data is still scarce. According to Cincinelli *et al.* (2017) the levels in the surface waters of Antarctica were lower than those already recorded in other seawaters worldwide. Moreover, Waller *et al.* (2017) suggests that plastics must have originated from outside this region, and the authors suggest standardized monitoring programs for the region as an urgent need.

3.3.5. Riverine sources of plastic pollution

Riverine records of plastic pollution date back to the 1990s, when Williams et al. reported plastic items in the River Taff, U.K. (Williams and Simmons, 1999). Since then studies in freshwater environments are rapidly advancing, as rivers act as an important input of plastics and microplastics (Horton *et al.*, 2017a; Lambert and Wagner, 2018) into the marine environment, and data were still scarce until recently (Eerkes-Medrano, *et al.*, 2016; Lambert and Wagner, 2018). Several potential pathways exist linked to inland littering, storm overflows, outflows from wastewater treatment plants (WWTP), households and industries, and even atmospheric deposition of fibers (Lambert and Wahner, 2018).

Microplastics densities in rivers are highly variable depending on the studied region, Asia being the continent with most microplastic particles in their rivers, ranging from 192 to 20,264 items km^{-2}, on average (Eerker-Medrano *et al.*, 2015; Lambert and Wagner, 2018). There is an urgent need for standardization of procedures and reporting units to make studies comparable. Moreover, some authors suggest that beaches connected to estuaries, rivers, or lakes should have regular monitoring programs to estimate inputs that could be used to improve models (Horton *et al.*, 2017b) and create management tools for policy makers to tackle this global environmental problem.

3.4. Occurrence of microplastics in marine organisms

Microplastic pollution poses a threat to marine biota, becoming available to a wide range of marine organisms (GESAMP, 2016). Microplastics are confused with prey or are ingested during passive water filtration and, after being ingested, they may be transferred from prey to predators. Ingestion of these microparticles involves a wide range of taxa, from microscopic zooplankton to large vertebrates (Lusher, 2015).

3.4.1. Invertebrates

Ingestion of microplastics occurs in organisms at the base of the food chain such as plankton (Frias *et al.*, 2014; Desforges *et al.*, 2015) as well as in polychaetes, bivalves, echinoderms, and decapods (Thompson *et al.*, 2004; Graham and Thompson, 2009; Murray and Cowie, 2011; Van Cauwenberghe and Janssen, 2014). Poriferans are the oldest extant metazoans (Larink and Westheide, 2006) but no studies reported microplastics in marine Porifera.

Regarding Cnidarians, microplastics have been detected on the exterior of octocorals (*Anthomastus* spp.) and on zoanthids in the SW Indian Ocean and Equatorial mid-Atlantic (Taylor *et al.*, 2016). In echinoderms, Taylor *et al.* (2016) observed microplastics in the holothuria (sea cucumber) from the Equatorial mid-Atlantic waters. In the phylum Annelida, microplastics have been reported in *Alitta virens* collected along the Nova Scotia's Eastern Shore (Mathalon and Hill, 2014) and in *Arenicola marina* collected along the French-Belgian-Dutch coastline (Van Cauwenberghe and Janssen, 2014).

In the case of zooplankton, their vertical migration can transport microplastics to predators occupying various depths of the water column (Wright *et al.*, 2013b). Ingestion of microplastics has been documented for copepods, medusae, euphausiids, salps, and fish larvae, which transfer these contaminants to higher trophic levels, posing a risk to secondary producers (Wright *et al.*, 2013b; Desforges *et al.*, 2015; Enders *et al.*, 2015; Clark *et al.*, 2016). However, information on microplastic ingestion by zooplankton is still very limited (Steer *et al.*, 2017). In Portuguese coastal waters, microplastics were identified in 61 % of zooplankton samples (n = 152 samples, with no identified species) (Frias *et al.*, 2014) while a study from the Northeast Pacific showed microplastic ingestion by 1 in 17 calanoid

copepods and 1 in 34 euphausiids, the majority of identified plastics being fibers (Desforges *et al.*, 2015). This type of microplastic has been further identified in zooplankton communities sampled from the South China Sea (Sun *et al.*, 2017), where 70 % were fibers. In this last study, the ingested microplastics varied from 2.83 to 103.49 particles m^{-3}, mainly ingested by copepods, accounting for 79 % of the total number of particles consumed. The uptake of microplastics by meroplankton including fish larvae has been considerably under-researched, though Steer *et al.* (2017) found that in the western English Channel 2.9 % of fish larvae (n = 347) were identified with particles, of which 66% were fibers, showing that planktonic fish larvae are also vulnerable to this pollution.

Molluscs, in particular bivalves, are of special interest since their filter-feeding activity exposes them directly to microplastics present in the water column. Li *et al.* (2015) investigated this in several commercial bivalves from a fishery market in China and reported the presence of fibers, fragments, and pellets in the tissues of all the selected species. The presence of microplastics in *Mytilus genus* was reported by Vandermeersch *et al.* (2015) in the Po estuary (Italy), Tagus estuary (Portugal), and in Amposta Ebro Delta (Spain). Specimens of *Crassostrea gigas* obtained from a market in Brittany (France) and a local market from California (USA) contained an average of 0.47 and 0.6 particles per g (wet weight), respectively (Van Cauwenberghe and Janssen, 2014; Rochman *et al.*, 2015). Crustacea likewise take up microplastics: Taylor *et al.* (2016) found them in the squat lobster and in the hermit crab, and they were also reported in 83 % of *Nephrops norvegicus*, 33.5 % of Lepas spp. and 13 % of *Eriocheir sinensis* specimens captured from the North Pacific Subtropical Gyre, Clyde Sea area and Baltic Sea (Murray and Cowie, 2011; Goldstein and Goodwin, 2013; Wójcik-Fudalewska *et al.*, 2016).

3.4.2. Vertebrates

While plastic litter has well recognized adverse effects on vertebrates (Derraik, 2002), and small debris ingestion has been reported in several vertebrates including fish, turtles, mammals, and seabirds (Lusher, 2015; Amélineau *et al.*, 2016; GESAMP, 2016), microplastics are more difficult to detect. Some studies considered that debris smaller than 0.5 cm may be generated by fragmentation of larger items

inside these organisms to produce plastics that fall into the microplastic category (Nicolau *et al.*, 2016).

Fish are one of the major protein sources for humans around the world, and microplastic ingestion by fish will constitute an important threat to human health (Bouwmeester *et al.*, 2015). Recently, microplastics were found in 7 % – 100 % of the digestive tracts of *Trachurus trachurus* and *Mullus surmuletus*, respectively, in the Portuguese coast (Neves *et al.*, 2015). The same authors reported that of all the fish found to ingest microplastics, 63.5 % were benthic and 36.5 % were pelagic species. In the English Channel, Lusher *et al.* (2013) examined five pelagic and five demersal species and found that 36.5 % contained plastics in the gastrointestinal tract, with an average (± standard deviation) number of pieces per fish of 1.90 ± 0.10. Güven *et al.* (2017) reported the presence of microplastics in stomach and/or intestines of 28 species from the Turkish Mediterranean Sea, where 58 % of the total sample contained plastics with an average of 2.36 particles per fish, including 75 % of captured *Argyrosomus regius* (benthopelagic), 66 % of *Mullus barbatus* (demersal), 65 % of *Pelates quadrilineatus* (reef-associated), and 35 % of *Liza aurata* (pelagic-neritic). In epipelagic fish from the North Pacific Gyre (Boerger *et al.*, 2010) and catfish species from Northeast Brazil (Possatto *et al.*, 2011), microplastic ingestion was reported to be 35 % and 23 %, respectively. In five commercial species sampled from the Central and North Adriatic Sea, Avio *et al.* (2015b) reported the presence of microplastics in 19 % of the pelagic species (*Sardina pilchardus*), 44 % and 100 % of the two benthopelagic species (*Squalus acanthia* and *Merlucius merlucius* and 64 % and 67 % of the two benthic species (*M. barbatus* and *Chelidonichthys lucernus*).

Few studies have quantified microplastics in marine mammals. The presence of these particles was reported in the stomachs and intestines of *Phoca vitulina* (Bravo Rebolledo *et al.*, 2013) and *Megaptera novaeangliae* (Besseling *et al.*, 2015) from the Netherlands coast and in the digestive track of *Mesoplodon mirus* in the North and West Coast of Ireland (Lusher, 2015).

Plastic ingestion rates in turtles and seabirds are useful environmental indicators of plastic pollution in the marine environment (Bost and Le Maho, 1993; Auman *et al.*, 2004; Schuyler *et al.*, 2014; Wilcox *et al.*, 2015). Their complex life histories make marine turtles particularly vulnerable to plastic debris (Schuyler *et al.*,

2014). The presence of these plastic particles was described in *Chelonia mydas* in the Southern Brazilian coast (Tourinho *et al.*, 2010) and in *Caretta caretta* in the Portuguese continental coast (Nicolau *et al.*, 2016). In the Mediterranean Sea, plastic fragments and pellets in *C. caretta* were described by Casale *et al.*, (2016) (Central Mediterranean Sea), Camedda *et al.* (2014) (Western Mediterranean Sea), and Campani *et al.* (2013) (Mediterranean Sea). Ingestion of plastic debris has also been widely reported globally for seabirds (Tourinho *et al.*, 2010; Bond *et al.*, 2013; Acampora *et al.*, 2014; Floren and Shugart, 2017; Furtado *et al.*, 2016; Acampora *et al.*, 2017). At least 50% of species are known to interact with marine plastic debris (Kühn *et al.*, 2015) and while entanglement or ingestion by larger fragments is known to lead to starvation, digestive tract physical damage and ultimately death in several organisms, microplastic ingestion generally does not affect seabirds so severely (Lusher, 2015). Some seabirds are known to be zooplanktivorous (*e.g.* Little auks; *Alle alle*). A recent study published by Amélineau *et al.* (2016) demonstrated that microplastics can be ingested by colour selectivity, suggesting that they are mistaken for prey items. However, transference from zooplankton to birds remains unclear. In general, monitoring of plastic debris in seabirds has been primarily achieved through the analysis of the stomach contents of dead animals and the effects of microplastics are less well understood.

3.5. Biological effects and toxicity of microplastics in marine species

The potential impacts of ingested microplastics are driven by their mechanical and chemical effects, the latter being influenced by the presence of additive and adsorbed organic chemicals. Mechanical effects include hindering mobility and clogging of the digestive tract, while chemical effects can include inflammation, hepatic stress, and decreased growth (Setälä *et al.*, 2016). The size of ingested plastic materials is related to the type, body size, and life stage of marine organisms (Cole *et al.*, 2013). Our existing knowledge on impacts of large-sized items of plastic litter on organisms (*e.g.* fish and seabirds) has the potential to inform us about the mechanisms of toxicity in organisms that ingest microplastics, although this remains to be proven (Ross and Morales-Caselles, 2015). The impacts of microplastics on marine organisms have recently been comprehensively studied and reviewed

(Lusher, 2015; Rochman *et al.*, 2015; Van Cauwenberghe *et al.*, 2015a; Anderson *et al.*, 2016; Rochman *et al.*, 2016; Solomon and Palanisami, 2016; Auta *et al.*, 2017).

3.5.1. Microalgae and marine bacteria

There have been few microplastic toxicity studies with microalgae to date. This may well reflect a perceived lack of potential for toxicological responses, and nanoplastics (NPs) have been more frequently investigated with this class of organisms. One recent study (Sjollema *et al.*, 2016) investigated the role of particle size (0.05, 0.5, and 6 μm) and physicochemical properties (negatively charged and uncharged) on the toxicity of polystyrene microplastics to the marine diatom *Thalassiosira pseudonana* and the marine flagellate *Dunaliella tertiolecta.* None of the treatments tested had significant effects on microalgal photosynthesis after 72 h, while microalgal growth was negatively affected (up to 45 %) by uncharged polystyrene particles, but only at high concentrations (250 mg/L). In another study (Zhang *et al.*, 2017), PVC microplastics (1 μm; up to 50 mg/L for 96 h) caused a significant growth inhibition (~ 40 %), and a decrease in both chlorophyll content and photosynthetic efficiency in the marine microalgae *Skeletonema costatum*. In contrast, PVC debris (1 mm) had no significant effect on the growth of microalgae. It is suggested that interactions between microplastics and microalgae such as adsorption and aggregation accounted for the observed toxic effects rather than shading (Zhang *et al.*, 2017). In addition, a number of experiments specifically studying NPs have been reported. The marine bacterium *Vibrio fischeri* was not found to exhibit any acute toxicity to two poly(methylmethacrylate)-based NPs (PMMA) with different surface chemistry (medium and hydrophobic) at concentrations ranging from 0.01 to 1000 mg/L (Booth *et al.*, 2016). Recent studies with carbon-based nanomaterials have high potential problems in the accurate quantification of chlorophyll in algae tests conducted with particulate materials (Hund-Rinke *et al.*, 2016; Farkas and Booth, 2017), an issue that should be carefully considered in studies looking at microplastics and NPs interactions with microalgae.

3.5.2. Zooplankton

Zooplankton, especially microzooplankton, are the main gazers of microalgae in the micrometer-size range. As they are the lowest trophic level to exhibit direct

ingestion of microplastics, combined with the ease of utilizing them in laboratories, an increasing number of toxicity studies have been conducted with them, with many reporting negative impacts on organism function and health. Copepods, in particular, have been the subject of a number of microplastics effects studies. The ingestion of microplastics depends on particle size and feeding strategy (Cole *et al.*, 2013; Lee *et al.*, 2013; Setälä *et al.*, 2014). Exposure of the copepod *Centropages typicus* to natural assemblage of algae with and without microplastics showed that high concentrations of microplastics (> 4000 particles/mL) significantly decreased algal feeding (Cole *et al.*, 2013). The survival and fecundity of the copepod *Tigriopus japonicus* were also negatively impacted at chronic exposure (96 h) to high concentrations of polystyrene microplastics (1.25 – 25 µg/mL) (Lee *et al.*, 2013) (Fig. 3.6). However, no acute toxicity was observed in either nauplii or adult copepods (*T. japonicus*) exposed to high concentrations of polystyrene microplastics at sizes of 0.05, 0.5, and 6 µm (Lee *et al.*, 2013). At lower exposure concentrations (75 particles/mL) of 20 µm polystyrene microplastics, energetic depletion and reduced reproduction were observed in the copepod *Calanus helgolandicus* (Cole *et al.*, 2015). They concluded that microplastics competed with food items for ingestion and that the copepods did not actively decline ingestion of nonnutritious particles. A particle size-dependent effect was observed for polystyrene microbeads (0.05, 0.5, and 6 µm) exposed to the monogonot rotifer *Brachionus koreanus*, with smaller particles eliciting increased responses of growth rate, fecundity lifespan, and reproduction time (Jeong *et al.*, 2016). Although a reduction in food uptake was observed for the marine isopod *Idotea emarginata* exposed to polyethylene microplastics over a 7-week period, there were no effects on survival, intermoult duration, or growth (Hämer *et al.*, 2014). The early life stages of many benthic organisms also pass through a planktonic stage, and a number of these have been subjected to microplastic and NP uptake and effects studies. The larvae of the sea urchin *Tripneustes gratilla* exposed to polyethylene MPs (5 days) exhibited reduced body width at the highest exposure tested in abundance (300 spheres/mL), but no effects were observed at environmentally relevant concentrations (Kaposi et al., 2014). Larvae of the sea urchin *Paracentrotus lividus* were exposed to polystyrene NPs with different surface characteristics (NH_2 and COOH). No embryotoxicity was observed for PS-COOH up to 50 µg/mL, whereas PS-NH_2 caused severe developmental defects (EC_{50} = 2.61 µg/mL 48 h post-fertilization) (Della Torre *et al.*,

2014). The results indicate that differences in surface chemistry can significantly influence the microplastic and nanoplastic toxicities.

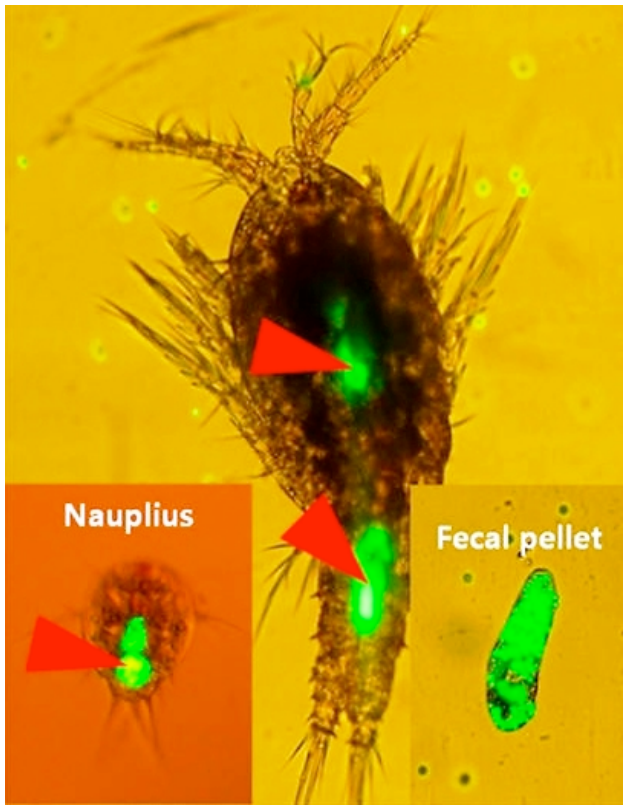

Figure 3.6. Polystyrene microplastic ingestion by adults and *nauplius of Tigriopus japonicus* and egestion in fecal pellets (Reprinted from Lee *et a*l., 2013, with permission of the publisher).

3.5.3. Benthic organisms

Many of the parent polymer materials in microplastics have densities higher than that of seawater, while the processes of aging and biofouling further increase the sedimentation of microplastics (Andrady, 2011). As a result, the sediment surface is a recipient for microplastics, suggesting that benthic species are at high risk to exposure and potential impacts of these particles. A positive relationship was observed between the polystyrene microplastic concentration in sediment and both uptake of microplastic particles and weight loss by the lugworm *A. marina,* and a reduction in feeding activity was observed at a polystyrene dose of 7.4 % dry weight (Besseling *et al.*, 2013). *A. marina* exposed to PVC microplastics for 4 weeks fed less, had reduced lipid reserves, and exhibited increased phagocytic activity and

inflammatory response (Wright *et al.*, 2013a). PVC microplastics were also found to increase susceptibility of *A. marina* to oxidative stress (Browne *et al.*, 2013). Beachhoppers (*Platorchestia smithi*) were observed to readily ingest microplastics, affecting their survival (Tosetto *et al.*, 2016). In contrast, the benthic marine amphipod *Corophium volutator* did not exhibit acute toxicity to two poly(methylmethacrylate)-based nanoplastics (PMMA) with different surface chemistry (medium and hydrophobic) at concentrations ranging from 0.01 to 500 mg/L, and showed no effects in a reburial test conducted after 10-day exposure (Booth *et al.*, 2016). Also, no significant effects were observed in adult sandhoppers (*Talitrus saltator*) exposed to polyethylene microplastics for 24 h followed by a 7-days depuration period (Ugolini *et al.*, 2013), while polystyrene microplastics did not elicit physical or behavioural effects in the common littoral crab (*Carcinus maenas*) over a 21-day exposure (Farrell and Nelson, 2013). Mussels are sedentary filter feeders commonly used as laboratory test species, and have been utilized in a number of studies investigating microplastics and NPs ingestion and toxicity. In the presence of polystyrene NPs, the blue mussel (*Mytilus edulis*) exhibited reduced filtering activity and production of pseudofaeces, which indicates a purging response to the low nutritional value of the microplastics (Wegner *et al.*, 2012). *M. edulis* also accumulated high-density polyethylene (HDPE), which led to an inflammatory response within 6 h of ingestion and destabilization of the lysosomal membrane after 96-h exposure (von Moos *et al.*, 2012). The Asian green mussel (*Perna viridis*) exposed for two 2-h time periods per day to PVC microplastics (1 – 50 µm) had decreased filtration and respiration rates after 44 days, and a decline in survival after 91 days (Rist *et al.*, 2016). The authors suggest that these negative effects resulted from prolonged periods of valve closure as a reaction to the presence of microplastics. In contrast to the above studies, ingestion of polystyrene microplastics (3.0 or 9.6 µm; 3 or 12 h exposure and 48 days of depuration) caused no significant effects on the oxidative status of haemolymph, viability, or phagocytic activity of the haemocytes, or filter-feeding activity in *M. edulis* (Browne *et al.*, 2008). Polystyrene microplastics (2 and 6 µm in diameter; 0.023 mg/L) induced in adult Pacific oysters (*C. gigas*) exposed for 2 months during their reproductive cycle significant decreases in oocyte number (−38 %), diameter (−5 %), and sperm velocity (−23 %) (Sussarellu *et al.*, 2016). Furthermore, D-larval yield and larval development of offspring derived

from exposed parents decreased by 41% and 18%, respectively (Sussarellu *et al.*, 2016).

3.5.4. Fish species

In fish, microplastics have been found to cause several effects, such as decreased predatory performance, endocrine disruption, hepatic stress, intestinal alterations, oxidative stress and damage, among other effects (Oliveira *et al.*, 2013; Rochman *et al.*, 2013b; Rochman *et al.*, 2014b; de Sá *et al.*, 2015; Ferreira *et al.*, 2016; Pedà *et al.*, 2016; Barboza *et al.*, 2018c). Juveniles of the common goby (*Pomatoschistus microps*), an inhabitant of brackish coastal waters, exposed for 96 h to polyethylene microplastics (1-5 μm) showed significantly reduced acetylcholinesterase (AChE) activity, but no significant effects were found for glutathione S-transferase activity or lipid peroxidation (Oliveira *et al.*, 2013). In a follow-up study, wild caught *P. microps* juveniles from two different populations inhabitaing estuaries with different environmental conditions including chemical contamination levels, showed that under simultaneous exposure to microplastics and *Artemia,* fish from the most contaminated estuary showed a significant reduction of the predatory performance (65 %) and efficiency (up to 50 %) in relation to fish from the less contaminated estuary (de Sá *et al.*, 2015). The distinct predatory performance of fish in relation to their provenience estuary, indicates that the developmental conditions may influence the capability of fish to discriminate microplastics from the real prey (de Sá *et al.*, 2015). The microplastic-induced reduction in food intake may decrease individual and population fitness (de Sá *et al.*, 2015). In addition, the presence of microplastics in the water influence the toxicity of pyrene (Oliveira *et al.*, 2013), cefalexin (Fonte *et al.*, 2016), and Cr(VI) (Luis *et al.*, 2015) to *P. microps*. Considering that the temperature variations are expected or are already occurring in several regions of the world as a consequence of global warming, Fonte *et al.* (2016) showed that the rise in water temperature (from 20°C to 25°C) may increase the microplastics-induced mortality (from 8% to 33%) in *P. microps* (Fonte *et al.*, 2016).

3.5.5. Effects on assemblages and communities

Effects on assemblages and communities within real habitats remain largely unknown (Browne *et al.*, 2015b). The first study investigating microplastics impacts on ecological communities (Green, 2016) reported the impacts of biodegradable and conventional microplastics (0.8 - 80 µg/L; 60 d) on the health and biological functioning of European flat oysters (*Ostrea edulis*) and the structure of associated macrofaunal assemblages were assessed in an outdoor mesocosm. Effects on the oysters were minimal, but benthic assemblage structures differed and species richness and the total number of organisms were ~1.2 and 1.5 times greater in control mesocosms than those exposed to high doses of microplastics (Green, 2016). The study indicates that repeated exposure to high concentrations of microplastics could reduce the abundance of benthic fauna (Green, 2016).

3.5.6. Other issues

3.5.6.1. Additives

Recently, there has been increasing interest in the role of plastic additive chemicals, many of which have known toxicity, on the microplastics-induced adverse effects (Meeker *et al.*, 2009; Teuten *et al.*, 2009). Modern plastic materials can contain a vast range of additives that provide specific properties (*e.g.* photostabilizers, plasticisers for flexibility, antioxidants, colour, and pigmentation, flame retardance, biocidal activity) (Meeker *et al.*, 2009; Lithner *et al.*, 2011; Kwon *et al.*, 2017). The leaching of these additive chemicals has been proposed as a potential contribution to microplastic induced effects in marine organisms (Teuten *et al.*, 2009; Koelmans *et al.*, 2014; Jang *et al.*, 2016; Kwon *et al.*, 2017). The toxicity of virgin and beach-stranded plastic pellets on sea urchin embryos (*Lytechinus variegatus*) were investigated by simulating the transfer of additive chemical compounds to the interstitial water and the water column (Nobre *et al.*, 2015). The virgin pellets were more toxic than the beached pellets, increasing anomalous embryonic development by 58.1 % and 66.5 %, respectively. Other studies (Browne *et al.*, 2013; Rochman *et al.*, 2014b) have utilized microplastics artificially contaminated with common additive chemicals. Triclosan (antimicrobial) and PBDE-47 (flame retardant) adsorbed to PVC microplastics were shown to transfer to the tissues of *A. marina*, leading to a reduction in feeding (Browne *et al.*, 2013).

Koelmans *et al.* (2014) assessed the leaching potential of nonylphenol and bisphenol A from microplastics in the intestinal tracts of *A. marina* and *Gadus morhua* (North Sea cod) using a biodynamic model. The conservative analysis showed that microplastic ingestion by lugworms yields nanoplastics and bisphenol A concentrations below their current global concentration ranges, and are therefore unlikely to constitute a relevant exposure pathway (Koelmans *et al.*, 2014). Migration and release of additive chemicals from plastics is a complicated process which is likely significantly influenced by environmental conditions and depend on the type of plastic and additive load (Teuten *et al.*, 2009). The available data is insufficient to be able to reach a consensus on this issue (Jang *et al.*, 2016).

3.5.6.2. Adsorbed pollutants and mixtures

Plastics are typically hydrophobic, which can lead to the adsorption of organic molecules to their surface (Teuten *et al.*, 2009; Frias *et al.*, 2010). Hydrophobic organic chemicals (HOCs) have been shown to have a greater affinity for a range of plastics (*e.g.* polyethylene, polypropylene, polyvinyl chloride) than for natural sediments (Teuten *et al.*, 2007), and have been detected on plastic pellets collected from the marine environment (Mato *et al.*, 2001; Rios *et al.*, 2007; Rochman *et al.*, 2013a; Zhang *et al.*, 2015). Recent studies indicate that microplastics are also able to sorb pharmaceuticals and personal care products (Wu *et al.*, 2016), and metals (Ashton, *et al.*, 2010; Holmes, *et al.*, 2012), among other chemicals. Furthermore, in marine ecosystems, organisms are generally exposed to mixtures of a high number of contaminants, and toxicological interactions between microplastics and other mixture components may occur in exposed organisms (Ferreira *et al.*, 2016; Fonte *et al.*, 2016).

The ingestion of plastics with sorbed contaminants has been suggested as a possible exposure route to very hazardous environmental contaminants (Mato *et al.*, 2001; Thompson *et al.*, 2004), and a recent review (Ziccardi *et al.*, 2016) has summarized the current state of knowledge. Studies (Chua *et al.*, 2014; Wardrop *et al.*, 2016) have shown that HOCs adsorbed to microplastics are bioavailable and can elicit toxicological responses. For example, fish exposed to a mixture of polyethylene with chemical pollutants sorbed from the marine environment bioaccumulated such pollutants, and as a result showed liver toxicity and disruption of the endocrine

system (Rochman *et al.*, 2013b). Interestingly, a recent study (Gandara e Silva *et al.*, 2016) comparing the toxicity of virgin and beached plastic pellet leachates to the brown mussel (*Perna perna*) indicated that both microplastic types caused embryo development toxicity, but beached pellets were more toxic than virgin pellets. The authors attributed this to the presence of contaminants adsorbed to the microplastic surface. However, careful control of exposure systems is necessary to ensure that any observed toxicological effects are derived from pollutants truly adsorbed to microplastic surfaces and not resulting from desorption and dissolution into the aqueous exposure media. Despite the concentrations of HOCs associated with microplastics that can be orders of magnitude greater than the surrounding seawater, the relative importance of microplastics as a route of exposure is difficult to quantify because aquatic organisms are typically exposed to HOCs from various compartments, such as water, sediment, and food. As a result, the relative importance of microplastics as an exposure route for HOCs must be considered in the context of other exposure routes. A comprehensive study (Bakir *et al.*, 2016) modelling the transfer of HOCs from polyvinyl chloride and polyethylene microplastics to a benthic invertebrate, a fish and a seabird suggested that this exposure route was negligible with respect to the combined intake from food and water. Ziccardi *et al.* (2016) also conclude that there is currently weak evidence to support the occurrence of ecologically significant adverse effects on aquatic life as a result of exposure to HOCs sorbed to microplastics. More data are needed to fully understand the relative importance of exposure to HOCs from microplastics compared with other exposure pathways. Microplastics also modulate the fate of other common contaminants (*e.g.* chromium, mercury, pyrene, fluoranthene) inside organisms, and interact with their toxic effects modifying their toxicity to marine species (*e.g.* Oliveira *et al.*, 2013; Luís *et al.*, 2015; Paul-Pont *et al.*, 2016; Barboza *et al.*, 2018c).

3.6. Analysis of microplastics in seawater

While it is relatively simple to observe larger marine plastics, it is more difficult to rigorously collect, isolate, identify, and quantify these particles in complex environmental matrices. Problems include the lack of technology to collect and quantify very small microplastics and nanoplastics (Andrady, 2017), sampling

difficulties for other microplastics such as bias toward or away from areas of microplastic accumulation and variation in collection methods among laboratories, as well as those of sample processing and reporting (Masura *et al.*, 2015; Rocha-Santos and Duarte, 2015). There is no single "standard" method to measure microplastics in environmental samples, leading to considerable uncertainty when comparing results among laboratories. Research groups have often developed their own procedures and protocols, although there are some common elements and approaches. Steps often include isolation of the microplastics from the environmental matrix (water, sediment, tissue), description of individual particles (size, shape, and colour), determination of polymer composition (most commonly by Fourier transform infrared spectroscopy – FTIR), segregating the microplastics into size classes, and quantification (by mass and or by number) (Claessens *et al.*, 2013; Cole *et al.*, 2014; Besley *et al.*, 2017; Zhao *et al.*, 2017). These steps are labour intensive and, since they often rely on visual identification of microplastics, are subject to the analyst's expertise and potential confirmation bias (Filella, 2015). Microplastics in the samples may be lost or altered (*e.g.* broken into smaller particles) during processing, and the samples may be contaminated with microplastics during collection and processing (especially by fibers from clothing) (Masura *et al.*, 2015). Finally, there is no accepted standard way to report the result of these analyses, again compromising the ability to compare distinct studies.

3.6.1. Polymer identification

Fingerprint techniques are very useful tools to provide polymer identification and characterization at a molecular level. One of those techniques is the micro-FTIR (μ-FTIR) that allows identification of different materials through the interaction between infrared radiation and matter (Käppler *et al.*, 2015).

The interactions are different for each material, resulting in fingerprint spectra with characteristic bands (Hummel, 2012). This method of vibrational spectroscopy is extremely sensitive to molecular structural changes (bending and stretching). When a microscope is coupled with the μ-FTIR spectrometer, it is possible to identify tiny fragments and fibers with a size range of micrometers (Afremow *et al.*, 1969; Hummel, 2012) – Figs. 3.7 and 3.8. The match between the micro- sample spectrum and reference database spectra assures the reliability of this technique. In order to identify a polymer with high certainty, the match between the sample and reference

spectra should be above 80 %. Also, it is important to identify correctly the characteristic bands, which sometimes is a process that needs to be done manually, by comparing the spectrum with reference material (Hummel, 2012). In order to facilitate the identification process, Table 3.2 was compiled, representing the principal infrared characteristic bands for microplastic sampling.

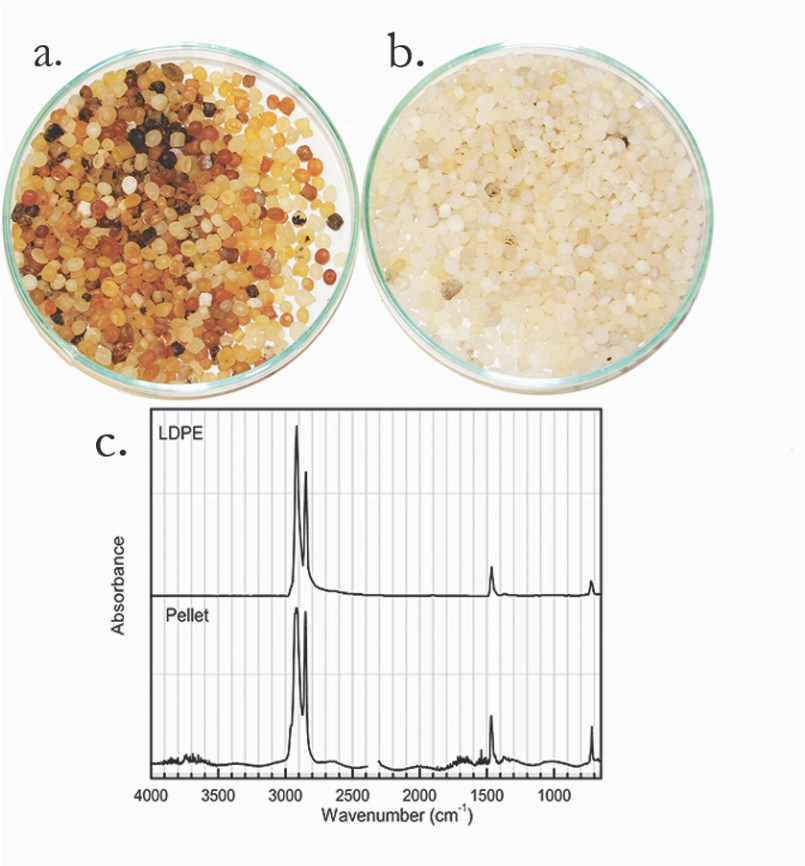

Figure 3.7. Aged (A) and virgin (B) resin pellets retrieved from sandy beaches and comparison of acquired spectrum with a reference spectrum for low-density polyethylene (LDPE, C) (kindly provided by João Frias).

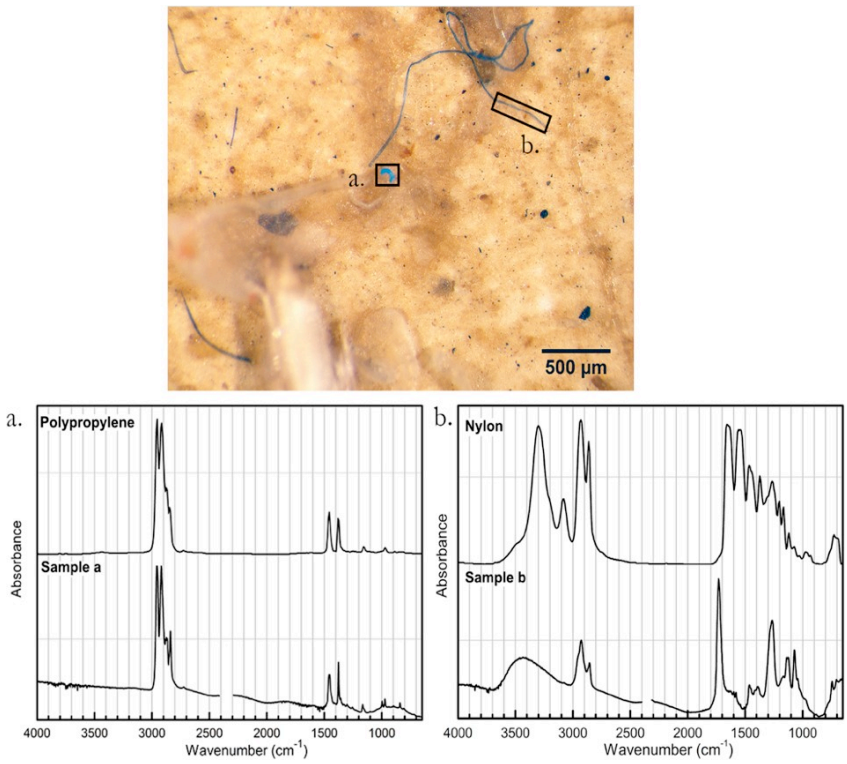

Figure 3.8. Microplastic samples and their FTIR spectra: (A) fragment identified as polypropylene and (B) fiber identified as nylon (kindly provided by João Frias).

Table 3.2. Infrared characteristic bands (cm^{-1}) for microplastic samples. Adapted from Hummel, 2002. [ν – stretching; δ_{as} – asymmetric bending; δ_s – symmetric bending; intensity band; vs – very strong; s – strong; m – medium; w – weak].

Compound	Characteristic band (cm^{-1})	Assignment
Polyethylene (PE)	2918 vs, 2850 s	$\nu_{as}(CH_2)$, $\nu_s(CH_2)$
	1472 w	$\delta_{as}(CH_2)$
Polypropylene (PP)	2960 vs, 2877 m	$\nu_{as}(CH_3)$, $\nu_s(CH_3)$
	2918 s, 2838 m	$\nu_{as}(CH_2)$, $\nu_s(CH_2)$
	1460 m	$\delta_{as}(CH_3)$
	1377 m	$\delta_s(CH_3)$
Polypropylene	2960 s, 2877 m	$\nu_{as}(CH_3)$, $\nu_s(CH_3)$
+	2918 vs, 2850 m, 2838 m	$\nu_{as}(CH_2)$, $\nu_s(CH_2)$
poly(ethylene:propylene)	1460 m	$\delta_{as}(CH_3)$
Copolymer Mix [PE+P(E:P)]	1377 m	$\delta_s(CH_3)$
Rayon	3650-3000 s	ν(OH)
(synthetic cellulose)	2990-2820 w	ν(CH)
	1425, 1372, 1320 m	δ(CH)
	1200-1000 vs	δ(C-OH) + δ(C-C) + ν(C-O-C) + ν(C-OH)
Polyvinyl acetate (PVAc)	2971, 2930 w	ν(CH)
	1738 vs	ν(C=O)
	1434 w	$\delta(CH_2)$
	1373 m	$\delta(CH_3)$
	1242 s	ν(COC)
Polyacrylonitrile (PAN)	2935 m, 2870 m	$\nu_{as}(CH_2)$, $\nu_s(CH_2)$
	2243 vs	ν_{as}(CN)
	1731 s	ν(C=O)
Polyvinyl chloride (PVC)	2970, 2912 m	ν(CH)
	1430 vs	$\delta(CH_2)$
	1330 m	$\delta(CH_3)$
	1250 s	δ(CH)
	692 m	ν(C-Cl)
Polyethylene-vinyl acetate (PEVA)	2920 vs, 2850 s	$\nu_{as}(CH_2)$, $\nu_s(CH_2)$
	1738 m	ν(C=O)
	1465 w	$\delta(CH_2)$
	1242 m	ν(COC)
Polystyrene (PS)	3060 w, 3026 m	ν(CH ring)
	2923 m, 2850 w	$\nu_{as}(CH_2)$, $\nu_s(CH_2)$
	1600 w, 1492 m, 1450 m	ν(CC ring)
	756 m, 699 vs	δ(CH ring)
Polyester (PES)	2966 w	ν(CH)
	1720 vs	ν(C=O)
	1408 m	δ(ring)
	1340 m	δ(CH)
	1261/1246 s, 1117/1102 s	ν(COC)
	1018 m, 728 m	δ(CH ring)
Polyamide 6	3300 s, 3082 w	ν(NH)
(Nylon 6)	2934 m, 2863 m	$\nu_{as}(CH_2)$, $\nu_s(CH_2)$
	1642 vs	ν(C=O)
	1544 vs	δ(NH)
	1463 m, 1371 m	δ(CH)
	1263 m	δ(CN)

3.7. Priorities and opportunities for future research

The lack of standardized methodologies, the ongoing debates concerning microplastics size limits, and use of different quantitative units to express their environmental abundance (mg/L, number.m^{-3}; number.m^{-2}; number.m^{-1}; number.g^{-1}; number.kg^{-1}), means there is a need to improve data comparisons (Hidalgo-Ruz *et al.*, 2012). Inter-calibration exercises (*e.g.* cruises to collect samples using different methodologies, different laboratory manipulation of the same sample in order to produce the same results, etc.) and workshops (*e.g.* establishment of laboratorial sampling criteria, debate on size limits, units to express densities, etc.) could enable scientists to greatly improve data collection for future comparisons. Also, polymer identification by spectroscopy techniques (FTIR and RAMAN), as well as research on physical and chemical properties of plastics, might be extremely valuable to improve oceanic models.

