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Microplastics as vectors of heavy metals for fish

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Resumo

Os ecossistemas marinhos têm sofrido inúmeras pressões resultantes das atividades antrópicas. Mais recentemente tem-se verificado uma preocupação crescente em relação à poluição por plásticos no ambiente marinho, que tem vindo a aumentar ao longo dos anos e é expectável que perdure por muitos mais. Os ecossistemas costeiros são considerados como sendo zonas muito vulneráveis à contaminação por plástico, uma vez que se encontram mais perto das fontes de poluição. Estima-se que cerca de 50-80% dos resíduos de plástico se encontrem nos ecossistemas costeiros. O plástico, é constituído por diversos materiais sintéticos que lhe conferem grande diversidade de formas e tamanhos. A sua durabilidade, utilização insustentável e má gestão do seu uso, têm permitido a sua crescente utilização na sociedade, tendo como consequência a contaminação de diversos habitats naturais. Não só a poluição pelo plástico apresenta graves consequências para o ambiente marinho, como, por exemplo, perda de biodiversidade, mas também constitui uma ameaça para a saúde humana.

Apesar das severas consequências da poluição por plásticos nos ecossistemas aquáticos, apenas na década de 1970s, surgiram os primeiros estudos sobre esta temática. Ao longo dos anos seguintes, este assunto ganhou interesse por parte dos média, do público e, principalmente, da comunidade científica. Recentemente o número de estudos acerca do mesmo aumentou drasticamente, abrangendo não só as áreas da biologia marinha, da toxicologia, da oceanografia e da ecologia, mas também, as áreas da bioquímica e da engenharia ambiental.

Sob condições ambientais normais, o plástico de grandes dimensões é degradado, através de processos físicos e químicos, em plásticos de reduzidas dimensões. Os microplásticos, são assim designados por possuírem dimensões inferiores a 5mm. Na última década, vários estudos evidenciaram a presença de microplásticos em diversos ecossistemas marinhos. A crescente preocupação com esta temática, levou ao desenvolvimento de estudos que permitem entender os perigos da contaminação do plástico nos organismos marinhos. Inicialmente, estes estudos foram focados na ingestão e emaranhamento de plásticos de grandes dimensões em tartarugas marinhas, aves marinhas e mamíferos marinhos. Atualmente, existem alguns estudos publicados acerca dos efeitos resultantes da ingestão dos microplásticos em outros organismos marinhos. Informação relativa à sua distribuição temporal e espacial também tem vindo a revelar-se bastante útil, fornecendo uma perspetiva mais global da distribuição e densidade dos microplásticos nos oceanos. Apesar das dificuldades em quantificar a concentração de plásticos nos oceanos, principalmente, devido ao custo elevado e ao tempo necessário, é fundamental entender quais as fontes e formas dos detritos, para que seja possível avaliar as transformações do mesmo após entrar no ambiente marinho. Estes aspetos são fundamentais para compreender os riscos e os impactos da contaminação dos oceanos pelo lixo marinho. Deste modo, tem sido possível entender quais as zonas de maior e menor acumulação de microplásticos, assim como, a evolução crescente da quantidade de microplásticos que acaba nos oceanos anualmente. Outro aspeto fundamental revelado pelos estudos diz respeito à capacidade que os microplásticos possuem em adsorver outros contaminantes, como metais pesados, aumentando, deste modo, o nível de toxicidade para os organismos que os ingerem. O reduzido tamanho e as características hidrofóbicas dos microplásticos permitem a adesão de poluentes orgânicos persistentes (POP's), que por sua vez, resistem à degradação química, fotolítica e biológica, persistindo no ambiente e contaminando os organismos marinhos. No entanto, muita informação ainda se encontra em falta referente aos efeitos celulares da contaminação por microplásticos nos organismos marinhos.

Este estudo pretende determinar, pela primeira vez, os efeitos da contaminação por microplásticos e o seu papel como vetores de metais pesados em peixes, tendo o sargo-comum (*Diplodus sargus*) como espécie modelo.

Foram recolhidos indivíduos juvenis nas poças intertidais de três praias no Cabo Raso, Cascais, transportados para laboratório, e posteriormente, colocados em aquários com condições controladas. Ao fim de um período de aclimatização de 10 dias, com alimentação com camarão, foi iniciada a experiência, na qual os indivíduos foram alimentados com 1) camarão (controlo), ou 2) camarão + 8 microesferas virgens (0,1% p/p poliestireno virgem) ou 3) camarão + microsferas de plástico contaminadas por metais pesados (0,1% p/p poliestireno virgem). Ambos os tratamentos, juntamente com o controlo, foram replicados por três aquários. Durante o período experimental foi registada a mortalidade diariamente. Após 30 dias de exposição, seis indivíduos foram retirados ao acaso dos respetivos aquários, pesados, medidos e congelados a -80°C, para análise posterior.

Foi determinada a condição corporal dos indivíduos (através do índice de Fulton-*K*) e a resposta ao stress celular (através dos biomarcadores de stress oxidativo), em vários tecidos, numa experiência a longo-prazo (30 dias). As atividades das enzimas Glutationa-S-Transferase (GST), Catalase (CAT), e Superoxide Dismutase (SOD) assim como a concentração de Peroxidação Lipídica (LPO) foram determinadas em diversos órgãos – fígado, intestino, músculo e brânquias. A concentração de Ubiquitina foi determinada apenas no fígado. O principal objetivo do presente trabalho foi determinar as respostas celulares dos diversos biomarcadores nos vários tecidos, para os vários tratamentos. Com a informação obtida foi possível avaliar o efeito de *stress* oxidativo provocado pela contaminação por plásticos e por metais pesados nos diversos tecidos.

O uso dos biomarcadores de *stress* oxidativo tem vindo a revelar-se extremamente importante na compreensão dos mecanismos celulares de defesa contra diversos tipos de *stress* (térmico, radiação UV e poluição). Existe já literatura acerca do *stress* oxidativo, em diversos organismos marinhos, sujeitos a diferentes condições de *stress*. Sabe-se que sob condições de *stress*, os organismos produzem e acumulam espécies reativas de oxigénio (ROS), causando um desequilíbrio de oxidantes e antioxidantes no organismo, levando a danos nas membranas lipídicas, nas proteínas e nos ácidos nucleicos. Como resposta, os organismos desenvolveram mecanismos de defesa para combater a toxicidade causada pelas espécies reativas de oxigénio. O equilíbrio entre a eliminação e a produção de ROS é mantida por antioxidantes e por enzimas. As enzimas antioxidantes (ex: GST, CAT, SOD) atuam de forma a proteger as células dos efeitos nocivos causados por ROS. Por outro lado, a LPO e as Ubiquitinas fornecem informação de danos ao nível das membranas celulares e de danos nas proteínas, respetivamente. A sua eficácia depende do estágio de desenvolvimentos e de outros aspetos fisiológicos dos organismos. Neste sentido, este estudo vem realçar a importância do papel dos biomarcadores de *stress* oxidativo na compreensão dos efeitos celulares da contaminação por metais pesados em peixes costeiros.

