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Dissertação de candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

Orientador – Professora Doutora Lúcia Maria das Candeias Guilhermino

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0.1. Abstract

Plastics have been increasingly found in aquatic environments. They are now considered as emerging pollutants of high concern, mainly because they are able to induce physical and chemical toxic effects on the biota, to act as vectors for the entrance of several other environmental contaminants, including some of high toxicity, they can also interact with the toxicity of other common environmental contaminants. Subsequently they can enter into the food chain and thus potentially affecting humans due to bioaccumulation and biomagnification phenomena.

The central goal of the present study was to investigate the effects of cadmium alone and in combination with microplastics on early juveniles (0+ age group) of the common goby (*Pomatoschistus microps*) in relation to long-term exposure to environmental contamination in previous developmental phases.

The following hypotheses were tested: (i) *P. microps* juveniles from the estuaries of Minho and Lima rivers, which have differences in several environmental conditions including in the levels of environmental contamination, have differences of sensitivity to cadmium; (ii) the presence of microplastics in the water influences the toxicity of cadmium to *P. microps* juveniles.

To test the first hypothesis, two 96 hs laboratory bioassays, one with juveniles from the Minho river estuary and the other with juveniles from the Lima river estuary, were carried out. In both bioassays, groups of juveniles were exposed to artificial seawater (ASW) only (control groups) and to six nominal concentrations of cadmium (from 1.6 to 50 mg/L). In each bioassay, 9 juveniles individually exposed were used per treatment. Effect criteria were: mortality, post-exposure predatory performance, the levels of lipid peroxidation (LPO) and the activity of the enzymes acetylcholinesterase (AChE), gluthatione *S*-transferases (GST) and Ethoxyresorufin-*O*-deethylase (EROD). Data (individual effect criteria) were analyzed using a two Analysis of Variance (2-ANOVA) with interaction (main factors: estuary from where

the fish came and treatment), a one way Analysis of Variance (ANOVA), and other statistical tests when appropriate. To test the second hypothesis, two other bioassays were carried out, one with the juveniles of the Minho estuary and the other with Lima estuary juveniles. In each bioassay, the treatments were: ASW water only (control group), 0.186 mg/L of microplastics alone, and 6 concentrations of cadmium (from 1.6 to 50 mg/L), each one combined with 0.186 mg/L of microplastics. The number of fish per treatment and the effect criteria were similar to the bioassays testing cadmium alone. Data (individual effect criteria) were analyzed using a two Analysis of Variance (2-ANOVA) with interaction (main factors: presence/absence of microplastics and cadmium concentrations), one way Analysis of Variance (ANOVA), and other statistical tests when appropriate.

The comparison of the bioassays testing cadmium alone indicated: significant differences ($p \leq 0.05$) between fish from distinct estuaries (Minho and Lima) in AChE, GST, LPO and EROD; significant differences among treatments ($p \leq 0.05$) in GST; and a significant interaction between the origin of the fish (estuary) and cadmium concentrations in AChE. Fish from the Minho estuary had higher mean levels of AChE, GST, LPO and EROD than juveniles from the Lima estuary. In juveniles of the Minho estuary, cadmium did not induced significant differences relatively to the control group. In juveniles of the Lima estuary, cadmium induced significant differences relatively to the control group in AChE and GST at concentrations equal to 1.6 mg/L and equal to 1.6 mg/L/higher than 6.3 mg/L, respectively. Overall, these findings indicate differences of sensitivity to cadmium between juveniles from distinct estuaries, thus corroborating our first hypothesis. Since the experimental conditions were similar for fish from both estuaries, these results suggest that exposure to different environmental conditions, including distinct pollution levels, in the original habitats during pre-developmental phases modulated the toxic effects of cadmium.

The results of the second pair of bioassays performed, indicated that microplastics combined with cadmium were able to induce toxic effects on fish from both estuaries by increasing AChE and decreasing LPO and EROD (Minho estuary fish), and increasing AChE, GST, LPO and decreasing in EROD in juveniles from the Lima estuary. The comparison of the bioassays carried out with juveniles of the Minho river estuary testing cadmium alone and in the presence of microplastics indicated: significant differences ($p \leq 0.05$) between treatments with and without microplastics in predatory performance, AChE, LPO, and EROD; significant differences among cadmium treatments ($p \leq 0.05$) in predatory performance, AChE, GST, LPO and EROD; and no significant ($p > 0.05$) difference in interaction between the origin of the fish (estuary) and cadmium concentrations. The comparison of the bioassays were carried out with juveniles of the Lima river estuary testing cadmium alone and in the presence of microplastics indicated: significant differences ($p \leq 0.05$) between the bioassays performed with and without microplastics in, AChE, GST, and LPO; significant differences among cadmium treatments ($p \leq 0.05$) in predatory performance, no significant ($p > 0.05$) differences in the interaction between the origin of the fish (estuary) and cadmium concentrations.

In juveniles of the Minho estuary, cadmium in the presence of microplastics induced significant differences relatively to the control group in AChE at concentrations equal to 12.5mg/L. In juveniles of the Lima estuary in the presence of microplastics, cadmium induced significant differences relatively to the control group in GST, at concentrations equal or higher than 12.5 mg/L. Overall, these findings indicate that the presence of microplastics influences the toxicity of cadmium to juveniles from distinct estuaries, thus corroborating our second hypothesis. These findings indicate toxicological interactions between microplastics and cadmium.

Into the future it would be interesting to verify the interaction of microplastics with the trophic chain, performing the same type of bioassay with organisms from different levels of it. It would be also interesting to include methalothionines as biomarkers because they are induced by metals, and so in this case the interaction of microplastics in relation to cadmium induction of these protein family could be eventually assessed.

Keywords: *Pomatoschistus microps*, microplastics, cadmium, biomarkers, predatory performance.

0.2. Resumo

Os plásticos têm vindo a ser cada vez mais encontrados no ambiente aquático. São considerados actualmente como poluentes emergentes, sobretudo porque são capazes de induzir efeitos nocivos de cariz físico e químico na biota e de agir como vectores para entrada de vários outros contaminantes ambientais, incluído alguns de alta toxicidade. Os plásticos possuem ainda a capacidade de interagir com a toxicidade de outros contaminantes ambientais comuns. Posteriormente podem entrar na cadeia alimentar e, portanto, potencialmente afetar seres humanos por processo de bioacumulação e bioampliação.

O objetivo central do presente estudo é investigar os efeitos do cádmio, isoladamente e em combinação com microplásticos, em juvenis (0+) do góbio comum (*Pomatoschistus microps*) em relação à sua exposição de longo prazo a contaminantes ambientais em fases de desenvolvimento prévias.

As seguintes hipóteses foram testadas: (i) juvenis de *P. microps* do estuário do rio Minho e Lima, que têm diferenças em várias condições ambientais, incluindo nos níveis de contaminação ambiental, têm diferenças de sensibilidade face ao cádmio; (ii) a presença de microplásticos na água influência a toxicidade do cádmio a juvenis de *P. microps*.

Para testar a primeira hipótese, dois bioensaios de 96 hs foram realizados em laboratório, um com juvenis do estuário do rio Minho e outro com juvenis do estuário do rio Lima. Em ambos os bioensaios os grupos de juvenis foram expostos apenas à água salgada artificial (grupo controle) e seis a 6 concentrações nominais de cádmio (de 1,6 até 50 mg/L). Em cada bioensaio, foram usados 9 juvenis individualmente expostos por cada tratamento. Os critérios de efeito utilizados foram: mortalidade, desempenho predatório, níveis de peroxidação lipídica (LPO), actividade da enzima acetilcolinesterase (AChE), glutationa *S*-transferase (GST) e etoxiresorufina-*O*-desetilase (EROD). Os dados (critérios de efeito) foram

analisados usando a análise de variância (2 Way-ANOVA) com interacção (factores principais: estuário de onde o peixe foi recolhido e concentrações de cádmio), outra análise de variância (One-way ANOVA) foi utilizada, sempre que apropriado outros testes estatísticos foram efectuados.

Para testar a segunda hipótese, outros dois bioensaios foram realizados, um com juvenis do estuário do rio Minho e outro com juvenis do estuário do rio Lima. Para cada bioensaio, os tratamentos usados foram: água do mar artificial (grupo controle), 0,186 mg/L de microplásticos apenas, e seis concentrações de cádmio (de 1,6 até 50 mg/L), cada uma destas concentrações possuía também 0,186 mg/L de microplásticos. O número de peixes por tratamento e os critérios de efeitos foram semelhantes ao bioensaio mencionado anteriormente.

Os dados (critérios de efeito) foram analisados usando a análise de variância (2 Way-ANOVA) com interacção (factores principais: presença/ausência de microplásticos e concentrações de cádmio), outra análise de variância (One-way ANOVA) foi utilizada, sempre que apropriado outros testes estatísticos foram efectuados.

A comparação dos bioensaios que testaram apenas o cádmio indicaram: diferenças significativas ($p \leq 0,05$) entre os peixes de estuários distintos (Minho e Lima) em AChE, GST, LPO e EROD; diferenças significativas entre os tratamentos ($p \leq 0,05$) em GST; e uma interação significativa entre a origem do peixe (estuário) e concentrações de cádmio em AChE. Os peixes do estuário do Minho apresentaram maiores níveis médios de AChE, GST, LPO e EROD do que os juvenis do estuário do rio Lima. Nos juvenis do estuário do Minho o cádmio não induziu diferenças significativas relativamente ao grupo de controlo.

Nos juvenis do estuário do Lima o cádmio induziu diferenças significativas relativamente ao grupo de controlo em AChE e GST em concentrações iguais a 1,6 mg/L e iguais a 1,6 mg/L/maiores do que 6,3 mg/L, respectivamente. Em geral,

estes resultados indicam diferenças de sensibilidade ao cádmio entre juvenis de estuários distintos, corroborando assim a nossa primeira hipótese. Uma vez que as condições experimentais foram similares para peixes de ambos os estuários, estes resultados sugerem que a exposição a diferentes condições ambientais, incluindo níveis de poluição distintos, nos habitats originais durante as fases de pré-desenvolvimento pode ter modulado os efeitos tóxicos do cádmio.

Os resultados do segundo par de bioensaios realizados, indicaram que microplásticos combinados com cádmio foram capazes de induzir efeitos tóxicos sobre os peixes de ambos os estuários, aumentando a AChE e diminuindo LPO e EROD (peixes do estuário do Minho), e o aumento da AChE, GST, LPO e diminuindo em EROD nos juvenis do estuário Lima. A comparação dos bioensaios realizados com juvenis do estuário do rio Minho testando cádmio sozinho e na presença de microplásticos indicou: diferenças significativas ($p \leq 0,05$) entre os tratamentos com e sem microplásticos no desempenho predatório, AChE, LPO, e EROD; diferenças significativas entre os tratamentos de cádmio ($p \leq 0,05$) no desempenho predatório, AChE, GST, LPO e EROD; e nenhuma ($p > 0,05$) diferença significativa relativamente à interacção da presença/ausência de microplásticos com as concentrações de cádmio. A comparação dos bioensaios realizados com juvenis do estuário do rio Lima testando o cádmio sozinho e na presença de microplásticos indicou: diferenças significativas ($p \leq 0,05$) entre os bioensaios realizados com e sem microplásticos em, AChE, GST e LPO; diferenças significativas entre os tratamentos de cádmio ($p \leq 0,05$) no desempenho predatório, não houveram diferenças significativas ($p > 0,05$) na interação entre a presença/ausência de microplásticos e as concentrações de cádmio.

Nos juvenis do estuário do Minho, o cádmio na presença de microplásticos induz diferenças significativas relativamente ao grupo controlo em AChE, a concentrações iguais 12,5 mg/L. Nos juvenis do estuário do Lima, na presença de

microplásticos o cádmio induziu diferenças significativas relativamente ao grupo de controlo em GST, a concentrações iguais ou superiores a 12,5 mg/L. No geral, estes resultados indicam que a presença de microplásticos influência a toxicidade do cádmio para juvenis dos dois estuários, corroborando, assim, a segunda hipótese. Estes resultados indicam interações toxicológicas entre microplásticos e cádmio.

No futuro, seria interessante verificar a interacção de microplásticos com a cadeia trófica, realizando o mesmo tipo de bioensaio com organismos provenientes de diferentes níveis tróficos. Seria também interessante incluir metalotioninas como biomarcadores, porque são induzidas por metais, podendo-se tentar averiguar se os microplásticos tem capacidade de alterar a indução desta família de proteínas.

Palavras-chave: *Pomatoschistus microps*, microplásticos, cádmio, biomarcadores, desempenho predatório.

0.3. List of abbreviations

AChE: Acetylcholinesterase

ASW: Artificial seawater

ATSDR: Agency for Toxic Substances and Disease Registry of the United States of America

BHT: 2,6-bis(1,1-dimethylethyl)-4-methylphenol

Cd: Cadmium

DNCB: 1-chloro-2 ,4-dinitrobenzene

P450: Cytochrome P450

DTNB: (5,5'-dithiobis-(2-nitrobenzoic acid)

EROD: Ethoxyresorufin *O*-deethylase

GSH: Glutathione

GST: Glutathione S-transferases

LC_{50}: Median Lethal Concentration – the concentration estimated to cause 50% of mortality in the studied population under the specific conditions of the bioassay

LPO: Lipid peroxidation

MPs: Microplastics

OECD: Organization for Economic Co-operation and Development

PAHs: Polycyclic aromatic hydrocarbons

PCBs: Polychlorinated biphenyls

TBARS: Thiobarbituric acid reactive substances

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0.6. Acknowledgments

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Chapter I – Introduction

1. Introduction

Estuaries are very important ecosystems from both ecological and economic perspectives. Generally, they have a great diversity of habitats suitable for a wide range of organisms, including species that can live only in these ecosystems, living their whole life-cycle in estuaries and others that spend in that habitat their crucial periods of life (e.g. reproduction, juvenile development, etc.) (NOAA, 2008). This high biodiversity, including economic important species, several other resources (e.g. sand for building, species for field crops fertilization, water) and strategic location (e.g. adjacent to the sea) makes the estuaries a god support for a wide range of crucial anthropogenic activities, such as fishery, tourism, maritime traffic, several types of industry, nearby urban settlements, among several others (Costanza *et al.*, 1993, Abrahim and Parker, 2002, Meire *et al.*, 2005). For this reasons, they have been selected for human settlements since remote times (Wolanski, 2007). Recent estimations indicate that about 60% of the human population lives near estuaries and other coastal areas (Lindeboom, 2002), and these areas contribute with about 90% of global fisheries (Wolanski *et al.*, 2004). As the result of these intense anthropogenic pressures, several estuaries in diverse regions of the world have been suffering from habitat lost and fragmentation, over-exploration of resources alterations due to climate changes (Roessig *et al.*, 2004), invasion by exotic species, environmental contamination by chemicals (Villanueva and Botello, 1998, Simonetti *et al.*, 2013), and several other negative impacts.