There is insufficient data available to allow strong conclusions regarding the impacts of microplastics on marine organisms. In particular, there is a need to generate more knowledge on the impacts of microplastics at the assemblage and ecosystem levels. Most studies have employed spherical, smooth-surfaced, "'pristine" primary microplastic reference materials to investigate their environmental fate and effects. In reality, microplastic particles encountered in the marine environment are "secondary" particles derived from the breakdown and degradation of larger plastic items; and are characterized by their irregular shape and complex surface morphologies. Fibers and fragments are the most commonly encountered in the world's oceans and seas and should therefore be the focus of future studies (Moore *et al.*, 2001; Lusher *et al.*, 2014; Thompson *et al.*, 2004). In addition, further research is needed on persistent bioaccumulative and toxic chemicals (PBT) adsorbed to microplastics, ecotoxicology bioassays to assess toxicological interactions (potentiation, addtition, synergism, antagonism) of microplastics and other chemicals of high concern, as well as experiments to investigate cumulative effects in marine species and potential biomagnification of microplastics and the chemicals that they often contain. These are required to understand many of the questions and processes that remain unknown. Such knowledge is crucial in order to be able to develop and implement effective management strategies (Thompson *et al.*, 2009).

New emerging challenges with growing concerns related to damage to benthic and costal habitats are being reported. Plastic is also affecting corals (Sheenan *et al.*, 2017) and microplastic paradigm is also related to human food safety (EFSA, 2016). Human food safety is demanding and after the reports on the ingestion and potential of microplastics to accumulate and biomagnify in the food webs (Boerger *et al.*, 2010; Setälä *et al.*, 2014) requires further investigation in relation to these particles. Moreover, microplastics were also found in drinking water and food items, such as honey, beer and salt. Thus, future research should also contemplate the quantification of these microparticles in other human food items (Liebezeit and Liebezeit, 2013, 2014; Karami *et al.*, 2016a; Schymanski, *et al.*, 2018).

Monitoring programs are fundamental (OSPAR, 2010), and when conducted in beaches and coastal areas are relatively economic. Due to technological reasons, costs rise for monitoring floating or sunken debris, yet data gathered is valuable to estimate accumulation areas and sinking rates of plastics and microplastics (Newman *et al.*, 2015; UNEP, 2016). Notwithstanding the efforts already made to estimate the costs associated with plastics (UNEP, 2014), cost-benefit evaluations could be improved to provide further financial and societal benefits. There are good examples of policy instruments that influence behaviour that actually reduce plastic consumption (Vaz *et al.*, 2009; Pahl and Wyles, 2017; Xanthos and Walker, 2017). Improvements in recycling and waste management processes can also be a catalyst with strong direct and indirect socio-economic and environmental implications.

3.8. Acknowledgments

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Chapter IV

Effects of microplastics alone and in mixture with mercury on juveniles of the European seabass, Dicentrarchus labrax (Linnaeus, 1758)

Chapter IV

Section 1. Brief introduction to the paradigm of microplastics-mercury mixtures in the marine environment and to the species tested in this Chapter

As indicated in the previous Chapters and exemplified in Fig. 4.1, because of their physical and chemical properties, microplastics generally accumulate several chemical contaminants present in the surrounding seawater, in addition to the chemicals previously incorporated during the plastic manufacturing process (Rochman, 2015). For example, mercury that is considered an ubiquitous pollutant of high concern, and commonly found at increased concentrations in several antropogenically impacted areas and in naturally enriched ones may sorb to microplastics also present in these areas. In fact, evidences suggest that microplastics are able to adsorb contaminants from the water (Rochman, 2015, Turner and Holmes, 2015). Furthermore, because in some countries mercury is still used in the production process of polyvinyl chloride (PVC) (Ren *et al.*, 2014), the resulting plastics are already contaminated by mercury before being released into the environment. In both cases, mercury-contaminated microplastics may be uptaken by the biota through microplastics that will be exposed to the metal thorough this additional route. Moreover, mercury-contaminated microplastics may travel for long distances transported mainly through water currents, air and long-distance migrations of contaminated organisms.

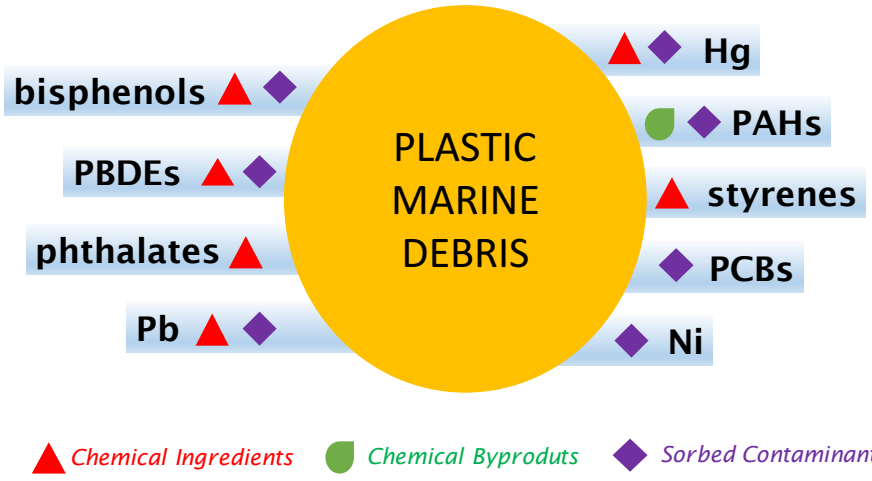

Figure 4.1. Examples of contaminants associated with marine plastic debris. Contaminants associated with marine debris include chemical ingredients, byproducts, and those that accumulate in the plastics from the surrounding ocean water in the marine environment (Adapted from Rochman, 2015).

Moeover, in the marine environment, the biota is generally exposed to mixtures of several different chemical contaminants (Kienzler *et al.*, 2016). Some of

these substances are considered priority pollutants and are regulated by governmental agencies, and international institutions and Conventions because of their toxicity and/or persistence in the environment, organisms and food webs. Moreover, environmental contaminants may be degraded and interact among them in the environment producing an even more wide range of compounds. Furthermore, after entering into organisms, environmental contaminants may be biotransformed producing metabolites with toxicity different from their parental compounds, and toxicological interactions may also occur (Ikenaka *et al.*, 2007; Jorgensen *et al.*, 2008; Paul-Pont *et al.*, 2016; Fonte *et al.*, 2016). Therefore, it is very important to investigate the effects caused by mixtures of chemicals, and several institutions included “mixtures” as a priority topic in their research programmes (*e.g.,* Horizon 2020 of the European Union).

Among the several environmental contaminants that may be present in marine ecosystems together with microplastics, mercury is of special interest for several reasons as previously introduced. In fact, mercury is one of the most hazardous contaminants that may be present in the marine environment occurring at increased concentrations in several regions around the world (Gworek *et al.*, 2016). It is considered to be among the highest priority environmental pollutants in the scope of the European Water Framework Directive and on a global scale (Namour *et al.*, 2010 EEA, 2018). Mercury enters into the environment through natural sources, primarily in the form of elemental mercury, including volcanic emissions, degassing from soils and volatilization from the ocean, as well as through anthropogenic sources derived from emissions of industrial processes, combustion sources and the disposal products containing mercury including: car parts, batteries, fluorescent bulbs, medical products, thermometers, and thermostats (EEA, 2018). Wet and dry deposition of mercury from the atmosphere is among the most significant sources of mercury in the marine environment (Batrakova et al., 2014*).* Mercury exists in different chemical and physical forms in seawater; two main forms are present, namely elemental mercury (Hg^0) dissolved as particulate ions (Hg^{2+} and Hg^{+}) or as methyl or ethyl-mercury ($MeHg^{+}$) in dissolved or particulate forms (Morel et al., 1998), as shown in Fig. 4.2. In this way, forms of mercury with relatively low toxicity can be transformed into forms with very high toxicity and can be accumulated and biomagnified in trophic

webs resulting in increased exposure of high trophic levels, including humans (Boudou and Ribeyre, 1997).

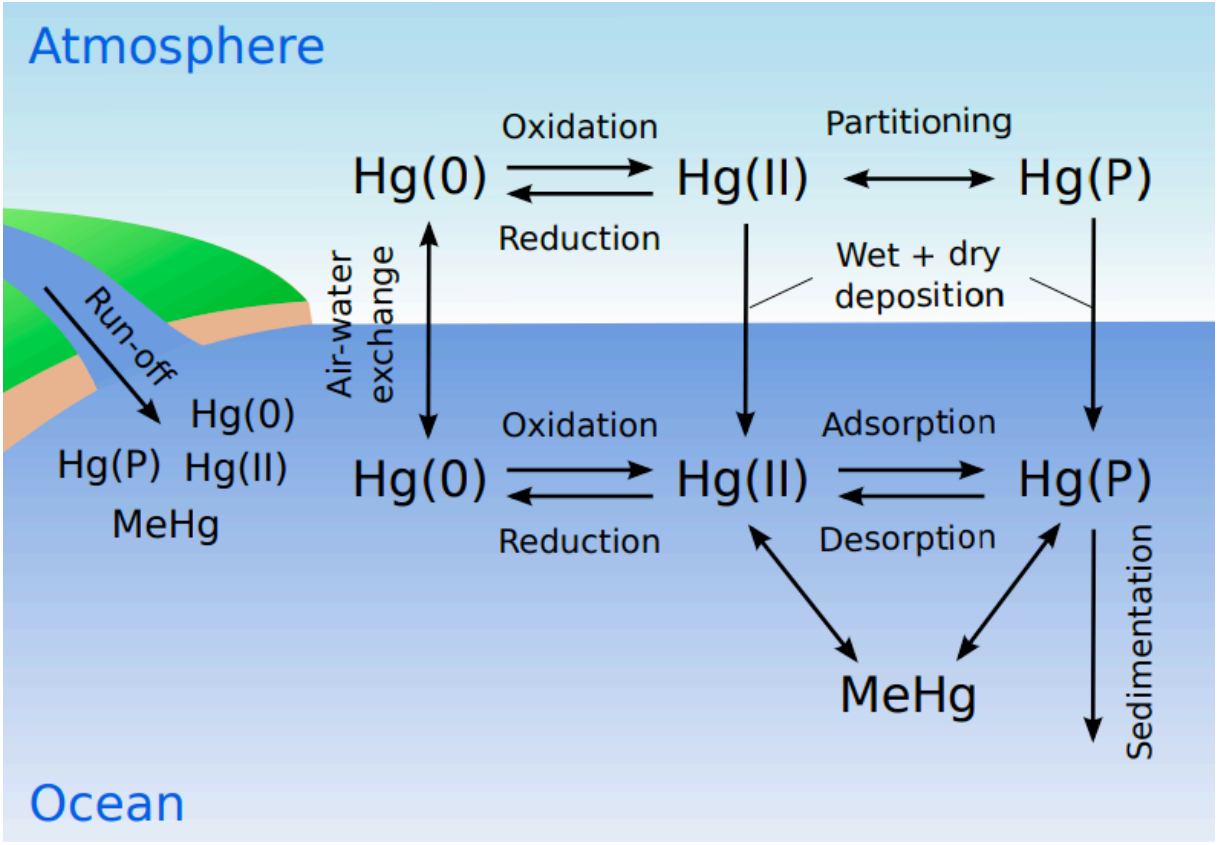

Figure 4.2. General scheme of mercury transformations in the ocean (Reprinted from Batrakova et al., 2014, with permission of the publisher).

The impacts of mercury on marine life are complex and affect a wide variety of tissues and organs. The most know and toxic form is metylmercury that causes neurotoxicity, among other toxic effects. In marine and esturine organisms, exposure to mercury can cause a wide range of adverse effects, such as: developmental and behavioral abnormalities, impaired reproduction, mortality, among several other toxic effects in animals of higher trophic levels, such as fish (Vieira *et al.*, 2009; Kidd *et al.*, 2012; Zhang *et al.*, 2017; Green and Planchart, 2018; Rumbold *et al.*, 2018).

Mercury also causes a wide range of adverse human health effects, including permanent damage to the nervous system, in particular the developing nervous system (Azevedo *et al.*, 2012; Clarson and Magos, 2006). Due to these effects, and also because mercury can be transferred from a mother to her unborn child and

through the human milk, among other reasons, newborns, children, pregnant woman and woman with child of bearing age are considered especially vulnerable populational groups (Bose-O'Reilly *et al.*, 2010; Starling *et al.*, 2015). In humans, exposure to mercury and mercury compounds can cause a variety of toxic effects including neurotoxicity, nephrotoxicity, teratogenicity and also produces profound cardiotoxicity (Clarson and Magos, 2006; Park *et al.*, 2012).

As in the marine environment, fish are likely exposed simultaneously to mercury and microplastics, and marine fish are an important component of a healthy human diet, the investigation of the potential effects induced by simultaneous exposure of fish to microplastics and mercury is of high importance and a gap of knowledge on this topic exists. Among fish, fish top predators of particular relevance regarding the potential effects induced by microplastic-mercury mixtures because the possible biomagnification of the substances. Moreover, those spending parts of their life-cycle in continental waters, such as estuaries and costal lagoons, of antropogenically impacted areas may be increased exposed to both microplastics and mercury.

The European sea bass (*Dicentrarchus labrax*) is a teleost fish found in the North-eastern Atlantic Ocean and throughout the Mediterranean and Black Seas (FAO, 2019). The distribution and life cycle of the European sea bass encompass environmentally-contrasted habitats (Volckaert *et al.*, 2008; Lopez *et al.*, 2015). *D. labrax* inhabits coastal waters, where reproduction occurs, but migrates offshore in colder weather and occurs in deep water during the Winter (Spitz *et al.*, 2003, Lopez *et al.*, 2015). However, it can also enter in brackish waters of estuarine areas and coastal lagoons, and occasionally rivers (Volckaert *et al.*, 2008. Tine *et al.*, 2014). Thus, *D. labrax* is an eurythermal fish (8 – 24 ^{o}C, or even up to 8 – 24 ^{o}C) with high tolerance to salinity changes (Volckaert *et al.*, 2008; Tine *et al.*, 2014). The juvenile stage occurs approximately 2 months after spawning. The growing larvae drift from the open sea to inshore, and eventually into creeks, backwaters, and estuaries. These habitats are used by juveniles for the next 4 – 5 years, before they mature and adopt the migratory movements of adults (Volckaert *et al.*, 2008; Tine *et al.*, 2014; Lopez *et al.*, 2015). The adult of *D. labrax* is a voracious opportunistic predator consuming small fish and a large variety of invertebrates. Larvae are equally

opportunistic predators, feeding in a lower size range (Volckaert *et al.*, 2008; Tine *et al.*, 2014; Pawson *et al.*, 2007; FAO, 2019).

D. labrax was selected as test organism in the present Thesis since it has been widely used as a model species in marine ecotoxicology (Gravato and Guilhermino, 2009, Hernandez-Moreno *et al.*, 2011; Mieiro *et al.*, 2014; Almeida *et al.*, 2015) and because it is a key species in European estuaries and in other marine ecosystems. Moreover, is an economically important species, highly appreciated for human consumption as food, and natural stocks are subject to intensive exploitation by professional and sport fisheries, thus raising future conservation and management issues (Tine *et al.*, 2014). Although the effects of mercury on *D. labrax* have been intensively studied (Weis, 2014), there was no information available in the literature regarding microplastics-mercury mixtures and their potential effects on fish species, providing support to this study.

Chapter IV

Section 2. Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, Dicentrarchus labrax (Linnaeus, 1758)

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(Article published and included in the Thesis with permission of Elsevier included in the Appendix A)

4.2.1. Abstract

Microplastics pollution is a global paradigm that raises concern in relation to environmental and human health. This study investigated toxic effects of microplastics and mercury in the European seabass (*Dicentrarchus labrax*), a marine fish widely used as food for humans. A short-term (96 h) laboratory bioassay was done by exposing juvenile fish to microplastics (0.26 and 0.69 mg/L), mercury (0.010 and 0.016 mg/L) and binary mixtures of the two substances using the same concentrations, through test media. Microplastics alone and mercury alone caused neurotoxicity through acetylcholinesterase (AChE) inhibition, increased lipid oxidation (LPO) in brain and muscle, and changed the activities of the energy-related enzymes lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH). All the mixtures caused significant inhibition of brain AChE activity (64 – 76 %), and significant increase of LPO levels in brain (2.9 – 3.4 fold) and muscle (2.2 – 2.9 fold) but not in a concentration-dependent manner; mixtures containing low and high concentrations of microplastics caused different effects on IDH and LDH activity. Mercury was found to accumulate in the brain and muscle, with bioaccumulation factors of 4 – 7 and 25 – 40, respectively. Moreover, in the analysis of mercury concentrations in both tissues, a significant interaction between mercury and microplastics was found. The decay of mercury in the water increased with microplastics concentration, and was higher in the presence of fish than in their absence. Overall, these results indicate that: microplastics influence the bioaccumulation of mercury by *D. labrax* juveniles; microplastics, mercury and their mixtures cause neurotoxicity, oxidative stress and damage, and changes in the activities of energy-related enzymes in juveniles of this species; mixtures with the lowest and highest concentrations of their components induced different effects on some biomarkers. These findings and other published in the literature raise concern regarding high level predators and humans consuming fish being exposed to microplastics and heavy metals, and highlight the need of more research on this topic.

Keywords: *Dicentrarchus labrax*, Microplastics, Mercury, Mixtures, Neurotoxicity, Bioaccumulation

4.2.2. Introduction

The presence of microplastics in the marine environment due to primary and secondary sources (*e.g.* pre-production pellets, synthetic textiles, cosmetics, fragmentation of plastic debris) has been reported worldwide (Cózar *et al.*, 2014; Barboza and Gimenez, 2015; van Sebille *et al.*, 2015). Since these particles have been found to induce adverse effects in a considerable variety of organisms (*e.g.* Avio *et al.*, 2015a; Gall and Thompson, 2015; Ferreira *et al.*, 2016; Ribeiro *et al.*, 2017), concerns regarding environmental, animal and human health exist (Thompson, 2015). Thus, regulations to monitor and investigate the problem to minimize its impacts have been implemented (*e.g.* European Marine Strategy Framework Directive).

Microplastics present in the environment can be ingested by different types of organisms (Fossi *et al.*, 2012; Besseling *et al.*, 2013; Goldstein and Goodwin, 2013; Frias *et al.*, 2014; de Sá *et al.*, 2015; Romeo *et al.*, 2015; Rummel *et al.*, 2016; Güven *et al.*, 2017) including species widely used in the human diet (Neves *et al.*, 2015; Rochman *et al.*, 2015; Battaglia *et al.*, 2016; Silva-Cavalcanti *et al.*, 2017). Microplastics can induce toxic effects *per se* (Oliveira *et al.*, 2013; Ferreira *et al.*, 2016). They may also contain very hazardous chemicals that are introduced in organisms when microplastics are taken up potentially leading to increased accumulation of these substances in food webs (Teuten *et al.*, 2009; Setälä *et al.*, 2014; Batel *et al.*, 2016). Thus, special concerns regarding top predators exist, especially because some of them are consumed by humans. In fish, microplastics have been found to cause several adverse effects, including decreased predatory performance, endocrine disruption, hepatic stress, intestinal alterations, oxidative stress, among others (Oliveira *et al.*, 2013; Rochman *et al.*, 2013b; de Sá *et al.*, 2015; Ferreira *et al.*, 2016; Pedà *et al.*, 2016).

A complex problem associated to microplastics is their capability to sorb and interact in other ways with other common environmental contaminants, such as metals (Ashton *et al.*, 2010; Holmes *et al.*, 2012; Rochman *et al.*, 2014a,b), pharmaceuticals (Wu *et al.*, 2016), and other contaminants (Rochman *et al.*, 2013a; Wang *et al.*, 2015; Tosetto *et al.*, 2016). Therefore, microplastics can influence the fate of these substances in the environment and in organisms, as well as their toxicity. For example, microplastics have been found to influence the localization, biotransformation and/or toxicity of polycyclic aromatic hydrocarbons (PAHs) and

polybrominated diphenyl ethers (PBDEs) in fish (Oliveira *et al.*, 2013; Rochman *et al.*, 2013b) and in other organisms (Chua *et al.*, 2014; Avio *et al.*, 2015a; Paul-Pont *et al.*, 2016), of pharmaceuticals and personal care products in fish (Fonte *et al.*, 2016; Wardrop *et al.*, 2016), and of metals in fish (Khan *et al.*, 2015; Luís *et al.*, 2015). However, more knowledge on such interactions in needed to assess the risks and increase the safety in the use and management of microplastics and other common environmental contaminants.

Estuaries and other coastal areas of industrial and urbanized impacted regions are considered microplastics hotspots (Isobe *et al.*, 2015; Gallagher *et al.*, 2016; Peters and Bratton, 2016). Such ecosystems are also contaminated with a high number of other chemicals, including several ubiquitous pollutants. Among these, mercury raises special concern mainly because is very toxic at low concentrations and its organic forms, methylmercury in particular, are biomagnified in trophic webs, increasing the risk of exposure and toxic effects on top predators and humans consuming them (Atchison *et al.*, 1987; Branco *et al.*, 2004; Carvalho *et al.*, 2008; Selin, 2009). In addition, because of its high degree of toxicity, mercury is listed as a priority hazardous substance under the scope of the United Nations Environment Programme (UNEP), United States Environmental Protection Agency (US EPA) and European Commission (EC).

To the best of our knowledge, the toxic effects resulting from the simultaneous exposure to microplastics and mercury through the water were not investigated before in fish. Thus, the goals of the present study were to investigate the short-term toxic effects of microplastics and mercury exposures, individually and in binary mixtures, on juveniles of the European seabass *Dicentrarchus labrax* (Linnaeus, 1758). *D. labrax* was selected as model species for this study mainly because it is a key species in several European estuaries and in other marine ecosystems, is used as food for humans being a very appreciated marine fish and therefore having a high commercial value, and recent studies have investigated the effects of microplastics in this species (*e.g* survival, growth and intestinal alterations) (Mazurais *et al.*, 2015; Pedà *et al.*, 2016).

4.2.3. Material and methods

4.2.3.1. Chemicals

Fluorescence red polymer microspheres, 1-5 µm diameter (lot number: 4-0906-0661), purchased from Cospheric − Innovations in Microtechnology (USA), were used as microplastics model. According to the manufacturer, the particles are spherical, red opaque, 1.3 g/cc density, and can be detected by spectrofluorimetry (excitation wavelength of 575 nm and emission wavelength of 607 nm). Mercury chloride (≥ 99.5 % pure, lot number: 031M0173 V) was purchased from Sigma-Aldrich (USA). The other chemicals used were all of the highest purity available and purchased from Sigma-Aldrich (USA) or Merck (Germany). The Bradford reagent used for protein determinations was from BIORAD (Germany).

4.2.3.2. Ethical issues

Experiments were conducted in accordance with ethical principles and other requirements of the Portuguese and European regulations for the protection of animals used for scientific purposes, including authorization of the Portuguese National Authority: "Direção Geral de Alimentação e Veterinária" (DGAV): 0421/000/000/2017, 014227, 31st May 2017. L. Guilhermino, L. R. Vieira and F. Carvalho are accredited by the DGAV as investigator/coordinator (equivalent to FELASA category C) to carry animal experimentation. The experiments were carried out in the CIIMAR bioterium that is accredited by DGAV for studies with aquatic animals.

4.2.3.3. Fish maintenance and acclimatization

Seabass juveniles were purchased from a saltwater fish aquaculture (Vigo, Spain) and acclimatized to laboratory conditions for 4 months. During this period, they were maintained in 2000 L tanks with aerated, biologically and UV-filtered seawater (salinity: 34 ± 1 g/L), hereafter indicated as water. Partial water renewal was made every week and water abiotic parameters (temperature, conductivity, salinity, dissolved oxygen, pH, ammonia, nitrates, and nitrites) were periodically monitored. During this period, fish were fed with commercial fish food (Tetramin®, Tetra, Germany). Fifteen days before the bioassay, fish were put in a room with control of temperature (19 ± 1 °C) and photoperiod (14 h light: 10 h dark), with water temperature maintained at 18 °C ± 1 °C. Here, they were maintained in 5 L glass

beakers (with 4 L of water), 1 animal per beaker, with continuous air supply. The water was changed every other day, the water parameters above mentioned were determined every day. Fish were fed *ad libitum* with commercial fish food (Tetramin®, Tetra, Germany) and observed two times per day. No mortality was observed during the acclimatization period. Forty-eight hours before the start of the bioassay, fish were transferred to beakers with clean water and feeding was stopped.

4.2.3.4. Preliminary assay without fish

Prior to the bioassay, a preliminary assay without fish was carried out to investigate the behavior of mercury and microplastics in the water. Briefly, the assay was carried out for 96 h; photoperiod, water temperature and salinity were as indicated in Section 4.2.3.3. Treatments were: 1 control (water only), 1 treatment containing a low microplastics concentration (MPs-L: 0.25 mg/L); 1 treatment containing a high microplastics concentration (MPs-H: 0.69 mg/L); 1 treatment containing a low mercury concentration (Hg-L: 0.005 mg/L); 1 treatment containing a high mercury concentration (Hg-H: 0.010 mg/L); and 4 binary mixtures containing microplastics and mercury simultaneously (MPs-L+ Hg-L; MPs-L + Hg-H; MPs-H + Hg-L; MPs-H + Hg-H). The concentrations of microplastics and mercury above indicated are the mean of mid-point actual concentrations in treatments containing the lowest or the highest concentrations of tested substances during the assay (determined as indicated in Sections 4.2.3.7 and 4.2.3.8.). The treatments containing the test substances (alone or in mixture) were prepared by diluting the appropriate volume of microplastics or/and mercury stock solutions (prepared in seawater) to obtain the desired final test concentration. Test beakers were 5 L glass beakers filled with 4 L of water, with continuous air supply and covered to prevent evaporation. Nine beakers were used per treatment and water was renewed each 24 h. Water samples for determination of microplastics and mercury concentrations were collected at the beginning and at the end of the bioassay, and at the time of water renewal in both clean and old water. Microplastics concentrations were determined immediately after sample collection (as described in Section 4.2.3.7), and samples for mercury analyses were kept at − 20 °C.

4.2.3.5. Bioassay

The bioassay was conducted under the conditions of temperature and photoperiod indicated in Section 4.2.3.3., also used in the preliminary assay (section 4.2.2.4). Test media was water (as in sections 4.2.3.3. and 4.2.3.4.). The exposure period was 96 h and no food was provided during the bioassay. Juvenile fish with a mean (± standard deviation − SD) of total length of 8.8 cm (± 0.295) and mean weight of 7.7 g (± 0.293) (determined at the end of the bioassay to avoid inducing extra stress to fish) were used. The experimental design included 9 treatments: 1 control (water only), 1 treatment containing a low microplastics concentration (MPs-L); 1 treatment containing a high microplastics concentration (MPs-H); 1 treatment containing a low mercury concentration (Hg-L); 1 treatment containing a high mercury concentration (Hg-H); and 4 binary mixtures containing microplastics and mercury simultaneously (MPs-L + Hg-L; MPs-L + Hg-H; MPs-H + Hg-L; MPs-H + Hg-H). The concentrations were the mean of mid-point actual concentrations in treatments containing the lowest and the highest concentrations during the bioassay (determined as indicated in Sections 4.2.3.7. and 4.2.3.8.), namely: 0.010 mg/L and 0.016 mg/L of mercury, respectively; 0.26 mg/L and 0.69 mg/L of microplastics, respectively. The concentrations of microplastics were selected based on results of previous studies with microplastics of the same size (*e.g.* Luis et al., 2015; Ferreira et al., 2016; Fonte et al., 2016) and on the results of the preliminary assay. The treatments containing the test substances (alone or in mixture) were prepared by diluting the appropriate volume of microplastics or/and mercury stock solutions (prepared in seawater) to obtain the desired final test concentration. Fish were randomly distributed per treatments, 9 fish per treatment, and they were exposed individually (*i.e.* 1 fish per beaker) in 5 L glass beakers (with 4 L of water), with continuous additional air supply. All the beakers were sealed to prevent evaporation and other possible sources of bias. Water was renewed each 24 h to decrease the possibility of microplastics concentration reduction in the water. Abiotic water parameters were measured in the beginning and at the end of the bioassay, and at the time of water renewal, in both clean and old water. Water samples for determination of microplastics and mercury concentrations were collected at the beginning and at the end of the bioassay, and at the time of water renewal in both clean and old water. The concentrations of microplastics in water samples were determined immediately (as indicated in Section 4.2.3.6.). Water samples for

determination of mercury were collected and stored in teflon bottles and stored at −20 °C until further analyses (as indicated in Section 4.2.3.7.). Microplastics concentrations were determined immediately (as indicated in Section 4.2.3.6.). At the end of the exposure period, the swimming behaviour endpoints were immediately determined for another study (described in Section 4.4 of the present Chapter), fish were measured (total body length) and weighed, and put back in their original exposure beakers and left to rest for 2 h to stabilize metabolic rates, preventing interference with the biomarkers used as effect criteria (Almeida *et al.*, 2010, 2015).

To investigate the effects of exposure to microplastics and/or mercury, a set of biomarkers, including enzymes involved in functions crucial for fish survival and performance and of oxidative damage, was employed: brain acetylcholinesterase (AChE) activity, muscle total cholinesterase (ChE) activity, brain lipid peroxidation (LPO) levels, muscle isocitrate dehydrogenase (IDH) activity, muscle lactate dehydrogenase (LDH) activity, muscle isocitrate dehydrogenase activity (IDH), and muscle LPO levels. Previous characterization of *D. labrax* cholinesterases in brain and muscle revealed that brain contains mainly AChE, whereas muscle has both AChE and pseudocholinesterase (Varò *et al.*, 2003). Thus, brain AChE activity was selected because it provides valuable information regarding neurotoxicity in the brain, and muscle ChE activity because it provides indication of potential neuromuscular cholinergic disruption. LDH and IDH activities were selected because they have key roles in the anaerobic and aerobic pathways of cellular energy production, respectively, and IDH is also important to maintain the cellular redox balance. LPO levels were selected as marker of lipid oxidative damage.

Fish were euthanized by decapitation under ice-cold induced anesthesia. No chemical anesthetics were used to avoid possible interactions with tested substances effects and/or interference with biomarker determinations. From each fish, the whole brain, five samples of dorsal muscle (with about 0.2 g each), the liver and the gills were isolated on ice. The liver and gills were frozen at − 80 °C separately, and used for another study (described in Section 4.4 of the presente Chapter). Brains were frozen individually at − 80 °C. The 5 muscle samples were 1 for ChE activity determinations, 1 for LDH activity determinations, 1 for IDH activity determinations, 1 for LPO level analyses and 1 for mercury concentration determinations. All muscle samples were frozen separately at −80 °C until further analyses. The further preparation of the biological tissues for biomarker analyses was done as described in

Almeida *et al.* (2010). Briefly, in the day of biomarker analyses, samples of each fish were desfrozen on ice. The whole brain of each fish was homogenized in cold phosphate-buffer (0.1 M, pH = 7.2, Ystral GmbH d-7801, Germany) and centrifuged at 4 °C (3300 g for 3 min) using a SIGMA 3 K 30 centrifuge (Germany). The supernatant was collected and divided into three samples: 1 for AChE determinations, 1 for brain LPO level analyses, and 1 for mercury concentration determinations). Samples for LDH determinations were frozen/desfrozen three times to disrupt the cell membrane and release the cytoplasmic enzyme. All samples for LDH and IDH were centrifuged at 4 °C (3300 g for 3 min, SIGMA 3 K 30 centrifuge, Germany). The supernatant of each sample was carefully collected. The protein content of each sample was determined by the Bradford method (Bradford, 1976) adapted to microplate (Guilhermino *et al.,* 1996), using bovine γ-globulin as protein standard. Then, the protein content of samples for AChE and ChE determinations was standardized to 0.5 mg/mL, those of samples for IDH and LDH determinations to 1 mg/mL (Almeida *et al.*, 2010). AChE and ChE activities were determined by the Ellman's method (Ellman *et al.*, 1961) adapted to microplate (Guilhermino *et al.*, 1996), using acethylthiocholine as substrate, and absorbance was read at 412 nm. IDH activity was determined according to Ellis and Goldberg (1971) adapted to microplate (Lima *et al.*, 2007), using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as substrate and absorbance was read at 340 nm. LDH activity was determined according to the method of Vassault (1983) adapted to microplate (Diamantino *et al.*, 2001), using pyruvate as substrate and absorbance was read at 340 nm. After the enzymatic determinations, the protein of the samples used for enzymatic determinations was determined again and used to express the enzymatic activities as nano moles of substrate hydrolyzed per min per mg of protein (nmol/min/mg protein). LPO levels were determined according to the method of Ohkawa (1979), by measuring (at 535 nm) the thiobarbituric acid reactive species (TBARS), and the values were expressed as nanomoles of TBARS per mg of protein (nmol TBARS/mg protein). All analyses were performed with a Spectramax® spectrophotometer (Molecular Devices, USA).

4.2.3.6. Determination of microplastics concentrations in the water

The actual concentrations of microplastics in the water were determined by spectrofluorometry, using 575 nm excitation and 607 nm emission wavelengths,

according to the properties of the microplastics indicated by the manufacturer. The procedure described in Luis *et al.* (2015) was followed with minor adaptations to the type of particles and water used. Briefly, 3 independent colloidal solutions with a microplastics concentration of 12 mg/L were prepared in water. Each solution was serially diluted 1:2 (v/v) with water to obtain additional solutions with nominal microplastics concentrations between 0.024 mg/L and 12 mg/L. The solutions with concentrations between 0.094 and 1.5 mg/L were used for the calibration curve, obtained by plotting the fluorescence readings against the corresponding nominal microplastics concentrations after discounting the blank values. A positive and significant correlation (Pearson's correlation coefficient − r) was found ($N = 15$, $r = 0.999$, $p = 0.000$). A linear regression model was fitted to the data: concentration of microplastics (mg/L) = 0.08 + 0.012 x fluorescence (F units) (Appendix B, Figure S-1). The actual microplastics concentration in water from different treatments of the bioassay were calculated from the calibration curve using the fluorescence readings made on clean and old water. The deviation (%) of microplastics actual concentrations in clean water relative to nominal ones was calculated as 100 – (actual microplastics concentration x 100 / nominal concentration) (Ferreira *et al.*, 2016). The potential decrease of microplastics concentrations in water along the interval of water renewal (24 h) was determined from the fluorescence readings of clean water (Cw) and old water (24 h) (Ow) as: decay (%) = 100 – (Ow x 100 / Cw) (Ferreira *et al.*, 2016). Because a considerable decay of microplastics concentrations in the water over 24 h was found (Appendix B, Table S-2), the mid-point of actual concentration at 0 h and 24 h per beaker was calculated and the total mean of mid-point concentrations in treatments containing each of the tested concentrations (*i.e.* low or high) were taken as the lowest and highest concentrations of the particles tested, respectively.

4.2.3.7. Determination of mercury concentrations in water and fish tissues, and bioaccumulation factors

Prior to mercury analyses, water samples containing microplastics were filtered with a nylon membrane syringe filter with a pore of 0.2 µm (Acrodisc®), for separation of aqueous solutions, and stored in Teflon tubes. From all the other water samples, sub-samples were taken with a nylon membrane syringe. In the day of mercury analyses, brain samples were desfrozen, agitated individually for 1 min in a

vortex mixer, and 0.100 mL were used for mercury analyses. In addition, muscle samples were defrozen and 0.30 mg of each sample was used for determination of mercury concentration.

Mercury concentrations were determined by atomic absorption spectrometry (AAS) using a silicon UV diode detector (AMA-254, LECO, Czech Republic), after pyrolysis of each sample following the procedure described by Costley et al. (2000). Samples were first dried at 120 °C prior to combustion at 680–700 °C in an oxygen atmosphere. The mercury vapor was collected in a gold amalgamator and after a pre-defined time (45 s) the gold amalgamator was heated at 900 °C. The released mercury was transported to a heated cuvette (120 °C) and then analyzed by AAS using a silicon UV diode detector. Procedural blanks were carried out between samples to avoid cross contamination. The accuracy of the analytical procedures was verified through the analysis of certified reference material (CRM), BCR 463 (mercury and methyl-mercury in tuna fish). In our analysis, the precision error, expressed as relative standard deviation of three replicate samples, was less than 5% (Appendix B, Table S-1).

The potential decrease of mercury concentrations in the water of the assays during the interval of water renewal (24 h), hereafter indicated as mercury decay, was determined from the concentration of mercury in clean water (Cw) and old water (Ow) as: decay (%) = 100 – (Ow x 100 / Cw) (Ferreira et al., 2016). Because a considerable decay of mercury concentrations in the water over 24 h was found (Tables 4.1 and 4.2), the mid-point of actual concentration at 0 h and 24 h per beaker was calculated and the total mean of mid-point concentrations in treatments containing the lowest and highest mercury concentrations were taken as the lowest and highest concentrations of the particles tested, respectively.

The mercury bioaccumulation factors (BAF) were determined in brain and muscle tissues according to Beldowska and Falkowska (2016) as: BAF = mercury concentration in the tissue (ppm) / mercury concentration in the water (ppm). For BAF calculations, the mean of the mercury concentration of the 9 fish per treatment, and the mean of the mercury concentrations in the water per treatment were used (the concentration of mercury in the water of each treatment was calculated as the mean of mid-point mercury concentrations in clean and old water of the 9 beakers of the treatment).

4.2.3.8. Statistical analyses of data

All data sets were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) before the Analysis of Variance (ANOVA). Data transformations were performed when these assumptions were not fulfilled (Zar, 1999). Then, for each parameter/variable, different treatments were compared using one-way analysis of variance (ANOVA), two-way ANOVA with interaction (2-ANOVA) or three-way ANOVA with interactions (3-ANOVA) as appropriate. The Tukey's multicomparison post-hoc test was used to discriminate statistically significant treatments when ANOVA indicated significant differences among treatments. The SPSS statistical analysis package (version 24.0) was used for all the statistical analyses, and the significance level was 0.05.

4.2.4. Results

4.2.4.1. Preliminary assay

In the preliminary assay (beakers without fish) the deviation of the actual microplastics concentrations relative to nominal ones ranged from 1 % to 10 % (Appendix B, Table S-2). In clean water, significant differences of fluorescence among treatments with different concentrations of microplastics, no significant differences between treatments containing mercury or not, and no significant interaction between the two factors were found (2-ANOVA, concentrations of microplastics: $F_{(2,79)} = 475.727$, $p = 0.000$; presence of mercury: $F_{(1,79)} = 0.879$, $p = 0.351$; interaction: $F_{(2,79)} = 1.296$, $p = 0.258$). The microplastics decay in the water after 24 h ranged from 25 to 38 %.

The water concentrations of mercury in clean and old water are indicated in Table 4.1. The decay of water mercury concentrations over 24 h ranged from 17 % to 73 % (Table 4.1), being higher in the presence of microplastics (30 – 73 %) than in the absence of these particles (17 – 21 %). Moreover, the highest decay was found in beakers containing the highest concentration of microplastics (69 and 73 %).

4.2.4.2. Bioassay

4.2.4.2.1. Actual concentrations of microplastics in the water

The actual concentrations of microplastics in the water at the beginning of the bioassay estimated from the linear model fitted to the microplastic calibration curve data show deviations from the nominal ones ranging from 1 to 15 % (Appendix B,

Table S-2). In clean water significant differences of fluorescence among treatments with different concentrations of microplastics, no significant differences between treatments containing mercury or not, and no significant interaction between the two factors were found (2-ANOVA, concentrations of microplastics: $F_{(2,79)} = 691.658$, $p = 0.000$; presence of mercury: $F_{(1,79)} = 0.406$, $p = 0.526$; interaction: $F_{(2,79)} = 0.600$, $p = 0.441$). The decrease of microplastics concentrations in the water after 24 h of fish exposure ranged from 27 % to 32 %.