Este trabalho revelou que a contaminação somente por microplásticos virgens leva a níveis baixos de *stress* oxidativo, já que se verificou um aumento apenas em um biomarcador, a GST, e apenas num dos tecidos testados, o músculo. Já a hipótese de que os microplásticos serão potenciais vetores de metais pesados, confirmou-se. O tratamento contendo microplásticos com metais pesados, levou a um aumento significativo nas atividades e concentrações da LPO no músculo, da GST nas brânquias, da CAT e da Ubiquitina no fígado. O intestino, por sua vez, foi o único tecido que não respondeu significativamente a nenhum biomarcador. Contrariamente, o músculo foi o tecido onde se observou uma resposta mais significativa dos biomarcadores analisados, nomeadamente a GST e LPO, o que sugere que este tecido pode ser o mais indicado para biomonitorizar este tipo de poluição. Foi registada uma ligeira mortalidade em todos os tratamentos, tendo ocorrido um aumento da mesma em ambas as experiências comparativamente com o controlo. O índice corporal Fulton-*K* não variou significativamente entre as

experiências, indicando que o *stress* causado aos indivíduos não foi severo o suficiente para induzir um decréscimo na condição corporal dos mesmos.

Concluindo, este projeto revela, pela primeira vez, o papel dos microplásticos como vetores de metais pesados em peixes, tendo-se obtido resultados significativos em quatro dos cinco biomarcadores de *stress* oxidativo testados, em vários tecidos. Tendo sido esta experiência realizada num período de 30 dias e com juvenis, permite-nos ter uma perspetiva dos possíveis danos celulares que poderão ocorrer num período de exposição mais prolongado a este tipo de contaminantes, e possíveis alterações no desenvolvimento e crescimento de indivíduos a partir desta fase do ciclo de vida.

Palavras-chave: Microplásticos, Metais pesados, *Stress* oxidativo, Biomarcadores, *Diplodus sargus*

Abstract

This project aimed, to determine if microplastic can act as vectors for heavy metal contamination in fish, using juvenile white seabream (*Diplodus sargus*) as model organism. It its known that heavy metal contamination affects the cellular systems of the contaminated organisms, and so, the purpose of this study was to investigate the cellular impacts of heavy metal contamination via microplastics in several tissues of the white seabream, in a long-term experience (30 days).

After being collected in their natural habitat, individuals were placed in aquaria under controlled conditions. Juveniles of *D. sargus* were fed with 1) shrimp (control), 2) shrimp containing virgin microplastic beads (0.1% w/w of virgin polystyrene) and 3) shrimp containing microplastic beads contaminated with Cu and Zn (0.1% w/w of virgin polystyrene). Whole-body (survival and Fulton-K index) and cellular stress (oxidative stress biomarkers) indicators were assessed in a long-term experiment (30 days). The enzymatic activities of superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) as well as Lipid peroxidation (LPO) concentration were determined in the liver, intestine, muscle and gills. Ubiquitin concentration was determined only in the liver.

Results showed the lack of cellular response after contamination with virgin microplastics, for the exception of GST in the muscle. Conversely, our data points for a connection between heavy metal contamination and oxidative stress, since we reported an increase in the activities and concentrations of LPO in the muscle, GST in the gills, CAT and an increase of Ubiquitin concentrations in the liver. The intestine was the only tissue that not responded significantly to any of the tested biomarkers. In contrast, the muscle was the tissue that responded to more biomarkers, namely GST and LPO, and therefore, it was considered the most suitable tissue for biomonitoring this type of pollution. A slight mortality was recorded in all treatments, with an increase occurring in both experiments compared to the control. The Fulton-*K* body index did not vary significantly between the experiments, indicating that the stress caused to the individuals was not severe enough to induce a decrease in their body condition.

Concluding, this work shows, for the first time, the oxidative stress of heavy metals contamination via microplastics, in fish, in a long-term experience. The role of microplastics as heavy metals vectors was clear in our study. Also, this project showed that *Diplodus sargus* is a good model-species for this kind of studies.

Key-words: Microplastics, Heavy metals, Oxidative stress, Biomarkers, *Diplodus sargus*

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CHAPTER 1

General Introduction

1. Plastics and microplastics: characterization, threats and distribution in the world's oceans

One of the most important current problems facing marine ecosystems is the impact of marine litter, with coastal ecosystems and food chains being most vulnerable as they are most exposed to the sources of litter (Setälä et al., 2016). World plastic production has increased significantly since 1950, from 1.5 million tons to 311 million tons in 2014 (Green, 2016). Oceans receive about 10% of the plastic annual production, with reports of plastic litter from poles to abyssal regions (Avio et al., 2015).

The term "plastic" is used to define materials consisting of synthetic polymers whose raw materials are hydrocarbons of natural origin, such as alcohol, natural gas, coal and oil (Derraik, 2002). Unfortunately, for the last years, the velocity of entrance of these polymers in the marine environment has been proportional to their production (Moore, 2008). Synthetic polymers can be very diverse: polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC), polyamide (PA), polyethylene terephthalate (PET), polyvinyl alcohol (PVA), being the most common. Their position in the water column depends on their density - polymers denser then seawater, sink and polymers with lower density float (Avio et al., 2016). This is important, because it defines its availability for interactions with marine wildlife.

Due to its physical and chemical properties, i.e., its small size and its difficulty in biodegrading, plastic persists and accumulates in the oceans (Lu et al., 2016; Pedà et al., 2016). Also, plastics capacity to adsorb other contaminants, makes them a potential vector for transferring other pollutants to the marine ecosystems, such as heavy metals. Both plastics and these contaminants are very difficult to degrade in the environment. Also, marine organisms cannot degrade these contaminants through digestion, which leads them to bioaccumulate in the food chains, potentially reaching higher trophic levels (Gregory, 1996; Rios et al., 2007).

Marine litter reaches the oceans in different ways. The main one's result from land-based sources, such as rivers, along with wind transportation and wastewater discharge. Other inputs of plastic marine litter into the oceans result from garbage dumping from fishing activities - where all types of material used, such as nets, lines and buoys are released to the sea - and from recreational vessels, such as cruises. Human consumption activities ranging from food to leisure also contribute to increase the amount of plastic in the oceans (Avio et al., 2016; Wilcox et al., 2016).

The deleterious effects of plastic litter in the oceans have been neglected for a long time, beginning to be noticed from the 1970's, when the scientific community started to pay attention to this matter (Andrady, 2011). More recently, several studies came out mainly focused on the entanglement and ingestion of plastic debris in marine mammals, seabirds and sea turtles (Derraik, 2002; Andrady, 2011).

