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Effects of microplastics and other contaminants in freshwater organisms

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Effects of microplastics and other contaminants in freshwater organisms

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

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Abstract

The contamination of aquatic ecosystems with microplastics (MP) is a global environmental problem of increasing concern among the scientific community, media and the general public. MP have been detected in freshwater ecosystems, including in sediments and in the water column. MP were also detected in freshwater fauna, suggesting uptake of MP by organisms. Thus, MP pollution is a threat to animal health. In natural ecosystems, organisms are exposed to MP and other contaminants simultaneously. Therefore, mixtures of MP with other pollutants can potentially increase the toxic effects on organisms in comparison to the compounds individually.

The objectives of the present study were: (1) to review the literature regarding the paradigm of MP in freshwater ecosystems; (2) to investigate the effects of model MP and deltamethrin (DLT), alone and in mixture, on the freshwater exotic invasive species *Corbicula fluminea*.

In the first phase of the work, a literature review on the paradigm of MP in freshwater ecosystems was made. Briefly, the topics covered were: (1) sources of MP present in freshwater ecosystems; (2) occurrence and distribution of MP in freshwater ecosystems; (3) uptake of MP in natural environments; (4) ecotoxicological effects of MP; (5) influence of MP on the toxicity of other chemical contaminants. The main findings were: (1) MP accumulate in freshwater environments; (2) it is difficult to compare the contamination of freshwater environments by MP due to the use of different quantitative units in distinct studies; (3) MP are present in organs and tissues of various freshwater organisms; (4) MP alone or in mixture with other contaminants can cause toxic effects on freshwater biota. Therefore, more knowledge on the effects of MP in freshwater organisms is needed, including in exotic invasive species such as *C. fluminea* that may be used as bioindicators in environmental field studies and as test organisms in laboratory bioassays avoiding the use of native species.

In the second phase of the study, a bioassay to investigate the combined effects of MP and DLT on *C. fluminea* was carried out. Bivalves were collected in the Minho River estuary (NW Iberian Peninsula). In 9 of the sampled bivalves, the activities of cholinesterases (ChE), isocitrate dehydrogenase, octopine dehydrogenase (ODH), catalase and glutathione *S*-transferases enzymes, and lipid peroxidation levels were determined shortly after arrival to the laboratory. The other bivalves were acclimated to laboratory conditions for 14 days. After the acclimatization period, 9 bivalves were used for biomarker determinations (those previously indicated). The remaining bivalves were used in the bioassay that was conducted for 8 days at room temperature, using dechlorinated tap water (DTW) as test medium and

24 h was test medium renewal period. Daily, the bivalves were fed with *Chlorella vulgaris* cells. Bivalves were exposed to the following treatments: control (DTW); control with solvent (DTW with 0.050 mg/L of acetone); DTW with 0.2 mg/L of MP; mixture of MP and acetone (DTW with 0.2 mg/L of MP + 0.050 mg/L of acetone); DTW with DLT (0.2 mg/L); DTW with 0.2 mg/L of MP and 0.2 mg/l of DLT (mixture). The MP and DTL concentrations indicated are the nominal ones. The exposure concentrations of MP in test media were also determined. The possible decrease of MP concentrations in test medium (potentially due to microalgae, bivalves, and interaction of test substances and of these with the solvent in test medium) was investigated. 18 bivalves were used per treatment (9 for biomarkers, 9 for DTL chemical determinations), except in the control where ten bivalves were used (9 for biomarkers, 1 for chemical analyses). After the exposure period, 9 bivalves from each treatment were used to determine the post-exposure filtration rate and the above indicated biomarkers. The other bivalves were used for DTL determinations. Concerning the control group, the mixture of MP and DLT increased the activity of ChE and ODH enzymes, suggesting alterations in cholinergic transmission and increased energy need under simultaneous exposure to MP and deltamethrin. No other significant alterations were found.

Overall, the work included in the present Master Thesis contributed to increase the knowledge on the effects of MP, other environmental contaminants, and mixtures on freshwater organisms.

Keywords: plastic pollution, microplastics, freshwater, deltamethrin, biomarkers, C*orbicula fluminea*

Resumo

A contaminação dos ecossistemas aquáticos com microplásticos (MP) é um problema ambiental global de crescente preocupação entre a comunidade científica, os meios de comunicação social e o público em geral. Os MP têm sido detetados em ecossistemas de água doce, quer em sedimentos quer na coluna de água. Os MP têm também sido detetados na fauna de água doce, sugerindo incorporação de MP por estes organismos. Assim, a poluição por MP constitui uma ameaça para a saúde animal. Nos ecossistemas naturais, os organismos são expostos a MP e outros contaminantes ambientais simultaneamente. As misturas de MP com outros poluentes podem potencialmente aumentar os efeitos tóxicos nos organismos, em comparação com os compostos individualmente.

Os objetivos do presente estudo foram: (1) rever a literatura sobre o paradigma dos MP em ecossistemas de água doce; (2) investigar os efeitos de um tipo de MP e da deltametrina (DLT), individualmente e em mistura, na espécie exótica de água doce *Corbicula fluminea*.

Numa primeira fase do estudo foi feita uma revisão bibliográfica sobre o paradigma dos MP em ecossistemas de água doce. Os temas abordados foram: (1) fontes dos MP presentes em ecossistemas de água doce; (2) ocorrência e distribuição de MP em ecossistemas de água doce; (3) a captação de MP por organismos em ambientes naturais; (4) efeitos ecotoxicológicos de MP; (5) influência de MP na toxicidade de outros contaminantes químicos. As principais conclusões foram: (1) os MP acumulam-se em ambientes de água doce; (2) é difícil comparar a concentração de MP em diferentes ecossistemas devido à utilização de diferentes unidades em estudos distintos; (2) os MP estão presentes em órgãos e tecidos de vários organismos de água doce; (3) os MP, individualmente e/ou em mistura com outros contaminantes ambientais, podem causar efeitos tóxicos na fauna de água doce. Porém, é ainda necessário mais conhecimento sobre os efeitos de MP em organismos de água doce, incluindo em espécies exóticas invasoras como *C. fluminea*, as quais podem ser utilizadas como bioindicadores em estudos de campo e como organismos-teste em bioensaios laboratoriais evitando a utilização de espécies autóctones.

Na segunda fase do estudo foi realizado um bioensaio para investigar os efeitos combinados de MP e DLT em *C. fluminea*. Os bivalves foram recolhidos no estuário do rio Minho (Península Ibérica, NW). Pouco tempo após a chegada ao laboratório, em 9 dos bivalves amostrados, foram determinadas as atividades das enzimas colinesterases (ChE), isocitrato desidrogenase, octopina desidrogenase (ODH), catalase e glutationa *S*-

transferases, bem como os níveis de peroxidaçãolipídica.. Os outros bivalves foram aclimatizados a condições laboratoriais durante 14 dias.

Após o período de aclimatização, 9 bivalves foram utilizados para determinar os biomarcadores anteriormente referidos. Os restantes bivalves foram utilizados no bioensaio, o qual foi efetuado durante 8 dias à temperatura ambiente, utilizando água da rede pública para consumo humano desclorada (DTW) como meio de teste e 24 h como período de renovação do meio de teste. Diariamente, os bivalves foram alimentados com células de *Chlorella vulgaris*. Os bivalves foram expostos aos seguintes tratamentos: controlo (DTW); controlo com solvente (DTW com (0,050 mg/L de acetona); DTW com 0,2 mg/L de MP; mistura de MP e acetona (DTW com 0,2 mg/L de MP + 0,050 mg/L de acetona); DTW com DLT (0,2 mg/L); DTW com 0,2 mg/L de MP e 0,2 mg/l de DLT (mistura) (0,2 mg/L MP + 0,1mg/L DLT). As concentrações de MP indicadas são as nominais. As concentrações de exposição dos MP foram também determinadas. A possível diminuição das concentrações de MP no meio de teste (potencialmente devida a microalgas, bivalves e interação entre as substâncias testadas e entre estas e o solvente no meio de ensaio) foi investigada. Foram utilizados 18 bivalves por tratamento (9 para determinação de biomarcadores, 9 para análises químicas de DLT), exceto no grupo de controlo onde foram utilizados 10 bivalves (9 para biomarcadores, 1 para análise químicas). Após o período de exposição, em 9 dos bivalves de cada tratamento foi determinada a taxa de filtração pós-exposição e os biomarcadores acima referidos. A mistura de MP e DLT causou um aumento na atividade das enzimas ChE e ODH em relação ao grupo controlo, sugerindo alterações na transmissão colinérgica e necessidade de mais energia sob exposição simultânea a MP e DTL. Não foram encontradas outras alterações significativas.

No seu conjunto, o trabalho incluído na presente dissertação contribuiu para aumentar o conhecimento sobre os efeitos de MP, outros contaminantes ambientais e misturas em organismos de água doce.

Palavras-chave: poluição com plásticos, microplásticos, água doce, deltametrina, biomarcadores, *Corbicula fluminea*

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List of abbreviations

ANCOVA - Analysis of Covariance

ANOVA - Analysis of Variance

AChE – Acetylcholinesterase enzyme

CAT – Catalase enzyme

ChE – Cholinesterase enzymes

Ctr - Control

Ctr-ACET - Acetone control

DLT - Deltamethrin

FR - Filtration rate

GR - Glutathione reductase enzyme

GPx - Glutathione peroxidase enzyme

GSH - Glutathione

GSSG - Glutathione disulfide

GST - Glutathione *S*-transferase enzymes

IDH - Isocitrate dehydrogenase enzyme

DL - Detection level

LPO - Lipid peroxidation

MP - Microplastics

NADH - Nicotinamide adenine dinucleotide

$NADP^+$ - Nicotinamide adenine dinucleotide phosphate

NADPH - Reduced form of nicotinamide adenine dinucleotide phosphate

ND - Not detected, bellow the detection level

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

ODH - Octopine dehydrogenase enzyme

PAHS - Polycyclic aromatic hydrocarbons

PCBs - Polychlorinated biphenyl

PE - Polyethylene

PET - Polyethylene terephthalate

POP - Persistent organic pollutants

PP - Polypropylene

PS - Polystyrene

PVC - Polyvinyl chloride

RFU - Relative fluorescence units

ROS - Reactive oxygen species

SD - Standard deviation

SEM - Standard error of the mean

SOD - Superoxide dismutase enzyme

TBARS - Thiobarbituric acid-reactive substances

WWTP – Wastewater treatment plant

Chapter 1. General Introduction

The increasing growth of the human population has been causing a new set of challenges, such as the need of increasing food production to feed all the persons and the accumulation of traditional and emerging contaminants in the environment (Godfray *et al*., 2010; Thompson *et al*., 2009).

Despite plastic production has been increased in recent years, reaching 360 million tons of plastic produced in 2018 (Plastics Europe, 2019), only half of the plastics are recycled or transported to landfill. The other part usually ends up in the environment, including aquatic ecosystems (Rochman *et al*., 2013). Estimates indicated that among the plastics present in the sea most (~ 80 %) derived from land-based sources, whereas sea-based sources contributed with ~ 20 % (Galafassi *et al.*, 2019). In natural environments, larger plastic debris can fragment originating micro-sized plastic particles (GESAMP, 2015). The contamination of aquatic ecosystems by small particles of plastic with less than 5 mm of size, known as microplastics (MP), is considered an environmental emerging threat that may affect ecosystems and human health (Thompson *et al*., 2009). MP are ubiquitously distributed in freshwater environments (Eriksen *et al*., 2013; Rodrigues *et al*., 2018; Yuan *et al*., 2019), they are present in sediments, in the water column, and have been also found in the digestive system and tissues of freshwater organisms (Jabeen *et al*., 2017; Li *et al*., 2019). MP can cause adverse impacts to aquatic organisms, such as on biomarkers related to neurotoxicity, oxidative stress and lipid peroxidation (Barboza *et al*., 2018; Martins *et al*., 2018; Oliveira *et al*., 2013). In the last decades, there has been an increasing number of studies about the paradigm of MP. Nevertheless, the knowledge is still limited, especially regarding the effects of MP in freshwater species, particularly in organisms exposed to mixtures of MP and other relevant contaminants in freshwater ecosystems. Thus, many questions about the impacts of MP on ecosystems, and in animal and human health remain open.

In many freshwater ecosystems, particularly in those located in agricultural areas or in their vicinity, pesticides are important contaminants (Afful *et al*., 2013; Xue *et al*., 2006). Pyrethroid insecticides are a good alternative in comparison with organochlorine, organophosphate and carbamate insecticides due to their relative low persistence in the environment, low toxicity to mammals and birds and low tendency to induce insect resistance (Köprücü *et al*, 2008a). Additionally, this class of insecticides has high effectiveness even when applied in low concentrations (Abdelkhalek *et al*., 2015). Nevertheless, these compounds can have adverse effects, such as the death of non-target species with negative implication for the ecosystem (Damalas *et al.*, 2011). Pyrethroids alter the normal function of the nervous system since they modify the kinetics of voltage-sensitive sodium channels (Chrustek *et al.*, 2018), among other effects.