4.2.4.2.2. Water mercury over 24 h and accumulation of mercury in *D. labrax*

In the bioassay, the decay of water mercury concentrations over 24 h ranged from 68 % to 91 % (Table 4.2). The highest decay was found in beakers containing the highest concentration of microplastics (90 – 91 %). The integrated analysis of the water mercury decay from the preliminary assay and the bioassay (3-ANOVA, fixed factors: mercury concentrations, microplastics concentrations, fish presence) indicated significant differences between the lowest and the highest concentrations of mercury ($F_{(1,36)} = 5.799$, $p = 0.021$), significant differences among microplastics concentrations ($F_{(2,36)} = 709.647$, $p = 0.000$), significant differences between beakers with and without fish ($F_{(2,36)} = 2343.375$, $p = 0.000$), significant interaction between mercury concentrations and microplastics concentrations ($F_{(2,36)} = 3.438$, $p = 0.043$), significant interaction between mercury concentrations and fish presence ($F_{(1,36)} = 17.441$, $p = 0.000$), significant interaction between microplastics concentrations and fish ($F_{(2,36)} = 75.439$, $p = 0.000$), and no significant interaction among the three fixed factors ($F_{(2,36)} = 0.735$, $p = 0.487$). The decay of mercury concentrations significantly increased with the microplastics concentration (mean ± SD): no microplastics 45 % (± 27 %); 0.26 mg/L of microplastics 54 % (± 25 %); 0.69 mg/L of microplastics 81% (± 10 %). The decay (mean ± SD) was significantly higher in the presence of fish (80 ± 9 %) than in their absence (40 ± 23 %).

Mercury was found in fish tissues at concentrations (mean ± SD) between 0.039 ± 0.019 µg/g and 0.079 ± 0.013 µg/g in the brain, and from 0.302 ± 0.065 µg/g to 0.501 ± 0.053 µg/g in the muscle (Table 4.2). For both tissues, significant ($p \leq 0.05$) differences among treatments with distinct microplastics concentrations, between treatments with different mercury concentrations, and significant ($p \leq 0.05$) interaction between mercury and microplastics concentrations were found (Appendix B, Table S-

3). Fish exposed to the highest mercury concentration had significantly higher mean concentrations of the metal in the brain and in the muscle than animals exposed to the lowest mercury concentration (Appendix B, Table S-3). Mercury BAF in the brain ranged from 4 to 7, whereas in the muscle ranged from 25 to 40 (Table 4.2).

4.2.4.2.3. Effects of microplastics alone and in mixture with mercury on *D. labrax*

The effects of microplastics, mercury and their mixtures on *D. labrax* biomarkers are shown in Fig. 4.3. Significant differences among treatments were found for brain AChE activity ($F_{(8,72)} = 16.017$, $p = 0.000$), brain LPO levels ($F_{(8,72)} = 34.301$, $p = 0.000$), muscle ChE activity ($F_{(8,72)} = 8.345$, $p = 0.000$), muscle LPO levels ($F_{(8,72)} = 17.866$, $p = 0.000$), muscle LDH activity ($F_{(8,72)} = 13.666$, $p = 0.000$) and muscle IDH activity ($F_{(8,72)} = 13.692$, $p = 0.000$). No significant differences between the control group and the treatment with the lowest concentration of microplastics alone were found (Fig. 4.3). In relation to the control group, animals exposed to the highest concentration of microplastics had significant inhibition of brain AChE activity (50%, Fig. 4.3-A) and of muscle IDH activity (50%, Fig. 4.3-F), significant increase of LPO levels in the brain (2.2 fold, Fig. 4.3-B) and in the muscle (2.1 fold, Fig. 4.3-D), significant induction of LDH activity (1.6 fold, Fig. 4.1-E), and no significant alterations of muscle ChE activity (Fig. 4.3-C). Regarding mercury, no significant differences in muscle ChE activity between the control and the treatments containing mercury alone were found (Fig. 4.3-C). Relative to the control group, fish exposed to both treatments containing mercury alone had significant inhibition of brain AChE (62 − 74%, Fig. 4.3-A), and significant increase of brain LPO levels (3.1 – 3.3 fold, Fig. 4.3-B) and of muscle LDH activity (1.4 fold, Fig. 4.3-E). In addition, the lowest concentration of mercury caused significant inhibition of IDH activity (44%, Fig. 4.3-F), whereas the highest concentration of mercury alone caused increase of muscle LPO levels (3.8 fold, Fig. 4.3-D). In relation to the control group, all the mixtures caused significant inhibition of brain AChE activity (64 – 76 %), and significant increase of LPO levels in both brain (2.9 – 3.4 fold) and muscle (2.2 – 2.9 fold) but not concentration-dependently; both mixtures containing the lowest concentration of microplastics significantly increased LDH activity (1.7 – 1.9 fold), whereas the other two mixtures did not; the mixture containing the lowest concentration of microplastics and the highest concentration of mercury significantly inhibited muscle ChE activity; and mixtures containing the highest microplastics

concentration significantly inhibited muscle IDH activity, whereas the two mixtures with the lowest concentration of microplastics did not. Moreover, the results of the integrated analysis of data indicated a significant interaction between microplastics and mercury for brain AChE activity, brain LPO levels, muscle ChE activity, muscle LDH activity, muscle IDH activity, and muscle LPO levels (Appendix B, Table S-4).

4.2.5. Discussion

4.2.5.1. Behaviour of microplastics and mercury in the water

The results of 2-ANOVA with interaction in the preliminary assay and in the bioassay indicate that the spectrofluorometric method used was able to discriminate the lowest concentration of microplastics from the highest one, and that the presence of mercury does not interfere significantly with fluorescence readings. Thus, the method was adequate to determine the concentrations of microplastics in the water in the presence of mercury, as found previously in other test media and in the presence of other chemical substances (Luís *et al.*, 2015; Ferreira *et al.*, 2016; Fonte *et al.*, 2016). Since in clean media the deviation of actual concentrations of microplastics from nominal ones ranged from 1 % to 15 % (Appendix B, Table S-2), the actual concentrations of microplastics did not differ significantly from the nominal ones (OECD, 2014) at the beginning of the bioassay. However, a considerable decay of microplastics concentrations over 24 h occurred, which may have been due adsorption to glass beaker walls, aggregation of the particles followed by sedimentation among others, as suggested in previous studies with comparable microplastics in artificial saltwater (Luis *et al.*, 2015; Ferreira *et al.*, 2016). Moreover, in beakers with fish the uptake of microplastics by animals may also have also occurred.

Table 4.1. Actual concentrations of mercury (Hg) in clean (0 h) and old (24 h) water of different treatments decay of water mercury concentrations over 24 h and mid-point concentrations during the preliminary assay without fish. The values are the mean and standard deviation of 9 replicates. Actual Hg Conc.: concentration of mercury determined in the water. Mid-point Hg conc.: mid-point concentration determined as: (actual concentration of Hg at 0 h + actual concentration of Hg at 24 h) / 2. MPs − microplastics. Hg − mercury. Total mean low − mean of Hg concentrations in beakers of all treatments containing the lowest concentration of Hg tested. Total mean high − mean of Hg concentrations in beakers of all treatments containing the highest concentration of Hg tested.

Treatments	0h Actual Hg conc. (mg/L)	24 Actual Hg conc. (mg/L)	Decay (%)	Mean mid-point Hg conc. in the water
Hg low	0.007 (± 0.0001)	0.006 (± 0.0003)	17 (± 4)	0.006 (± 0.0001)
Hg high	0.013 (± 0.0002)	0.010 (± 0.0002)	21 (± 2)	0.011 (± 0.0002)
MP low + Hg low	0.007 (± 0.0002)	0.005 (± 0.0001)	33 (± 4)	0.006 (± 0.0002)
MP low + Hg high	0.013 (± 0.0001)	0.009 (± 0.0002)	30 (± 2)	0.011 (± 0.0001)
MP high + Hg low	0.007 (± 0.0004)	0.002 (± 0.0001)	73 (± 2)	0.004 (± 0.0004)
MP high + Hg high	0.013 (± 0.0002)	0.004 (± 0.0001)	69 (± 1)	0.008 (± 0.0002)
Total mean low				0.005 (± 0.0013)
Total mean high				0.010 (± 0.0018)

Table 4.2. Mean and standard deviation (within brackets) of mercury (Hg) concentrations in the water, and in *Dicentrarchus labrax* brain and muscle (wet weight) after 9 exposure to treatments containing the metal, mercury decay over 24 h and bioaccumulation factors (BAF). Actual Hg Conc.: concentration of mercury determined in the w 0 h or 24 h. Mid-point Hg conc.: mid-point concentration, corresponding to the estimated exposure concentration during the bioassay, determined as: (actual concentration at 0 h + actual concentration of Hg at 24 h) / 2. MPs − microplastics. Hg − mercury. Total mean low − mean of Hg concentrations in beakers of all treatments containing the concentration of Hg tested. Total mean high − mean of Hg concentrations in beakers of all treatments containing the highest concentration of Hg tested.

	Actual mercury concentrations and decay in the water				Concentrations of mercury in *Dicentrarchus labrax* (brain and muscle)			
Treatments	0h Actual Hg conc. (mg/L)	24 Actual Hg conc. (mg/L)	Decay (%)	Mean mid-point Hg conc. in the water	Brain Hg conc. (µg/g)	BAF brain	Muscle Hg conc. (µg/g)	BAF muscle
	WATER				TISSUES			
Hg low	0.017 (± 0.0007)	0.006 (± 0.0003)	68 (± 3)	0.011 (± 0.0003)	0.067 (± 0.010)	7	0.404 (± 0.049)	40
Hg high	0.027 (±0.0006)	0.007 (±0.0003)	74 (± 1)	0.017 (± 0.0002)	0.073 (± 0.014)	5	0.441 (± 0.093)	28
MP low + Hg low	0.017 (±0.0003)	0.004 (±0.0004)	76 (± 3)	0.010 (± 0.0001)	0.039 (± 0.019)	4	0.302 (± 0.065)	30
MP low + Hg high	0.027 (±0.0003)	0.005 (±0.0008)	82 (± 3)	0.016 (± 0.0005)	0.073 (± 0.007)	5	0.401 (± 0.049)	25
MP high + Hg low	0.017 (±0.0004)	0.002 (±0.0008)	91 (± 5)	0.009 (± 0.0004)	0.055 (± 0.012)	6	0.310 (± 0.072)	31
MP high + Hg high	0.027 (±0.0005)	0.003 (±0.007)	90 (± 3)	0.015 (± 0.0003)	0.079 (± 0.013)	5	0.501 (± 0.053)	31
Total mean low				0.010 (± 0.0008)				
Total mean high				0.016 (± 0.0009)				

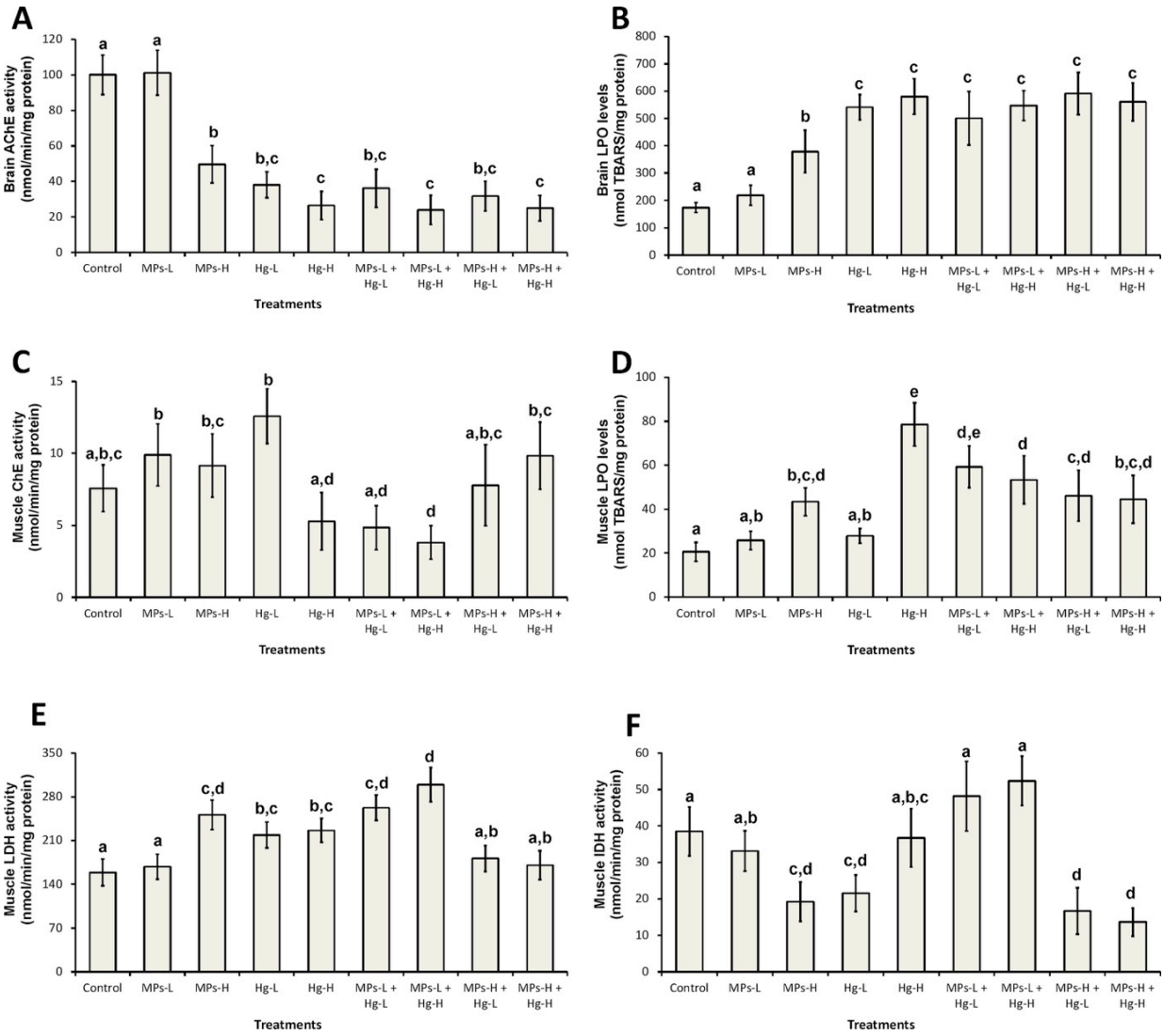

Figure 4.3. Effects of microplastics and mercury on biomarkers of neurotoxicity and oxidative damage, and energy-related enzymes of *Dicentrarchus labrax*. A) brain AChE activity; (B) brain LPO levels; (C) muscle ChE activity; (D) muscle LPO levels; (E) muscle LDH activity; (F) muscle IDH activity. Values are the mean of 9 fish per treatment with the corresponding standard error bars. Different letters indicate statistically significant differences between treatments ($p < 0.05$, Tukey test). MPs-L − low microplastics concentration; MPs-H − high microplastics concentration; Hg-L − low mercury concentration; Hg-H − high mercury concentration.

The increase of mercury decay at increasing concentrations of microplastics and the significant interaction between mercury and microplastics suggest that mercury sorbs to microplastics, as found for other metals (Ashton *et al.*, 2010; Holmes *et al.*, 2012, Holmes *et al.*, 2014; Rochman *et al.*, 2014a,b). The significant interaction between microplastics and fish presence suggests that both factors acting together influence the mercury decay. One hypothesis for this to occur is that fish take up microplastics containing mercury from the water.

4.2.5.2. Bioaccumulation of mercury in D. labrax

The results of Table 4.2 indicate that fish take up mercury from water and that it reaches the brain and muscles. Mercury accumulated more in the muscle than in the brain (Table 4.2). Moreover, the analyses of mercury concentrations in the brain and in the muscle in relation to microplastics concentration in the water (Appendix B, Table S-3) indicate that microplastics influence the mercury concentration in brain and muscle of fish. These findings increase the concern regarding the health of fish inhabiting ecosystems contaminated with relatively high concentrations of both microplastics and mercury, and the risks to their predators including humans consuming contaminated species, as found in previous studies with microplastics and other substances (Rochman *et al.*, 2014a,b; Fonte *et al.*, 2016; Paul-Pont *et al.*, 2016; Wardrop *et al.*, 2016).

4.2.5.3. Neurotoxicity responses, oxidative damage and changes in the activity of energy-related enzymes

The significant inhibition of brain AChE caused by the highest concentration of microplastics alone (Fig. 4.3-A) indicates neurotoxicity, in good agreement with previous studies in other species (Oliveira *et al.*, 2013; Avio *et al.*, 2015a; Luís *et al.*, 2015; Ribeiro *et al.*, 2017). The lack of inhibition of the enzyme at 0.26 mg/L and the high inhibition (50 %) at 0.69 mg/L of microplastics suggests a relationship that is not concentration-dependent in good agreement with previous studies on other fish (Oliveira *et al.*, 2013). This may have occurred because the inhibition is caused by indirect effects (Oliveira *et al.*, 2013) or because the anti-cholinesterase effects start to be induced at concentrations higher than 0.26 mg/L. The increase of brain LPO levels induced by microplastics (Fig. 4.3-B) indicates that these particles cause oxidative stress and lipid damage. Thus, microplastics cause neurotoxicity in *D. labrax* juveniles through AChE inhibition and lipid peroxidation damage. Microplastics-induced lipid peroxidation was also found in the muscle (Fig. 4.3-D). Moreover, microplastics also caused induction of LDH suggesting increased use of the anaerobic pathway of energy production likely to get additional energy to face chemical stress. The increase of this pathway under chemical stress has been observed in several organisms exposed to other environmental contaminants (Firat *et al.*, 2011; Oliveira *et al.*, 2012). The inhibition of IDH activity caused by

microplastics may have contributed to oxidative stress and damage of muscle because this enzyme is important to the maintenance of cellular redox balance.

The inhibition of brain AChE caused by mercury alone (62 – 74 %, Fig. 4.3-A) indicates neurotoxicity. Mercury-induced ChE inhibition was also found in other fish (Vieira *et al.*, 2009; Richetti *et al.*, 2011; Jesus *et al.*, 2013). Mercury alone also caused lipid peroxidation in brain and muscle, in good agreement with the well-known oxidative stress and damage caused by this metal (Elbaz *et al.*, 2010; Seppänen *et al.*, 2004). The increase of LDH activity suggests induction of the anaerobic pathway of energy production, as previously found in other organisms exposed to mercury (Radhakrishnaiah *et al.*, 1993; Vieira *et al.*, 2009). However, to understand potential changes in pathways of energy production other parameters, such as citrate synthase (CS) and cytochrome c oxidase (COX), need to be studied.

All mixtures caused neurotoxicity through AChE inhibition and lipid peroxidation, and also lipid peroxidation in the muscle. The results of AChE, IDH and LDH activity, and LPO levels in brain and muscle (Fig. 4.3), and the significant interaction between microplastics and mercury in all the biomarkers (Appendix B, Table S-4) suggest toxicological interactions between microplastics and mercury in *D. labrax* juveniles. Mixtures containing the highest concentration of microplastics caused some effects different from those caused by mixtures containing the lowest concentration of these particles. In fact, mixtures with the highest concentration of microplastics inhibited IDH activity and had no significant effects on LDH activity, whereas the opposite was found for mixtures with the lowest microplastics concentration. Effects different at low and highest concentrations of mixture components were reported in other organisms exposed to distinct types of mixtures (Juhel *et al.*, 2017).

4.2.6. Conclusions

After 96 h of exposure to mercury through the water, *D. labrax* juveniles accumulated the metal in the brain and in the muscle, with BAF of 4 − 7 and 25 − 40, respectively. Microplastics likely sorbed mercury from the water and influenced the bioaccumulation of mercury in fish tissues. Microplastics alone and mercury alone caused neurotoxicity, lipid peroxidation in brain and muscle, and changed the activity of energy-related enzymes (LDH and IDH). Overall, microplastics-mercury mixtures

caused effects on the same biomarkers but evidence of toxicological interactions was found. Moreover, mixtures containing low and high concentrations of microplastics caused different effects on IDH and LDH activity. Therefore, the findings of this study highlight the importance of further investigating the combined effects of microplastics and mercury in *D. labrax* and other aquatic species, especially those used for human consumption, since these substances are ubiquitous pollutants and their combined effects may adversely affect wild populations, ecosystem functions, and human health.

4.2.7. Acknowledgements

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Chapter IV

Section 3. Microplastics increase mercury bioconcentration in gills and bioaccumulation in the liver, and cause oxidative stress and damage in Dicentrarchus labrax juveniles

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(Article published and included in the Thesis with permission of Springer Nature included in the Appendix A)

4.3.1. Abstract

The presence of microplastics and several other pollutants in the marine environment is of growing concern. However, the knowledge on the toxicity of mixtures containing microplastics and other contaminants to marine species is still scarce. The main goals of this study were to investigate the oxidative stress and lipid oxidative damage potentially induced by 96 h of exposure to mercury (0.010 and 0.016 mg/L), microplastics (0.26 and 0.69 mg/L), and mixtures of the two substances (same concentrations, full factorial) in the gills and liver of *D. labrax* juveniles, and the possible influence of microplastics on mercury bioconcentration (gills) and bioaccumulation (liver). The results indicate that the presence of microplastics in the water increased the concentration of mercury in gills and liver of *D. labrax* juveniles. Microplastics and mercury, alone and in mixtures, caused oxidative stress in both organs. Based on the total induction of antioxidant enzymatic activity, the type of toxicological interaction in fish exposed to the mixture containing the lowest concentration of the two substances was addition in gills, and addition or synergism in the liver. These results stress the need to further address the role of microplastics in the bioconcentration, bioaccumulation, and toxicity of other environmental contaminants in different species.

4.3.2. Introduction

Over the last few years, microplastics have been found in the environment worldwide, including enclosed water bodies and remote areas (Suaria *et al.*, 2016; Waller *et al.*, 2017) and are now considered global pollutants of priority study (Barboza and Gimemez, 2015; Auta *et al.*, 2017; Oliveira *et al.*, 2018). Such particles result either from the fragmentation of larger plastic debris in the environment or from specifically produced micro- or nanosized plastics used for several purposes (*e.g.* pre-production pellets, cleaning agents, textiles, cosmetics and personal care products) (Duis and Coors, 2016).

The levels of microplastics in aquatic environments are diverse, such as 2.46 particles/m^3 in the Northeast Atlantic Ocean (Lusher *et al.*, 2014), 0.0032 to 1.18 particles/m^3 in the Ross Sea (Antarctica) (Cincinelli *et al.*, 2017), 0.028 particles/m^3 in the Tamar Estuary, UK (Sadri *et al.*, 2014), 300 ng/mL in the North Pacific subtropical gyre (Goldstein *et al.*, 2012), and high abundances and

concentrations have been found in polluted areas such as 228 particles/m^2 in the Coastline of Qatar Gulf (Abayomi *et al.*, 2017), 324 particles/m^3 in the Israeli Mediterranean coastal waters (van der Hal *et al.*, 2017), and average concentrations of 1.56 ± 1.64 and 5.51 ± 9.09 mg/L in lakes and wetlands (Lasee *et al.*, 2017). Data on the microplastics concentration found in the environment are often difficult to compare due to the lack of standardized sampling methodologies, normalization units and expression of data (Avio *et al.*, 2017).

Due to their small size, microplastics are in the size range of food particles normally ingested by several aquatic animals (Au *et al.*, 2017). The reasons for the ingestion of these small particles include their accidental consumption by aquatic filter feeders (Germanov *et al.*, 2018), and active selection (*e.g.* confusion of microplastics with a prey), since many species are attracted to these microparticles based on their attributes such as shape and colour (de Sá *et al.*, 2015; Ory *et al.*, 2018a) and through sensory signals (*i.e.* visual or olfactory cues) (Savoca *et al.*, 2016). Microplastics are also ingested indirectly as a result of trophic transfer, when contaminated prey are consumed by their predators (Farrel and Nelson, 2013; Santana *et al.*, 2017). After ingestion or after crossing the gills, microplastics absorption and distribution through the circulatory system can occur, and if so the particles may be incorporated into different tissues and cells (Barboza *et al.*, 2018a). This can result in several types of effects, such as: behavior alterations, predatory performance reduction, neurotoxicity, inflammation, hepatic stress, metabolic disorders, decreased growth, among others (Rochman *et al.*, 2013b; Ferreira *et al.*, 2016; Pedà *et al.*, 2016; Imhof *et al.*, 2017a; Barboza *et al.*, 2018c, d). Moreover, the uptake of microplastics contaminated with other environmental contaminants has been suggested as a possible additional exposure route to several chemicals harmful to aquatic organisms including styrene, metals, phthalates, bisphenol A, polychlorinated biphenyls and polycyclic aromatic hydrocarbons (Koelmans *et al.*, 2014; Hahladakis *et al.*, 2018). For this reason, the potential for microplastics and associated contaminants to undergo bioaccumulation and trophic transfer is high (Au *et al.*, 2017).

The accumulation of environmental contaminants by microplastics is likely important in ecosystems contaminated with complex mixtures of chemicals such as estuaries impacted by strong industrial, urban and/or agricultural surroundings. This may cause adverse effects on the biota of these systems, including important

marine species such as the European seabass *Dicenthrarchus labrax* (Linnaeus, 1758) that spends part of its life cycle within estuaries before reaching maturity (Almeida *et al.*, 2010). The ingestion of microplastics by *D. labrax* from an estuarine ecosystem was recently reported (Bessa *et al.*, 2018). In this species, exposure to microplastics can cause several adverse effects, including behavioral changes, intestinal alterations, and neurotoxicity (Mazurais *et al.*, 2015; Pedà *et al.*, 2016; Barboza *et al.*, 2018c,d). Moreover, the exposure of *D. labrax* juveniles to mixtures of microplastics and mercury (another common contaminant of high concern found in different concentrations in the environment such as 0.5 to 200 ng/L in the North Sea (Schmidt, 1991), 39 to 430 ng/L in the Wuli Estuary, China (Wang *et al.*, 2009), and 990 to 27,060 ng/L in the Mediterranean Sea (Nasfi, 1995) was found to reduce the swimming performance, cause neurotoxicity, and induce changes in the activity of energy-related enzymes (Barboza *et al.*, 2018c,d).

To complement these studies, the oxidative stress and lipid oxidative damage potentially induced by 96 h of exposure to mercury (0.010 and 0.016 mg/L), microplastics (0.26 and 0.69 mg/L), and mixtures of the two substances (same concentrations, full factorial) in the gills and liver of *D. labrax* juveniles, and the possible influence of microplastics on mercury bioconcentration (gills) and bioaccumulation (liver) were investigated. In this study, “bioconcentration” was used to refer the direct uptake of microplastics from the water by the gills, whereas “bioaccumulation” was used to indicate the accumulation in the liver after absorption (through all exposure routes), distribution, storage and elimination.

4.3.3. *Material and methods*

4.3.3.1. Chemicals

Fluorescent red polymer microspheres (1–5 μm diameter) were used as microplastics particles and were purchased from Cospheric – Innovations in Microtechnology (USA). According to manufacturer indications, 1 mg of the product contains about 1.836E + 8 spheres (estimate made for an average of 2 μm diameter). Mercury chloride (≥ 99.5 % pure) was purchased from Sigma-Aldrich (USA). The Bradford reagent used for protein determinations was from BIORAD (Germany). All the other chemicals for biomarkers determinations were of the highest purity available and purchased from Sigma-Aldrich (USA) or Merck (Germany).

4.3.3.2. Ethical issues

Experiments were authorized by the Portuguese National Authority for Animal Health ("Direção Geral de Agricultura e Veterinária" - DGAV) and conducted according to the ethical principles and other requirements of Portuguese and EU regulations for the protection of animals used for scientific purposes. L. Guilhermino and L. R. Vieira are accredited by the DGAV as investigator/coordinator (equivalent to FELASA category C) to carry animal experimentation. The experiments were carried out in the CIIMAR bioterium, which is accredited by DGAV for studies with aquatic animals.

4.3.3.3. Bioassay

The test species, *Dicentrarchus labrax,* was selected for this study because of its wide use for human consumption, high commercial value, important ecological functions, and wide use in ecotoxicological studies (Gravato and Guilhermino, 2009; Vinagre *et al.*, 2012). The juveniles used were measured at (mean ± standard deviation - SD) 7.75 ± 0.293 cm (total length) and 8.82 ± 0.295 g (body wet weight – w.w.). The experimental design, fish exposure and tissue isolation are described in detail in Barboza *et al.* (2018c), included in the present Thesis (Chapter IV, Section 4.2.3. Briefly, fish purchased from an aquaculture were acclimatized to laboratory conditions in a room with controlled temperature and photoperiod (19 ± 1 ºC, photoperiod: 14 h light: 10 h dark), in UV-filtered seawater (salinity: 34 ± 1 g/L). After this period, 81 *D. labrax* juveniles were randomly distributed per 9 treatments (9 fish per treatment). Our schematic procedure of experiment is shown in Fig. 4.4.

The exposure period was 96h and no food was provided to fish during the experiment. Test beakers were glass, filled with 4L of filtered water and continuous additional air supply. Water was renewed (*i.e.* completely replaced) every 24h. Water samples for determination of mercury and microplastics concentrations were collected at the beginning and the end of the bioassay and at each water renewal, including the collection of both clean and old water. Water samples were stored at – 20ºC until further analyses. After 96 h of exposure, samples of gills and liver were collected from each fish as indicated in Barboza *et al.* (2018c) and stored at – 80ºC. Both concentration of microplastics and both concentrations of mercury tested are ecologically relevant (Nasfi, 1995; Goldstein *et al.*, 2012; Lasee *et al.*, 2017). The

higher concentration of microplastics tested (0.69 mg/L) is lower than those reported for some polluted waters (Lasee *et al.*, 2017).

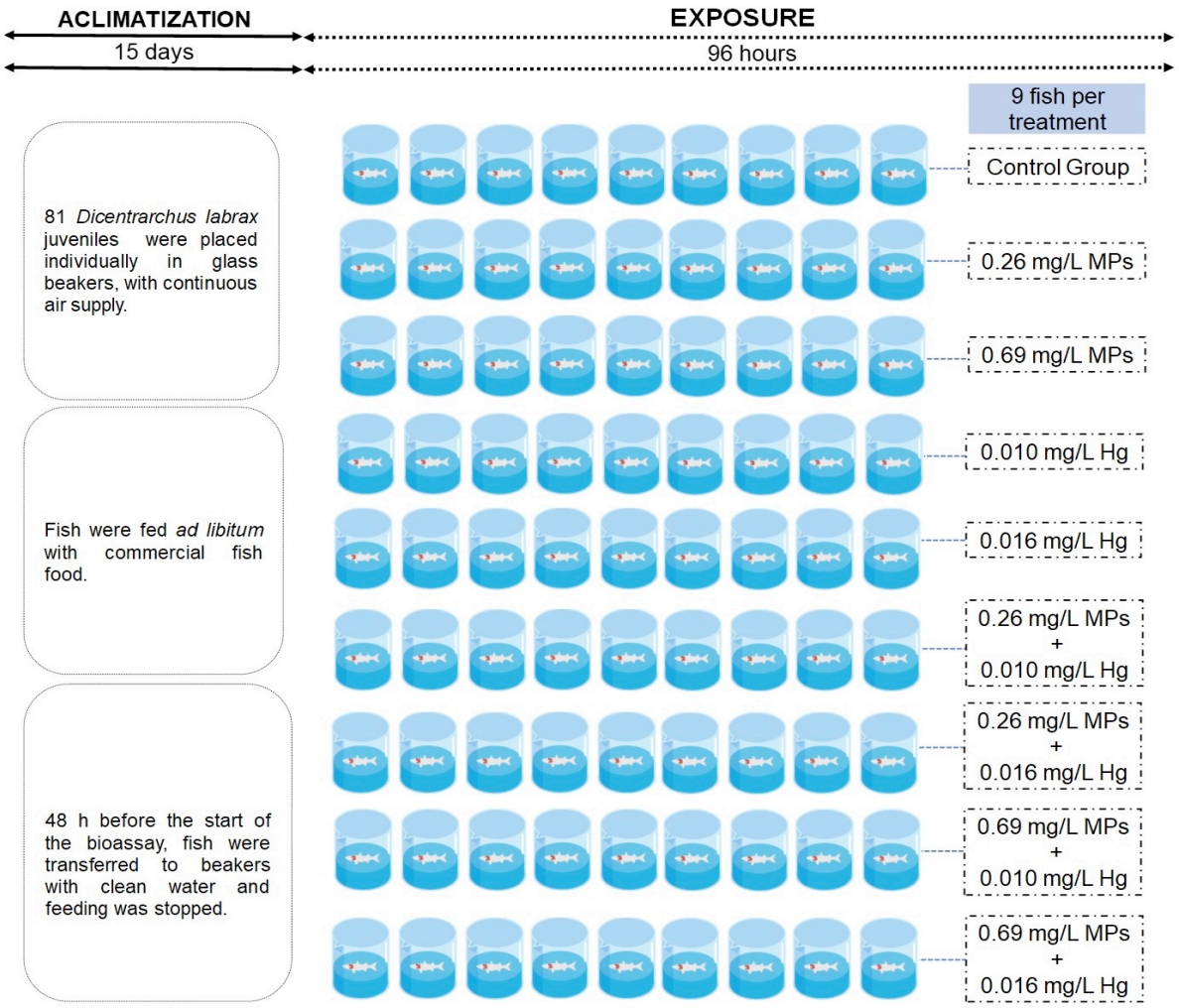

Figure 4.4. Experimental design scheme.

4.3.3.4. Biomarkers determination

Several biomarkers involved in important physiological functions related to fish health status maintenance were measured, namely gill and liver superoxide dismutase (SOD) activity, gill and liver catalase (CAT) activity, gill and liver glutathione peroxidase (GPx) activity, gill and liver glutathione reductase (GR) activity, gill and liver glutathione-S-transferase (GST) activity and gill and liver lipid peroxidation (LPO) levels. Antioxidant enzymes including SOD, CAT, GPx, GR and GST were selected because they usually act in a coordinated manner in order to

ensure the optimal protection against oxidative stress. LPO levels were selected as marker of oxidative damage to lipids. On the day of the analyses, liver and gill samples (1:10 g wt/v) were homogenized in phosphate buffer (pH 7.4, 0.1 M). Homogenates were divided into aliquots to analyse LPO and total mercury concentration. One aliquot was used for enzymatic activity assays following post-mitochondrial fraction isolation (centrifugation for 20 min at 10,000g at 4 ^{o}C). All biomarkers and protein determinations were made at 25 ºC. The protein content of the samples was determined by the Bradford method (Bradford, 1976) adapted to microplate (Guilhermino *et al.*, 1996). Then, it was standardized to 0.3 mg/mL (GST samples) or to 1 mg/mL (LPO, SOD, CAT, GPx and GR samples). LPO levels were determined by quantification of thiobarbituric acid reactive substances (TBARS) at 535 nm (Ohkawa, 1979). GST activity was determined at 340 nm (Habig *et al.*, 1974) adapted to microplate (Frasco *et al.*, 2002). SOD, GPx, GR activities were determined by the techniques of Flohé and Ötting (1984), Flohé and Gunzler (1984) and Carlberg and Mannervik (1985), respectively, with adaptations (Lima *et al.*, 2007). CAT activity was determined according to Clairborne (1985) at 240 nm. All analyses were performed in a Spectramax® spectrophotometer (Molecular Devices, USA). LPO levels were expressed in nanomoles of TBARS per mg of protein (nmol TBARS/mg protein). SOD activity was expressed in one unit per mg of protein (U/mg protein). CAT activity was expressed in micromoles per mg of protein (µmol/min/mg protein). GPx, GR and GST activities were expressed in nanomoles per mg of protein (nmol/min/mg protein).

4.3.3.5. Mercury concentrations and bioaccumulation factors

The preparation of water and tissue samples for mercury analyses is described in detail in Barboza *et al.* (2018c). Briefly, water samples containing microplastics were filtered with a nylon membrane syringe filter with a pore size of 0.2 µm (Acrodisc®) and stored in Teflon tubes for further analysis. Liver and gills samples were thawed individually, agitated for 1 min in a vortex mixer, after which 0.100 mL were collected for analysis. Mercury concentrations in water and tissues samples were determined by atomic absorption spectrometry (AAS) using a silicon UV diode detector (AMA-254, LECO, Czech Republic) as described in detail in Barboza *et al.* (2018c). The accuracy of the analytical procedure was verified through the analysis of a certified reference material (CRM), BCR 463 (mercury and

methyl-mercury in tuna fish). The mercury bioconcentration factors (BCF) and mercury bioaccumulation factors (BAF) were determined according to Beldowska and Falkowska (2016) as: BCF = mercury concentration in the gills (ppm) / mercury concentration in the water (ppm); BAF = mercury concentration in the liver (ppm) / mercury concentration in the water (ppm). The mercury concentrations in the water are given in detail in Barboza et al. (2018c) and according to these results the mean water ± SD exposure concentrations during the interval of water renewal were 0.010 ± 0.0008 mg/L and 0.016 ± 0.0009 mg/L in treatments with the lowest and the highest mercury concentrations, respectively. Mean values were used to calculate the BCF and BAF factors in fish exposed to treatments containing the lowest or the highest mercury concentrations, respectively.

4.3.3.6. Water microplastics concentrations

Water microplastics concentrations were determined in clean and old water by spectrofluorimetry following Luís *et al.* (2015), with adaptations to the type of water and microplastics used. Between water renewals (every 24h), the mean (± SD) microplastic exposure concentration was 0.26 ± 0.028 mg/L and 0.69 ± 0.036 mg/L in treatments containing the lowest and the highest concentrations of the particles, respectively (Barboza *et al.*, 2018c).

4.3.3.7. Statistical analyses of data

Statistical analyses were performed using the SPSS statistical analysis package (version 24.0). For each data set, normality of distribution and equality of variance were checked by Shapiro-Wilk test and Levene's test, respectively. When these assumptions were not fulfilled, Analysis of Variance (ANOVA) was preceded by data transformation (Zar, 1999). Each data set was analysed through one-way ANOVA (1-ANOVA) or two-way ANOVA with interaction (2-ANOVA) followed by the Tukey's multiple comparisons test when statistical significant differences were found. When ANOVA assumptions could not be achieved even after data transformation, the non-parametric Kruskal-Wallis test was used, followed by a nonparametric multiple comparisons test (using Dunn's procedure with a Bonferroni adjustment when significant differences were found). Differences between treatments were considered significant a p-level ≤ 0.05.

4.3.4. Results and Discussion

4.3.4.1. Mercury concentrations, bioconcentration and bioaccumulation factors, and influence of microplastics

The concentrations of mercury (mean ± SD) in gills ranged from 1.519 ± 0.369 µg/g to 4.825 ± 0.881 µg/g, whereas in the liver they ranged from 2.571 ± 0.903 µg/g to 8.169 ± 1.398 µg/g (Table 4.3). The bioconcentration factors (BCF) in gills ranged from 152 ± 37 to 302 ± 55 and the bioaccumulation factors (BAF) in the liver ranged from 257 ± 86 to 511 ± 80 (Table 4.3). Thus, fish uptake the metal from the water, bioconcentrate it in gills and accumulate it in the liver. These findings are in good agreement with previous studies reporting accumulation of mercury by *D. labrax* (Mieiro *et al*., 2014; Barboza *et al*., 2018c).

Significant differences in the concentrations of mercury among distinct treatments were found for both gills ($\chi^2_{(5)}$ = 36.384, p = 0.000) and liver ($\chi^2_{(5)}$ = 33.084, p = 0.000). Significant differences in gill BCF ($\chi^2_{(5)}$ = 28.066, p = 0.000) and liver BAF ($\chi^2_{(5)}$ = 27.287, p = 0.000) among fish exposed to distinct treatments were also found. In fish exposed to mercury alone, the concentration of metal in both gills and liver was significantly higher in fish exposed to water containing 0.016 mg/L of mercury than in fish exposed to treatments containing 0.010 mg/L of mercury (Table 4.3). Thus, the accumulation of mercury depends on the water exposure concentration. The comparison of the BCF and BAF factors obtained in the present study in fish exposed to mercury alone (Table 4.3) with those determined previously in brain (BAF = 5 and 7) and muscle (BAF = 28 and 40) tissues (Barboza *et al*., 2018c) indicates the following decreasing order of mercury accumulation or bioconcentration in tissues of *D. labrax* juveniles: liver > gills > muscle > brain.