Microplastics have been described as plastic particles with a length of less than 5 mm (Watts et al, 2014) and are considered the predominant form of marine litter in the oceans (Mazurais et al., 2015). These can be classified into two groups: primary microplastics – initially small in size and secondary microplastics – fragmented from large plastics (Watts et al., 2014; Setälä et al., 2016). Primary microplastics can be found in cosmetics containing exfoliating beads, which use has been increasing

drastically since its appearance in the 1980's. Primary microplastics are also used in the production of air blasting technology to remove paint from metal surfaces, which involves different synthetic particles, such as acrylic, melamine and polyester, that throughout time will be contaminated with heavy metals. Microspheres are also used in medicine industry for pharmaceutical products distribution (Cole et al., 2011; Duis and Coors, 2016; Galloway et al., 2017). Secondary microplastics are formed by the breakdown into smaller pieces of bigger sized plastics, due to the action of mechanical, photo (oxidative) and biological processes that change plastics properties over time (Cole et al., 2011). This degradation, usually begins with photooxidation, due to the action of UV radiation. The combination of hydrolytic seawater properties and oxidative atmosphere properties, allows the fragmentation of polymers of lower molecular weight, forming smaller fragments (Moore, 2008; Duis and Coors, 2016; Galloway et al., 2017). The mechanical action of waves in marine environment and the biodegradation role by bacteria and fungi also contribute to the fragmentation of plastics (Browne et al., 2007).

Studies regarding spatial distribution and abundance of microplastics in the world's oceans are mainly focused on the interactions between microplastics and organisms in productive waters (Kanhai et al., 2017). Even though monitoring plastics' abundance in marine ecosystems is very difficult, expensive and requires time to gather information (Lusher et al., 2014), some studies have already come out with valuable information on spatial and temporal distribution of plastic debris in world's oceans (Eriksen et al., 2014; Lusher et al., 2014; Van Sebille et al., 2015; Kanhai et al., 2017).

Over the last 40 years, the concentration of microplastics has increased drastically (Browne et al., 2007). Microplastics can be found in most marine ecosystems, from open sea to seafloor (Kanhai et al., 2017). Their abundance in the marine environment is dependent on oceanographic conditions, such as oceans currents, tides, wind and waves action (Cole et al., 2015a). The estimated abundance of microplastics comprises concentrations between 3 m^{-3} particles up to very high levels of 102 000 particles m^{-3} in case of highly contaminated areas (Setälä et al., 2016). These highest contaminated areas are the convergence zones of five sub-tropical gyres - North Pacific, North Atlantic, South Pacific, South Atlantic, Indian Ocean, - which support low marine biodiversity (Kanhai et al., 2017). A study by Eriksen et al. (2014) reveals an oceanography model of spatial distribution and density not only of microplastics, but also of mesoplastics and macroplastics in all five sub-tropical gyres along with Mediterranean Sea, Australian coast and Bay of Bengal. The databased used was gathered by several expeditions conducted between 2007-2013. The results reinforced that plastics are spread out in all world's oceans. All three types of plastics were found in all ocean regions, with sub-tropical gyres being reported as accumulation zones and ocean coast as areas of migration. This type of studies is important since it provides a global perspective of the distribution of plastics pollution in all marine ecosystems and the oceanography processes that are involved in its distribution. It also allows to connect information of other global plastic mass inventories and study the evolution of its spatial and temporal distribution and density. In this study, models estimated that are floating at sea at least 5.25 trillion plastics particles weighing 268,940 tons. With this information, it is possible to predicted areas in world's oceans where plastics damage in marine organisms could be possibly higher. This leads to interesting studies about the interactions and the biological effects of plastic pollution in marine biota.

Several studies have been carried out to understand the dangers that microplastics present in many marine organisms. Ingestion of microplastics has been evident in several groups of organisms, including zooplankton, invertebrates, bivalves and fish (Mazurais et al., 2015; Pedà et al., 2016). Entanglement and ingestion, followed by chemical contamination resulting of ingestion are being reported as the main causes of exposure to marine litter that compromise the welfare of marine animals

(Wilcox et al., 2016). The ingestion of microplastics causes several damages to the organism itself, such as physiological injuries, energetic deficiency, feeding reduction and death (Cole et al., 2015b).

The emerging concern of microplastics severe impacts in world's oceans, led to its inclusion in legal instruments, such as EU Marine Strategy Framework Directive (MSFD 2008/56/EC), Water Framework Directive (WFD, 20/60/EC) and NOAA Marine Debris Program (Wagner at al., 2014; Cole et al., 2015b). The aim of these policies is to understand its impacts in marine life and ecosystems, as well as minimize future inputs of plastic in the oceans (Eerkes-Medrano et al., 2015). Studies already demonstrated that there are differences in contaminants between freshwater resources and marine systems. This instruments goal is to improve the good environmental state of marine waters (covered by Descriptor 11 of MSFD) by 1) monitoring the presence of microplastics in freshwater systems and its sources, 2) assess and evaluate the biological effects of microplastics exposure and 3) understand possible interactions between microplastics and other contaminants. Despite all the efforts in surveying and monitoring microplastics, it is yet in lack a better understanding of its effects in freshwater systems, and so, it is necessary to improve and/or develop new framework to manage microplastics (Wagner at al., 2014).

2. Biomarkers of oxidative stress: ROS and biochemical defensive mechanisms

Biomarkers have been studied since 1980s, and they are currently, considered useful tools for ecological risk assessment (Beliaeff and Burgeot, 2002). The use of biomarkers is important in providing information about the effects of exposure of contaminants to the aquatic ecosystem. Contaminants, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzofurans (PCDFs) and dibenzo-p-dioxins (PCDDs) and heavy metals can be adsorbed by the surface of the plastics (Cole, 2014; Avio et al., 2016). A biomarker is usually defined as any biological response, from molecular - through cellular and physiological responses - to behavioural changes, which may be related to exposure or to toxic effects of environmental chemicals (Pawles et al., 2013). Biomarkers should indicate that an organism has been exposed to a pollutant - exposure biomarkers - as well as the magnitude of the body response to the pollutant - biomarkers of effect or stress (Cajaraville et al., 2000). Therefore, biomarkers are very important tools for environmental assessment, since they provide information regarding biological effects of contaminants and environmental stress (Beliaeff and Burgeot, 2002).

Reactive oxygen species (ROS) production is a consequence of oxidative stress in marine organisms induced by one or several contaminants (Pandey et al., 2003). Since ROS are important in cellular signalling, they are produced in normal circumstances. Although, its increase can induce oxidative stress, and consequently, proteins, lipids and DNA damage (Madeira, 2016). Hence, all organisms have developed a cellular mechanism to defend themselves against ROS toxicity (Pandey et al., 2003). This mechanism includes the production of antioxidant enzymes, molecular chaperones and proteolytic systems. All three mechanisms have different functions. While antioxidant enzymes play a critical role in reducing and/or maintaining the ROS levels in organism cells, molecular chaperones repair oxidatively damaged proteins, and proteolytic systems, such as ubiquitin, marking them for degradation via the proteasome system (Van der Oost et al., 2003; Shang and Taylor, 2011; Madeira, 2016). Superoxide dismutase (SOD), Glutathione-S-Transferase(GST) and Catalase (CAT) are some of the antioxidant enzymes that counteract the deleterious effects of free radicals (Van der Oost et al., 2003). Below it is briefly described the biochemical role of all biomarkers of oxidative stress against ROS toxicity used in this study:

Superoxide dismutase

Superoxide dismutase is an important enzyme of the antioxidant defense mechanism. SOD is a group of metalloenzymes containing copper and zinc, or manganese, or iron with different cellular distributions that metabolizes the reactive superoxide anions ($O_2^{-\bullet}$) in molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), which is a less reactive ROS (Beyer and Fridovich, 1987; Van der Oost et al., 2003; Lesser, 2006; Monteiro et al., 2006).

