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Toxicity of mixtures of cyanobacteria and microplastics in aquatic organisms

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FACULDADE DE CIÊNCIAS

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Toxicity of mixtures of cyanobacteria and microplastics in aquatic organisms

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Abstract

Over the years, it has been observed an increase contamination of aquatic environments, where the presence of natural toxins and microplastics is increasingly frequent and at higher concentrations in these environments, frequently co-exist.

Organisms such as cyanobacteria and dinoflagellates may produce the toxins. Cyanobacteria are ubiquitous and in certain environmental conditions have an exponential population growth forming blooms. *Microcystis aeruginosa* is very common in freshwater environments and may produce bioactive compounds such as microcystin-LR, a hepatotoxic cyanotoxin. Toxic effects have already been described in many organisms exposed to these cyanobacteria and cyanotoxin. The effects caused by the cyanobacteria are not only due to MC-LR but also to other compounds produced by *M. aeruginosa*.

Microplastics are ubiquitous synthetic particles up to 5mm with a lot of different physic-chemical properties such as size, shape, density, polymer, among others. These particles were already shown to be toxic to many organisms and their effects are variable according to their properties.

The aim of this of this study was to investigate the toxicity of the mixtures of *M. aeruginosa* extracts and microplastics on *Daphnia magna*.

An acute bioassay was carried out with four concentrations of MPs, four concentrations of *M. aeruginosa* extracts and four binary mixtures of both, in which immobilization was used as effect criteria. Then a chronic bioassay of 21 days was carried out with three concentrations of MPS, three concentrations of *M. aeruginosa* extracts and three binary mixtures of both, in which mortality, growth and reproduction parameters were used as effect criteria. OECD guidelines for *D. magna* were followed to perform these bioassays, with the necessary adjustments.

In addition to the results of exposure to contaminants alone that indicate a toxic effect, as already described by other authors, the results evidence some interactions of both contaminants.

Resumo

Ao longo dos anos, tem sido observado um aumento da contaminação dos ambientes aquáticos, onde a presença de toxinas naturais e microplásticos é cada vez mais frequente e em concentrações mais altas, coexistindo com frequência, sendo que estas toxinas podem ser produzidas por cianobactérias e dinoflagelados. As cianobactérias são omnipresentes e, em certas condições ambientais, têm um crescimento populacional exponencial, formando blooms. A *Microcystis aeruginosa* é uma cianobactéria muito comum em ambientes de água doce e pode produzir compostos bioativos como a microcistina-LR, uma cianotoxina hepatotóxica. Estas cianobactérias e cianotoxinas já mostraram ser tóxicas para vários organismos e foi demonstrado que o efeito da cianobactéria não se deve apenas à cianotoxina.

Os microplásticos são partículas sintéticas omnipresentes com dimensões até 5 mm e muitas propriedades físico-químicas diferentes, como tamanho, forma, densidade, polímero, entre outros. Estas partículas já mostraram ser tóxicas para vários organismos e os seus efeitos podem variar consoante as suas propriedades.

O objetivo deste estudo foi investigar a toxicidade das misturas de extratos de *M. aeruginosa* e de microplásticos sobre *Daphnia magna*.

Um bioensaio agudo foi realizado com quatro concentrações de MPs, quatro concentrações de extratos de *M. aeruginosa* e quatro misturas binárias de ambos, nos quais a imobilização foi utilizada como critério de efeito. Em seguida, foi realizado um bioensaio crónico de 21 dias com três concentrações de MPs, três concentrações de extratos de *M. aeruginosa* e três misturas binárias de ambos, nos quais a mortalidade, o crescimento e os parâmetros reprodutivos foram utilizados como critério de efeito. As diretrizes da OCDE para D. magna foram seguidas para realizar esses bioensaios, com os ajustes necessários.

Além dos resultados da exposição aos contaminantes isolados, que indicam um efeito tóxico na *D. magna*, como já descrito por outros autores, os resultados evidenciaram algumas interações entre os contaminantes.

Os resultados obtidos foram ponderados e debatidos em relação à saúde ambiental bem como nas possíveis implicações no bem-estar e saúde humana.

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Abbreviations List

ASTM – American Society for Testing and Materials
BPA – Bisphenol A
HPLC – High Performance Liquid Chromatography
MBL – Marine Biological Laboratory
MEoH – Methanol
MPs – Microplastics
NPs – Nanoplstics
PE – Polystyrene
TFA – Trifluoroacetic acid
u.p. – ultra pure

1. Introduction

The human population growth and the consequent increase in demand of food and other products have led to the development of several industries and agriculture, which naturally have had a severe impact on the environment. This development has also resulted in the increase of pollution and natural toxins in the environment due to eutrophication.

Microcytis aeruginosa is one of the most common types of cyanobacteria present in freshwater ecosystems where bloom formations are most frequent. It produces one of the most common cyanotoxins, namely microcystin-LR. This toxin induces adverse effects on aquatic organisms, such as macrophytes, zooplancton, and fish.

Microplastics (MPs) are persistent emergent pollutants that have the capacity to adsorb other contaminants present in the environment.

1.1. Cyanobacteria

Cyanobacteria are gram-negative bacteria (Lindsay et al., 2006), previously known as blue-green *algae*. They are ubiquitous and have the capacity to produce secondary metabolites. Some of these compounds are bioactive and toxic and known as cyanotoxins (Carmichael, 1992; Dao et al., 2010), which were reported for the first time around 120 years ago (Pflugmacher et al., 1998). According to their predominant biological target, cyanotoxins can be classified as hepatotoxins, neurotoxins, endotoxins, cytotoxins, dermatoxins, or irritant toxins (Lindsay et al., 2006) with hepatotoxins and neurotoxins being most frequently found in aquatic ecosystems.

The changes in biotic and abiotic parameters of the aquatic environments, such as input of nutrients (mainly of phosphorus and nitrogen), intensity of light, temperature, increasing carbon dioxide (CO_2) and climate change, among others, cause eutrophication of these environments leading to cyanobacteria bloom formation (Freitas et al., 2014; Ortiz-Rodríguez et al., 2012; Ortiz-Rodríguez and Wiegand, 2010; Pflugmacher et al., 1998). Although cyanobacteria exist in marine and freshwater environments, blooms are more frequent in freshwater environments, especially in lakes and water reservoirs. Blooms induce some abiotic changes degrading water quality (Anna et al., 2014) and when senescence of the bloom and cell lysis occurs, toxins are released in the water (Dao et al., 2010). Therefore, blooms are important shapers of aquatic communities and overpower them, frequently (Ortiz-Rodríguez and Wiegand, 2010).

Cyanobacteria and cyanotoxins of different types are commonly found co-existing in the environment (Freitas et al., 2014) and are hazardous and stressful to ecosystems health (Pflugmacher et al., 1998). They can induce death of the co-existing organisms due to the lack of oxygen that is used for respiration, interfering or inhibiting feeding, affecting growth, reproduction and survival, among others, negatively affecting zooplankton (Sadler and von Elert, 2014), other species, whether they are wild or domestic organisms, and humans. Blooms are an ecological hazard (Yang et al., 2012) representing a risk to human and environmental health (Ortiz-Rodríguez and Wiegand, 2010), for example blooms can destroy ecological balance by heavily decreasing zooplankton communities. The frequency and intensity of blooms has been increasing (Freitas et al., 2014) and is expected to keep this trend (Yang et al., 2012).

There are some strategies to attempt to remove or reduce cyanobacteria cells in the environment such as coagulation, flocculation, sedimentation, filtration, clarification, flotation, algaecides, ozone, photolysis and ultrasounds. However, some are not completely efficient and do not totally supress the proliferation or remove the toxin. Their efficiency is probably decreased during blooms and some of them may cause severe side effects.

As mentioned above, hepatotoxins are the most common type of cyanotoxins, of which microcystins are the most produced (Lindsay et al., 2006) and reported (Pflugmacher et al., 1998) either in freshwater or marine environments (Ortiz-Rodríguez et al., 2012). *M. aeruginosa* is one of the most common cyanobacteria species and can be described as dominant among many cyanobacteriain blooms. It produces microcystins, but this type of toxin is not the only toxic compound produced by *M.aeruginosa*.

Microcystins are hepatotoxins (Carmichael, 1992), the most common, hazardous, prevalent and studied type to be found in freshwater (Ortiz-Rodríguez and Wiegand, 2010). Produced by species of *Microcystis*, *Nostoc*, *Anabaena*, *Planktothrix*, *Oscilatoria* and *Aphanizonemon*, among others, these toxins are cyclic heptapeptides (Pflugmacher et al., 1998) with two amino acids which vary from the general structure, originating more than 250 variants already described in literature.

The cell lysis, or senescence, and decay of blooms release microcystins into the aquatic environment (Anna et al., 2014; Pflugmacher et al., 1998). Once in the environment, they are bioavailable to be taken up by organisms in these ecosystems. Although biodilution is seen in first consumers (Sadler and von Elert,

2014) and no biomagnification through the food chain to higher trophic levels has been observed, ingested toxins are actively absorbed in the digestive tract and some researchers describe signs of bioaccumulation (Liu et al., 2016; Martins and Vasconcelos, 2009).

Microcystins effects have been studied worldwide having as one major effect, inhibition of phosphatase proteins type pp1 and pp2A (MacKintosh et al., 1990) leading to cell damage and oxidative stress, alterations on liver enzymes, promotion of tumours, intrahepatic bleeding, apoptosis or necrosis and, as a result, lethal hepatoptosis and death (Pflugmacher et al., 1998). The risks associated with the exposure to these toxins are not only to aquatic organisms, such as macrophytes, zooplankton and fish, (Rojo et al., 2013) but to mammals, birds and even humans as well, constituting a cause of concern not only economically but also in terms of environmental and public health. Intoxications leading to death were described for the first time in the 19th century affecting cattle (Francis, 1878). Human lethal intoxications were also reported in the case of Caruaru, Brazil, where 60 patients of dialysis treated with contaminated water died (Pouira, 1998).

Microcystin-LR (MC-LR) is the most common, potent, toxic and studied microcystin and presents a low hydrophobicity (Sadler and von Elert, 2014). Beyond the general effects of microcystins, like pp1 and pp2 inhibition and oxidative stress (Martins and Vasconcelos, 2009; Schmidt et al., 2014) microcystin-LR causes alterations in the energy metabolism by binding to ATPases, feeding inhibition, decrease of growth, disruption of regulation systems and mortality, depending on the concentration and the organism (Liu et al., 2016; Martins and Vasconcelos, 2009; Schmidt et al., 2014)). The hazard associated to these toxins demands a frequent monitoring of their concentration in the water. The measured concentration values of microcystins dissolved in aquatic environments are normally under 10 μg/L. Nevertheless, during cell lyses of a large-scale bloom, concentrations can reach much higher values (Yang et al., 2012). (Carmichael, 1992) describes the occurrence of microcystins in 80% of samples of which 4% were above the limit and two of those were in treated drinking water between 1996 and 1998. Sivonen and Jones, 1999 reported a concentration of 200 μg/L and the highest values described are >1800μg/L during the decay of big blooms. The intensity of the effects on organisms varies depending on what they are exposed to, being more toxic when exposed to extracts with cells than only extracts or the pure toxin (Freitas et al., 2014).

Researchers have attempted to find a 100 % efficient way to fight bloom formations and diminish or remove the toxins of the environment. As mentioned above, most of physical strategies remove cyanobacteria cells but are not able to remove cyanotoxins dissolved in water – sonication in higher frequencies and longer time is the most efficient strategy. Sonication destroys cells vacuoles and leads to radical oxidation by oxidising the toxin. At low frequencies it is able to damage cells decreasing proliferation and growth, but at high frequencies it inactivates these processes. The concentration of toxin dissolved increase with the frequencies used (Park et al., 2017a); however increasing time of sonication from 10 to 30 minutes slightly decreased the concentrations of dissolved toxin. This process is the one that uses less manipulation, considered effective and environmentally relevant but not sufficient considering the ecological risk of the toxin (MC-LR) released through the process (Park et al., 2017a).

Chemical strategies are the most adequate to remove the toxins and between the more effective there are advanced oxidation processes (AOPs) such as UV/H_2O_2, UV/O_3, TiO_2 photocatalysis and Fenton process. Fenton process was shown to have a good cost-efficient relation, be fast and 100% effective under lab conditions, as well as, environmentally sound since, during the process, hydrogen peroxide (H_2O_2) turns into water (H_2O) and oxygen (O_2). When tested in real aquatic ecosystems, the efficiency decreased due to the abiotic parameters and the competition for the compounds (Park et al., 2017b).

There aren't many biological strategies described in the literature, only an enzymatic pathway and grazers. By the enzymatic pathway there are three enzymes, extracted from bacteria, with the capacity to degradate microcystin-LR. Relatively to grazers, these could be seen as a biological strategy but they are not very effective, once grazers are capable of reducing the toxin in the medium or decrease *M.aeruginosa* ingested and are not acutely toxic for *D. magna*, however they induce the same effect as starvation in these cladocerans (Zhang et al., 2009). These three types of strategies were shown to be not totally effective separately nevertheless, the combination of the strategies appears to be promising.

1.2. Microplastics

Plastics are used intensively in our modern life (Jemec et al., 2016), including in water and food containers, clothes, furniture and diverse technologies, among several other uses. The use of plastic has had a huge increase in the last decades (Canniff and Hoang, 2018) from 1,7 million tons produced in 1950 to 288, 299, 311, 322 and 335 million tons in 2012, 2013, 2014, 2015 and 2016, respectively (Plastics Europe, 2016; Plastics Europe, 2017). The most demanded types of plastics are polystyrene (PE), polyethylene, polypropylene, polyvinyl chloride, polyurethane, polypropylene and polyethylene terephthalate. Among these, PE is not only the most produced (Mattsson et al., 2015) but also one of the most abundant in the environment.

The plastics present in aquatic environments may have different origins and pathways such as direct disposal, through wind, water treatment plants, accidental disposal from factories near the rivers and via other aquatic systems, namely rivers, a big source of marine ecosystems pollution . Although the total amount of plastic litter present in aquatic environments remains unknown. Some estimates indicate that in 2010 4,8 to 12,7 million tons entered into aquatic environments , and that plastic litter represents about 60 to 80% of the total aquatic litter.

MPs are plastic particles with size lower than 5 mm (Imhof et al., 2017). They have variable physical and chemical characteristics such as size, shape, type of polymer, coating agents and functional groups, largely influencing the MPs tendency to adsorb or release other contaminants (Rehse et al., 2016). MPs are generally divided in primary and secondary. Primary MPs are particles produced to have a size in the micro or nano range that enters into in the environment already as MPs (Jemec et al., 2016). They are used in the cosmetics and personal care products and in several other industries. Secondary MPs result from the breakdown and transformation of larger plastics in the environment (Jemec et al., 2016) that occur mainly through photo-oxidation caused by UV radiation, mechanical abrasion, biological degradation, disintegration (Mattsson et al., 2015) and temperature changes.

MPs are being increasingly found in marine and freshwater environments (Jemec et al., 2016; Scherer et al., 2017). The most part of them have irregular and different shapes (Frydkjær et al., 2017) as the result of their environmental degradation along hundreds of years. The distribution of these particles, in marine environments, is heterogeneous (Cole et al., 2011) and their fate is poorly

understood. In the marine environment, MPs accumulate mainly in ocean gyres and coastal zones (Cole et al., 2011), including estuarine zones and shorelines. In freshwater ecosystems they have been found in rivers, lakes, streams and other systems even remote ones. The distribution of MPs in the water column and sediments is influenced by their characteristics like density and size.

The abundance and concentration of MPs that have been found in freshwater ecosystems is very variable. For example, studies in the literature report hundreds to thousands particles per square kilometre, and concentrations from 0.00012 particles/L, up to 0.32 particles/L in the water. In sediments of freshwater ecosystems, they were found from 1.2 particles/kg up to 3763 particles/kg (Horton et al., 2017).

MPs may be ingested by a large number of marine and freshwater organisms (Rist et al., 2017), depending on many factors of these particles and of the organisms, such as size of MPs, type of feeding, among others. After ingestion or uptake by other ways, MPs, can enter into the circulatory system (Browne et al., 2008; Guilhermino et al., 2018) and reach several organs including the brain (Kashiwada, 2006). The biological effects of MPs depend of several factors such as the type, size and other properties of the particles, exposure conditions (concentration, time, other conditions), organism size and sensitivity, (Canniff and Hoang, 2018). MPs were shown to decrease the feeding rate and food ingestion of several species, which in long-term could lead to starvation and death. Moreover, MPs have been found to cause metabolic alterations, decreased growth and weight, depletion of energy reserves, decreased fertility, oxidative stress and damage, neurotoxicity, inflammation, behaviour alterations, teratogenicity, and death caused as consequence of effects other than starvation, among others. Furthermore, MPs can be transferred from one trophic level to the upper one.

MPs present in the environment generally contain other chemicals of high concern due to their toxicity that were incorporated in the MPs during their manufacture, use and during their permanence in the environment. Thus, the effects induced by particles of the same type may change and be considerably different (Pacheco et al., 2018) according the other chemicals that they contain. The effects of MPs may also change due to the desorption of some components and additives of MPs, among other processes. Thus, MPs may introduce or increase the concentrations of such chemicals in aquatic systems and food and food webs. Moreover, in case of simultaneous exposure to MPs and other

contaminants, toxicological interactions inside exposed organisms may occur (Ma et al., 2016; Pacheco et al., 2018).