Fish exposed to the metal alone had significantly lower mercury concentrations in gills than those exposed to the same concentration of mercury in combination with microplastics (Table 4.3). In the liver, a comparable situation occurred, but only in relation to the highest concentration of mercury tested (Table 4.3). Thus, the presence of microplastics had influence on the mercury concentrations in gills and liver. Such influence of microplastics may have been due to several processes. For exemple (Fig. 4.5), microplastics may adsorb mercury from the water and act as an additional exposure route to the metal. Because microplastics are frequently stocked in gills of aquatic animals (Watts *et al*., 2014; Oliveira *et al*., 2018) if the microplastics uptaken by fish though the gills had

mercury adsorbed this could have result in increased concentrations of the metal in the gills exposed to the mixtures. Moreover, in the gills, release of the metal from the particles and absorption of at least part of it may have occurred leading to increased accumulation of mercury also in other organs such as the liver. A comparable process may have occurred in the digestive system (Fig. 4.5) also contributing to increase the mercury concentrations in the liver. Previous studies indicating that mercury adsorbs to microplastic virgin pellets provide support to this hypothesis (Turner and Holmes, 2015). In addition to the processes discussed above, the presence of microplastics in the gills may have interfered with the mechanisms regulating the uptake and elimination of the metal locally. Additionally, the presence of the particles in the gills may have decreased the oxygen uptake leading to hypoxia, subsequent reduction of the aerobic cellular energy production, as hypothesized for *Daphnia magna* exposed to the same type of microplastics (Pacheco *et al*., 2018). If so, the elimination of mercury may have been reduced in fish exposed to mixtures due to shortage of energy available.

4.3.4.2. Oxidative stress and damage induced by microplastics, mercury and their mixtures

Significant differences ($p \leq 0.05$) in all the oxidative stress and damage biomarkers among treatments were found in both gills and liver (complete results in Appendix C, Table S-1). The anti-oxidant enzymes with significantly increased activity are shown in Fig. 4.6.

In relation to the control group, fish exposed to 0.26 mg/L of microplastics alone had significantly increased superoxide dismutase (SOD) activity (1.6 fold) in gills (Fig. 4.6 A), and significantly increased SOD and catalase (CAT) activities (3.4-fold of total anti-oxidant enzymatic induction, hereafter indicated as total induction) in the liver (Fig. 4.6 B). The induction of these anti-oxidant enzymes was probably enough to cope with the oxidative stress induced by the lowest concentration of microplastics tested because no significant increase of lipid peroxidation (LPO) levels was observed (Fig. 4.7). Fish exposed to the highest concentration of microplastics alone (0.69 mg/L), had significant induction of CAT, glutathione-S-transferase (GST) and SOD, resulting in a total induction of 4.8 fold. Despite the induction of two additional enzymes, the LPO levels were significantly increased (Fig. 4.7-A) indicating that lipid oxidative damage in gills occurred. In the liver, fish

exposed to 0.69 mg/L of microplastics alone, had significantly induced activities of SOD, CAT, GST, glutathione peroxidase (GPx) and glutathione reductase (GR), resulting in a total induction of 8.3 fold which was enough to avoid lipid oxidative damage in this organ (Fig. 4.7-B). Overall, these results indicate that microplastics induced oxidative stress in both gills and liver at concentrations ≥ 0.26 mg/L and lipid oxidative damage in gills at 0.69 mg/L. This may have been caused by indirect effects resulting from physical damage caused by the particles themselves and/or by additives that the microplastics likely contain. The microplastics-induced oxidative stress and damage found here are in agreement with the microplastic-induced oxidative stress and damage in brain and muscle of *D. labrax* juveniles previously described (Barboza *et al*., 2018c). Oxidative stress induced by different types of microplastics was also reported in other species, such as the fish *Danio rerio* (Lu et al., 2016), the bivalves *Scrobicularia plana* (Ribeiro *et al*., 2017), and *Corbicula fluminea* (Oliveira *et al*., 2018), and the rotifer *Brachionus koreanus* (Jeong *et al*., 2016).

In relation to the control group, fish exposed to the lowest concentration of mercury alone (0.010 mg/L) showed significant induction of SOD, CAT and GST activities in both gills and liver, in a total induction of 5.6 and 5.2 fold, respectively (Fig. 4.6-A, 4.6-B), and no significant changes in LPO levels (Fig. 4.7-A, 4.7-B). Exposure to 0.016 mg/L of mercury alone resulted in a higher induction of SOD, CAT and GST activities in gills (total induction of 7.4 fold). In the liver, mercury exposure caused the additional induction of GPx and GR activities, with a total induction of 11.3 fold (Fig. 4.6-B). In both organs, no significant increase of LPO levels occurred (Fig. 4.7). Therefore, exposure to mercury (0.010 mg/L and 0.016 mg/L) caused oxidative stress in *D. labrax* juveniles but did not result in lipid oxidative damage. Oxidative stress is a well-known effect of mercury previously reported in *D. labrax* (Mieiro et al., 2014; Barboza *et al*., 2018c) and other fish species (Vieira *et al*., 2009; Monteiro *et al*., 2010; Cappello *et al*., 2016).

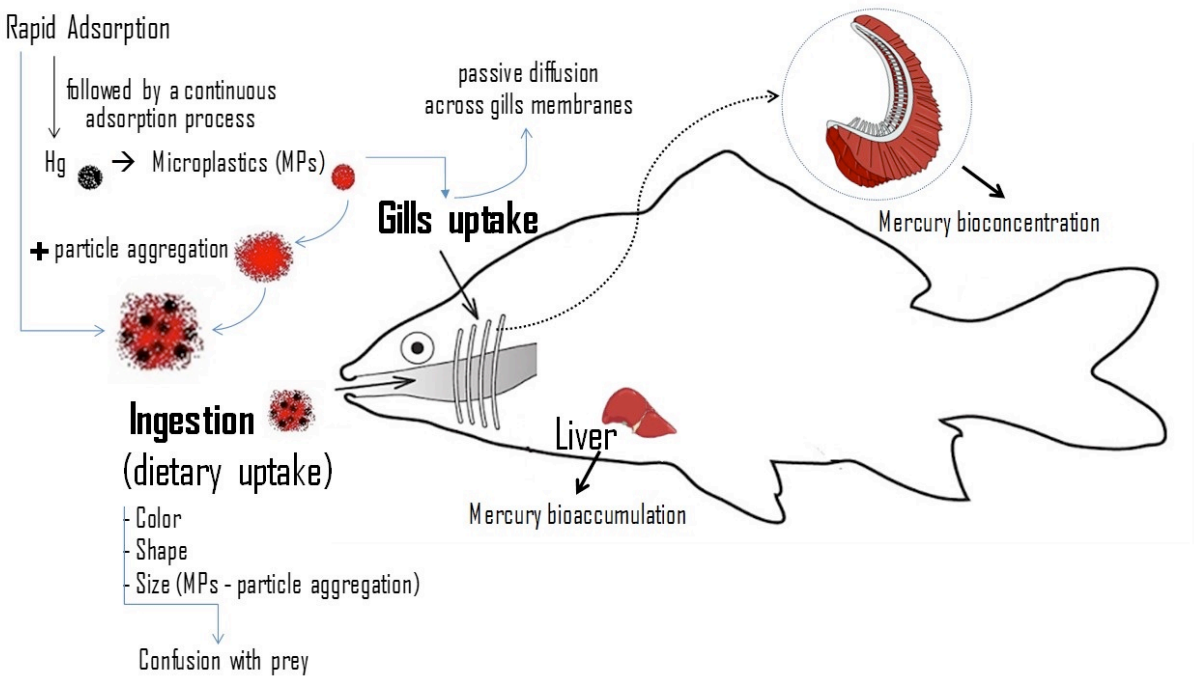

Figure 4.5. Potential influence of microplastics on mercury bioconcentration and bioaccumulation by fish.

All the mixtures tested induced the activity of three anti-oxidant enzymes in gills (SOD, CAT and GST) and five in the liver (SOD, CAT, GPx, GR and GST) (Fig. 4.6). The mixture containing the lowest concentration of microplastics and the highest concentration of mercury also caused a significant increase of LPO levels in gills (Fig. 4.7-A), suggesting toxicological interactions between the two substances in *D. labrax* juveniles. Thus, with the exception of this mixture, the induction of anti-oxidant enzymes was likely enough to prevent the occurrence of lipid oxidative damage. The results of 2-ANOVA (complete results in Appendix C, Table S-2) carried out with some gills (CAT, GPx, GST and LPO) and liver (SOD, CAT, GST and LPO) biomarkers, also indicated significant interaction ($p \leq 0.05$) between microplastics and mercury suggesting toxicological interactions between microplastics and mercury in *D. labrax* juveniles. Moreover, in gills, the total induction of anti-oxidant enzymatic activity caused by the mixture containing the lowest concentrations of microplastics and mercury tested (7.1 fold) was comparable to the sum of the total induction caused by the same concentrations of the substances individually (1.6 + 5.6 = 7.2 fold). In the liver, the same mixture induced a higher total induction (10.8 fold) than the sum of the total induction caused by microplastics and mercury individually (3.4 + 5.2 = 8.6 fold). These results suggest that the type of

toxicological interaction may be addition in gills, and addition or synergism in the liver. At high concentrations of one or both mixture components it was not possible to draw conclusions about the type of interaction because, after a certain level, the induction of anti-oxidant enzymes does not necessary increase with the increase of the exposure concentrations. This is a well-known behaviour of anti-oxidant enzymes towards a high number of environmental contaminants that is often indicated as “bell-shape behaviour” (Vieira et al., 2009; Almeida et al., 2012).

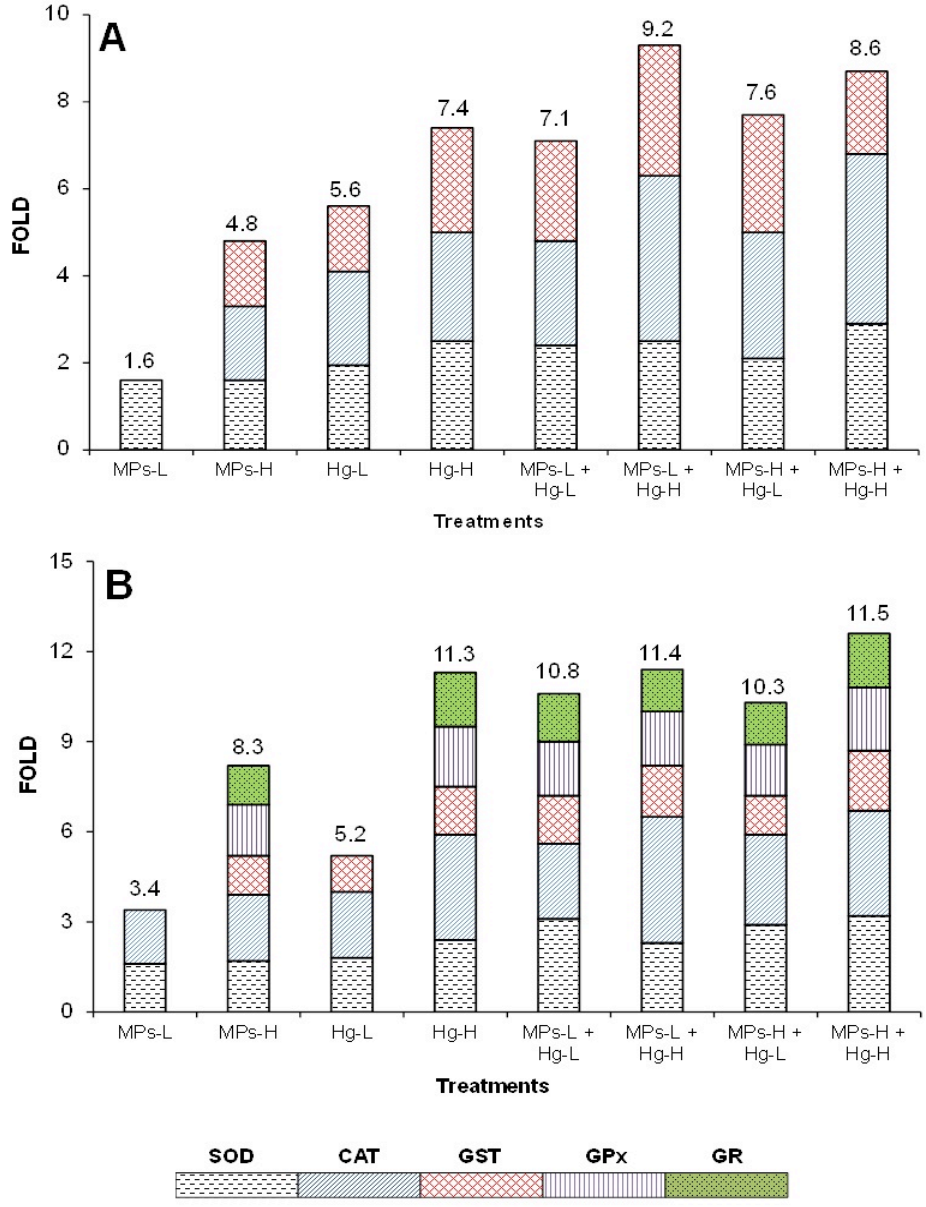

Figure 4.6. Contribution of enzymes superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) in the antioxidant defense system of *Dicentrarchus labrax (*A – gills; B – liver). Numbers above the columns indicate the total induction (fold).

Table 4.3. Concentrations of mercury (Hg) in *Dicentrarchus labrax* gills and liver (µg/g wet weight), bioconcentration factors (BCF) and bioaccumulation factors (BFA) after 96 hours of exposure. In the columns of concentrations, BCF and BAF, the values are the mean and standard deviation of nine replicates (fish) after discounting the mean of control group. For each data set (*i.e.* gills or liver mercury concentrations, BCF and BAF) different letters in the post-hoc test columns indicate statistical significant differences (Kruskal-Wallis test + non-parametric multicomparison test, $p \leq 0.05$).

TREATMENTS	Gills Hg Conc. (µg/g)	Post-hoc test	BCF gills	Post-hoc test	Liver Hg Conc. (µg/g)	Post-hoc test	BAF liver	Post-hoc test
Hg low	1.519 (± 0.369)	A	152 (± 37)	a	3.127 (± 0.753)	A	313 (± 75)	a
Hg high	2.836 (± 0.535)	B	177 (± 33)	a,b	5.419 (± 1.826)	B	339 (± 92)	a
MPs low + Hg low	2.670 (± 0.918)	B	267 (± 92)	b,c	2.571 (± 0.903)	A	257 (± 86)	a
MPs low + Hg high	4.310 (± 0.965)	C	269 (± 60)	b,c	4.370 (± 2.296)	A,B	273 (± 96)	a
MPs high + Hg low	2.995 (± 1.158)	B	300 (± 86)	c	5.040 (± 1.179)	B	504 (± 87)	b
MPs high + Hg high	4.825 (± 0.881)	C	302 (± 55)	c	8.169 (± 1.398)	C	511 (± 80)	b

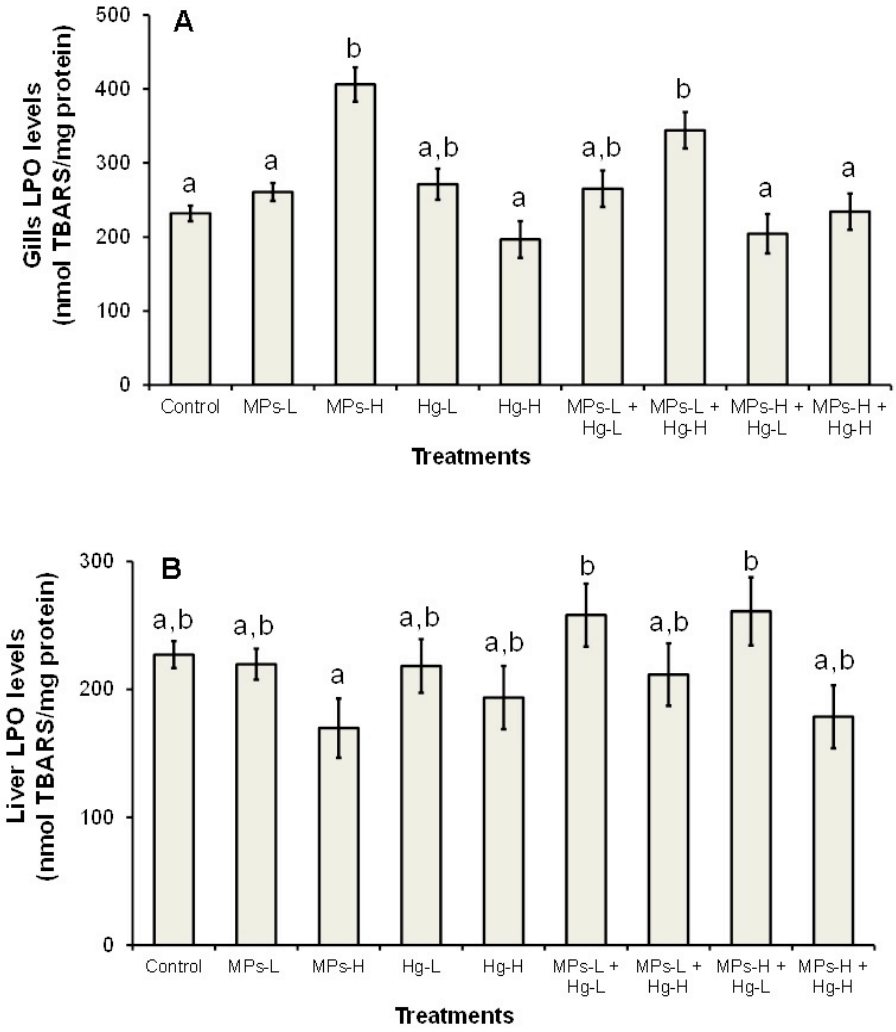

Figure 4.7. Gills (A) and liver (B) lipid peroxidation (LPO) in *Dicentrarchus labrax* exposed for 96 h to microplastics (MPs), mercury (Hg) or mixtures of the two substances. The values are the mean per treatment (9 animals) with corresponding standard error bars (SEM). Different letters indicate statistically significant differences between treatments ($p \leq 0.05$, Tukey test).

4.3.5. Conclusions

The concentrations of mercury in both gills and liver of *D. labrax* juveniles were significantly higher in the presence of microplastics than in their absence, indicating that microplastics influence the bioconcentration of the metal in gills and its bioaccumulation in the liver. The concentrations of microplastics and mercury tested, alone and in mixture, caused oxidative stress in gills and liver of *D. labrax* juveniles. Additionally, the highest concentration of microplastics caused lipid oxidative damage in gills. In fish exposed to mixtures, evidences of toxicological interactions between microplastics and mercury were found. At low concentrations of both mixture

components and based on the total induction of anti-oxidant enzymes activity, the type of toxicological interaction likely is addition in gills, and addition or synergism in the liver. These findings stress the need of further investigating the influence of microplastics in the bioconcentration, bioaccumulation, absorption, elimination and toxicity of other environmental contaminants in different species.

4.3.6. Acknowledgements

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Chapter IV

Section 4. Single and combined effects of microplastics and mercury on juveniles of the European seabass (Dicentrarchus labrax): changes in behavioural responses and reduction of swimming velocity and resistance time

Published as: Barboza, L.G.A., Vieira, L.R., Guilhermino, L., 2018. Single and combined effects of microplastics and mercury on juveniles of the European seabass (*Dicentrarchus labrax*): changes in behavioural responses and reduction of swimming velocity and resistance time. *Environmental Pollution 236*, 1014-1019 pp.

(Article published and included in the Thesis with permission of Elsevier included in the Appendix A)

4.4.1. Abstract

Microplastics and mercury are environmental pollutants of great concern. The main goal of the present study was to investigate the effects of these pollutants, both individually and in binary mixtures, on the swimming performance of juvenile European seabass, *Dicentrarchus labrax*. Microplastics alone, mercury alone and all the mixtures caused significant reduction of the swimming velocity and resistance time of fish. Moreover, changes in behavioural responses including lethargic and erratic swimming behaviour were observed. These results highlight that fish behavioural responses can be used as sensitive endpoint to establish the effects of contamination by microplastics and also emphasizes the need to assess the combined effects of microplastics and other environmental contaminants, with special attention to the effects on behavioural responses in fish and other aquatic species.

Keywords: European sea bass; microplastics; mixtures; swimming performance, behavior

4.4.2. Introduction

Aquatic ecosystems are frequently impacted by anthropogenic pollution (Connon *et al.*, 2012; Imhof *et al.*, 201b). Over the past decades, a large number of pollutants have been widely introduced in marine and freshwater environments threatening the health and integrity of these ecosystems around the world (Chaudhry and Malik, 2017). Among the environmental pollutants present in aquatic ecosystems, microplastics and mercury are both of great concern.

Microplastics were identified as an important emerging threat (Barboza and Gimenez, 2015; Browne *et al.*, 2007; Andrady, 2017). Their ability to interact with other contaminants (*e.g.* chemicals, bacteria and organic matter) increases concern related to their transference and potential effects on the biota (Fossi *et al.*, 2016; Galloway *et al.*, 2017; Santana *et al.*, 2017). Mercury is among the most extensively studied environmental pollutants (Gworek *et al.*, 2016). It is extremely toxic even at low concentrations and bioaccumulative (Wolfe *et al.*, 1998; Branco *et al.*, 2017). Mercury and microplastics, individually, are known to cause several damages (*e.g.* physiological, behavioural, physical and chemical) to aquatic organisms, such as effects on growth and development (Lee *et al.*, 2017; Lo and Chan, 2018), reduction

in feeding efficiency (de Sá *et al.*, 2015), intestinal blockage (Wright *et al.*, 2013a), alterations in swimming behaviour (Webber and Haines, 2003; Vieira *et al.*, 2009), interference on reproductive success (Frederick and Jayasena, 2011; Sussarellu *et al.*, 2016), oxidative stress (Monteiro *et al.*, 2010; Jeong *et al.*, 2017), neurotoxicity (Vieira *et al.*, 2009; Avio *et al.*, 2015a; Barboza *et al.*, 2018c) among others.

However, contaminant exposure in aquatic environments rarely consists of a single substance and thus, represents a big problem for aquatic organisms. Currently, the understanding of the effects of these complex mixtures of contaminants and their possible interactions is still very limited (Kienzler *et al.*, 2016), especially in relation to interactions of different contaminants with microplastics and how they affect organisms. The toxic effects resulting from the simultaneous exposure to microplastics and mercury through the water were investigated recently in fish (Barboza *et al.*, 2018c). In addition, other studies also report that the presence of microplastics in the water influences the toxicity of different pollutants such as pyrene (Oliveira *et al.*, 2013), cephalexin (Fonte *et al.*, 2016) and phenanthrene (Karami *et al.*, 2016b) to fish species. Despite the studies conducted in recent years, several important questions related to the fate of microplastics in organisms and ecosystems, and toxicological and ecological effects and interactions remain open (Guilhermino *et al.*, 2018), including questions regarding the interactions of these contaminants and potential effects on behavioural responses of fish and other aquatic organisms.

Behavioural responses have been widely used in aquatic toxicity assessments, representing a practical diagnosis of many complex processes that might be affected by a wide range of contaminants (Scott and Sloman, 2004; Barbe *et al.*, 2014). Effects on behaviour can occur at much lower exposure concentrations than expected for traditional toxicological endpoints (Melvin and Wilson, 2013). Therefore, due to their rapidity and sensitivity, behavioural analyses are often used to establish effects from contamination in comparison with the standard LC_{50} approach which is often used in ecotoxicology and may be ideal for studying the effects of different pollutants (Scott and Sloman, 2004; Boyle *et al.*, 2013). Among aquatic organisms, fish are widely used to investigate the effects of different pollutants on behavioural responses and are considered a great model for this purpose (Scott and Sloman, 2004). In this framework, swimming performance tests of fish have been

used with success to study effects of several contaminants, such as metals (Atchison *et al.*, 1987; Vieira *et al.*, 2009; Puga *et al.*, 2016) microplastics (Chen *et al.*, 2017; Tosetto *et al.*, 2017), PAHs (Lucas *et al.*, 2016), pesticides (Ballesteros *et al.*, 2009; Hernández-Moreno *et al.*, 2011) among others and represent a good indicator to measure the toxic effects of environmental contaminants (Tierney, 2011; Calfee *et al.*, 2016).

The swimming performance of fish is often assessed by measuring the swimming velocity and resistance time (Howard, 1975; Miller, 1980). The first parameter is related to velocity and distance traveled during the movement of fish while the second is related with to the ability of fish keep swimming when subjected to resistance, such as water flow (Hymel *et al.*, 2002; Barbieri, 2007). Other variables which can be evaluated include frequency and angle of swimming against flow, position in the water column and type of swimming behaviour (NOAA, 2015). Thus, to assess the effects of short-term exposure to microplastics and mercury both individually and in binary mixtures on the swimming performance of fish, we conducted a laboratory experiment using European seabass (*Dicentrarchus labrax*) as a model organism. *D. labrax* was chosen because it is widely employed to assess the effects of environmental pollutants on behavioural responses (*e.g.* Gravato and Guilhermino, 2009; Almeida *et al.*, 2010; Hernández-Moreno *et al.*, 2011). More specifically, we asked the following questions: a) how does microplastics and mercury alone and in binary mixtures affect the swimming velocity and resistance time of fish when swimming against the water flow? and b) are there any changes in behavioural responses of fish after exposure to low and high concentrations of microplastics and mercury, alone and in mixtures?

*4.*4.3. *Material and methods*

4.4.3.1. Chemicals, fish maintenance acclimatization and ethics

Fluorescent red polymer microspheres (1 - 5 µm diameter) were purchased from Cospheric – Innovations in Microtechnology (USA). Mercury chloride (≥ 99.5 % pure) was purchased from Sigma-Aldrich (USA). European seabass juveniles were purchased from a saltwater fish aquiculture (Vigo, Spain) and acclimatized to laboratory conditions as described in Barboza *et al.* (2018c). Forty-eight hours before the start of the bioassay, fish were transferred to glass beakers with clean water. The

experiment was carried out at the CIIMAR bioterium that is accredited “Direção Geral de Alimentação e Veterinária” (DGAV), Portugal, for studies with aquatic animals, conducted in accordance with ethical principles and regulations. L. Guilhermino and L.R. Vieira are accredited by DGAV to conduct animal experimentation (FELASA category C equivalent) and the study had DGAV authorization.

4.4.3.2. Bioassay

After the acclimatization period, 81 seabass juveniles were exposed individually (*i.e.* 1 fish per beaker) during 96 h (short-term bioassay) in 5 L glass beakers (with 4 L of water), with continuous aeration, and no food was provided during the bioassay. Room temperature was maintained at 19 ± 1 °C and photoperiod (14 h light: 10 h dark), with water temperature at 18 ± 1 °C. Water was renewed at each 24 h to decrease the potential microplastics aggregation and possible reduction of microplastics concentration in the water. In addition, water abiotic parameters were measured every day. Fish were randomly distributed per 9 treatments (9 fish per treatment): control (water only), two concentrations of microplastics alone 0.26 mg/L (MPs-low) and 0.69 mg/L (MPs-high), two concentrations of mercury alone 0.010 mg/L (Hg-low) and 0.016 mg/L (Hg-high), and all the mixtures in a full factorial experimental design (*i.e.*, MPs-low + Hg-low; MPs-low + Hg-high; MPs-high + Hg-low; MPs-high + Hg-high) as described in Barboza *et al.* (2018c). At the end of the exposure period, the post-exposure swimming performance of fish was assessed by determining the swimming velocity and resistance time (as indicated in section *4.4.3.3.*).

4.4.3.3. Swimming performance

The swimming performance of fish was assessed for each fish individually according to previously developed protocols (Gravato and Guilhermino, 2009), using the device described by Almeida *et al.* (2015) (Fig. 4.8). Briefly, immediately after the exposure period, fish were placed in a constant counter-current flow system (16 L/min) which simulates fish swimming against the water flow. The distance (d) covered by fish when swimming against the water flow and the time (t) they needed to cover that distance were recorded and used to calculate the swimming velocity (SV) as: SV (m/s) = d (m) / t (s). The resistance time (s) was determined by measuring the time the fish resisted (after reaching the end of the system or not

progressing in distance) until being dragged away by the water flow (Vieira *et al.*, 2009; Almeida *et al.*, 2015). After being dragged by the water flow each fish was put back into their original exposure beaker and left there for 2 h to stabilize metabolic rates (Almeida *et al.*, 2015). To eliminate impacts of human observers on fish behaviour, observers stood behind a screen. An Olympus® Digital Camera Full HD with 42x Wide Optical Zoom was used to film the swimming performance of each fish. At the end of the bioassay, individual body weight and total length were recorded and fish were immediately euthanized by decapitation under ice-cold induced anesthesia and different tissues (brain, muscle, liver and gills) were isolated to be used in another study. Videos were analyzed to verify possible behaviour changes.

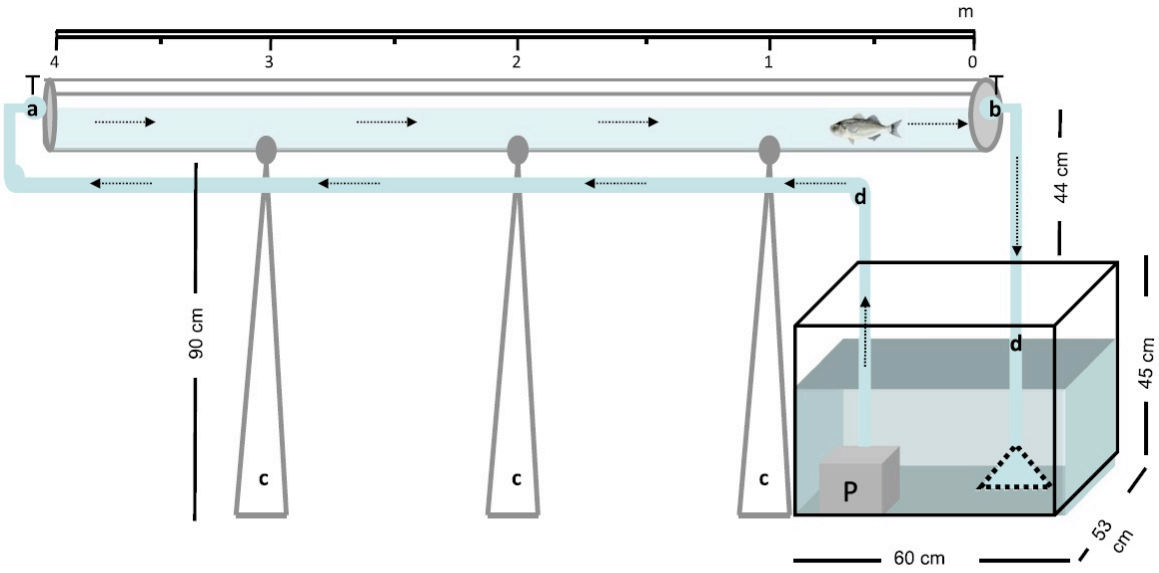

Figure 4.8. Behaviour device used to assess the swimming performance of fish composed of a 4-m-long acrylic transparent flume, opened on the top and supported by three metal structures (c). The water is driven from the pool through the system with a water pump (P) via connection hoses (d), and the water flux is controlled by two valves at both sides of the tube (a,b). Arrows represent water flow direction, and fish indicates both starting point position and swimming direction in the countercurrent system (Reprinted from Almeida *et al.*, 2015, with permission of the publisher).

4.4.3.4. Statistical analyses of data

First, the possible effects of length or body weight of fish on swimming performance was examined using the analysis of covariance (ANCOVA) (dependent variable: swimming velocity or resistance time; fixed factor: treatments; covariate (total length; body weight)). To avoid the negative effect of collinearity, the possible

correlation between weight and length was verified *a priori* (Pearson's correlation). Because we found no effect of the covariate, for each parameter (swimming velocity or resistance time), different treatments were compared using one-way analysis of variance (ANOVA). In addition, two-way ANOVA with interaction was used to investigate the possible interaction between microplastics and mercury on swimming velocity or resistance time of *D. labrax*. The Tukey's multicomparison post-hoc test was used to discriminate statistically significant treatments when ANOVA indicated significant differences among treatments. The assumptions for use of ANCOVA and ANOVA were checked *a priori* (Zar, 1999). Statistical significance was accepted at $p \leq 0.05$ for all analyses. Statistical analyses were performed using the SPSS statistical analysis package (version 24.0).

4.4.4. Results

4.4.4.1. General conditions

No fish mortality occurred. Water abiotic parameters were stable throughout the experiment (Appendix D, Tables S-1). Mean and standard deviation (SD) of total length and body weight were 7.75 ± 0.293 cm and 8.82 ± 0.295 g, respectively. Total length and body weight were positively and significantly correlated (Pearson's correlation coefficient - r) with each other ($N = 81$, $r = 0.994$, $p = 0.000$) and thus, to avoid the negative effect of collinearity, one of them was selected for the analysis of covariance. Significant differences among treatments, no significant differences among body weight, and no significant interaction between the two factors were found for swimming velocity (ANCOVA, treatments: $F_{(8,63)} = 2.134$, $p = 0.045$; body weight (covariate): $F_{(1,63)} = 0.499$, $p = 0.483$; interaction: $F_{(8,63)} = 0.984$, $p = 0.457$) and resistance time (ANCOVA, treatments: $F_{(8,63)} = 3.743$, $p = 0.001$; body weight (covariate): $F_{(1,63)} = 0.136$, $p = 0.713$; interaction: $F_{(8,63)} = 2.015$, $p = 0.07$), indicating that the covariable tested did not differ significantly among treatments and also did not influence the swimming performance of fish.

4.4.4.2. Single and combined effects of microplastics and mercury on juveniles of the European seabass

The effects of microplastics, mercury and their mixtures on *Dicentrarchus labrax* swimming performance are shown in Fig. 4.9. Significant differences were found among different treatments for the swimming velocity ($F_{(8,72)} = 28.481$,

$p = 0.000$) and resistance time ($F_{(8,72)} = 620.658$, $p = 0.000$). Post-hoc comparisons using the Tukey test indicated that fish exposed to the different treatments showed more differences between them ($p < 0.05$) to resist the water flow (Fig. 4.9-B) than to cover a distance as a function of time (Fig. 4.9-A). Relative to the control group, significant reduction in swimming velocity was observed (Fig. 4.9-A) in fish exposed to the highest concentration of microplastics (64 %), both treatments containing mercury alone (53 – 76 %) and all the mixtures (80 – 87 %). No significant differences in swimming velocity between the control and the treatments containing the lowest concentration of microplastics alone were found. In addition, in relation to the control group, significant reduction in resistance time was observed (Fig. 4.9-B) in animals exposed to both treatments containing microplastics alone (5 – 28 %), mercury alone (45 – 53 %) and all the mixtures (52 – 64 %). Moreover, the results of the integrated analysis of data indicated a significant interaction between microplastics and mercury for swimming velocity and resistance time (Appendix D, Tables S-2). At least one type of change in behavioural responses in fish exposed to the lowest and highest concentration of microplastics and mercury alone and in all the mixtures were observed, including lethargic and erratic swimming behaviour, such as swimming upside down, erratic jumping and loss of swimming control. In addition, signs of rapid fatigue were observed in fish exposed to all mixtures (Table 4.4).

Table 4.4. Changes in behavioural responses of *Dicentrarchus labrax* after 96 h of exposure to microplastics and mercury alone and in mixtures. SUD: swimming upside down; EJ: erratic jumping; LSC: loss of swimming control; RF: signs of rapid fatigue.

Treatments	**Behavioural responses**
Control	-
MPs low	SUD
MPs high	LSC
Hg low	SUD; LSC
Hg high	SUD; EJ; LSC
MPs low + Hg low	SUD; LSC, RF
MPs low + Hg high	EJ; LSC, RF
MPs high + Hg low	SUD; LSC; RF
MPs high + Hg high	EJ; LSC; RF

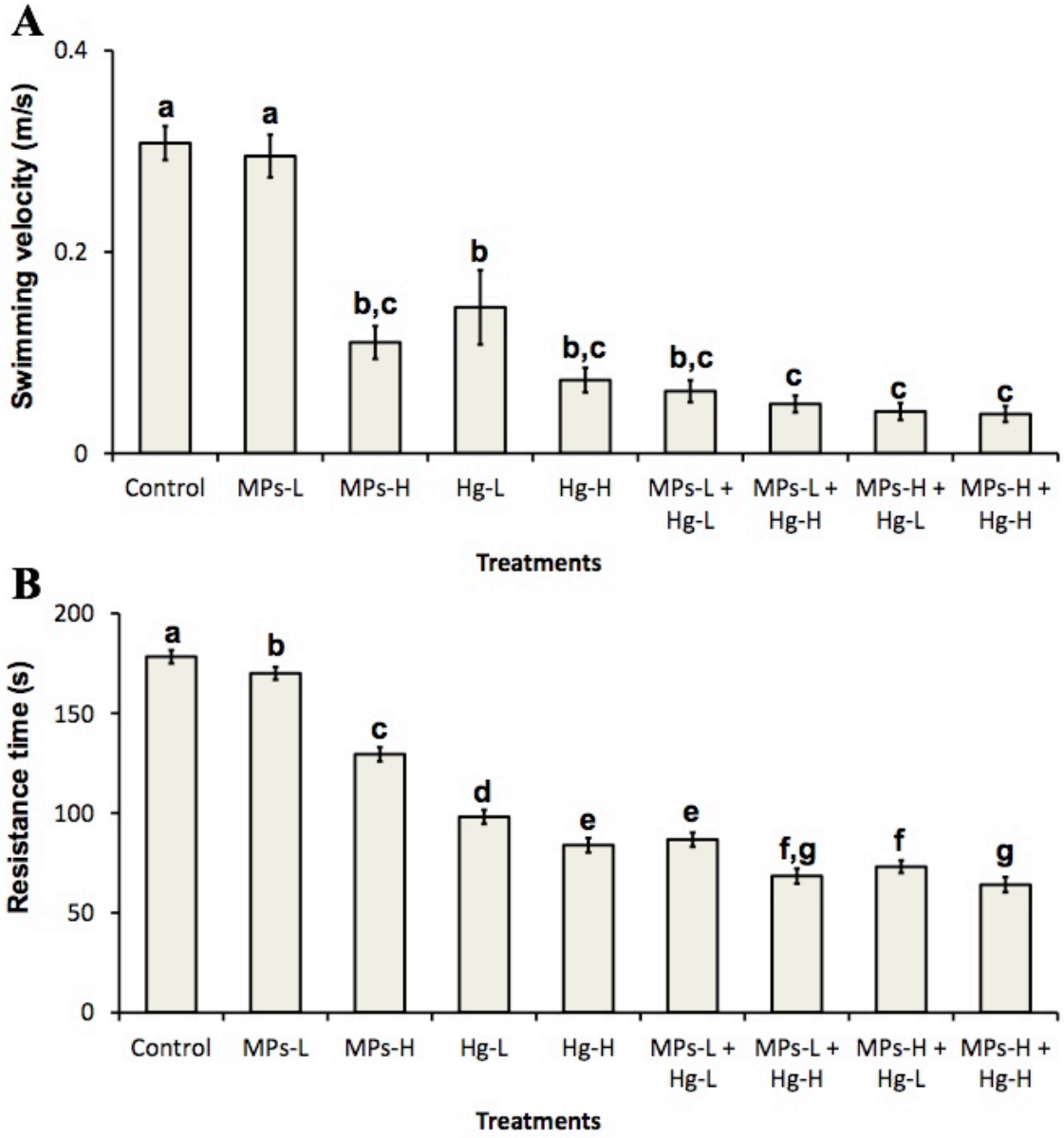

Figure 4.9. Swimming velocity (m/s) and resistance time (s) of *Dicentrarchus labrax* after 96 h exposure to microplastics and mercury alone and in mixtures. Values are the mean of 9 fish per treatment with the corresponding standard error bars. Different letters indicate statistical significant differences between treatments ($p < 0.05$, Tukey test). MPs-L – low microplastics concentration; MPs-H – high microplastics concentration; Hg-L – low mercury concentration; Hg-H – high mercury concentration.