Deltamethrin (DLT) is a pyrethroid insecticide that is widely used in agriculture and forestry, which can protect the crops of a wide range of insect pests (Köprücü *et al.*, 2008a). It is also applied in aquaculture in treatment baths to combat parasites (Langford *et al.*, 2014; Tucca *et al.*, 2017), among other uses. DLT enters into freshwater ecosystems mainly through runoff of agriculture fields, and domestic and industrial effluents (Ullah *et al.*, 2019b). It has relatively low solubility in water (<0.002 mg/L at 25 °C) and a log $_{Kow}$ of 6.20 (NCBI, 2020). Thus, when it is released to the water of natural ecosystems, it tends to adsorb to water suspended solids and sediments (NCBI, 2020). Many studies reported the presence of DLT in surface waters of different parts of the world. In Table 1, some studies that reported concentrations of DLT in freshwater bodies are indicated. The concentrations documented in these studies ranged from 33 to 1140000 ng/L.

Table 1 - Examples of deltamethrin concentrations in surface water of freshwater environments reported in the literature. ND – Not detected; DL – Detection level – 0.10 ng/L.

Country	**Location**	**Concentration**		**Reference**
Pakistan	Chenab river	ND – 108	ng/L	(Riaz *et al.*, 2018)
Pakistan	Ravi river	33 – 450	ng/L	(Mahboob *et al.*, 2015)
China	GuanTing Reservoir	ND – 6.26	ng/L	(Xue *et al.*, 2006)
Nigeria	Benue river	280000 – 1140000	ng/L	(Akan *et al.*, 2015)
Ghana	Weija lake	<DL – 0.70	ng/L	(Afful *et al.*, 2013)
South Africa	Lourens River	1400	ng/L	(Dabrowski *et al.*, 2002)

DLT enters in the body of aquatic organisms mainly through gills, skin and the ingestion of contaminated water and prey (Chrustek *et al.*, 2018). DLT metabolization occurs mainly in the liver, where it is hydrolyzed and finally excreted in free form. However, the biotransformation of deltamethrin is not an easy process due to this compound has high lipophilicity (Zhang *et al*., 2019a). In literature, many studies reported the toxic effects of DLT in various aquatic species. In fish, exposure to DLT can cause behavioural changes, decrease of acetylcholinesterase (AChE) activity, decrease of glutathione (GSH) levels and increase glutathione *S*-transferases (GST) activity in fish tissues as documented in the freshwater fish *Channa punctana* (Bhattacharjee *et al.*, 2020; Singh *et al.*, 2018). Alak *et al*. (2019) reported a significant decrease of AChE activity and a reduction in the gene expression of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) in brain tissue of the rainbow trout (*Oncorhynchus mykiss*). In silver carp (*Hypophthalmichthys molitrix*), the exposure to DLT led to behaviour alteration which might be associated with inhibition of AChE activity and alterations in the activity of oxidative stress biomarkers (Ullah *et al.*, 2019a). Toxic effects of DLT have been also documented in crustaceans and other invertebrates. For example, exposure to DLT caused oxidative stress

and damage in the Chinese mitten crab (*Eriocheir sinensis)* (Hong *et al.*, 2018; Zhang *et al*., 2019a). Oliveira *et al*. (2012), reported a decrease of the swimming velocity of the common prawn *Palaemon serratus* after exposure to DLT. In *Daphnia magna*, the exposure to DLT has adverse impacts on the reproduction capacity, such as the decrease of juvenile production (Toumi *et al.,* 2013). In freshwater mussels, Kontreczky *et al.* (1997) found a reduction of filtration activity in *Anodonta cygnea L*. and *Unio elongatulus eucirrus* exposure to DLT caused a significant decrease on glutathione levels and catalase activity in the digestive gland and gills (Köprücü *et al.*, 2008b). No further studies were found about the toxic effects of DLT on molluscs. Thus, the knowledge about this topic is still limited and more research is needed.

Bioinvasions are a natural evolutionary process of species (Ochocki *et al*., 2017). However, the introduction of several species into new territories has been accelerated due to diverse human activities. In aquatic ecosystems, the principal cause of bioinvasions is the globalization of trade (Binimelis *et al*., 2007; Karatayev *et al*., 2007). Exotic invasive species are an important threat to the biodiversity of freshwater ecosystems (Karatayev *et al*., 2007). Moreover, among freshwater animals, bivalve invasive species can disrupt trophic chains and alter nutrient fluxes (Vaughn *et al*., 2001).

Corbicula fluminea, also known as Asiatic claim, is a native species of Asia, Africa and Australia (Crespo *et al*., 2015). In the last decades, this species has been spreading worldwide (Molina *et al*., 2015) causing negative ecotoxicological impacts in many ecosystems (Oliveira *et al*., 2015; Sousa *et al*., 2008). *C. fluminea* has the capacity to tolerate diverse abiotic changes and environmental conditions and recover more rapidly from negative impacts than native species (Oliveira *et al*., 2015; Sousa *et al*., 2008), which can explain the success of its bioinvasions. Moreover, this species of bivalve is consumed by humans in several regions of the globe, which may help in the control of bioinvasions. *C. fluminea* has a high filtration rate which contributes for the rapid concentrations of contaminants inside of its body (Bolam *et al*., 2019). Thus, is a good model organism to be used in ecotoxicological and other studies (Guilhermino *et al*., 2018; Oliveira *et al*., 2015; Sousa *et al*., 2008). The use of C. fluminea as model organism in scientific investigations also contributes to control the invasions and do not have significant implications for the ecosystem (Guilhermino *et al*., 2018; Oliveira *et al*., 2018).

1.1 Objectives of the Master Thesis

The main goals of this MSc Thesis were: (1) to review the literature regarding the paradigm of MP in freshwater ecosystems and summarize the main findings; (2) to

investigate the effects of model MP and DLT, alone and in mixture, on the freshwater exotic invasive species *Corbicula fluminea*.

DLT was selected for this study because is widely used in agriculture and aquaculture (Langford *et al.*, 2014; Tucca *et al.*, 2017), has been detected in the water column of freshwater environments (Afful *et al.*, 2013; Riaz *et al.*, 2018), and is able to induce toxic effects on freshwater organisms (Köprücü *et al.*, 2008b; Singh *et al.*, 2018).

C. fluminea was selected as test organism because it is an exotic invasive species in Europe and many freshwater ecosystems of other regions (Sousa *et al*., 2008), it can cause adverse impacts in invaded ecosystems and is a threat to several native species (Oliveira *et al*., 2015), it is consumed as food by humans in several regions (Guilhermino *et al*., 2018), and it is an adequate test organism for use in ecotoxicity bioassays (Guilhermino *et al.*, 2018; Oliveira *et al*., 2018).

1.2 Master Thesis structure

The Master Thesis is divided into 5 chapters. Chapter 1 is the general introduction, which includes a brief introduction about the problem of the contamination of aquatic environments by MP and pesticides, the main objectives of the work done, and the structure of the Master Thesis. Chapter 2 corresponds to a review of the literature published on the impacts of MP in freshwater environments. In Chapter 3, the toxicological effects of MP and DLT, alone and in mixture, were investigated using the freshwater exotic invasive bivalve *Corbicula fluminea* as biological model. In chapter 4, the main conclusions and future work perspectives are presented. Finally, Chapter 5 is the list of references.

Chapter 2. Impacts of microplastics in freshwater environments

2.1 Introduction

The term “microplastic” was first applied to microscopic plastic particles collected in the water column and sediments of estuaries (Thompson *et al.*, 2004). Since then the term MP was used to describe small plastic particles with millimetre size range (GESAMP, 2015). In 2009, MP were defined as plastic particles smaller than 5 mm by the National Oceanic and Atmospheric Administration (NOAA) (Arthur *et al*., 2009).

According to their source, MP can be classified as primary and secondary. Primary MP particles are intentionally produced to have small dimensions (micro- or nano- scales) for different industrial and domestic applications (Cole *et al*., 2011). Some examples are plastic microbeads in personal care and cosmetic products, scrubs for abrasive blast cleaning, plastic powders used in moulding and raw materials for plastic production (GESAMP, 2015; Wang *et al.*, 2020). Secondary MP result from the fragmentation of larger plastic pieces by UV radiation, mechanical abrasion, hydrolysis and biological degradation (GESAMP, 2015).

Wastewater treatment plants (WWTP) are the main source of MP into aquatic environments. Therefore, these particles are transported by rivers, wind and rainwater and accumulate in freshwater systems (Galafassi *et al.*, 2019). In natural ecosystems, organisms are exposed to MP and a variety of other contaminants that can be uptake and potentially cause toxic effects (Rochman *et al*., 2013). Despite the increasing number of studies about the paradigm of MP, many questions remain open.

This chapter compiles information about the sources of MP present in freshwater ecosystems, occurrence and distribution of MP in freshwater ecosystems, uptake of MP in natural environments, ecotoxicological effects of MP and influence of MP on the toxicity of chemical contaminants.

2.2 Sources of microplastics present in freshwater ecosystems

MP can enter in the freshwater environment, mainly, through household sewage discharge or runoff from landfills (Du *et al*., 2020). Additionally, rivers, wind and rainwater have a fundamental role in the distribution of plastic into lakes, seas and oceans (Galafassi *et al*., 2019). WWTP collect water from a variety of users, from domestic to industrial, thus the MP present in the effluent can have various sources and proprieties. Moreover, clothes washing have an important impact on the release of fibers into natural environments through WWTP. Browne *et al*. (2011) report that 1900 fibers per item may come out during the

washing process. The removal efficiency of a WWTP is dependent on the design of the plant, the application of secondary or tertiary treatment and their technology. Murphy *et al*. (2016) reported a removal efficiency of 98,41% in a WWTP with secondary treatment. Although, in WWTP with tertiary treatment, the removal efficiency can reach 99% (Carr *et al.*, 2016). Despite modern WWTPs can remove a large percentage of MP from the final effluent, there is still a considerable amount of MP disposal into the environment. When WWTP exceed the treatment facilities handling capacity, during episodes of intense rains, the untreated wastewater is discharged. Despite these events occasionally occur, a significant amount of MP and other contaminants are released to the environment (Galafassi *et al*., 2019). Furthermore, during primary and secondary treatment, MP present in the wastewater with a higher density than water is retained in sewage sludge (Mintening *et al*. 2017). In many countries, the sewage sludges are applied in the soil as emending agent and fertilizer in agriculture (Nizzetto *et al*., 2016). Due to this procedure and the use of plastics, agriculture is one of the main anthropogenic activities that contribute to MP pollution (Akdogan *et al.*, 2019). The MP present in the soil are transported to the water through the runoff and wind erosion (Galafassi *et al*., 2019). Anthropogenic activities such as lettering, municipal solid waste collection and disposal process are secondary sources of MP pollution (Akdogan *et al.*, 2019; UNEP, 2015).

2.3 Occurrence and distribution of microplastics in freshwater ecosystems

MP have been detected in various aquatic environments, such as surface water, sediment, beaches, benthic zones and ice core from polar regions (Obbard *et al*., 2014; Rodrigues *et al*., 2018; Ballent *et al*., 2016).

Kummu *et al*. (2011) estimate that half of the world's population lives closer than 3 km to a surface freshwater body. Thus, freshwater systems are highly impacted by MP pollution due to the proximity areas of high human population density (Xu *et al*., 2020). Therefore, it is important to protect the freshwater bodies because the human population depends on rivers and lakes for many activities, namely to agriculture and to supply water to the households (Kummu *et al*., 2011). There are a series of studies assessing the MP concentrations both in marine and freshwater environments. Table 2 presents a summary of recent studies that report MP in freshwater environments globally, such as lakes and rivers. There is spatial variability in the distribution and abundance of MP globally. It is still difficult to assess the real concentrations in freshwater systems and compare with each other because there is not a unique protocol to identify and quantify MP particles and one

uniformized unit to express the results. Thus, is important to define a standard methodology to understand the extension of the MP pollution and their potential risk for the environment and human health (Mendoza *et al*., 2019).

MP have different shapes, sizes, colours, densities and chemical compositions which may influence their transport and environmental fate (Nizzetto *et al*., 2016). It is possible to have an idea of the sources of MP through their colour. For example, transparent fibres might originate from the breakdown of fishing lines or nets, while coloured particles derived from abrasion or fragmentation of some plastic, such as clothing and packaging (Wang *et al*., 2020). In aquatic environments, MP with a higher density (polystyrene (PS), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polyester) tend to accumulate in sediments. MP with a lighter density float in the water surface (polypropylene (PP), light density polyethylene (PE)) and are likely to be transported by rivers into oceans (Haegerbaeumer *et al*., 2019; Wu *et al.*, 2019). During the residence time in aquatic systems, MP can form aggregations between MP particles, with other detritus or form biofilms, which alter the MP density and their fate in the environment (Wang *et al*., 2020). Also, MP can be a vector of transport of toxic substances and persistent contaminants (polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and heavy metals) added during MP manufacture or incorporated in the water environment due to the large surface area and hydrophobicity of the particles (Rochman *et al.*, 2013).

