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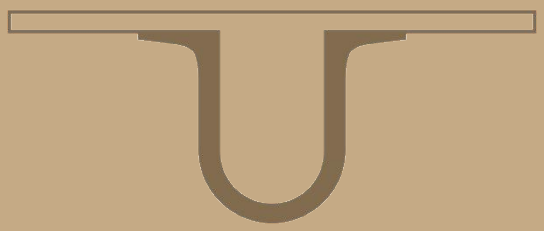

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# THE EFFECT OF MICROPLASTIC ON TERRESTRIAL AND AQUATIC ORGANISMS

VOLUME 1

Dissertação no âmbito do Mestrado em Ecologia orientada pelo Professor Doutor José Paulo Sousa e pelo Doutor Tiago Natal de Luz e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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**Resumo**

Os microplásticos (<5 mm) são resultado da atividade antropogénicas e constituem um problema em ambientes marinhos, de água doce e terrestres. No entanto, há uma escassez de informação sobre o impacto dos microplásticos no solo e nos organismos de água doce. O presente trabalho estudou os riscos potenciais do microplástico polietileno em concentrações de até 10% para as espécies padrão de invertebrados de solo *Eisenia andrei*, *Folsomia candida* e *Enchytraeus crypticus* e o efeito de polietileno e poliestireno nas concentrações de 0,37 mg/mL e 0,0005 mg/mL, respetivamente sobre a lentilha de água doce *Lemna minor* e o anfípode *Gammarus pulex* através da transferência trófica de microplásticos de lentilha de água pré-exposta aos microplásticos em condições de laboratório.

A reprodução de *E. andrei* e *E. crypticus* foi significativamente reduzida e nenhum outro efeito foi detetado nos organismos do solo após a sua exposição a microplásticos. Os dados sugerem que minhocas e enquitreídeos podem alocar energia para manter a biomassa, a fim de melhorar a resistência contra MPs, condicionando assim a sua reprodução.

Não foram detetados efeitos negativos de microplásticos no crescimento e desempenho fotossintético da lentilha *L. minor* após 7 dias de exposição. A experiência de transferência trófica não mostrou acumulação consistente de microplásticos no intestino de anfípodos, porém pequenas quantidades de partículas de microplásticos foram transferidas das lentilhas para os anfípodos, sugerindo que, embora a baixo nível, os microplásticos podem ser transferidos de plantas para animais.

**Palavras-chave**: *Eisenia andrei*; *Enchytraeus cripticus*; *Folsomia candida*; Reprodução; Histopatologia; Consumo de oxigénio; *Lemna minor*; taxa de crescimento relativo; *Gammarus pulex*; transferência trófica.

**Abstract**

Microplastics (<5 mm) are one of the growing anthropogenic litters in marine, freshwater and terrestrial environments. However, there is a scarcity of information on the impact of microplastics to soil and freshwater organisms. The present work studied the potential risks of the microplastic polyethylene at concentrations up to 10 % to the standard soil invertebrate species *Eisenia andrei*, *Folsomia candida* and *Enchytraeus crypticus* and the effect of polyethylene and polystyrene at concentrations 0.37 mg/mL and 0.0005 mg/mL, respectively on the freshwater duckweed *Lemna minor* and the amphipod *Gammarus pulex* through trophic transfer of microplastics from duckweed pre-exposed to the microplastics in laboratory conditions.

The reproduction of *E. andrei* and *E. crypticus* significantly decreased and no other effects were detected in the soil organisms after their exposure to microplastics. Data suggested that earthworms and enchytraeids might have allocated energy into keeping biomass in order to improve resistance against MPs, conditioning by this way its reproduction.

No negative effects of microplastics were detected on growth and photosynthetic performance of duckweed *L.minor* after 7 days of exposure. The trophic transfer experiment did not show consistent accumulation of microplastics in gut of amphipods, however small amounts of microplastic particles were transferred from the duckweed to the amphipods suggesting that, although at low level, microplastics can be transferred from plants to animals.

**Keywords**: *Eisenia andrei; Enchyatraeus cripticus; Folsomia candida*; Reproduction; Histopathology; Oxygen consumption; *Lemna minor*; relative growth rate; *Gammarus pulex*; trophic transfer.

**Thesis framework**

The thesis is divided into three chapters. Chapter I is composed by a general introduction that comprises information regarding microplastic pollution and consequences worldwide. This chapter addresses the sources of microplastic pollution, accumulation in the environment and the impacts of microplastics to biota in general.