4.4.5. Discussion

Although the effects of mercury on behaviour of fish species have been intensively studied (Weis, 2014) few studies have investigated the effects of microplastics on behavioural responses of these animals. In this context, recent

studies have shown that microplastics can reduce the predatory performance of fish (de Sá *et al.*, 2015). However, fish may not have its personality altered if the ingestion of these microparticles is through a contaminated prey (Tosetto *et al.*, 2017). Microplastics also did not affect the swimming distance of zebrafish larvae in the darkness (Chen *et al.*, 2017). To the best of our knowledge, this study is the first to investigate the effects caused by microplastics on the swimming velocity and resistance time of fish species. Our results indicate that a short-term exposure to microplastics alone may cause reduction in the swimming performance of fish (Fig. 4.9-A, 4.9-B). Thus, the presence of microplastics in aquatic environments may be capable of compromising the swimming performance of these animals. In the present study, the reduction of swimming velocity and resistance time was also observed in fish exposed to both concentrations of mercury alone. Similar results were obtained by Hilmy *et al.* (1987), Vieira *et al.* (2009) and Puga *et al.* (2016) reporting reduced swimming performance of fish after exposure to this substance.

Our results also suggest that the simultaneous exposure to microplastics and mercury may also cause reduction of swimming velocity and resistance time of fish. Moreover, evidences of toxicological interactions between these substances in *D. labrax* juveniles were found. The significant differences observed among treatments containing microplastics alone, mercury alone and their respective mixtures (Fig. 4.9-B) suggest that the interaction of these substances may affect the swimming performance of these animals. It is known that microplastics may adsorb mercury and other substances from the water (Turner and Holmes, 2015; Barboza *et al.*, 2018c). Some of these substances are known to alter the normal behaviour patterns of fish, including, ability to escape from predators, modification in the schooling and social behaviour, among others (Scott and Sloman, 2004). Thus, the capacity of microplastics to adsorb metals (Ashton *et al.*, 2010; Holmes *et al.*, 2012; Turner and Holmes, 2015; Wang *et al.*, 2017), pathogens and other pollutants (Teuten *et al.*, 2007; Frias *et al.*, 2010; Rochman *et al.*, 2013a; Kirstein *et al.*, 2016; Viršek *et al.*, 2017) from water and leach out chemicals and harmful pollutants in the marine environment (Koelmans *et al.*, 2016; Hartmann *et al.*, 2017) raises concerns about how different contaminants may interact with these microparticles and then potentially desorb into animals and affect them (Browne *et al.*, 2013; Bakir *et al.*, 2014; Koelmans *et al.*, 2014).

The behavioural changes observed in fish exposed to low and high concentrations of microplastics and mercury alone and in all the mixtures (Table 4.4), indicates the sensitivity of these individuals to these substances. Lethargic and erratic swimming behaviour such as those observed in the present study are commonly reported in fish species exposed to environmental contaminants (Berntssen *et al.*, 2003; Scott and Sloman, 2004; Ololade and Oginni, 2010; Brown *et al.*, 2017; Chen *et al.*, 2017). Changes in behavioural responses induced by toxic agents represent one of the most sensitive indicators of environmental stress, and thus, can be a first indication of fish health status since swimming capacity is usually affected before the death of the organism (Barbieri, 2007; Calfee *et al.*, 2016).

In general, our results showed that the short-term exposure to microplastics and mercury, both individually and in mixtures may cause changes in behavioural responses and affect the swimming velocity and resistance time of fish. These results may be due to negative effects induced on metabolic, endocrine and nervous systems that are known to be targets of microplastics and mercury (Weis, 2014; Ferrarini *et al.*, 2016; Chen *et al.*, 2017; Qiu *et al.*, 2017). The most commonly observed links with behavioural disruption include cholinesterase inhibition, altered brain neurotransmitter levels, sensory deprivation, and impaired thyroid hormone levels (Weis *et al.*, 2001; Scott and Sloman, 2004). Thus, knowing that microplastics and mercury can affect these systems (Berntssen *et al.*, 2003; Vieira *et al.*, 2009; Oliveira *et al.*, 2013; Rochman *et al.*, 2014a; Lu *et al.*, 2016; Barboza *et al.*, 2018c) different mechanisms may be involved in the behavioural responses observed in the present study.

Our results provide an initial contribution on the effects of microplastics combined with another contaminant of global concern on swimming performance of fish. In aquatic ecosystems, any alterations in swimming performance such as those observed in the present study, can have significant implications for fish species (*e.g.* capture prey and escape from predators, protecting territory, reproduction, migration, and dispersal) and consequently may affect the success of wild populations. The results obtained in this study may also contribute to risk analysis models of microplastics and their complex mixtures. However, for a balanced risk assessment, future studies should also contemplate the use of wild-caught animals, in order to compare if the effects on these organisms resemble the effects observed in laboratory animals (*e.g.* from aquaculture) (Koelmans *et al.*, 2016). Moreover, chronic

bioassays should also be performed to determine whether effects caused by microplastics alone or in mixtures on the swimming performance of fish are temporary or permanent, once is particularly important in terms of overall impacts on fitness and survival in the wild (Weis, 2014). Lastly, the possible effects of interaction between microplastics and other pollutants frequently found in aquatic environments on swimming performance in other fish species and aquatic organisms, including wild-caught animals, deserve further investigation.

4.4.6. Acknowledgements

This study was carried out in the scope of the project “PLASTICGLOBAL – Assessment of plastic-mediated chemicals transfer in food webs of deep, coastal and estuarine ecosystems under global change scenarios”, cofunded by the Fundação para a Ciência e a Tecnologia, I.P. (FCT), Portugal, with national funds (FCT/MCTES, “orçamento de Estado”, project reference PTDC/MAR-PRO/1851/2014) and the European Regional Development Fund (ERDF) through the COMPETE 2020 (POCI-01-0145-FEDER-016885) and Lisboa 2020 (LISBOA-01-0145-FEDER-016885) programmes. The study was also supported by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT and ERDF in the framework of the programme Portugal 2020 to CIIMAR, and by the Institute of Biomedical Sciences of Abel Salazar of the University of Porto (ICBAS), Portugal (Department of Populations Study, Laboratory of Ecotoxicology – ECOTOX).

Chapter V

Microplastic in wild fish from North East Atlantic Ocean in relation to neurotoxicity and other toxic effects, and estimated human exposure to microplastics through fish consumption

Subbmited as: Barboza, L.G.A., Lopes, C., Oliveira, P., Raimundo, J., Caetano, M., Vale, C., Guilhermino, L. Microplastic in wild fish from North East Atlantic Ocean in relation to neurotoxicity and other toxic effects, and estimated human exposure to microplastics through fish consumption (*under review*).

5.1. Abstract

Microplastics (MP) contamination and effect biomarkers were investigated in fish (*Dicentrarchus labrax*, *Trachurus trachurus*, *Scomber colias*), from North East Atlantic Ocean and aimed at being sold for human food consumption. From the 150 analysed fish (50 per species), 49 % had MP. In fish from the 3 species, MP in the gastrointestinal tract, gills and dorsal muscle were found. Fish with MP had significantly ($p \leq 0.05$) higher lipid peroxidation levels in the brain, dorsal muscle and gills, and increased brain acetylcholinesterase activity than fish where no MP were found. These results suggest lipid oxidative damage in gills and muscle, and neurotoxicity through lipid oxidative damage and acetylcholinesterase induction in relation to MP and/or MP-associated chemicals (MP-AC) exposure. From the 150 fish analysed, 32 % had MP in dorsal muscle, with a total mean (± SD) of 0.054 ± 0.099 MP items/g. Based on this mean and on EFSA recommendation for fish consumption by adults or the general population, human consumers of *D. labrax*, *T. trachurus*, *S. colias* may intake 842 MP items/year from fish consumption only. Based on the mean of MP in fish muscle and data (EUMOFA, NOAA) of fish consumption per capita in selected European and American countries, the estimated intake of microplastics through fish consumption ranged from 518 to 3078 MP items/year/capita. Other human food items (*e.g.* other seafood species, salt, honey) are known to be contaminated by MP, whereas others are likely too (*e.g.* meet from terrestrial animals, vegetables). Therefore, human exposure to MP through food is probably much higher than the estimates based on fish consumption only. Moreover, humans are also exposed to MP and MP-AC through other routes (*e.g.* air, water) during the entire life. Furthermore, a wide range of toxic effects (*e.g.* neurotoxicity, immunotoxicity, reproductive and transgerational effects) caused by MP and MP-AC in animals have been documented. Thus, microplastics pollution and its effects should be further investigated and addressed according the WHO One Health approach.

5.2. Introduction

The contamination of the marine environment by microplastics is currently recognized as a global threat of great concern. The abundance and worldwide distribution of microplastics in marine ecosystems have been increasingly documented (Cózar *et al.*, 2014; Suaria *et al.*, 2016; Auta *et al.*, 2017; Paul-Pont *et*

al., 2018; Frias and Nash, 2019). Estimates point to over 35,000 tons of microplastics afloat at seas and oceans (Eriksen *et al.*, 2014). Sediments of diferent regions are also contaminated by microplastics (Van Cauwenberghe *et al.*, 2013; Woodall *et al.*, 2014; Young and Elliott, 2016; Zhao *et al.*, 2018; Zhang *et al.*, 2019). The small size and relatively low density of microplastics contribute to their long-range transport (Cózar *et al.*, 2017; Barboza *et al.*, 2019b) and global distribution.

Microplastics can remain for many years in the marine and other environments, at least part of them being available to a wide range of organisms (Cole *et al.*, 2011; Barboza *et al.*, 2019b). After entering into organisms, microplastics may induce physical and chemical toxicity, including genotoxicity, neurotoxicity, oxidative stress and damage, changes in behavior, reproductive impairment, mortality, population growth rate decrease, transgerational effects, among several others (Avio *et al.*, 2015; Fonte *et al.*, 2016; Ribeiro *et al.*, 2017; de Sá *et al.*, 2018; Barboza *et al.*, 2018b,d; Guilhermino *et al.*, 2018; Yin *et al.*, 2018; Yu *et al.*, 2018; Qiao *et al.*, 2019; Zhu *et al.*, 2019). The negative effects may be due to the particles themself, to additives incorporated during the manufacture of plastic products, to chemicals incorporated during microplastic use (*e.g.* as abrasives), and/or to environmental contaminants adsorbed to plastic debris during their permanence in the environment (Teuten *et al.*, 2009; Frias *et al.*, 2010; Silva *et al.*, 2016; Beiras *et al.*, 2018; Hahladakis *et al.*, 2018; Vedolin *et al.*, 2018).

Marine fish uptake microplastics from the seawater passively when water enters through the mouth or gills (Barboza *et al.*, 2018, Collard *et al.*, 2017b), and actively because they confuse some microplastics with prey due to colour, size, shape and other similarities (de Sá *et al.*, 2015; Ory *et al.*, 2018a,b). The first records of plastic ingestion by fish date back to the 1970s (Carpenter *et al.*, 1972) and since then the occurence of microplastics in the gastrointestinal tract of several fish species has been extensively documented (Jovanović, 2017; Barboza *et al.*, 2018a; Rezania *et al.*, 2018). Microplastics were found in fish at different stages of their life cycle (larvae, juvenile and adult) (Romeo *et al.*, 2015; Steer *et al.*, 2017; Bessa *et al.*, 2018; Collicutt *et al.*, 2019), in distinct components of trophic guilds (*e.g.* planktivorous, omnivorous, carnivorous, herbivorous) (Mizraji *et al.*, 2017; Ory *et al.*, 2018a; Baalkhuyur *et al.*, 2018; Herrera *et al.*, 2019) and in species of different habitats (*e.g.* pelagic, benthic, coral reef, seagrass) (Lusher *et al.*, 2013; Neves *et*

al., 2015; Güven *et al.*, 2017; Forrest and Hindell, 2018; Garnier *et al.*, 2019; Giani *et al.*, 2019). In addition to economic, and animal and environmental health negative impacts that the contamination of fish by microplastics has, the presence of microplastics in species consumed as food by humans is a risk to human food safety and health (Wright and Kelly, 2017; Barboza *et al.*, 2018a).

As microplastics and associated chemicals are a treat to animal, environmental and human health, the global pollution by microplastics and its effects should be addressed according to World Health Organization (WHO) One Health approach. Therefore, the goals of the present study were (i) to investigate the microplastic contamination of fish (*Dicentrarchus labrax*, *Trachurus trachurus* and *Scomber colias*) from North East Atlantic Ocean (North West Portuguese coastal waters) on sale for human consumption; (ii) to assess the potential neurotoxic effects and lipid oxidative damage in fish in relation to microplastics contamination; and (iii) based on the microplastics found in the main edible tissue (dorsal muscle) of the three investigated species, to estimate the human exposure to microplastics through the consumption of fish as food, contributing to improve the bases for human health risk assessment of microplastics.

5.3. Material and Methods

5.3.1. Sample collection and preparation

The present sudy investigated specimens of the European seabass (*D. labrax*), the Atlantic horse mackerel (*T. trachurus*) and Atlantic chub mackerel (*S. colias*) captured in March and April 2018 in Northwest (NW) Portuguese coastal waters (continental shelf), North East (NE) Atlantic Ocean. Fish were landed in Matosinhos port to be sold for human consumption as food. These species were selected for the present study because they are very much appreciated and consumed as food by humans in Europe (EUMOFA, 2018) and other regions. Fifty specimens of each species were transported to the laboratory within 30 min after landing to be analysed. In the laboratory, the total body length (cm) and weigth (g) of each specimen was determined. The mean ± standard deviation (SD) of fish total body lenght and weight were, respectively: 31 ± 1 cm and 343 ± 23 g for *D. labrax*; 29 ± 2 cm and 228 ± 19 g for *T. trachurus*; and 37 ± 1 cm and 344 ± 8 g for *S. colias*. Subsequently, from each fish, the whole gastrointestinal tract, three brachial arcs (hereafter indicated as gills) and 10 g of the dorsal muscle were isolated and used to

assess their contamination by microplastics. Moreover, from each fish, the whole brain, one brachial arc and 5 g of dorsal muscle were also isolated as indicated in Barboza et al. (2018c) for determination of biomarkers. The liver and the rest of dorsal muscle were collected for another study. All samples were stored individually at − 80 °C until further analyses.

5.3.2. Microplastics isolation and visual characterization

To each sample, a volume of a 10% KOH solution (prepared in ultra-pure water) corresponding to three folds of its volume was added. Gastrointestinal tract and dorsal muscle samples were incubated at 60 ºC for 24 h (Dehaut *et al.*, 2016), and gill samples were incubated at 40 °C for 72 h (Karami *et al.*, 2017) to digest the organic material. Density separation was not performed to preserve all types of microplastics (Abbasi *et al.*, 2018). After the incubation period, the remaining liquid was vacuum filtered through glass-microfiber filter membranes (pore size 1.2 µm, Munktell & Filtrak GmbH, Germany). Filters were sealed in glass Petri dishes and oven-dried at 40 °C for 24 h (Drying oven EV50, Raypa, Spain). Then, filter membranes were analysed and photographed in a stereomicroscope with an integrated CMOS camera (LEICA S9i, Leica Microsystems GmbH, Germany). All the plastic items recovered from the samples were sorted and quantified by colour (blue, black, whitish, yellow, red/pink), shape (fragments - irregular pieces; pellets - spherical and ovoid debris; fibers - thin and elongated pieces) (Karami *et al.*, 2017; Frias *et al.*, 2018), and size based on their largest cross section measured using the ImageJ software available in https://imagej.nih.gov/ij/ (< 100 µm; 101-150 µm; 151-500 µm; 501-1500 µm; 1501-3000 µm; 3001-5000 µm). The number of microplastics in the gastrointestinal tract and in gills was expressed as the number of microplastic items per individual (MP items/individual). The amount of microplastics in the dorsal muscle was expressed in microplastic items per g of tissue (MP items/g).

5.3.3. Contamination control

Tissue samples were prepared and analysed in a laboratory with restricted access and previously cleaned to prevent contamination by microplastics from other sources. Clean cotton laboratory coats and nitrile gloves were worn during all the steps of the procedure. All work surfaces and dissection materials were cleaned with ethanol 70 % before use and in-between individual samples to prevent cross-

contamination. The outer part of the fish was rinsed twice with ultra-pure water and once with ethanol to eliminate any potential particles attached to fish body surface as descibed in Karami *et al.* (2017). In all procedures, three clean Petri dishes were placed next to the work area and analysed as procedural blank controls. In addition, during digestion procedures, three procedural blanks (without tissues, containing ultra-pure water as substitute of fish sample) were analysed in parallel with the digested fish samples. Such blanks were included to assess any potential contamination from laboratory atmosphere during digestion procedures that might have occurred despite all the care taken.

5.3.4. Determination of biomarkers in fish

Based on fish length and weight, the Fulton's condition factor (Fulton's K) was determined according to Lloret *et al.* (2002). The other biomarkers used were: brain acetylcholinesterase (AChE) activity as indicative of neurofunction; muscle total cholinesterases (ChE) activity as indicative of neuromuscular function; brain, muscle and gills lipid peroxidation (LPO) levels as indicative of lipid peroxidation damage.

The procedures for sample preparation and determination of the biomarkers used are described in detail in previous papers (*e.g.* Guilhermino *et al.*, 1996; Barboza *et al.*, 2018c). Briefly, AChE and ChE activities were determined by the Elman's technique (Ellman *et al.*, 1961) adapted to microplate (Guilhermino *et al.*, 1996), using acetylcholine as substrate and readings at 412 nm, and expressed in nanomoles of substrate hydrolysed per minute per mg of protein (nmol/min/mg protein). LPO levels were determined through the quantification of thiobarbituric acid reactive substances (TBARS) at 535 nm according to Ohkawa (1979) with punctual modifications (Torres *et al.*, 2002), and expressed in nanomoles of TBARS per mg of protein (nmol TBARS/mg protein). The protein content of the samples was determined at 600 nm by the Bradford method (Bradford, 1976) adapted to microplate (Frasco and Guilhermino, 2002), using bovine gama globulin as protein standard. All biomarker and protein determinations were carried out at 25ºC using a Spectramax® spectrophotometer (Molecular Devices, USA).

5.3.5. Estimated human exposure to microplastics through fish consumption

Two approaches to estimate the human exposure to microplastics through fish consumption were used. The first one was based on the recomendations of the

European Food Safety Authority (EFSA) regarding fish consumption: 1 year old children - 40 g per week; 2-6 year old children - 50 g per week; > 6 year old children - 200 g per week; adults or the general population - 300 g per week (EFSA, 2014). The second one was based on data from the European Market Observatory for Fisheries and Aquaculture Products (EUMOFA) and National Marine Fisheries Service (NOAA) regarding human consumption of fish per capita in Portugal (57000 g/year/capita) and in the main importer countries of fish from Portugal, namely Spain (47700 g/year/capita), Italy (31100 g/year/capita), USA (21400 g/year/capita) and Brazil (9600 g/year/capita) (EUMOFA, 2018; NOAA, 2018). The estimated human intake of microplastics (indicated as MP in A, B, C and D bellow) from fish was based on EFSA recomendations (A, B) or EUMOFA and NOAA data (C, D) and on the total mean of the number of microplastics in dorsal muscle considering the three species of fish and including fish where microplastics were not found (*i.e.* total number of microploastics found in muscle tissue / 150 specimens):

(A) Human MP intake per week (MP items/week): mean of MP items in the muscle tissue (MP items/g) x recommended fish food intake per week (g)

(B) Human MP intake per year (MP items/year): mean of MP items in the muscle tissue (MP items/g) x recommended fish food intake per week (g) x number weeks per year (52)

(C) Human MP intake per week per capita (MP items/week/capita): mean of MP items in the muscle tissue (MP items/g) x consumption of fish per week per capita in the selected country(g)

(D) Human MP intake per year per capita (MP items/year/capita): mean of MP items in the muscle tissue (MP items/g) x consumption of fish per year per capita in the selected county (g)

5.3.6. Statistical analyses of data

Statistical analyses were performed using the SPSS statistical analysis package (version 24.0) and the statistical significance level was 0.05. For each

species and biomarker, the Student's *t*-test was used to compare fish found to have microplastics with fish where no microplastics were found. However, it should be mentioned that because not all the fish body was analysed regarding the presence of microplastics, the contamination of fish where no microplastics were found cannot be completely excluded. Neverthless, if no microplastics where found in the gastrointestinal tract or in the analysed portion of gills and dorsal muscle, likely these fish were considerably less contaminated by microplastics than those found to have particles in the parts of the body analysed. For simplicity, the two groups of fish will be hereafter indicated as "fish with microplastics" and "fish without microplastics".

5.4. Results

5.4.1. Microplastics in fish

No microplastics were found in any of the blanks analysed.

Microplastics were found in 73 of the 150 examined fish (49 %): 52 fish (35 %) had microplastics in the gastrointestinal tract, 54 fish (36 %) had microplastics in the gills and 48 fish (32 %) had microplastics in the dorsal muscle. Microplastics were found in all species: 42 % of the 50 *D. labrax*; 42 % of the 50 *T. trachurus* individuals; and 62 % of the 50 *S. colias* (Fig. 5.1).

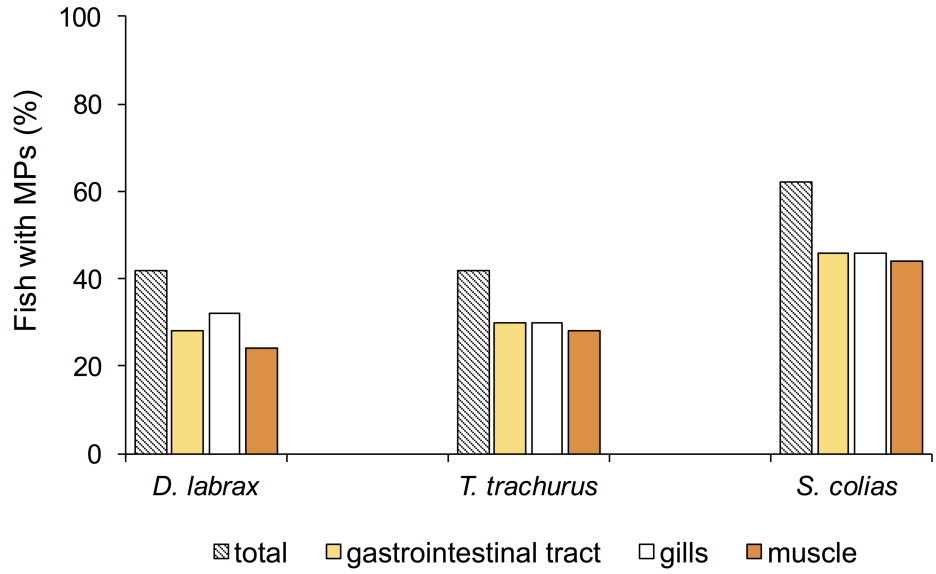

Figure 5.1. Percentage of *Dicentrarchus labrax* (N = 50), *Trachurus trachurus* (N = 50), and *Scomber colias* (N = 50) having microplastics (MPs) in the gastrointestinal tract (GT), gills (GI), dorsal muscle (MU), or in any of these sites (TOTAL).

A total of 368 microplastic items were recovered from the 150 specimens: 175 microplastics from the gastrointestinal tract (48 %), 112 items from the gills (30 %) and 81 from the muscle (22 %). Considering the 50 animals of each species analised, the mean (± SD) of the number of microplastics was: 1.3 ± 2.5 MP items/individual in the gastrointestinal tract, 0.8 ± 1.4 MP items/individual in gills and 0.4 ± 0.7 MP items/g in the dorsal muscle of *D. labrax*; 1.0 ± 1.9 MP items/individual in the gastrointestinal tract, 0.7 ± 1.4 MP items/individual in gills and 0.7 ± 1.3 MP items/g in the dorsal muscle of *T. trachurus*; 1.2 ± 1.6 MP items/individual in the gastrointestinal tract, 0.7 ± 1.0 MP items/individual in gills and 0.6 ± 0.8 MP items/g in the dorsal muscle of *S. colias*. Considering the three species (N = 150), the total mean (± SD) of the number of microplastics in the gastrointestinal tract, gills and dorsal muscle was 1.2 ± 2.0 items/individual, 0.7 ± 1.2 items/individual and 0.054 ± 0.099 items/g of tissue, respectively.

Considering the colour of the microplastics (Fig. 5.2), *D. labrax* specimens had microplastics of 5 colours: blue (67 %), whitish (15 %), black (9 %), red/pink (6 %) and yellow (3 %). *T. trachurus* specimens had blue (90 %) and whitish (10 %) microplastics. *S. colias* specimens had microplastics of 4 colours: blue (79 %), whitish (11 %), black (5 %) and red/pink (5 %).

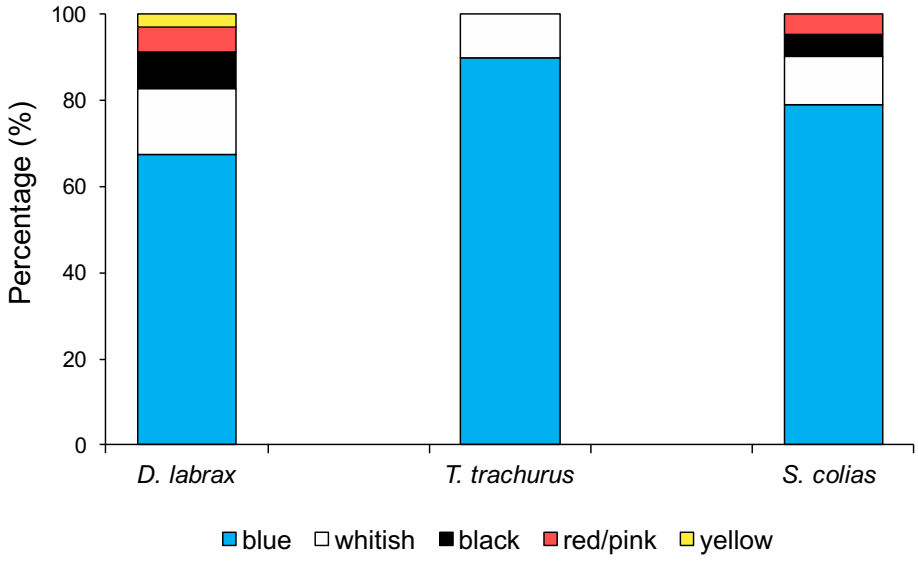

Figure 5.2. Percentage of microplastics (fragments + pellets + fibers) found in *Dicentrarchus labrax, Trachurus trachurus* and *Scomber colias* categorized by colour.

The shape of the microplastics recovered from fish samples were fibers, fragments and pellets (Fig. 5.3 and Fig. 5.4). From the total number (368) of microplastics recovered from fish, 199 items (54 %) were fibers, 167 items (45 %) were fragments and 2 items (1 %) were pellets. Fibers and fragments were found in all the species and types of samples, whereas the 2 pellets were only found in the gastrointestinal tract of *T. trachurus* and *S. colias* (Fig. 5.4). *D. labrax* specimens had more fibers (≥ 66 %) than fragments (≥ 10 %) in the gastrointestinal tract, gills and muscle (Fig. 5.4). *T. trachurus* and *S. colias* specimens had more fragments (76 %) than fibers (22 %) and pellets (2 %) in the gastrointestinal tract, more fibers than fragments in the gills and approximately the same percentage of fibers and fragments in the muscle (Fig. 5.4).

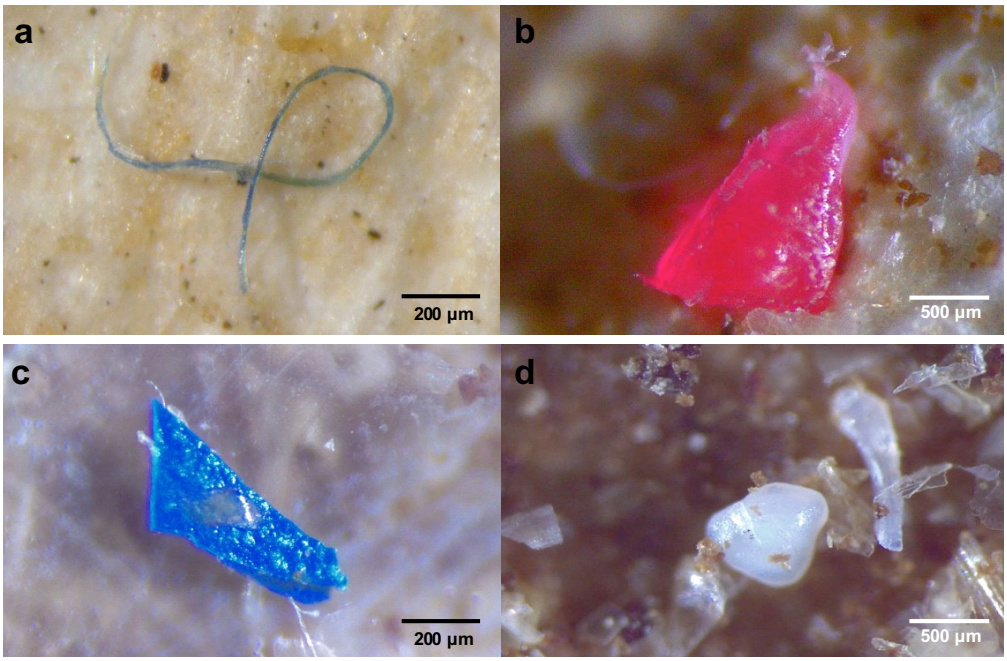

Figure 5.3. Examples of microplastics recovered from *Dicentrarchus labrax, Trachurus trachurus* and *Scomber colias.* (a – fiber; b and c – fragment; d – pellet) (Photos: Gabriel Barboza).

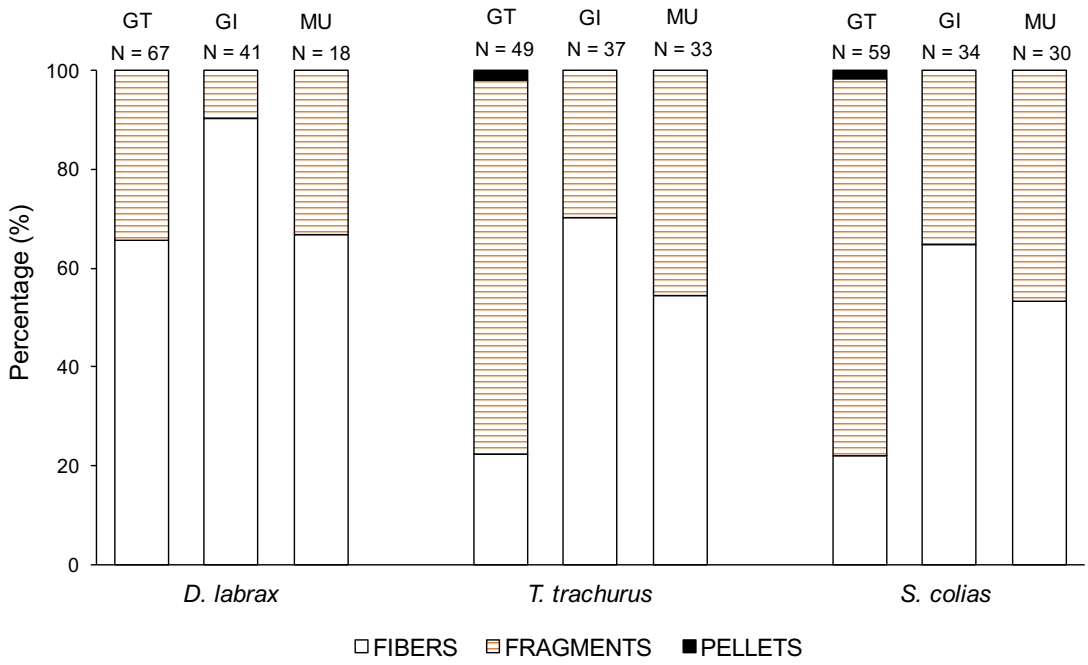

Figure 5.4. Percentage of microplastics found in *Dicentrarchus labrax*, *Trachurus trachurus* and *Scomber colias* gastrointestinal tract (GT), gills (GI) and dorsal muscle (MU) categorized by shape.

Based on microplastic size, all the species had more fibers in the size range 501 - 1500 µm in the gastrointestinal tract (≥ 36 %) and 151 - 500 µm in gills (≥ 50 %) than fibers of other size ranges. In the dorsal muscle, *D. labrax* and *S. colias* had more fibers in the size range 501 - 1500 µm (≥ 58 %) and *T. trachurus* in the size range 151 - 500 µm (39 %) than other size ranges (Fig. 5-A). In all the species, fragments lower than 100 µm were more abundante in dorsal muscle (≥ 67 %) than other fragments, and fragments between 101 - 150 µm were more abundant in gills (≥ 45 %) than fragments of other size ranges. In the gastrointestinal tract, the most part of fragments were in the size range 151 - 500 µm (≥ 32 %) in *D. labrax* and *T. trachurus*, and in the size range 501 - 1500 µm (36 %) in *S. colias* (Fig. 5-B).

5.4.2. Fish biomarkers

In all the species, no significant ($p > 0.05$) differences in lenght, weight and Fulton's K between fish with and without microplastics were found (Table 5.1).

Regarding brain AChE activity and LPO levels in brain, muscle and gills significant differences between fish with and without microplastics were found in all the species (Table 5.1). Fish with microplatics had significantly higher brain AChE activity (2 fold) and increased LPO levels (2 fold) in the brain, muscle (2 fold) and

gills (1 fold) than fish without microplastics (Table 5.1). No significant diferences in muscle ChE activity between fish with and without microplastics were found in any of the species (Table 5.1).

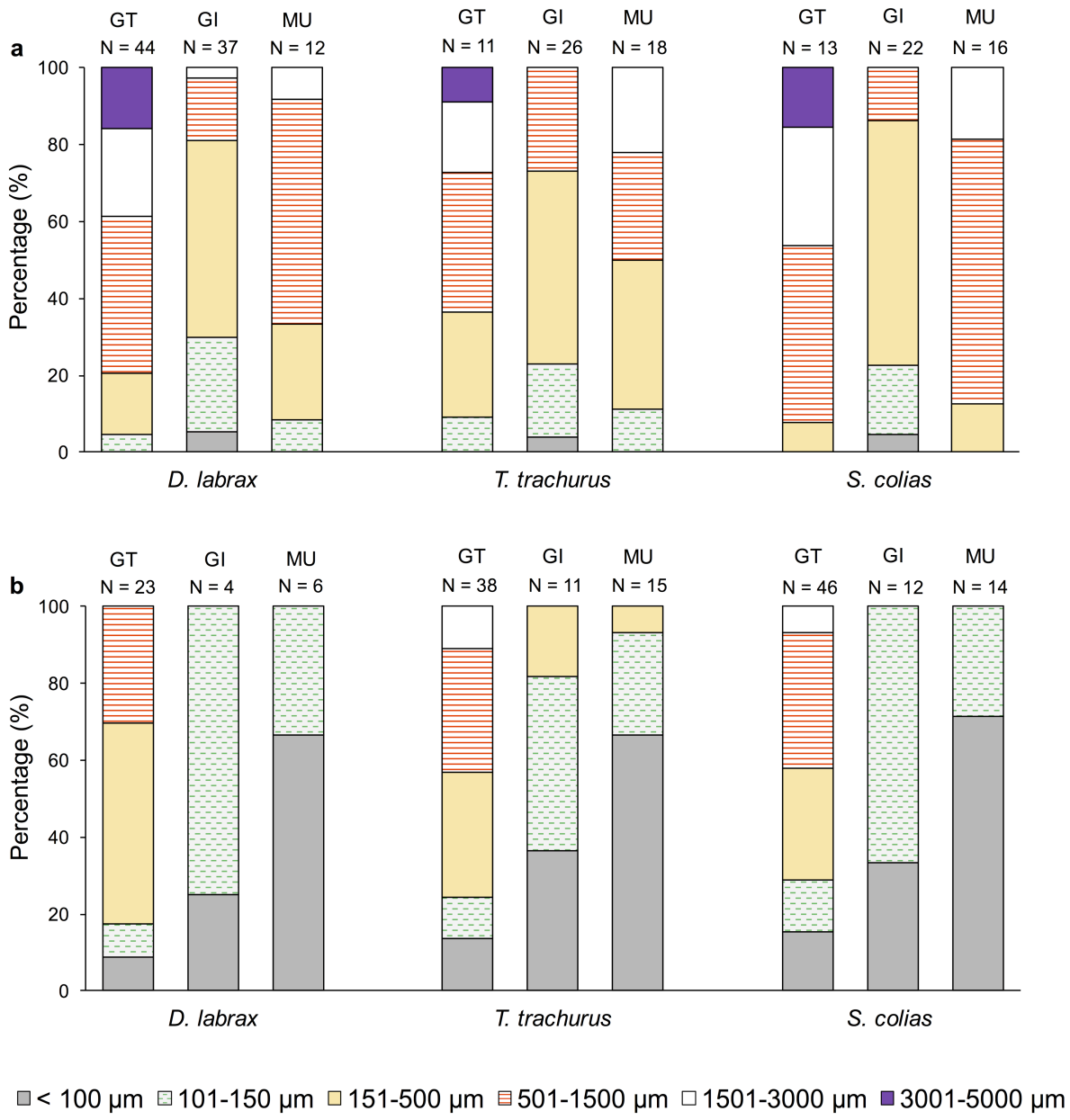

Figure 5.5. Percentage of microplastics found in *Dicentrarchus labrax*, *Trachurus trachurus* and *Scomber colias* gastrointestinal tract (GT), gills (GI) and dorsal muscle (MU) categorized by size classes (a – fibers; b – fragments).

5.4.3. Estimated intake of microplastics by humans consuming fish

Based on the total mean of microplastics found in fish muscle (0.054 MP items/g tissue, N = 150) and on the the weekly intake of fish recommended for distinct human populational groups by EFSA (EFSA, 2014), the estimated intake of microplastics by human consumers per year ranged from 112 MP items/year (1 year old children) to 842 MP items/year (adults or the general population) as shown in Table 5.2. Additionally, based on the total mean of microplastics found in fish muscle and on the consumption of fish per capita in each of the selected countries (EUMOFA, 2018; NOAA, 2018), the estimated human intake of microplastics through fish consumption (Table 5.3) ranged from 518 MP items/year/capita (Brazil) to 3078 MP items/year/capita (Portugal).

5.5. Discussion

5.5.1. Microplastics in fish

In this study, microplastics were found in a considerable percentage of *D. labrax* (42 %), *T. trachurus* (42 %) and *S. colias* (62 %) specimens from Portuguese coastal waters (NE Atlantic Ocean). The NE Atlantic Ocean water is contaminated with microplastics (Lusher *et al.*, 2014; Maes *et al.*, 2017; Murphy *et al.*, 2017; Hernández-González *et al.*, 2018; Courtene-Jones *et al.*, 2019), as well as zooplankton and sediment samples from the Portuguese shelf (Frias *et al.*, 2014, Antunes *et al.*, 2018). Therefore, microplastics may have been uptaken by fish directly from the seawater passively (*e.g.* gill water filtration) and actively (*i.e.* ingested by confusion with prey), and through the ingestion of contaminated prey, as suggested in previous studies with fish (Lusher *et al.*, 2013; de Sá *et al.*, 2015; Ory *et al.*, 2018 a,b). Moreover, fish may have also uptake microplastics from the nets used for their capture, as pointed out before (Lusher *et al.*, 2013). *S. colias* had a higher percentage of microplastic contamination (62 %) than the other species (42 %). This difference may be due to some distinct ecological features (*e.g.* time spend in areas more close to the shore, feedind ecology), physiological differences (*e.g.* water filtration rates, elimination processes), among others.