Table 2 - Global reports of microplastics (MP) concentrations in freshwater environments

Country	Location	Sample	Levels of MP	Size range	Type of debris	Reference
Uganda	Northern Lake Victoria	Lake sediment	0 - 14.5 particles/kg	0.3 - 1.0 mm	Fragment, filament, film and foam	(Egessa *et al*., 2020)
China	Estuary of the Yellow River	Surface water	930 - 497 item/L	5 - <0.2 mm	Fibers, fragments and particles	(Han *et al*., 2020)
China	Lakes along the Yangtze River Basin	Surface water Sediment	240 - 1800 items/m^3 90 - 580 items/kg	5 - <0.1 mm	Fiber and fragment	(Li *et al*., 2019)
China	Lake Ulansuhai	Surface water	1760 - 10.120 n/m^3	5 - <0.5 mm	Fiber, film, fragment and grain	(Wang *et al*., 2019)
China	Poyang Lake	Surface water Sediment	5 - 34 items/L 54 - 506 items/kg	0.1 - 0.5 mm	Fibres, films, fragments, and pellets	(Yuan *et al*., 2019)
China	Pearl River	Surface water	0.017 - 29.697 n/m^3	0.355 - 4.75 mm	Polystyrene foam, hard fragment and fibre	(Cheung *et al*., 2018)
China	Three Gorges Reservoir	Surface water Sediments	1597 - 12.611 n/m^3 25 - 300 n/kg	5 - <0.5 mm	Fiber, fragments, pellets ang film	(Di *et al*., 2018)
China	Guangzhou section of the Pearl River	Surface water Sediments	379 - 7924 items/m^3 80 - 9597 items/kg	5 - 0.5 mm	Fiber, Fragment and film	(Lin *et al*., 2018)
China	Dongting Lake Hong Lake	Surface water	900 - 2800 n/m^3 1250 - 4650 n/m^3	5.0 - 0.05 mm	Fiber, granule and film	(Wang *et al*., 2018)
Mongolia	Lake Hovsgol	Surface water	997 - 44.435 particles/km^2	> 4.75 - 0.333 mm	Fragment, film, fiber, foam and pellet	(Free *et al*., 2014)
Thailand	Chao Phraya River at Tha Pra Chan, Bangkok	Surface water Sediments	17.9 - 120.7 mg/m^3 1.3 - 6.4 mg/Kg	5.0 - 0.05 mm 0.3 - 0.05 mm	Fragment, fibers, pallet and film Fibers and fragment	(Ta *et al*., 2020)
Vietnam	Saigon River	Surface water	172.000 - 519.000 items/m^3 10 - 223 items/m^3	4650 -50 µm >500 - 0.5 µm	Fiber Fragment	(Lahens *et al*., 2018)

Mexico	Todos Santos Bay	Surface water Sediment	0.01 - 0.70 particles/m^3 85 - 2494 particles/0.1 m^2	Not specified	Fragment and fibers Fragment and fibers	(Ramírez-Álvarez *et al*., 2020)
Canada	Lake Winnipeg	Surface water	53.000 - 748.000 particles/km^2	Not specified	Fiber, fragment, film and foam	(Anderson *et al*., 2017)
USA	Snake and Lower Columbia Rivers	Surface water	0 - 5.405 particles/L	500 - 100 µm	Fragments, films and beads	(Kapp *et al*., 2018)
USA	Great lakes	Surface water	0 - 450.000 particles/Km^2	> 4.75 - 0.355 mm	Fragment, film, foam, pellet and line	(Eriksen *et al*., 2013)
Portugal	Antuã River	Surface water Sediments	58 - 1265 items/m^3 18 - 629 items/Kg	Not specified	Fragments, pellets, films, foam and fiber	(Rodrigues *et al*., 2018)
Italy	Lake Maggiore Lake Iseo Lake Garda	Surface water	39000 particles/ km^2 40000 particles/km^2 25000 particles/km^2	1 - 5 mm	Fragments, balls, filaments, sheets and pallets	(Sighicelli *et al*., 2018)
UK	Thames river basin	Sediment	18.5 - 66 particles per 100 g	1 - 4 mm	Fragment, fiber and film	(Horton *et al*., 2017a)
Italy	Lake Bolsena Lake Chiusi	Surface water	0.82 - 4.42 particles/m^3 2.68 - 3.36 particles/m^3	5.0 - 0.3 mm	Fragment and fiber	(Fischer et al., 2016)
France	River Seine, urban area	Surface water	3 - 106 particles/m^3	100 - 5000 µm	Not specified	(Dris *et al*., 2015)
Switzerland	Lake Geneva, Lake Constance, Lake Maggiore, Lake Neuchâtel, Lake Zurich and Lake Brienz	Surface water Sediment	220000 - 11000 particles/Km^2 20 - 7200 particles/m^2	Not specified	Fragments, pellets, cosmetic beads, lines, fibres, films and foams	(Faure *et al*., 2015)

2.4 Uptake of microplastics in natural environments

The aquatic organism can be exposed to MP directly when uptake MP through the environment matrix, such as water, sediment or air. Indirect exposure occurs when organisms uptake MP through the food chain by the consumption of species that accumulate the contaminant (Du *et al.*, 2020). Studies reported the presence of MP in a diverse group of aquatic organisms, including fish, crustaceans, molluscs and invertebrates (Table 3). Freshwater mussels and benthic invertebrates accumulate MP mainly from sediments (Li *et al.*, 2020), while fish uptake MP directly confusing the particles with natural food items due to their small size or indirectly via the consumption of other organisms that contain MP (Romeo *et al*., 2015). MP particles can also adhere to the skin or translocate to the gills of fish (Abbasi *et al*., 2018). The ingestion of MP can cause harmful effects to the organisms, thus, rapid depuration is a strategy adopted by many aquatic organisms to minimize the potentially harmful effects of MP, resulting in the elimination of part of MP particles (Birnstiel *et al*., 2019).

Table 3 - Summary of the studies reporting the presence of microplastics (MP) particles in freshwater species.

Species	**Organism**	**Parts**	**Concentration of MP**	**Size range**	**Types of MP**	**Reference**
Gambusia holbrooki	Fish	Body Head	0.60 items/individual 0.11 items/individual	0.09 - 4.86 mm 0.22 - 2.01mm	Fiber, fragment, film, and pellet Fiber and fragment	(Su *et al*., 2019)
Platycephalus indicus	Fish	Whole body	21.8 items/ individual	<100 - 1000 mm	Fiber	(Abbasi *et al*., 2018)
Saurida tumbil	Fish	Whole body	13.5 items/individual	<100 - 1000 mm	Fiber	(Abbasi *et al*., 2018)
Sillago sihama	Fish	Whole body	14.1 items/individual	<100 - 1000 mm	Fiber	(Abbasi *et al*., 2018)
Cynoglossus abbreviatus	Fish	Whole body	12.0 items/individual	<100 - 1000 mm	Fiber	(Abbasi *et al*., 2018)
Squalius cephalus	Fish	Stomach	0.23 - 3.33 items/g stomach contentes	<0.5 - >1 mm	Fiber and fragment	(Collard *et al*., 2018)
Rutilus rutilus	Fish	Gut	0.69 particles/individual	<5 mm	Fiber, fragment and pallet	(Horton *et al*., 2018)
Cyprinus carpio	Fish	Gastrointestinal tract, intestine and stomach	2.5 items/individual	2 - 25 mm	Fiber	(Jabeen *et al*., 2017)
Carassius auratus	Fish	Gastrointestinal tract, intestine and stomach	1.9 items/individual	<2 - 25 mm	Fiber and pallet	(Jabeen *et al*., 2017)
Hypophthalmichthys molitrix	Fish	Gastrointestinal tract, intestine and stomach	3.8 items/individual	<2 - 25 mm	Fiber, fragment and pallet	(Jabeen *et al*., 2017)
Pseudorasbora parva	Fish	Gastrointestinal tract, intestine and stomach	2.5 items/individual	<2 - 25 mm	Fiber and fragment	(Jabeen *et al*., 2017)
Megalobrama amblycephala	Fish	Gastrointestinal tract, intestine and stomach	1.8 items/individual	<2 - 25 mm	Fiber and fragment	(Jabeen *et al*., 2017)
Hemiculter bleekeri	Fish	Gastrointestinal tract, intestine and stomach	2.1 items/individual	<2 - 25 mm	Fiber	(Jabeen *et al*., 2017)

Hoplosternum littorale	Fish	Gut	3.6 particles/individual	<1 - >12 mm	Fiber and soft and hard plastics	(Silva-Cavalcanti *et al*., 2017)
Paratya australiensis	Crustacean	Whole body	0.52 items/individual	0.190 - 4.214 mm	Fiber	(Nan *et al*., 2020)
Penaeus semisulcatus	Crustacean	Whole body	7.8 items/individual	<100 - 1000 mm	Fiber	(Abbasi *et al*., 2018)
Crangon crangon	Crustacean	Whole body	1.23 items/individual	200 - 1000 µm	Fiber	(Devriese *et al*., 2015)
Corbicula fluminea	Mollusc	Soft tissue	0.4 - 5.0 items/individual	0.021-4.02 mm	Fiber	(Su *et al*., 2018)
Mytilus edulis	Mollusc	Soft tissue	0.36 particles/g tissue	5 - >25 µm	Not specified	(Van Cauwenberghe *et al*., 2014)
Crassostrea gigas	Mollusc	Soft tissue	0.47 particles/g tissue	5 - >25 µm	Not specified	(Van Cauwenberghe *et al*., 2014)
Baetidae, Heptageniidae and Hydropsychidae	Insect	Whole body	0.14 MP mg/tissue	Not specified	Not specified	(Windsor *et al*., 2019)
Tubifex	Annelid	Whole body	129 particles/g tissue	50 - 4500 µm	Fiber and fragments	(Hurley *et al*., 2017)

2.5 Ecotoxicological effects of microplastics

When MP are uptake by organisms, they may be excreted, retained, blocking the digestive tract, or translocated across the gastro-intestinal membranes and consequently transported through the circulatory system into different tissues and cells (Du *et al*., 2020). A previous study conducted to investigate the translocation of MP in the mussel *Mytilus edulis* reported that particles were translocated from the gut to the circulatory system (Browne *et al.*, 2008). In agreement with this finding, translocation of MP has also been found in the liver of anchovy *Engraulis encrasicolus (*Collard *et al*., 2017*) and fish Danio rerio (Lu et al., 2016)* and *Mugil chephalus (Avio et al., 2015).* Despite these studies, further investigation is needed to better understand the mechanisms of translocation.

Previous studies conducted in freshwater fish showed that the exposure to MP leads to physiologic alterations and inflammation in intestinal and gill tissues (Jabeen *et al*., 2018; Limonta *et al.*, 2019; Mak *et al.*, 2019; Qiao *et al.*, 2019). Experiments in *D. magna* exposed to micro-sized particles showed an increase of parental mortality, decrease in the number of broods released and reduction in the number of juveniles (Felten *et al.*, 2020; Martins *et al*., 2018; Pacheco, *et al.*, 2018; Schür *et al.*, 2020). Alterations of behaviour namely, seizures, lethargic and erratic swimming and morphologic changes were reported in fish exposed to MP (Mak *et al.*, 2019). Also, various studies conducted on fish (Ding *et al*., 2018; Iheanacho *et al.*, 2020; Mak *et al.*, 2019) and molluscs *(*Guilhermino *et al.*, 2018; Oliveira *et al.*, 2018) reported that MP caused neurotoxicity. MP may cause an increase of lipid peroxidation and oxidative stress, as reported in many freshwater species (Guilhermino *et al.*, 2018; Hamed *et al.*, 2020; Iheanacho *et al.*, 2020; Oliveira *et al*., 2018; Qiao *et al.*, 2019; Zhang *et al.*, 2019b). Moreover, in the last years, some studies research the toxic effects of MP in the gene expression (Imhof *et al.*, 2017; LeMoine *et al.*, 2018; Limonta *et al.*, 2019; Mak *et al.*, 2019).

Overall, the effects of exposure to MP can be physical, through the clogging the intestinal tract of organisms and tissue and skin damage or biochemical which induces oxidative stress and lipid peroxidation, inflammation, growth and development inhibition, impacts in food intake, reproductive capacity and gene expression. Despite all the research conducted in the last years, the knowledge about the paradigm of MP is still limited for freshwater species.