Catalase

Catalase is an antioxidant enzyme that is present in the peroxisomes of most aerobic cells and plays an important role in fatty acid metabolism (Van der Oost et al., 2003). Hydrogen peroxide is formed in the eukaryotic cell as a by-product of various oxidase and superoxide dismutase reactions. Like Glutathione peroxidase, Catalase also decomposes hydrogen peroxide into water and oxygen in a two-step reaction (Johansson et al., 1988; Madeira, 2016).

Glutathione-S-Transferase

Glutathione-S-Transferase is an enzyme that catalyse the conjugation of GSH with numerous electrophilic compounds that are involved in the detoxification of reactive and oxygen radicals. GST major role is to defend cells, DNA and lipids against oxidative damage, since generates less toxic and more water-soluble products (Van der Oost et al., 2003; Menezes et al., 2006; Monteiro et al., 2006). Glutathione peroxidase and S-transferase detoxify lipid peroxides into alcohols (Madeira, 2016). There are several electrophilic metabolites that act as substrate, being 1-chloro-2,4-dinitrobenzene (CDNB) considered the most important one (Mannervik et al., 1988). The increase of GST activity has been reported in fish after contaminants exposure (Van der Oost et al., 2003).

Lipid peroxidation

Lipid peroxidation is considered one of the most important mechanisms of cellular damage (Lesser, 2006). It generates several products, such as malondialdehyde, that can react with DNA and protein leading to a toxic and mutagenic outcome (Marnett, 1999; Lesser, 2006). Lipid peroxidation occurs when there is oxidation of free radicals in polyunsaturated fatty acids (Gutteridge, 1995). This ROS attack leads to a chain reaction that causes cell membrane destruction. Cellular functions get compromised due to the inactivation of enzymes, receptors and transport proteins (Madeira, 2016).

The biochemical role of detoxification enzymes provides valuable information and so, it is very often used in studies that aim to understand the effects of environmental contaminants (Monteiro et al., 2006).

Ubiquitin

The ubiquitin-proteasome pathway it is involved in diverse cellular regulation processes, including transcription, transduction, differentiation and apoptosis (Shang et al., 1997; Madeira, 2016). The major role of the ubiquitin-proteasome pathway is to eliminate misfolded proteins which have lost their function, by a process called ubiquitinylation which involves several proteins (Shang et al., 1997; Madeira, 2016). Substrate proteins are attached by several ubiquitin molecules and are later degraded by the 26S proteasome (Shang and Taylor, 2011).

3. Model organism: *Diplodus sargus*

The model organism chosen for the present study was *Diplodus sargus* (Linnaeus, 1758), a demersal fish that occupies different niches, like shallow rocky reefs, sandy sea beds and estuaries (Summerer et al., 2001; Bargelloni et al., 2005). Its distribution is very diverse, being the Mediterranean Sea and the Atlantic Ocean its key areas. Although, it is also possible to find *D.sargus* in the Caribbean, Indian Ocean, Gulf of Mexico, Red sea and Persian Gulf (Summerer et al., 2001). *D. sargus*, plays an important role in shallow water ecosystems – areas of spawning and nursery - and its importance in aquaculture has increased over the last years (Bargelloni et al., 2005). This species can reach 12 years of age and a maximum length of 45 cm, and its diet is mainly composed by gastropods and crustaceans (Pajuelo and Lorenzo, 2002; Leitão et al., 2007). According to the IUCN Red List of Threatened Species, *D.sargus* is not threatened, being categorized as of least concern. Its wide distribution, abundance in shallow waters, commercial status, and easy adaptation to captivity make this a potential model species for the study presented in this dissertation.

4. Dissertation aims

This thesis focuses on the effects of microplastics and heavy metals on the juvenile white seabream (*Diplodus sargus*). The main aim is to determine the role of microplastics as vectors of heavy metals for coastal fish. Whole-body (survival and Fulton-*K* index) and cellular stress response (oxidative stress biomarkers) were assessed in a long-term experiment (30-days). Oxidative stress biomarkers were analysed in various tissues.

Studies, such as the present one, are crucial in understanding the impacts of contaminants via microplastics in marine ecosystems. Since microplastics pollution is a recent concern issue, it is necessary the development of studies that improve the knowledge of microplastics physiological and biochemical effects on marine organisms and its consequences for the marine ecosystems.

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CHAPTER 2

Microplastics as vectors of heavy metals for fish

Abstract

Microplastics have already proven their ability to carry a wide variety of contaminants, including heavy metals. The aim of this study was to investigate the role of microplastics as vectors of heavy metals for fish, using a common coastal fish species, the white seabream *Diplodus sargus*. Organisms were collected in intertidal pools of the west Portuguese coast. They were kept in aquaria and fed with 1) shrimp, 2) shrimp containing microplastic beads (0.1% w/w of virgin polystyrene) and 3) shrimp containing microplastic beads contaminated with Cu and Zn (0.1% w/w of virgin polystyrene). Whole-body (survival and Fulton-*K* index) and cellular stress (oxidative stress biomarkers) indicators were assessed in a long-term experiment (30 days). The activities of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and malondialdehyde (MDA) formation were determined in multiple tissues: liver, intestine, muscle and gills. Ubiquitin concentration was determined only in the liver. Our findings showed significant increases in the activities of GST in the gills, MDA in the muscle, CAT and ubiquitin levels in the liver in fish exposed to microplastics contaminated with heavy metals. The exposure solely to virgin polystyrene microplastic beads did not reveal significant responses to the tested biomarkers, with the exception of an increase of GST in the muscle. Mortality rates were higher in both treatments compared with the control. The results of this study highlight the role of microplastics as vectors of heavy metals for coastal fish. Alterations in some antioxidant enzymes (GST, CAT) LPO and ubiquitin levels reflect the oxidative damage caused by heavy metal toxicity in *D.sargus*. In addition, this study reinforces the importance of using biomarkers of oxidative stress in biomonitoring aquatic pollution.

Key-words: Microplastics**,** Heavy metals, Oxidative stress, Biomarkers

Introduction

The amount of plastic debris in marine ecosystems have been increasing over the last 50 years (Cole et al., 2015). The consecutive loss of biodiversity has been diagnosed as one of the consequences of the presence of marine litter (Eerkes-Medrano et al., 2015). Depending on the size of plastic litter, its effects in marine organisms will differ. Plastic items of big size tend to cause entanglement to a diverse number of species – from invertebrates, to fish, mammals, seabirds and sea turtles – while, smaller sized plastic items are usually ingested by several marine organisms, leading to gut problems and contamination, caused by the absorption of chemicals from the plastic items (Eerkes-Medrano et al., 2015). Plastic debris are composed by synthetic polymers that persist in the environment for thousands of years due to its difficulty to degrade. Under the influence of UV radiation and degradation by weathering processes, big sized plastics will gradually break down into smaller pieces (Moore, 2008; Lonnstedt and Eklov, 2016).