MPs were already found in several marine and freshwater organisms of different trophic levels, such as shrimps, molluscs and fish in general, some of them consumed by humans. Thus, in addition to the environmental concerns, a high preoccupation regarding human food safety. In addition to aquatic organisms consumed as food by humans, MPs were also found in commercial salt, honey and other products consumed by humans (Karami et al., 2017). Furthermore, humans may be exposed to MPs by other routes, including air, contaminated water (Karbalaei et al., 2018), and vegetables.

Thus, it is important to increase the safety of use and management of MPs mainly because of: (i) their increasing production and presence in the environment, (ii) the biological and ecological risks associated to MPs exposure, and (iii) the technological difficulties in relation to their environmental monitoring and environmental and human risk assessment. Measures have been taken to reduce and remove this emerging contaminant from aquatic ecosystems and to find more effective ways of doing so. For example, there is a substantial number of actions to collect plastics from beaches and remove plastics from waters and (Paço et al., 2017) found a marine fungus that was capable of degrading plastics from environmental waters. However, considering the global scale of the MPs paradigm, these efforts are still not enough and there is a lot of work to be done.

1.3. *Daphnia magna*

Daphnia magna is a freshwater planktonic crustacean, of the Cladocera order, commonly known as the water flea. These organisms have a body length up to 5 mm and a transparent chitinous carapace (Freitas et al., 2014). *D. magna* reproduces by cyclic parthenogenesis and sexual reproduction, in adverse conditions. *D. magna* is often considered a non-selective filter feeding, filtering particles of different sizes up to some mm, however some authors defend that their filtering presents some selectivity.

D. magna is widely distributed (Ortiz-Rodríguez et al., 2012) and has an important ecological value transferring energy and nutrients from the primary producers (phytoplankton) to higher consumer levels in the freshwater food chains (Liu et al., 2011; Ortiz-Rodríguez and Wiegand, 2010). This species feeds mainly on suspended particles regulating algae and bacteria population growth.

D. magna is commonly used in ecotoxicological bioassays due to several favourable characteristics for use as test organisms, such as high sensitivity to several pollutants, relative rapid life cycle, possibility of maintaining the reproduction by parthenogenesis continuously, high reproductive output, and size allowing the observation of several effect criteria by naked eye.

1.4. Effects of microcystin on *D. magna*

D. magna has different responses when exposed to purified MC-LR, extracts, *M.aeruginosa* cells, samples from different blooms and MC-LR mixed with other compounds, and the main effects were concentration and time dependency. Acute exposure to MC-LR causes alterations in biotransformation, antioxidant and energetic parameters, however no mortality is observed (Ortiz-Rodríguez and Wiegand, 2010).

Chronic exposure to the purified toxin causes alteration in biotransformation leading to bioaccumulation of the toxin, in antioxidant and energetic parameters, alterations in cells, at low concentrations it does not affect reproduction and mortality increasing with the concentration (Chen et al., 2005). Exposure to extracts or *M.aeruginosa* also leads to alterations in growth, reproduction parameters and survival time (Tarczynska et al., 2001). In transgenerational studies, neonates from mothers exposed for more days were able to have better response when exposed to the toxin than neonates from mothers only exposed for one day (Ortiz-Rodríguez et al., 2012).

When *D. magna* is exposed to MC-LR alone and to mixtures of MC-LR and other compounds from the same cyanobacteria, alterations in the organisms are not due only to the toxin (Wiegand et al., 2002). MC-LR and other cyanotoxin extracts showed no alteration on the effects, only a slight change in food ingestion (Park et al., 2017b), MC-LR and other compounds found in the water during bloom decay, such as ammonia (Yang et al., 2012; Zhu et al., 2015), have some synergistic and antagonist alterations in reproduction parameters and alter neonates survival and reproduction parameters in higher concentrations exposure, and as polyunsaturated fat acids (Reinikainen et al., 2001) alter survival rate.

1.5. Effects of microplastics on *Daphnia magna*

D. magna is able to ingest MPs of different types and sizes (Kokalj et al., 2018; Rist et al., 2017). MPs longer than 1.4 mm were found in their gut after laboratorial exposure (Jemec et al., 2016). It is also able to some or total egestion of MPs (Frydkjær et al., 2017; Rist et al., 2017). MPs were also found in D. *magna* brood chamber, eggs and embryos.

MPs show different effects in *D. magna* considering size, type, shape, mixtures, concentration, environmental stressors, duration of exposure, among others.

In this species, acute exposure to some MPs caused immobilization (Frydkjær et al., 2017; Rehse et al., 2016) and mortality (Jemec et al., 2016), whereas comparable exposure to other MPs did not (Rehse et al., 2018). Chronic studies with some types of MPs reported accumulation of the particles, alterations of food intake, and, decrease of growth (Besseling et al., 2014), decrease of the reproductive success (Besseling et al., 2014; Martins and Guilhermino, 2018; Pacheco et al., 2018), among other effects. However, chronic studies with other MPs did not found significant effects on reproductive parameters, neither mortality (Canniff and Hoang, 2018; Rist et al., 2017). Furthermore, transgenerational exposure of *D. magna* populations to MPs for two generations lead to population extinction, and exposure for one generation caused decrease of the population growth rate that was not reverted after three generations in medium without MPs (Martins and Guilhermino, 2018).

The combined effects of MPs and other contaminants in *D. magna* were also investigated. MPs were found to modulate the toxicity of some other contaminants to *D. magna* and toxicological interactions were found in some cases. For example, in a chronic mixture bioassay, *D. magna* was exposed to gold nanoparticles, MPs and their mixtures; mixtures were found to be more toxic than MPs and gold nanoparticles individually and toxicological interactions were found, namely antagonism at low concentrations of mixture components, and synergism at high ones (Pacheco et al., 2018). In another study, in a chronic mixture bioassay, *D. magna* was exposed to nickel, MPs with different functional groups and their mixtures, results showed that functional group lead to different level of alterations of the effects caused by nickel alone (Kim et al., 2017). In another study, *D. magna* was chronically exposed in a mixture bioassay to bisphenol A (BPA), MPs and their mixtures; mixtures were found to be more toxic than MPs alone but less toxic than BPA alone (Rehse et al., 2018). In another

study, in a 14 days mixture bioassay, *D. magna* was exposed to phenanthrene, nanoplastics (NPs), MPs and mixtures of phenanthrene with NPs and with MPs; mixtures showed that NPs increased bioaccumulation of phenanthrene but MPs did not alter bioaccumulation of the contaminant (Ma et al., 2016).

Overall, the results published in the literature showed that the effects of MPs on D. magna depend of the properties of the tested particles, organism developmental stage, and environmental conditions. Therefore, considering the high diversity of MPs present in the environment a considerable amount of work still needs to be done, especially regarding long-term exposure. Moreover, the works on the combined effects of MPs and other contaminants highlight the need of more research. A particularly important gap of knowledge is on the effects resulting from the simultaneous exposure to MPs and cyanobacteria toxins.

1.6. Objectives and outline of the Master Thesis

The central objective of this study was to investigate the effects of the simultaneous exposure to MPs and *M. aeruginosa* extracts on *D. magna*. The hypothesis tested was: exposure to mixtures of microplastics and *M. aeruginosa* extracts alters the effects of exposure to these contaminants alone.

The MSc Thesis is divided in six sections: Introduction, Material and Methods, Results, Discussion, Conclusions and Future Perspectives, and References. In the Introduction, the environmental contamination by *M. aeruginosa* and MPs and its implications to environmental and human health, and the organism tested D. magna, are briefly introduced. In the Material and Methods section, the procedures used are described. In the results section, the results are described presented through tables and figures. In Discussion section, results are compared to the ones already described in the exiting literature. In the Conclusion and Future Perspectives section, the most important results and the next steps of this field of study considered important are briefly described. In References section, all the literature read and used is presented.

2. Material and Methods

2.1. Chemicals

Red fluorescent plastic microspheres, polymer of unknown composition, were purchased from Cospheric-Innovation in Microtechnology (USA) (lot number 4-1006-1053). According to the information provided by the supplier, the particles have a diameter of 1 to 5 μm (with a mean diameter of ~ 2 μm), 1 mg of the product corresponds approximately to 1.84 $x10^8$ particles (based on an average diameter of 2 μm), the particles have 1.3 g/cm^3 density, 575nm and 607 nm excitation and emission wavelengths, respectively. These particular MPs were selected because their toxicity to *D. magna*, and their characterization in solutions of hard water of the American Society for Testing and Materials (ASTM, 1980), hereafter indicated as ASTM, were previously investigated (Martins and Guilhermino, 2018; Pacheco et al., 2018), providing baseline knowledge for the present study.

Chemicals (analytical grade) used for *Daphnia magna* and *Chlorella vulgaris* media preparation were all purchased from Sigma-Aldrich (USA) or Merck (Germany). Chemicals used for the extraction and quantification of MC-LR in *M. aeruginosa* extracts were purchased from Sigma-Aldrich (USA) or Merck (Germany).

2.2. Standardization of the procedures to measure the actual concentrations and behaviour of microplastics in ASTM hard water

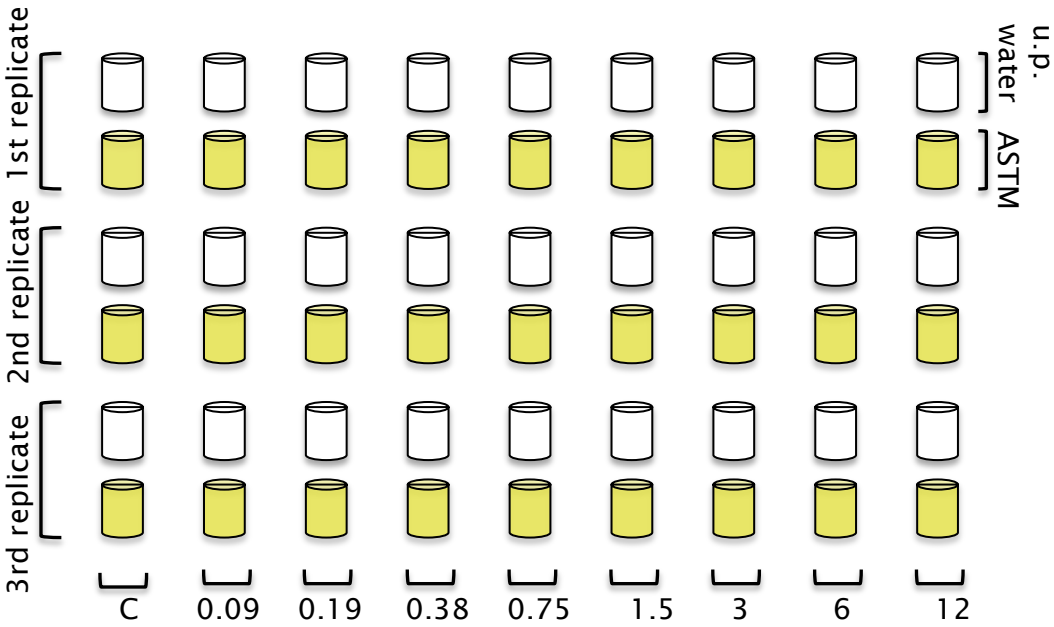

Figure 7: Microplastics (MP) calibration curve and behaviour assay design - 9 treatments (control and 8 concentrations of MPs 0.09; 0.19; 0.38; 0.75; 1,5; 3; 6 and 12 mg/L, nominal concentrations) with three replicates per treatment and per test media (ultra-pure water and ASTM).

To standardise the procedures for the assay, especially regarding the determination of the actual concentrations of MPs in test medium and their potential decrease during the exposure periods (hereafter indicated as MPs decay), a preliminary assay was carried out for 48 h following the general procedure of (Luís et al., 2015) with adaptations regarding the properties of the MPs used and ASTM (Pacheco et al., 2018). Two aqueous mediums were compared for validation purposes: ultra-pure (u.p.) water and ASTM.

For each medium, treatments were: a control (u.p. water or ASTM) and eight MPs colloidal solutions were prepared in u.p. water or ASTM. MPs solutions were prepared through serial dilutions 1:2 (v/v) ranging from 12 to 0.19875 mg/L, nominal concentrations (Figure 1). Three independent replicates per treatment and test medium were used. Immediately after preparation, the fluorescence of each solution was read (JASCO, FP-6200 spectrofluorometer, Netherlands), using 575 and 607 nm as excitation and emission wavelengths, respectively (0 h readings). All the treatments were maintained in a PGC1400 Bronson Climate (Bronson Incubator Services B.V., The Netherlands) at 20 ± 1ºC,

40% humidity and 16 : 8 h light : dark photoperiod. The fluorescence of all treatments was measured again after 24 h and 48 h, with previous resuspension of MPs by a vortex.

The fluorescence values obtained at 0 h were used to obtain a calibration curve of fluorescence *versus* nominal concentrations of MPs. The actual concentrations of MPs in test media of the bioassays were posteriorly calculated based on the linear regression model adjusted to the calibration curve. Data was also used to select the medium renewal interval during the bioassays.

2.3. *Chlorella vulgaris* cultures

Chlorella vulgaris, cultured for more than 20 years in the Laboratory of Ecotoxicology of ICBAS (ECOTOX) and by the team of Ecotoxicology, Stress Ecology and Environmental Health (ECOTOX) of CIIMAR, was used as food for *D. magna*. *C. vulgaris* cultures were maintained in glass beakers of 5 L filled with 4 L of culture, generally as indicated in Guilhermino *et al.* (1999). Briefly, all the glass material used was previously decontaminated with nitric acid at 20 % and rinsed with distilled water. Beakers were filled with 4 L of Marine Biological Laboratory medium (MBL) (Stein, 1973) autoclaved (AJC, Uniclave 88, Portugal) at 121 ºC for 60 minutes. After cooling, in a sterile environment with the help of Bunsen burner, 0,5 mL of a solution of vitamins and 10 mL of *C. vulgaris* inoculum (up to 50 mL sample, collected at the seventh day, during the exponential stage, stored at 4 ºC and used to start a new culture) were added. At the 7th day of growth, when the culture reached an exponential phase, inoculums and half of the culture were collected. The beaker was refilled with the same volume of sterile medium and the process of collection of algae was repeated every 2 days.

After collection, the microalgae samples used to prepare *D. magna* feeding solution were centrifuged at 3500 rpm for 7 minutes (KUBOTA 5400, Japan). The supernatant was eliminated and the resulting pellet was mixed with ASTM enriched with a vitamins solution (Bradley et al., 1993) and used as culture medium for *D. magna*. The obtained solution was analysed by spectrophotometry (Jenway, 6405 UV/Vis spectrophotometer, UK) at 440 nm after 1:10 dilution (0.4 mL *algae* and 3.6 mL ASTM with vitamins). The volume of solution used to feed the organisms (containing an adequate number of cells) was calculated through linear regression model adjusted to a calibration curve of absorbance *versus C. vulgaris* cell concentration, previously calculated and available in the laboratory.

The feeding solutions were stored at 4 ºC for a maximum of three days (fresh food) or frozen at -20 ºC for a maximum of three months. *D. magna* was preferably fed with fresh feeding solution and fed with frozen feeding solution only when there was no fresh feeding solution available.

2.4. *D. magna* cultures

D. magna was chosen for this study because these are freshwater organisms with a central role in ecosystems, non-selective filterers and so are exposed to both contaminants via feeding, which makes them good test organisms.

D. magna (clone A, *sensus* Baird et al., 1989) used in this work was from cultures maintained in the ECOTOX for more than 20 years. They were maintained as indicated in continuous parthenogenic reproduction in a constant climate chamber (BINDER, KBF-S 720, Germany) at 20±1 ºC, 40 % humidity and 16 h : 8 h light : dark photoperiod. Briefly, female *D. magna* were maintained individually in 200 mL beakers filled with 100 mL of ASTM supplemented with vitamins B1, B12 and H (Bradley et al., 1993) and 4 mL of *Ascophyllum nodosum* extract (organic supplement) (Baird et al., 1989) and fed with *C. vulgaris* 5 days a week (algae density: $3x10^5$ cells/mL/daphnid corresponding to 0,322 mg of carbon/daphnid/day, (Guilhermino et al., 1999). The culture medium was renewed three days a week and the cultures were always started with daughters from the 3rd to 5th broods picked randomly from parents that were healthy (Guilhermino et al., 1999). To check the health *status* of *D. magna* cultures and their suitability to be used as test organisms in toxicity bioassays, a toxicity test with the reference substance potassium dichromate as performed as recommended by the OECD Guideline 202 (OECD, 2004).

2.5. Potassium dichromate test

According to the OECD guideline 202 (OECD, 2004), is convenient to carry out an acute bioassay with a reference substance (e.g. potassium dichromate) before toxicity testing with *D. magna.*

A stock solution of potassium dichromate in ASTM without vitamins was prepared. From this solution, a first solution with a concentration of 8 mg/L of potassium dichromate was prepared, of which 5 additional solutions with potassium dichromate concentrations of 4, 2, 1, 0.5 and 0.25 mg/L) were prepared by successive 1:2 (v/v) dilutions in ASTM without vitamins. A control

(ASTM without vitamins) was also included in the experimental design. The design of this test included 28 glass beakers of 100 mL filled with 50 mL of test solution, four beakers per treatment, with twenty *D. magna* per concentration (five per beaker). Beakers were maintained in a PGC1400 Bronson Climate (Bronson Incubator Services B.V., The Netherland) at 20±1 ºC, 40 % humidity and 16 : 8 h light : dark photoperiod. *D. magna* used were juvenile females (3rd brood) with more than 6 h and less than 24 h of age. The exposure period was 24 h, no food was provided to organisms, and the test was carried out in static conditions. The physicochemical parameters measured were water temperature, pH, dissolved oxygen and conductivity, measured at 0 h and 24 h (HACH HQ40d multi, U.S.A., and multi-parameter WTW Multi 340i/SET, Germany).