Among many pressures that estuaries face, pollution is a major one, especially in heavy anthropogenically impacted areas, where these ecosystems are generally contaminated by a high number of chemical substances. Estuaries may receive environmental contaminants through river, urban/industrial effluent discharges, from the sea, from runoff of adjacent fields, among several other processes (Kennish, 1997).

Many of these environmental contaminants persist for long periods in estuarine water, where they may accumulate in different environmental compartments (e.g. sediments) and in the biota. Once they have entered in the food chain, some may be biomagnified (Mwashote, 2003, Kojadinovic *et al.*, 2007), increasing the risk of toxic effects on high-level predators and humans who consume them (Ababneh and Al-Momani, 2013). Common environmental contaminants of estuaries are metals, several types of pesticides, polycyclic aromatic hydrocarbons (PAHs), as polychlorinated biphenyls (PCBs), nutrients, among several others (Wang *et al.*, 2008). The contamination of estuaries by these chemicals is very concerning because they can reduce the biodiversity (e.g. through the elimination of most sensitive species), change ecosystem functioning (e.g. decrease of nutrients cycling through negative impacts of decomposers) and decrease ecosystem services (e.g. reduction of fisheries due to the decline of populations of edible species) (Chapman and Wang, 2001, YSEBAERT *et al.*, 2003, EPA, 2011). In addition to the well-known contaminants of estuaries, the presence of several other substances has been increasingly found in estuaries in the last decades. They are known as "emerging contaminants of concern" and include several types of chemicals of wide use, such as microplastics, pharmaceuticals, nanoparticles and nanomaterial's, among others (Richardson *et al.*, 2005, Moore, 2006, Farré *et al.*, 2008, Klaine *et al.*, 2008, Kahru and Dubourguier, 2010, Cole *et al.*, 2011). Microplastics are now considered ubiquitous pollutants in the marine environment, and more knowledge on their effects on marine organisms are needed (Andrady, 2011, Cole *et al.*, 2011, Galgani *et al.*, 2014), especially in relation to their potential toxicological interaction with other environmental contaminants (Monserrat *et al.*, 2007). This topic is relevant in the case of estuaries, because in industrialized and/or densely populated areas, they are contaminated by a wide range of different substances and the

concentration of microplastics is expected to be high in these ecosystems (Thompson *et al.*, 2004, Cole *et al.*, 2011).

1.1. Cadmium

Cadmium (Cd) is a metal that was discovered in 1817 by Friedrich Strohmeyer. It is considered a ubiquitous environmental contaminant of high concern due to its toxicity even at low concentrations (Matovic *et al.*, 2011).

Cadmium and some of its compounds are classified as human carcinogens (IARC, 1993). In humans it can accumulate in the kidney (Klaassen and Liu, 1997), liver (Novelli *et al.*, 1998) and testicles (Shen and Sangiah, 1995). In humans and other mammals, cadmium can induce nephrotoxicity (Adams *et al.*, 1969), osteotoxicity (Kazantzis, 2004), carcinogenesis (Mussalo-Rauhamaa *et al.*, 1986), DNA damage (Waalkes and Poirier, 1984), among other toxic effects. It may be accumulated by several organisms, including plants (Grant *et al.*, 1997) and fish (Asagba *et al.*, 2008, Ababneh and Al-Momani, 2013), and thus it can induce chronic toxicity in humans through the consumption of these species (Ababneh and Al-Momani, 2013).

Cadmium is a natural substance, however their environmental background levels may be considered increased as the result of natural processes (e.g. volcanic eruptions) and anthropogenic activities, such as incineration, agriculture, mining, and combustion of coal and oil (Matovic *et al.*, 2011). Several studies have reported environmental concentrations of cadmium in environmental compartments. For example, cadmium concentrations ranging from 0.1 to 10 ng/L have been found in open sea water, however considerably higher concentrations can be found in estuaries and other coastal systems, reaching around 100 ng/L in heavy contaminated areas (Kennish, 1997, ICdA, 2014). Marine organisms can uptake cadmium both from the environment (water and sediment) and from contaminated

food (Baldisserotto *et al.*, 2005). Moreover, several marine organisms bioconcentrate and bioaccumulate cadmium, for example, cadmium concentrations between 5.7 and 38.7 mg/Kg have been found in scallops (*Argopecten irradians*) from (Shenzhen, China) (Pan and Wang, 2008), 1 and 5 mg/kg (range of values that were recorded in several coastal zones around the globe) in mussels (*Mytilus edulis*) (Kennish, 1997), and between 46 and 50 mg/Kg in oysters (*Crassostrea virginica*) from the Pearl River in China (Fang *et al.*, 2003). There are several studies in Portugal about cadmium contamination, concentrations in sediment (Soares *et al.*, 1999) have reported contamination of the river Ave, Este and Trofa, among others (the average cadmium of the sites considered in this study was 2.89 mg/Kg); concentration of cadmium in aquatic species, there are studies reporting concentrations of cadmium in mussels (*Mytilus edulis*) (Stenner and Nickless, 1974) along the Portuguese coast reporting values 2-4 µg/g dry weight; there are also studies that have reported concentrations of 150 µ/g on common octopus (*Octopus vulgaris*) that were collected in Matosinhos area and there are several studies on fish like (Vieira *et al.*, 2011) where the average concentration of cadmium in several fishes was analyzed, 0.0056 ± 0.0029 (0.0017-0.0151 mg/Kg wet weight), 0.0084 ± 0.0036 (0.0031-0.0144 mg/Kg wet weight) and 0.0075 ± 0.0029 mg/Kg (0.0030-0.0141 mg/kg wet weight) were the values reported for sardine (*Sardina Pilchardus*), chub mackerel (*Scomber japonicus*) and horse mackerel (*Trachurus trachurus*), respectively.

In fish, cadmium can induce mortality and there are some studies about the median lethal concentrations (LC_{50}) in fish species, such: 173.78 mg/L in the rita (*Rita rita)* after (Ghosh and Mukhopadhyay, 2000); 121.8 mg/L in the common carp (*Cyprinius carpio*) after 96 h of exposure (Muley *et al.*, 2000); 14.95 mg/L in spotted snakehead (*Channa punctata)* after 96 h of exposure (Tiwari *et al.*, 2011); 96.57 ppm in mozambique tilapia (*Oreochromis mossambicus*) after 96 h of

exposure (Benjamin and Thatheyus, 2012); 50.41 ppm in fossil cat (*Heteropneustes fossilis)* after 96 h of exposure (Jain and Kumar, 2010); and 30.4 mg/L in guppy (*Poecilia reticulata*) after 96 h of exposure (Yilmaz *et al.*, 2004). Other toxic effects induced in fish by cadmium include, changes of metabolic patterns (Almeida *et al.*, 2001), growth inhibition (Lemaire and Lemaire, 1992), decrease of the reproduction (Lin *et al.*, 2000), among others.

1.2. Microplastics

Since the introduction of plastics in the market, their global production has been continuously increasing, reaching about 57 Megatons in Europe and 288 Megatons worldwide in the year of 2012 (Plastics Europe, 2013). Plastic polymers are very suitable materials for use in a wide range of industrial applications (Plastics Europe, 2010, Ribic *et al.*, 2010), their production costs are relatively low and thus their introduction/applications in several goods and devices significantly contributed to make more affordable products to a wide range of consumers from everywhere in the world (Andrady, 2011). As the result of plastic production, use and non-appropriate disposal, plastic materials are continuously entering into the environment (Andrady, 2011). Most part of them takes a long time to degrade, and the other part is degraded in a very slow rate, so plastics have been accumulated in the environment over time, reaching enormous quantities in several areas (Derraik, 2002, Barnes *et al.*, 2009, Ryan *et al.*, 2009). Thus, at the present, the environmental contamination by plastics is a major pollution problem at global level, despite the recycling and other efforts that have been done to reduce their environmental amounts.

In the marine environment plastics can suffer degradation that can be initiated trough sunlight (particularly ultraviolet light), this process is considered efficient when these plastics are exposed to air, however when floating in the sea, this

process occurs slowly due to lower temperatures and oxygen concentrations (Andrady *et al.*, 1993, Andrady, 1998).

The main process of ecotoxicological interest probably is their low and progressive fragmentation into smaller particles reaching the micro size, and they are known as secondary microplastics (Zitko and Hanlon, 1991). In addition to these, there are microplastics specifically produced to have micro or nano sizes, which are known as primary microplastics, such microplastics are used in a wide range of industries for several purposes such cosmetic products like facial cleansers or exfoliants (Zitko and Hanlon, 1991, Galgani *et al.*, 2010).

Microplastics, recently defined as plastic particles with a size lower than (≤ 5mm) (NOAA, 2014), have been found everywhere in the marine environment and are now considered marine pollutants of high concern (Thompson *et al.*, 2004, Ryan *et al.*, 2009). Their concentrations are particularly high in ocean gyres (Cole *et al.*, 2011), in estuaries and other coastal ecosystems of highly impacted areas. Along the Belgian coast (in a harbor sediment sample) where found concentrations of 390 particles Kg^{-1} dry sediment (Claessens *et al.*, 2011). The studies made in Portuguese coasts begin to appear and reported microplastics concentrations of 392.85 items m^{-2} and 28.6 items m^{-2} in Cova the Alfarroba and Bordeira beaches respectively, the high difference between values may be explained due to differences in the populations near the locations studied (Bordeira Beach have significant less population density than Cova de Alfarroba) (Martins and Sobral, 2011).

These microplastics that are present in the environment may contain other chemicals, (including some of high concern due to their potential toxic effects), incorporated during their production to give them specific properties, as substances to improve fire resistance, to improve performance, endurance, among others (Browne *et al.*, 2007). For example the leaching of additives which are

incorporated in the raw plastic as nonylphenol or polyorominateddiphenyl ethers (Browne *et al.*, 2007), and other outdoor pollutants that have adhered to the surface of this material (Cole *et al.*, 2011) are matters of environmental concern (Barnes *et al.*, 2009). The property of adhesion is also being target of scientific concern due to adherence of metals to the surface of plastics, first studies are from (Ashton *et al.*, 2010, Holmes *et al.*, 2012) that were made with polyethylene pellets and reported a surprising (since the pellets don´t have charge) adhesion of metals to the surface of these structures.

The small size of microplastics debris makes them "available" to a broad spectrum of marine organisms, this is why this subject is also attracting scientific attention (Derraik, 2002, Thompson *et al.*, 2004, Barnes *et al.*, 2009, Fendall and Sewell, 2009, Lozano and Mouat, 2009). In this regard several studies began to appear reporting the intake of micro-plastics in various levels of the food chain (Browne *et al.*, 2008), the species that lie at the bottom of the food chain do not have the ability to discern the type of food that are being ingested, making them more likely to the ingestion of microplastics (Moore, 2008). So latterly are beginning to appear several studies of the possible effects that microplastics can cause to the individuals who have ingested it such, mortality, morbidity, impact on reproductive capacity (Zarfl *et al.*, 2011), it is known that plastics cause-oriented mechanical effects in small animals (Barnes *et al.*, 2009, Fendall and Sewell, 2009).

As stated above several marine species uptake microplastics from the water, including zooplankton (Cole *et al.*, 2013), crustaceans (Murray and Cowie, 2011), mollusks (Browne *et al.*, 2008), fish (Davison and Asch, 2011), seabirds (van Franeker *et al.*, 2011), possibly also whales (Fossi *et al.*, 2012) among others. In a large extent, the ingestion occurs by confusion with food (Browne *et al.*, 2008, Moore, 2008). Microplastics of different types (mainly polyethylene, polypropylene and polyvinylchloride) and size have been found in the gastrointestinal track of

marine fish from different regions (Carpenter and Smith, 1972, Frias *et al.*, 2010, Lusher *et al.*, 2013, Wright *et al.*, 2013). Laboratorial studies with the lugworm, *Arenicola marina* (L.) (Besseling *et al.*, 2013) also indicated the ingestion of microplastics. In fish, microplastics may cause physical and chemical toxic effects. Physical effects include blockages, internal abrasions (Talsness *et al.*, 2009), oriented mechanical effects (Fendall and Sewell, 2009) among others. Physical harm can be caused by the presence of microplastics themselves but there are other process that can cause chemical toxicity which is the exposition to additives that lixiviate directly from these material and may subsequently interfere with important biological mechanisms which may eventually lead to carcinogenesis and endocrine disruption (Mato *et al.*, 2001, Barnes *et al.*, 2009), examples of such materials commonly used as additives are polybrominated diphenyl ethers and the constituent monomer of phthalates, the bisphenol which are considered to be endocrine disruptors: they mimic some chemicals, competing with the synthesis of several hormones (Meeker *et al.*, 2009).

A few number of recent studies suggest toxicological interactions between microplastics and other environmental contaminants. For example, polyethylene plastic microspheres (1–5 mm) were found to influencing the toxicity of pyrene to the common goby (*P. microps*), possibly by modifying pyrene biotransformation (Oliveira *et al.*, 2013). Therefore, this is now considered a priority topic of research in marine ecotoxicology.

The growing awareness of the potential pollution by plastics and MPs of the aquatic environment has led to the Illinois in the U.S.A. to become the pioneer state in the world taking the decision to prohibit plastic exfoliating microbeads by the year of 2019 (Macdonald, 2014).

1.3. Bioassays with fish and effect criteria

Bioassays with fish have been used for decades for several purposes, including to assess the effects and risks of chemical substances. For example, bioassays with fish are recommended in the OCDE guidelines for testing of chemicals (OECD, 1993), including acute bioassays with juvenile fish (OECD, 2013b), fish growth bioassays (OECD, 2000), tests with embryos (OECD, 2013a), among others. Several species can be used, both marine and freshwater ones, including standard organisms and wild ones after proper acclimatization to laboratorial conditions. In fish bioassays, a considerable number of effect criteria can be used, including mortality, growth, reproduction, behaviour, subindividual biomarkers, among several others.

Either used alone or in combination with other effect criteria, fish behaviour is generally of high value because it may connect sub-individual changes with potential effects at population level (Atchison *et al.*, 1987). Fish behaviour is crucial to escape to predators (Gotceitas and Colgan, 1990), mate (Reebs, 2008), vigilance (Krause and Godin, 1996), among several others. Changes in behaviour induced by chemicals often cause a reduction of individual and population fitness, thus, the use of behavioural parameters may considerable increase the ecological relevance of the bioassays (Weis and Candelmo, 2012) and may allow the detection of subtle effects caused by pollutants affecting at least the individual level at concentrations lower than those needed to cause mortality (Graham and Thompson, 2009).