2.6 Influence of microplastics on the toxicity of chemical contaminants

During the plastic and MP manufacturing process, chemicals are added to plastics to enhance plastic properties. These chemicals, known as additives, can be flame retardants, phthalates used as plasticizers, stabilizers, antioxidants, antimicrobials, among others (Thompson *et al.*, 2009). These chemical additives can leach out of the plastic over time due to being weakly bound to the polymer (Horton *et al.*, 2017b). Also, UV radiation and temperature changes can facilitate the process of release of additives from the MP (Andrady, 2011). Some studies using leaching experiments reported that plastic additives are toxic for various aquatic organisms. Studies using *D. magna* as biologic model reported that the chemical substances leached from plastics caused immobility and an increase of mortality (Lithner *et al*., 2009; Lithner *et al*., 2012). Silva *et al.* (2016) reported abnormal development of the embryo of brown mussel (*Perna perna*) exposed to leachates from virgin and beached plastic pellets.

Moreover, in freshwater ecosystems, MP can adsorb various contaminants (e.g. heavy metals, PAHs, PCBs, persistent organic pollutants (POPs) and pharmaceuticals) present in the water and sediments due to MP large specific surface area, small particle size and hydrophobic properties (Rochman *et al*., 2013). This process can be more significant in freshwater environments because concentrations of these chemicals are expected to be higher than in marine systems due to proximity to the sources of discharge of industrial and domestic wastewater (Xu *et al*., 2020). Research on the effects of MP combined with other contaminants began in the last years. PAHs in combination with MP show effects in swimming behaviour and morphological and lethal effects in embryos of various freshwater species (Bartonitz *et al.*, 2020; Batel *et al.*,2018; Pannetier *et al*., 2019). The combined exposure to PCBs and MP caused morphologic alterations in tissues of the mollusc *C. fluminea* (Rochman *et al*., 2017). Some studies reported adverse effects in freshwater species exposure to MP and heavy metals. For example, the exposure of the bivalve *C. fluminea* to MP and mercury cause a decrease of filtration rate and induced oxidative stress and lipid peroxidation (Oliveira *et al.*, 2018). An increase of oxidative damage and inflammation of tissue were reported in discus fish (*Symphysodon aequifasciatus*) (Lu *et al*., 2018) and zebrafish (*D. rerio*) (Wen *et al.*, 2018) exposure to MP and cadmium. Moreover, pharmaceuticals in combination with MP showed toxicological effects in various freshwater species (Guilhermino *et al*., 2018; Qu *et al.*, 2020).

2.7 Conclusions

The extensive use of plastics over the last years resulted in a major environmental problem. Due to their high durability, plastics have been accumulating in freshwater ecosystems environments and some have been fragmenting over time into MP. Such MP may induce several types of adverse effects in exposed organisms as reported in several studies. Thus, the presence of MP in freshwater ecosystems put in risk the organisms living on these habitats. Different quantitative units are used, which makes difficult the comparison between studies. There is still a lack of knowledge of the ecotoxicological effects of MP in freshwater ecosystems. MP can adsorb other chemical pollutants present in the water and release them as well as additives inside the organisms. Moreover, in natural freshwater ecosystems, the biota is commonly exposed to several contaminants in addition to MP, and the toxic effects of such mixtures are still poorly understood. Thus, more studies on the interaction of MP with other contaminants in freshwater environments are needed.

Chapter 3. Effects of microplastics and deltamethrin, alone and in mixture, on the freshwater bivalve *Corbicula fluminea*

3.1 Introduction

DLT is a pyrethroid insecticide used in agriculture and aquaculture and its use has been increasing in recent years (Köprücü *et al.*, 2008a). The pollution of freshwater systems with DLT, mainly near agricultural areas, can put at risk many organisms since this insecticide has already been detected in the water column of rivers and lakes (Dabrowski *et al.*,2002; Riaz *et al.*, 2018; Xue *et al.*, 2006). DLT can cause a wide range of adverse effects in freshwater species, such as behavioural changes, neurotoxicity, oxidative stress and decrease of the reproduction capacity (Bhattacharjee *et al.*, 2020; Toumi *et al.,* 2013).

MP are also important contaminants in many freshwater ecosystems all over the world (Xu *et al*., 2020). Freshwater organisms uptake MP from water and sediments and accumulate them (Devriese *et al*., 2015; Jabeen *et al*., 2017; Su *et al*., 2018). The exposure to MP cause adverse effects in freshwater species, namely in *D. rerio* (Mak *et al.*, 2019), *D. magna* (Martins *et al*., 2018; Pacheco, *et al.*, 2018) and *C. fluminea* (Guilhermino *et al*., 2018; Oliveira *et al*., 2018). In natural environments, animals are exposed simultaneously to different contaminants, that can potentially cause toxic effects (Rochman *et al*., 2013). Felten *et al*. (2020) reported a decreased number of broods and neonates per surviving adult in *D. magna* exposed to a mixture of MP and DLT. Nevertheless, more investigation is needed to understand the impacts of MP in freshwater environments.

The main goal of the experimental work was to investigate the effects of MP and DLT, alone and in mixture, on the freshwater bivalve *Corbicula fluminea.*

3.2 Material and Methods

First, an assay without bivalves to select the interval of test medium renewal was carried out. Based on the findings of this assay, a complete test was done. The following biomarkers were used as effect criteria: filtration rate (FR), the activity of the enzymes cholinesterases (ChE), isocitrate dehydrogenase enzyme (IDH), octopine dehydrogenase enzyme (ODH), GST, CAT and lipid peroxidation (LPO) levels. FR was selected because it indicates the capacity of animals to intake food and is associated with the respiratory function. Therefore, this biomarker was selected because feeding is crucial for bivalve's fitness and is indicative of predator-prey relationships, that occurs in natural ecosystems. Also, studies show that MP cause significant effects on filtration rate in *C. fluminea* (Guilhermino *et al*., 2018; Oliveira *et al.*, 2018). The enzymatic biomarkers selected are involved in important physiological functions. ChE activity was determined because neurotransmission is fundamental in behaviour functions (Guilhermino *et al.*, 2018). In bivalves, this enzyme has an important role in shell opening and closing (Oliveira *et al*.,

2018). Several studies reported a decrease of ChE activity in mussels exposed to different types of MP (Guilhermino *et al.*, 2018; Oliveira *et al*., 2018; Ribeiro *et al.*, 2017). ODH and IDH are involved in the production of cellular energy and is involved in the cell redox balance. The enzymes, CAT and GST belong to the antioxidant system, as well as GST which is involved in phase II of biotransformation. Köprücü *et al*., 2008b shows a significant reduction in CAT activity in digestive gland and gills of the freshwater bivalve *Unio elongatulus eucirrus after* exposure to DLT. LPO levels were selected because are indicative of lipid peroxidation. A previous study showed that DLT increased the LPO levels in the bivalve *Unio elongatulus eucirrus* (Köprücü *et al.*, 2008b).

3.2.1 Chemicals

DLT was purchased from Sigma-Aldrich (CAS number: 52918-63-5). MP were purchased from Cospheric – Innovations in Microtechnology (U.S.A.) (lot number: 4-1006-1053). They were fluorescent polymer red microspheres, with a diameter between 1 and 5 µm, a density of 1.3 g/cc, an excitation wavelength of 575 and emission wavelength of 607 nm, as indicated by the manufacturer. The Bradford reagent used for protein quantification was purchased from Bio-Rad Laboratories (Germany). All chemicals used for biomarker determinations were purchased from Sigma-Aldrich (Germany) or Merck (Germany).

3.2.2 C. fluminea sampling and acclimation

Adult specimens of *C. fluminea* were collected in the upper part of Minho River estuary (NW Iberian Peninsula, ~42º03′22.51″N, 8º32′22.51″W) with an adapted rake. Bivalves were transported to the laboratory in thermally isolated boxes with water from the collection site (Oliveira *et al*., 2015). In the laboratory, all the bivalves were measured with a calliper. The mean (± SD) of shell length of the bivalves used in the study were 2,3 ± 0,1 cm. In the laboratory, nine bivalves were sacrificed, and the tissues of each animal were isolated and frozen at -80 ºC, for biomarker determinations. Bivalves aimed at being used in the bioassay were maintained in tanks with 20 L of dechlorinated tap water (DTW) for human consumption and continuous aeration was supplied. The tanks were maintained in a room for 14 days. During this period, the medium was renewed at each 48 h and the animals were fed daily with *Chlorella vulgaris* in a concentration of 8 x 10^5 cells/day/bivalve (Oliveira *et al*., 2018). After the acclimation period, nine animals were sacrificed on ice and the tissues of each were frozen individually at −80 °C, for further biomarker analyses. The remaining bivalves were used for the bioassay.

3.2.3 Experimental design

In the assay without bivalves and the bioassay, test medium was dechlorinated tap water for human consumption (DTW) and was added 0,67 mL of *C. vulgaris* cells in a concentration of 8 x 10^5 cells/day/bivalve to feed the bivalves. Six treatments were tested: control (only DTW); control with acetone (DTW with 0.05 mL/L of acetone, the same concentration that DLT treatments contained); DTW with 0.2 mg/L of MP; DTW with 0.2 mg/L of MP and 0.05 mL/L of acetone; DTW with 0.02 mL/L of DTL; and DTW with 0.2 mg/L of MP and 0.02 mL/L of DTL. The indicated concentrations of acetone, MP and DTL are nominal the ones. The concentrations of MP and DLT tested were chosen based on previous studies (Guilhermino *et al*., 2018; Köprücü *et al.*, 2008b). The concentration of acetone in the test medium selected to dissolve DLT in water was based on the guideline nº 203 (OECD, 2019). According to this guideline, the final concentration of a solvent in the test medium should not exceed 0.1 mL/L of solvent. For the preparation of treatments with MP, a stock solution with a concentration of 80 mg/L was prepared in ultra-pure water. This solution was diluted in clean medium to obtain the corresponding test medium. The treatments with DLT were prepared by dilution of a stock solution of 4 g/L, previously prepared in acetone, into a clean medium. Glass beakers were filled with 1L of the corresponding test medium and continuous air supply was provided.

The assay without bivalves was conducted during 48 h and 3 beakers were used per treatment. At 0, 24 and 48 h, the abiotic parameters (pH, dissolved oxygen and temperature) were measured with a Multi 340i/Set, Wissenschaftlich-Technische Werkstätten GmbH (Germany) multi-parametric. Samples of the test medium were collected to determine the concentration of MP and DLT. The samples for MP determination were immediately analysed by fluorescence reading, the remain samples were frozen at -20 ºC, for future determination of DLT concentration.

The bioassay was carried out for 8 days. Two bivalves were added to each beaker. 18 bivalves were used per treatment: 9 for biomarkers and 9 for determination of DLT concentrations in the whole body. However, due to space limitations, in the control treatment were used 10 bivalves: 9 for biomarkers and 1 for determination of DLT concentrations. The test medium was renewed at each 24 h. Daily, in freshly prepared medium and old medium (medium with 24 h) the abiotic parameters (pH, dissolved oxygen and temperature) were measured. At each medium renewal, samples of the old and fresh solution test were collected to determinate the concentration of MP and DLT, as previously described for the assay without bivalves. After 8 days of exposure, the soft body and the

shell of 9 bivalves of each treatment and one bivalve from the control group were isolated and stored at -20 ºC, for further determination of DLT concentrations. The remaining 9 bivalves were used for determination of biomarkers and observation for MP localization in the digestive system, using a stereoscope (213628 Nikon, Japan).

3.2.4 Determination of microplastic concentration in test media

The actual concentrations of MP were determined by spectrofluorimetry as described in Luís *et al.* (2015) and adapted by Guilhermino *et al*. (2018). To obtain calibration curves to determine the MP concentrations in test media, three solutions of MP, with a concentration of 5 mg/L, were performed in deionized water, dechlorinated tap water and dechlorinated tap water and a mixture of *C. vulgaris* cells (with the same concentration used for feeding the animals during the bioassay). Each solution was serial diluted (1:2, v:v) with DTW to obtain a final series of solutions with MP concentrations of: 5; 2.5; 1.25; 0.625; 0.313; 0.156; 0.078 and 0.039 mg/L. The procedure was repeated in two different days to obtain 3 independent replicates of each solution and take into consideration some potential variation. The fluorescence of all the solutions was measured, using a spectrofluorimeter (Spectramax® M2, Molecular Devices, U.S.A.), using wavelengths of excitation and emission of 575 and 607 nm, respectively. For each data set (i.e. three series of each solution type), the correlation between the log of MP nominal concentrations and the corresponding log fluorescence values was investigated and a linear model was fitted to the data. The linear models obtained were used to determine the actual concentrations of MP in test medium from the fluorescence values. The deviation of the actual concentrations of MP regarding the nominal ones was calculated as described by Guilhermino *et al*. (2018):

$$MP\ deviation\ (\%) = \left|100 - \frac{actual\ concentration\ x\ 100}{nominal\ concentration}\right|$$

The decrease of MP concentrations during the interval of test media renewal (hereafter indicated as MP decay), was calculated as described by Guilhermino *et al*. (2018), from the MP concentrations in fresh and old test medium as:

$$MP\ decay\ (\%) = 100 - \frac{MP\ concentrations\ of\ old\ test\ medium\ x\ 100}{MP\ concentrations\ of\ fresh\ test\ medium}$$

Because the MP decay in test media was higher than 20% (as shown in the Results and Discussion section), the estimated exposure concentrations of MP during the bioassay were calculated from the geometric means of the actual concentrations of fresh and old media collected from the individual beakers at each time of medium renewal (OECD, 2011).