2.6. *M. aeruginosa* culture

The strain of *M. aeruginosa* used was isolated in 1991 from Mira lake, Portugal by Dr. Vítor Vasconcelos. Since then it has been maintained in laboratory conditions in the CIIMAR cyanobacteria collection. *M. aeruginosa* was initially cultured in a culture flask filled with 100mL of autoclaved (Tuttnauer, 3870ELV, The Netherlands) Z8 medium. After reaching the end of the exponential growth phase it was amplified to a beaker filled with 800mL of medium, then to a NALGENE filled with 8 L and finally to a NALGENE filled with 20 L of medium. The medium and the material used to grow the culture was previously autoclaved (Tuttnauer, 3870ELV, The Netherlands) at 121 ºC for 20 minutes. To avoid contaminations, all amplification occurred in a flow chamber (Telstar, Bio II Advance) and samples were collected regularly to ensure the viability of the culture. The cultures were maintained in a room at 25.0 ºC, a photoperiod of 16 h : 8 h light : dark and in aerated medium when in NALGENEs.

2.7. Cyanobacteria culture harvest

At the end of the exponential phase, the cultures were harvested and centrifuged (Thermo SCIENTIFIC, Sorvall BIOS 16 Bioprocessing Centrifuge, Germany) for 15 min at 4700 rpm and 4 ºC. The supernatant was eliminated and with the help of some deionized water the pellet was collected to 100 mL containers and stored at -80 ºC. The pellets were further lyophilized at -80 ºC in a freeze-dryer (Telstar, LyoQuest) until it was completely dry. The resultant biomass was weighted and stored.

2.8. Improvement of extraction method

Different methods of extraction were used to compare efficiency and differences between extractions with different solvents. Samples of 50 mg of freeze-dried cells (biomass) were dissolved in 10 mL of solvent and ultrasonicated (Vibro Cell, V50, USA) an ice-water bath at 60 Hertz (Hz).

The first extraction was prepared with different solvents with the same extraction method to test differences between solvents. Two samples were prepared with methanol 50% (MeOH 50 %) (sample 1) and other two with u.p. water (sample 2). The second extraction was performed to compare efficiency of extraction using the same solvent and different sonication and different centrifugation times. Two samples with the same extraction method used in the first time (sample 2), one sample with different centrifugation time (sample 3) and other sample with different sonication time (sample 4).

Samples 1, 2 and 3 were ultrasonicated in 5 cycles of 1 minute each, with 1-minute breaks between cycles and sample 4 was ultrasonicated for 3 cycles of 10 minutes with 10 minutes breaks. Samples 1, 2 and 4 were centrifugated 1 min at 10000 rpm and 4 ºC and sample 3 was centrifugated 20 minutes at 4000 rpm and 4 ºC. The specifications of each test are summarized in Table 1.

Table 2: Different methods of extraction performed

<table>
<tr><th></th><th>Method</th><th>Solvent</th><th>Ultrasonication</th><th>Centrifugation</th></tr>
<tr><td rowspan="4">First extraction</td><td>1</td><td rowspan="2">MeOH 50%</td><td rowspan="6">5 x 1 min</td><td rowspan="4">10000 rpm
1 min</td></tr>
<tr><td>1</td></tr>
<tr><td>2</td><td rowspan="5">u.p. water</td></tr>
<tr><td>2</td></tr>
<tr><td rowspan="3">Second extraction</td><td>2</td></tr>
<tr><td>2</td></tr>
<tr><td>3</td><td>4000 rpm
20 min</td></tr>
<tr><td>4</td><td>3 x 10 min</td><td>10000 rpm
1 min</td></tr>
</table>

After this process, the supernatant was transferred to new beakers, 5 mL of the same solvent was added to the pellet and left to rest. After 24 hours the process of extraction was repeated. In the end, the supernatant was collected and the pellet was discarded. The samples were frozen and then lyophilized at -80 ºC.

The obtained extract was resuspended in 2 mL of 50 % of MeOH with 0.1% TFA and 50 % of u.p. water with 0.1 % TFAand ultra-sonicated for 1 minute. Finally, it was centrifuged at 4 ºC and 10000 rpm for 1 minute and the supernatant obtained (extract) was further quantified.

2.9. Extract preparation

As referred in the Introduction, section 1.1. extracts of cyanobacteria were shown to be more toxic than pure toxin, so, and to make a more environmentally relevant study, it was decided to use *M. aeruginosa* extracts instead of pure MC-LR.

The *M.aeruginosa* extract was prepared according to (Ramanan et al., 2000) with some modifications. A sample of 10 g was taken from the homogenized freeze-dried cells and divided in two samples of 5g to get higher disruption efficiency and the maximum concentration of MC-LR possible. A volume of 150 mL of u.p. water was added to each sample. Ultrasonication in five cycles of 1minute with 1-minute breaks was then conducted in an ice bath at 60 Hertz (Hz). The solutions obtained were centrifuged at 10000 rpm for 1 minute. The supernatant was collected in a glass flask and some u.p. water was added to the pellet. The process was repeated every 24 h for six days. After cell disruption and centrifugation, some of the water was dried in rotary evaporator to concentrate the sample. The solution obtained was too concentrated and so, three samples were taken and diluted 10, 20 and 100 times, while the rest of the solution was frozen at -20 ºC to avoid degradation. The toxin of these samples was then quantified by high precision liquid chromatography (HPLC).

2.10. Extract quantification

Two solutions were prepared, (1) u.p. water with 0.1% of trifluoracetic acid (TFA) and (2) MeOH with 0.1% TFA and then filtered by vacuum (GH polypro 47 mm, 0.2 μm filters) and degassed for 5 minutes in an ultrasound bath. These solutions make up the mobile phase.

The samples were quantified by high precision liquid chromatography with diode array detectors (HPLC-PDA or HPLC-DAD) (alliance, Waters e2695) with a

Merck Lichrospher 100 RP-18 endcapped column (5 μm, 250 x 4 mm) at 45ºC. The PAD range was 210 to 4000 nm and it was fixed at 238 nm. HPLC run the number of samples plus a blank and two different standards (one of pure MC-LR and one from an environmental sample). Each sample was injected in duplicate, 20 μL each time, and the runs were of 20 minutes with a 0.9 mL/min flow with the gradient of solutions as shown in Table 2. The Empower 2 Chromatography Data Software was used for calculations and reporting peak information.

Table 3: Gradient program of the mobile phase

Time (min)	MeOH + 0.1% TFA	u.p. water + 0.1% TFA
0	55	45
5	65	35
10	80	20
15	100	0
15.1	55	45
20	55	45

2.11. Acute toxicity of microplastics, *M. aeruginosa* extract and their mixtures to *D. magna*

To investigate the acute toxicity of MPs, toxic *M. aeruginosa* extract and their mixtures to *D. magna*, a 48 h bioassay was carried out. Generally, it followed the OECD guideline nº 202 for acute toxicity testing with *D. magna* (OECD, 2004) with adaptations to a mixture design and other punctual changes as further described.

The acute bioassay was carried out in a chamber (PGC1400 Bronson Climate, Bronson Incubator Services B.V., The Netherlands), at 20 ºC and a photoperiod of 16 h light : 8 h dark. It was carried out in semi-static conditions with no food. Test medium was ASTM (without vitamins or organic supplement) and it was renewed at each 24 h.

D. magna juvenile females (> 6 h and < 24 h old, 5th brood) were exposed in 100 mL glass beakers, filled with 50 mL of test solution. The experimental design (Figure 2) included 13 treatments: control (ASTM), four concentrations of MPs (0.2, 1, 5 and 25 mg/L, nominal concentrations), four concentrations of *M. aeruginosa* extract (0.250, 1, 4 and 16 mg/L, nominal concentrations of MC-LR) and four binary mixture nominal concentrations (0.2 mg/L MPs + 0.250 mg/L of

MC-LR, 1 mg/L MPs + 1 mg/L of MC-LR, 5 mg/L MPs + 4 mg/L of MC-LR and 25 mg/L MPs + 16 mg/L of MC-LR). Twenty animals were exposed per treatment (5 daphnids per beaker. The effect criterion was mortality, recognised by the immobilization (not able to swim within for 15 seconds under bright light, after gentle agitation of the beaker, even if still able to move their antennae). Organisms were observed at 0 h, 24 h and 48 h to observe (naked eye) their behaviour, record the number of immobilizations per beaker. A stereo microscope (NIKON, SMZ800) to investigate the potential accumulation of MPs in the gut of the organisms at the end of the bioassay.

To prepare the treatments contain MPs, a stock solution of MPs in ASTM was first prepared. The treatments were prepared by serial dilution of this MPs stock solution. *M. aeruginosa* extracts were used as prepared during extraction, using only u.p. water and no other solvents, the volume of extracts needed to each treatment were calculated to obtain the required concentration of MC-LR.

Physicochemical parameters, namely water temperature, pH, conductivity and dissolved oxygen were measured (HACH HQ40d multi, U.S.A., and multi-parameter WTW Multi 340i/SET, Germany) at the beginning of the bioassay (0 h), and after 24 h and 48 h of exposure.

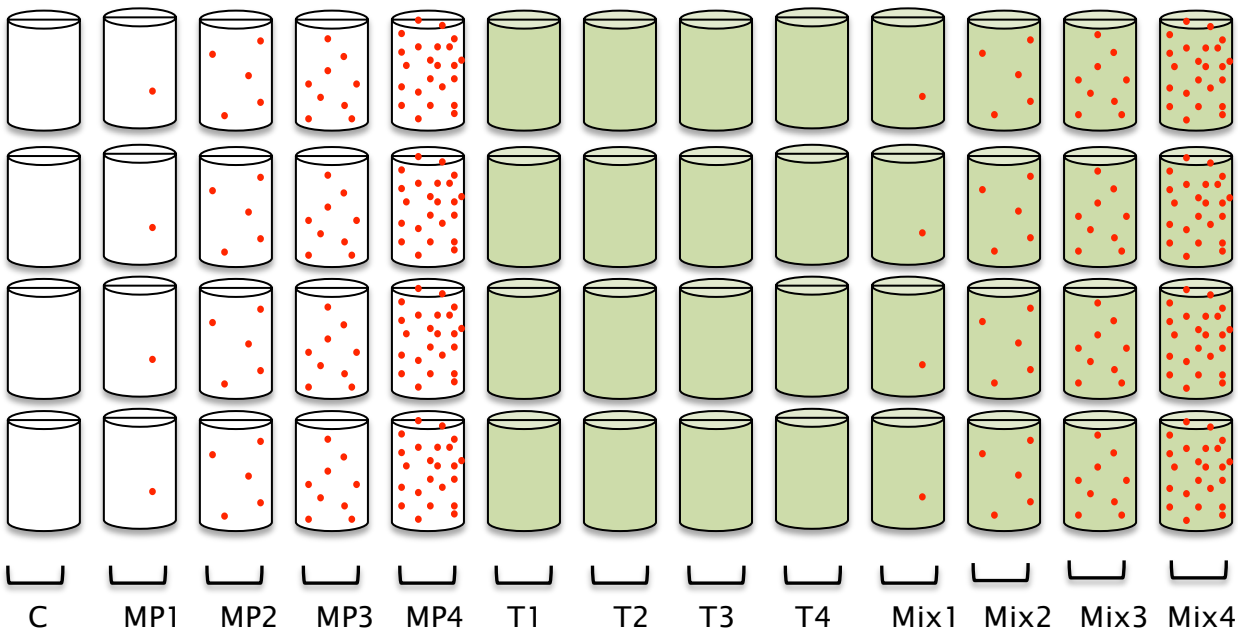

Figure 8: Experimental design to investigate the effects of microplastics (MPs), *M. aeruginosa* extracts (MC-LR), and their mixtures. C- control, MP1 – 0.2 mg/L of MPs, MP2 – 1 mg/L of MPs, MP3 – 5 mg/L of MPs, MP4 – 25 mg/L of MPs, T1 – 0.250 mg/L of MC-LR, T2 – 1 mg/L of MC-LR, T3 – 4 mg/L of MC-LR, T4 – 16 mg/L of MC-LR, Mix1 – 0.2 mg/L of MPs + 0.250 mg/L of MC-LR, Mix2 – 1 mg/L of MPs + 1 mg/L of MC-LR, Mix3 – 5 mg/L of MPs + 4 mg/L of MC-LR, Mix4 - 25 mg/L of MPs + 16 mg/L of MC-LR.

2.12. Chronic toxicity of microplastics, toxic *M.aeruginosa* extracts and their mixtures to *D. magna*

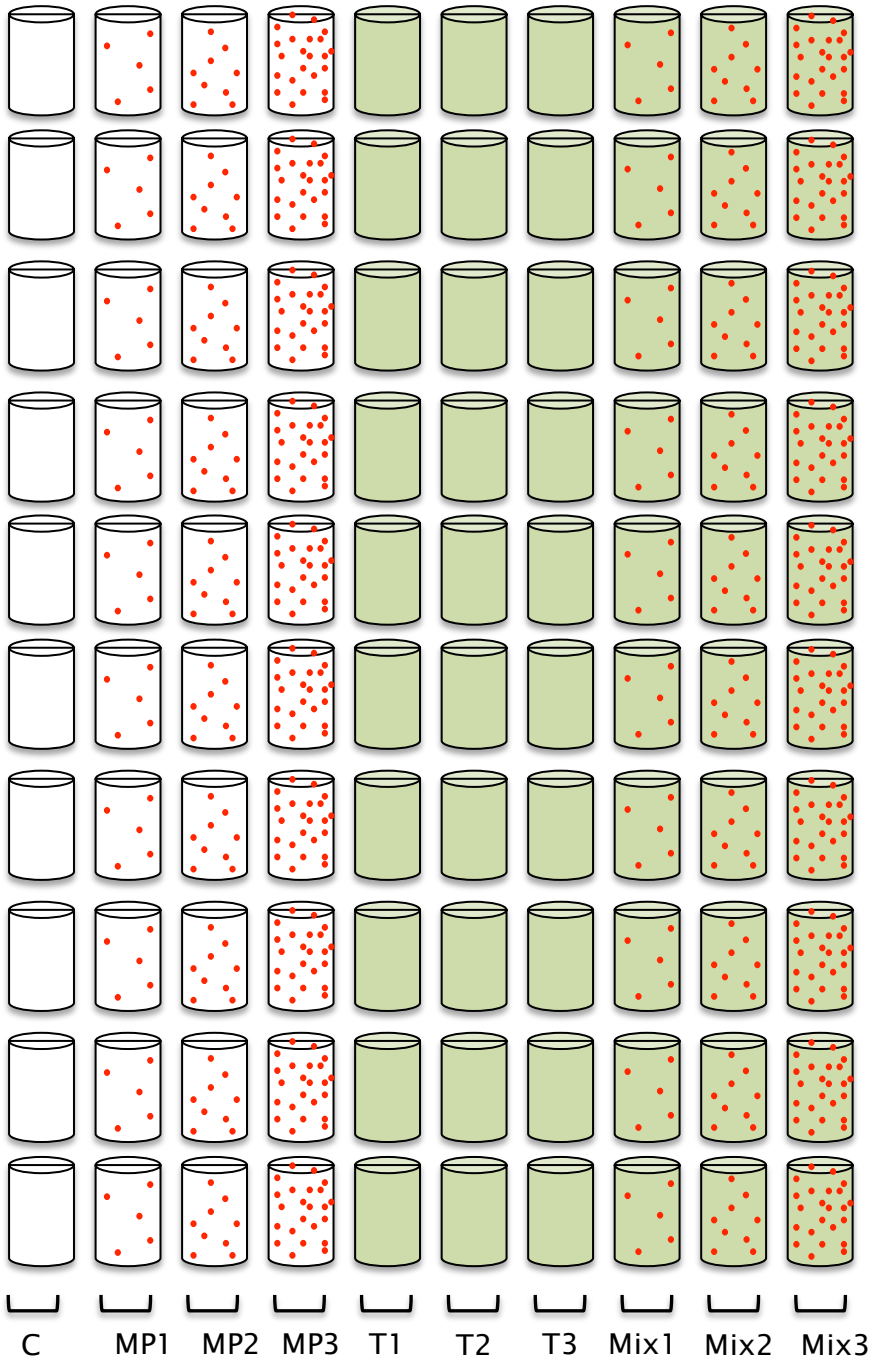

Figure 9: Experimental design to investigate the effects of microplastics (MPs), *M. aeruginosa* extracts (MC-LR), and their mixtures. C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, T3 – 100 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR, Mix3 – 0.2 mg/L of MPs + 100 μg/L of MC-LR.

To assess the chronic toxicity of MPs, toxic *M.aeruginosa* extracts and their mixtures to *D. magna*, a 21-day bioassay was carried out, generally according to the OECD guideline 211 (OECD, 2012), with adaptations to a mixture design and other punctual modifications as further described. The chronic bioassay was conducted in a climatized chamber (PGC1400 Bronson Climate, Bronson Incubator Services B.V., The Netherlands) at 20 ± 1ºC under a 16 h light : 8 h dark photoperiod. Test medium was ASTM with vitamins (0.1 ml/L) and *A. nodosum* extract (40 ml/L) as described in section 2.4.), and it was renewed at each 24 h.