In the last decades, the use of biomarkers as effect criteria in fish bioassays has been intense. Several definitions of the term biomarker have been provided over the time. A considerable part of them consider a biomarker as a biological response to stress exposure, considered at sub-individual level, indicating a change relatively to a level considered normal for the studied population. Biomarkers allow the detection of adverse effects early in time, allow the integration of adverse

effects induced in time and space, the assessment of mechanisms of toxicity and biotransformation, and the integrated effects induced by multi-stressors (Oost *et al.*, 2003).

Acetylcholinesterase is the enzyme that degrades the neurotransmitter acetylcholine in the cholinergic synapses of vertebrates and invertebrates (Guilhermino *et al.*, 1998). Several environmental contaminants are able to change the activity of this enzyme, including anticholinesterase pesticides (e.g. organophosphate and carbamate insecticides), metals, surfactants and detergents, among several others (Monteiro *et al.*, 2005, Moreira and Guilhermino, 2005).

Glutathione *S*-transferases (GSTs) are a family of enzymes which are responsible for the catalyzing of the glutathione (GSH) in the reduced form to subtracts allowing the posterior detoxification, being also important in the prevention of the lipid peroxidation (George, 1994). This family of enzymes is useful as biomarker of environmental contamination and its used in this regard, as for instance (Moreira and Guilhermino, 2005).

Lipid peroxidation (LPO) is a process that refers to oxidative damage of the lipids, free radicals remove electrons from lipids of cell membranes resulting in damage for these structures, in fish exposed to metals its expected lipid peroxidation increase reflecting oxidative stress (Vieira *et al.*, 2009).

EROD (7-ethoxyresorufin *O*-deethylase) activity its effective to analyze the response of the cytochromes P450, and its widespread in fish assessments of exposure to chemicals (Whyte *et al.*, 2000, Oost *et al.*, 2003).

1.4. Objectives and outline of the Thesis

The central objective of the present thesis was to investigate the toxic effects of cadmium alone and in combination with microplastics on early juveniles (0^+ age group) of the common goby (*Pomatoschistus microps*) in relation to previous long-

term exposure to environmental contamination. The following hypothesis were tested:

(i) *P. microps* juveniles from the estuaries of Minho and Lima rivers, which have differences in several environmental conditions including in the levels of environmental contamination, have differences of sensitivity do cadmium;

(ii) The presence of microplastics in the water influences the toxicity of cadmium to *P. microps* juveniles.

P. microps was selected as test organism for this study because it has a wide distribution ranging from North Europe to Mediterranean coasts; it is an important intermediary predator in the ecosystems where it inhabits, controlling several zooplankton populations while in the larvae and early juvenile phase and it is a crucial prey for higher predators during its entire life cycle, including several fish consumed by humans; these reason make it a suitable sentinel species and a good test organism; the workgroup also have a considerable experience of working with it in both laboratorial and field studies (Monteiro *et al.*, 2005, Monteiro *et al.*, 2007, Vieira *et al.*, 2008, Vieira *et al.*, 2009, Guimaraes *et al.*, 2012, Oliveira *et al.*, 2012, Oliveira *et al.*, 2013). Cadmium was selected as one of the test substances mainly because is an ubiquitous environmental contaminant that is very toxicity both to wild organisms and humans (Matovic *et al.*, 2011), and because several metals, including heavy (such as cadmium) have been found in microplastics (Ashton *et al.*, 2010, Cole *et al.*, 2011, Holmes *et al.*, 2012). Polyethylene plastic microsphere were selected as microplastic model because polyethylene is one of the most produced type of plastics, being also one of the main types of microplastic polymers found in the environment and in marine biota (Frias *et al.*, 2010, Roy *et al.*, 2011, Rochman *et al.*, 2013).

This Thesis is divided in 5 Chapters: Introduction (Chapter I), Material and Methods (Chapter II), Results/Discussion (Chapter III), Conclusion (Chapter IV) and Reference

List (Chapter V). Chapter I corresponds to the introduction where the problem of estuarine contamination by chemicals, especially cadmium and microplastics, and fish bioassays are introduced; this section finish with the presentation of the objectives and outline of the Thesis. In Chapter II, the methods and procedures used during the experimental work and in data analysis are described. In Chapter III, the results obtained are indicated and discussed. In Chapter IV, conclusions and future work perspectives and presented. Finally, Chapter V corresponds to the reference list.

Chapter II – Material and Methods

2. Material and Methods

2.1. Chemical substances

The test substances used were cadmium chloride hemi (pentahydrate) (CAS number: 7790-78-5; ≤0.005% impurities) that was purchased from Merck (Germany), and fluorescent red polyethylene microspheres, 1-5 µm of diameter, which were purchased by Cospheric (California, USA). The salt used to prepare the artificial seawater (ASW) was, purchased from PRODAC (Italy). All the other reagents used were purchased from Sigma-Aldrich Chemical (Germany), with the exception of the Bradford reagent that was purchased from Bio-Rad (Germany).

2.2. Collection of *P. microps* and acclimatization to laboratory conditions

P. microps early juveniles (0^+ age group) were collected in the estuaries of Minho and Lima (NW of the Iberian coast), in one site per estuary: site A in the Minho River estuary: about 41°53'31"N, 8°49'28"W; site B in the Lima River estuary: about 41°41'11.41"N, 8°49'20.42"W. These estuaries were selected as a source of test organisms for this study because they are neighbor estuaries, with several comparable characteristics but having also important environmental differences, mainly in the background levels of environmental contamination (Guimaraes *et al.*, 2012). While Minho estuary is considered as low impacted (Ferreira *et al.*, 2003), Lima estuary have several sources of pollution such as the harbour and shipyard, industrial and urban effluents among others (Carvalho *et al.*, 2009).

Fish were collected with a hand operated net at low tide. After capture, the juveniles were transported to the laboratory as soon as possible in isolated thermal boxes with aeration containing water from the sampling site. They were acclimatized for at least 10 days to laboratory conditions in 60 L aquaria with air supply and filtration systems (EHEIM® classic 350, Germany), with a mean of 300 fish per

aquarium, and 15 L of water from the sampling site. Gradually (i.e. in about 4 days), the water was progressively replaced by artificial seawater (ASW). The ASW was prepared by dissolving marine salt (PRODAC, Italy) into distilled water until reaching 19 $g.L^{-1}$ of salinity (digital refractometer Hanna HI 96822, USA). Fish were feed *ad libitum"*, with "tropic mix food" (AQUAPEX, Portugal) and the ASW was renewed every two days. They were maintained at room temperature under a photoperiod of 16 hours light (L) and 8 hours dark (D) and a temperature ranging from 18°c to 26°c (HACH 40d field case probe, USA). Water, pH, temperature and dissolved oxygen were checked 3 times a week (HACH 40d field case probe, USA, and HANNA® HI 96822 digital refractometer, USA).

2.3. Bioassays to assess the acute toxicity of cadmium

Two acute 96h bioassays, one with juveniles from the Minho estuary and the other with juveniles from the Lima estuary, were carried out, following in general the OECD guideline nº 203 for acute toxicity testing with juvenile fish (OECD, 1993), with some modifications as described below. Early *P. microps* juveniles are very sensitive to manipulation. Thus fish were measured (digital caliper) and weighted (Analytical balance, Kern ABS-N, Kern & Sohn GmbH, Germany) only at the end of the bioassays. The bioassays were carried out in in the conditions of photoperiod as previously described (section 2.2). The temperature of water ranged from 20 °c to 24 °c (HACH 40d field case probe, USA). A

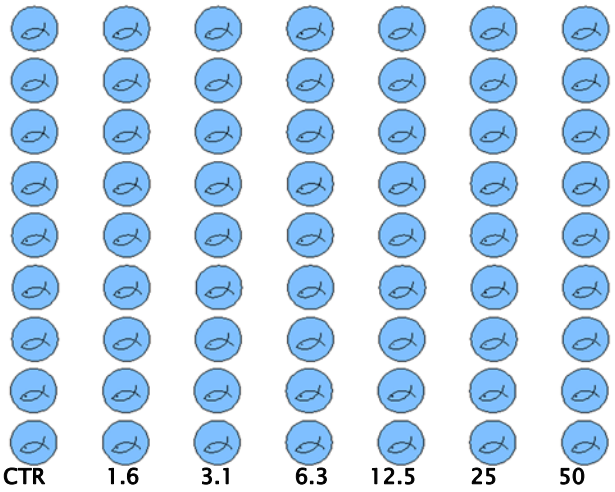

Figure1 – Schematic representation of the experimental design including a control treatment (artificial seawater) and 6 cadmium treatments with nominal (Cd) concentrations ranging from 1.6 to 50 (mg/L).

stock solution of cadmium chloride in ultra-pure (u.p.) water was prepared with a concentration of 50 g/L (Cd concentration). Test solutions containing cadmium were prepared by serial dilution from the stock solution in ASW prepared (as described in section 2.2), which was used as test medium. The experimental design (Figure 1) included a control (ASW only) and six cadmium treatments with the following nominal concentrations; 50.0, 25.0, 12.5, 6.3, 3.1, 1.6 mg/L of cadmium. Fish were exposed individually in 1000 mL test beakers with 500 mL of test medium (ASW or ASW + cadmium), with air supply. In each bioassay nine fish were used per treatment. Feeding was stopped 24 h before the begging of the bioassays and no food was provided during the exposure period. The effect criteria were mortality, predatory performance and AChE, GST, LPO and EROD biomarkers. At the beginning of the assay and at each 24 h, the following parameters were determined in test media: pH, dissolved oxygen (D.O.), and temperature (HACH 40d field case probe, USA). Mortality was recorded at each 24 h and was recognized when no movement of the fish was noticed or if there is no mechanical response to a gentle touch from a plastic pipette. At the end of the bioassay, post-exposure predatory performance was assessed as described below (section 2.5).

2.4. Bioassays to assess the acute toxicity of cadmium in the presence of MPs

The conditions and procedures of the bioassays (one with fish from the Minho estuary and another with fish from the Lima estuary) were similar to those described for bioassays with cadmium alone, with the differences further indicated. In each bioassay, treatments were: control (ASW only); MP (0.185 mg/L); Cd (50 mg/L) + MP (185 µg/L); Cd (25 mg/L) + MP (185 µg/L); Cd (12.5 mg/L) + MP (185 µg/L); Cd (6.3 mg/L) + MP (185 µg/L); Cd (3.1 mg/L) + MP (185 µg/L); and Cd (1.6 mg/L) + MP (185 µg/L). Nine fish were used per treatment in each bioassay. In

addition, 3 test beakers with MP (185 µg/L) and 3 test beakers with MP (185 µg/L) and cadmium (50 mg/L Cd), in both cases without fish, were included in the experimental design to determine the behaviour of the test substances during the exposure period in the absence of fish. All the other procedures and conditions were made as indicated in sections 2.2 and 2.3.

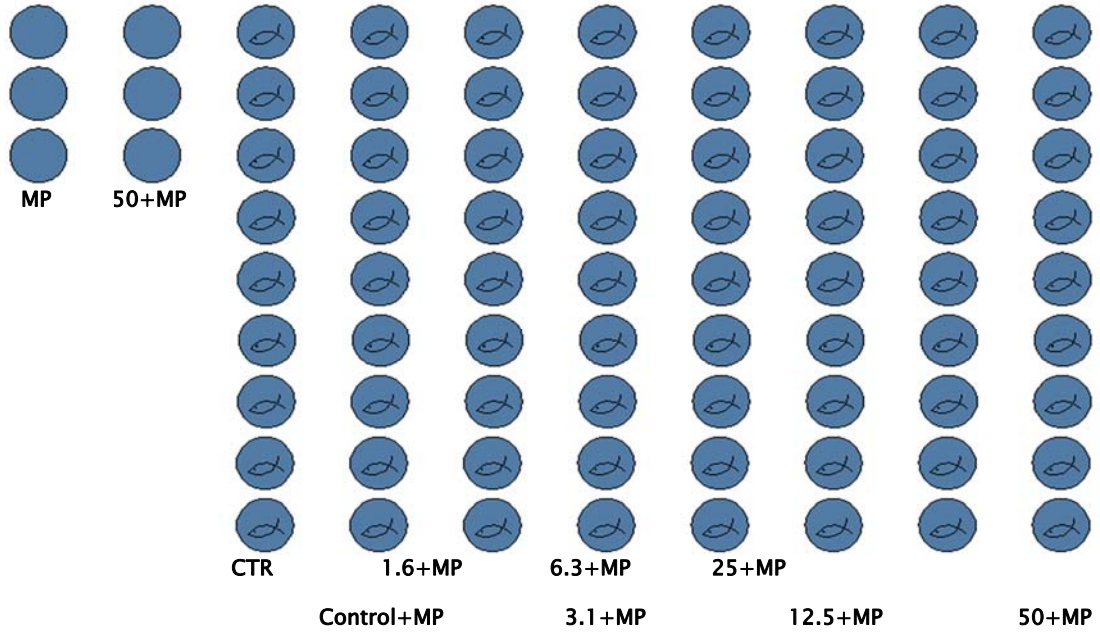

Figure 2 – Schematic representation of the experimental design. The treatments were: control (CTR) – artificial seawater (ASW) only; ASW + MP (185 µg/L) and several concentrations of cadmium (mg/L) + MP (185 µg/L) in artificial seawater.

2.5. Post-exposure predatory performance

Immediately after the 96h of exposure, a post-exposure predatory performance bioassay was performed following a protocol previously developed by the team. For this, each fish was carefully transferred to a prey-exposure chamber containing 300 mL of ASW where it was allowed to rest for 5 min. Then, 30 *Artemia franciscana* nauplii (48 hours old after hatching were introduced in this container with the ASW. After 3 minutes, the fish was removed and placed back into its original test solution, afterwards the number of nauplii remaining in prey-exposure chamber was counted. The number of the nauplii ingested by the fish (B) was determined as

the difference between the initial number of nauplii offered to the fish (A = 30) and the number of nauplii reaming in the chamber after fish removal. The fish predatory performance was determined as: B x100/A.

2.6. Biomarkers determination

After the post-exposure predatory performance test and 2 hs of resting in their original exposure solution, fish were measured, weighted and sacrificed by decapitation under cold-induced anesthesia. No other anesthetics were used to avoid interference with biomarkers determinations. From each fish, the head and the body were isolated separately on ice, and then stored separately in 1.5mL microtubes, then they were kept at -80°C (SANYO® MDF-U500VXC-PA ultralow freezer) until further analysis.