Chapter II addresses the issues regarding microplastic contamination in soil environments and the negative effects of microplastics to soil biota. This chapter gives all the information regarding the study on soil invertebrates developed and comprises methods, results, discussion and conclusion.

Chapter III addresses the microplastic contamination in freshwaters and the impact of microplastics to freshwater biota. In this chapter, all the experiments with freshwater organisms developed are described and discussed. The chapter contains methods, results, discussion and conclusion.

# CHAPTER I
# GENERAL INTRODUCTION

# 1 CHAPTER I

## General introduction

### Plastic pollution overview

Plastic pollution is an emerging global environmental concern. Plastics are materials based on polymers and various types of chemicals are added to adjust them into different purposes. Plastic materials within polymer classifications can vary in structure depending on the types and quantities of chemicals used in the production (Wagner & Lambert, 2018).

The cheap price, durability and plasticity make plastic materials suitable for variety of products (Cozar *et al.*, 2014). Nowadays, we use plastic materials in household, clothing, packaging and many other products we use, might contain some amount of plastic products. The most dominated plastics in market are polyethylene (PE, high and low density), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyurethane (PUR) and polyethylene terephthalate (PET) (Alison *et al.*, 2015). The worldwide production of plastic materials in 2015 accounted for 322 million tons in comparison to 1.5 million tons in 1950 (see **Fig. 1**). In 65 years plastic production increased rapidly with annual growth 8.6 % (Growth, 2015). Moreover, 50 % of plastic products are made just for a single use (Thompson *et al.*, 2009). Alone in Europe, in 2014 only 29.7 % of plastic materials were recycled, and landfilling is the still first option in many European countries (PlasticsEurope 2016).

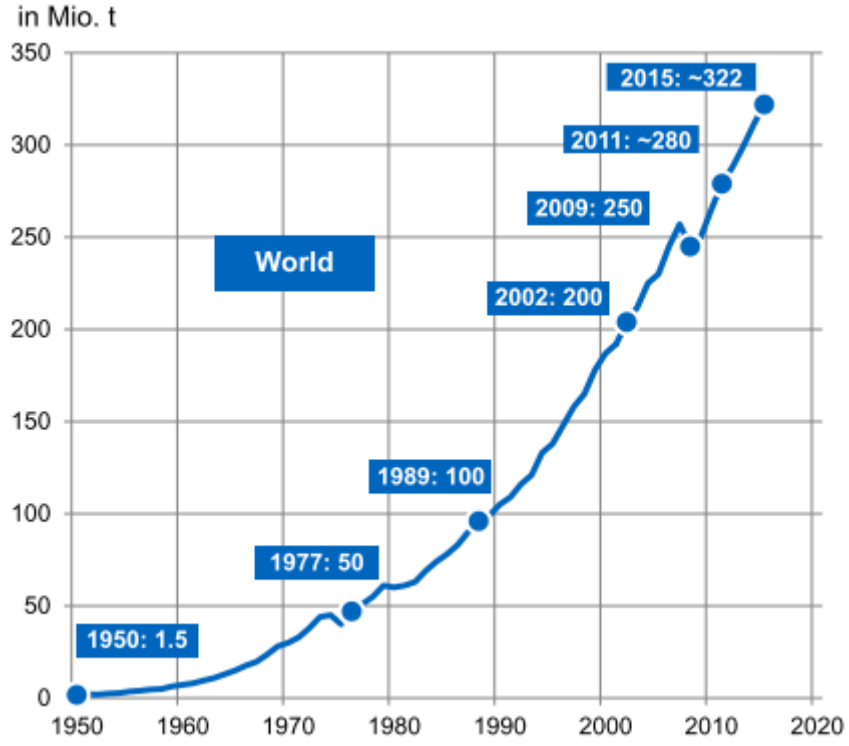


**Fig. 1. Plastic production between 1950 and 2015 (Growth, 2015)**

## 1.1 Microplastics pollution as an emerging concern

In contemporary society, humankind faced a new environmental issue – microplastic pollution. Microplastic debris is one of the growing anthropogenic litters in marine, freshwater and terrestrial environments (Lassen *et al.,* 2015). According to the National Oceanic and Atmospheric Administration (NOAA), microplastic (MP) has been defined as a plastic material less than 5 mm in size (Wright *et al.,* 2013). MPs occur in the environments due to release of primary MPs, which are manufactured in small size or due to fragmentations of larger plastic materials. Once microplastics enter the environments, it is almost impossible to remove them. Because, nowadays used wastewater treating plants do not have efficient techniques to detect tiny MPs (Browne *et al.,* 2011). Moreover, after they end up in deep seas or oceans it is even not possible to eliminate them.