Microplastics, are small plastic debris - microscopic plastic beads, fragments or fibres - with less than 5mm of dimension and they are considered the most common form of marine litter, comprising about 85% of it (Cole, 2014., Katzenberger and Thorpe, 2015). Due to its ubiquity and persistence, microplastics are found in different environments, from coastal ecosystems to open sea, from sea surface to sediments in the sea floor (Gutow et al., 2016). Microplastics adsorb other chemical contaminants, such as persistent organic pollutants (POP's), which increases its toxicity and makes them become more harmful to marine organisms that are expose to them (Cole, 2014). Microplastics are found in between sand grains owing to their similar dimension, which makes them available to be ingested by benthic invertebrates at the based level of food webs, and transferred to higher trophic levels (Hall et al., 2015; Gutow et al., 2016).

The increase in marine pollution, leads to the need to develop methods to identify the risks of chemical pollution and to measure the magnitude of this chemicals in marine organisms (Cajaraville et al., 2000). Biomarkers have been used as a measure to investigate the biological impacts of a contaminant exposure (Pauwels et al., 2013). Biomarkers are commonly referred to as "early warning signals" because they can detect early biological responses in organisms, weather it is at a biochemical, physiological, molecular or cellular level as a response of exposure to one or several contaminants (Cajaraville et al., 2000; Pauwels et al., 2013).

Heavy metal contamination induces oxidative stress, since it promotes the increase of cellular concentration of reactive oxygen species (ROS) (Atli et al., 2006; Farombi et al., 2007). The deficiency of antioxidants and the increasing of ROS causes cellular damage to the contaminated organism. DNA damaging and protein oxidation along with lipid peroxidation production are consequences of ROS reacting with biological molecules (Monteiro et al., 2006). In response to the prejudicial effects of oxidative stress, organisms developed defensive mechanisms to neutralize the ROS impact. Defensive antioxidant enzymes such as catalase, superoxide dismutase and peroxidases can decrease the number of free radicals rehabilitating the cellular damage (Vinodhini and Narayanan, 2008; Madeira et al., 2015).

The increasing concern regarding marine pollution has been followed by the development of studies about this emerging issue. The studies of microplastics formation and its distribution in worlds' oceans is well documented (Andrady, 2011; Eriksen et al., 2014; Avio et al., 2016). Microplastics ingestion is already documented in fishes (Neves et al., 2015; Pedà et al., 2016), invertebrate marine organisms, such as oysters (Cole and Galloway, 2015), corals (Hall et al., 2015), cetaceans (Fossi et al., 2012; Lusher et al., 2015), marine mammals (Eriksson and Burton, 2003) and zooplankton (Cole, 2014; Cole et al., 2015). However, most of this literature was produced in a physiological view. A biochemical perspective of contamination resulting from microplastics exposure using biomarkers response in marine organisms is yet poorly documented except for the works of Fossi et al., (2016); Avio et al.,

(2015); Oliveira et al., (2013); von Moos et al., (2012); Karami et al., (2016), which focused on biomarkers analyses in different animal tissues resulting of microplastic contamination of several groups of animals - whales, mussels and fishes.

Diplodus sargus (white seabream), is a common coastal fish that can be found in the Mediterranean and in the northeastern Atlantic regions – from Senegal to Azores, Madeira and Canary Islands (Summerer, 2001). *D. sargus* is a very important fish species in the fishing industry, it is one of the most widely consumed fishes in the Mediterranean area and it has a very high commercial value (Gonçalves, 2000). It is also important ecologically, since it plays a crucial role in shallow and coastal environments. *D. sargus* is a common species in various coastal habitats within its distribution area and thus it has potential as an indicator of habitat quality (Lloret and Planes, 2003), its easy adaptation to captivity also makes it a good model to study environmental contamination.

The aim of this study was to investigate the role of microplastics as vectors of heavy metals for fish, using a common coastal fish species, the white seabream *D. sargus*.

Juveniles of *D. sargus* were fed with 1) shrimp (control), 2) shrimp containing microplastic beads (0.1% w/w of virgin polystyrene) 3) shrimp containing microplastic beads contaminated with Cu and Zn (0.1% w/w of virgin polystyrene). Whole-body (survival and Fulton-*K* index) and cellular stress (oxidative stress biomarkers) biomarkers were assessed in a long-term experiment (30 days). The activities of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), malondialdehyde (MDA) formation and ubiquitin levels were determined in the liver, intestine, muscle and gills.

Materials and methods

Microplastics characterization

In this study two different microplastic beads were used: virgin polystyrene beads (Styropor® P 326, BASF, Ludwigshafen, Germany) and the same microplastic beads covered with a paint containing heavy metals - Copper(I)-oxide (25-50%) and zinc-oxide (10-25%) (antifouling paint Micron Optima Base YBA953 and Micron Optima Activator YBA953, International Paint Ltd., Southampton, UK). Virgin microplastic beads were briefly dipped in paint to produce these heavy metals covered microplastic beads. Each microplastic bead weighted 0.275mg and had a diameter between 0.7 and 0.9 mm.

Collection of fish and experimental setup

Diplodus sargus were collected in Cabo Raso, Portuguese west coast (38° 42′N, 9° 29′W) in July of 2016 in the intertidal pools of 3 adjacent rocky beaches, during low tides. The specimens were collected using hand nets ($n = 62$). Then, they were transported in tanks with sea water from the sampling site to the laboratory and housed in 96 l tanks with continuous aerated seawater. The fish were randomly placed in a re-circulated system using nine aquaria (25L volume), with aerated seawater (dissolved O_2 between 95% to 100%), a constant temperature of 20°C (± 0.5°C), salinity 33±2‰. They were acclimated for 10 days, being fed once a day *ad libitum* with shrimps and commercial fish food. After the acclimation period, they were fed once a day according to each treatment. Microplastic beads were mixed within the food (2.19g of shrimp) in concentrations of 0.1% (= 8 microplastic beads). In all treatments, food was supplemented with commercial fish food to avoid nutritional deficiencies eventually caused by an exclusive shrimp feeding. This experiment, had, in total, three treatments: 1) control, 2) shrimp containing microplastic beads (0.1% w/w of virgin polystyrene) 3) shrimp containing microplastic beads contaminated with Cu and Zn (0.1% w/w of virgin polystyrene). For each treatment, three aquaria were used. Individuals were starved for 24h before sampling. At $t = 30$ days six individuals

from each treatment were chosen randomly from aquaria and immediately measured and weighed and then frozen at -80°C, until further analysis. Condition was estimated based on Fulton's *K*, according to the equation:

$$K = 100Mt/Lt^3$$

where Mt is total wet mass (mg) and Lt is total length (mm) (Ricker, 1975).