Four biomarkers were used in the present study: AChE activity as indicative of neurotoxic effects; EROD activity as indicative of phase I biotransformation alterations; GST activity as indicative of phase II biotransformation alterations and/or oxidative stress; and LPO levels as indicative of lipid peroxidation damage. The number of biomarkers was limited by the small size of the fish. In the day of the analysis, fish head and remaining body samples were defrosted on ice. Head samples were used for AChE determinations. Body samples were used for GST, LPO and EROD determinations. To each head, 500mL of cold phosphate buffer (0.1M, pH=7.2) were added; the sample was then homogenized on ice with a Ystral, Ballrechten-Dottingen homogenizer (Germany) for 40 seconds and then centrifuged (Eppendorf 5804r refrigerated centrifuge, Germany) at 3300g for 3 minutes at 4°c. The supernatant was carefully collected and used for AChE determinations. Relatively to the body samples, 700mL of phosphate buffer (0.1M, pH = 7.4) were added to each sample. From each sample, 250 mL were removed to a microtube containing 4µL of butylated hydroxytoluene (BHT) 4 % in methanol.

These samples were used to determine LPO levels. The remaining volume of body sample (450 mL) was centrifuged at 10000 g for 20 min at 4 °c (Eppendorf 5804r refrigerated centrifuge, Germany), the supernatant was carefully recovered and used for determination of GST activity and EROD activity.

Before AChE and GST enzymatic analysis, the protein content of the samples was determined by the Bradford technique (Bradford, 1976) adapted to microplate (Frasco and Guilhermino, 2002). Briefly, in the microplate, a calibration curve was made using bovine β-globulin as standard protein. From this solution, 0.010 mL, 0.005 and 0.002 mL were placed in successive column wells of the microplate (4 wells of each); 0.005 mL and 0.008 mL of u.p. water, respectively were added to these column wells, an additional series containing 0.010 mL of u.p. water was included. In the remaining microplate wells (also in quadruplicate), 0.010 mL of each sample were placed. Then, 0.250 mL of diluted Bradford reagent (using the dilution of 1mL of Bio-Rad for each 4 mL of u.p. water) were added to each microplate well. After 15 min in the dark, the absorbance was read at 600 nm in a microplate spectrophotometer (BIO-TEK®, Powerwave 340, USA). The protein content of each sample was determined from the calibration curve and standardized to 0.5 mg/mL.

The activity of AChE was determined by the Ellman's technique (Elman et al., 1961) adapted to microplate (Guilhermino *et al.*, 1996). Briefly, a solution of acetylthiocholine (0.075 M) was prepared in u.p. water in dark conditions, maintained at 4°c and used for no more than two weeks (solution A). A solution of (5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) 10 mM was prepared, for that 0.0198 g of DTND with 0.0075 g of $NaHCO_3$ were diluted in phosphate buffer (0.1M, pH= 7.2) in dark conditions it was maintained at 4°c and used for no more than two weeks (solution B). Before the enzymatic determinations, a reaction solution was prepared by adding 0.2 mL of the solution A and 1 mL of the solution B to 30mL

of phosphate buffer. Then, 0.050 mL of each homogenized head sample was placed in a microplate well, and 0.250 mL of the reaction solution were added to these wells. From each sample, 4 replicates were made. One column of the microplate was left blank, and two others contained the reaction solution only. The absorbance was read at 412nm during 5 minutes in a spectrophotometer microplate reader (BIO-TEK®, Powerwave 340, USA) for 5 minutes. The activity of the enzyme was determined from the slope of the linear part of the curve, after determination of the protein content of the samples at the end of the enzymatic analysis, using the procedure previously indicated. The enzymatic activity was expressed in nanomoles of hydrolysed substrate per minute per mg of protein (nmol/min/mg protein). In the experimental conditions used, the supernatant of *P. microps* head homogenates contain mainly AChE and not other esterases (Monteiro et al., 2005).

To GST activity is measured due to the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (DNCB). It was assessed with the post-mitochondrial fraction, following the Habig method (Habig *et al.*, 1974), adapted to microplates (Frasco and Guilhermino, 2002). The overall procedure is similar of that used in the AChE assessment. The main differences are among the phosphate buffer (in this case, 0.1 M, pH=6.5) and the reaction solution (in this case, 1.5 mL of DNCB, 9ml of GSH and 49.5 mL of phosphate buffer (0.1 M, pH= 6.5)). The wavelength used to read the absorbance (read for 5 minutes) was 340 nm (BIOTEK Powerwave 340). The results were expressed in function of the protein (nanomoles of substrate hydrolyzed per minute per mg of protein).

The LPO levels were assessed by quantification of thiobarbituric acid reactive substances (TBARS) and the results were expressed in nmol TBARS/g tissue (Ohkawa *et al.*, 1979). The procedure consists in the pipetting of 200 µL from the fish homogenate to an tube with 15mL capacity, after this process in the tube are

added 1000 µL of TCA (12%) then 800 µL of the Tris-HCl (60 nM) solution with DTPA (0.1 mM) were added and, finally 1000 µL of TBA (0.78%) were the last solution used in this stage. This set of reactants is then heated in a bain marie for one hour at 100° c. Subsequently 2 mL of the solution are transferred to 2 mL microtubes and centrifuged at 11500 rpm for 5 minutes at 25 ° c. The microtubes must be removed for a stand in the dark then the supernatant were used for absorbance reading performed on the spectrophotometer (JENWAY 6405 UV/Vis Spectrophotometer) at 535 nm.

The protocol that was used to quantify EROD was the one described in by (Burke and Mayer, 1974), the activity is measured is the increase of resorufin production, then the florescence is read. Summarizing 100 µL of the sample (fish homogenate) were pipetted directly into the cuvette of the spectrofluorometer, subsequently was added reaction buffer (1000µL) and NADPH (10 µL) immediately after the addition of the last reagent, the kinetics were read on the spectrofluorometer (Jasco® FP-6200) with excitation wavelength of 530 nm and emission of 585nm for a period of time of 10 minutes.

2.7. Statistical analysis of data

Data from the predation performance and individual biomarkers were checked for the basic requirements of the Analysis of Variance (ANOVA), namely homogeneity of variances (Bartlett's test) and normality of distribution (Kolmogorov-Smirnov normality test) (Zar, 1999). For the predatory performance, data transformations needed to be carried out, so the percentage were transformed into arcsin values. Then, for each parameter, a two-way ANOVA with interaction was carried out with (estuary and treatment as main factors, or presence of microplastics and cadmium concentrations as main factors). When significant differences were found after ANOVA analysis, the Tukey test was carried for each factor, comparing each

treatment, to identify statistical significant different treatments. The IBM SPSS Statistics, version 20 was used for all the statistical analysis. In all the statistical analysis the significance level was 0.05.

Chapter III – Results/Discussion

3. Results/Discussion

For better interpretation of the results of the bioassays performed, they will now be separated in four:

Bioassay 1 (B1) – Bioassay performed with *P. microps* from the Minho estuary with cadmium concentrations.

Bioassay 2 (B2) – Bioassay performed with *P. microps* from the Lima estuary with cadmium concentrations.

Bioassay 3 (B3) – Bioassay performed with *P. microps* from the Minho estuary with a mixture of cadmium concentrations and microplastics.

Bioassay 4 (B4) – Bioassay performed with *P. microps* from the Lima estuary with a mixture of cadmium concentrations and microplastics.

B1 and B2	Cadmium Concentration (mg/L)	B3 and B4	Cadmium Concentration (mg/L) + MPs Concentration (µg/L)
Control	0	**Control**	0
Conc.1	1.56	**Only MPs**	185 of MPs
Conc.2	3.13	**Conc.1 + MPs**	1.56 + 185 of MPs
Conc.3	6.25	**Conc.2 + MPs**	3.13 + 185 of MPs
Conc.4	12.5	**Conc.3 + MPs**	6.25 + 185 of MPs
Conc.5	25	**Conc.4 + MPs**	12.5 + 185 of MPs
Conc.6	50	**Conc.5 + MPs**	25 + 185 of MPs
		Conc.6 + MPs	50 + 185 of MPs

Table 1 – Concentrations of cadmium and MPS used in the different bioassays performed.

As previously stated two hypothesis will be tested:

(i) *P. microps* juveniles from the estuaries of Minho and Lima rivers, which have differences in several environmental conditions including in the levels of environmental contamination, have differences of sensitivity do cadmium;

(ii) the presence of microplastics in the water influences the toxicity of cadmium to *P. microps* juveniles.

Note that for the purposes mentioned, the criteria will be evaluated and compared, predatory performance and several enzymes (Acetylcholinesterase (AChE), Glutathione S-transferase (GST), Lipid peroxidation (LPO), Ethoxyresorufin-O-deethylase (EROD)).

So B1 (Minho) and B2 (Lima) assays will be compared in this section to verify the first hypothesis.

The second hypothesis will be verified by the comparison (predatory performance, AChE, GST, LPO and EROD) between B1 (Minho) – B3 (Minho + MPs) and B2 (Lima) – B4 (Lima + MPs).

3.1. Measured parameters and mortality

There was control of certain parameters over the four bioassays, and these are shown in the Table 2.

The protocol followed has been mentioned above (OECD, 1993). The data obtained from the measure of the parameters is in accordance with what has been stipulated as required, as pH have always maintained between 6 and 8.5. The temperature oscillation aimed to recreate the natural environment of the fishes (day-night cycle), this was not possible because the effective temperature range obtained was not in accordance with the temperature from the natural environment.

Parameters B1	**0 Hours**	**24 Hours**	**46 Hours**	**72 Hours**	**96 Hours**	**Diference (96h-0h)**
pH	8.37 (0.055)	8.39 (0.053)	8.44 (0.033)	8.39 (0.071)	8.43 (0.050)	0.06 (0.005)
Dissolved oxigen_mg/L	10.32 (0.052)	10.37 (0.048)	10.11 (0.035)	10.18 (0.057)	10.31 (0.045)	0.01 (0.007)
Temperature (ºc)	Average temperature registered during the assay: 21.9. (Minimum: 19.7 and maximum 23.2)					
Relative Humidity	Average relative humidity registered during the assay: 38.9. (Minimum: 35.0 and maximum 44.7)					
Parameters B2	**0 Hours**	**24 Hours**	**46 Hours**	**72 Hours**	**96 Hours**	**Diference (96h-0h)**
pH	8.33 (0.046)	8.29 (0.072)	8.29 (0.017)	8.30 (0.029)	8.32 (0.039)	0.01 (0.007)
Dissolved oxigen_mg/L	10.33 (0.054)	9.68 (0.079)	9.67 (0.073)	9.30 (0.178)	9.61 (0.060)	0.72 (0.006)

Temperature (ºc)	Average temperature registered during the assay: 18.5 (Minimum: 17.7 and maximum 20.2)					
Relative Humidity	Average relative humidity registered during the assay: 38.9. (Minimum: 32.6 and maximum 46.8)					
Parameters B3	**0 Hours**	**24 Hours**	**46 Hours**	**72 Hours**	**96 Hours**	**Diference (96h-0h)**
pH	8.29 (0.025)	8.30 (0.017)	8.30 (0.030)	8.34 (0.044)	8.34 (0.052)	0.05 (0.027)
Dissolved oxigen_mg/L	9.68 (0.079)	9.67 (0.073)	9.56 (0.104)	9.59 (0.101)	9.40 (0.178)	0.28 (0.099)
Temperature (ºc)	Average temperature registered during the assay: 23.0 (Minimum: 21.0 and maximum 23.9)					
Relative Humidity	Average relative humidity registered during the assay: 59.0 (Minimum: 52.9 and maximum 67.7)					
Parameters B4	**0 Hours**	**24 Hours**	**46 Hours**	**72 Hours**	**96 Hours**	**Diference (96h-0h)**
pH	8.32 (0.062)	8.37 (0.065)	8.39 (0.045)	8.44 (0.029)	8.44 (0.042)	0.11 (0.020)
Dissolved oxigen_mg/L	10.32 (0.052)	10.37 (0.048)	10.11 (0.034)	10.33 (0.054)	10.35 (0.061)	0.03 (0.009)
Temperature (ºc)	Average temperature registered during the assay: 22.6 (Minimum: 22.2 and maximum 23.5)					
Relative Humidity	Average relative humidity registered during the assay: 55.7 (Minimum: 44.7 and maximum 64.8)					

Table 2 – Different parameters measured during all the bioassays performed, values in parenthesis represent the standard deviation.

Mortality

Mortality_B1	**24h**	**48h**	**72h**	**96h**	**T(%)**
Control	0	0	0	0	0
1.56	0	0	4	0	44
3.13	0	1	0	1	22
6.25	1	1	0	2	44
12.5	0	2	1	1	44
25	1	5	2	1	100
50	5	4	0	0	100
Mortality_B2	**24h**	**48h**	**72h**	**96h**	**T(%)**
Control	0	0	0	1	11
1.56	0	0	0	4	44
3.13	0	1	1	1	33
6.25	0	1	1	1	33
12.5	0	1	0	1	22
25	0	5	2	0	89
50	3	5	1	0	100

Mortality_B3	**24h**	**48h**	**72h**	**96h**	**T(%)**
Control	0	0	0	0	0
Only MPs	0	0	0	0	0
1.56	0	2	1	2	56
3.13	1	1	0	0	22
6.25	0	3	1	0	44
12.5	0	2	1	1	44
25	3	4	1	1	100
50	8	1	0	0	100
Mortality_B4	**24h**	**48h**	**72h**	**96h**	**T(%)**
Control	0	0	0	0	0
Only MPs	0	0	0	1	11
1.56	0	0	1	1	22
3.13	1	1	1	1	44
6.25	0	2	2	0	44
12.5	0	1	0	0	11
25	6	0	1	2	100
50	6	3	0	0	100

Table 3 – Mortalities occurred throughout the different bioassays, the total is shown in percentage.

Of the four bioassays, B1- Minho, B2- Lima, B3- Minho + MPs, B4- Lima + MPs, there was no mortality verified in the controls of B1, B3 and B4 and so they can be considered valid, according to protocol (OECD, 1993). Unfortunately, in the bioassay B2 a fatality occurred in the control (Table 3), exceeding in 1% the 10% limit set by the protocol followed. After the bioassays a statistical analysis was performed in order to obtain the LC50 values, in this case however no relevant statistical differences were found because all the values of LC50 (from B1, B2, B3 and B4) intersect themselves on the confidence intervals.

Measurement of fish

B1 and B2

The fish used in bioassays had a total length of (2.3±0.2; 2.2±0.2 cm), and a weight of (0.14 ± 0.056; 0.12 ± 0.035 g), fishes from Minho and Lima estuaries, respectively.

B3 and B4

The fish used in bioassays had a total length of (2.2±0.2; 2.1±0.2 cm), and a weight of (0.11 ± 0.040; 0.12 ± 0.042), fishes from Minho + MPs and Lima + MPs bioassays respectively.

There are five important considerations in this section:

- One fish from the B2 bioassay (control) was moribund in the end of the assay, so it was not considered in the predatory performance, AChE, GST, LPO and EROD assessments.