The experimental design (Figure 3) included 10 treatments: control (ASTM with vitamin and *A. nodosum* extract), three concentrations of MPs (0.02, 0.1 and 0.2 mg/L, nominal concentrations, respectively), three concentrations of *M.aeruginosa* extracts (0.001, 0.01 and 0.1 mg/L of MC-LR, nominal concentration, respectively) and three binary mixture nominal concentrations (0.02 mg/L MPs + 0.001 mg/L of MC-LR, 0.1 mg/L MPs + 0.01 mg/L of MC-LR, 0.2 mg/L MPs + 0.1 mg/L of MC-LR). Treatments were prepared as indicated in section 2.11. but using ASTM with vitamins and *A. nodosum* extract. The bioassay was started with juvenile females (> 6 h and < 24 h old, 3rd brood). They were exposed individually (1 female per beaker) in 100 mL glass beakers, filled with 50 mL of test solution. During the exposure period, organisms were fed with *C. vulgaris* (3 x 10^5 cells/mL/daphnid, corresponding to 0.322mg of carbon/daphnid/day (Guilhermino et al., 1999). The effect criteria were growth, reproduction and mortality (if occurring and determined through the immobilization for 15 seconds under bright light). Growth was calculated as the difference in length of the exopodite of the right basiopodite (Figure 4) of the last and first moults released during the bioassay and maintained at – 20 ºC. This measure is adequate to evaluate *D. magna* growth (Soares, 1989). The reproductive parameters were: mean of the age (days) of first brood release per female and treatment (hereafter indicated as 1st Brood), mean of the total number of broods produced per female per treatment (hereafter indicated as Broods), mean of the total offspring produced per female and treatment (hereafter indicated as total offspring), mean of the number of mobile juveniles produced per female and treatment (hereafter indicated as mobile juveniles), mean of the number of immobile juveniles produced per female and treatment (hereafter indicated as immobile juveniles) and the mean of the number of aborted eggs produced per female and treatment (hereafter indicated as aborted eggs).

A stereo microscope (NIKON, SMZ800) was used to measure the exopodites (Figure 4) and to investigate the possible accumulation of microplastics in animals after the end of the bioassay.

Figure 10: Detail of a daphnid antenae with identified exopodite.

2.13. Statistical analyses

Data are presented as the mean and standard error of the mean (SEM). In acute bioassays, the 50 % effective concentrations (EC_{50}, respectively) were calculated from the toxicity curves (log of nominal concentrations *versus* corresponding probit transformed percentages of response). Chronic bioassay data sets were tested for normality of distribution (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) and if necessary, data transformations were performed (Zar, 1999). When normal distribution and homogeneity of variances were obtained, the Analysis of Variance (ANOVA) followed by the Tuckey's multi-comparison test was used. When these ANOVA assumptions were not achieved even after transformation, a non-parametric Krukal-Wallis test followed by Tuckey-type nonparametric multi-comparison test (Zar, 1999) were used. Significance level was 0.05 and the SPSS Statistic package, version 25 (IBM, USA) was used for all the statistical analyses.

3. Results

3.1. HPLC chromatograms

Chromatograms obtained from standards were analysed first to observe the time of retention of the toxin and the different peaks that appeared.

In the chromatogram of pure MC-LR standard the only peak was from the toxin (Figure 5). In the chromatogram of the standard obtained from an environmental sample the toxin retention time was the same but there were observed more peaks from other compounds of the sample (Figure 6).

In the extracted samples chromatogram the peak with the same retention time from the standards and other peaks were observed (Figure 7). The peak corresponding to MC-LR was analysed by measuring his area. The area value was used to calculate MC-LR volume and concentrations using Excel.

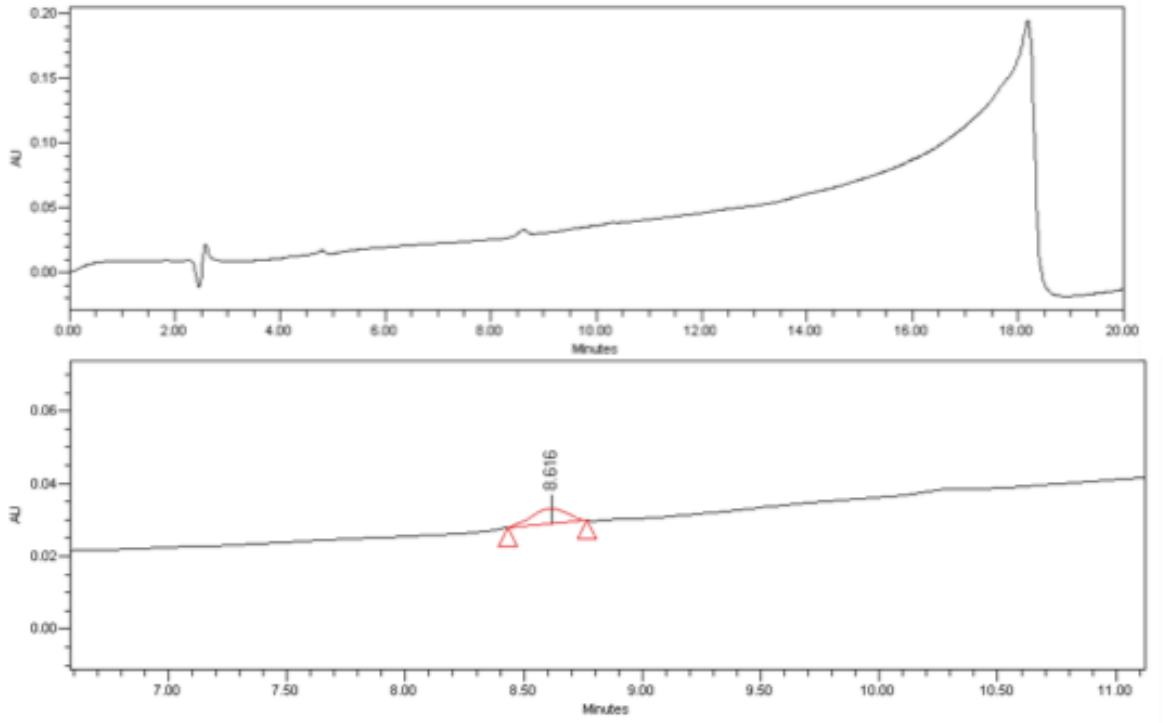

Figure 11: Chromatogram obtained through HPLC of a standard of pure MC-LR, with respective peak at 8.616 min.

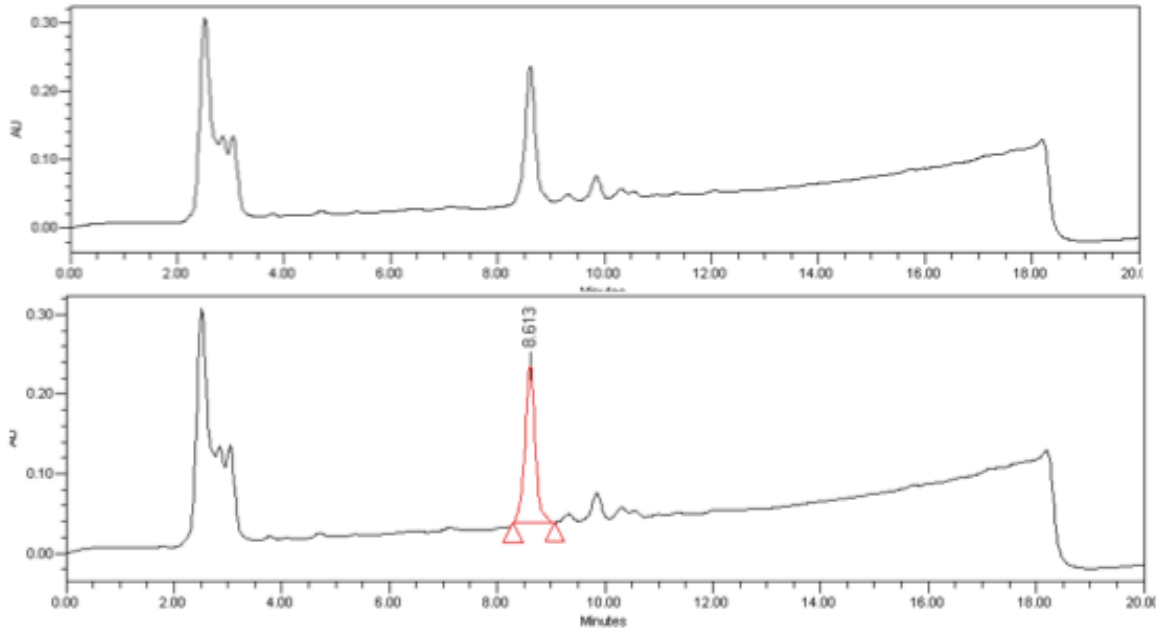

Figure 12: Chromatogram obtained through HPLC of a standard obtained from an environmental sample, with MC-LR peak at 8.613 min and other peaks of isomers after.

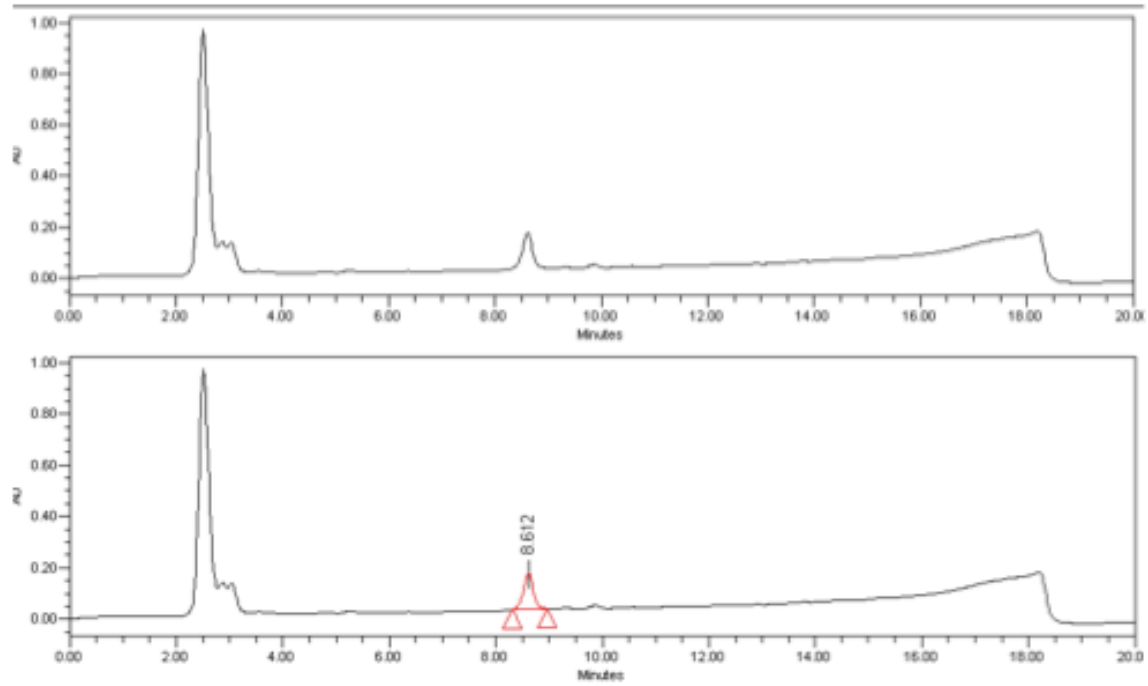

Figure 7: Chromatogram obtained through HPLC of a sample, with MC-LR peak at 8.612 min and other peaks of isomers after.

3.2. Improvement of Extraction Method

The data obtained by the chromatograms and analysed in Excel showed that extraction method 2 was the most effective extracting MC-LR, as shown in Table 3. The two extractions were performed in two samples collected in different times; hence the results can't be compared between extractions.

Table 4: MC-LR obtained from each method of extraction in μg/g of dry cells

	Method	MC-LR concentration (μg/g)
First extraction	1	801.47
	1	785.94
	2	967.66
	2	957.32
Second extraction	2	2452.90
	3	2298.75
	4	2263.80

3.3. Extract Quantification

After analysis of the calculated data dilution 10 and 100 times were eliminated for having values outside of standards range. The data of MC-LR concentration and total MC-LR obtained from the selected sample were multiplied by the dilution factor (Table 4).

This data was used to calculate the volume of sample to each exposure treatment considering the MC-LR concentration pretended.

Table 5: Concentration of MC-LR in the sample extracted (μg/g), total MC-LR extracted (μg) and concentration of MC-LR in the solution obtained (μg/mL), calculated by the analysis of chromatograms.

Dilution	MC-LR concentration (μg/g)	Total MC-LR (μg)	MC-LR concentration (μg/mL)
20	2014.32	20143.22	98.26

3.4. Standardization of the procedures to measure the actual concentrations and behaviour of microplastics in ASTM hard water

Table 6: Nominal concentrations of microplastics in freshly prepared solutions of different media and respective decay (decrease of microplastics suspended in media) after 24 h.

	Nominal concentration (mg/L)	Decay 24h (%)
u.p. water	0.09	13.3
	0.19	7.0
	0.38	7.1
	0.75	5.3
	1.5	5.2
	3	3.2
	6	2.9
	12	1.0
ASTM	0.09	10.1
	0.19	22.1
	0.38	5.4
	0.75	3.1
	1.5	6.8
	3	5.6
	6	2.5
	12	11.4

Microplastics decay observed in u.p. water and ASTM was different. At 24 h the decay of MPs in UP H_2O ranged from 1.0 % to 13.3 % and in ASTM ranged from 2.5 % to 22.1 % (Table 5). These values were considerably higher when solutions were not resuspended and homogenized. The decay observed at 24 h,

based on OECD guidelines, was sufficient to support the change of test solutions at every 24 h during the bioassays. At 0.047 mg/L the fluorescence was equal to the control, meaning that spectrofluorometer was not sensible to this or lower concentrations.

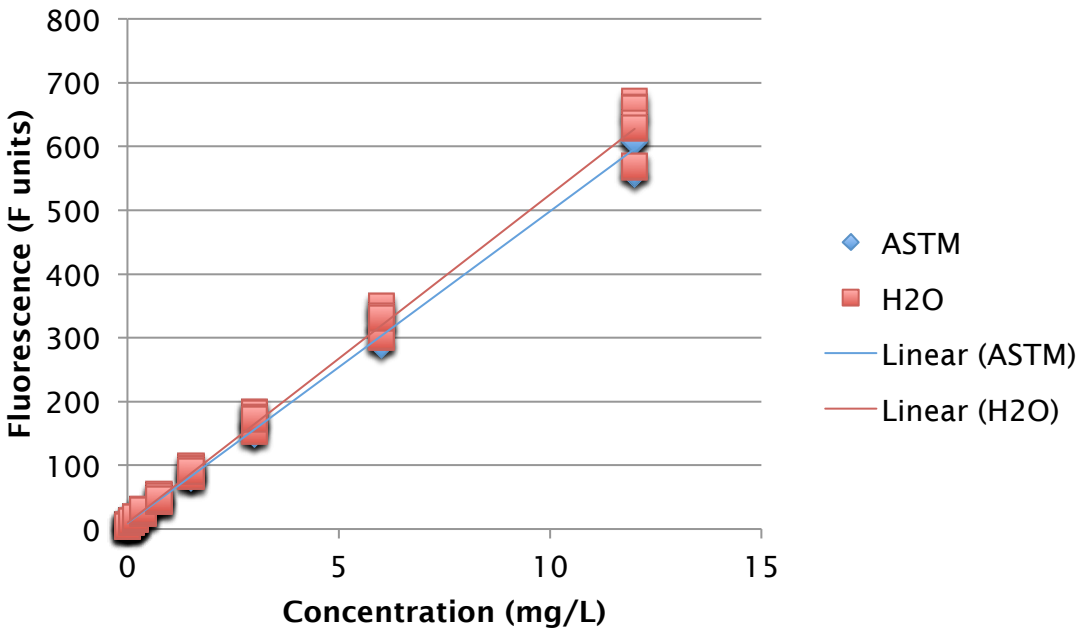

Figure 13: Fluorescence curves for the tested concentrations (0, 0.09, 0.19, 0.38, 0.75, 1.5, 3, 6, and 12 mg/L) in both media used with respective linear regression model.

Figure 8 shows the differences of between fluorescence of microplastics at the same concentrations in u.p. water and ASTM at 0h. These differences are noticeable through the trend line adjusted to the fluorescence of each medium.

The linear regression model fitted to ASTM was y= 49.047x + 2.7595 (y- fluorescence in F units; x- concentration of microplastics in mg/L). This model was used to calculate real concentrations used during the bioassays, as shown in Table 6 and Table 7. As described above the equipment was not sensible to the lowest concentration used. It was also observed, during the acute bioassay, that 25 mg/L of microplastics was over the range of spectrofluorometer.

Table 7: Acute bioassay: nominal and actual concentrations of microplastics in freshly prepared solutions, respective percentage of deviation (difference between nominal actual concentrations), maximum and minimum concentration of exposure to microplastic only treatments. The values of actual concentrations presented are the mean and respective SEM.