Biomarkers analysis

The fish organs (liver, gills, muscle and intestine) were removed and transferred into microtubes (1.5ml volume). All samples were homogenized in 1 ml of sodium phosphate buffer solution (pH 7.4). Before biomarkers analysis all homogenates were centrifuged at 4°C for 15 min at 10,000 x g (refrigerated centrifuge model Himac CT15RE, Hitachi Koki Co. Ltd., Japan) and frozen immediately at -80°C. Protein content and enzymatic analysis were determined in 96-well microplates as follows:

Lipid peroxides assay

The assay was performed using TBARS (thiobarbituric acid reactive substances), measured as malondialdehyde (MDA) content as previously described by Uchiyama and Mihara (1978). In Brief, 5µl of each sample processed as previously described were added to 45µl of 50mM monobasic sodium phosphate buffer. Then, to each microtube were added a mix contained 12.5µl of SDS 8.1%, 93.5µl of trichloroacetic acid 20%, 93.5µl of thriobarbituric acid 1% and 50.5µl of Mili-Q grade ultrapure water. Then, microtubes were agitaded using a vortex for 30s. Afterwards, the lid of each microtube was punctured with a needle, placed in boiling water for 10 min and then transferred into ice to cool. After cooling, 62.5µl of Mili-Q grade ultrapure water was added to each microtube. Then, the microtubes were centrifuged at 7,000 x g for 5 min at 4°C (refrigerated centrifuge model Himac CT15RE, Hitachi Koki Co. Ltd., Japan). Duplicates of 150µl of the supernatant were placed in 96-well microplate wells and the absorbance was read at 530nm. To quantify the lipid peroxides, a 12-point calibration curve(0-0.3µM) was calculated using malondialdehyde standards.

ELISA (ubiquitin)

Total ubiquitin was quantified using a indirect Enzyme Linked Immunosorbent Assay (ELISA) as described by Madeira et al., (2014). Two replicates of 50µl of each sample were transferred to the microplates' wells and incubated overnight at 4°C. Afterwards, the microplates were washed 3x in PBS 0.05% Tween-20 and then blocked by adding 200µl of 1% BSA (Bovine Serum Albumin, Sigma-Aldrich, USA) in PBS and incubated at 37°C for 90 min. Primary antibody (Ub P4D1, sc-8017, HRP conjugate, Santa Cruz, USA) was diluted in 1% BSA in PBS. Then, microplates were washed again with PBS-Tween and 50µl of primary antibody's solution (diluted in 1% BSA in PBS to 0.5 µg/mL) was added to each well followed by microplates incubation during 90 min at 37°C. After another washing step, 50µl of diluted (1µg/mL in 1% BSA) secondary antibody (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) were added to each well followed by (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) incubation at 37°C for 90 min. Then, 100µl of substrate (TMB/E, Temecula California, Merck Millipore) was added to each well and incubated for 15min at room temperature. 50µl of stop solution (0.5M HCl) was added to each well and the absorbance was read at 450nm in a 96-well microplate reader (BIO-RAD, Benchmark, USA). A calibration curve was calculated using five dilutions (0.05-0.8 $\mu g.mL^{-1}$) of purified ubiquitin.

Determination of enzymes activity

The enzymatic assay of Glutathione S-Transferase (GST), was carried out previously described by Habig et al., (1974), and adapted for a 96-well microplate using the CNDB (1-Chloro-2,4-dinitrobenzene) as substrate. The absorbance was read at 340nm in a 96-well microplate reader (BIO-RAD, Benchmark, USA), at each minute during six minutes. GST activity was calculated using the molar extinction coefficient for CDNB of $5.3^{\varepsilon mM-1}$.

Catalase activity was measured according to Johansson and Borg (1988). The absorbance was read at 540nm in a 96-well microplate reader (BIO-RAD, Benchmark, USA). Catalase activity was determined using a 7-point calibration curve (0-75µM) calculated from formaldehyde standards.

Superoxide dismutase (SOD) activity, using nitroblue tetrazolium (NBT) and xanthine oxidase (XOD), was determined according to the method described by Sun et al., (1988). After reading the absorbance at 550 nm, SOD activity was calculated as the %inhibition:

$$\frac{(\text{Abs560/min negative control} - \text{Abs560/min sample})}{(\text{Abs560/min negative control})} \text{ X } 100$$

Protein quantification

Total protein content was determined by the Bradford method (Bradford, 1976). A calibration curve was obtained using BSA (Bovine Serum Albumin) standards (0-2 mg/ml). All results were divided by the total amount of protein, to express results per mg of total protein.

Statistical Analyses

Data was analysed for normality and homoscedasticity through Shapiro-Wilk and Levene's test, respectively. One-way ANOVAs were performed for each tissue (liver, intestine, muscle and gills) in each biomarker- lipid peroxidation, catalase, superoxide dismutase, glutathione-S-transferase and ubiquitin - in order to detect significant differences between treatments. For significant results ($p<0.05$), the Tukey *post-hoc* test was performed. Statistics were carried out using the Software Statistica (Version 8.0 StatSoft Inc., USA).

Results

Juvenile *D. sargus* showed some mortality in all treatments. Controls showed the lowest mortality rate (11,1%) but it increased to 16,6% in the microplastics treatment and in the microplastics contaminated with heavy metals (Fig.2.1). Total length (cm) and weight (g) of individuals were (mean ± SD) 36.5 ± 4.2/0.795 ± 0.28 for the control, 38.8 ± 4.8/1 ± 0.39 for the treatment 0.1% and 32.2 ± 6.2/ 0.512 ± 0.28 for the treatment 0.1%HM. Fulton's K condition index did not vary significantly among the experimental treatments (Fig.2.2). The exposure of *D. sargus* to microplastic beads resulted in a significant increase of GST in the muscle (Table 2.1, Fig. 2.3). All other stress biomarkers presented no significant response, in all tissues examined (Fig. 2.3). Microplastics contaminated with heavy metals significantly affected four out of the five biomarkers tested (Table 2.1), with the exception of superoxide dismutase, which was not affected by microplastics with heavy metals in any tissue. The microplastics with heavy metals treatment lead to a significant increase in LPO in the muscle, GST in the gills, CAT and Ubiquitin in the liver (Fig. 2.3). Also, intestine was the only tissue that did not responded significantly to any of the oxidative stress biomarkers and muscle was the only that responded significantly to more than one biomarker (GST and LPO). GST, CAT and LPO responded significantly to one of the four analysed tissues (Fig. 2.3).

Table 2.1 One-way ANOVA results for glutathione-S-transferase, catalase, superoxide dismutase, lipid peroxidation and ubiquitin in liver, intestine, muscle and gills accounting for treatment of *Diplodus sargus.* Significant values on bold.