- There was just one individual that survived in the Concentration 5 (25 mg/L of cadmium) in the bioassay B2 and for this reason it was not considered in the statistical analysis for not have statistical relevance.

- An individual bioassay B4 (only MPs) was inadvertently killed in the process performed for predatory performance, so it was not considered in the Predatory performance, AChE, GST, LPO and EROD assessments.
- The data of the predatory performance has suffered transformation from percentage to arcsin to be subjected to statistical analysis.
- In the EROD assessment 3 individuals of each treatment were joined in order to obtain relevant activity.

3.2. Experiment to assess whether there are differences in susceptibility to cadmium between fish from Minho and Lima Estuaries

To evaluate this hypothesis, in this section, individuals from the estuaries of Minho (B1) and Lima (B2) without microplastics will be compared (predatory performance, AChE, GST, LPO and EROD). As previously stated in section 2.2 Minho estuary is considered as reference while the estuary of Lima has several sources of pollution including an harbor, one shipyard, industrial and urban effluents among others.

Predatory performance was carried out in order to evaluate the influence of the toxic in the predation capacity of individuals. If possible it would be also analyzed the difference between estuaries i.e. between the Minho and Lima estuaries.

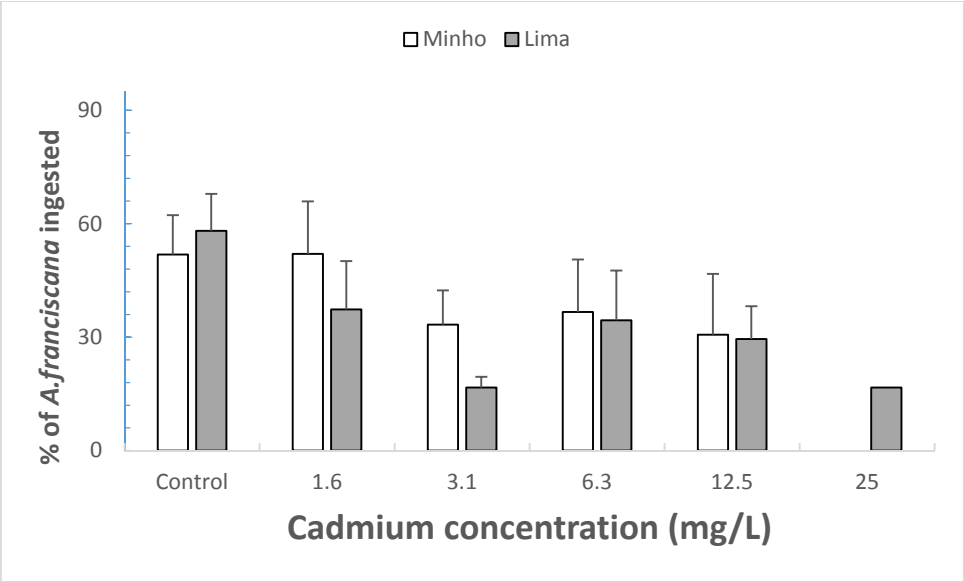

Figure 3 – Comparison of the percentage (nauplii ingested) between the bioassays from Minho (B1) and Lima (B2) estuaries. Values shown represent the average of the percentage of nauplii ingested by each treatment, the associated error is represented.

Factor	Estuary	N	Average ± standard error	F	P
Estuary	Minho	31	38.403 ± 3.554	$F_{(1, 52)}$= 0.472	P > 0.05
	Lima	31	34.984 ± 3.481		
Treatment	Control	16	47.618 ± 4.846	$F_{(4, 52)}$= 2.100	P > 0.05
	Conc1	10	40.442 ± 6.081		
	Conc2	13	29.136 ± 5.349		
	Conc3	11	34.872 ± 5.822		
	Conc4	12	31.400 ± 5.630		
Interaction				$F_{(4, 52)}$= 0.508	P > 0.05

Table 4 – Results of the statistical analysis (Two-way ANOVA) (data were transformed from percentage to arcsin values) of the nauplii ingested from the fishes of the two bioassays.

In the Figure 3 is represented the mean of the percentage of nauplli ingested by fish that survived the bioassay B1 and B2 (Minho and Lima estuaries without microplastics). The value shown on each bar of the graph is the average from each concentration and respective associated error.

After statistical analysis (Table 4) no significant differences were found in the following factors: estuary, treatment and interaction between estuary and treatment.

Although it appears that the percentage of ingested nauplii decrease over the concentrations tested, the standard deviation and associated error do not permit to obtain significant differences.

An one way ANOVA was performed separately to each estuary, $F_{(4,26)}= 0.632$, $p>0.05$ (Minho) and $F_{(4,26)}= 2.085$, $p>0.05$ (Lima).

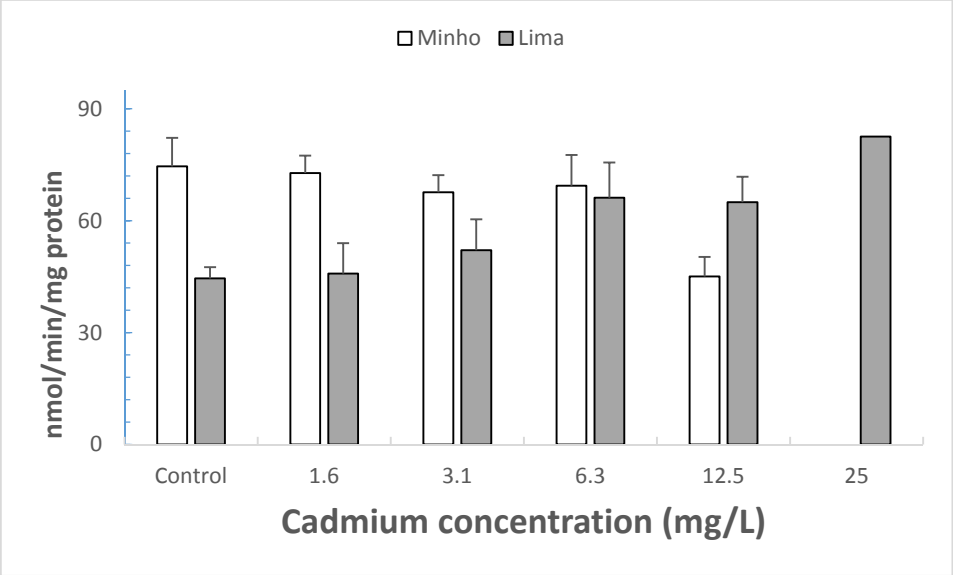

Figure 4 – Comparison of the AChE activity between the bioassays from Minho (B1) and Lima (B2) estuaries. Values shown represent the average of the AChE values by each treatment, the associated error is represented.

Factor	Estuary	N	Average ± standard error	F	P
Estuary	Minho	31	65.865 ± 2.734	$F_{(1, 52)}= 8.499$	$P \leq 0.05$
	Lima	31	54.709 ± 2.677		
Treatment	Control	16	59.544 ± 3.727	$F_{(4, 52)}= 1.089$	$P > 0.05$
	Conc1	10	59.289 ± 4.678		
	Conc2	13	59.849 ± 4.115		
	Conc3	11	67.750 ± 4.479		
	Conc4	12	55.005 ± 4.331		
Interaction				$F_{(4, 52)}= 5.771$	$P \leq 0.05$

Table 5 – Results of the statistical analysis (Two-way ANOVA) of the AChE activity from the fishes of the two bioassays.

In the Figure 4 are represented the average values obtained in AChE activity that was assessed in the homogenized head of *P. microps* in the individuals of B1 and

B2 (Minho and Lima estuaries without microplastics), the error shown is the associated error.

After statistical analysis (Table 5) no significant differences were found among treatments. However significant differences were found between the interaction of Minho and Lima estuaries and between these two estuaries.

This analysis indicated that fishes from Minho estuary have higher values of AChE than fishes from Lima estuary, suggesting that fishes from the Lima estuary are exposed to anticholinesterase agents. This results are in agreement with a previous study performed by (Guimaraes *et al.*, 2012), this study also demonstrated a lower activity of AChE in places that were supposed to have a higher level of contamination than Minho estuary that was considered as reference.

An one way ANOVA was performed separately to each estuary, $F_{(4,26)}= 2.312$, $p>0.05$ (Minho) and $F_{(4,26)}= 6.630$, $p\leq0.05$ (Lima). So in fish from the Lima estuary, significant differences among fish exposed to different treatments were found, with concentrations equal to 1.6 mg/L or higher than 6.3 mg/L inducing effects significantly different from the control group.

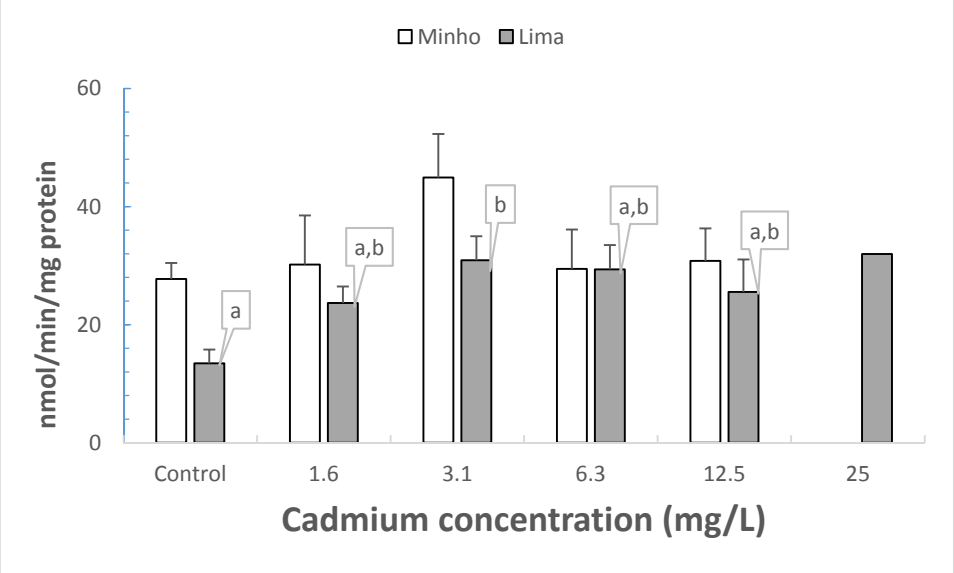

Figure 5 – Comparison of the GST activity between the bioassays from Minho (B1) and Lima (B2) estuaries. Values shown represent the average of the GST values by each treatment, the associated error is represented. Tukey test is representative of the One way ANOVA that was performed separately to each estuary.

Factor	Estuary	N	Average ± standard error	F	P	Tukey
Estuary	Minho	31	32.615 ± 2.562	$F_{(1, 52)}$= 5.362	P ≤ 0.05	
	Lima	31	24.313 ± 2.509			
Treatment	Control	16	19.907 ± 3.492	$F_{(4, 52)}$= 3.038	P ≤ 0.05	a
	Conc1	10	26.919 ± 4.383			a,b
	Conc2	13	37.914 ± 3.856			b
	Conc3	11	29.402 ± 4.196			a,b
	Conc4	12	28.180 ± 4.058			a,b
Interaction				$F_{(4, 52)}$= 0.689	P > 0.05	

Table 6 – Results of the statistical analysis (Two-way ANOVA) of the GST activity from the fishes of the two bioassays.

In the Figure 5 are represented the average values obtained in GST activity that was assessed in the homogenized body of *P. microps* in the individuals from B1 and B2 (Minho and Lima estuaries without microplastics), the error shown is the associated error.

After statistical analysis (Table 6) no significant differences were found in the interaction between estuaries and treatment. However significant differences were found between Minho and Lima estuaries and between treatments. This analysis indicate that fishes from Minho have higher values of GST than those form Lima estuary which may suggest different levels of contamination between the two locations, these contaminants may possibly function as enzyme inhibitors.

An one way ANOVA was performed separately for each estuary, $F_{(4, 26)} = 1.296$, $p>0.05$ (Minho) and $F_{(4, 26)} = 3.068$, $p\leq0.05$ (Lima). So in fish from the Lima estuary, significant differences among fish exposed to different treatments were found, with concentrations equal to 3.1 mg/L of cadmium inducing effects significantly different from the control group.

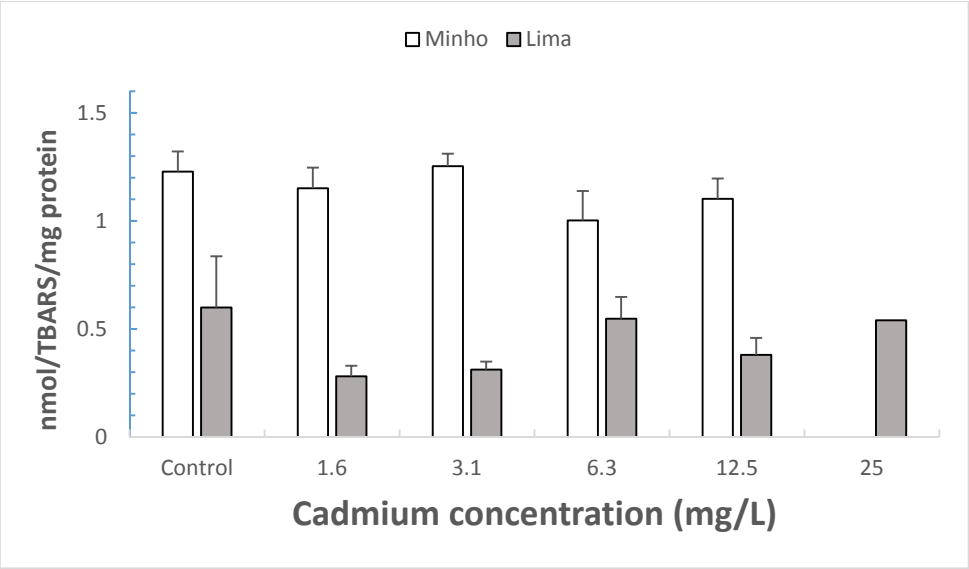

Figure 6 – Comparison of the LPO values between the bioassays from Minho (B1) and Lima (B2) estuaries. Values shown represent the average of the LPO values by each treatment, the associated error is represented.

Factor	Estuary	N	Average ± standard error	F	P
Estuary	Minho	31	1.148 ± 0.059	$F_{(1, 52)}$= 77.481	P ≤ 0.05
	Lima	31	0.424 ± 0.058		
Treatment	Control	16	0.914 ± 0.080	$F_{(4, 52)}$= 0.804	P > 0.05
	Conc1	10	0.716 ± 0.101		
	Conc2	13	0.783 ± 0.088		
	Conc3	11	0.775 ± 0.096		
	Conc4	12	0.741 ± 0.093		
Interaction				$F_{(4, 52)}$= 1.086	P > 0.05

Table 7 – Results of the statistical analysis (Two-way ANOVA) of the LPO values from the fishes of the two bioassays.