3.2.5 Biomarkers analysis

After the exposure period, the FR of each bivalve was determined as described by Coughlan (1969). Glass beakers were filled with 250 ml of clean medium containing *C. vulgaris* cells with a concentration of 2 x 10^6 cells/mL. The bivalves were put in the beakers (1 per beaker) supplied with aeration. Samples of the medium of each beaker were collected at the beginning of the test and 1 h later. The optical density (OD) of these samples was read at 440 nm, using a Spectramax® M2 spectrophotometer to determine the microalgae cell concentration, using the following calibration curve: OD = -155820 + cells concentrations x 13144324. The FR was calculated using the following formula: FR = (V/nt) x ln (Ci/Cf); *V* - volume of test medium (mL); *n* - number of animals per beaker; *t* - time (h), *Ci* - concentration of microalgae at the beginning and *Cf* - microalgae concentration after 1 h; and was expressed in mL of algal suspension/h/bivalve.

After filtration rate determination, the tissues of each bivalve were isolated on ice. The adductor mussel was collected to 1 mL of potassium-phosphate buffer (0.1 M, pH = 7.2) for ChE analysis. The foot was divided into two, one portion was put in Tris buffer (20 mM, pH = 7.5) with ethylenediaminetetraacetic acid disodium salt dihydrate (1 mM) and Dithiothreitol (1 mM) and the other portion was put in Tris hidroxymethil-aminomethan buffer (50 mM, pH = 7.8) to ODH and IDH analysis, respectively. Gills were collected to potassium-phosphate buffer (0.1 M, pH = 7.4) for GST, CAT and LPO determinations. All the samples were frozen at -80 ºC, until further analysis.

In the day of biomarker analysis, the samples were defrosted on ice and prepared as indicated in Oliveira *et al*. (2015). Briefly, the samples were homogenized in the respective buffers, using a Ystral GmbH d-7801 (Dottingen, Germany) homogenizer. The samples of adductor mussel and foot were centrifuged at 3300 g for 3 min at 4 ºC. The gill's samples were divided into two parts. To 250 µL of the homogenate were added 4 µL of a 4% solution of butylated hydroxytoluene and these samples were stored at -80 ºC, for further determinations of LPO levels. The remaining homogenate was centrifuged at 10000 g for 10 min at 4 ºC, for determination of the enzymatic activity of GST and CAT. After centrifugations, the supernatants were collected, and their protein content was quantified by the Bradford method (Bradford, 1976) adapted for microplate by Frasco *et al.* (2002). Bovine γ-globuline was used to prepare a standard curve which provides a comparison for the measurement of the samples. In a microplate was pipetted 10 µL of the sample in triplicate and added 250 µL of the Bradford reagent. After an incubation of 15 minutes, the absorbance was read at 600 nm. Before biomarker determinations, protein concentrations in the supernatants of tissue homogenates was adjusted: 1 mg/mL for ChE, ODH and IDH analysis and 4 mg/mL for GST and CAT.

The activity of ChE was determined by Ellman's technique (Ellman *et al.*, 1961) adapted to microplate (Guilhermino *et al.*, 1996). This method uses acetylthiocholine as subtract, which is hydrolysed and the production rate of thiocholine is measured. The continuous reaction of the thiol with the 5:5-dithiobis-2-nitrobenzoate ion produces the 5-thio-2-nitro-benzoic acid. The production of this yellow anion was measured at 412 nm. ODH activity was determinate using the method of Livingstone *et al*. (1990) adapted to microplate (Lima *et al.*, 2007). This method is based on the measurement at 340 nm, of the consumption of pyruvate due to the oxidation of nicotinamide adenine dinucleotide (NADH). IDH was determinate by measuring the production of NADPH through the reduction of $NADP^+$, at 340 nm. This technique was described by Ellis *et al.* (1971) and adapted to microplate by Lima *et al.* (2007). GST activity was measured by the method described by *Habig et al.* (1974) adapted to microplate by Frasco *et al.* (2002). GST catalyzes the conjugation of 1-chloro-2, 4-dinitrobenzene with reduced GSH. The product of this reaction is dinitrophenyl thioether which was measured at 340 nm. CAT activity was determined measuring the decrease in absorbance at 240 nm caused by the decomposition of hydrogen peroxide in molecular oxygen and water catalysed for this enzyme, as described by Clairborne (1985). LPO levels were determined by the quantification of thiobarbituric acid-reactive substances (TBARS) at 535 nm. This method described by Bird *et al.* (1984) and Ohkawa *et al*. (1979) and is based on the reaction of 2-thiobarbituric acid (TBA) with the degradation products of free radical attack. The enzymatic activities of ChE, IDH, ODH and GST were expressed in nmol/min, the CAT activity in µmol/min and the LPO levels in TBARS.

After biomarker determination, protein content was quantified, and the values obtained were used to express the enzymatic activities and LPO levels. The enzymatic activities were expressed in nmol of substrate hydrolysed per minute per mg of protein (nmol/min/ mg protein), except CAT that was expressed in µmol/min/ mg protein. LPO levels were expressed in nmol TBARS/mg protein. For the protein, ChE, IDH, ODH, GST, and LPO determinations were used a spectrophotometer microplate reader (PowerWave 340, BioTek Instruments, Inc., USA) and a spectrophotometer (Jasco V-630, Japan) was used for CAT analysis.

3.2.6 Statistical analyses

The results are indicated as the mean ± standard deviation (SD) or as the mean ± standard error of the mean (SEM). Each abiotic and biomarker data set was checked for normality of distribution (Shapiro-Wilks) and homogeneity of variances (Levene test).

Appropriate transformations were applied when the data do not have a normal distribution or/ and homogeneity of variances.

The Pearson's correlation coefficient was used to investigate the correlation between fluorescence and nominal MP concentrations of each calibration curve data, and a linear regression model was fitted to the data. The Analysis of Co-variance (ANCOVA) was used to compare pairs of calibration curves.

The one-way Analyses of Variance (1-ANOVA) was used to compare biomarkers (each biomarker data set separately) determined in bivalves immediately after arrival from the field, after 14 days of acclimatization in the laboratory, and after 14 days of acclimatization with 8 additional days of exposure in the same conditions (control of the bioassay). When significant differences were found, the Tukey's multiple comparison test was used to discriminate significant differences. When ANOVA assumptions were not achieved after data transformation, the non-parametric Kruskal-Wallis test was used, followed by a nonparametric multiple comparisons test comparable to the Tukey's test (Zar, 2010) when significant differences were found.

Regarding the data of the bioassay, each biomarker data set with normal distribution and homogeneity of variances was analysed by two-way Analyses of Variance with interaction (2-ANOVA) using the MP (presence/absence) and DTL (presence/absence) as fixed factors. The biomarker data sets for which the 2-ANOVA detected significant differences, were also analysed through the Tukey test to compare all the individual treatments. Biomarker data sets for which normal distribution and/or homogeneity of variances could not be achieved even with data transformation were analysed by the non-parametric Kruskal-Wallis test followed by a nonparametric multiple comparisons test comparable to the Tukey's test (Zar, 2010) when significant differences were found.

Data sets of the actual concentrations of MP in test medium were analysed by three-way Analyses of Variance with interactions (3-ANOVA) using the following fixed factors: acetone (presence/absence), DTL (presence/absence) and time (0h, 24h, 48h), followed by the Tukey multiple comparisons test. When the ANOVA assumptions were not full field the non-parametric Kruskal-Wallis test was used, followed by a nonparametric multiple comparisons test (Zar, 2010).

The significant level was 0.05. The software IBM SPSS Statistics v26.0 for Windows (IBM®, U.S.A.) was used for statistical analyses. The nonparametric multiple comparisons test was calculated as described by Zar (2010).

3.3 Results and Discussion

During the assay without bivalves, the pH and temperature variation in individual beakers was always lower than 0.4 pH units and 0.8 ºC, respectively, and the dissolved oxygen in test media was always higher than 6.88 mg/L.

During the bioassay, the correspondent variation of pH and temperature was always lower than 0.82 pH units and 2.5 ºC, in individual beakers. The dissolved oxygen in test media was always higher than 6.00 mg/L. Mortality was registered in two bivalves during the bioassay, one in day 6 in the treatment with MP and acetone and the other in day 7 in the treatment containing MP only. These animals were immediately removed from the test and the corresponding data of these bivalves were not included in the statistical analysis. No *C. fluminea* mortality was recorded in the controls of the bioassay.

3.3.1 Calibration curves

For all the serial sets of solutions (3 replicate series for each type of solutions) significant and positive correlation between the log of concentrations and the log of fluorescence values were found, as shown in Table 4 and in Figure S 1 of the Annexes where the models fitted to each calibration curve were also found.

Table 4 - Linear regression model, coefficient of determination and Pearson's correlation coefficient of each curve performed in deionized water, dechlorinated tap water and dechlorinated tap water and algae. MP conc. log. – Microplastic concentration logarithm; Fluor. log. – Fluorescence logarithm

			Pearson's correlation coefficient			
Calibration curve		**Linear regression model**	**p**	**N**	**r**	**R (%)**
1	Deionized water	MP conc. log. = -1.869 + 1.033 x fluor. log.	< 0.001	24	0.984	96.9
2	Dechlorinated tap water	MP conc. log. = -1.653 + 0.960 x fluor. log.	< 0.001	24	0.982	96.5
3	Dechlorinated tap water + Algae	MP conc. log. = -1.720 + 0.914 x fluor. log.	< 0.001	24	0.980	96.1

ANCOVA showed a significant difference ($F_{(1,45)} = 9.219$); $p \leq 0.05$) between the curves performed with deionized water and dechlorinated tap water (1 and 2). Thus, the type of water used does influence the fluorescence readings and has implications for the calculation of MP actual concentrations. This analysis was not used in comparisons with the calibration curve 3 because ANCOVA requirements could not be achieved (the dada does not have homogeneity of variances). The calibration curve 3 was used to calculate the

MP concentrations in the test medium because in the bioassay the clean medium was dechlorinated tap water and *C. vulgaris* cells.

3.3.2 Microplastics in test media

3.3.2.1 Assay without bivalves

The fluorescence values and the actual concentrations of MP (mg/L) in the test medium at 0, 24 and 48 h are shown in Table 5.

Table 5 – Fluorescence and actual concentrations of microplastics in test medium at 0, 24 and 48 h. The values are the mean and the corresponding standard deviation. Ctr – control group; MP – microplastics; ACET - acetone DLT – deltamethrin; AC – actual concentrations; N – number of samples analysed; FLUOR 0 h – fluorescence at 0 h; FLUOR 24 h – fluorescence after 24 h; FLUOR 48 h – fluorescence after 48 h; RFU – relative fluorescence units; Dev. – deviation of microplastics actual concentrations relatively to nominal ones at 0 h; Decay 24 h – reduction of the microplastics concentration in test media during 24 h; Decay 48 h – reduction of the microplastics concentration in test media during 48 h.

Treatment	N	Fluor 0 h (RFU)	MP AC 0h (mg/L)	Dev. (%)	N	Fluor 24 h (RFU)	MP AC 24h (mg/L)	Decay 24 h (%)	N	Fluor 48 h (RFU)	MP AC 48h (mg/L)	Decay 48 h (%)
Ctr	3	0	0	-	3	0	0	-	3	0	0	-
MP only	3	6.12 ± 0.77	0.10 ± 0.01	50	3	4.94 ± 0.93	0.08 ± 0.01	17	3	3.09± 0.08	0.05 ± 0.01	47
MP+ACET	3	6.38 ± 0.21	0.10 ± 0.00	48	3	4.74 ± 1.43	0.08 ± 0.02	24	3	4.33 ± 0.23	0.07 ± 0.00	30
MP+DLT	3	6.84 ± 1.00	0.14 ± 0.01	32	3	6.87 ± 1.29	0.11 ± 0.02	18	3	4.76 ± 0.41	0.08 ± 0.01	41

At 0h, the mean (± SD) concentration of MP in test medium (Table 5) was 0.10 ± 0.01 mg/L in treatments containing only MP, 0.10 ± 0.00 mg/L in treatments with MP and acetone and 0.14 ± 0.01 mg/L in treatments containing the mixture of MP and DLT. The corresponding values of MP concentrations in test medium at 24 h was 0.08 ± 0.02 mg/L, 0.08 ± 0.02 mg/L and 0.11 ± 0.01 mg/L, respectively and at 48 h was 0.05 ± 0.01 mg/L, 0.07 ± 0.02 mg/L and 0.08 ± 0.02 mg/L, respectively. In test medium at 0, 24 and 48 h of the control tretment MP were not detected.