Table 5.1. Mean ± standard deviation (SD) of total body length, body weight, Fulton condition index (Fulton), brain acetylcholinesterase activity (AChE-B), muscle cholinesterase activity (ChE-M), lipid peroxidation levels in brain (LPO-B), muscle (LPO-M) and gills (LPO-G) in *Dicentrarchus labrax, Trachurus trachurus*, and *Scomber colias*, in groups of fish with (MP) and without (No) microplastics. N = number of individuals per group. Enzymatic activities are expressed in nmol/min/mg protein. LPO levels are expressed in nmol TBARS/mg protein. * indicates statistical significant differences between groups of fish with and without microplastics (Student's *t* test, $p \leq 0.05$).

		Dicentrarchus labrax			*Trachurus trachurus*			*Scomber colias*		
Biomarker	Level	N	Mean ± SD	*t* test	N	Mean ± SD	*t* test	N	Mean ± DF	*t* test
Length (cm)	No	29	32 ± 2	$t_{(48)} = 0.500$	29	29 ± 2	$t_{(48)} = -1.768$	19	37 ± 1	$t_{(48)} = 1.034$
	MP	21	31 ± 2	p = 0.619	21	30 ± 1	p = 0.083	31	36 ± 1	p = 0.306
Weight (g)	No	29	342 ± 23	$t_{(48)} = -0.299$	29	226 ± 19	$t_{(48)} = -1.129$	19	347 ± 10	$t_{(48)} = 1.859$
	MP	21	344 ± 23	p = 0.766	21	232 ± 19	p = 0.264	31	343 ± 7	p = 0.069
Fulton	No	29	1 ± 0.11	$t_{(48)} = -0.959$	29	1 ± 0.08	$t_{48} = -1.287$	19	1 ± 0.03	$t_{(48)} = -0.269$
	MP	21	1 ± 0.10	p = 0.343	21	1 ± 0.11	p = 0.204	31	1 ± 0.02	p = 0.789
AChE-B	No	29	4 ± 2	$t_{(22.084)} = -3.587$	29	4 ± 1	$t_{(20.168)} = -3.063$	19	4 ± 1	$t_{(30.942)} = -4.371$
	MP	21	9 ± 7	p = 0.002*	21	12 ± 12	p = 0.006*	31	11 ± 8	p ≤ 0.001*
ChE-M	No	29	2 ± 1	$t_{(48)} = 0.078$	29	2 ± 1	$t_{48} = -0.137$	19	1 ± 0.21	$t_{(48)} = -0.228$
	MP	21	2 ± 1	p = 0.938	21	2 ± 1	p = 0.892	31	1 ± 0.43	p = 0.352
LPO-B	No	29	183 ± 58	$t_{(21.146)} = -3.061$	29	152 ± 36	$t_{(20.438)} = -3.549$	19	141 ± 32	$t_{(36.398)} = -5.005$
	MP	21	381 ± 292	p = 0.006*	21	380 ± 93	p = 0.002*	31	256 ± 121	p ≤ 0.001*
LPO-M	No	29	6 ± 2	$t_{(21.054)} = -2.738$	29	12 ± 2	$t_{(20.025)} = -2.457$	19	4 ± 1	$t_{(32.117)} = -4.454$
	MP	21	12 ± 10	p = 0.012*	21	49 ± 69	p = 0.023*	31	10 ± 8	p ≤ 0.001*
LPO-G	No	29	212 ± 14	$t_{(28.594)} = -2.955$	29	188 ± 9	$t_{(21.432)} = -3.458$	19	180 ± 7	$t_{(41.626)} = -2.826$
	MP	21	230 ± 25	p = 0.006*	21	221 ± 43	p = 0.002*	31	192 ± 20	p = 0.007*

Table 5.2. Estimated human intake of microplastics from fish consumption based on the microplastics found in *Dicentrarchus labrax, Trachurus trachurus* and *Scomber colias* and on EFSA recommendations for fish consumption per week by children of different age groups, and adults or the general population.

	Children			Adults or the general population (≥ 18 y)
	(1 y)	(2-6 y)	(>6 y)	
g fish muscle/week	40 g	50 g	200 g	300 g
MP items/week	2	3	11	16
g fish muscle/year	2080 g	2600 g	10400 g	15600 g
MP items/year	112	140	562	842

Table 5.3. Estimated human intake of microplastics from fish consumption based on the microplastics found in *Dicentrarchus labrax, Trachurus trachurus* and *Scomber colias* and on per capita consumption of fish in Portugal and in the largest importer countries of fish from Portugal.

	Portugal	Importer countries			
		Spain	Italy	United States	Brazil
Per capita consumption (Kg/year/capita)	57.0 Kg	47.7 Kg	31.1 Kg	21.4 Kg	9.6 Kg
g fish muscle/week/capita	1096 g	917 g	598 g	412 g	185 g
MP items/week/capita	59	50	32	22	10
g fish muscle/year/capita	57000 g	47700 g	31100 g	21400 g	9600 g
MP items/year/capita	3078	2576	1679	1156	518

The pollution of Portuguese coastal waters by microplastics may result from local inputs of plastic materials (*e.g.* lost nets and other fishery materials), mobilization of microplastics from sediments that are known to contain microplastics (Frias *et al.*, 2016), especially during storms, from continental sources in the Portuguese coast, including beaches and estuaries, where microplastics were documented (Frias *et al.*, 2010, 2014; Antunes *et al.*, 2018; Bessa *et al.*, 2018; Rodrigues *et al.*, 2019), and from far way transported by ocean currents, organisms and other ways.

The presence of microplastics in the gastrointestinal tract, gills and muscle of *D. labrax, T. trachurus* and *S. colias* from Portuguese coastal waters is in agreement with the presence of microplastics in the gastrointestinal tract (Rochman *et al.*, 2015; Jabeen *et al.*, 2017; Baalkhuyur *et al.*, 2018; Ferreira *et al.*, 2018; Pozo *et al.*, 2019), muscle (Abbasi *et al.*, 2018; Akhbarizadeh *et al.*, 2018) and gills (Collard *et al.*, 2017b; Karami *et al.*, 2017; Abbasi *et al.*, 2018) of fish from other regions. Moreover, the percentage of fish that had microplastics in the gastrointestinal tract (35 % of 150 fish) is in the range of corresponding values reported in the literature, such as: 19.8 % of 263 fish from Portuguese coastal waters (Neves *et al.*, 2015), 38 % of 120 fish from the Mondego River estuary in Portugal (Bessa *et al.*, 2018), 58 % of 1337 fish from the Mediterranean Sea (Güven *et al.*, 2017) and 65 % of 178 fish from the Red Sea (Baalkhuyur *et al.*, 2018). Nevertheless, it should be mentioned that the direct comparison among several of these studies is difficult due to diferences in the methods used to isolate and quantify microplastics, the amount of tissue investigated, among other sources of variability.

Blue was the predominant colour of the microplastics found in *D. labrax*, *T. trachurus* and *S. colias*, in good agreement with previous studies with fish (Neves et al., 2015) and mammals (Hernández-González *et al.*, 2018) from the NE Atlantic Ocean. The predominance of blue over other colours found in the present study may have been due to a higher abundance of blue microplastics in seawater, a higher contamination of fish prey by blue microplastics, and/or to preferential active ingestion of blue microplastics by fish because they mistake them more with food than microplastics of other colours. Blue microplastics were the most abundant ones in NE Atlantic seawater (Lusher *et al.*, 2014) and sediment samples (Woodal et al., 2014). Being more abundant, blue microplastics have a higher probability of be uptaken by fish and their prey than microplastics of other colours. All the investigated

species are visual predators, colour is an important clue for prey perception by this type of predators, and microplastics may be ingested by confusion with prey with colour likely playing an important role (de Sá *et al.*, 2015). Therefore, *D. labrax*, *T. trachurus* and *S. colias* may have also actively ingested mainly blue microplastics because this is the colour of their most important or preferential prey (*e.g.* Bessa *et al.*, 2018; Ory *et al.*, 2018a; Herrera *et al.*, 2019). For example, Herrera *et al.* (2019) suggested that blue was the predominant colour of microplastics found in *S. colias* from Canary Islands coasts, possibly because they feed on local copepods, and some of them are blue. Moreover, in deep waters, fish prey may look bluewish when seen against light coming from water surface, since on reaching a depth of 100 m or more, light's blue component becomes completely predominant in the ocean (Blaxter, 1980; Archer, 1995). The second most frequent colour of microplastics recovered from the analysed fish was whithish. As for blue microplastics, this may be due to a high abundance of whithish microplastics in NE Atlantic Ocean seawater, high contamination of prey by whitish microplastics, and active ingestion by fish due to confusion with whithish prey.

All the microplastics recovered from *T. trachurus* were blue or whitish, whereas in the other species more colours were found, although at very low percentages. Differences in feeding ecology and other ecological characteristics may have contributed to this finding. For example, species spending more time in areas closer to the shore probably will be exposed to a higher diversity of microplastic colours (due to recent inputs) than species preferentially staying far from the coast likely being exposed mainly to aged microplastics that often have lost their original colour during their permanence in seawater. All the species were captured in waters of the Portuguese continental shelf. In the range of size of the fish analysed, *T. trachurus* feeds mainly on zooplankton, especially on crustaceans (*Nicthyphanes couchii*, *Meganyctiphanes norvegica and Euthemisto bispinosa*), but they also prey on fish, mainly on the blue whiting *Micromesistus poutassou* and on squid, *Allotheuthis spp*. (Murta *et al.*, 1993; Olaso *et al.*, 1999). *T. trachurus* generally stays in deep waters far from the shore, and typical spends the day in bottom/mid water moving to the surface at night to feed (Murta *et al.*, 1993). In the continental shelf of NE the Atlantic Ocean, *D. labrax* of size range comparable to analysed specimens are mainly piscivorous and preferentially feed on smaller pelagic fish, mainly mackerel (*Scomber scombrus*), sardine (*Sardina pilchardus*), anchovy (*Engraulis*

encrasicolus) and scads (*Trachurus spp.*), but they also feed on cephalopods and crustaceans (Spitz *et al.*, 2013). Often, *D. labrax* of size comparable to the investigated fish is found relative close to the shore and in estuaries, except in the winter when they generally migrate to deeper waters. Regarding *S. colias* of size comparable to the analysed range, in Portuguese coastal waters it feeds mainly on zooplankton (mainly *Calanus helgolandicus* and *Centropages chierchiae*) but also ingests phytoplankton, fish eggs, cephalopods, and small pelagic fish (Martins *et al.*, 2013; Garrido *et al.*, 2015). Generally, *S. colias* is found more close to the shore than *T. trachurus* adults. Therefore, ecological differences may explain at least partially the distinct diversity of microplastic colours between *T. trachurus* and the other species.

From the total number of microplastics recovered (all species), only 2 pellets (1 %) were found, suggesting that pellets are considerably less abundant in NE Atlantic Ocean water than fibers and fragments. These results are in good agreement with previous findings in NE Atlantic Ocean water (Lusher *et al.*, 2014). Fibers were more abundant in fish (54 %) than fragments, in agreement with other studies, such as: 66 % in fish from Portuguese coastal waters (Neves *et al.*, 2015), 97 % in fish from the Mondego River estuary, central coast of Portugal (Bessa *et al.*, 2018), 68 % in fish from the English Channnel (Lusher *et al.*, 2013), 70 % in fish from the Mediterranean Sea (Güven *et al.*, 2017) and 74 % in fish from Canary Islands coast (Herrera *et al.*, 2019).

Fibers uptaken by the investigated fish may have come from ropes, nets and other materials associated to fishery diretly input into marine waters, and also from continental sources (*e.g.* washing machines, textile industry, harbour industry, river/estuarine fishery). The predominance of fibers over fragments in gills of all the species suggests that fibers are more abundant in seawater of fish habitat because microplastics present in gills were uptaken through passive water filtration. However, the relative percentage of fibers and fragments in the gastrointestinal tract reveal differences among species and suggests contribution of active and preferential ingestion of microplastics with particular shape by fish. In addition to colour, shape is also important to prey-perception by visual predator fish (Blaxter, 1980). Threfore, *D. labrax* may mistake fibers with food more than fragments because it feeds preferentially on smaller fish that have alongated shape, whereas the opposite happens with *T. trachurus* and *S. colias* because they feed mainly on zooplankton

species and several of them have more spherical shapes. In addition to shape, other processes may contribute to differences in the predominant type of microplastics in fish gastrointestinal tract among species (*e.g.* differences in grastrointestinal absorption and elimination rates of fibers and fragments; differences among species in such rates).

As shown in Fig. 5.5, microplastics of different size ranges were found in fish gastrointestinal tract. Microplastics present in the gastrointestinal tract were uptaken through fish mouth and thus both large and very small particles were able to enter. Also, as previously discussed, fish likely ingested some microplastics actively (confusion with prey), and fish prey may also contain microplastics. Size contributes to prey perception by visual predators and microplastics with size comparable to prey are more prone to be actively ingested by fish (Galloway *et al.*, 2017; Lehtiniemi *et al.*, 2018). As all the species analysed are visual predators, possibly they ingested relative large microplastics with size comparable to some of their prey actively. Several studies (*e.g.* de Sá *et al.*, 2015; Ory *et al.*, 2018 a,b) also suggest that at least part of microplastics ingested by fish are uptaken actively because they were taken as food. In addition to colour, shape and size, odour may also contribute to microplastic active ingestion by fish (van der Lingen, 1994; Markic *et al.*, 2018). Indead, during their long permanence in the marine environment, microplastics may adquire odours similar to prey eliciting predatory behaviour (Savoca *et al.*, 2017; Procter *et al.*, 2019). Laboratory studies suggest that particles with size < 1230 μm may elicit fish feeding behaviour more by chemical stimulation than by visual stimulation (van der Lingen, 1994). The analysed specimens had microplastic fragments (96 % in *D. labrax*, 89 % in *T. trachurus* and 93 % in *S. colias*) and fibers (59 % in *D. labrax*, 73 % in *T. trachurus* and 54 % in *S. colias*) lower than 1230 μm in the gastrointestinal tract. Thus, it is possible that part of them were also ingested due to chemically-induced feeding stimulation.

After ingestion, some microplastics were likely internalized, others may have been retained in the gastrointestinal tract, whereas the remaining ones were likely eliminated. Microplastics retention in the gastrointestinal tract can cause false food satiation leading to decreased food consumption, intestinal obstruction and physical injury ultimately resulting in death (Carpenter *et al.*, 1972; Derraik, 2002; Ryan *et al.*, 2009; Duis and Coors, 2016; Jovanović, 2017). Moreover, in the gastrointestinal tract, release of chemicals adsorbed to microplastics may occur leading to the entry

of such substances into the blood streem. Also, in the digestive system of aquatic animals, microplastics can be fragmented into smaller particles (Dawson *et al.*, 2018) facilitating internalization. Elimination of microplastics from the gastrointestinal tract along with faeces occurs (Karakolis *et al.*, 2018).

The microplastics found in gills resulted from their retention in this organ during water filtration. This process and the uptake of microplastics through gills depend of microplastic size, and of the morphology and efficiency of the filtering apparatus (Collard *et al.*, 2017b). Data of Fig. 5.5 indicate retention of microplastics with size < 100 μm up to 3000 μm in gills of the studied species. Microplastics stuck in gills may decrease respiratory efficiency leading to hypoxia (Movahedinia *et al.*, 2012). Moreover, microplastics can cause physical damage in gills, such as breakage of filaments (Jabeen *et al.*, 2018), facilitating the entry of microplastics and other particles, and increasing the probability of infections (Movahedinia *et al.*, 2012; Jabeen *et al.*, 2018). Gill damage, hypoxia and infections may ultimately lead to death.

The presence of microplastics in dorsal muscle of all the analysed species indicates internalization of the particles. As absorption of microplastics lower than 150 μm may occur (EFSA, 2016), likely the most part of the microplastics with size in this range found in fish dorsal muscle resulted from absorption in the gastrointestinal tract and gills (Fig. 5.6). Fish may also uptake very small microplastics through the skin, especially when they have skin alterations or lesions (Handy *et al.*, 2008; Abbasi *et al.*, 2018). Thus, although skin alterations lesions were not noticed during the physical visual observation of fish, the possibility of uptake through skin cannot be excluded at least for the smallest microplastics found in dorsal muscle. As shown in Fig. 5, large fibers (up to 2363 μm) and large fragments (up to 490 μm) were also found in the dorsal muscle of fish from the species analysed, indicating that somehow they entered into fish body and reached internal tissues. Some of the fibers were very thin and thus their absorption could have occurred. Regarding other large fibers and fragments, they could have entered through the skin if it was damaged even if damage was not evident by naked eye observation. Another possibility is uptake through lesions in the gastrointestinal tract or in gills that fish may had due to long-term contact with microplastics (Jabeen *et al.*, 2018) or other abiotic or biotic stressors in their natural habitat. Moreover, fibers uptake by fagocytosis may have occurred. The presence of large microplastics (> 1000 μm and > 5000 μm in fish

muscle was reported before (Abbasi *et al.*, 2018; Akhbarizadeh *et al.*, 2018) but the mechanisms involved were not clearly demonstrated yet. Indeed, this deserves further investigation as the presence of relatively large fibers in fish muscle raises additional concerns regarding the microplastic paradigm.

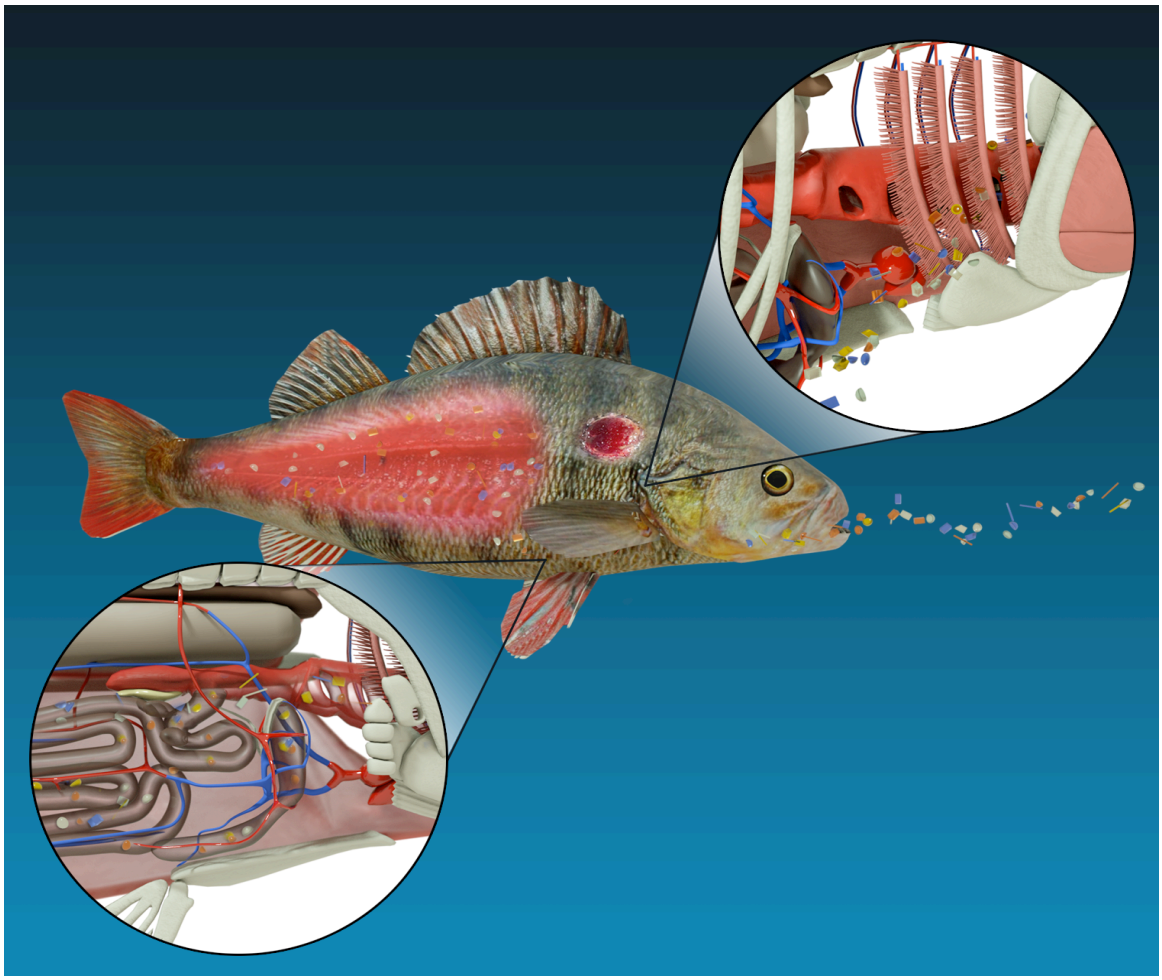

Figure 5.6. Conceptual model illustrating capture, retention and internalization of microplastics by fish species (kindly designed by Ella Maru).

Independently of the mechanisms involved in microplastic internalization, their presence in dorsal muscle also indicates that after entering into the blood circulation, they were distributed through the body and stored in muscle tissue. Possibly, smaller microplastics entered into muscle cells, whereas larger ones remained in the intersticial tissue. During fish body distribution, probably some microplastics reached other internal tissues and organs too. The fate of microplastics inside the fish body is not yet clearly understood (Jovanović, 2017; Abbasi *et al.*, 2018) but the size, chemical composition, charge and molecular weight, among other properties of the

particles, likely influence it (Collard *et al.*, 2017a). Some microplastics can reach internal tissues and organs (*e.g.* liver), as evidencied in the present work and other studies (Collard *et al.*, 2017a; Abbasi *et al.*, 2018, Akhbarizadeh *et al.*, 2018). Moreover, at least nanoplastics are able to cross the blood-brain barrier and enter in the brain (Kashiwada *et al.*, 2016; Mattsson *et al.*, 2017). These findings raise concern on the potential long-term accumulation of microplastics in the body of animals and humans, and more studies are needed.

5.5.2. Fish biomarkers

Since in all the investigated species no significant differences of body length, body weight and Fulton's condition factor between fish with and without microplastics were found, the two groups can be compared regarding the biomarkers investigated.

Increased LPO levels indicate lipid peroxidation damage. Therefore, fish with microplastics had more lipid peroxidation in brain, gills and muscle than fish without microplastics. Lipid oxidative damage can lead to a wide range of adverse effects. Gill lipid peroxidation damage may compromise respiration, biotransformation of xenobiotics in gills, among other crucial processes (Evans, 1987; Pandey et al., 2008). Lipid peroxidation in muscle may disrupt muscular (*e.g.* cellular energy production) and neuromuscular functions resulting in deficit of energy, problems of movement coordination, decrease of the swimming performance and several other adverse effects (Vieira *et al.*, 2009). Lipid peroxidation damage in the brain may cause the disruption of membranes of pre-synaptic vesicles containing neurotransmitters resulting in increased levels of neurotransmitters into synaptic clefs (Hilfiker *et al.*, 1999), among other types of neurotoxicity (Bradbury *et al.*, 2008). Several laboratory studies documented lipid oxidative stress and damage induced by microplastics in several fish species, such as *D. labrax* (Barboza *et al.*, 2018b,c), *P. microps* (Ferreira *et al.*, 2016) and *S. aequifasciatus* (Wen *et al.*, 2018), and other aquatic species (*e.g.* Ribeiro *et al.*, 2017; Guilhermino *et al.*, 2018; Oliveira *et al.*, 2018; Yu *et al.*, 2018). These findings support the hypothesis of a relation between fish contamination by microplastics and increased lipid oxidative damage suggested by the results obtained in *D. labrax*, *T. trachurus* and *S. colias*.

In addition to brain lipid oxidative damage (~ 2 fold LPO increase), fish with microplastics also had increased AChE activity in the brain (~ 2 fold). Lipid

oxidative damage may have caused rupture of membranes of vesicles containing acetylcholine in pre-synaptic neurons resulting in increased release of the neurotransmitter into cholinergic synaptic clefts and overstimulation of post-synaptic receptors. To deal with this toxic effect, AChE production may have been induced. Moreover, if lipid peroxidation damage was of relatively low magnitude, as suggested by the ~ 2 fold increase of LPO levels found, and long-term exposure to low concentrations of LPO inducers continued as probably occurred in NE Atlantic Ocean sewater, fish may have gradually increased their AChE activity basal levels to degradate increased concentrations of acetylcholine in synaptic clefts caused by lipid peroxidation. Increase of AChE activity under exposure to low concentrations of AChE inhibitors in an attempt to cope with the excess of acetylcholine in the synaptic cleft is known to occur (NRC, 1982), including in fish (Jurkowski *et al.*, 1979). Therefore, it is likely that AChE induction may also occur under long-term exposure to low concentration of environmental contaminants causing brain lipid oxidation damage and excess of acetylcholine in cholinergic synaptic clefts. Indepently of the mechanisms involved, increased AChE activity in the brain indicates neurologic alterations, with potential negative effects on individual fitness (*e.g.* increased energetic demands, discoordination, confusion, visual impairment). The laboratory studies published so far showed that microplastics can cause AChE and ChE induction (*e.g.* Deng *et al.*, 2017), no significant effect or inhibition (e.g. Oliveira *et al.*, 2013; Avio *et al.*, 2015; Luis *et al.*, 2015; Ribeiro *et al.*, 2017; Barboza *et al.*, 2018c, Ding *et al.*, 2018; Oliveira *et al.*, 2018; Yu *et al.*, 2018), dependending of the species, developmental stage, type of microplastics, other contaminants simultaneous present, and environmental conditions tested. However, it should be mentioned that in these studies, animals were exposed to microplastics for periods considerably shorter than in real scenarios where animals are exposed to such pollutants for generations. Also, the concentrations of microplastics tested are higher than those expected to occur in the area of the NE Atlantic Ocean inhabitated by the fish investigated here. Moreover, several chemicals stimulate biological responses at low concentrations and inhibit them at high concentrations, including some anticholinesterase agents.

Although the presence of microplastics in fish without particles in the gastrointestinal tract, gills and dorsal muscle cannot be excluded because not all the fish body was analised, such fish were less contaminated by microplastics than

fish with microplastics. All the fish from the same species were captured in the same area approximately at the same time, therefore their exposure to other contaminants, including lipid peroxidation and AChE activity inducers, was comparable, as well as capture-induced stress. After capture, fish maintenance and handling was the same. Thus, the results of biomarkers suggest that microplastics and/or associated chemicals caused muscle and gill lipid peroxidation damage, and neurotoxicity through lipid oxidative damage and AChE activity induction, decreasing fish individual fitness with potential negative effects at population level. Moreover, fish with decreased fitness are more prone to be infected by pathogenic and non-pathogenic agents, contributing to population fitness decrease. Furthermore, fish with decreased health status have poor nutritional quality for their predators and human consumers, and their infection by pathogenic agents is a treath to animal, environmental and public health. Thus, microplastic contamination of wild fish and other animals and its relation with biomarker alterations indicative of adverse biological and ecological effects needs further and urgent research.

5.5.3. Estimated intake of microplastics by humans consuming fish

Fish meal is an important component of a healthy human diet. However, the consumption of fish containing microplastics may represent a risk to human health especially in areas where fish consumption is high or in regions reported to be contaminated with large number of these small debris (Barboza *et al.*, 2018a).

The estimates made in the present study based on EFSA recommedations of fish consumption (EFSA, 2014; Table 5.2) indicate that adults or the general population eating 300 g of the analysed species per week will intake a mean of 16 MP items/ week or 842 MP items/year, corresponding to 0.054 MP items/g/week and 2.8 MP items/g/year. These values are comparable to those previously estimated for humans consuming fish species from the Persian Gulf, namely 17 MP items/week or 877MP items/year, corresponding to 0.056 MP items/g/week and 2.9 MP items/g/year (Akhbarizadeh *et al.*, 2018).

Based on fish consumption per capita (Table 5.3), our estimates suggest that the ingestion of microplastics by humans via consumption of fish may have high levels in individuals of countries where fish consumption is high, as in several European countries, including Portugal, the country with the highest consumption of

fishery and aquaculture products in Europe, and one the largest in the world (EUMOFA, 2018). In addition to fish, humans intake other food items known to be contaminated with microplastics (*e.g.* shellfish, salt, sugar and honey) (Liebezeit and Liebezeit, 2013; van Cauwenberghe and Janssen, 2014; Rochman *et al.*, 2015; Kim *et al.*, 2018; Peixoto *et al.*, 2019), and food contamination by microplastics during food preparation and meal consumption likely is a common situation (Catarino, *et al.*, 2018). Moreover, atmospheric fallout of microplastics may also result in deposition on the skin and inhalation, resulting in dermal exposure, airway and interstitial lung diseases, among other adverse effects with unknown consequences to human health (Wright and Kelly, 2017; Prata, 2018). Therefore, the exposure to microplastics might occur by several routes (*i.e.* ingestion, absorption by the skin or oral inhalation) and thus, the human uptake of these small items likely is considerable higher than the estimates based on fish consumption only.

Recently, microplastics were found in human stools for the first time (Schwabl *et al.*, 2018) indicating that humans indead ingest and eliminate these particles. Properties of microplastics likely affecting retention and clearance rates in the human body are the size, shape, polymer type, surface chemistry and charge, and other chemicals that the ingested microplastics may have (Smith *et al.*, 2018). After ingestion, absorption of microplastics may occur. The cellular uptake of microplastics may be strongly influenced by their interactions with surrounding biological components, such as proteins, phospholipids, or carbohydrates, as with nanoplastics (Lehner *et al.*, 2019). It has been assumed that only microplastics smaller than 150 µm can be absorbed by the human body (EFSA, 2016). If so and supposing that only the dorsal muscle was eaten, 46 % of the microplastics recovered from this tissue in the present study could have been absorbed by human consumers. In mussels from UK supermarkets, a corresponding estimate was ~ 40 – 60 % of the microplastics recovered from these animals (Li *et al.*, 2018). Although still provisory, it was estimated that > 90 % of the ingested micro- and nanoplastics are eliminated through the human body's excretory system (Smith *et al.*, 2018). Considering the global pollution by microplastics, the toxic effects that have been found in animals, and the risks to humans, more research on human exposure to microplastics and on the toxicity of these particles to humans are urgently needed.

5.6. Conclusions

This study provides evidences of microplastics contamination of fish species (*D. labrax*, *T. trucharus* and *S. colias*) captured in Portuguese coastal waters of the continental shelf (NE Atlantic Ocean) and aimed at being sold for human food consumption. All the analysed species had microplastics in the gastrointestinal tract, dorsal muscle and gills indicating contamination of fish by microplastics. Lipid oxidative damage in the brain, muscle and gills and increased brain AChE activity in fish containing microplastics were found. These findings indicate oxidative damage in gills and muscle, and neurotoxicity due to lipid peroxidation damage and increased AChE activity, and suggest relation between these alterations and the contamination of fish by microplastics. Moreover, the presence of microplastics in edible tissues of fish (*i.e.* dorsal muscle) highlight the need of further assessment of human food contamination by these particles, and the need of more research on the toxicity of microplastics to humans. Based on the mean of microplastics found in *D. labrax*, *T. trachurus* and *S. colias* and the recommendations of EFSA regarding fish intake, the estimated human dose intake (children with different ages, and adults or the general population) ranged from 112 to 842 microplastic items/g/year. The estimates of microplastics intake per year/capita for different countries showed that the exposure to microplastics through fish consumption may indeed be considerably higher in countries where fish consumption is high, such as Portugal (3078 microplastic items/year/capita). These estimates may contribute to the establishement of microplastic daily intake limits and to improve the basis for human risk assessment of microplastics. Moreover, the findings of the present study and several other available in the literature highlight the need of more research on microplastics and their effects following the WHO One Health approach.

5.7. Acknowledgements

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Chapter VI

Marine microplastic debris: an emerging issue for food security, food safety and human health

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6.1. Abstract

Recent studies have demonstrated the negative impacts of microplastics on wildlife. Therefore, the presence of microplastics in marine species for human consumption and the high intake of seafood (fish and shellfish) in some countries cause concern about the potential effects of microplastics on human health. In this brief review, the evidence of seafood contamination by microplastics is reviewed, and the potential consequences of the presence of microplastics in the marine environment for human food security, food safety and health are discussed. Furthermore, challenges and gaps in knowledge are identified. The knowledge on the adverse effects on human health due to the consumption of marine organisms containing microplastics is very limited, difficult to assess and still controversial. Thus, assessment of the risk posed to humans is challenging. Research is urgently needed, especially regarding the potential exposure and associated health risk to micro- and nano-sized plastics.

Keywords: Emerging food contaminants, microplastics, additives, seafood safety, toxicity, human health

6.2. Introduction

Plastics have been found worldwide in the marine environment, with estimates pointing to > 5 trillion of plastic debris (over 250,000 tons) afloat at sea (Eriksen *et al.*, 2014). A considerable amount of such plastic debris comes from continental sources entering into the marine environment mainly through rivers (Lebreton *et al.*, 2017), industrial and urban effluents, and runoff of beach sediments and neighbor fields. The other part, results from direct inputs, such as offshore industrial activities (*e.g.* oil and gas extraction, aquaculture), loss of nets in fisheries and litter released during sea activities, including tourism.

Among plastic litter, microplastics are of special concern regarding the environmental, animal and human health mainly due to their small size, the lack of technology available to quantify the presence of the smallest microplastics in the environment, and their potential to cause adverse effects on the marine biota and humans.

Microplastics have been defined as small pieces of plastic less than five millimeters in size with no lower limit established (GESAMP, 2016). The microplastics

present in the marine environment result from the fragmentation of larger plastic debris or may be introduced into the water and sediments already as micro- or nano-sized particles. Examples of microplastics are pre-production pellets and components of diverse products, such as fragments of fishing gear, packages and drink bottles, synthetic textiles, car tyres, paints, cosmetics and personal care products (*e.g.* facial cleaners, bath gels, toothpaste), and electronic equipment among others (Fendall and Sewell, 2009; Andrady, 2011; GESAMP, 2016). Consequently, microplastics encompass a very heterogeneous assemblage of particles that vary in size, shape, and chemical composition, among other properties (Hidalgo-Ruz *et al.*, 2012; Andrady, 2017).

Microplastics have been found worldwide, are highly persistent in the environment and are, therefore, accumulating in different marine ecosystems at increasing rates (Woodall *et al.*, 2014; Sebille *et al.*, 2015; Suaria *et al.*, 2016; Cózar *et al.*, 2017; van Waller *et al.*, 2017). Ocean gyres, estuaries and other coastal areas of heavily anthropogenic impacted regions are the ecosystems most polluted with these types of particles (Cózar *et al.*, 2014; Eriksen *et al.*, 2014; Galgani *et al.*, 2015; Peters and Bratton, 2016; Frère *et al.*, 2017).

Microplastics can be uptaken by a wide range of marine organisms by different processes (Lusher, 2015; GESAMP, 2016; Foley *et al.*, 2018). Among these, ingestion is believed to be a main microplastics exposure route for several marine species. In some cases, microplastics are ingested because they are confounded with prey, but ingestion through passive water filtration and deposit feeding activity also occur (de Sá *et al.*, 2015; Luís *et al.*, 2015; Naji *et al.*, 2018). After ingestion, microplastics absorption, distribution through the circulatory system, and entrance into different tissues and cells can occur, potentially resulting in several types of adverse effects (von Moos *et al.*, 2012; Wright *et al.*, 2013a; Pedà *et al.*, 2016; Avio *et al.*, 2017; Chae and An, 2017; Foley *et al.*, 2018). Such effects may be caused by the particles (*e.g.* physical damage or reaction to it and their chemical components) or chemicals added during the particle manufacturing or sorb to the microplastics during their use or permanence in the environment (Hartmann *et al.*, 2017). Moreover, microplastics (Farrell and Nelson, 2013; Mattsson *et al.*, 2017; Santana *et al.*, 2017), as well as the chemicals they contain (Hartmann *et al.*, 2017), can be transferred from marine prey to predators.

Microplastic ingestion has been observed in a range of animals of commercial interest that are consumed by humans as food, including fish (*e.g.* Atlantic cod, Atlantic horse mackerel; European pilchard, red mullet, European sea bass), bivalves (*e.g.* mussels, oysters), and crustaceans (*e.g.* brown shrimp) (Lusher *et al.*, 2013; van Cauwenberghe and Janssen, 2014; Avio *et al.*, 2015b; Devriese *et al.*, 2015; Bellas *et al.*, 2016; Brate *et al.*, 2016; Güven *et al.*, 2017; Bessa *et al.*, 2018). In addition to animals from wild populations, those from aquaculture can also ingest microplastics (Cheung *et al.*, 2018; Renzi *et al.*, 2018). For example, bivalves cultured in estuaries and coastal lagoons are prone to ingest microplastics because the water and sediments of many such areas are contaminated with these particles (Lusher *et al.*, 2017). Furthermore, aquaculture systems where fish, shrimps or other farmed species are fed with feeding materials produced from fish and other animals (*e.g.* fishmeal) may be contaminated with microplastics present in these products (GESAMP, 2016). The presence of plastic debris has also been detected in seafood sold for human consumption, as well as in fish and shellfish purchased from markets (*e.g.* Li *et al.*, 2015; Neves *et al.*, 2015; Rochman *et al.*, 2015; Karami *et al.*, 2017a). This evidence raises concerns regarding the ingestion of microplastics by humans through the consumption of marine species contaminated with these particles as food and the potential effects on the human health.

Knowledge about the effects of microplastics on the human health through the consumption of fish and shellfish is still in its infancy and requires further investigation (Law and Thompson, 2014; Barboza and Gimenez, 2015; Rist *et al.*, 2018). Therefore, our objective was to provide an overview of the evidence and potential risks associated with the presence of microplastics in the marine environment, integrating a dimension on the implications for human food security, food safety and health. Thus, the literature providing evidence of the presence of microplastics in human seafood and other food items was reviewed and discussed, and challenges and gaps in knowledge were identified.

6.3. Evidence of microplastics presence

6.3.1. Seafood

Despite the growing number of scientific investigations into the occurrence, transport, and distribution of microplastics in the marine environment and their adverse effects on marine life (Barboza and Gimenez, 2015), researchers have only

recently begun to consider the potential effects on human health. Research has shown that shellfish (including crustaceans and bivalves), and a high variety of commercially important fish species are often contaminated with microplastics (Table 6.1), being a potential route through which human consumers become exposed to these particles and the chemicals they contain (Bouwmeester *et al.*, 2015; GESAMP, 2016). For example, among the 25 species contributing mostly to global sea fishing (FAO, 2016), 11 were found to contain microplastics.

Van Cauwenberghe and Janssen (2014) were among the first researchers to estimate the potential exposure of humans to microplastics through the ingestion of seafood contaminated by these particles. They calculated that in European countries with high shellfish consumption, consumers ingest up to 11,000 microplastic particles (size range 5 – 1000 µm) per year, whereas in countries with low shellfish consumption, consumers ingest an average of 1,800 microplastics per year (van Cauwenberghe and Janssen, 2014), which is still a considerable exposure. Considering shrimp consumption only, estimates indicate about 175 microplastic particles (size range 200 – 1000 µm) per person per year (Devriese *et al.*, 2015). Regarding mussels consumed as food by humans, microplastics were found in *Mytilus edulis* and *M. galloprovincialis* from five European countries (France, Italy, Denmark, Spain and The Netherlands) (Vandermeersch *et al.*, 2015). In commercial mussels from Belgium, the number of microplastic particles varied from three to five fibers per 10 g of mussels (de Witte *et al.*, 2014). In other regions, several studies also reported the presence of microplastics in marine molluscs consumed as food by humans. For example, a study of microplastics in commercial bivalves in China reported that the average number of microplastics (size range 5 – 5000 µm) varied from 2 to 11 items per g and from 4 to 57 items per individual bivalve (Li *et al.*, 2015). In five shellfish species (including gastropods and bivalves) of the Persian Gulf, 3.7 to 17.7 particles per individual were found (Naji *et al.*, 2018). Concerning fish, microplastics were found in the Atlantic cod (*Gadus morhua*), the European hake (*Merluccius merluccius*), the Red mullet (*Mullus barbatus*) and the European pilchard (*Sardina pilchardus*) from several localities (*e.g.* Avio *et al.*, 2015b; Bellas *et al.*, 2016; Brate *et al.*, 2016; Liboiron *et al.*, 2016; Rummel *et al.*, 2016; Compa *et al.*, 2018). Rochman *et al.* (2015) demonstrated the presence of microplastics (size > 500 µm) in 9 % and 28 % of the gastrointestinal tracts from fish sold at

markets in the USA and Indonesia, respectively, with an average number of plastic pieces of 0.5 per individual fish in the USA samples and 1.4 in the Indonesian samples. Miranda and Carvalho-Souza (2016) also found microplastics in the digestive tract of two important species of edible fish (*Scomberomorus cavalla* and *Rhizoprionodon lalandii*) caught along the eastern coast of Brazil, and Neves *et al.* (2015) detected microplastics in 19.8 % of commercial fish from the Portuguese coast. Moreover, microplastics have been detected in the stomachs of commercially important fish from the Mediterranean (Romeo *et al.*, 2015), and in the gastrointestinal tract and liver of anchovies and sardines that sometimes are totally consumed (*i.e.* the entire fish) (Avio *et al.*, 2015b; Collard *et al.*, 2017a; Compa *et al.*, 2018).