In the Figure 6 are represented the average values obtained from LPO levels that were assessed in the homogenized body of *P. microps* in the individuals from B1 and B2 (Minho and Lima estuaries without microplastics), the error shown is the associated error.

After statistical analysis (Table 7) no significant differences were found in the following factors: treatment and interaction between estuary and treatment. However significant differences between the Minho and Lima estuaries were found. This analysis indicate that fishes from Minho estuary have higher values of LPO than those form Lima estuary which may suggest different levels of contamination

between these two locations, the previous plausible exposure to contaminants could possibly have resulted in a lower response from individuals from the Lima estuary to lipid peroxidation.

An one way ANOVA was performed separately to each estuary, $F_{(4,26)}= 0.885$, $p>0.05$ (Minho) and $F_{(4,26)}= 0.929$, $p>0.05$ (Lima).

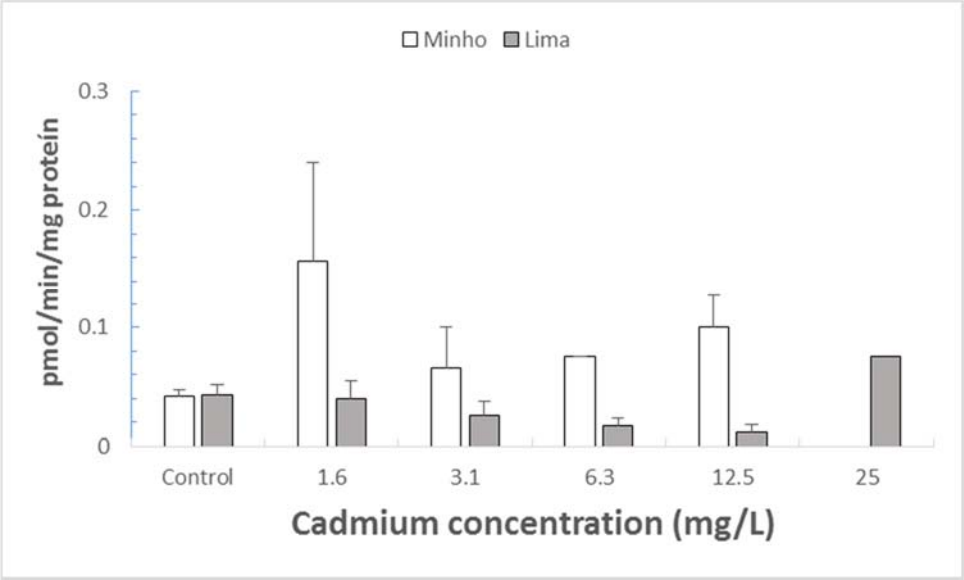

Figure 7 – Comparison of the EROD values between the bioassays from Minho (B1) and Lima (B2) estuaries. Values shown represent the average of the EROD values by each treatment, the associated error is represented.

Factor	Estuary	N	Average ± standard error	F	P
Estuary	Minho	11	0.088 ± 0.013	$F_{(1,11)}= 10.489$	P ≤ 0.05
	Lima	11	0.037 ± 0.013		
Treatment	Control	5	0.042 ± 0.019	$F_{(5,11)}= 1.251$	P > 0.05
	Conc1	4	0.098 ± 0.21		
	Conc2	4	0.046 ± 0.021		
	Conc3	4	0.046 ± 0.021		
	Conc4	4	0.059 ± 0.021		
Interaction				$F_{(4,11)}=1.239$	P > 0.05

Table 8 – Results of the statistical analysis (Two-way ANOVA) of the EROD values from the fishes from the two bioassays.

In the Figure 7 are represented the average values obtained in EROD activity that were assessed in the homogenized body of *P. microps* in the individuals from B1 and B2 (Minho and Lima estuaries without microplastics), the error shown is the associated error.

After statistical analysis (Table 8) no significant differences were found in the following factors: Treatment and interaction between estuary and treatment. However significant differences between the Minho and Lima estuaries were found. This analysis indicate that fishes from Minho estuary have higher values of EROD than those form Lima estuary which may suggest different levels of contamination between the two locations.

An one way ANOVA was performed separately to each estuary, $F_{(4,6)}= 0.1.438$, $p>0.05$ (Minho) and $F_{(4,26)}= 3.103$, $p>0.05$ (Lima).

3.3. Experiment to assess if the presence of microplastics in the water does not influence the acute toxicity of cadmium to *P. microps* juveniles

3.3.1. Comparison between bioassays B1 and B3 (Minho and Minho + MPs)

To evaluate this hypothesis on this section individuals from Minho estuary (B1) will be compared with individuals of Minho estuary with microplastics (B3) (predatory performance, AChE, GST, LPO and EROD).

If possible it would be also analyzed the difference between presence/absence of MPs i.e. between the Minho and Minho + MPs bioassys.

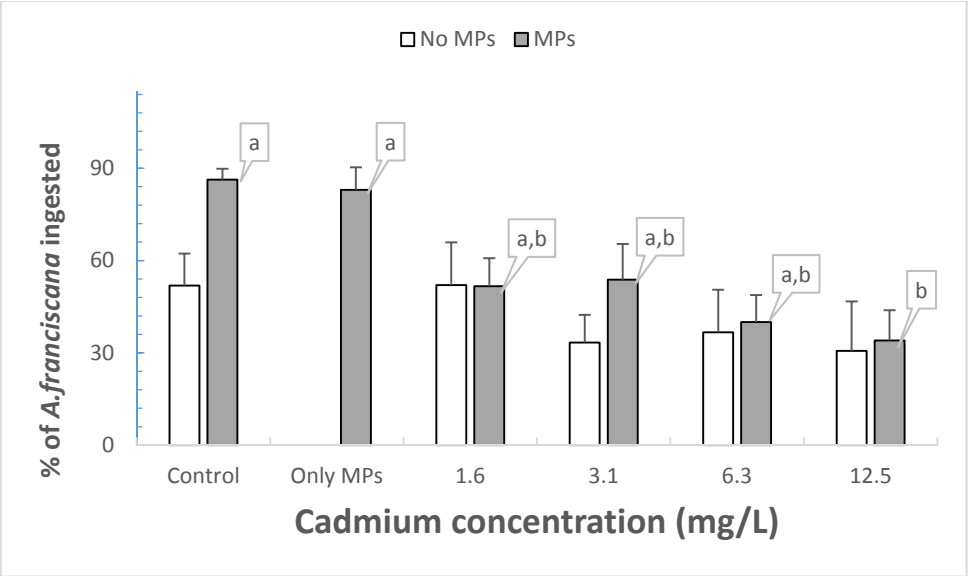

Figure 8 – Comparison of the percentage (nauplii ingested) between the bioassays from Minho (B1) and Minho + MPS (B3). Values shown represent the average of the percentage of nauplii ingested by each treatment, the associated error is represented. Tukey test is representative of the One way ANOVA that was performed separately to bioassay.

Factor		N	Average ± standard error	F	P	Tukey
	No MPs	40	40.940 ± 3.422	$F_{(1, 52)}$= 0.759	P > 0.05	
	MPs	21	41.963 ± 4.262			
Treatment	Control	18	57.515 ± 4.513	$F_{(4, 52)}$= 3.456	P ≤ 0.05	a
	Conc1	9	46.280 ± 6.422			a,b
	Conc2	14	41.166 ± 5.117			a,b
	Conc3	10	36.873 ± 6.055			a,b
	Conc4	10	33.200 ± 6.055			b
Interaction				$F_{(3, 52)}$= 0.270	P > 0.05	

Table 9 – Results of the statistical analysis (Two-way ANOVA) (data were transformed from percentage to arcsin values) of the nauplii ingested from the fishes from of the two bioassays.

In the Figure 8 is represented mean of the percentage of *A.franciscana* ingested by fish that survived the bioassay B1 and B3 (Minho without and Minho with microplastics, respectively). The value shown on each bar of the graph is the average from each concentration and respective associated error. After statistical analysis (Table 9) no significant differences were found in the following factors: presence and absence of MPs and interaction between the presence/absence of MPs with treatments. However significant differences among treatments have been

found, so, concentrations equal to 12.5 mg/L inducing effects significantly different from the control group, due to this fact it appears that cadmium has the ability to inhibit predatory performance in this concentration.

An one way ANOVA was performed separately to each bioassay, $F_{(4,36)}= 0.632$, $p>0.05$ (Minho without MPs) and $F_{(5,33)}= 7.416$, $p\leq0.05$ (Minho with MPs). So in fish from the Minho + MPs bioassay, significant differences among fish exposed to different treatments were found, with concentrations equal to 12.5 mg/L inducing effects significantly different from the control group.

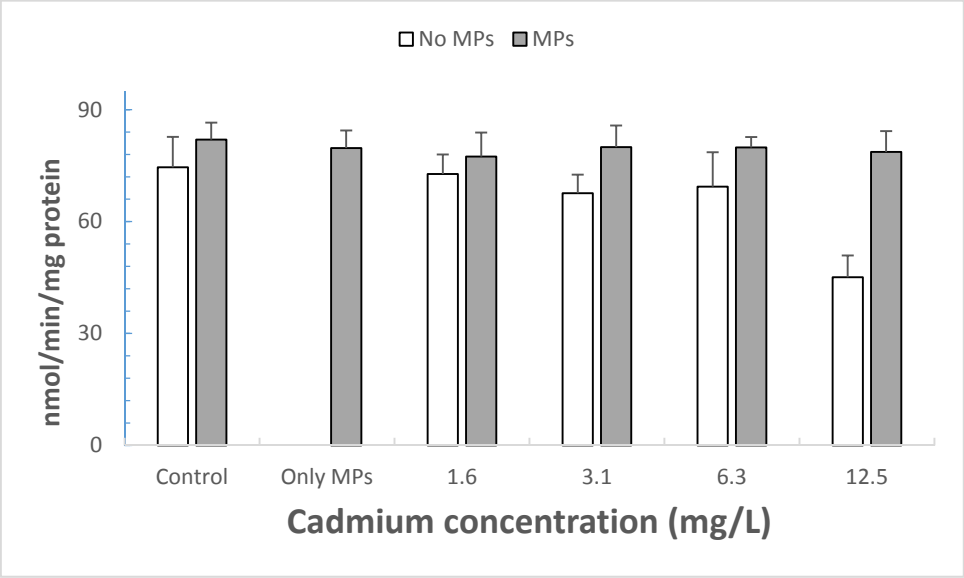

Figure 9 – Comparison of the AChE activity between the bioassays from Minho (B1) and Minho + MPs (B3) estuaries. Values shown represent the average of the AChE values by each treatment, the associated error is represented.

Factor		N	Average ± standard error	F	P
	No MPs	40	66.607 ± 2.846	$F_{(1, 52)}= 9.604$	P ≤ 0.05
	MPs	21	78.985 ± 3.546		
Treatment	Control	18	78.262 ± 3.754	$F_{(4, 52)}= 3.192$	P ≤ 0.05
	Conc1	9	75.094 ± 5.342		
	Conc2	14	73.780 ± 4.257		
	Conc3	10	74.620 ± 5.037		
	Conc4	10	61.862 ± 5.037		
Interaction				$F_{(3, 52)}= 1.544$	P > 0.05

Table 10 – Results of the statistical analysis (Two-way ANOVA) of the AChE activity from the fishes of the two bioassays.

In the Figure 9 are represented the average values (separated by concentrations) obtained in AChE activity that was assessed in the homogenized head of *P. microps* in the individuals of B1 and B3 (Minho without and Minho with microplastics, respectively), the error shown is the associated error.

After statistical analysis (Table 10) no significant differences were found in the interaction between the presence/absence of MPs with treatments. However significant differences between presence and absence of MPs and among treatments have been found. This analysis indicate that fishes of the Minho + MPs bioassay have higher values of AChE than those form Minho bioassay, which suggest interference of MPs in the enzyme functioning.

An one way ANOVA was performed separately to each bioassay, $F_{(4,36)}= 2.312$, $p>0.05$ (Minho without MPs) and $F_{(5,33)}= 0.081$, $p>0.05$ (Minho with MPs).

These results are in contradiction to a previous study performed by (Oliveira *et al.*, 2013) where inhibition of AChE was detected, in pyrene combined with MPs.

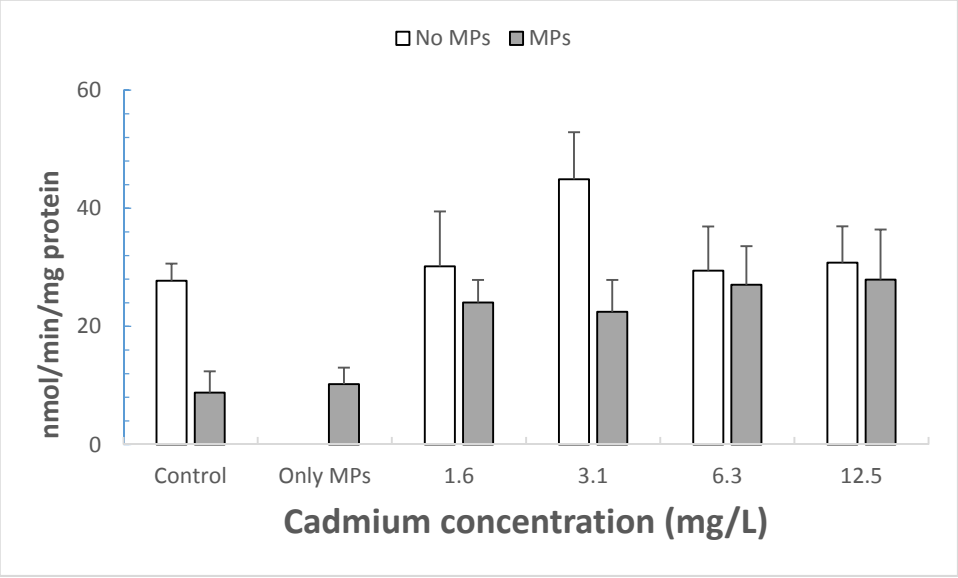

Figure 10 – Comparison of the GST activity between the bioassays from Minho (B1) and Minho + MPs (B3) assays. Values shown represent the average of the GST values by each treatment, the associated error is represented.

Factor		N	Average ± standard error	F	P
	No MPs	40	30.722 ± 2.838	$F_{(1, 52)}$= 2.958	P > 0.05
	MPs	21	25.374 ± 3.535		
Treatment	Control	18	18.267 ± 3.743	$F_{(4, 52)}$= 2.777	P ≤ 0.05
	Conc1	9	27.106 ± 5.327		
	Conc2	14	33.696 ± 4.244		
	Conc3	10	28.255 ± 5.022		
	Conc4	10	29.362 ± 5.022		
Interaction				$F_{(2, 52)}$= 1.120	P > 0.05

Table 11 – Results of the statistical analysis (Two-way ANOVA) of the GST values from the fishes of the two bioassays.