The deviation of MP concentrations regarding the nominal concentrations of MP in the test medium at 0 h (Table 5) was of 50% in the treatments containing only MP, 48% in the treatments with MP and acetone, and 32% in the treatments with the mixture of MP and DLT. Thus, the deviation of actual concentrations of MP from nominal ones raged from 32 to 52% and the actual concentration of MP differ significantly from the nominal ones, because the concentrations of test substance during the assay should be at least 80% of the nominal concentration (OCDE, 2014).

The decay of MP concentrations in the test medium at 24 h (Table 5) was of 17% in the treatments containing MP alone, 24% in the treatments with MP and acetone, and 18%

in the treatments with the mixture of MP and DLT. The corresponding percentages in the test medium after 48 h were of 47%, 30% and 41%.

The results of three-way ANOVA performed to investigate if the presence of acetone, DLT and the time influenced the MP concentrations determined in test medium are presented in Table 6.

Table 6 – Results of three-way ANOVA and Tukey's test. Different letters indicate a significant difference at 0.05. MP – Microplastics; ACET – Acetone; DLT – Deltamethrin.

Factor or interaction	**Level or type of interaction**	**N**	**Mean ± SD and Tukey results**	**3-ANOVA results**
ACET presence	With ACET Without ACET	18 9	0.10 ± 0.03 0.08 ± 0.02	$F_{(1, 18)} = 0.915$ $p = 0.351$
DLT presence	With DLT Without DLT	9 18	0.11 ± 0.03 0.08 ± 0.02	$F_{(1, 18)} = 10.169$ $p = 0.005$
Time	0 h 24 h 48 h	9 9 9	0.11 ± 0.02 a 0.09 ± 0.02 a 0.07 ± 0.01 b	$F_{(2, 18)} = 21.531$ $p < 0.001$
Interaction	ACET and time	-	-	$F_{(2, 18)} = 0.534$ $p = 0.595$
Interaction	DLT and time	-	-	$F_{(2, 18)} = 1.856$ $p = 0.185$

Acetone had no significant effects, thus it did not interfere with MP concentrations determination. DLT had a significant effect suggesting interference of this compound with the determinations of MP concentrations. Time had also a significant effect, with significant differences at 48 h. Therefore, the test medium should be renewed at 24 h because no significant differences were showed between 0 and 24 h (Table 6) and decay of MP in test medium at 48 h was higher than at 24 h (Table 5). Previous studies reported analogous percentages of MP decay at 24 h using the same type of MP particles in beakers without animals (Guilhermino *et al.*, 2018; Oliveira *et al.*, 2018). The causes for the MP decay can be due to the aggregation of the particles and/or sedimentation and sorption of the particles in the internal walls of the beakers (Luís *et al.*, 2015).

3.3.2.2 Bioassay

In Table 7 are represented the fluorescence values and the actual concentrations of MP (mg/L) in fresh and old test medium.

Table 7 - Fluorescence and actual concentrations of microplastics in fresh (0 h) and old (24 h) test medium. The values are the mean with the corresponding standard deviation. Ctr – control group; MP – microplastics; ACET – acetone; DLT – deltamethrin; AC – actual concentrations; EEC – MP estimated exposure concentrations during the bioassay; N – number of samples analysed; FLUOR 0 h – fluorescence at 0 h; FLUOR 24 h – fluorescence after 24 h; RFU – relative fluorescence units; Dev. – deviation of microplastics actual concentrations relatively to nominal ones; Decay – reduction of the microplastics concentration in test media during 24 h.

Treatment	N	Fluor 0 h (RFU)	MP AC 0h (mg/L)	Dev. (%)	N	Fluor 24 h (RFU)	MP AC 24 h (mg/L)	Decay (%)	N	MP EEC (mg/L)
Ctr	40	0	0	-	40	0	0	-	-	-
MP only	72	10.55 ± 3.90	0.16 ± 0.06	28	58	4.91 ± 2.08	0.08 ± 0.03	50	58	0.13 ± 0.03
MP+ACET	72	11.38 ± 3.17	0.18 ± 0.04	21	59	4.08 ± 1.81	0.07 ± 0.03	59	59	0.12 ± 0.02
MP+DLT	72	11.00 ± 3.58	0.17 ± 0.05	25	64	4.37 ± 2.61	0.07 ± 0.04	56	64	0.12 ± 0.03

In fresh (0 h) test medium, the mean (± SD) of MP concentration (Table 7) was 0.16 ± 0.06 mg/L in treatments with MP alone, 0.18 ± 0.04 mg/L in treatments with MP and acetone and 0.17 ± 0.05 mg/L in treatments containing the mixture of MP and DLT. In old (24 h) test medium, the mean (± SD) of MP concentration was 0.08 ± 0,03 mg/L, 0.07 ± 0.03 mg/L and 0.07 ± 0.04 mg/L, respectively. Both in fresh and old test medium in the control treatments were not detected MP.

The deviation of MP concentrations relative to the nominal ones in the test medium (Table 7) at 0 h was of 28% in the treatments containing only MP, 21% in the beakers with MP and acetone, and 25% in the beakers with the mixture of MP and DLT. Because in the fresh medium the deviation of actual concentrations of MP raged from 21 to 28% from nominal ones, the actual concentration of MP differs from the nominal ones. According to OCDE (2014), if the deviation from the nominal concentration is higher than 20%, the mean concentrations of exposure should be calculated.

The decay of MP concentrations in old media (Table 7) at 24 h was of 50% in the treatments containing only MP, 59% in the mixture of MP and acetone and 56% in the treatments with the mixture of MP and DLT. In relation with the results obtained in the medium at 24, in the assay without bivalves, the values of the decay were higher because, among the other factors described early in section 3.3.2.1, MP could have been uptake by the bivalves or/ and MP may have formed aggregates with algae and sedimented.

The results of the Kruskal-Wallis and the non-parametric multiple comparisons test performed to investigate significant differences between the treatments in fresh and old test medium are represented in Table 8.

Table 8 - Results of Kruskal-Wallis and non-parametric multiple comparisons test. Different letters indicate a significant difference. The significant level is 0.05. MP – Microplastics; ACET – Acetone; DLT – Deltamethrin.

Fresh media (0 h)			
Treatments	**N**	**Mean ± SD**	**Kruskal -Wallis**
MP	72	0.16 ± 0.06	X^2 (2) = 2.004 p = 0.367
MP+ACET	72	0.18 ± 0.04	
MP+DLT	72	0.17 ± 0.05	
Old media (24 h)			
Treatments	**N**	**Mean ± SD**	**Kruskal -Wallis**
MP	58	0.08 ± 0.03	X^2 (2) = 4.342 p = 0.114
MP+ACET	59	0.07 ± 0.03	
MP+DLT	64	0.07 ± 0.04	
MP EEC			
Treatments	**N**	**Mean ± SD**	**Kruskal -Wallis**
MP	58	0.13 ± 0.03	X^2 (2) = 0.47 p = 0.790
MP+ACET	59	0.12 ± 0.02	
MP+DLT	64	0.12 ± 0.03	

In fresh medium, no significant differences were found between the test medium of the treatments tested. Thus, acetone and/or DLT have not influence fluorescence readings. In old test medium, no significant differences between the test medium with 24 h were found suggesting that interaction between MP, acetone and DLT did not occur.

The means (± SD) of the values of the estimated exposure concentration of MP in each beaker previously calculated are also summarized in Table 8. The estimated exposure concentration of MP was 0.13 ± 0.03 mg/L, 0.12 ± 0.02 mg/L and 0.12 ± 0.03 mg/L in treatments containing only MP, MP and acetone and mixture of MP and DLT, respectively. No significant differences between the treatments were found. Since concentrations of 5.51 mg/L were reported in freshwater systems (Lasee *et al.*, 2017), the real concentrations of MP tested in this study have ecological relevance.

3.3.3 Biomarkers

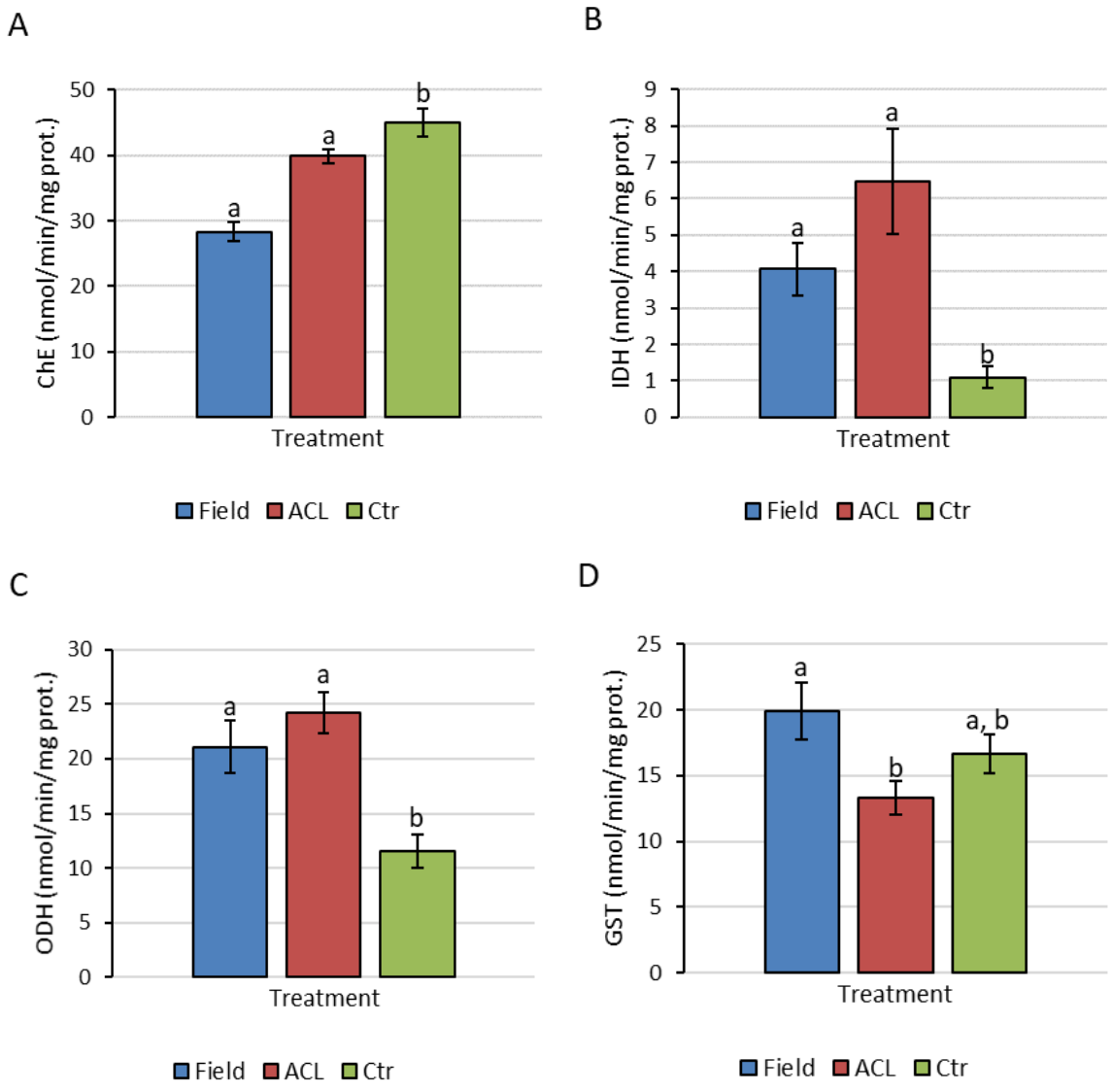
The results of the biomarkers (ChE, IDH, ODH, GST and CAT activity and LPO levels) determined in groups of bivalves immediately after arrival to the laboratory, after the acclimation period, and in the control group of the bioassay after additional 8 days are shown in Figure 1. The complete results of the statistical analyses of this data per biomarker are shown in Table 9.

Table 9 - Biomarkers determined in Corbicula fluminea after arrival from the field (field), after the acclimation period (ACL) and after the bioassay in the control group (Ctr). Values are the mean and the corresponding standard error. The biomarkers are: Cholinesterase enzymes (ChE) activity; NADP-dependent isocitrate dehydrogenase (IDH) activity; Octopine dehydrogenase (ODH) activity; Glutathione S-transferases (GST) activity; Catalase (CAT) activity and Lipid peroxidation (LPO) levels. The significant differences between treatments are identified by different letters (one-way ANOVA + Tukey's test or Kruskal-Wallis + non parametric multiple comparison test; $p \leq 0.05$).