	Nominal concentration (mg/L)	Actual concentration (mg/L)	Deviation (%)	Minimum (mg/L)	Maximum (mg/L)
MP1	0.2	0.20 ± 0.01	0	0.12	0.27
MP2	1	1.34 ± 0.11	34	0.96	2.08
MP3	5	6.63 ± 0.47	32.6	5.07	10.47
MP4	25	-	-	-	-

Table 8: Chronic bioassay: nominal and actual concentrations of microplastics in freshly prepared solutions, respective percentage of deviation (difference between nominal actual concentrations), maximum and minimum concentration of exposure to microplastic only treatments. The values of actual concentrations presented are the mean and respective SEM.

	Nominal concentration (mg/L)	Actual concentration (mg/L)	Deviation (%)	Minimum (mg/L)	Maximum (mg/L)
MP1	0.02	-	-	-	-
MP2	0.1	0.101	1	0.076	0.153
MP3	0.2	0.236	18	0.137	0.459

3.5. Potassium Dichromate Test

Physico-chemical parameters (temperature, pH and dissolved O_2), during potassium dichromate test, followed OECD Guidelines for acute bioassays in *Daphnia magna.* Condutivity was always higher than 584 μS/cm.

LC_{50} calculated from data collected at 24 h was 1.166 mg/L, which is inside the range in the guidelines, from 0.6 to 2.1 mg/L. The obtained LC_{50} determined that *D.magna* were healthy and appropriate for acute bioassays.

Table 9: Percentage of mortality of *D. magna* at 24h of exposure to Potassium Dicromate (C- control; D1- 0.25 mg/L; D2- 0.5 mg/L; D3- 1 mg/L; D4- 2 mg/L; D5- 4 mg/L and D6- 8 mg/L).

	C	D1	D2	D3	D4	D5	D6
Death (%)	0	0	0	70	85	100	100

3.6. Acute toxicity of microplastics, *M. aeruginosa* extract and their mixtures to *D. magna*

In acute bioassay all the requirements and validity criteria were full filled. In this bioassay temperature, pH, dissolved O_2 and conductivity were always higher than 20.0 ºC; 6.72 pH units; 7.4 mg/L and 563 μS/cm and the highest variations were 0.85 ºC; 2.13 pH units, 1.35 mg/L and 42 μS/cm, respectively.

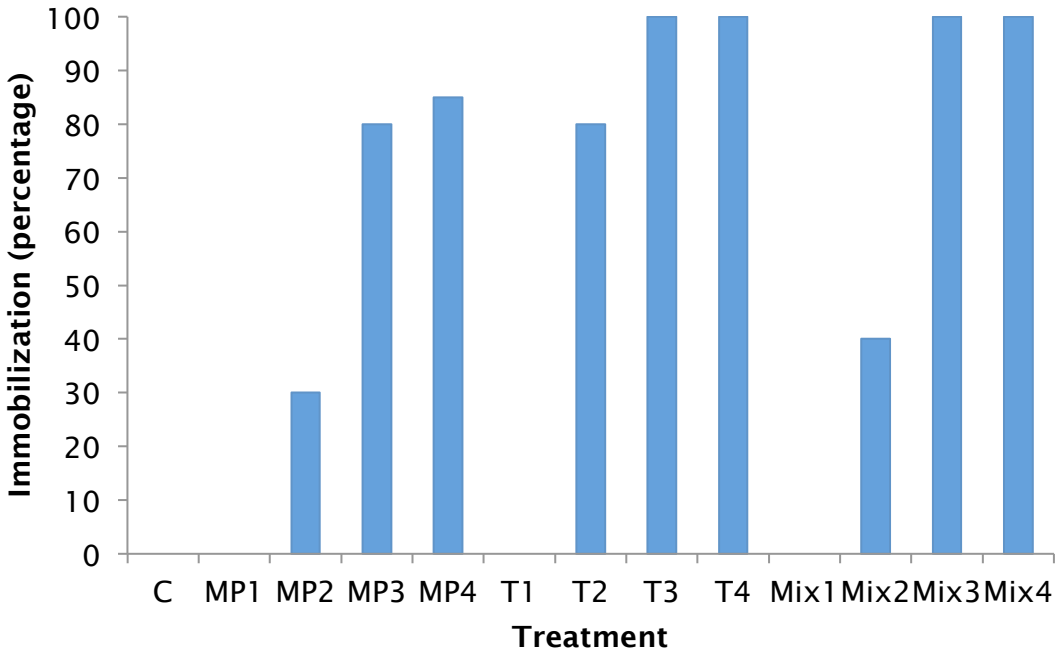

Figure 14: Percentage of mortality at 48 h of *D. magna* exposed to microplastics , *M. aeruginosa* extract and mixtures of both. C (control), MP1 (0,2 mg/L of MP), MP2 (1 mg/L of MP), MP3 (5 mg/L of MP), MP4 (25 mg/L of MP), T1 (0,25 mg/L of MC-LR), T2 (1 mg/L of MC-LR), T3 (4 mg/L of MC-LR), T4 (16 mg/L of MC-LR), Mix1 (0,2 mg/L of MP + 0,25 mg/L of MC-LR), Mix2 (1 mg/L of MP + 1 mg/L of MC-LR), Mix3 (5 mg/L of MP + 4 mg/L of MC-LR), Mix4 (25 mg/L of MP + 16 mg/L of MC-LR).

Immobilization of *D.magna* at 24 h was observed in all treatments, except in the control, in the two lowest concentrations of microplastics (MP1 and MP2), in the lowest concentration of MC-LR (T1) and in the lowest concentration of mixtures (Mix1). Microplastics treatments containing 5 mg/L and 25 mg/L of microplastics induced 10 % and 5 % of immobilization, respectively. Treatments of *M.aeruginosa* extract containing 1 mg/L, 4 mg/L and 16 mg/L of MC-LR induced 70 %, 100 % and 100 % of immobilization, respectively. Mixture treatments containing 1 mg/L of microplastics and 1 mg/L of MC-LR, 5 mg/L of

microplastics and 4 mg/L of MC-LR, and 25 mg/L of microplastics and 16 mg/L of MC-LR induced 20 %, 100 % and 100 % of immobilization, respectively.

Immobilization of *D. magna* at 48 h (Figure 9) was observed in all treatments, except in the control, the lowest concentration of microplastics (MP1), the lowest concentration of MC-LR (T1) and the lowest concentration of mixtures (Mix1). Microplastics treatments containing 1 mg/L, 5 mg/L and 25 mg/L of microplastics induced 30 % 80 % and 85 % of immobilization, respectively. Treatments of *M.aeruginosa* extract containing 1 mg/L, 4 mg/L and 16 mg/L of MC-LR induced 80 %, 100 % and 100 % of immobilization, respectively. Mixture treatments containing 1 mg/L of microplastics and 1 mg/L of MC-LR, 5 mg/L of microplastics and 4 mg/L of MC-LR, and 25 mg/L of microplastics and 16 mg/L of MC-LR induced 40 %, 100 % and 100 % of immobilization, respectively.

The data obtained from 24 h exposure to MP did not show a dose-response effect. The analysis of the remaining data of 24 h exposure and of the 48h exposure data did not obtain Log Probit model results. Due to these data, EC_{50} of 24 h and 48 h could not be calculated.

Through the observation of Figure 9 obtained by 48h data it was observed a dose-response effect in microplastics, MC-LR and mixture of microplastics and MC-LR exposure. Once that the exposure to the lowest concentrations of microplastics (MP1, 0.200 mg/L), of MC-LR (T1, 0.250 mg/L) and of mixture of microplastics and MC-LR (Mix1, 0.200 mg/L of microplastics + 0.250 mg/L of MC-LR), did not show immobilization and the two highest concentration of MC-LR (T3 and T4, 4 and 16 mg/L, respectively) and of mixtures of microplastics and MC-LR (Mix3 and Mix4, 5 mg/L of microplastics + 4 mg/L of MC-LR and 25 mg/L of microplastics + 16 mg/L of MC-LR, respectively) showed 100 % of immobilization, only MP2, T2 and Mix2 could be compared to presuppose the acute effect of the mixture. Comparing MP2 and T2 to Mix2 immobilization values, it is presupposed that mixture of microplastics and *M.aeruginosa* extracts has an antagonistic effect.

3.6.1. Presence of Microplastics

In the organisms exposed only to MPs retention of these particles was observed (Figure 11 and Figure 12). In the mixtures some MPs were observed in the organisms gut but in much lower concentrations (Figure 13).

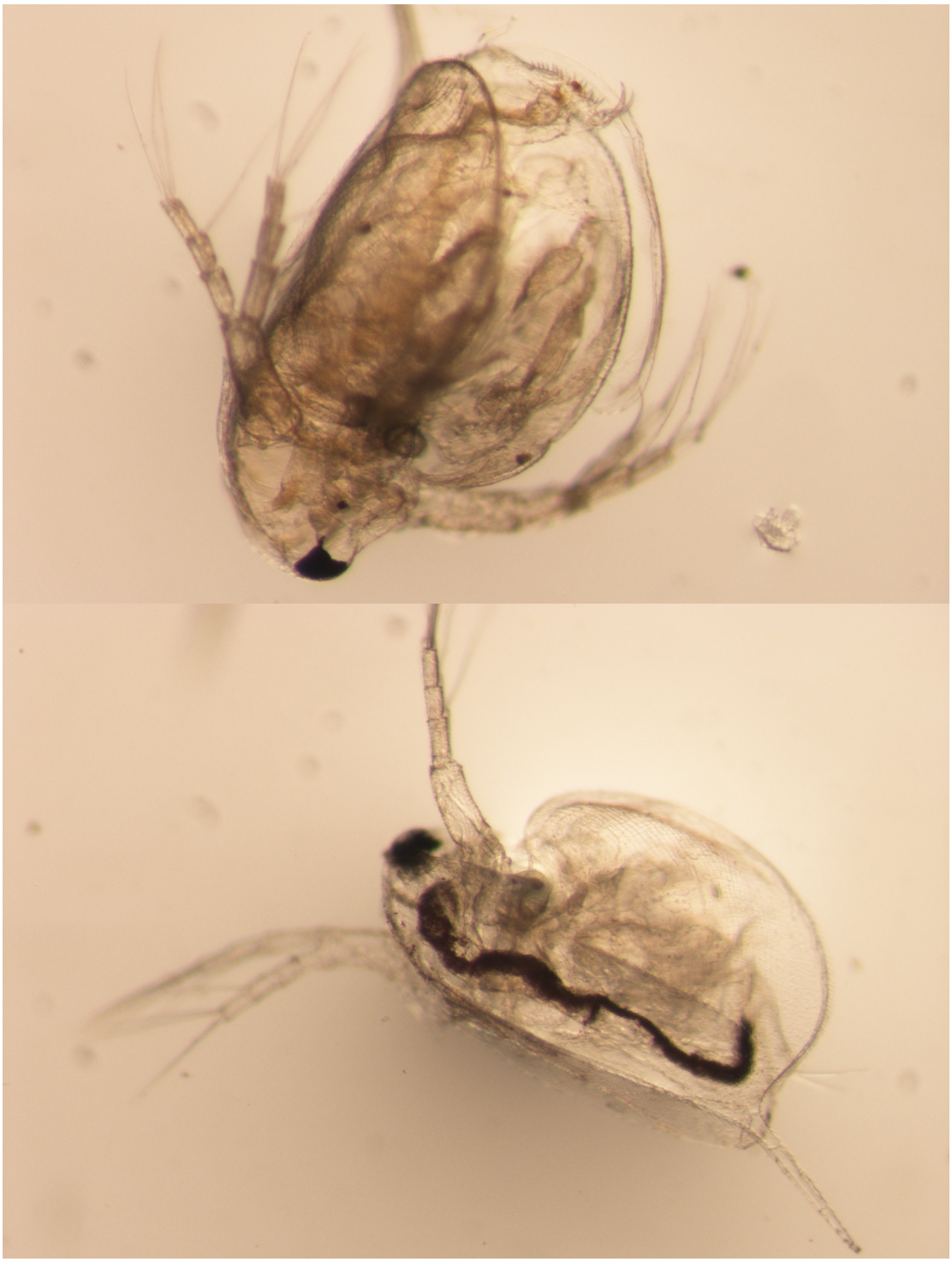

Figure 15: *D. magna* with empty gut and *D. magna* with the gut full of microplastics exposed to diferente treatments for 48 h, obtained by stereo microscopy.

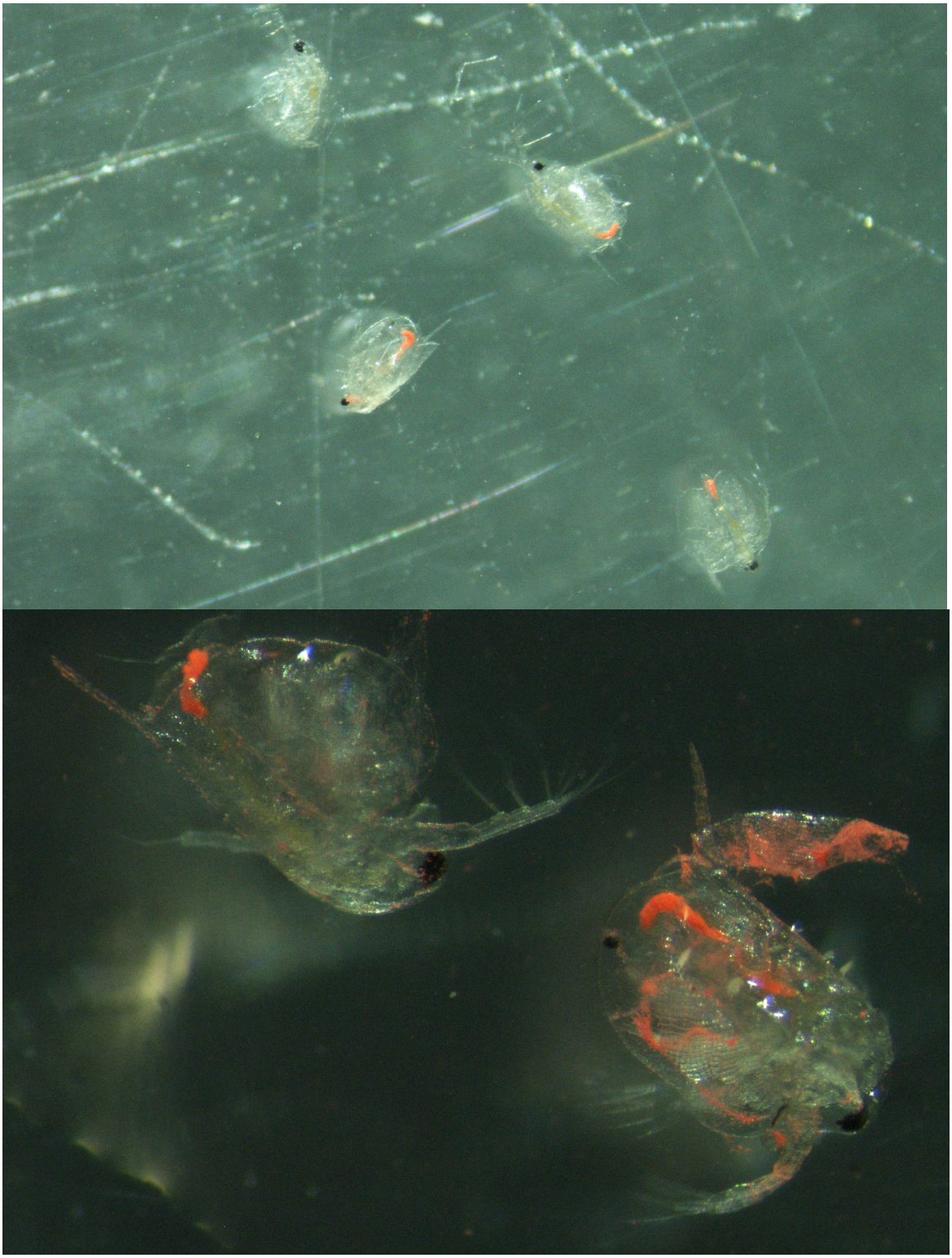

Figure 16: Multiple *D. magna* with different accumulation of microplastics in their guts when exposed to the same concentration of microplastics only for 48 h; and two *D. magna* with microplastics in their guts and outside of their body when exposed to microplastics only for 48 h.