In the Figure 10 are represented the average values obtained in GST activity that was assessed in the homogenized body of *P. microps* in the individuals of B1 and B3 (Minho without and Minho with microplastics, respectively), the error shown is the standard error.

After statistical analysis (Table 11) no significant differences were found in the presence/absence of MPs and in the interaction between the presence/absence of MPs with treatments. However significant differences among treatments have been found. Cadmium seems to induce GST activity.

An one way ANOVA was performed separately to each bioassay, $F_{(4,36)}$= 1.296, $p>0.05$ (Minho without MPs) and $F_{(5,33)}$= 3.172, $p\leq0.05$ (Minho with MPs).

Although there are have been significant differences among treatments, the Tukey test had not statistic power to discriminate differences between treatments.

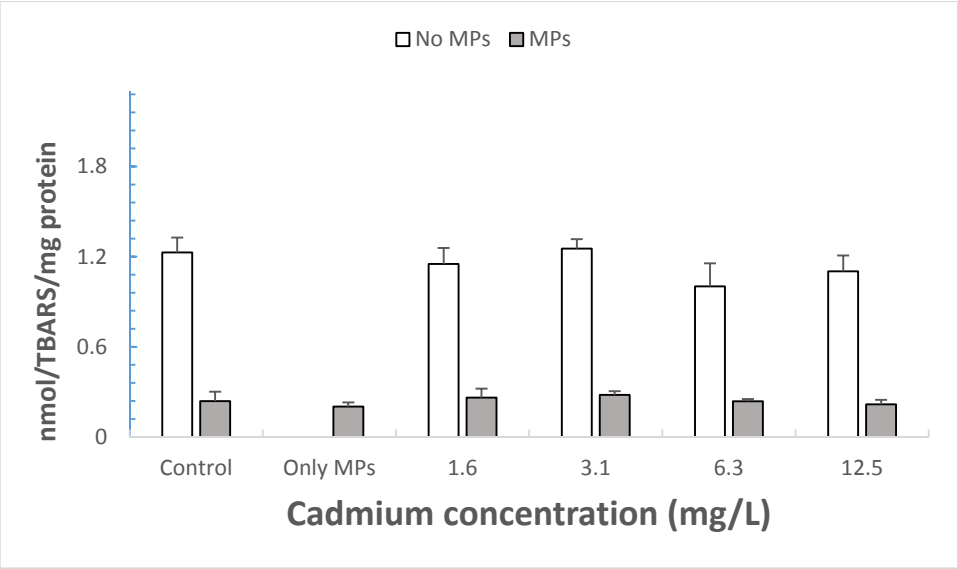

Figure 11 – Comparison of the LPO values between the bioassays from Minho (B1) and Minho + MPs (B3) assays. Values shown represent the average of the LPO values by each treatment, the associated error is represented.

Factor		N	Average ± standard error	F	P
	No MPs	40	1.049 ± 0.063	$F_{(1, 52)}$= 63.646	P ≤ 0.05
	MPs	21	0.249 ± 0.079		
Treatment	Control	18	0.734 ± 0.084	$F_{(4, 52)}$= 3.417	P ≤ 0.05
	Conc1	9	0.707 ± 0.119		
	Conc2	14	0.767 ± 0.095		
	Conc3	10	0.620 ± 0.112		
	Conc4	10	0.660 ± 0.112		
Interaction				$F_{(3, 52)}$= 0.167	P > 0.05

Table 12 – Results of the statistical analysis (Two-way ANOVA) of the LPO values from the fishes of the two bioassays.

In the Figure 11 are represented the average values (separated by concentrations) obtained from LPO levels that were assessed in the homogenized body of *P. microps* in the individuals of B1 and B3 (Minho without and Minho with microplastics, respectively), the error shown is the standard error.

After statistical analysis (Table 12) no significant differences were found in the interaction between the presence/absence of MPs with treatments. However significant differences between the presence/absence of MPs and among treatments have been found.

An one way ANOVA was performed separately to each bioassay, $F_{(4,36)}$= 0.885, $p>0.05$ (Minho without MPs) and $F_{(5,33)}$= 0.438, $p>0.05$ (Minho with MPs).

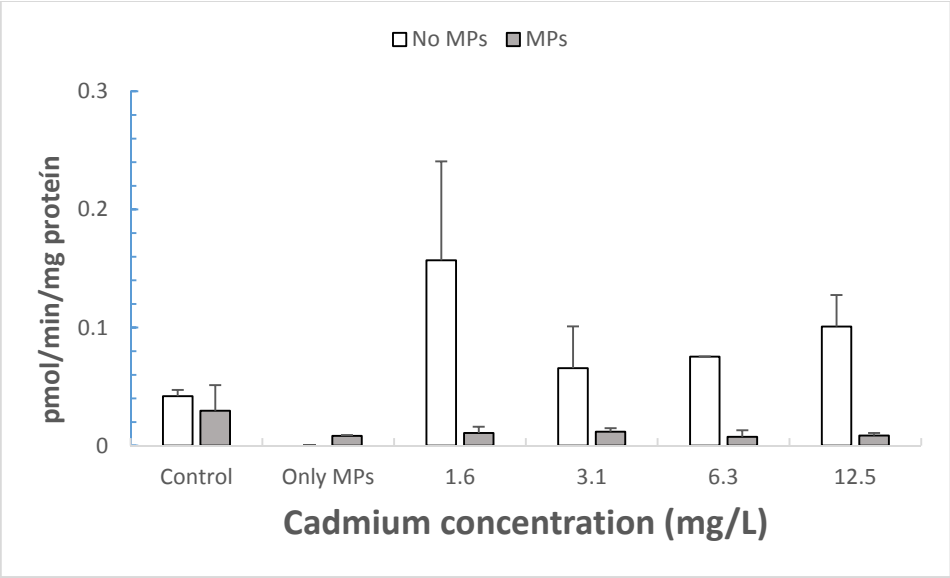

Figure 12 – Comparison of the EROD values between the bioassays from Minho (B1) and Minho + MPs (B3) assays. Values shown represent the average of the EROD values by each treatment, the associated error is represented.

Factor		N	Average ± standard error	F	P
	No MPs	14	0.087 ± 0.011	$F_{(1,14)}$= 22.273	P ≤ 0.05
	MPs	9	0.010 ± 0.013		
Treatment	Control	6	0.036 ± 0.016	$F_{(4,14)}$= 3.186	P ≤ 0.05
	Conc1	4	0.084 ± 0.019		
	Conc2	5	0.039 ± 0.018		
	Conc3	4	0.041 ± 0.019		
	Conc4	4	0.055 ± 0.019		
Interaction				$F_{(3,14)}$=1.141	P > 0.05

Table 13 – Results of the statistical analysis (Two-way ANOVA) of the EROD values from the fishes of the two bioassays.

In the Figure 12 are represented the average values (separated by concentrations) obtained in EROD activity that were assessed in the homogenized body of *P. microps* in the individuals of B1 and B3 (Minho without and Minho with microplastics, respectively), the error shown is the standard error.

After statistical analysis (Table 13) no significant differences were found in the interaction between the presence/absence of MPs with treatments. However significant differences between the presence/absence of MPs and treatments have been found.

An one way ANOVA was performed separately to each bioassay, $F_{(4,6)}$= 1.438, p>0.05 (Minho without MPs) and $F_{(5,9)}$= 0.615, p>0.05 (Minho with MPs).

This analysis indicate that fishes of the Minho bioassay have higher values of EROD than those form Minho + MPs bioassay.

3.3.2. Comparison between bioassays B2 and B4 (Lima and Lima + MPs)

To verify if the microplastics interfere with the effect of criteria used, in the estuary of Lima, in this section bioassays B2 (Lima without MPs) and B4 (Lima with MPs) will be compared.

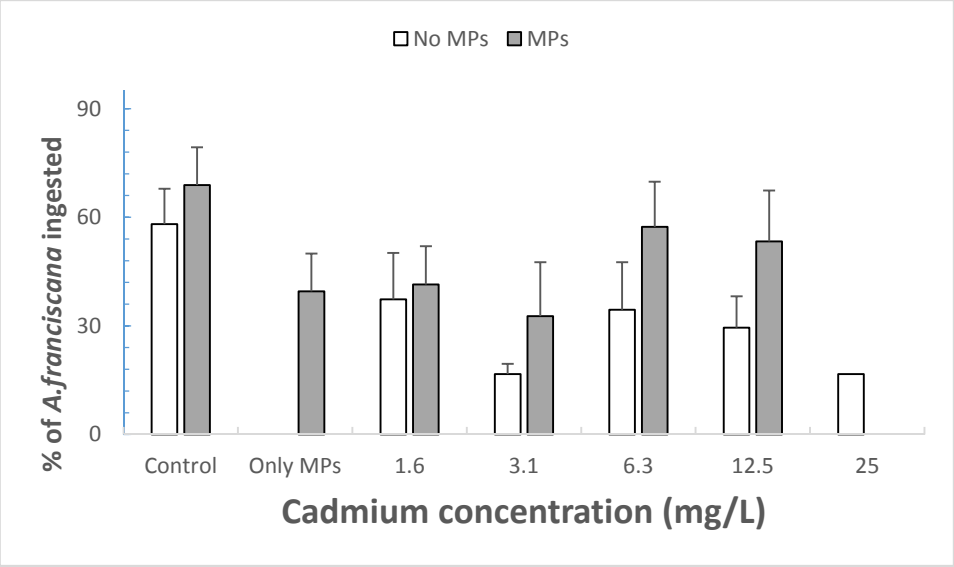

Figure 13 – Comparison of the percentage (nauplii ingested) between the bioassays from Lima (B2) and Lima + MPS (B4). Values shown represent the average of the percentage of nauplii ingested by each treatment, the associated error is represented.

Factor		N	Average ± standard error	F	P	Tukey
	No MPs	39	36.964 ± 3.671	$F_{(1, 55)}$= 2.450	P > 0.05	
	MPs	25	41.622 ± 4.294			
Treatment	Control	16	56.806 ± 5.255	$F_{(4, 55)}$= 4.047	P ≤ 0.05	a
	Conc1	12	37.489 ± 6.154			a,b
	Conc2	11	27.711 ± 6.364			b
	Conc3	11	41.911 ± 6.364			a,b
	Conc4	14	40.140 ± 5.676			a,b
Interaction				$F_{(3, 55)}$= 0.072	P > 0.05	

Table 14 – Results of the statistical analysis (Two-way ANOVA) (data were transformed from percentage to arcsin values) of the nauplii ingested from the fishes of the two bioassays.

In the Figure 13 is represented the mean of the percentage of *A.franciscana* ingested by fish that survived the bioassay B2 and B4 (Lima without and Lima with microplastics, respectively). The value shown on each bar of the graph is the average from each concentration and respective standard error.

After statistical analysis (Table 14) no significant differences were found between the presence/absence of MPs and in the interaction between the presence/absence of MPs with treatments. However significant differences among and treatments have been found, in this case in concentration of 3.1mg/L, cadmium seems to inhibit predatory behavior.

An one way ANOVA was performed separately to each bioassay, $F_{(4,26)}= 2.085$, $p>0.05$ (Lima without MPs) and $F_{(5,35)}= 1.510$, $p>0.05$ (Lima with MPs).

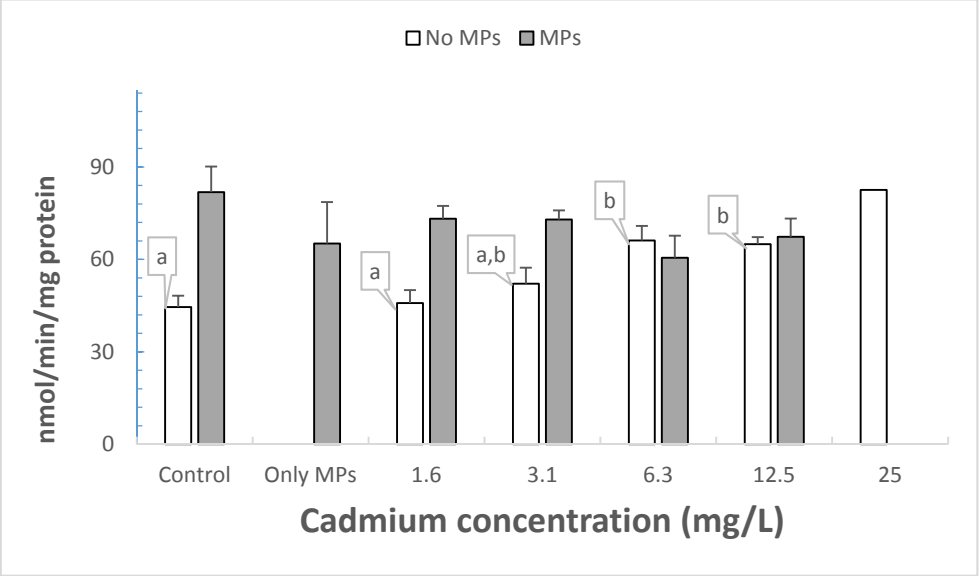

Figure 14 – Comparison of the AChE activity between the bioassays from Lima (B2) and Lima + MPs (B4) estuaries. Values shown represent the average of the AChE values by each treatment, the associated error is represented. Tukey test is representative of the One way ANOVA that was performed separately to bioassay.

Factor		N	Average ± standard error	F	P
	No MPs	39	59.033 ± 3.065	$F_{(1, 55)}$= 4.676	P ≤ 0.05
	MPs	25	68.510 ± 3.585		
Treatment	Control	16	65.512 ± 4.387	$F_{(4, 55)}$= 0.732	P > 0.05
	Conc1	12	59.513 ± 5.138		
	Conc2	11	62.522 ± 5.313		
	Conc3	11	63.340 ± 5.313		
	Conc4	14	66.471 ± 4.739		
Interaction				$F_{(3, 55)}$= 2.262	P > 0.05

Table 15 – Results of the statistical analysis (Two-way ANOVA) of the AChE activity from the fishes of the two bioassays.

In the Figure 14 are represented the average values obtained in AChE activity that was assessed in the homogenized head of *P. microps* in the individuals of B2 and B4 (Lima without and Lima with microplastics, respectively). The value shown on each bar on the graph is the average of each concentration and respective standard error.

After statistical analysis (Table 15) no significant differences were found between treatments and in the interaction between the presence/absence of MPs with treatments. However significant differences between the presence/absence of MPs were found.