Biomarker	Treatment	N	Mean ± SEM		Statistical analyses
ChE	Field	9	28.33 ± 1.44	a	X^2 (2) = 18.437 p < 0.001
	ACL	9	39.86 ± 1.08	a	
	Ctr	9	44.94 ± 2.15	b	
IDH	Field	9	4.06 ± 0.72	a	$F_{(2, 25)}$ = 18.807 p < 0,001
	ACL	9	6.49 ± 1.45	a	
	Ctr	8	1.09 ± 0.29	b	
ODH	Field	9	21.10 ± 2.37	a	$F_{(2, 26)}$ = 11.459 p < 0.001
	ACL	9	24.26 ± 1.90	a	
	Ctr	9	11.55 ± 1.51	b	
GST	Field	9	19.91 ± 2.19	a	$F_{(2, 26)}$ = 3.75 p = 0.038
	ACL	9	13.35 ± 1.27	b	
	Ctr	9	16.68 ± 1.47	a, b	
CAT	Field	9	10.26 ± 2.65		$F_{(2, 26)}$ = 0.069 p = 0.934
	ACL	9	6.61 ± 0.67		
	Ctr	9	7.90 ± 1.15		
LPO	Field	9	0.025 ± 0.001	a	$F_{(2, 26)}$ = 15.6 p < 0.001
	ACL	9	0.073 ± 0.004	b	
	Ctr	9	0.037 ± 0.002	a	

Significant differences in all the biomarkers among the three groups of bivalves were found, except in CAT activity (Table 9). In relation with the means determined immediately after arrival from the field, the group of bivalves where the biomarkers were determined after a 14-day acclimation period had lower GST activity (Figure 1 D) and higher LPO levels (Figure 1 F), and no significant differences in any of the other biomarkers (Figure 1 A-C and E). Higher LPO levels after 14 days of acclimatization suggest that the animals could be in adaptation to the laboratory conditions. Nevertheless, a period of acclimatization of 14 days has been also suggested in other studies conducted on *C. fluminea* (Guilhermino *et al*., 2018; Oliveira *et al*., 2015; Oliveira *et al*., 2018). Despite the increase of LPO levels, the activity of the antioxidant enzyme CAT (Fig 1 E) was not significantly changed indicating that it was not induced in response to increased oxidative stress levels. The reduction of GST activity after acclimation (Figure 1 D) suggests exposure of the bivalves in the field to environmental contaminants that were GST inducers, with a decrease of the enzyme activity in the laboratory because the exposure stop. Bivalves of the bioassay control group that were 22 days in the laboratory, had higher ChE activity than bivalves of the other two groups

(Figure 1 A). These results suggest that in the field, animals could have been exposed to neurotoxic contaminants leading to ChE activity inhibition, and the enzyme activity was higher after a period in the laboratory because the exposure to anticholinesterase agents did not occur. Also, lower activity of IDH (Figure 1 B) and ODH (Figure 1 C) was found in bivalves of the bioassay control group in comparison with the two other groups, suggesting a reduction of the use of aerobic and anaerobic pathways of cellular energy production. Concerning the bivalves of the acclimatization period, lower LPO (Figure 1 F) levels in the control group may indicate a reduction of oxidative stress and lipid oxidation damage. Because additional energy is needed for the detoxification process, the lower activity of IDH and ODH, previous described, suggests that less energy is required to combat the oxidative stress (Oliveira *et al*., 2012).



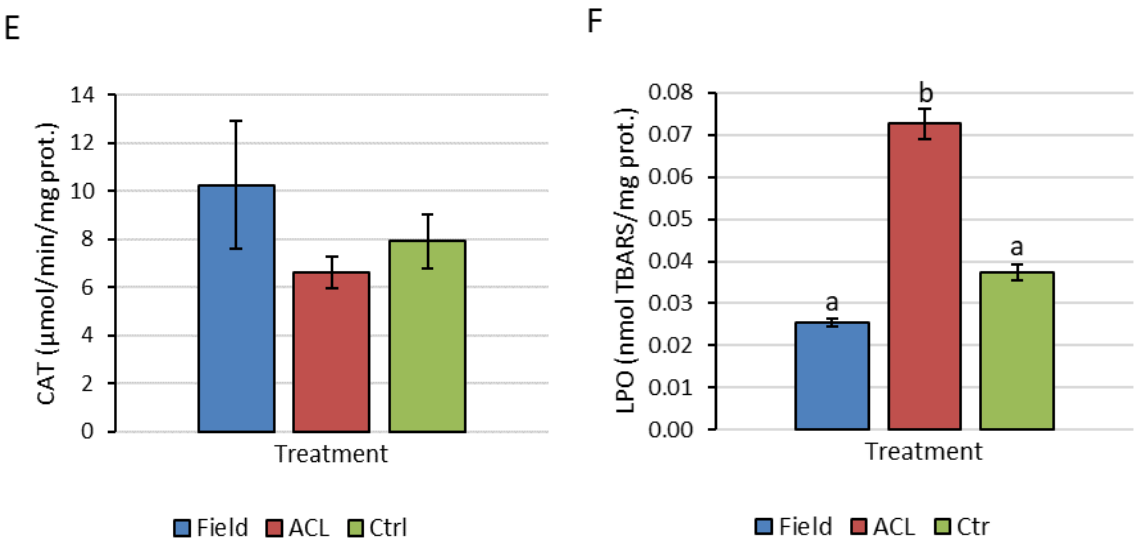


Figure 1 - Biomarkers determined in Corbicula fluminea after arrival from the field (field), after the acclimation period (ACL) and on the control group after the bioassay (Ctr). Values are the mean and the corresponding standard error. A - Cholinesterase enzymes (ChE) activity; B - Isocitrate dehydrogenase (IDH) activity; C - Octopine dehydrogenase (ODH) activity; D - Glutathione S-transferases (GST) activity; E - Catalase (CAT) activity; F - Lipid peroxidation (LPO) levels. The significant differences between treatments are identified by different letters above the bars (one-way ANOVA and the Tukey's test or Kruskal-Wallis and the non-parametric multiple comparison test). The significant level is 0.05

The results of the biomarkers determined at the end of the bioassay carried out to investigate the effects of MP, DTL and its mixture are shown in Figure 2. The results of the two-way ANOVA carried out with each biomarker data set are shown in Table 10.

Significant differences were not found in any biomarker between bivalves exposed to MP and those not exposed to MP (Table 10). These results indicate that MP alone and/or in mixture with DTL did not cause toxic effects in *C. fluminea*. Significant differences in ODH and LPO between bivalves exposed to DLT and not exposed to DTL (Table 10) were obtained. These results indicate that DLT alone and/or in mixture with MP can cause toxic effects to the bivalves. A significant interaction between MP and DTL in ODH (Table 10) were found, suggesting that toxicological interactions may have occurred in bivalves exposed to the MP-DTL mixture. All the other differences and interactions were not significant. Table 10 does do not include information on ChE, IDH and FR because their data could not be analysed through two-way ANOVA as the requirements for its use could not be achieved.

Table 10 - Results of the two-way ANOVA performed to investigate the effects of microplastics (MP) and deltamethrin (DLT) on biomarkers of Corbicula flumínea. Values are the mean and the corresponding standard error. The biomarkers are: Glutathione S-transferases (GST) activity; Octopine dehydrogenase (ODH) activity; Lipid peroxidation (LPO) levels and Catalase (CAT) activity. The significant level is 0.05.

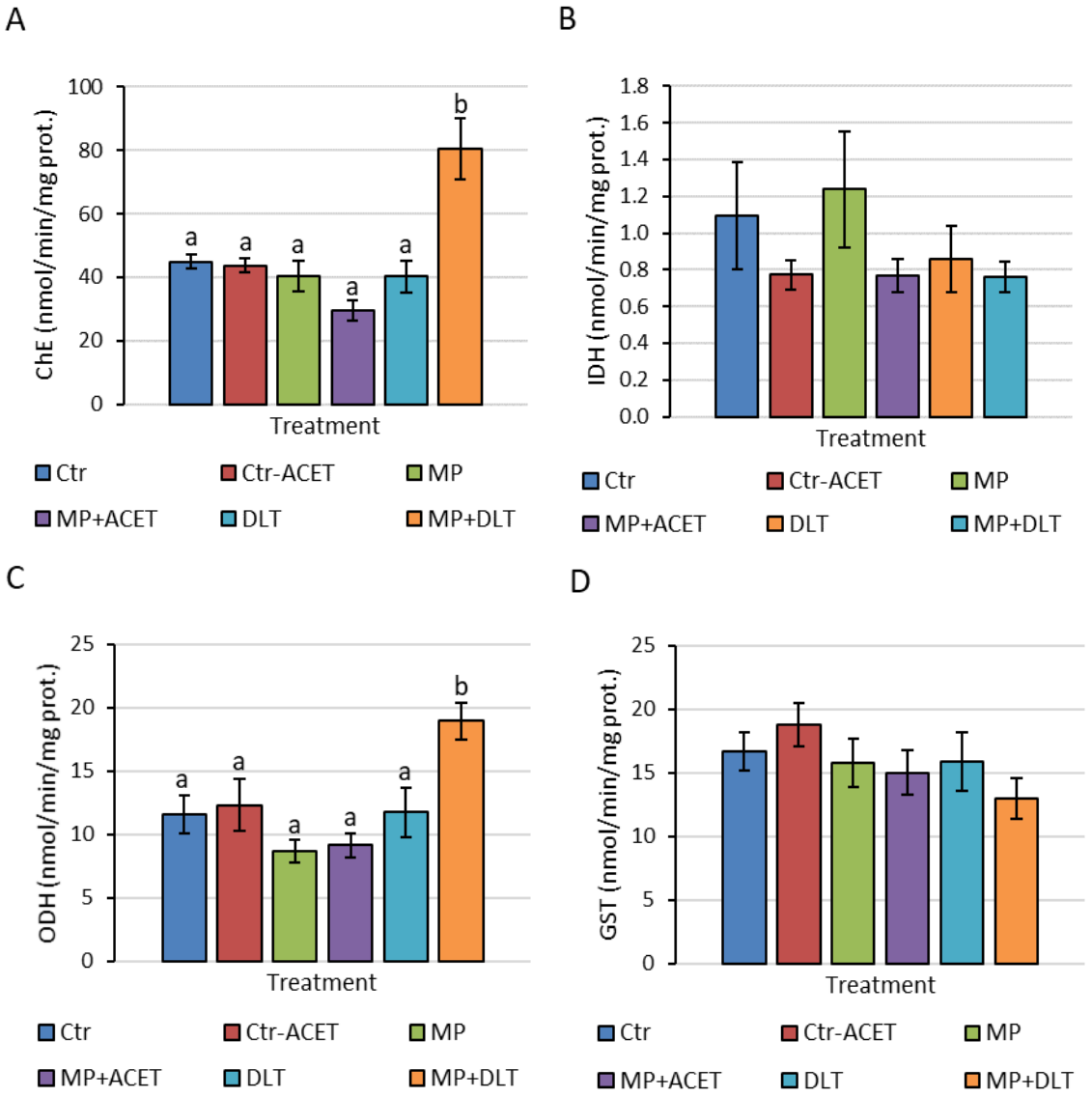
Biomarker	Factor	Level	N	Mean ± SD	Two-way ANOVA results
ODH	MP	No MP With MP	27 27	11.87 ± 5.37 12.26 ± 5.80	$F_{(1, 50)} = 2.553$, $p = 0.116$
	DTL	No DTL With DTL	36 18	10.43 ± 4.40 15.34 ± 6.23	$F_{(1, 50)} = 14.16$, $p < 0.001$
	Interaction				$F_{(1, 50)} = 15.294$, $p < 0.001$
GST	MP	No MP With MP	27 27	17.11 ± 5.49 14.61 ± 5.18	$F_{(1, 50)} = 2.828$, $p = 0.099$
	DTL	No DTL With DTL	36 18	16.55 ± 5.10 14.46 ± 5.95	$F_{(1, 50)} = 1.841$, $p = 0.181$
	Interaction				$F_{(1, 50)} = 0.033$, $p = 0.856$
CAT	MP	No MP With MP	27 27	8.04 ± 4.08 6.74 ± 3.06	$F_{(1, 50)} = 1.033$, $p = 0.314$
	DTL	No DTL With DTL	36 18	7.87 ± 3.98 6.43 ± 2.65	$F_{(1, 50)} = 0.689$, $p = 0.411$
	Interaction				$F_{(1, 50)} = 0.200$, $p = 0.657$
LPO	MP	No MP With MP	27 27	0.030 ± 0.014 0.027 ± 0.009	$F_{(1, 50)} = 0.367$, $p = 0.547$
	DTL	No DTL With DTL	36 18	0.030 ± 0.012 0.024 ± 0.010	$F_{(1, 50)} = 4.535$, $p = 0.038$
	Interaction				$F_{(1, 50)} = 0.024$, $p = 0.879$

The results of the Tukey test comparing bivalves of all the treatments are indicated in Figure 2. No significant differences were found between the control group and the acetone control group in any of the biomarkers analysed. Thus, the concentration of acetone used as solvent did not cause toxic effects in *C. fluminea,* which is in good agreement with previous findings in the same species (Guilhermino *et al.*, 2018).