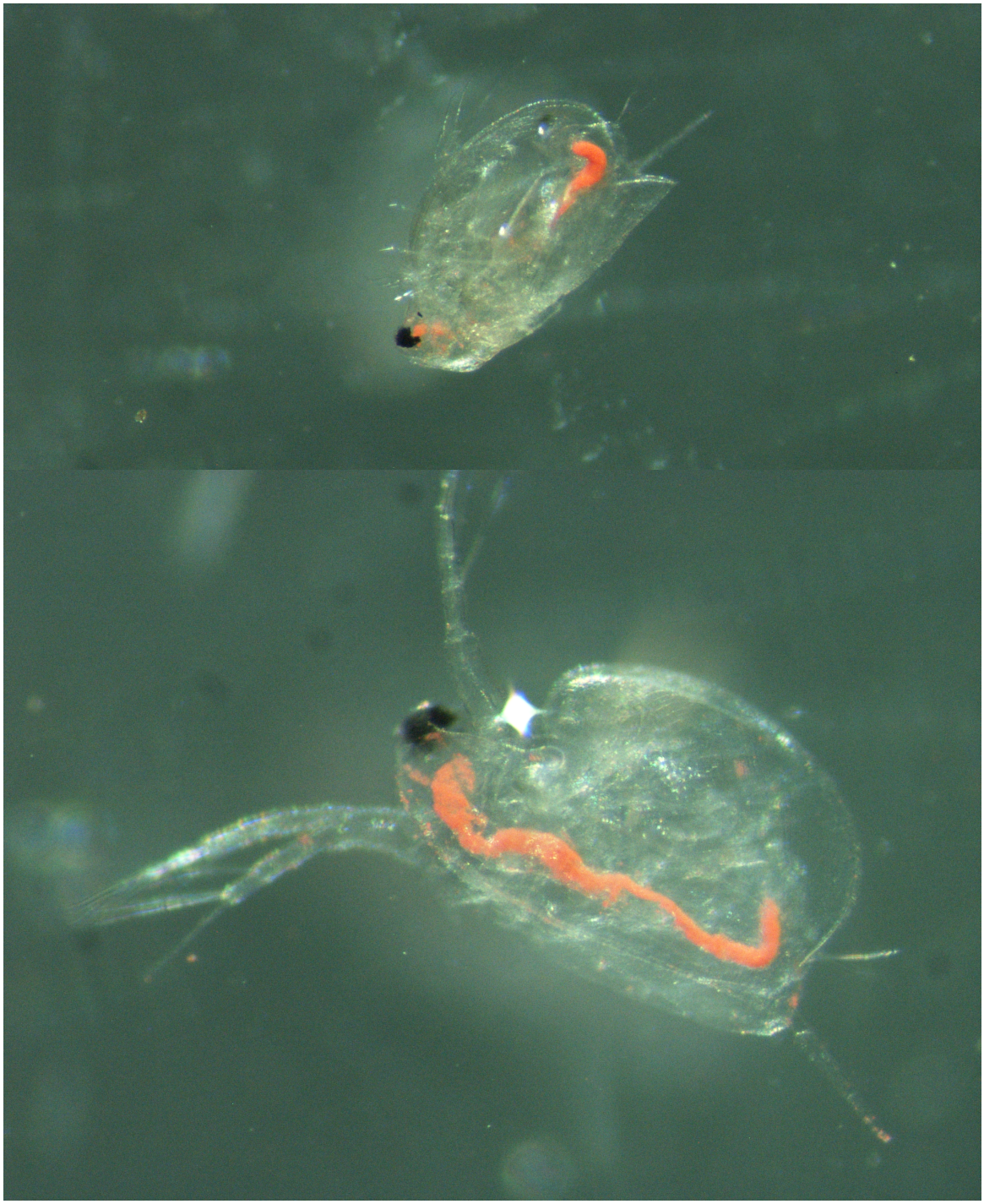

Figure 17: Individual *D. magna* with different accumulation of microplastics in their guts when exposed to microplastics only for 48 h.

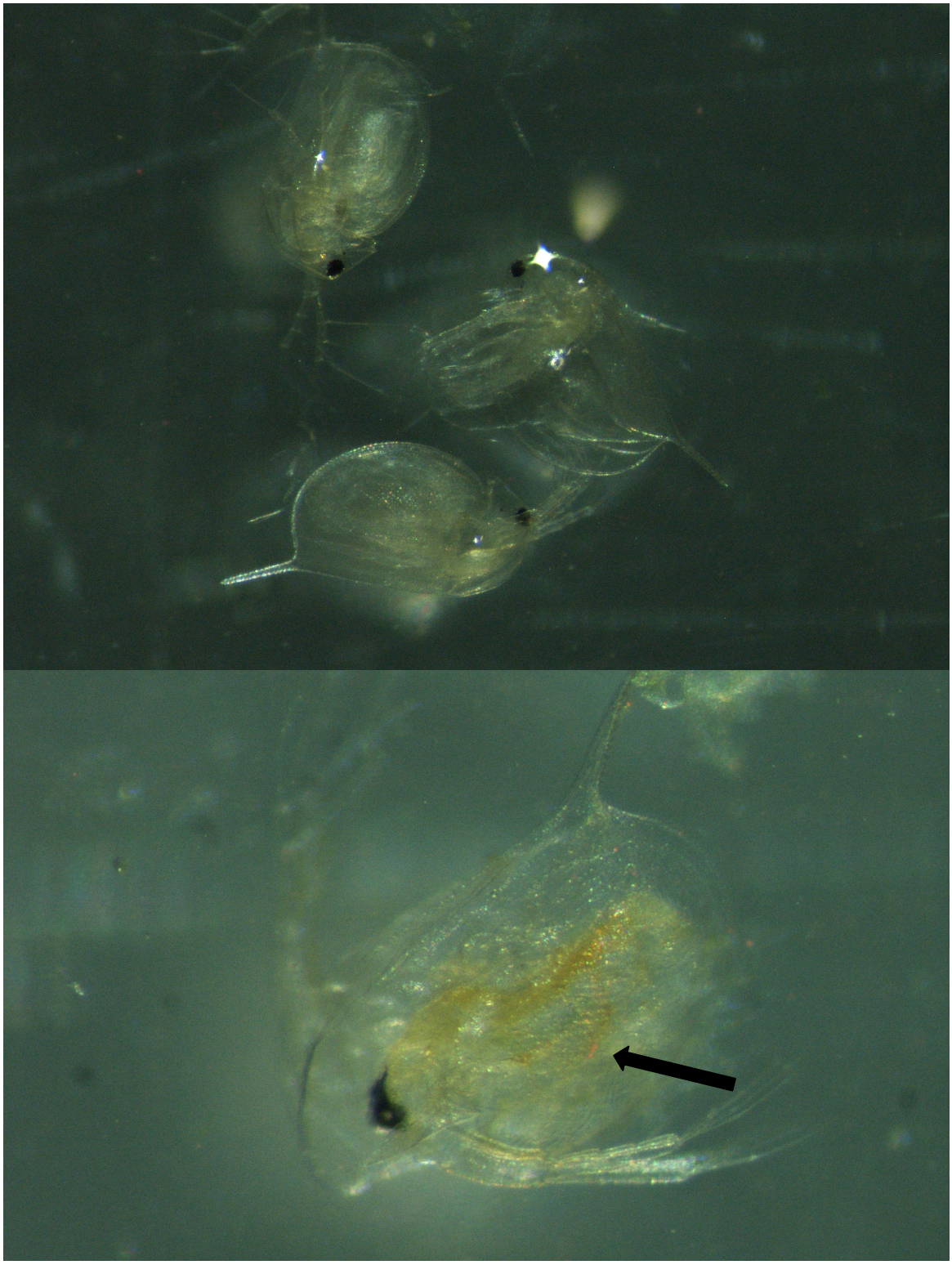

Figure 18: Multiple *D. magna* showing no signs of in or out accumulation of microplastics when exposed to mixtures of microplastics and *M.aeruginosa* extracts for 48 h; and individual *D. magna* with signs of accumulation of microplastics when exposed to mixtures of microplastics and *M.aeruginosa* extracts for 48 h.

3.7. Chronic toxicity of microplastics, toxic *M.aeruginosa* extracts and their mixtures to *D. magna*

In chronic bioassay all the requirements and validity criteria were full filled. In this bioassay temperature, pH, dissolved O_2 and conductivity were always higher than 20.0 ºC; 8 pH units; 6.785 mg/L and 550 μS/cm and the highest variations were 1.7 ºC; 0.785 pH units, 2.62 mg/L and 47.5 μS/cm, respectively.

During the bioassay there were two accidental deaths due to manipulation, one of the control (C) and one of the lowest mixture (Mix1), considering the total number of organisms (N) of each of these treatments to 9. These deaths were not considered to calculate mortality (Table 9).

Table 10: Percentage of mortality per treatment. N- total number of initial female parents used in each treatment not considering female parents that had acidental deaths. C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, T3 – 100 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR, Mix3 – 0.2 mg/L of MPs + 100 μg/L of MC-LR.

	N	Mortality (%)
C	9	0
MP1	10	50
MP2	10	60
MP3	10	40
T1	10	0
T2	10	20
T3	10	100
Mix1	9	11.1
Mix2	10	10
Mix3	10	100

The results presented hereafter were calculated using the data collected only from parental females that did not die during the bioassay, except in sub-chapter 3.7.5. in which the results presented are the day of first brood release and the total number of offspring in first brood, calculated using data from all the treatments.

In Table 10 it is presented the mean and respective standard error (SEM) of all the treatments and results of statistical analysis of each parameter. Results from statistical analysis show significant differences in all the parameters except in the number of aborted eggs. The following data, represented in Figure 14, Figure 15, Figure 16, Figure 17, Figure 18, Figure 19, Figure 20 and Figure 21 present the mean, respective SEM and significant differences between treatments, when existing, for each parameter.

Table 11: Mean and respective SEM of somatic growth (Growth), days until first brood release (1st Brood), total broods released (Broods), total offspring (total), mobile juveniles (Total), immobile juveniles (Immobile) and aborted eggs (Eggs). C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR.

	C		MP1		MP2		MP3		T1		T2		Mix1		Mix2	
Growth	19.0	±0.0	18.1	±0.5	17.9	±0.2	17.0	±0.4	18.9	±0.4	19.0	0.3	19.5	±0.1	19,4	±0.3
	H_3= 17.501; p= 0.014															
1st Brood (days)	8.1	±0.3	7.8	±0.2	7.7	±0.2	8.1	±0.3	7.2	±0.1	5.8	±0.5	8.4	±0.2	8.2	±0.3
	H_3= 18.783; p= 0.009															
Broods (number)	5.8	±0.2	5.2	±0.2	6.0	±0.0	6.0	±0.3	6.3	±0.2	5.6	±0.3	5.0	±0.0	5.3	±0.2
	H_3= 31.432; p= 0.000															
Total	108.2	±6.8	74.6	±4.6	89.3	±3.7	96.7	±7.7	98.9	±8.0	129.6	±7.3	89.1	±3.9	119.8	±5.3
	$F_{7,51}$= 7.504; p= 0.000															
Mobile	108.0	±7.0	72.0	±4.9	86.8	±4.7	91.8	±7.9	96.4	±8.2	126.5	±6.4	87.5	±3.9	116.6	±5.2
	$F_{7,51}$= 8.217; p= 0.000															
Immobile	0.2	±0.2	2.4	±1.2	2.5	±2.2	4.7	±3.1	2.4	±0.6	3.0	±0.9	1.5	±0.3	2.9	±0.8
	H_3= 15,359; p= 0.032															
Eggs	0.0	±0.0	0.2	±0.2	0.0	±0.0	0.1	±0.2	0.1	±0.1	0.1	±0.1	0,1	±0.1	0.2	±0.1
	H_3= 3,108; p= 0.875															

3.7.1. Mortality

During this bioassay mortality was observed in all the treatments except in the control and in the lowest concentration of MC-LR (T1, 0.001 mg/L). MPs treatments containing 0.02 mg/L, 0.1 mg/L and 0.2 mg/L of microplastics induced 50 %, 60 % and 40 % of mortality, respectively. Treatments of *M. aeruginosa* extract containing 10 μg/L and 100 μg/L of MC-LR induced 20 % and 100% of mortality, respectively. Mixture treatments containing 0.02 mg/L of MPs + 0.001 mg/L of MC-LR, 0.1 mg/L of MPs + 0.01 mg/L of MC-LR and 0.2 mg/L of MPs + 0.1 mg/L of MC-LR induced 11,1 %, 10 % and 100 % of immobilization, respectively.

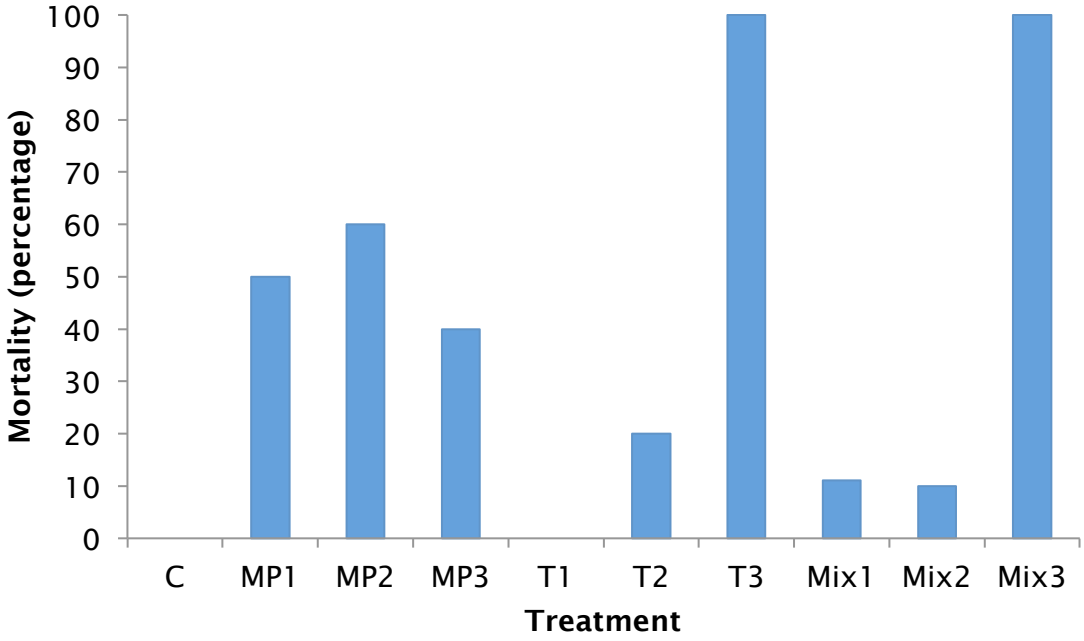

Figure 19: Percentage of mortality of *D. magna* exposed for 21 days to microplastics , *M. aeruginosa* extract and mixtures of both. C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, T3 – 100 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR, Mix3 – 0.2 mg/L of MPs + 100 μg/L of MC-LR.

3.7.2. Growth

After the chronic bioassay, differences of growth between treatments were observed (Figure 15). In microplastics and mixture treatments, it was observed a decrease in growth with the increase of the concentrations, while in *M. aeruginosa* extract treatments it was observed an increase in growth with the increase in concentration. The growth of the organisms in microplastics treatments was lower when compared with all the others, in *M. aeruginosa* extract treatments was lower and equal to the control and in mixture treatments the growth was higher when compared to other treatments. The only significant differences in growth were between MP3 and Mix2.

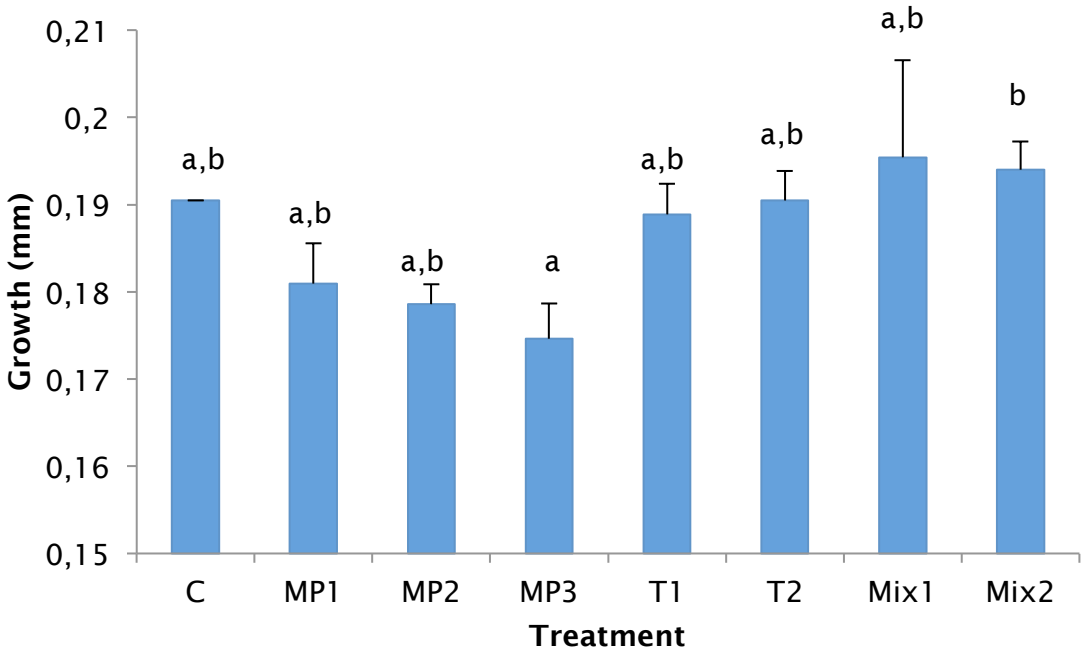

Figure 20: Mean and respective SEM of growth of *D. magna* exposed for 21 days to microplastics , *M. aeruginosa* extract and mixtures of both. C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR. Different letters indicate statistically significant differences.

3.7.3. Reproduction parameters

Exposure to microplastics and to mixtures did not show a dose-response effect. Exposure to MC-LR, although not significant, showed anticipation with the increase of concentrations. The day of first brood had no significant differences between the treatments.