	GST		CAT		SOD		LPO		Ub	
	F	P	F	P	F	P	F	P	F	P
Liver	0.71	0.511	4.00	**0.044**	2.52	0.121	0.95	0.411	5.99	**0.015**
Intestine	1.97	0.174	1.27	0.309	0.87	0.443	1.12	0.349		
Muscle	4.96	**0.022**	3.58	0.053	3.09	0.079	39.10	**0.000**		
Gills	5.23	**0.020**	1.83	0.197	0.84	0.452	2.76	0.097		

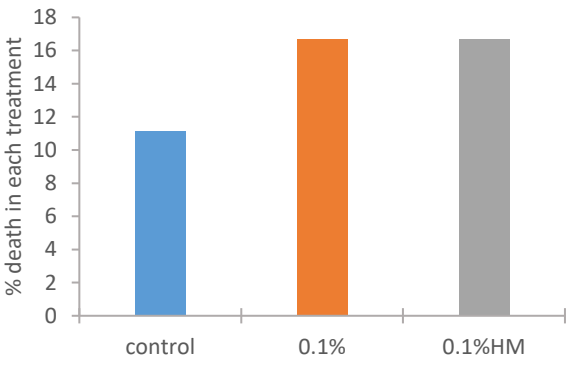

Fig.2.1 Mortality (%) in juvenile *Diplodus sargus*, after exposure to a concentration of 0.1% of virgin microplastics (0.1%) and 0.1% of heavy metal contaminated microplastics (0.1%HM).

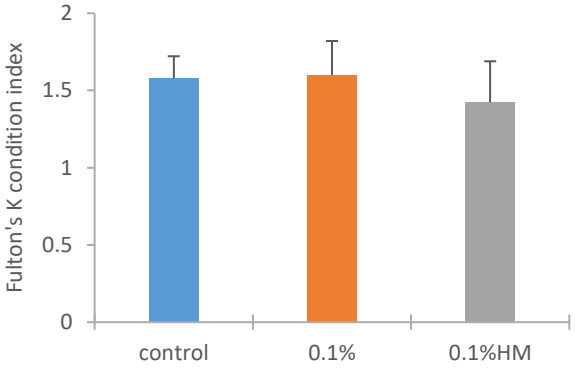

Fig.2.2 Fulton's K condition index in juvenile *Diplodus sargus*, after exposure to a concentration of 0.1% of virgin microplastics (0.1%) and 0.1% of heavy metal contaminated microplastics (0.1%HM).

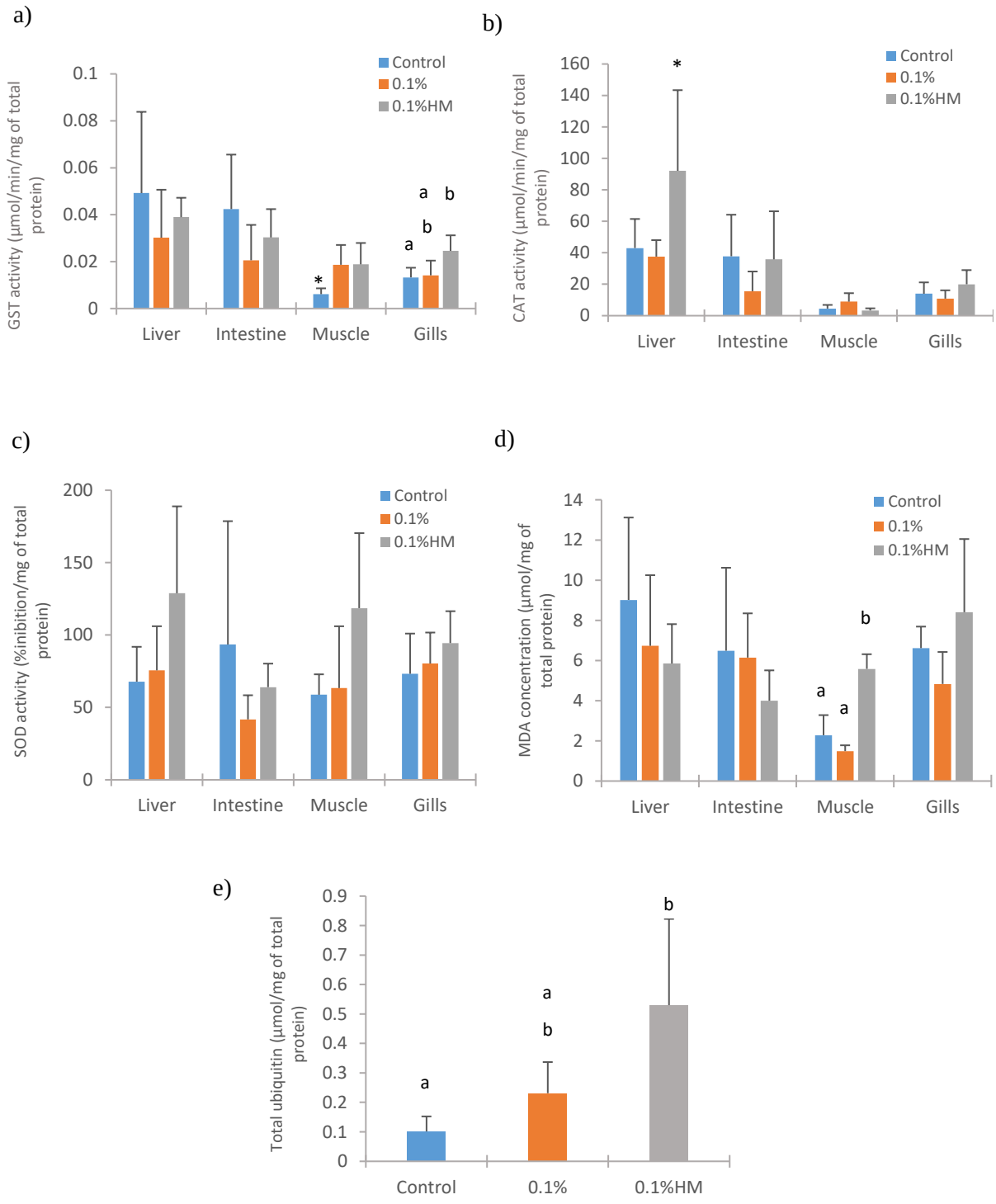

Fig.2.3 Levels (mean + SD) of antioxidant enzymes measured in several organs of juvenile *Diplodus sargus* exposed to a concentration of 0.1% of virgin microplastics (0.1%) and 0.1% of heavy metal contaminated microplastics (0.1%HM). **a)** Glutathione-S-Transferase (GST) activity **b)** Catalase (CAT) activity **c)** Superoxide dismutase (SOD) activity **d)** Lipid peroxidation **e)** total ubiquitin in the liver.

Discussion

This study aimed to analyse the role of microplastics as vectors of heavy metals for fish, using a whole-body and a cellular level complementary approach. The whole-body analyses showed a slight increase in mortality, caused by the exposure to microplastics. Exposing the fish to microplastics contaminated with heavy metals did not produce further mortality. No significant differences were detected in terms of body condition assessed by the Fultons-*K* index for any of the treatments, meaning that the stress caused to the individuals was not severe enough to cause any decrease in their general body condition. This study showed that microplastics *per se* can elicit some cellular stress, as shown by the increase of GST activity in the muscle. The role of microplastics as vectors of heavy metals for fish was clear, with significant responses by four out of the five stress biomarkers analysed and in various tissues. To the best of our knowledge this is the first study that revealed the cellular stress caused by microplastics contaminated with heavy metals, in fish, over a long-term experiment.