Although the occurrence of microplastics in the gastrointestinal tract of fish does not provide direct evidence for human exposure since this organ is usually not consumed (Wright and Kelly, 2017), generally seafood species that we eat whole (*e.g.* some molluscs and crustaceans, and small or juvenile phases of fish) pose a greater threat to seafood contamination than for example gutted fish or peeled shrimp. However, the presence of microplastics in the eviscerated flesh (whole fish excluding the viscera and gills) of two commonly consumed dried fish species (*Chelon subviridis* and *Johnius belangerii*) was significantly higher than excised organs (viscera and gills), evidencing that the evisceration does not necessarily eliminate the risk of microplastics intake by human consumers (Karami *et al.*, 2017a). Moreover, the presence of microplastics was recently detected in the muscle of commercially important species of fish (Abbasi *et al.*, 2018; Akhbarizadeh *et al.*, 2018) and of a crustacean (Abbasi *et al.*, 2018). These findings raise concerns about possible implications for human consumers.

Table 6.1. Summary of studies reporting the occurrence of microplastics in shellfish and fish of commercial interest as food.

SHELLFISH

SPECIES NAME	LEVELS OF MP	SIZE RANGE	PARTS	TYPES OF DEBRIS	LOCATION	SOURCE
Alectryonella plicatula	10.78 ± 4.07 particles/individual	5 – 5000 µm	soft tissue	fibers, fragments, pellets	China *From local fish market*	Li *et al.*, 2015
Amiantis umbonella	6 particles/individual	10 – 5000 µm	soft tissue	fibers, fragments, pellets, film	Coastal water of The Persian Gulf, Iran, Asia	Naji *et al.*, 2018
Amiantis purpuratus	6 particles/individual	10 – 5000 µm	soft tissue	fibers, fragments, pellets, film	Coastal water of The Persian Gulf, Iran, Asia	Naji *et al.*, 2018
Cerithidea cingulata	12 particles/individual	10 – 5000 µm	soft tissue	fibers, fragments, pellets, film	Coastal water of The Persian Gulf, Iran, Asia	Naji *et al.*, 2018
Crangon crangon	0.68 particles/g individual	200 – 1000 µm	whole shrimp and peeled shrimp (abdominal muscle tissue)	fibers	Belgium	Devriese *et al.*, 2015
Crassostrea gigas	0.6 particles/g individual	> 500 µm	entire tissue	fibers	California, USA *From local market*	Rochman *et al.*, 2015
	0.47 particles/g individual	5 – 25 µm	soft tissue	not specified	Atlantic Ocean *Market from Brittany, France*	Van Cauwenberghe and Janssen, 2014
Cyclina sinensis	4.82 ± 2.17 particles/individual	5 – 5000 µm	soft tissue	fibers, fragments, pellets	China *From local fish market*	Li *et al.*, 2015
Eriocheir sinensis	13% ind. with MP	not specified	stomachs	fragments, filaments	Baltic coastal	Wójcik-Fudalewska *et al.*, 2016
Meretrix lusoria	9.22 particles/individual	5 – 5000 µm	soft tissue	fibers, fragments, pellets	China *From local fish market*	Li *et al.*, 2015
Mytilus edulis	0.36 ± 0.07 particles/g	5 – 25 µm	soft tissue	not specified	North Sea	Van Cauwenberghe and Janssen, 2014
Mytilus	4.33 ± 2.62 particles/	5 – 5000 µm	soft tissue	fibers,	China	Li *et al.*, 2015

galloprovincialis	individual			fragments, pellets	*From local fish market*	
	6.2–7.2 particle/g	760 – 6000 µm	valves, hepatopancreas and gills	filaments	Italy *From maricultured and natural stocks*	Renzi *et al.*, 2018
Mytilus spp.	3.2 ± 0.52 particles/ individual	200 - >2000 µm	soft tissue	fibers	Scottish coast	Catarino *et al.*, 2018
Modiolus modiolus	3.5 ± 1.29 particles/ individual	200 - >2000 µm	soft tissue	fibers	Scottish coast	Catarino *et al.*, 2018
Nephrops norvegicus	83% ind. with MP	not specified	stomach	filaments	Clyde, UK	Murray and Cowie, 2011
Penaeus semisulcatus	7.8 particles/individual	< 100 – > 1000 µm	muscle, skin	fibers	Musa estuary, Persian Gulf	Abbasi *et al.*, 2018
Patinopecten yessoensis	57.17 ± 17.34 particles/individual	5 – 5000 µm	soft tissue	fibers, fragments, pellets	China *From local fish market*	Li *et al.*, 2015
Perna perna	26.7 % ind. with MP	not specified	digestive tract and entire tissue	fibers	Santos Estuary, Brazil	Santana *et al.*, 2016
Pinctada radiata	11 particles/individual	10 – 5000 µm	soft tissue	fibers, fragments, pellets, film	Coastal water of The Persian Gulf, Iran, Asia	Naji *et al.*, 2018
Ruditapes philippinarum	5.72 ± 2.86 particles/individual	5 – 5000 µm	soft tissue	fibers, fragments, pellets	China *From local fish market*	Li *et al.*, 2015
Scapharca subcrenata	45 ± 14.98 particles/individual	5 – 5000 µm	soft tissue	fibers, fragments, pellets	China *From local fish market*	Li *et al.*, 2015
Sinonovacula constricta	14.33 ± 2.21 particles/individual	5 – 5000 µm	soft tissue	fibers, fragments	China *From local fish market*	Li *et al.*, 2015
Tegillarca granosa	5.33 ± 2.21 particles/individual	5 – 5000 µm	soft tissue	fibers, fragments	China *From local fish market*	Li *et al.*, 2015
Thais mutabilis	3 particles/individual	10 – 5000 µm	soft tissue	fibers, fragments, pellets, film	Coastal water of The Persian Gulf, Iran, Asia	Naji *et al.*, 2018

FISH						
SPECIES NAME	LEVELS OF MP (n) and (%) with mp	SIZE RANGE	PARTS	TYPES OF DEBRIS	LOCATION	SOURCE
Acanthurus gahhm	10; 100 %	2700 µm (mean)	gastrointestinal tract	fibers, film, fishing thread	Saudi Arabian Red Sea coast	Baalkhuyur *et al.*, 2018
Alepes djedaba	20; 100 % (8.00±1.22 item/10 g fish muscle)	< 100 - 5000 µm	muscle	fibers, fragments, pellets	Northeast of Persian Gulf	Akhbarizadeh *et al.*, 2018
Argyrosomus regius	5; 60 %	217 – 4810 µm	gastrointestinal tract	fibers, fragments	Portuguese Coast **From local market*	Neves *et al.*, 2015
	51; 75 %	> 9.07 µm	gastrointestinal tract	fibers, hard plastic, nylon	Mediterranean Sea	Güven *et al.*, 2017
Atherinopsis californiensis	7; 29 %	> 500 µm	gastrointestinal tract	fibers, fragments	California, USA *From local market*	Rochman *et al.*, 2015
Brama brama	3; 33 %	217 – 4810 µm	gastrointestinal tract	fibers	Portuguese Coast **From local market*	Neves *et al.*, 2015
Cetengraulis mysticetus	30; 3.3 %	≤ 1100 µm	gut	fragment	Southeast Pacific Ocean	Ory *et al.*, 2018a
*Clupea harengus*****	566; 2 %	> 1000 µm	gastrointestinal tract	fibers, fragments	North Sea	Foekema *et al.*, 2013
Cynoglossus abbreviatus	11; 12 (mean/individual)	< 100 – > 1000 µm	muscle, gut, gills, liver, skin	fibers, fragments	Musa estuary, Persian Gulf	Abbasi *et al.*, 2018
Cynoscion acoupa	552; 51 %	< 5000 µm	gut	Filaments, hard microplastics	Goiana estuary, Brazil	Ferreira *et al.*, 2018
*Decapterus macrosoma*****	17; 29 %	> 500 µm	gastrointestinal tract	fragments, styrofoam	Eastern Indonesia *From local market*	Rochman *et al.*, 2015
*Decapterus muroadsi*****	20; 80 %	5000 µm	gut	fragments	South Pacific	Ory *et al.*, 2017
Dentex macrophthalmus	1; 100 %	217 – 4810 µm	gastrointestinal tract	fibers	Portuguese Coast **From local market*	Neves *et al.*, 2015
Dicentrarchus labrax	40; 23 %	≤ 1000 – 5000 µm	gastrointestinal tract	fibers, fragments	Mondego estuary, Portugal	Bessa *et al.*, 2018
Diplodus vulgaris	40; 73 %	≤ 1000 – 5000 µm	gastrointestinal tract	fibers, fragments	Mondego estuary, Portugal	Bessa *et al.*, 2018

Engraulis encrasicolus	10; 80 %	124 – 438 µm	liver	not specified	Mediterranean Sea	Collard *et al.*, 2017a
	105; 15.24 %	not specified	gastrointestinal tract	fibers, fragments	Mediterranean Sea	Compa *et al.*, 2018
*Engraulis japonicus***	64; 77 %	10 – 500 µm	gastrointestinal tract	fragments, bead, filament, foam	Tokyo Bay	Tanaka and Takada, 2016
Engraulis mordax	10; 30 %	> 500 µm	gastrointestinal tract	fiber, film, monofilament	California, USA *From local market*	Rochman *et al.*, 2015
Epinephelus areolatus	5; 20 %	1800 µm (mean)	gastrointestinal tract	fibers, film, fishing thread	Saudi Arabian Red Sea coast	Baalkhuyur *et al.*, 2018
Epinephelus chlorostigma	3; 33.33 %	1900 µm (mean)	gastrointestinal tract	fibers, film, fishing thread	Saudi Arabian Red Sea coast	Baalkhuyur *et al.*, 2018
Epinephelus coioides	20; 100 % (7.75±2.16 item/10 g fish muscle)	< 100 - 5000 µm	muscle	fibers, fragments, pellets	Northeast of Persian Gulf	Akhbarizadeh *et al.*, 2018
*Gadus morhua****	80; 13 %	> 1000 µm	gastrointestinal tract	fibers, fragments	North Sea	Foekema *et al.*, 2013
	74; 1.4 %	< 5000 µm	gastrointestinal tract	fibers, fragments, film	Baltic Sea	Rummel *et al.*, 2016
	205; 2.4 %	2800 – 4200 µm	gastrointestinal tract	fragments	Coast of Canada	Liboiron *et al.*, 2016
	302; 18.8 %	< 5000 – > 20000 µm	stomach	fibers, fragments, granule, film	Norwegian coast	Bråte *et al.*, 2016
Lethrinus microdon	10; 20 %	1480 µm (mean)	gastrointestinal tract	fibers, film, fishing thread	Saudi Arabian Red Sea coast	Baalkhuyur *et al.*, 2018
Lipocheilus carnolabrum	7; 28.57 %	1870 µm (mean)	gastrointestinal tract	fibers, film, fishing thread	Saudi Arabian Red Sea coast	Baalkhuyur *et al.*, 2018
Lutjanus kasmira	10; 16.67 %	2160 µm (mean)	gastrointestinal tract	fibers, film, fishing thread	Saudi Arabian Red Sea coast	Baalkhuyur *et al.*, 2018
Merlangius merlangus	50; 32 %	1000 – 2000 µm	gastrointestinal tract	fibers, fragments, beads	English Channel	Lusher *et al.*, 2013
Merluccius merluccius	12; 29 %	217 – 4810 µm	gastrointestinal tract	fibers	Portuguese Coast	Neves *et al.*, 2015

	3; 100 %	10 – 5000 µm	gastrointestinal tract	fragments, line, film, pellet	Adriatic Sea	Avio *et al.*, 2015b
	12; 16.7 %	380 – 3100 µm	stomach	fragments, fibers, film, spheres	Spanish Atlantic	Bellas *et al.*, 2017
*Micromesistius poutassou***	27; 51.9 %	1000 – 2000 µm	gastrointestinal tract	fibers, fragments, beads	English Channel	Lusher *et al.*, 2013
Morone saxatilis	7; 29 %	> 500 µm	gastrointestinal tract	fibers, film, foam	California, USA *From local market*	Rochman *et al.*, 2015
Mugil cephalus	30; 60 % (wild)	< 2000 – > 5000 µm	gastrointestinal tract	fibers, fragments, sheet	Hong Kong Coast	Cheung *et al.*, 2018
	30; 16.7 % (captive)	< 2000 – 5000 µm	gastrointestinal tract	fibers	Hong Kong *From fish farms*	Cheung *et al.*, 2018
Mullus barbatus	11; 64 %	10 – 5000 µm	gastrointestinal tract	fragments, line, film, pellet	Adriatic Sea	Avio *et al.*, 2015b
	207; 66 %	> 9.07 µm	stomach and intestine	fibers, hard plastic, nylon	Mediterranean Sea	Güven *et al.*, 2017
	128; 18.8 %	380 – 3100 µm	stomach	fragments, fibers, film	Mediterranean coast	Bellas *et al.*, 2017
Mullus surmuletus	4; 100 %	217 – 4810 µm	gastrointestinal tract	fibers	Portuguese Coast	Neves *et al.*, 2015
	51; 35 and 49 %	> 9.07 µm	gastrointestinal tract	fibers, hard plastic, nylon	Mediterranean Sea	Güven *et al.*, 2017
Odontesthes regia	9; 11.1 %	not specified	gut	fragments	Southeast Pacific Ocean	Ory *et al.*, 2018a
Oncorhynchus tshawytscha	4; 25 %	> 500 µm	gastrointestinal tract	fibers	California, USA *From local market*	Rochman *et al.*, 2015
Opisthonema libertate	40; 5 %	≤ 3700 µm	gut	thread	Southeast Pacific Ocean	Ory *et al.*, 2018a
Parascolopsis eriomma	5; 60 %	1380 µm (mean)	gastrointestinal tract	fibers, film, fishing thread	Saudi Arabian Red Sea coast	Baalkhuyur *et al.*, 2018
Platycephalus indicus	16; 100 % (18.5±4.55 item/10 g fish muscle)	< 100 - 5000 µm	muscle	fibers, fragments, pellets	Northeast of Persian Gulf	Akhbarizadeh *et al.*, 2018
	12; 21.8 %	< 100 – > 1000 µm	muscle, gut,	fibers	Musa estuary, Persian	Abbasi *et al.*, 2018

			gills, liver, skin		Gulf	
Platichthys flesus	40; 13 %	≤ 1000 – 5000 µm	gastrointestinal tract	fibers, fragments	Mondego estuary, Portugal	Bessa *et al.*, 2018
Plectorhinchus gaterinus	6; 33.33 %	3310 µm (mean)	gastrointestinal tract	fibers, film, fishing thread	Saudi Arabian Red Sea coast	Baalkhuyur *et al.*, 2018
Pristipomoides multidens	10; 20 %	3800 µm (mean)	gastrointestinal tract	fibers, film, fishing thread	Saudi Arabian Red Sea coast	Baalkhuyur *et al.*, 2018
Rastrelliger kanagurta	10; 56 %	> 500 µm	gastrointestinal tract	fragments, film, monofilament	Eastern Indonesia *From local market*	Rochman *et al.*, 2015
Rhizoprionodon lalandii	6; 33 %	1000 – 5000 µm	stomach	pellets	Northeastern Brazil	Miranda and de Carvalho-Souza, 2016
*Sardinella longiceps*****	10; 60 %	500 – 3000 µm	gut	fragments	Indian Coast	Sulochanan *et al.*, 2014
*Sardina pilchardus*****	99; 19 %	10 – 5000 µm	gastrointestinal tract	fragments, line, film, pellet	Adriatic Sea	Avio *et al.*, 2015b
	7; 57 %	> 9.07 µm	gastrointestinal tract	fibers, hard plastic, nylon	Mediterranean Sea	Güven *et al.*, 2017
	2; 100 %	124 – 438 µm	liver	not specified	Mediterranean Sea	Collard *et al.*, 2017a
	105; 14.28%	not specified	gastrointestinal tract	fibers, fragments	Mediterranean Sea	Compa *et al.*, 2018
Saurida tumbil	4; 13.5 %	< 100 – > 1000 µm	muscle, gut, gills, liver, skin	fibers, fragments	Musa estuary, Persian Gulf	Abbasi *et al.*, 2018
Sillago sihama	17; 14.1 %	< 100 – > 1000 µm	muscle, gut, gills, liver, skin	fibers, fragments	Musa estuary, Persian Gulf	Abbasi *et al.*, 2018
Scyliorhinus canicula	20; 5 %	1500 µm	stomach	micro-bead	North Sea	Smith, 2018
	72; 15.3 %	380 – 3100 µm	stomach	fragments, fibers, film	Mediterranean coasts	Bellas *et al.*, 2017
*Scomberomorus cavalla*****	8; 62.5 %	1000 – 5000 µm	stomach	pellets	Northeastern Brazil	Miranda and de Carvalho-Souza, 2016
*Scomber japonicus*****	7; 71 %	> 9.07 µm	gastrointestinal tract	fibers, hard plastic, nylon	Mediterranean Sea	Güven *et al.*, 2017
	35; 31 %	217 – 4810 µm	gastrointestinal tract	fragments, fibers	Portuguese Coast	Neves *et al.*, 2015
	30; 3.3 %	≤ 2100 µm	gut	fragment	Southeast Pacific Ocean	Ory *et al.*, 2018a

*Scomber scombrus***	13; 31 %	217 – 4810 µm	gastrointestinal tract	fragments, fibers	Portuguese Coast	Neves *et al.*, 2015
	13; 30.8 %	< 5000 µm	gastrointestinal tract	fibers, fragments, film	Baltic Sea	Rummel *et al.*, 2016
Siganus canaliculatus	3; 29 %	> 500 µm	gastrointestinal tract	monofilament	Eastern Indonesia *From local market*	Rochman *et al.*, 2015
Solea solea	533; 95 %	< 100 – 500 µm	gastrointestinal tract	fibers, fragments	Adriatic Sea	Pellini *et al.*, 2018
Sparus aurata	110; 44 %	> 9.07 µm	gastrointestinal tract	fibers, hard plastic, nylon	Mediterranean Sea	Güven *et al.*, 2017
Spratelloides gracilis	4; 40 %	> 500 µm	gastrointestinal tract	fragments	Eastern Indonesia *From local market*	Rochman *et al.*, 2015
Sphyraena jello	15; 100 %	< 100 - 5000 µm	muscle	fibers, fragments	Northeast of Persian Gulf	Akhbarizadeh *et al.*, 2018
Thalassoma rueppellii	12; 8.33 %	1930µm (mean)	gastrointestinal tract	fibers, film, fishing thread	Saudi Arabian Red Sea coast	Baalkhuyur *et al.*, 2018
Thunnus alalunga	131; 12.9 %	< 5000 µm	stomach	fragments	Mediterranean Sea	Romeo *et al.*, 2015
Thunnus thynnus	34; 34.4 %	< 5000 µm	stomach	fragments	Mediterranean Sea	Romeo *et al.*, 2015
Trachurus trachurus	56; 28.6 %	1000 – 2000 µm	gastrointestinal tract	fibers, fragments, beads	English Channel	Lusher *et al.*, 2013
Trigla lyra	31; 19 %	217 – 4810 µm	gastrointestinal tract	fragments, fibers	Portuguese Coast	Neves *et al.*, 2015
Xiphias gladius	56; 12.5 %	< 5000 µm	stomach	fragments	Mediterranean Sea	Romeo *et al.*, 2015
Zeus faber	1; 100 %	217 – 4810 µm	gastrointestinal tract	fibers	Portuguese Coast	Neves *et al.*, 2015
	42; 47.6 %	1000 – 2000 µm	gastrointestinal tract	fibers, fragments, beads	English Channel	Lusher *et al.*, 2013
Clupea harengus	400; 0.25 % Two plastic particles were found in only 1 (*Sprattus sprattus*) out	> 20 µm	gastrointestinal tract	spherical particles	North Sea	Hermsen *et al.*, 2017
Limanda limanda						

Merlangius merlangus	of 400 individuals					
*Sprattus sprattus*****						
Chelon subviridis	30; Between 0 and 3 pigments and MP particles were isolated from each individual fish.	1 – 1000 µm	eviscerated flesh (whole fish excluding the viscera and gills) and excised organs (viscera and gills)	fragments, filaments, films	Malaysia **From local market*	Karami *et al.*, 2017a
Johnius belangerii						
Rastrelliger kanagurta						
Stolephorus waitei						

[****Indicates that this species is included in the list of the most commonly caught marine species worldwide according to FAO (2016)].

6.3.2. Other products consumed as food by humans or used in human food preparation

It should be highlighted that data on plastic fragments in food products are available in the European Commission's Rapid Alert System for Food and Feed (RASFF)'s portal and on the European Food Safety Authority's (EFSA) website. The RASFF and the EFSA report the presence of these contaminants, classified as foreign bodies, in a wide variety of human food items (ESFA, 2016; RASFF, 2015). The literature also provides several records of the presence of microplastics and other synthetic microparticles in human food and ingredients to prepare it, and in human drinking water. For example, microplastics were found in canned sardines and sprats (Karami *et al.*, 2018), salt (Yang *et al.*, 2015; Iñiguez *et al.*, 2017; Karami *et al.*, 2017b; Gündoğdu, 2018; Kosuth *et al.*, 2018), beer (Liebezeit and Liebezeit, 2014; Kosuth *et al.*, 2018), honey and sugar (Liebezeit and Liebezeit, 2013). Moreover, drinking water distributed in plastic bottles, glass bottles and beverage cartons obtained from grocery stores in Germany were also found to contain microplastics (Schymanski *et al.*, 2018) as does tap water from different countries (Kosuth *et al.*, 2018) (Table 6.2). Therefore, the occurrence of microplastics in other food items increases concern about the risks associated with ingestion and long-term exposure to multiple microplastic sources (Karami *et al.*, 2018). Despite the growing research interest in the occurrence of microplastics in seafood and other human food items, the information available is still limited to some regions around the world. More research is required to evaluate the presence of microplastics in consumed marine species, including edible tissues, especially from areas with high concentrations of these contaminants in the water and sediment. Qualitative and quantitative data are needed, including on the type, size, and components of microplastics.

Novel approaches to identify, isolate and quantify very small microplastic particles in tissues, seawater and sediment samples, and harmonization and standardization approaches are required to improve exposure quantification. Moreover, quality assurance methods, standardization and harmonization during the processing of samples are fundamental to ensuring an adequate comparison of data (Wesch *et al.*, 2017). Furthermore, in relation to the presence of microplastics in seafood and other food items, there is currently no regulatory framework (EFSA, 2016) that is needed to increase human food safety.

Table 6.2. Summary of studies reporting the occurrence of microplastics in other food items and drinking water.

OTHER FOOD ITEMS						
ITEM	LEVELS OF MP		SIZE RANGE	TYPES OF DEBRIS	LOCATION	SOURCE
Beer	24; 100 %	2 – 79 fibers/L, 12 – 109 fragments/L 2 – 66 granules/L	not specified	fibers, fragments, granules	Germany *From local supermarkets*	Liebezeit and Liebezeit, 2014
	12; 100 %	0 – 14.3 particles/L	100 – 5000 µm	fibers, fragments	USA *Purchased from Minneapolis, Duluth, Alpena, Michigan and Rochester (liquor stores, breweries)*	Kosuth *et al.*, 2018
Honey	19; 100 %	166 ± 147 fibers/kg of honey 9 ± 9 fragments / kg of honey	10 – 20 µm	fibers, fragments	Germany, France, Italy, Spain and Mexico *From local supermarkets or producers*	Liebezeit and Liebezeit, 2013
Sugar	5; 100 %	217 ± 123 fibers /kg of sugar 32 ± 7 fragments /kg of sugar	10 – 20 µm	fibers, fragments	*From local supermarkets*	
Salt	15; 100 %	550 – 681 particles/kg of sea salts 43 – 364 particles/kg of lake salts 7 – 204 particles/kg of rock/well salts	45 – 4300 µm	fragments, fibers, pellets, sheets	China *From local supermarkets*	Yang *et al.*, 2015
	17; 94 %	1 – 10 particles/kg of salt	> 149 µm	fragments, filaments, films	Australia, France, Iran, Japan, Malaysia, New Zealand, Portugal, South Africa *From local supermarkets*	Karami *et al.*, 2017b
	21; 100 %	50 – 280 particles/kg of salt	10 – 3500 µm	fibers	Spanish salt producers	Iñiguez *et al.*, 2017
	16; 100 %	16–84 item/kg in sea salt 8–102 item/kg in lake salt 9–16 item/kg in rock salt	20 – 5000 µm	fibers, fragments, films	Turkish *From local supermarkets*	Gündoğdu, 2018

	12; 100 %	46.7 – 806 particles/kg of salt	100 – 5000 µm	fibers, fragments	USA *Purchased from grocery stores and specialty shops in* Minneapolis (Salt ID – North Sea Salt; Celtic Sea salt; Sicilian Sea Salt; Mediterranean Sea Salt; Utah Sea Salt; Himalayan Rock Salt; Hawaiian Sea Salt; Baja Sea Salt; Atlantic Sea Salt; Pacific Sea Salt)	Kosuth *et al.*, 2018
Canned sardines and sprats	20; 20 %	not specified	190 – 3800 µm	fragments, filaments, films	Purchased from Australian and Malaysian markets and manufactured in Canada, Germany, Iran, Japan, Latvia, Malaysia, Morocco, Poland, Portugal, Russia, Scotland, Thailand, and Vietnam	Karami *et al.*, 2018
DRINKING WATER						
Mineral water	38, 100 %	2 – 44 particles/L in single-use plastic bottles 28 – 241 particles/L in returnable plastic bottles 4 – 156 particles/L in glass bottles 5 – 20 particles/L in beverage cartons	1 – 500 µm	fragments	Grocery stores from Germany	Schymanski *et al.*, 2018
Tap water and bottle water*	159; 81 %	0 – 61 particles/L	100 – 5000 µm	fibers, fragments, films	Cuba, Ecuador, England, France, Germany, India, Indonesia, Ireland, Italy, Lebanon, Slovakia, Switzerland, Uganda, USA * From USA	Kosuth *et al.*, 2018

6.4. Implications for the environment and human food security

It is now well-known that microplastics are highly persistent in the environment and are accumulating in different ecosystems at increasing rates (Andrady, 2017). For this reason, microplastics are considered an emerging issue of great concern. However, uncertainty and variability in the data are considered as one of the main factors that hinder a realistic assessment of the environmental risks associated with these microparticles. Thus, the real environmental risks of microplastics remain uncertain (Koelmans *et al.*, 2017b).

In recent years, laboratory experiments provided important results showing marine organisms ingest and uptake microplastics, that microplastics and the chemicals they contain induce adverse effects and are accumulated in a high number of species, that microplastics interact with the toxic effects of other environmental contaminants and other stressors, and that trophic transfer of microplastics and chemicals associated with them occurs. Several of the organisms that were investigated are keystone species in the ecosystems where they occur; thus their populations are crucial to the functioning of these ecosystems (Au *et al.*, 2017; Luis *et al.*, 2015).

Recent studies have documented the trophic transfer of microplastics in the wild (Welden *et al.*, 2018) and in laboratory conditions (Farrell and Nelson, 2013; Setälä *et al.*, 2014; Mattsson *et al.*, 2017; Nelms *et al.*, 2018), suggesting that micro- and nano-sized plastics can be transferred within different food webs. These findings raise concerns regarding the bioaccumulation and biomagnification of microplastics, increasing the risks and toxic effects mainly to top predators (Fonte *et al.*, 2016; Carbery *et al.*, 2018; Ferreira *et al.*, 2018).

Regarding adverse effects, laboratory experiments have shown various effects on marine animals caused by exposure to microplastics, such as mortality (Luis *et al.*, 2015; Gray and Weinstein, 2017), reduced feeding rate, body mass, and metabolic rate (Welden and Cowie, 2016), reduced allocation of energy for growth (Farrell and Nelson, 2013), decreased predatory performance (de Sá *et al.*, 2015), changes in behavioral responses and reduced swimming performance (Barboza *et al.*, 2018d), decreased fertilization and larval abnormalities (Martínez-Gómez *et al.*, 2017), neurotoxicity due to acetycholinesterase inhibition and oxidative stress (Oliveira *et al.*, 2013; Avio *et al.*, 2015a; Ribeiro *et al.*, 2017; Barboza *et al.,* 2018c), intestinal damage (Pedà *et al.*, 2016) and several other adverse effects (Wright *et al.*,

2013a; Foley *et al.*, 2018). All these evidences indicate that in the wild, especially in areas with high concentrations of plastic debris (*e.g.* heavily industrialized and urbanized areas and oceanic gyres), populations may be negatively affected and at least some of them could decrease over time, with potentially adverse consequences for environmental health, biodiversity conservation, ecosystem services, and human food security (in terms of reduced food availability for the human population). Thus, to properly assess and manage the risks, more studies on the effects of microplastics are needed, with special focus on the long-term effects induced by the exposure to ecologically relevant concentrations of microplastics commonly found in the environment.

6.5. Implications for human food safety

In the marine environment, microplastics may act as vehicles for chemicals, including those intentionally added during their manufacturing process, as well as environmental contaminants that may be adsorbed on to their surface during their use and permanence into the environment, such as styrene, toxic metals, phthalates, bisphenol A (BPA), polychlorinated biphenyls (PCB) and polycyclic aromatic hydrocarbons (PAHs) (Teuten *et al.*, 2009; Ashton *et al.*, 2010; Bakir *et al.*, 2012; Holmes *et al.*, 2012; Oliveira *et al.*, 2013; Rochman *et al.*, 2014a; Barboza *et al.*, 2018c; Hahladakis *et al.*, 2018; Massos and Turner, 2017). It should also be stressed that a wide range of chemical products used in plastic manufacturing are recognized as very toxic to animals and humans (*e.g.* carcinogens, endocrine disruptors, neurotoxic chemicals) (Thompson et al., 2009; Galloway and Lewis, 2016; Wright and Kelly, 2017; Hahladakis *et al.*, 2018). Moreover, pollutants and additives can be transferred from ingested microplastics to animal tissues and cause impairment of key functions that normally sustain health and biodiversity (Bakir *et al.*, 2014). For example, plastic particles may be toxic to organisms due to physical damage caused by small particles adsorbed to membranes and also if they cross the membrane by altering cellular functioning (Bhattacharya *et al.*, 2010; von Moos *et al.*, 2012). Additionally, several of the chemicals associated with microplastics may accumulate and biomagnify in marine trophic webs (Amiard-Triquet *et al.*, 1993; Kelly *et al.*, 2007). This increases the risk of toxic effects of these chemicals, especially to top predators and humans consuming species contaminated with microplastics or with chemicals released from these particles after their ingestion (Koelmans *et al.*, 2016;

Hartmann *et al.*, 2017; Hermabessiere *et al.*, 2017). Phthalates and bisphenol A, for example, should receive particular attention because their toxicity has been proven in animal studies and because of their ubiquitous presence in the environment and the human body (Vom Saal *et al.*, 2008; Koch and Calafat, 2009; Thompson *et al.*, 2009; Koelmans *et al.*, 2014). Regarding chemicals adsorbed to microplastics in the environment, the ability of these particles to adsorb very toxic metals has been demonstrated in some studies (Ashton *et al.*, 2010; Holmes *et al.*, 2012; Vedolin *et al.*, 2018). Among these metals, mercury is of special relevance because it is a global pollutant, is a common contaminant in the marine environment occurring at increased concentrations in several regions, is highly toxic to animals and humans, is accumulated by a high number of organisms, and some of its organic forms, particularly methylmercury, biomagnify in trophic webs (Eagles-Smith *et al.*, 2018).

In addition to chemicals, microbes and other organisms that have been found on plastic debris, generally described as the "plastisphere" (Zettler *et al.*, 2013), are of particular concern regarding the spread of exotic invasive species and pathogens. Some of these communities have been found to include pathogenic organisms, such as *Vibrio spp.* (*e.g.* de Tender *et al.*, 2015; Keswani *et al.*, 2016; Kirstein *et al.*, 2016), *Escherichia coli, Stenotrophomonas maltophilia, Bacillus cereus* (van der Meulen *et al.*, 2014) and *Aeromonas salmonicida* (Virsek *et al.*, 2017). Therefore, it has been suggested that plastic debris may increase the global risk of human and animal diseases via new contamination/infection routes, introduction of pathogens and their vectors into new areas through the environmental spread of microplastics or migrations of organisms contaminated with the pathogens mediated via microplastics (Keswani *et al.*, 2016). Additionally, the "plastisphere" may also include exotic invasive species (pathogens or not) that may contribute to loss of biodiversity and other negative ecological and economic impacts (Zettler *et al.*, 2013).

Available information on the presence of microplastics and their additives, associated pollutants and pathogens in fish and seafood, as well as the potential effects on human health, is still very scanty (Seltenrich, 2015; USEPA, 2015; GESAMP, 2016; Vethaak and Leslie, 2016). Although there is laboratory evidence that microplastics may increase the effects of chemical contaminants in fish, for example (Rochman *et al.*, 2013b; Pedà *et al.*, 2016; Barboza *et al.*, 2018c; Rainieri *et al.*, 2018), there is little evidence from field studies that the ingestion of microplastics

affects the bioaccumulation of pollutants (Lohmann, 2017). As predicted by chemical partitioning models, the relative importance of contaminants exposure mediated by microplastics compared to other exposure pathways may be limited (Koelmans *et al.*, 2013; Bakir *et al.*, 2016; GESAMP, 2016). Indeed, to date, at the current observed microplastic concentrations, there is little evidence to suggest that microplastics may increase the chemical contamination of seafood when compared with other environmental sources (*i.e.* water, sediments, food web) (Koelmans *et al.*, 2014; GESAMP, 2016; Koelmans *et al.*, 2016; Lohmann, 2017; Pittura *et al.*, 2018). This is confirmed by a recent field study with seabirds off the coast of Norway that showed only a negligible impact of ingested microplastics on tissue concentrations of POPs (Herzke *et al.*, 2016).

Considering the high concentrations of additives or contaminants reported in microplastics and their potential release from the microplastics upon ingestion, the internationally peer-reviewed expert panel reports by EFSA (2016) and Lusher *et al.* (2017) calculate that microplastics may have a negligible effect on the exposure to some pollutants and additives considering the total dietary exposure of humans. However, given the uncertainties surrounding this issue (*e.g.* assumptions in modeling exercises, the analytical challenges of measuring micro- and nano-sized microplastics in environmental matrices including seafood), the contribution of plastic-derived chemicals to the human diet should receive continued attention in future research.

The transfer of pathogens from ingested plastics to humans is still speculative. It is currently unknown to what extent plastic debris is involved in the spread of infectious diseases to humans. However, the survival of these pathogenic organisms on plastic debris has not been extensively studied, and understanding pathogen transmission and infection disease risks via the consumption of seafood will require further studies.

Other critical issues regarding animal, ecosystem and human health are the toxicological interactions between microplastics and other environmental contaminants of concern, as well as the influence of alterations due to global climate changes, especially temperature variations, on such interactions. Several studies with marine organisms published in recent years have been showing that microplastics influence the toxicity (increasing, changing the type or the pattern of the

effects) of a wide diversity of pollutants, such as polycyclic hydrocarbons (Oliveira *et al.*, 2013), metals (Luis *et al.*, 2015; Barboza *et al.*, 2018c) and pharmaceuticals (Fonte *et al.*, 2016). Moreover, temperature variation, especially temperature rise, has been found to influence such toxicological interactions (Ferreira *et al.*, 2016; Fonte *et al.*, 2016). The properties and concentrations of the microplastics and other chemicals tested, the conditions of the bioassays, and the tested species influence the findings reported. Therefore, more research on this topic is also needed.

6.6. Implications for human health

Even though scientific evidence demonstrates the presence of microplastics in several food products, there is no information available about the fate of microplastics in the human body following ingestion of the particles (Wright and Kelly, 2017; Rist *et al.*, 2018). In this context, adverse effects on human health are still controversial and not well understood. Thus, several important questions remain open, such as if microplastics play a role in the development of cancer in marine animals and, by extension, in humans (Erren *et al.,* 2015); what are the long-term effects of human exposure to microplastics considering the simultaneous exposure to such particles through several routes (Wright and Kelly, 2017), among several others.

Scientists speculate that microplastics with size bigger than 150 µm probably will not be absorbed while microplastics smaller than 150 µm may translocate from the gut cavity to the lymph and circulatory system, causing systemic exposure. However, the absorption of these microplastics is expected to be limited (≤ 0.3%). Only microplastics with size ≤ 20 µm would be able to penetrate into organs while the smallest fraction (0.1 > 10 µm) would be able to access all organs, cross cell membranes, the blood-brain barrier and the placenta – Fig. 6.1 (von Moos *et al.*, 2012; Browne *et al.*, 2008; Bouwmeester *et al.*, 2015; Galloway, 2015; EFSA, 2016; Lusher *et al.*, 2017). If so, it is possible that the distribution of microplastics in secondary tissues, such as liver, muscle, and brain, may occur (Wright and Kelly, 2017). Moreover, it is expected that micro- and nanoplastic interactions with the immune system may potentially lead to immunotoxicity and consequently trigger adverse effects (*i.e.* immunosuppression, immune activation and abnormal inflammatory responses) (Lusher *et al.*, 2017; Wright and Kelly, 2017). Recently, *in vitro* studies with cerebral and epithelial human cells evidenced for the first time the

potential of micro- (10 µm) and nano-plastics (40–250 nm) to cause cytotoxic effects at cell level in terms of oxidative stress (Schirinzi *et al.*, 2017), reinforcing the scientific speculations on the possible consequences for human health.

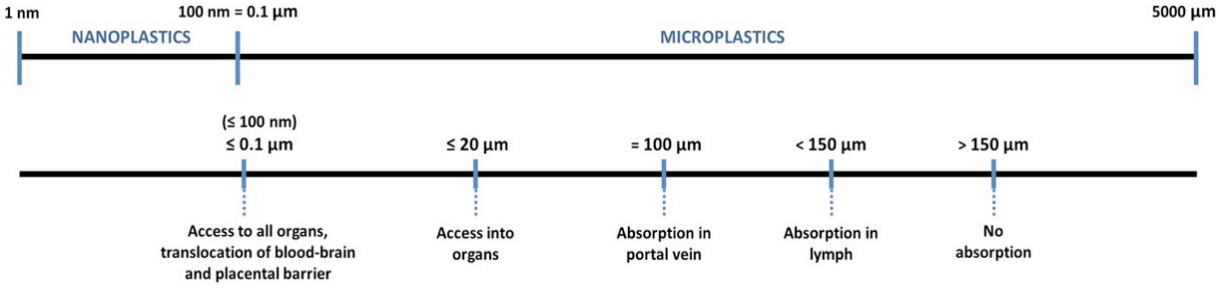

Figure 6.1. Fate of micro- and nanoplastics in mammalian bodies (adapted from Lusher *et al.*, 2017).