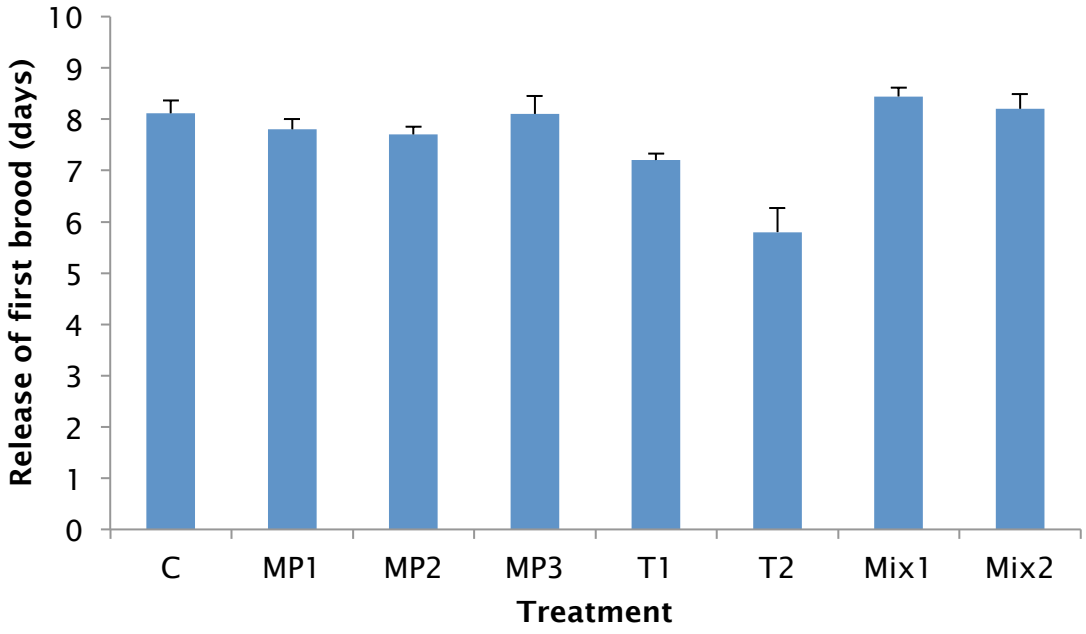

Figure 21: Mean and respective SEM of day of release of first brood of *D. magna* exposed for 21 days to microplastics , *M. aeruginosa* extract and mixtures of both. C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR.

The microplastic treatments showed a decrease in the lowest concentration, while the other concentrations did not differ from control. The *M.aeruginosa* extract treatments showed a higher number of broods in the lowest concentration, compared to the control, with a tendency to decrease, while the mixture treatments showed lower number of broods, compared to the control, with a tendency to increase. Only Mix1 showed significant differences with control, but T1 showed significant differences with MP1, Mix1 and Mix2.

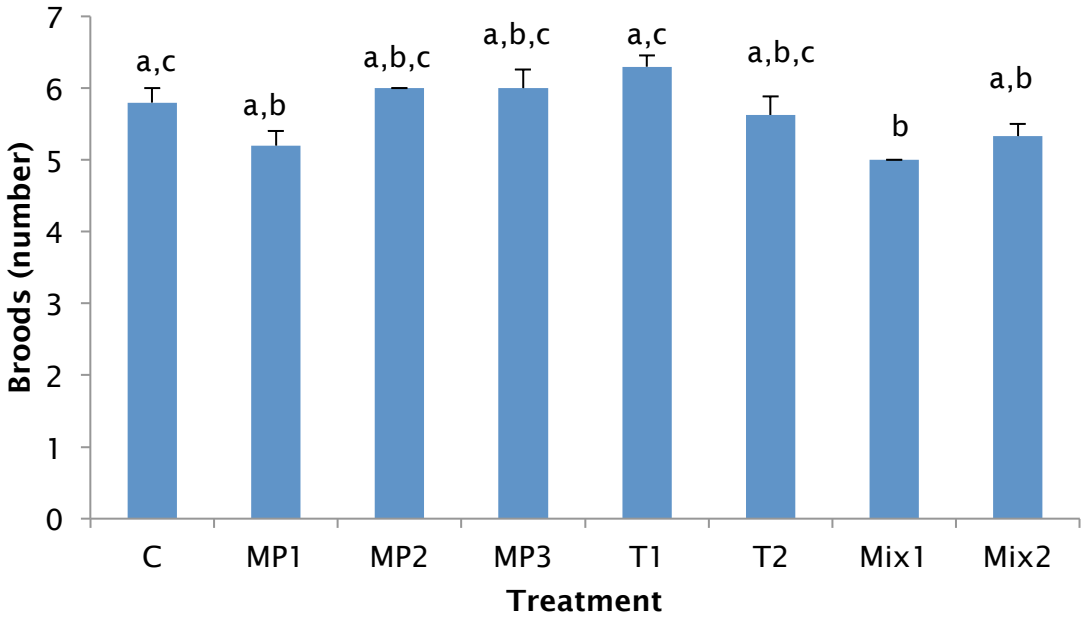

Figure 22: Mean and respective SEM of number of broods of *D. magna* exposed for 21 days to microplastics , *M. aeruginosa* extract and mixtures of both. C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR. Different letters indicate statistically significant differences.

The total number of offspring and the number of mobile juveniles increased with the increase of concentration when exposed to microplastics, *M. aeruginosa* extracts and mixture of both. These numbers were lower than the control, except in MC2 and Mix2. The only treatment significantly different to the control was MP1 in both of the parameters.

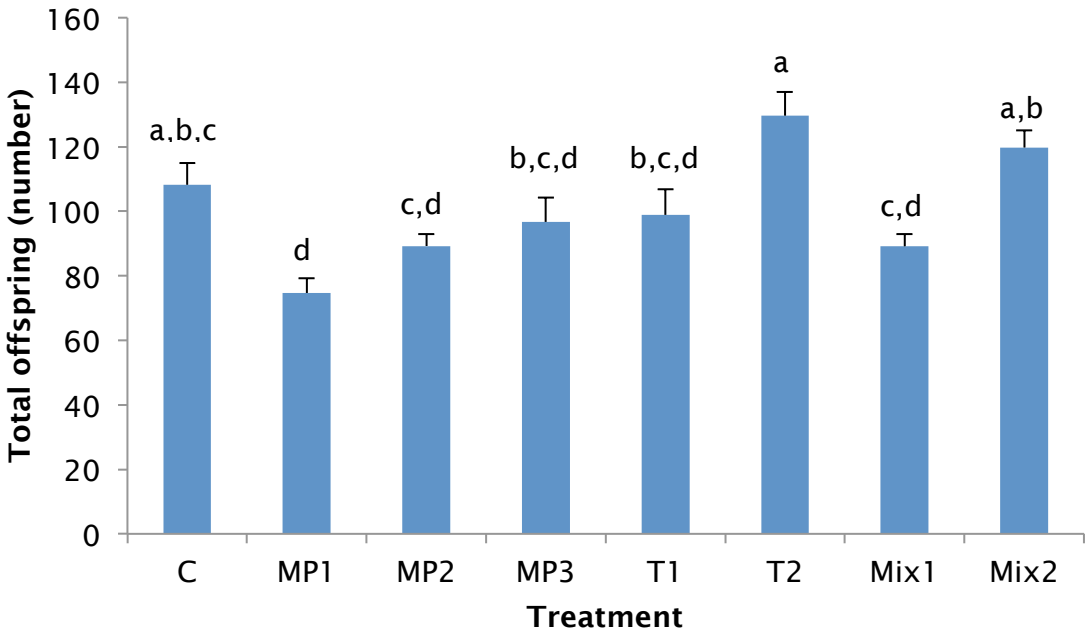

Figure 23: Mean and respective SEM of number total of offspring of *D. magna* exposed for 21 days to microplastics , *M. aeruginosa* extract and mixtures of both. C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR. Different letters indicate statistically significant differences.

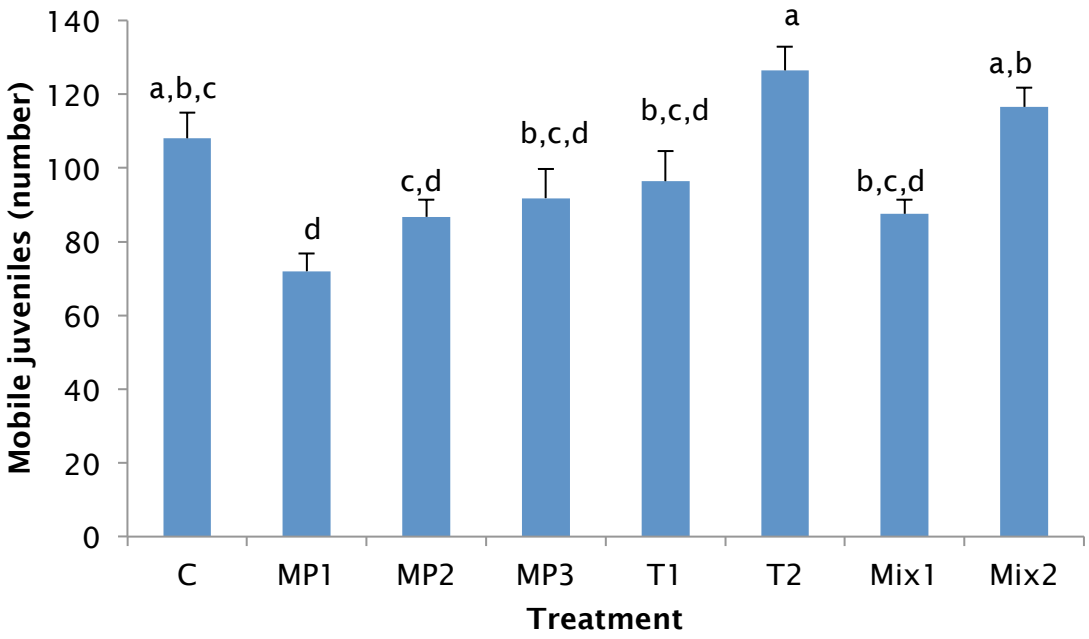

Figure 24: Mean and respective SEM of number of mobile of juveniles of *D. magna* exposed for 21 days to microplastics , *M. aeruginosa* extract and mixtures of both. C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR. Different letters indicate statistically significant differences.

The number of immobile juveniles increased with the increase of concentration when exposed to microplastics, *M. aeruginosa* extracts and mixture. These numbers were higher than the control in all other treatments. There were significant differences between the control and T2.

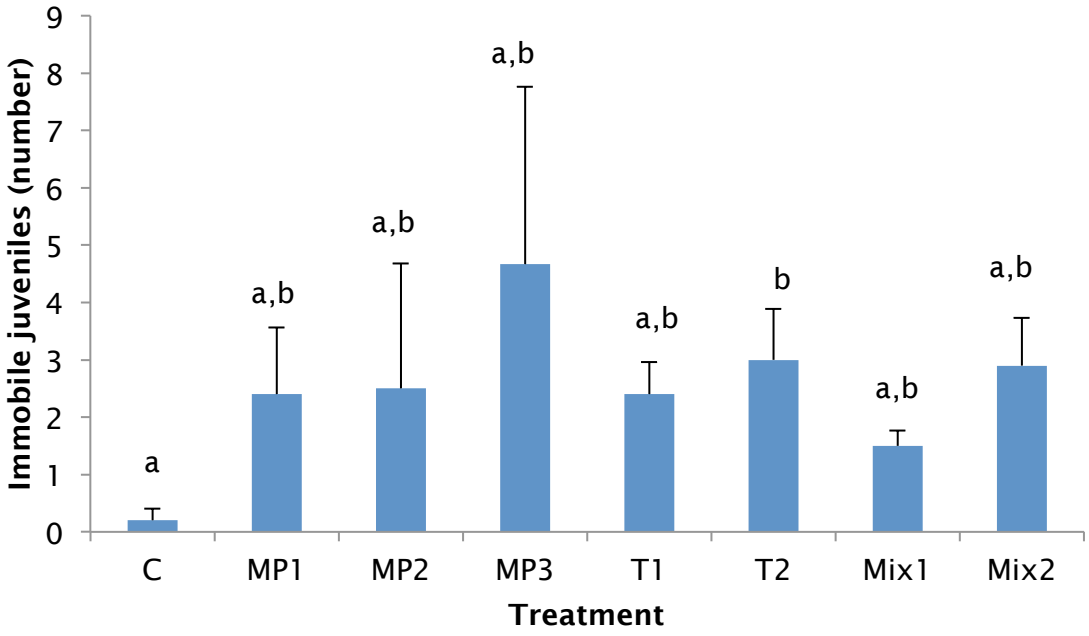

Figure 25: Mean and respective SEM of number of immobile of juveniles of *D. magna* exposed for 21 days to microplastics , *M. aeruginosa* extract and mixtures of both. C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR. Different letters indicate statistically significant differences.

There were no aborted eggs in the control and in the mean microplastics concentration (MP2). No significant differences of the number of aborted eggs were observed between all the other treatments.

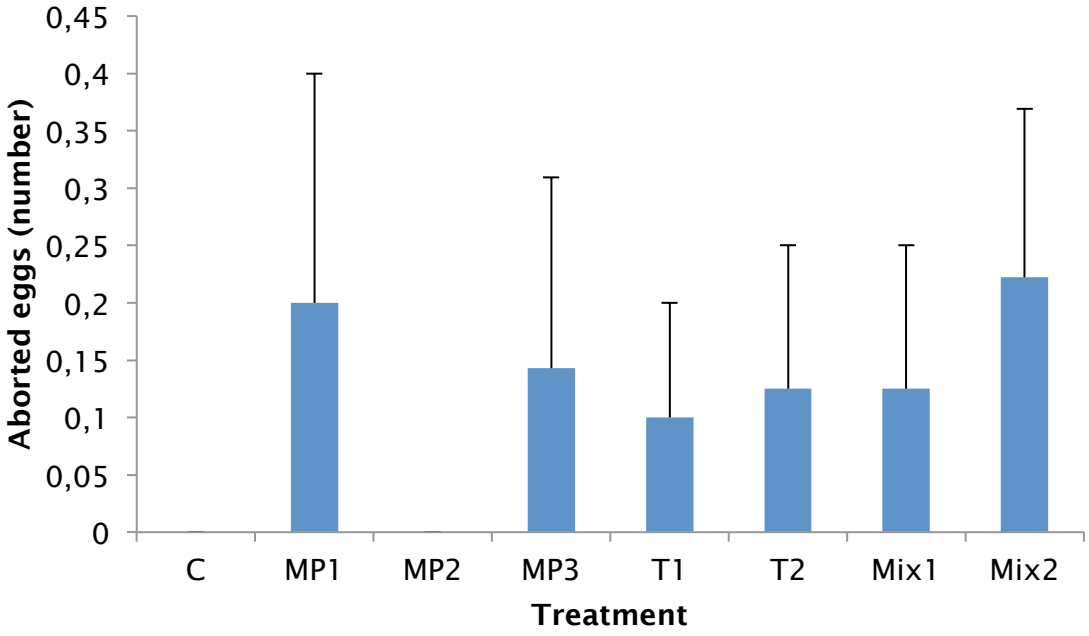

Figure 26: Mean and respective SEM of number of aborted eggs of *D. magna* exposed for 21 days to microplastics , *M. aeruginosa* extract and mixtures of both. C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR.

3.7.4. Presence of Microplastics

In some of the surviving organisms from MPs exposure it was observed retention of these particles in low concentrations (Figure 22). In mixtures exposure retention was only observed in one parental female and in low concentrations (Figure 23).

Figure 27: *D. magna* with signs of accumulation of microplastics in the guts when exposed to microplastics only, for 21 days and *D. magna* with signs of obstruction of the guts when exposed to microplastics only, for 21 days.

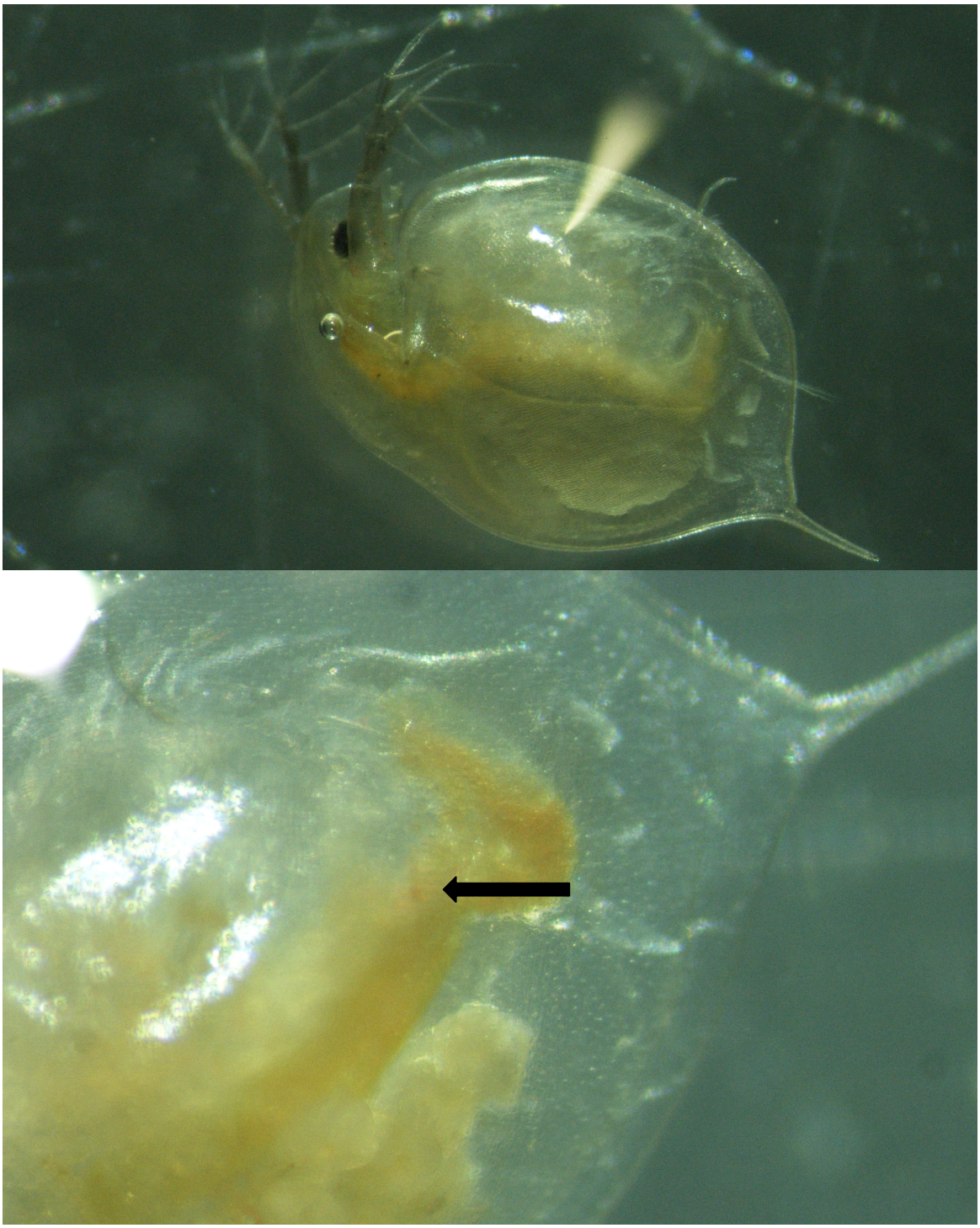

Figure 28: *D. magna* showing no signs of in or out accumulation of microplastics when exposed to mixtures of microplastics and *M. aeruginosa* extracts for 21 days; and *D. magna* with signs of accumulation of microplastics when exposed to mixtures of microplastics and *M.aeruginosa* extracts for 21 days.