The exposure to the treatments with MP (alone) and DLT (alone) did not cause significant alterations in any biomarker. Although not significant, bivalves exposed to these treatments showed a decrease in LPO (Figure 2 F) levels of 26% and 31%, respectively. Same results are reported in other species of bivalves exposed to MP where the highest LPO levels have been found in the control groups (Nobre *et al.*, 2020; Ribeiro *et al.*, 2017). Since a significant increase in antioxidant enzymes did not occur, the decrease of LPO levels can be explained for a positive response of the antioxidant

system that neutralized the excess of reactive oxygen species (ROS) and the redox homeostasis was restored (Trestrail *et al.*, 2020).

Relatively to the control, a significant increase in ChE (79%) and ODH (64%) activity (Figure 2 A and C, respectively) was found in bivalves exposed to the mixture of MP and DLT. This find suggests toxic interactions between the components of the mixture on *C. fluminea*, despite no significant effects occurred on bivalves exposure to DLT and MP individually. Moreover, the increase ChE and ODH activity indicating that cholinergic transmission increase and more energy was needed to the biologic processes. The mixture did not cause other significant effects on *C. fluminea*.



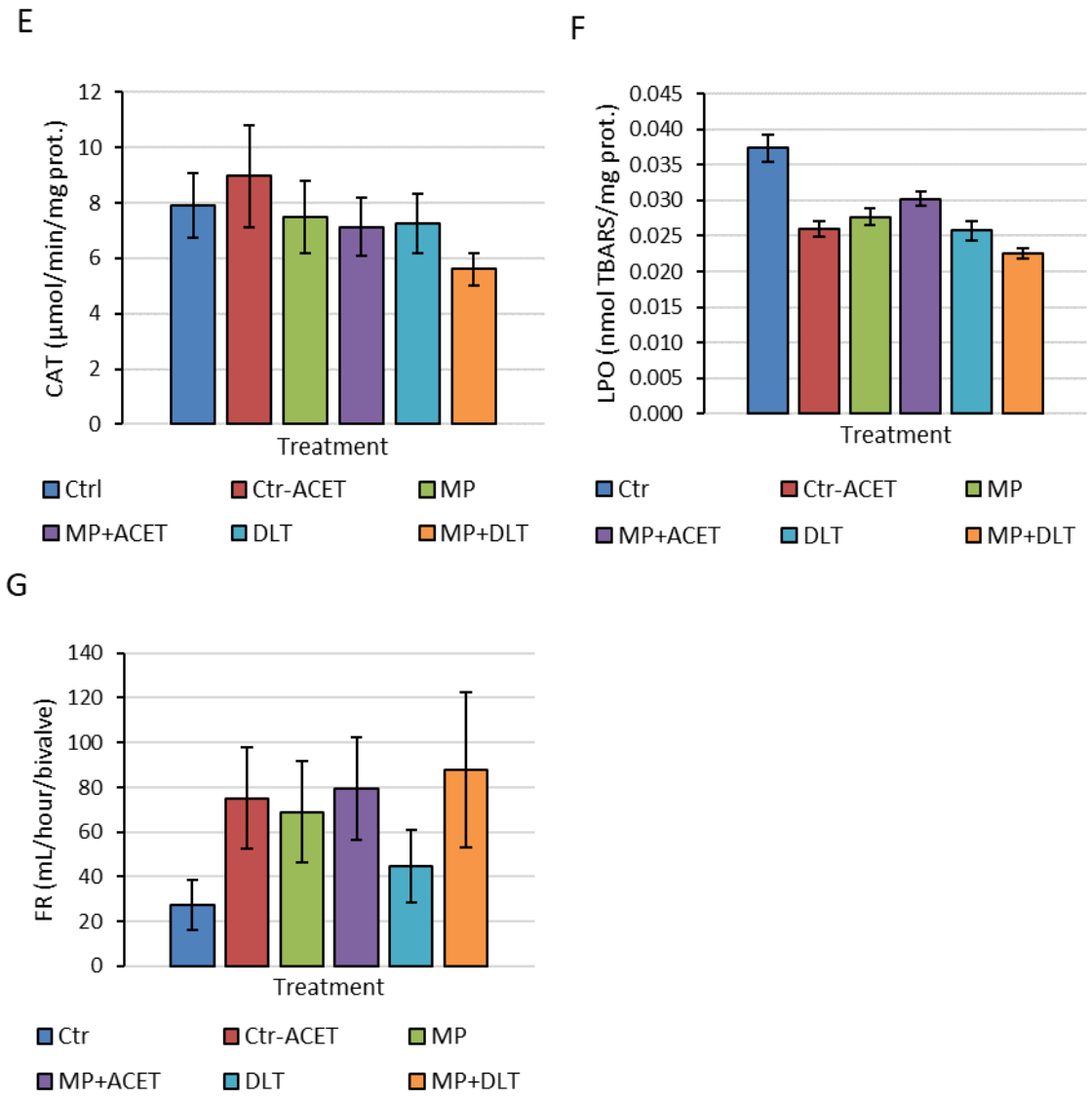


Figure 2 - Biomarkers determined in Corbicula fluminea after exposure to the treatments: Ctr – Control; C-ACET – Acetone control; MP – Microplastics; MP+ACET – Microplastics + acetone; DLT – Deltamethrin; MP + DLT – Microplastics + deltamethrin. Values are the mean and the corresponding standard error. A - Cholinesterase enzymes (ChE) activity; B - Isocitrate dehydrogenase (IDH) activity; C - Octopine dehydrogenase (ODH) activity; D - Glutathione S-transferases (GST) activity; E - Catalase (CAT) activity; F - Lipid peroxidation (LPO) levels; G - Filtration rate (FR). Values are the mean and the corresponding standard error. The significant differences between treatments are identified by different letters above the bars (ANOVA and the Tukey's test or Kruskal-Wallis and the non-parametric multiple comparison test). The significant level is 0,05.

3.3.4 Conclusions

In the assay without bivalves conducted to select the interval of test medium renewal, the presence of DLT and time have a significant effect in the determinations of MP concentrations. Because significant differences were found in MP concentration in the medium at 48 h and the decay of MP in test medium at 48 h was higher than at 24 h, it was chosen to renew the test medium at each 24h.

The results of the bioassay indicate that the 8 days of exposure of *C. fluminea* to MP and DLT alone did not cause significant differences in the biomarkers analysed. Although not significant a decrease in LPO levels was observed concerning the control group. In comparison with the control, a significant increase was shown in ChE and ODH activity, suggesting toxic interactions between MP and DLT in *C. fluminea*

Considering the contamination by MP and pesticides in freshwater ecosystems and the simultaneous exposure of organisms to various contaminants, this can result in toxicological interactions that potentially increase toxic effects on animals. Thus, more research about the interaction of MP with other contaminants is needed to understand the risks to the environment and human health.

Chapter 4. Conclusions and final remarks

In recent years, due to their extensive use, plastics are an environmental pollutant, which has the potential to endanger ecosystems. For these reasons, it is an emerging contaminant that concerns the scientific community.

In chapter 2 of this MSc thesis were reviewed the literature regarding the contamination of freshwater environments and the toxicologic effects of MP alone and in combination with other contaminants in freshwater organisms. This research showed that MP are ubiquitous in the freshwater environments and they can be ingested for a range of diverse species. The exposure to MP can cause adverse effects in organisms and mixtures of MP with other contaminants can potentially increase the impacts. Furthermore, this review identifies some topics that need further investigation, such as the importance of the use of standard methodologies to quantify MP abundance in an environmental matrix; the toxic effects of MP in the freshwater species; the effects of mixtures of MP with other contaminants in freshwater species and the implications of the MP pollution at an ecosystem level and the potential risks for the environment.

In Chapter 3 were carry out a bioassay to investigate the effects of model MP and DLT, alone and in mixture, on *C. flumínea.* In an initial phase of the bioassay, was carry out an assay without animals to investigate possible decrease of MP concentrations in test medium due to interactions of test substances and solvent in test medium. The presence of DLT has a significant in MP concentrations. Also, the test medium at 48 showed a significant effect in MP determinations. Therefore, 24 h was the time chosen for the medium test renewal in the bioassay. In the bioassay, *C. fluminea* was exposed to 0.2 mg/L of MP and 0.2 mg/L of DLT, individually and in mixture. Concerning to the toxic effects on *C. fluminea*, the exposure to the mixture caused a significant increase of ChE and ODH activity, suggesting alterations in cholinergic transmission and increased of the energy needed to biologic processes.

Overall, the main conclusions of this MSc Thesis are: (1) MP pollution is a threat to freshwater environments and (2) mixtures of MP and DLT cause toxic effects to the freshwater bivalve *C. fluminea*.

This MSc Thesis contributed to the knowledge of the effects of MPs and mixtures with other contaminants in the health of freshwater organisms. Because there is still a lack of knowledge in this topic, further investigation is needed to understand the potential risks of MP for the environment, at an ecosystemic level and also for human health.

Chapter 5. References

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Annexes

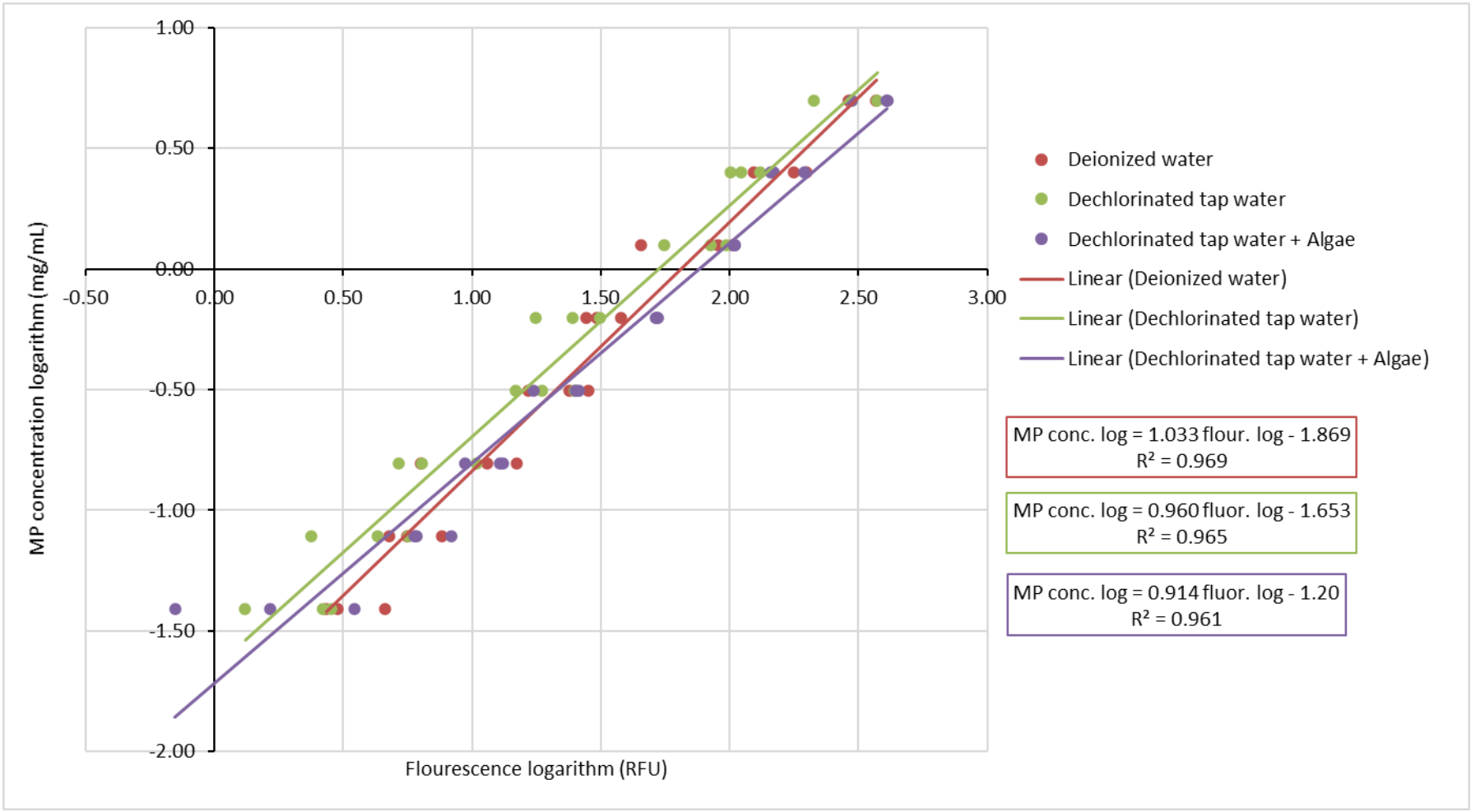

Figure S 1 - Calibration curves of the microplastic solutions performed in deionized water, dechlorinated tap water and dechlorinated tap water and a mixture of microalgae cells: logarithm of concentration of microplastics versus logarithm of fluorescence.