The present study demonstrated, for the first time, that heavy metal contaminated microplastics have a high oxidative-stress-inducing potential in *D.sargus*. A period of 30 days of exposure to heavy metal contaminated microplastics beads was enough to induce significant alterations in antioxidant enzymes such as GST, CAT and LPO concentrations, as well as ubiquitin levels. Given that the individuals used were 0-group juveniles, this small time-frame for cellular stress is highly relevant since it means that if such small juveniles spend even just a small part of their life-cycle in contaminated areas, they can be affected by this kind of pollution.

An interesting result of our work was the fact that the presence of virgin microplastics did not elicit the oxidative stress biomarkers response, for the exception of GST in muscle. The treatment with virgin microplastics worked as a second control. The major biomarkers response is due to the heavy metal contaminated microplastics. The reason for virgin microplastics not triggering the oxidative response, could indicate that the concentration of microplastic beads and the time of exposure were not enough to create stressful conditions and thus, to induce oxidative cellular damage.

Detoxification enzymes, such as GST, protect cells against ROS-induced damage, since it eliminates reactive compounds by forming conjugates with glutathione and eliminating them as mercapturic acid (Rodriguez-Ariza et al., 1991). In fish, antioxidant enzymes have been shown to be either induced or inhibited by copper, depending on the dose and the species. Zebrafish showed induction of CAT and GST within two weeks after copper exposure (Paris-Palacios et al., 2000), and otherwise, both GST and CAT were inhibited in carp after 96 hours of a higher dose of copper exposure (Dautremepuits et al., 2002). In our study, significant GST response occurred in the muscle for treatment with virgin microplastics. Since GST was the only biomarker to respond significantly to the treatment with virgin microplastics, this might indicate that this biomarker could be particularly interesting to study only plastic contamination. CAT activity responded significantly to the treatment with contaminated microplastics in the liver, which suggest the accumulation of H_2O_2 but not enough to make CAT activities inhibit in this tissue. Dautremepuits et al. (2004), also reported increases in CAT activity in the liver of carp after copper exposure.

Farombi et al. (2007) reported an increase of MDA in gills in African Cat fish (*Clarias gariepinus*) from the Ogun River, located closed to major industries in Nigeria, as well as Pandey et al. (2008), after exposing the freshwater fish *Channa punctata* to a mix of contaminants (Cu, Cd, Fe and Ni). Although, this MDA increase in the gills was not detected in the present study. Since lipid peroxidation results from oxidation of free radicals in polyunsaturated fatty acids, gills membrane damage probably results from the corrosion of polyunsaturated fatty acids compromising the water transport and the osmoregulatory functions of gills. Otherwise, our data showed significant increase of MDA concentrations in the treatment with contaminated microplastics in the muscle of *D.sargus*. In a study by Avci et al. (2005) increases of MDA concentrations in muscle were also reported in the fish *Silurus glanis* from a very contaminated river. On the other hand, a decreasing of MDA concentrations

in fish liver was reported, similarly to what occurred in our study. This is in accordance with previous studies that suggested that the liver has a stronger defensive mechanism than other tissues (Di Giulio et al., 1989; Radi and Matkovics, 1988). Also, since the muscle was the tissue that responded more clearly to more biomarkers (GST and LPO), we suggest that is the most indicated tissue for biomonitoring this type of contamination.

In our work, SOD activity did not responded to heavy metal contamination, as happened in the study by Maria et al. (2009), where SOD also showed lower antioxidant enzymes activities in wild juvenile *Dicentrarchus labrax* in Ria de Aveiro, and yet they associated this low response to a possible inhibition of the antioxidant system due to contaminants chemical structure and/or concentrations. Also, SOD inhibition in carp was showed after 46 hours of heavy metal exposure (Varanka et al., 2001), owing to enzyme deactivation.

Ubiquitin targets irreversibly damaged proteins to be degraded in the proteasome, preventing cytotoxic aggregations (Hofman and Somero, 1995). Under environmental stress several enzymes are damaged, losing their function. This triggers an increase of ubiquitin levels due to high number of denatured enzymes that are removed from the cell (Woo et al., 2009). This happened in our study, since significant ubiquitin increase occurred in the treatment with contaminated microplastics in the liver of *D.sargus*. This suggests that heavy metals toxicity in the liver induced formations of abnormal proteins, and may lead to protein degradation. This is in accordance with a study by Heier et al. (2013), which also reported hepatic increases in ubiquitin level after exposure to heavy metals in Atlantic Salmon (*Salmo solar*). Since Ubiquitin is an indicator of irreversible damage to protein it can be concluded that the oxidative stress provoked by the contaminated microplastics was considerably high.

Conclusion

The present study shows that microplastics are heavy metals vectors, compromising the health of the marine organisms and the environment. The exposure to virgin microplastics and microplastics contaminated with heavy metals caused different effects at the biomarkers response levels. Exposure to microplastics contaminated with heavy metals affected the liver, intestine, muscle and gills of *D. sargus* leading to an oxidative stress response. The present investigation highlights the importance of using oxidative stress biomarkers to investigate the oxidative effects of heavy metal contamination in fish. In addition, this work showed that *D.sargus* is a good bioindicator species of heavy metal contamination associated with microplastics, since this study produced a response after 30 days of exposure to contaminants.

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CHAPTER 3

Final conclusions and future perspectives

Marine pollution is a major environmental problem that affects all marine ecosystems and biota. Microplastics pollution is, currently, one of the most dangerous types of marine pollution and is an extremely relevant issue of concern. Microplastics ubiquity makes its presence inevitable in the world's oceans, and its consequences to aquatic organisms are severe. Also, the toxicity of chemicals associated to this type of plastics have begun to raise concern regarding the effects of contamination in marine organisms as a result of its ingestion.

The present study is the first long-term study that shows the role of microplastics as vectors of heavy metal toxicity in fish, contributing to the body of knowledge regarding the risks associated with microplastic pollution. Another important characteristic of this study is the integration of a complete set of biomarkers (GST, CAT, LPO, SOD and Ubiquitin) to assess the oxidative stress response of several tissues of fish juveniles, which had not been previously investigated.

The development of studies like this one is extremely important since there is a major lack of information regarding biochemical effects of heavy metal contamination by microplastics ingestion in marine organisms. The amount of literature regarding this issue is low, especially regarding long-term experiments, and so, hopefully this work will lead to the development of future studies regarding this matter.

Cellular responses may undergo the influence of genetics and environmental conditions. In this sense, it would be interesting in the future to have a more global perspective of how cellular response to environmental pollutants contaminants differ among species and among habitats. Studies like this, contribute to the understanding of how oxidative stress is induced in coastal marine organisms and what are the most vulnerable tissues to oxidative stress. Additionally, this study has the potential to contribute to the improvement of legal instruments that aim to biomonitor marine pollution and its effects on marine life, in order to minimize the future inputs of plastic in the oceans.