3.7.5. T3 and Mix3

All the female parents of the treatment with the highest MC-LR concentration (T3, 0.1 mg/L of MC-LR) and the treatment with the highest concentrations mixture (Mix3, 0.2 mg/L of microplastics + 0.1 mg/L of MC-LR) died before the end of the bioassay. However, some of these organisms survived until after first brood or even until after second brood.

During the bioassay it was observed a decrease of growth of the organism exposed in these treatments, which led to a delay of maturation causing a delay of first brood as observed in Figure 24. The delay was more pronounced when exposed to Mix3 than when exposed to T3.

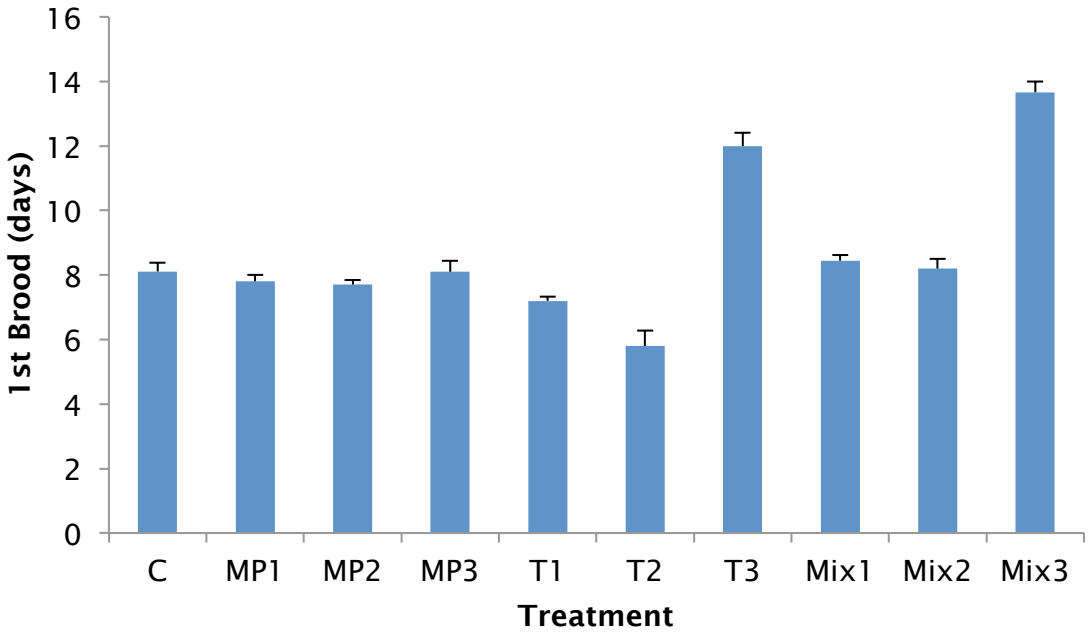

Figure 29: Mean and respective SEM of day of release of first brood of *D. magna* exposed for 21 days to microplastics , *M. aeruginosa* extract and mixtures of both.C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, T3 – 100 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR, Mix3 – 0.2 mg/L of MPs + 100 μg/L of MC-LR.

In these treatments it was also observed a decrease of the total number of offspring in the first brood, compared to all the other treatments as observed in Figure 25. This decrease was more pronounced in Mix3 than in T3.

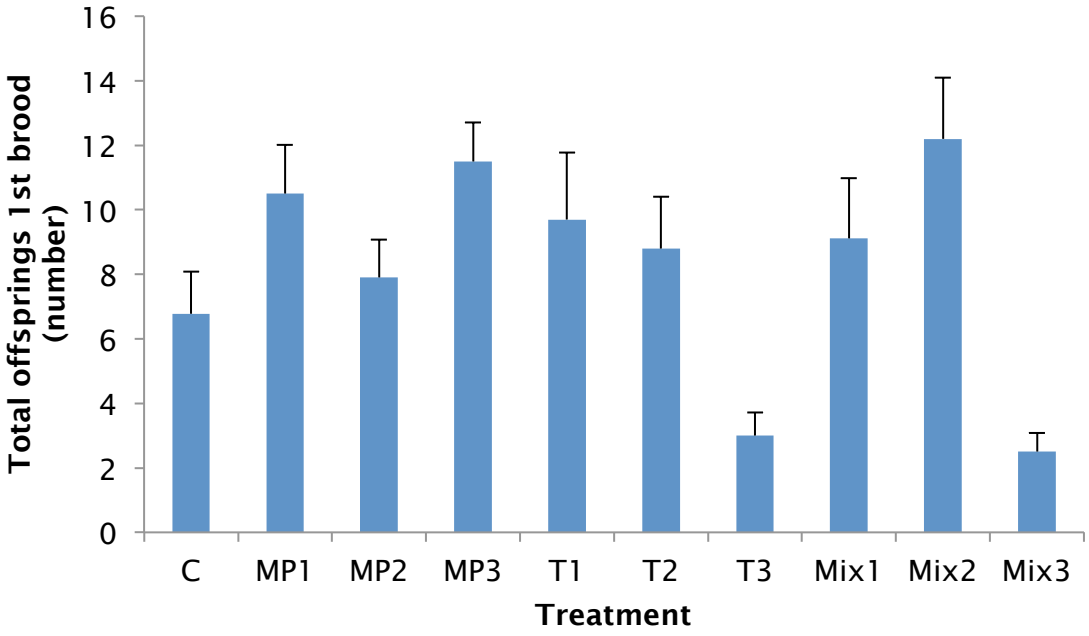

Figure 30: Mean and respective SEM of number of offspring of first brood of *D. magna* exposed for 21 days to microplastics , *M. aeruginosa* extract and mixtures of both.C - control, MP1 – 0.02 mg/L of MPs, MP2 – 0.1 mg/L of MPs, MP3 – 0.2 mg/L of MPs, T1 – 1 μg/L of MC-LR, T2 – 10 μg/L of MC-LR, T3 – 100 μg/L of MC-LR, Mix1 – 0.02 mg/L of MPs + 1 μg/L of MC-LR, Mix2 – 0.1 mg/L of MPs + 10 μg/L of MC-LR, Mix3 – 0.2 mg/L of MPs + 100 μg/L of MC-LR.

4. Discussion

4.1. *M. aeruginosa* extract

At 48 h of the acute bioassay *M. aeruginosa* had a dose-effect response, yet EC_{50} could not be calculated, due to data distribution.

M. aeruginosa extract, after 21days exposure, caused mortality of *D.magna* parental female, did not affect growth, but showed toxicity in reproductive parameters. There were significant differences in the number of broods between the lowest concentration of MC-LR and lowest concentration of microplastics and mixtures, significant differences were also observed between the media MC-LR concentration and control at the number of immobile offspring. Mortality of these organisms when exposed to MC-LR was already described by (Dao et al., 2010) in the same range of concentrations. The exposure to this toxin did not affect the growth in the two lowest concentrations, however in the highest concentration *D. magna* were much smaller until their death, the decrease of growth of these organisms has already been described by (Dao et al., 2010) when exposed to pure MC-LR and by (Becker, 2010) when exposed to *M.aeruginosa*. The release of the first brood was earlier up to approximately 2 days, comparing to control, in the two lower concentrations, data supported by (Dao et al., 2010), however in the highest concentration the day of release was delayed by 4 days, comparing to control, which had already been described by (Becker, 2010; Chen et al., 2005; Dao et al., 2010). On one hand number of broods was slightly higher in the lowest concentration and no differences were observed in media concentration as in (Chen et al., 2005), on the other hand at the highest concentration there were signs of a lower number of broods, if demonstrated it would be supported by (Becker, 2010) that exposure to *M. aeruginosa* leads to a decrease of broods. The total offspring and mobile number of juveniles showed a slight decrease in the lowest concentration and an increase in the media concentration; in the first brood the number of offspring of parental females exposed to the highest concentration was lower than all the other treatments. These values did not lead to any conclusion, in the literature is described a decrease of offspring (Becker, 2010; Chen et al., 2005; Dao et al., 2010) when exposed to higher concentrations of MC-LR or to *M. aeruginosa* cells. The number of immobile juveniles increased when compared to control having significant differences in the media concentration. The number of aborted eggs increased with the concentration but there was no significance in these values, this was also observed by (Dao et al.,

2010). These results caused by microcystins can be due to alteration of enzymes level. *D. magna* are able to form MC-LR-GSH conjugate showed by an increase of GST activity (Sadler and von Elert, 2014) but at high levels of toxin levels of GST decrease (Chen et al., 2005) which leads to the assumption that the concentration of MC-LR reached a limit that the detoxification system could take. MC-LR also leads to oxidative stress (Chen et al., 2005; Sadler and von Elert, 2014). The stress caused by the toxin leads to an increase of LDH (Chen et al., 2005) and a decrease of energy for growth and reproduction that is allocated to fight the stress, detoxification and repair of damages a caused by the toxin.

The use of *M. aeruginosa* extracts with disrupted cells in the concentrations used in the chronic bioassay can be considered environmentally relevant, once pure toxin alone does not occur in the environment and the concentrations used were already found in the environment. The data obtained from exposure to 1mg/L of MC-LR in acute bioassay deserves special attention once this concentration is between the range of concentrations found in a heavily polluted environment during the decay of a bloom.

4.2. Microplastics

In the acute bioassay microplastics showed a non monotonic response of immobilization at 24 h as previously described by (Jemec et al., 2016) and a dose-effect response at 48 h, yet EC_{50} could not be calculated.

After 21 days of exposure of *D. magna* to microplastics it was observed mortality of parental females, reduction of growth and a toxic response in reproduction. Mortality in chronic bioassays was previously described by (Martins and Guilhermino, 2018; Ogonowski et al., 2016; Pacheco et al., 2018), however some authors describe that no mortality was observed as in (Canniff and Hoang, 2018). In this bioassay mortality had a non monotonic response as described by (Jemec et al., 2016) in an acute bioassay. Growth was reduced from 5 to 8,3% compared to control but these differences were not significant. Reduction of growth had already been described in (Besseling et al., 2014) and in (Ogonowski et al., 2016) at low algal concentration, yet the opposite was observed at high algal concentration. Toxicity was observed in all reproductive parameters except in the number of aborted eggs. In some parameters differences were only observed in the lowest concentration, in the other parameters differences were more severe in these concentration, however there were no significant differences to control. Unlike to what (Pacheco et al., 2018) observed and in

agreement to (Ogonowski et al., 2016), in the lowest concentration it was observed a earlier 1st brood. The number of broods decreased as observed by (Pacheco et al., 2018) and by (Ogonowski et al., 2016) with secondary microplastics, on the other hand this author observed an increase of broods with primary microplastics exposure. There was a decrease of total and mobile neonates, significant at the lowest concentration, data supported by (Besseling et al., 2014) in organisms exposed to nanoplastics, by (Pacheco et al., 2018) in organisms exposed to pristine microplastics and by (Ogonowski et al., 2016) in organisms exposed to primary and secondary microplastics. The number of immobile juveniles increased, on average, with the concentration, immobile juveniles were also observed by (Pacheco et al., 2018). Aborted eggs were observed, unlike in (Martins and Guilhermino, 2018). These results caused by microplastics can be due to physical or chemical effects.

Physical effects are resulting effects of the accumulation of microplastics in guts, gills and organisms body. The accumulation of MPs on organisms body can lead to a decrease of mobility and consequent decrease of food ingestion. The effects of accumulation in the guts may lead to a false food satiation causing a decrease in the intake of food already described by (Rist et al., 2017). The reduction of food intake leads to bad nutrition of the parental females and to a reduction of energy available. The bad nutrition of the mothers leads to negative effects on the offspring. In cases of chemical stress *D. magna* can re-allocate the energy, whose destiny was reproduction, to destoxification and repair damages caused by the stress (Martins et al., 2013). The accumulation of MP in gills can impact the consumption of O_2 that was already described.

Chemical effects are resulting effects of the release of chemical compounds contained by MPs, some of them with a known endocrine disruption effect (Pacheco et al., 2018).

Concentrations of microplastics used in the chronic bioassay are higher than the concentrations usually found in aquatic environments, but they are lower than concentrations found in heavily contaminated lakes. That said, it is conclude that concentrations used can be considered environmentally relevant.

Furthermore, once secondary microplastics, whose are more toxic than primary microplastics (Frydkjær et al., 2017; Ogonowski et al., 2016), are more commonly found in aquatic environments, it is expected more pronounced effects in these organism in the environment when exposed to same concentrations.

4.3. Mixture

Acute bioassay with mixtures showed a dose-effect response, however, due to the distribution of data, it was not possible to calculate EC_{50}. However, comparing the first concentration to cause mortality of each contaminant to the mixture of both concentrations, it is presupposed an antagonistic effect of these mixture.

In the chronic bioassay, mixtures caused mortality, alteration in growth and reproductive toxicity. These mixtures show no significant differences to control, except in the number of broods, significant differences are also shown in growth between MP3 and Mix2 and in the number of broods between T1 and Mix1, treatments with the same concentration of MC-LR, and between T1 and Mix2. Mortality between the two lowest concentrations of mixtures did not show differences but at the highest was much higher reaching 100 %. Growth was higher than control, from 1 to 2 % higher, but showed a tendency to decrease. The release of first brood was slightly delayed in the two lowest concentrations but in the highest concentration it was delayed by approximately 5.7 days. The number of broods was decreased in comparison to all the other treatments. The total number and number of mobile juveniles was between the values obtained from exposure to microplastics and *M. aeruginosa* extracts, comparing to control it was lower in the lowest concentration and higher in the media concentration of mixtures. Immobile juveniles showed a tendency to increase as in the exposure to contaminants alone. The number of aborted eggs increased comparing to control.

The observation of microplastics in daphnids gut when exposed to mixtures was much lower than when exposed to microplastics only, however it occurred. This decrease of accumulation was already expected, once the existence of food or the increase of *algae* concentration leads to a higher egestion (Scherer et al., 2017).

Data obtained in the parameters: day of first brood in the highest concentration (0.2 mg/L of MPs + 0.1 mg/L of MC-LR) and the total number of offspring, number of mobile juveniles and number of aborted eggs in the two lowest concentrations (0.02 mg/L of MPs + 0.001 mg/L of MC-LR and 0.1 mg/L of MPs + 0.01 mg/L of MC-LR); shows an amplification of the effects of the exposure to *M. aeruginosa* extracts. This could be explained with the ingestion of MPs that adhere the toxin or other compounds of the cyanobacteria and indirectly lead to an ingestion of higher concentrations, leading to higher toxicity.

Chronic bioassay was considered environmental relevant once both contaminants were already observed in these concentrations in aquatic environments and because they co-exist in the same environments.

5. Conclusion and future perspectives

Based on the initial hypothesis and the initially defined objectives, throughout this MSc Thesis, it is sought to understand if exposure to mixture of *M. aeruginosa* and MPs evidence interactions of both contaminants when compared to the effects of exposure to these contaminants separately.

It was possible, through the results obtained, to prove that these contaminants have toxic effects in *D. magna* when exposed acutely and chronically.

The results obtained from exposure to the mixture of the contaminants evidenced their interactions in the bioassays. In the acute bioassay, an antagonistic effect can be presupposed. However, more studies are required with different concentrations, in a middle range from the one used. In the chronic bioassay, mixtures pointed to an amplification of the effects of *M. aeruginosa* in some parameters, but more studies are required for better understanding of these interactions.

Of highlight the co-existence of these contaminants and of what is known about their bioaccumulation in aquatic organisms, the results obtained increased the concern of environmental and human health and serve as an alert to an eminent danger.

In the future will be relevant to study the acute effect in a reduced range of concentrations of the ones used, study the mechanisms of these interactions and try to understand it, as well as study the possible bioaccumulation throughout the food chain.

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