Therefore, the knowledge in this field is still very limited and there is little evidence on the impact on human health from eating microplastics. A major challenge regarding this point is that we do not know the amounts of very small microplastics, including those with a size able to enter cells, in the water, sediments, organisms and air; thus the assessment of biota and human exposure is not possible. It should be noted that the microplastics encountered in the commercial species of all studies mentioned in Table 6.1 were limited to particles in the (upper) micro-size range. From these studies, it can be concluded that, in general, the prevalence of microplastics in seafood is typically low, suggesting that dietary exposure is likely to be low. However, it is worth noting that we are vulnerable to other exposures, such as airborne microplastics (Prata, 2018). In this regard, it has recently been demonstrated that the potential for human ingestion of fibers resulting from domestic dust during a meal may be higher than fiber intake through consumption of mussels (Catarino *et al.*, 2018). Based on the above considerations, although there have been efforts in the attempt to estimate the human intake of microplastics, actual exposure will fall within vast margins and may, for this reason, remain difficult to quantify in practice (Santillo *et al.*, 2017). Furthermore, our understanding of the risks that microplastics pose to human health is still in the early stages (Koelmans *et al.*, 2017a); thus a proper risk assessment is not yet possible. In this way, adopting food safety risk analysis frameworks to evaluate hazards and risks to consumers posed by seafood contaminated with microplastics is of extreme

necessity (Lusher *et al.*, 2017). An analysis and assessment of the potential health risk of microplastics for humans should include the dietary exposure from a variety of foods across the total diet (GESAMP, 2016), and the best understanding of various parameters such as particle size, polymeric composition, particle shape, surface area, density, persistence, sorbed pollutants, additive content and toxicological consequences is a prerequisite to proper risk assessment (Hale, 2018).

Thus, the subsequent effects of microplastics on human health should be viewed with caution, since there is a large discrepancy between the current knowledge based on scientific evidence of the real implications for human health and the magnitude of the problem that has been addressed by the media (Rist *et al.*, 2018; Wright and Kelly, 2017). The researchers face several challenges that need to be explored and clarified, and further research is needed to understand the effects of these particles on the human body. In this way, knowledge about the real effects of microplastics on human health is an area for research that should be explored in the coming years.

6.7. Final remarks

The contamination of oceans by microplastics is of concern not only because of the ecological impacts but also because they may compromise food security, food safety and consequently human health. The presence of microplastics in species used for human consumption is a global problem and we are vulnerable to microplastic exposure through the consumption of seafood and other human food items, as well as through other routes such as air. Nevertheless, information on the occurrence of microplastics in these products is scarce, the exposure levels are in general largely unknown, and the potential effects on consumers are poorly understood. This information is necessary to provide a basis for a sound risk assessment. Understanding the processes and mechanisms involved in the entry and assimilation of microplastics in human tissues and their potential effects on human health is a priority research area and should be explored in the coming years. In this regard, we identified some challenges or knowledge gaps in this field (Box 1).

Box 1
Challenges and gaps of knowledge regarding microplastics and implications for human food security, food safety and health.

✔ Since microplastic concentrations are expected to increase in future, it will be increasingly important to regularly assess levels of microplastics in seafood and other food items.

✔ It is important to quantify the presence of microplastics in edible tissues of fish and shellfish. Also, the quantification in edible echinoderms, tunicates and algae also deserves investigation since in several countries they are widely consumed.

✔ Continuous monitoring programs will be required to evaluate the presence of microplastics in environmental compartments and thus avoid the reduction of global fish and shellfish stocks.

✔ Research also should focus on the contributing chemical and microbiological hazards and risks associated with ingested microplastics and in improving methods to evaluate the intake and translocation of these particles in humans.

✔ It is important to adopt food safety risk analysis frameworks to evaluate hazards and risks to consumers of fish, shellfish and food items contaminated with microplastics.

✔ There is a great need to study the assimilation of a range of microplastic sizes and compositions into human tissues and in the development of techniques capable of identifying the presence of microplastics in the human body (*e.g.* biopsies and tissue banks).

✔ Another area that deserves urgent attention is the presence of nano-sized plastics in seafood on which there is even less data in the literature.

✔ Research on analytical methods, toxicokinetics, and toxicity of micro- and nano-sized plastics is needed to improve the understanding of their potential impacts on seafood safety and human health.

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Chapter VII

General discussion

Given their persistence in the environment, plastics and microplastics have become a global environmental concern and a potential risk to animal and human populations. Moreover, microplastics are now considered global pollutants of priority study (GESAMP, 2016; Gallo *et al.*, 2018; Frias and Nash, 2019).

At the begining of the presente Thesis, to understand the state of the art regarding the contamination of the marine environment by plastics and micropastics and its biological effects, and identify specific topics deserving further investigation, two revisions of the literature, one on plastics and the other on microplastics, were performed (Chapter II and Chapter III, respectively). The Chapter II showed the threats of the marine environment contamination by macroplastics to wildlife and the environment, the global distribution of plastics in the world's oceans and seas and identified global actions and initiatives to reduce the impact of plastic litter in the marine environment. Chapter III showed that microplastics are ubiquitous in the world's oceans and seas, that they can be uptaken by a wide range of marine organisms and cause adverse effects, and that they can interact with the toxic effects of other environmental contaminants (*e.g.* metals, PAHs, nanomaterials, pharmaceuticals, among other) and other stressors (*e.g.* temperature). Moreover, this study identified several topics of special concern and deserving further investigation, such as: the toxic effects of microplatics on marine fish; the effects induced by mixtures of microplastics and other common contaminants of high concern on marine organisms; the possible influence of microplastics in the bioaccumulation of other common environmental contaminants by marine organisms; the contamination of fish species used for human consumption as food by microplastics and the resulting human exposure to microplastics through fish intake; and the implications of the global contamination by microplastics to animal, environmental and human health. Therefore, these topics were further investigated in the scope of the present Thesis.

In Chapter IV, the short-term (96 h) toxicity of microplastics and mercury, individually and in binary mixtures, to *D. labrax* juveniles were investigated. Mercury was selected because is an ubiquous pollutant of particular concern, is very toxic, can accumulate in organisms and humans, and its organic forms, particularly methylmercury, biomagnify in trophic webs. In a first phase of this experimental study, the behaviour of microplastics and mercury in the water were investigated. The results indicated that microplastics adsorb mercury from the seawater (Chapter

IV, Sections *4.2.4.1. and 4.2.4.2.*) suggesting binding of the metal to microplastic particles, and thus interaction between the two substances in the water (as hypothesized in Figure 4.5, Chapter IV, Section 3) likely influencing the mercury availability to fish. Moreover, the presence of microplastics had influence on the mercury bioconcentration in gills and bioaccumulation in the liver, muscle and brain of *D. labrax* (Chapter IV, Sections 4.2 and 4.3). Regarding biological effects on *D. labrax* juveniles, microplastics, mercury and their mixtures induced: neurotoxicity through brain acetylcholinesterase (AChE) activity inhibition, oxidative stress and lipid peroxidation damage; neuromuscular impairment through muscle cholinesterase (ChE) activity inhibition, oxidative stress and damage, and changes in cellular energy production enzymes (Chapter IV, Section 4.2); oxidative stress and lipid peroxidation damage in gills and liver (Chapter IV, Section 4.3), and decrease of the swimming velocity and time of resistance when swimming against the water flow (Chapter IV, Section 4.4).

Swimming capability and performance is dependent of several physiological functions (Oliveira *et al.*, 2013; Scoot and Sloman, 2004). Neurofunction and neuromuscular transmission are particular important because they are crucial to fish orientation and movement coordination (Lurman *et al.*, 2009). Considering that the swimming performance of fish can be negatively influenced by the impairment of neurotransmission processes in brain and muscle (Vieira et al., 2009), caused for example by the inhibition of brain and/or muscle enzymes activity, the results of the studies included in Chapter IV suggest that the reduction of the swimming performance observed in *D. labrax* juveniles exposed to microplastics, mercury and their mixtures was primarily due to the inhibition of brain AChE and muscle ChE activity. As other functions, swimming requires energy that must be produced at cellular level by anaerobic and/or aerobic pathways (Oliveira *et al.*, 2013). In this way, changes in the normal activity of the enzymes lactate dehydrogenase (LDH), involved in the anaerobic pathway of cellular energy production, and isocitrate dehydrogenase activity (IDH), involved in the aerobic pathway of cellular energy production, have been used as indicative of alterations in the pathways of cellular energy production induced by toxicants (Vieira *et al.*, 2009; Gorbatiuk, 2013). In the studies of Chapter IV, LDH activity was found to be increased in *D. labrax* juveniles after exposure to both substances alone and in mixtures, suggesting increased use of the anaerobic pathway of energy production likely to get additional energy

rapidely to face chemical stress. As a consequence of extra energy requirements to face such stress, energy available to other functions may be reduced. Therefore, it is also possible that in this work, fish need to allocate energy from locomotion and other functions to face chemical stress, resulting in a decreased swimming performance. Moreover, the inhibition of IDH activity observed may have contributed to oxidative stress and damage of muscle because this enzyme is important to the maintenance of cellular redox balance. It is well known that oxidative damage occurs when the antioxidant system is not able to remove the reactive oxygen species (ROS). High concentrations of ROS may cause adverse changes in cellular components such as lipids, proteins and DNA (Davies, 2001). Thus, organisms have defense mechanisms that can prevent the formation of ROS, react with these reactive intermediates, as well as repair the damages caused by them (Sies, 1993; Martínez-Alvarez *et al.*, 2005). However, when the defense antioxidant system is insufficient or inactivated, lipid peroxidation occurs, being this one of the most used indicator of oxidative damage (Gutteridge, 1995; Yadav, *et al.*, 2015). In Section 4.3, the results indicated induction of antioxidant enzymes activity, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione *S*-transferase (GST) in response to exposure to microplastics, mercury and their mixtures. The induction of these antioxidant enzymes in the liver was probably enough to cope with the oxidative stress induced by these substances because lipid peroxidation levels (LPO) did not increase significantly. However, in gills the induction of anti-oxidant enzymes was not enough to cope with the oxidative stress induced by the toxicants and lipidic oxidative damage occurred, as indicated by the increased LPO levels. The increase of brain and muscle LPO levels (Section 4.2) also indicated that the response of the antioxidant system was not sufficient to cope with the oxidative stress and thus oxidative damage occurred in the brain and muscle.

In fish, swimming performance is considered an ecologically relevant parameter because its impairment can cause mortality, growth delay and reduction of reproduction and therefore have a direct impact on the intrinsic population growth rate (Gravato and Guilhermino, 2009; Almeida et al., 2010; Vieira et al., 2008). In fish, it is also a sensitive biomarker because usually is affected before death occurrence (Scoot and Sloman, 2004; Calfee *et al.*, 2016). Therefore, it is important to know if and under what conditions of exposure is possible to observe behavioural

changes in fish species. In Chapter IV (Section 4), the results indicated that both microplastics and mercury, can cause decrease of fish swimming performance, including lethargic and erratic swimming behaviour, such as swimming upside down, erratic jumping and loss of swimming control. Moreover, mixtures of the two substances also induced signs of rapid fatigue. Under natural conditions, these behavioural changes may have serious repercussions on feeding, reproduction and survival of fish species and consequently may affect the success of wild populations.

In real scenarios, wild species are exposed simultaneously to mixtures of microplastics and other environmental contaminants by several routes and along their time-life. The results of this Thesis (Sections 4.2, 4.3 and 4.4) also indicated toxicological interactions between microplastics and mercury, highlighting the importance of investigating the combined effects of microplastics and other common contaminants on fish and other aquatic species.

In addition to their ecological relevance, fish are also an important food resource to humans. Therefore, in Chapter V the occurrence of microplastics in three fish species (*Dicentrarchus labrax*, *Trachurus trachurus*, *Scomber colias*) captured in Portuguese waters (NE Atlantic Ocean), landed in Matosinhos Port and aimed at being sold for human food consumption was investigated in relation to fish biomarkers (AChE, CHE, LPO levels), and the human exposure to microplastics through fish consumption was estimated. In general, the results indicated a considerable contamination of fish by microplastics, lipid oxidative damage, in the brain, muscle and gills, and neurologic alterations. Although the contributions of other stressors can not be excluded, such results suggest relation between these alterations and the contamination of fish by microplastics. As indicated by the findings of Chapter V and Chapter VI, humans ingest microplastics through fish and other seafood consumption. In addition, Chaper VI highlighted that the presence of microplastics in the marine environment has implications for human food security and safety, and for human health and wellbeing, and that human exposure through multiple routes of exposure (especially food, drinking water and air) increases the concern about the risks associated with the long-term exposure microplastics.

Chapter VIII

Concluding remarks and future perspectives

In general, the studies included in the present Thesis showed the global contamination of the marine environment by plastics and microplastics, that microplastics can be uptaken by fish and other organisms and induce diverse types of adverse effects, that microplastics influence the toxicity of other common contaminants in animals exposed to mixtures containing these particles, that fish used for human consumption contain microplastics, and that the environmental contamination by microplastics has implications to animal, environmental and human health.

The main conclusions of this Thesis are: a) microplastics are able to interact with mercury, another contaminant of great concern; b) exposure to microplastics and associated contaminants may cause adverse effects on fish health; c) microplastics can induce toxic effects *per se* and cause neurotoxicity, energy-related changes, and oxidative stress and damage; d) microplastics influence the toxicity of other contaminants in fish exposed to mixtures containing these particles; e) commercial fish species captured in Portuguese coastal waters (NE Atlantic Ocean) have microplastics in the gastrointestinal tract, dorsal muscle and gills; and f) the estimated exposure of the general European population (children of diferente ages, adults and the general population through marine fish consumption may range from 112 to 842 microplastic items/year, but may be higher in subpopulations consuming higher doses of fish.

This Thesis contributed to a better knowledge of the effects of microplastics and mixtures containing these particles on fish health and individual fitness, as well as to the environmental and human potential risks arising from the contamination of fish by microplastics. It contributed to the advance of knowledge in several specific topics in the scope of the microplastics paradigm and identified research needs, including in relation to the potential risks to human health. Regarding this topic, the review corresponding to Chapter VI was one of the two reference documents in a recent call for research projects in The Netherlands (research programme Microplastics & Health, ZonMw, The Netherlands) and therefore several gaps of knowledge identified in the review are expected to be adressed in the projects that will be funded through this programme.

In the continuation of the studies conducted in the scope of the present Thesis, further investigation is needed to improve our understanding and manage the risks inherent to the environmental contamination by microplastics. Some of the

priority topics are: a) long-term effects of microplastics on fish and other species; b) the relationships between the physico-chemical properties of microplastics and their toxic effects; c) the impact of ingested microplastics, leached plastic additives and adsorbed pollutants on marine biota; d) the potential for biomagnification of microplastics and microplastic-associated chemicals; e) the toxicological interactions between microplastics and other environmental contaminants of concern (including monomers, additives, byproducts and sorbed contaminants), as well as the influence of alterations due to global climate changes, especially temperature variations, on such interactions; f) the assessment of the occurrence of microplastics in human seafood and other food items and the effects of these particles on human food safety and security, and human health and wellbeing.

Chapter IX

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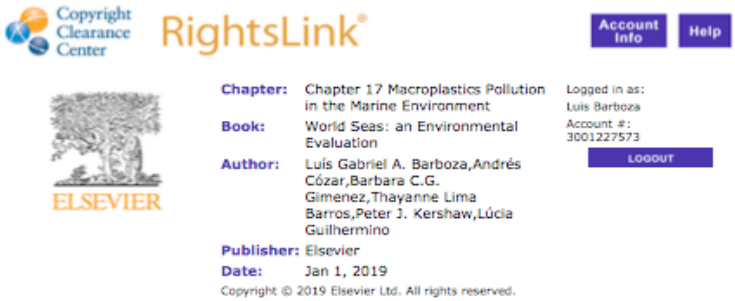
APPENDICES

♦ Appendix A
- Authorizations to include published book chapters and articles in the Thesis



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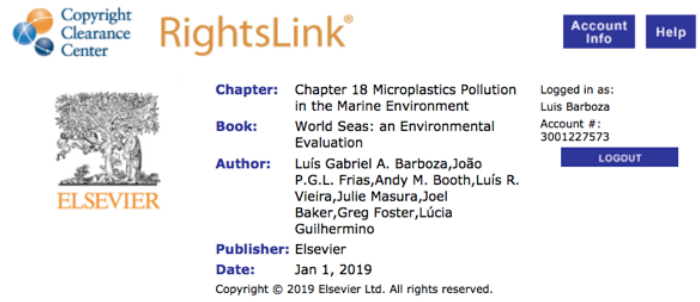

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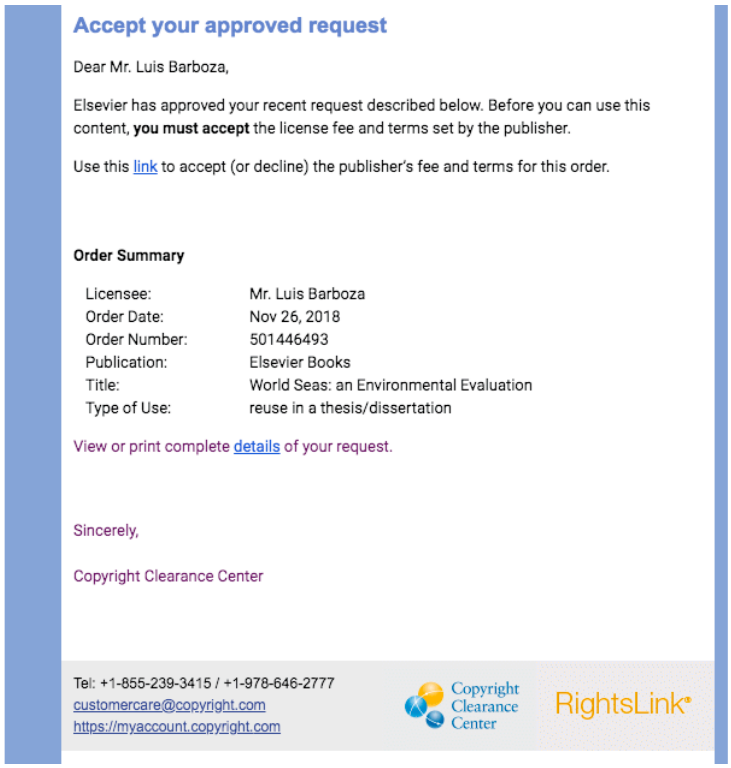

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Microplastics increase mercury bioconcentration in gills and bioaccumulation in the liver, and cause oxidative stress and damage in *Dicentrarchus labrax* juveniles

Luís Gabriel Antão Barboza, Luís Russo Vieira, Vasco Branco, Cristina Carvalho & Lúcia Guilhermino

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Marine Pollution Bulletin

Volume 133, August 2018, Pages 336-348

Review

Marine microplastic debris: An emerging issue for food security, food safety and human health

Luís Gabriel Antão Barboza [a, b, c], A. Dick Vethaak [d, e], Beatriz R.B.O. Lavorante [a, b], Anne-Katrine Lundebye [f], Lúcia Guilhermino [a, b]

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♦ Appendix B

– Supplementary information Chapter IV, Section 2

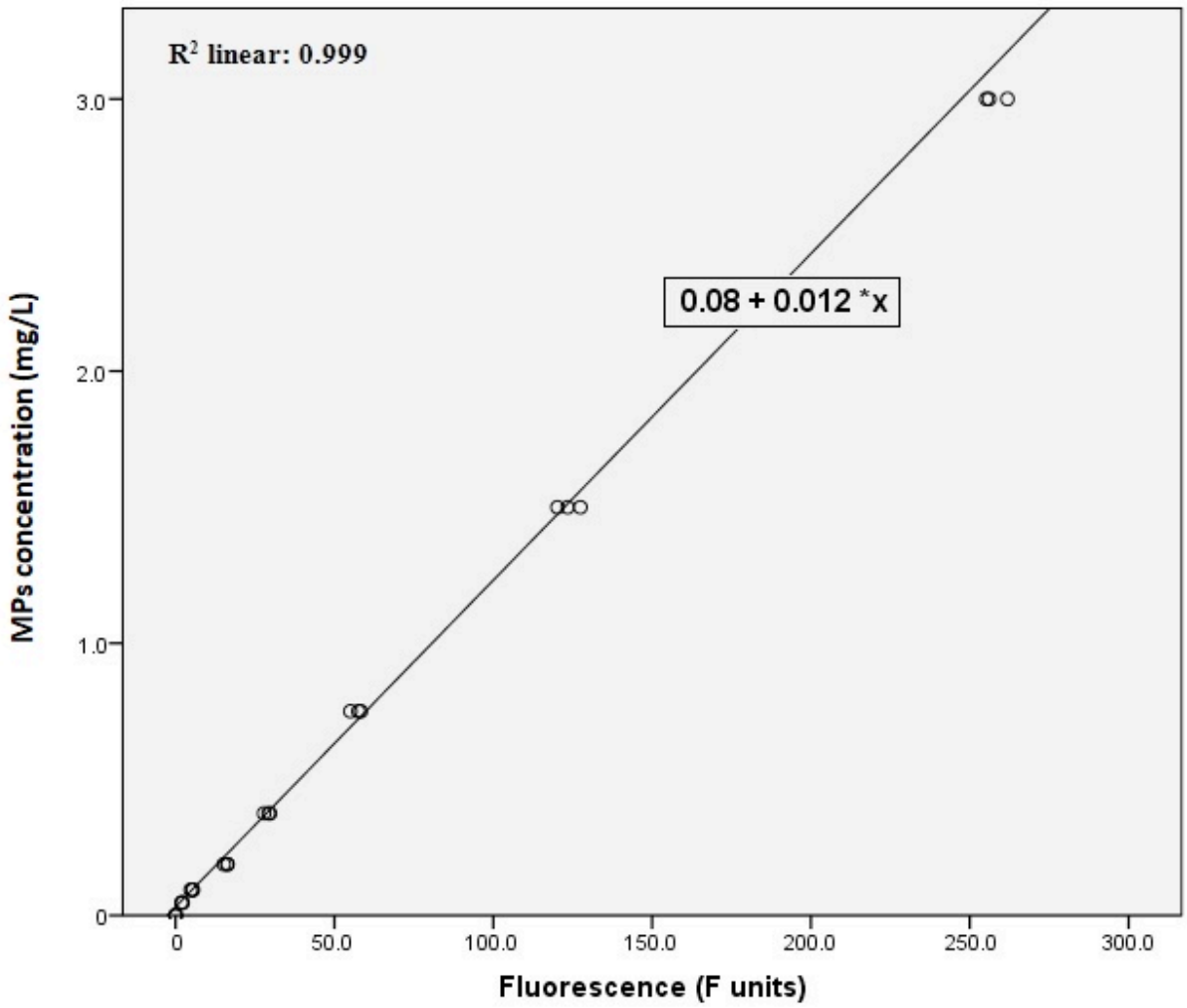

Figure S-1. Curve fluorescence versus concentration of microplastics (MPs) in filtered seawater and linear regression model: MPs concentration (mg/L) = 0.08 + 0.012 x fluorescence (F units). The results are the fluorescence of three independent replicates per nominal MPs concentration.

Table S-1. Obtained and certified concentrations (µg/g dry weight) in certified reference materials (CRM).

CRM		Hg (µg/g)
BCR 463	Certified	2.85 ± 0.16
	Obtained	2.68 ± 0.11

Table S-2. Curve fluorescence versus concentration of microplastics (MPs) in filtered seawater and linear regression model: MPs concentration (mg/L) = 0.08 + 0.012 x fluorescence (F units). The results are the fluorescence of three independent replicates per nominal MPs concentration. Table S-2. The actual concentrations of microplastics was estimated from the fluorescence readings (Fluor.) at freshly prepared test media using the linear model: actual MPs concentration (mg/L) = 0.08 + 0.012 x fluorescence (fluorescence units). Mid-point MPs conc.: mid-point concentration determined as: (actual concentration of MPs at 0 h + actual concentration of MPs at 24 h) / 2. The values are the mean of 12 samples from distinct test beakers with the corresponding standard error within brackets. The percentage of deviation (Dev.) of the actual concentrations (Conc.) from the nominal ones was calculated as: Dev. = 100 – (actual microplastics concentration x 100 / nominal concentration). The decay was determined was determined from the fluorescence readings of clean water (Cw) and old water (24 h) (Ow) as: decay (%) = 100 – (Ow x 100 / Cw). Conc. – concentration; Fluor – Fluorescence.

Presence of fish	Nominal MP conc. (mg/L)	Nominal Hg conc. (mg/L)	Fluor. 0 h (F units)	Fluor. 24 h (F units)	Mid-point actual MPs Conc. (mg/L)	Deviation (%)	Decay (%) 24 h
PRELIMINARY ASSAY							
No Fish	0.30	0	15.811 (±3.532)	11.120 (±1.956)	0.242 (±0.027)	10	30
		0.007	18.852 (±4.651)	11.687 (±2.602)	0.263 (±0.029)	1	38
		0.013	16.159 (±6.169)	11.428 (±2.998	0.246 (±0.040)	9	29
	0.80	0	58.002 (±6.736)	36.798 (±8.964)	0.649 (±0.077)	3	37
		0.007	57.728 (±11.824)	36.321 (±6.530)	0.644 (±0.089)	3	37
		0.013	57.649 (±6.390)	43.147 (±7.115)	0.685 (±0.067)	4	25
Total mean low					0.25 (±0.033)		
Total mean high					0.69 (±0.078)		
BIOASSAY							
Fish	0.30	0	15.627 (±1.579)	11.336 (±1.563)	0.242 (±0.017)	11	27
		0.017	19.293 (±2.818)	13.098 (±0.816)	0.274 (±0.021)	4	32
		0.027	17.986 (±3.100)	12.990 (±2.652)	0.266 (±0.033)	1	28
	0.80	0	61.141 (±3.022)	41.430 (±2.771)	0.695 (±0.029)	2	32
		0.017	59.274 (±4.075)	40.586 (±2.515)	0.679 (±0.021)	1	32
		0.027	60.688 (±5.310)	41.229 (±4.558)	0.692 (±0.052)	15	32
Total mean low					0.26 (±0.028)		
Total mean high					0.69 (±0.036)		

Table S-3. Results of two-way ANOVA with interaction comparing the effects of microplastics concentration and mercury concentration in the water on *Dicentrarchus labrax* mercury body burden.

Parameter	Factor	Level (mg/L)	Mean ± SD	Tukey test	F	p
Brain Hg Concentration	MPs	0	0.070 ± 0.012	a	$F_{(2, 48)} = 5.859$	0.005
		0.26	0.056 ± 0.022	b		
		0.69	0.067 ± 0.017	b		
	Hg	low	0.054 ± 0.018		$F_{(1, 48)} = 36.810$	0.000
		high	0.075 ± 0.012			
	MPs x Hg				$F_{(2, 48)} = 5.393$	0.008
Muscle Hg Concentration	MPs	0	0.422 ± 0.075	a	$F_{(2, 48)} = 5.759$	0.006
		0.26	0.352 ± 0.076	b		
		0.69	0.406 ± 0.116	a		
	Hg	low	0.339 ± 0.076		$F_{(1, 48)} = 37.465$	0.000
		high	0.448 ± 0.078			
	MPs x Hg				$F_{(2, 48)} = 6.377$	0.003

Table S-4. Results of the two-way Analysis of Variance with interaction investigating the effects of microplastics and mercury on several biological parameters of *Dicentrarchus labrax* comparing the effects of microplastics concentration and mercury concentration on the effects criteria at the end of the exposure period (96 hours). Fixed factors: microplastics concentrations (0.26 mg/L and 0.69 mg/L) and mercury concentrations (0.010 mg/L and 0.016 mg/L). SD – standard deviation.

Parameter	Factor	Level (mg/L)	Mean ± SD	Tukey test	F	p
Brain AChE	MPs	0	1.651 ± 0.2888	a	$F_{(2.72)}$ =5.445	0.006
		0.26	1.615 ± 0.3448	a		
		0.69	1.487 ± 0.2582	b		
	Hg	0	1.885 ± 0.1955	A		0.000
		0.010	1.508 ± 0.2244	B	$F_{(2.72)}$ = 53.209	
		0.016	1.362 ± 0.1218	C		
	MPs x Hg				$F_{(4.72)}$ = 2.707	0.037
Muscle ChE	MPs	0	0.880 ± 0.2088	a	$F_{(2.72)}$ = 7.690	0.001
		0.26	0.728 ± 0.2352	b		
		0.69	0.896 ± 0.2128	a		
	Hg	0	0.920 ± 0.1581	A	$F_{(2.72)}$ = 8.106	0.001
		0.010	0.853 ± 0.2593	A		
		0.016	0.731 ± 0.2258	B		
	MPs x Hg				$F_{(4.72)}$ = 8.793	0.000
Brain LPO	MPs	0	431.634 ± 209.706	a	$F_{(2.72)}$ = 9.402	0.000
		0.26	422.206 ± 161.442	a		
		0.69	510.127 ± 119.471	b		
	Hg	0	257.120 ± 82.672	A	$F_{(2.72)}$ =	0.000

		0.010	562.583 ± 104.562	B	118.424	
		0.016	454.655 ± 170.129	B		
	MPs x Hg				$F_{(4.72)}$ = 4.689	0.002
Muscle LPO	MPs	0	42.335 ± 29.142	a	$F_{(2.72)}$ = 0.596	0.554
		0.26	46.151 ± 18.210	a		
		0.69	44.628 ± 13.914	a		
	Hg	0	29.951 ± 16.599	A	$F_{(2.72)}$ = 33.555	0.000
		0.010	44.382 ± 16.222	B		
		0.016	58.781 ± 20.294	C		
	MPs x Hg				$F_{(4.72)}$ = 18.658	0.000
Muscle LDH	MPs	0	201.389 ± 36.388	a	$F_{(2.72)}$ = 7.943	0.001
		0.26	240.073 ± 75.751	b		
		0.69	201.082 ± 60.460	a		
	Hg	0	189.467 ± 59.046	A	$F_{(2.72)}$ = 7.730	0.001
		0.010	220.949 ± 47.854	B		
		0.016	232.128 ± 70.493	B		
	MPs x Hg				$F_{(4.72)}$ =18.896	0.000
Muscle IDH	MPs	0	32.260 ± 16.201	a	$F_{(2.72)}$ = 41.166	
		0.26	44.571 ± 13.582	b		
		0.69	16.502 ± 6.961	c		
	Hg	0	30.285 ± 11.150	A	$F_{(2.72)}$ = 1.646	0.200
		0.010	28.804 ± 16.921	A		
		0.016	34.246 ± 21.900	A		
	MPs x Hg				$F_{(4.72)}$ = 5.979	0.000

♦ Appendix C – Supplementary information Chapter IV, Section 3

Table S-1. Results of the one-way ANOVA or Kruskal-Wallis indicating significant differences among treatments for all biomarkers ($p \leq 0.05$).

Biomarker	Mean ± SD	Statistical analyses
SOD (gills)	28.039 ± 9.053	$\chi^2_{(8)} = 56.805$; $p = 0.000$
CAT (gills)	211.912 ± 23.913	$F_{(8,72)} = 108.339$; $p = 0.000$
GPx (gills)	5.383 ± 2.082	$F_{(8,72)} = 2.846$; $p = 0.008$
GR (gills)	10.443 ± 2.769	$\chi^2_{(8)} = 23.368$; $p = 0.000$
GST (gills)	101.508 ± 39.485	$F_{(8,72)} = 52.798$; $p = 0.000$
LPO (gills)	267.212 ± 142.743	$F_{(8,72)} = 2.703$; $p = 0.012$
SOD (liver)	31.491 ± 11.944	$F_{(8,72)} = 22.909$; $p = 0.000$
CAT (liver)	42.384 ± 17.676	$F_{(8,72)} = 23.275$; $p = 0.000$
GPx (liver)	24.226 ± 7.534	$\chi^2_{(8)} = 48.558$; $p = 0.000$
GR (liver)	19.919 ± 5.227	$\chi^2_{(8)} = 58.735$; $p = 0.000$
GST (liver)	163.908 ± 39.480	$F_{(8,72)} = 39.613$; $p = 0.000$
LPO (liver)	215.289 ± 61.875	$F_{(8,72)} = 2.767$; $p = 0.010$

Table S-2. Results of the two-way Analysis of Variance with interaction investigating the effects of microplastics and mercury on several biological parameters of *Dicentrarchus labrax* comparing the effects of microplastics concentration and mercury concentration on the effects criteria at the end of the exposure period (96 hours). (SOD and GR in the gills and GPx and GR in the liver could not be analysed because the ANOVA assumptions were not fulfilled). Fixed factors: microplastics concentrations (0.26 mg/L and 0.69 mg/L) and mercury concentrations (0.010 mg/L and 0.016 mg/L). SD – standard deviation.

Parameter	Factor	Level (mg/L)	Mean ± SD	Tukey test	F	p
Gills CAT	MPs	0	103.254 ± 37.679	a	$F_{(2.72)}$ =5.445	0.000
		0.26	128.560 ± 67.097	b		
		0.69	153. 370 ± 53.731	c		
	Hg	0	65.127 ± 19.69	A	$F_{(2.72)}$ = 283.558	0.000
		0.010	135. 295 ± 25.180	B		
		0.016	184. 761 ± 39.820	C		
	MPs x Hg				$F_{(4.72)}$ = 7.562	0.000
Gills GPx	MPs	0	5.111 ± 2.100	a,b	$F_{(2.72)}$ = 4.689	0.012
		0.26	6.281 ± 1.866	b		
		0.69	4.757 ± 2.030	a		
	Hg	0	5.894 ± 1.858	A	$F_{(2.72)}$ = 4.999	0.009
		0.010	4.433 ± 2.074	B		
		0.016	5.821 ± 2.043	A		
	MPs x Hg				$F_{(4.72)}$ = 0.847	0.500
Gills GST	MPs	0	86. 640 ± 33.019	a	$F_{(2.72)}$ = 18.088	0.000
		0.26	110.739 ± 49.172	b		
		0.69	107.143 ± 30.624	b		
	Hg	0	61.541 ± 15.074	A	$F_{(2.72)}$ = 132.543	0.000
		0.010	115.130 ± 32.150	B		
		0.016	127.851 ± 31.049	C		
	MPs x Hg				$F_{(4.72)}$ =30.282	0.000
Gills LPO	MPs	0	233.47 ± 109.731	a	$F_{(2.72)}$ = 1.663	0.197
		0.26	290.170 ± 148.530	a		
		0.69	281.695 ± 136.53	a		
	Hg	0	299.786 ± 157.793	A	$F_{(2.72)}$ = 1.367	0.261
		0.010	247.079 ± 106.125	A		

		0.016	258.475 ± 133.667	A		
	MPs x Hg				$F_{(4.72)}$ = 3.890	0.006
	MPs	0	24.572 ± 9.106	a	$F_{(2.72)}$ = 23.891	0.000
		0.26	33.039 ± 11.769	b		
		0.69	36.863 ± 11.633	b		
Liver SOD	Hg	0	20.355 ± 5.713	A	$F_{(2.72)}$ = 56.178	0.000
		0.010	37.152 ± 10.476	B		
		0.016	36.967 ± 10.166	B		
	MPs x Hg				$F_{(4.72)}$ = 5.783	0.000
	MPs	0	35.197 ± 19.747	a	$F_{(2.72)}$ = 10.853	0.000
		0.26	45.558 ± 18.423	b		
		0.69	46.397 ± 12.298	b		
	Hg	0	26.760 ± 10.514	A	$F_{(2.72)}$ = 74.566	0.000
Liver CAT		0.010	41.015 ± 11.630	B		
		0.016	59.377 ± 12.661	C		
	MPs x Hg				$F_{(4.72)}$ = 3.840	0.007
		0	145.609 ± 31.986	a	$F_{(2.72)}$ = 21.531	0.000
	MPs	0.26	170.920 ± 37.142	b		
		0.69	175.193 ± 43.154	b		
Liver GST	Hg	0	130.081 ± 21.150	A	$F_{(2.72)}$ = 116.184	0.000
		0.010	157.984 ± 29.879	B		
		0.016	203.658 ± 24.296	C		
	MPs x Hg				$F_{(4.72)}$ =10.369	0.000
	MPs	0	212.977 ± 64.797	a	$F_{(2.72)}$ = 1.507	0.228
		0.26	229.772 ± 59.533	a		
		0.69	203.119 ± 60.499	a		
Liver LPO	Hg	0	205.484 ± 66.116	A	$F_{(2.72)}$ = 6.045	0.004
		0.010	245.811 ± 54.539	B		
		0.016	194.572 ± 54.050	A		
	MPs x Hg				$F_{(4.72)}$ = 1.758	0.147

♦ Appendix D – Supplementary information Chapter IV, Section 4

Table S-1. Water abiotic parameters measured at each 0 h and 24 h. The results are the mean and standard deviation (SD) of nine replicates.

	Temperature (°C)		Dissolved Oxygen (mg/L)		Conductivity (µs/cm)		Salinity (ppm)		pH	
Treatments	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
Control	19.2±0.15	18.2±0.09	10.2±0.10	10.1±0.05	52.1±0.22	52.0±0.25	34.1±0.12	33.8±0.16	8.1±0.15	8.2±0.03
MP low	19.7±0.16	18.2±0.10	10.3±0.10	10.2±0.04	52.3±0.27	52.0±0.25	34.1±0.11	33.9±0.16	8.2±0.02	8.2±0.02
MP high	19.5±0.18	18.2±0.16	10.2±0.08	10.2±0.08	52.6±0.25	51.9±0.26	34.6±0.15	33.8±0.19	8.2±0.02	8.2±0.02
Hg low	19.6±0.15	18.2±0.16	10.3±0.10	10.2±0.08	52.8±0.21	51.9±0.26	34.0±0.12	33.8±0.19	8.2±0.03	8.2±0.02
Hg high	19.3±0.15	18.1±0.08	10.3±0.09	10.2±0.08	52.0±0.18	52.0±0.20	34.1±0.11	33.9±0.15	8.2±0.02	8.2±0.02
MP-L+ Hg-L	19.8±0.14	18.1±0.09	10.3±0.10	10.2±0.08	52.6±0.21	51.9±0.26	34.5±0.11	33.9±0.15	8.1±0.01	8.2±0.02
MP-L + Hg-H	19.7±0.13	18.2±0.09	10.3±0.09	10.2±0.08	51.9±0.19	51.9±0.25	34.1±0.11	33.8±0.17	8.1±0.01	8.2±0.02
MP-H + Hg-L	19.5±0.13	18.2±0.09	10.3±0.09	10.2±0.08	52.0±0.26	52.0±0.25	34.5±0.11	33.8±0.18	8.2±0.02	8.2±0.02
MP-H + Hg-H	19.7±0.13	18.3±0.08	10.3±0.08	10.2±0.08	52.5±0.23	52.0±0.26	34.0±0.11	33.8±0.17	8.1±0.02	8.2±0.02

Table S-2. Results of the two-way Analysis of Variance with interaction investigating the effects of microplastics and mercury on swimming performance parameters of *Dicentrarchus labrax* at the end of the exposure period (96 hours). The mean and standard deviation (SD) displayed refer to the original data. Fixed factors: MPs concentrations (low: 0.26 mg/L; high: 0.69 mg/L) and Hg concentrations (low: 0.010 mg/L; high: 0.016 mg/L).

Parameter	Factor	Level	Mean ± SD	N	Tukey test	F	p
Swimming velocity (m/s)	MPs	0	0.175 ± 0.132	27	A	$F_{(2.72)} = 24.293$	0.000
		low	0.135 ± 0.123	27	B		
		high	0.064 ± 0.044	27	C		
	Hg	0	0.238 ± 0.106	27	a		0.000
		low	0.083 ± 0.092	27	b	$F_{(2.72)} = 74.531$	
		high	0.054 ± 0.029	27	b		
	MPs x Hg					$F_{(4.72)} = 7.551$	0.000
Resistance time (s)	MPs	0	120 ± 42.6	27	A	$F_{(2.72)} = 246.167$	0.000
		low	108 ± 45.4	27	B		
		high	89 ± 29.9	27	C		
	Hg	0	159 ± 22.0	27	a	$F_{(2.72)} = 2169.603$	0.000
		low	86 ± 11.6	27	b		
		high	72 ± 10.6	27	c		
	MPs x Hg					$F_{(4.72)} = 33.432$	0.000