An one way ANOVA was performed separately to each bioassay, $F_{(4,26)}$= 6.638, $p \leq 0.05$ (Lima without MPs) and $F_{(5,35)}$= 0.853, $p > 0.05$ (Lima with MPs). So in Lima without MPs bioassay for concentrations equal or higher than 6.3 mg/L were verified effects significantly different from the control group.

These results are in contradiction to a previous study performed by (Oliveira *et al.*, 2013) where inhibition of AChE was detected, in pyrene combined with MPs.

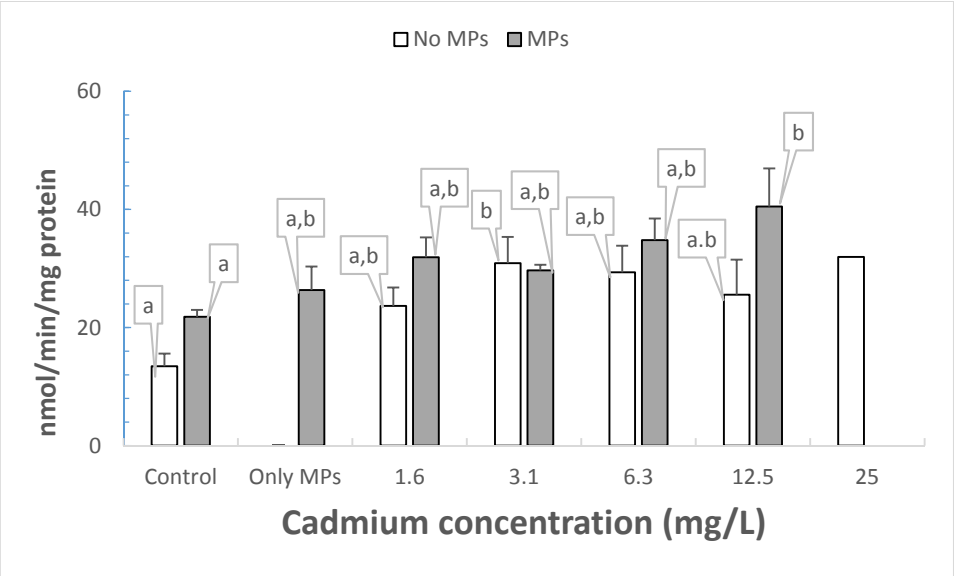

Figure 15 – Comparison of the GST activity between the bioassays from Lima (B2) and Lima + MPs (B4) assays. Values shown represent the average of the GST values by each treatment, the associated error is represented. Tukey test is representative of the One way ANOVA that was performed separately to bioassay.

Factor		N	Average ± standard error	F	P
	No MPs	39	25.175 ± 1.854	$F_{(1, 55)}$= 5.286	P ≤ 0.05
	MPs	25	34.230 ± 2.174		
Treatment	Control	16	17.566 ± 2.660	$F_{(4, 55)}$= 2.301	P > 0.05
	Conc1	12	27.788 ± 3.115		
	Conc2	11	30.294 ± 3.221		
	Conc3	11	32.089 ± 3.221		
	Conc4	14	32.444 ± 2.873		
Interaction				$F_{(3, 55)}$= 1.401	P > 0.05

Table 16 – Results of the statistical (Two-way ANOVA) analysis of the GST values from the fishes of the two bioassays.

In the Figure 15 are represented the average values obtained in GST activity that was assessed in the homogenized body of *P. microps* in the individuals of B2 and B4 (Lima without and Lima with microplastics, respectively). The value shown on each bar on the graph is the average of each concentration and respective standard error.

After statistical analysis (Table 16) no significant differences were found between treatments and in the interaction between the presence/absence of MPs with treatments. However significant differences between the presence/absence of MPs were found.

An one way ANOVA was performed separately to each bioassay, $F_{(4,26)}= 3.068$, $p \leq 0.05$ (Lima without MPs) and $F_{(5,35)}= 3.452$, $p \leq 0.05$ (Lima with MPs). So in Lima without MPs bioassay for concentrations equal to 6.1 mg/L were verified effects significantly different from the control group and for Lima with MPs were verified effects significantly different from the control group for concentrations of 12.5 mg/L.

This analysis possibly indicate that fishes of the Lima + MPs bioassay have higher values of GST than those form Lima bioassay.

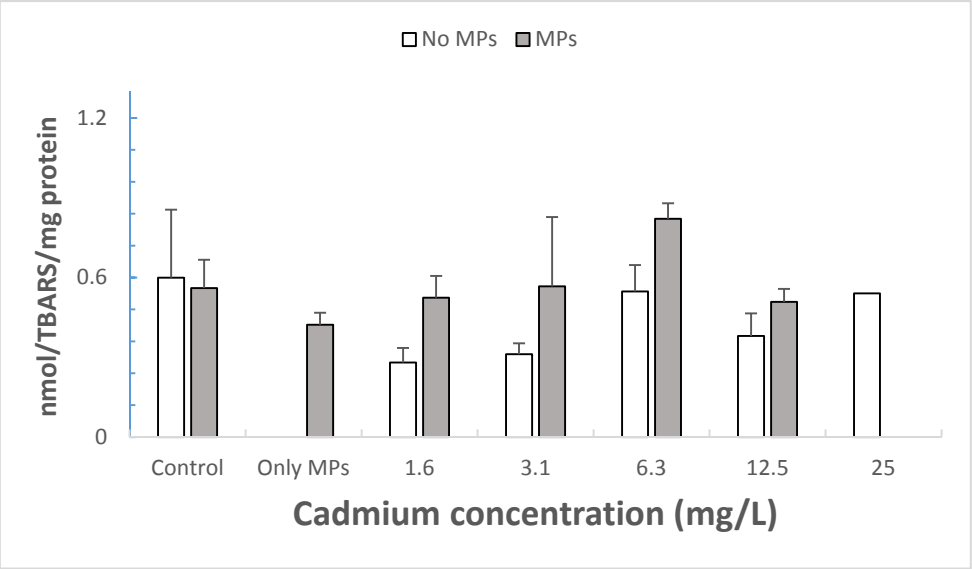

Figure 16 – Comparison of the LPO values between the bioassays from Lima (B2) and Lima + MPs (B4) estuaries. Values shown represent the average of the LPO values by each treatment, the associated error is represented.

Factor		N	Average ± standard error	F	P
	No MPs	39	0.423 ± 0.057	$F_{(1, 55)}= 5.345$	$P \leq 0.05$
	MPs	25	0.605 ± 0.067		
Treatment	Control	16	0.577 ± 0.082	$F_{(4, 55)}= 2.072$	$P > 0.05$
	Conc1	12	0.402 ± 0.096		
	Conc2	11	0.439 ± 0.099		
	Conc3	11	0.684 ± 0.099		
	Conc4	14	0.453 ± 0.088		
Interaction				$F_{(4, 55)}= 0.166$	$P > 0.05$

Table 17 – Results of the statistical analysis of the LPO values from the fishes of the two bioassays.

In the Figure 16 are represented the average values (separated by concentrations) obtained from LPO levels that were assessed in the homogenized body of *P. microps* in the individuals of B2 and B4 (Lima without and Lima with microplastics, respectively). The value shown on each bar on the graph is the average of each concentration and respective standard error.

After statistical analysis (Table 17) no significant differences were found between treatments and in the interaction between the presence/absence of MPs with treatments. However significant differences between the presence/absence of MPs were found.

Since differences were detected between the presence/absence of MPs a one way ANOVA was performed separately to each bioassay, $F_{(4,26)}= 0.929$, $p>0.05$ (Lima without MPs) and $F_{(5,35)}= 1.407$, $p>0.05$ (Lima with MPs).

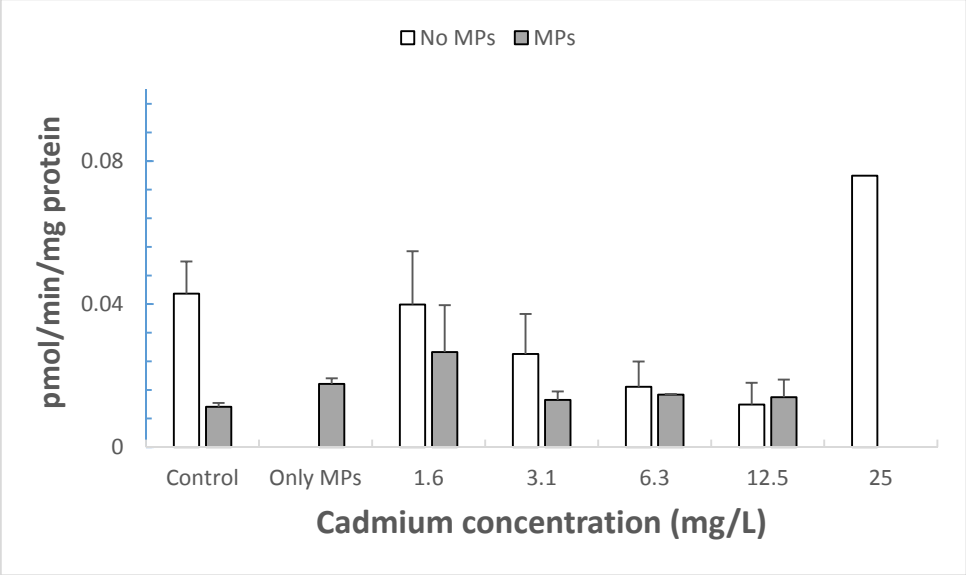

Figure 17 – Comparison of the EROD values between the bioassays from Lima (B2) and Lima + MPs (B4) estuaries. Values shown represent the average of the EROD values by each treatment, the associated error is represented.

Factor		N	Average ± standard error	F	P
	No MPs	13	0.025 ± 0.005	$F_{(1,14)}$= 1.161	P > 0.05
	MPs	10	0.017 ± 0.005		
Treatment	Control	5	0.024 ± 0.007	$F_{(4,14)}$= 0.976	P > 0.05
	Conc1	5	0.033 ± 0.007		
	Conc2	4	0.020 ± 0.008		
	Conc3	4	0.016 ± 0.008		
	Conc4	5	0.016 ± 0.007		
Interaction Estuary*Treatment				$F_{(3,14)}$= 0.146	P > 0.05

Table 18 – Results of the statistical analysis of the EROD values from the fishes of the two bioassays.

In the Figure 17 are represented the average values (separated by concentrations) obtained in EROD activity that were assessed in the homogenized body of *P. microps* in the individuals of B2 and B4 (Lima without and Lima with microplastics, respectively). The value shown on each bar on the graph is the average of each concentration and respective standard error.

After statistical analysis (Table 18) no significant differences were found between the presence/absence of MPs, treatments and in the interaction between the presence/absence of MPs with treatments.

A one way ANOVA was performed separately to each bioassay, $F_{(4,5)}$= 1.427, $p>0.05$ (Lima without MPs) and $F_{(5,10)}$= 0.724, $p>0.05$ (Lima with MPs)

Chapter IV – Conclusion and future work perspectives

4. Conclusion and future work perspectives

After this work there are to be considered relevant conclusions:

Considering the comparison between B1 and B2 (juveniles exposed to cadmium from estuaries of Minho and Lima respectively):

Significant differences were found ($p \leq 0.05$); between fishes from distinct estuaries (Minho and Lima) in AChE, GST, LPO and EROD; significant differences were found among treatments ($p \leq 0.05$) in GST; and a significant interaction between the origin of the fish (estuary) and cadmium concentrations in AChE. Fish from the Minho estuary had higher mean levels of AChE, GST, LPO and EROD than juveniles from the Lima river estuary. In juveniles of the Minho estuary, cadmium did not induced significant differences relatively to the control group. In juveniles of the Lima estuary, cadmium induced significant differences relatively to the control group in AChE and GST at concentrations equal to 1.6 mg/L and equal to 1.6 mg/L/higher than 6.3 mg/L, respectively. Overall, these findings indicate differences of sensitivity to cadmium between juveniles from distinct estuaries, thus corroborating our first hypothesis. Since the experimental conditions were similar for fish from both estuaries, these results suggest that exposure to different environmental conditions, including distinct pollution levels, in the original habitats during pre-developmental phases modulated the toxic effects of cadmium.

Considering the comparison between B1-B3 (Minho - Minho + MPs) and B2-B4 (Lima - Lima + MPs) (juveniles exposed to cadmium and cadmium combined with MPs):

This set of comparisons indicated that microplastics combined with cadmium were able to induce toxic effects on fish from both estuaries by increasing AChE and

decreasing LPO and EROD (Minho estuary fish), and increasing AChE, GST, LPO and decreasing in EROD in juveniles from the Lima estuary. The comparison of the bioassays carried out with juveniles of the Minho river estuary testing cadmium alone and in the presence of microplastics indicated: significant differences ($p \leq 0.05$) between treatments with and without microplastics in predatory performance, AChE, LPO, and EROD; significant differences among cadmium treatments ($p \leq 0.05$) in predatory performance, AChE, GST, LPO and EROD; and no significant ($p > 0.05$) difference in interaction between the origin of the fish (estuary) and cadmium concentrations. The comparison of the bioassays carried out with juveniles of the Lima river estuary testing cadmium alone and in the presence of microplastics indicated: significant differences ($p \leq 0.05$) between the bioassays performed with and without microplastics in, AChE, GST, and LPO; significant differences among cadmium treatments ($p \leq 0.05$) in predatory performance, no significant ($p > 0.05$) differences in the interaction between the origin of the fish (estuary) and cadmium concentrations.

In juveniles of the Minho estuary, cadmium in the presence of microplastics induced significant differences relatively to the control group in AChE at concentrations equal to 12.5mg/L. In juveniles of the Lima estuary in the presence of microplastics, cadmium induced significant differences relatively to the control group in GST, at concentrations equal or higher than 12.5 mg/L. Overall, these findings indicate that the presence of microplastics influences the toxicity of cadmium to juveniles from distinct estuaries, thus corroborating our second hypothesis. These findings indicate toxicological interactions between microplastics and cadmium.

The number of significant differences found between the comparisons with and without microplastics seem to indicate it interference in several biomarkers, which suggests the existence of phenomena such as adhesion of metal to plastic, that

after may suffer uptake to the fish and then remain their the body with a higher toxicity. However more studies are needed in order to understand the true interaction that microplastics have with metals and metabolic functions.

Ideally if performed in future the bioassays will be conducted in the same season and in the shortest period of time possible, however for several reasons such was not possible in this case (bioassays were performed in the spring and in the summer).

Looking ahead it would be interesting to verify the interaction of microplastics with the trophic chain, performing the same type of bioassay with organisms from different levels of it. It would be also interesting to include methalothionines as biomarkers because they are induced by metals, and so in this case the interaction of microplastics in relation to cadmium induction of these protein family could be eventually assessed.

Chapter V – Reference list

5. Reference list

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