Microplastics have been found in the world's oceans, in freshwaters and in soils and in the most distant islands. For example, Hawaii Island's Kamilo Beach sediment contained in average 3.3 % (w/w) of microplastics with a maximum concentration 30.2 % (w/w) (Carson *et al*., 2011). Also, high concentrations of microplastics were found in ocean gyres, Eriksen *et al*. (2014) have shown MP contamination in all five subtropical gyres of world oceans (North Pacific, North Atlantic, South Pacific, South Atlantic, Indian Ocean). According to their predicted model, it is estimated that at least 5.25 trillions of plastic debris in mass 268,940 tons are floating at sea, and from this 35,540 tons are MPs in size < 4.75 mm. (Eriksen *et al.,* 2014). Similar to that study, Cozar *et al.* (2014) has estimated that the world oceans contain between 7000 and 35000 tons of MPs (Cozar *et al.*, 2014).

If we take into account that plastic materials fragmentize due to abiotic and biotic factors, then in the future, amounts of MP might increase several times.

### 1.1.1 Primary Microplastics

Primary MPs are products manufactured in size < 5 mm, and they could enter the environment directly. Primary MPs produced to satisfy several needs, such as air blasting technologies (Out *et al.*, 2013), in cosmetics, such as toothpaste and scrubs (Leslie, 2014). These skin care products might contain up to 12 % of microplastic particles in size between 450 and 800 μm (Gouin *et al.,* 2015). All over the world products with microplastics are highly used. For instance in 2012, in Denmark 29 tons of skin care products with additions of microplastics were sold (Lassen *et al.,* 2015). Eventually all these products with microplastics after usage end up in wastewater treatment plants. Taking into account that there are no ways to detect tiny MPs,

wastewaters are the biggest contributors of MPs into terrestrial and aquatic environments (Browne *et al.,* 2011).

Another source of primary MP is raw materials for production of plastic materials. This industry is the largest use of primary MPs, it accounts for almost 550,000 tons per year (Lassen *et al.*, 2015). Pre-production plastics could be in form of pellets, plastic powders and they can enter the environment during transportation, storage, loading and due to accidental losses as well as inappropriate handling (Duis and Coors, 2016). As a consequences, the beaches near plastic industries were identified with the highest concentrations of pre-production pellets (Van Cauwenberghe *et al.*, 2015).

### 1.1.2 Secondary Microplastics

The commercialization of plastic industry, lack of waste management and simple careless littering are the most important sources of microplastics in the environment. Secondary MPs are debris of larger plastic materials, fragmented into sizes less than 5 mm. Secondary MPs are generated after fragmentation of larger plastic materials due to different environmental factors, such as UV radiation and other abiotic factors (Out *et al.,* 2013). Moreover, any mechanical forces such as animal bite and human activity might cause fragmentation of plastics materials. (Alison *et al.,* 2015).

However, until today, there are no models are able to predict the precise time of plastic fragmentation in different environments. Also, there is very few knowledge about the behavior of plastic due to abiotic factors such as salinity, temperature and impact of chemicals used in production of plastics.

## 1.2 Increasing concern: Microplastics in freshwater and terrestrial environments

Microplastic contamination is a well-known threat to marine environment. However, it has been estimated that 80 % of plastic loads are land based and only 20 % of plastic loads are oceanic sources (Allsopp *et al.,* 2006). Although, microplastic related researches are mainly focused on marine environments. Therefore, there is a lack of information on the abundance of MP in freshwater environments and terrestrial ecosystems. It has been estimated that from all researches on microplastics, only 3.7 % of papers on MPs in freshwaters (Wagner and Lambert, 2018). The information about MPs in terrestrial systems is even scarce. However, the fragmentation processes of plastic materials might be faster in soils and shallow lakes than in the oceans. It might due to drastic temperature changes in soils and higher sun exposer in shallow lakes (Horton *et al.*, 2017).

Existing knowledge on MP considers terrestrial and freshwater ecosystems as a source of microplastics to the oceans (Horton *et al.,* 2017). For instance, River Danube was found to discharge into the Black Sea up to 4.2 t of plastic per day (Lechner *et al.,* 2014). Another study estimated that annual MP input to the oceans via rivers is between 1.15 and 2.41 million t (Lebreton *et al.,* 2017).

MPs contamination in soils is an emerging concern. Soil seems to be a long term sink for microplastics (Zubris and Richards, 2005; Nizzetto *et al.,* 2016), huge sources of MPs derive to soils from wastewaters treatment plants. It has been estimated that the annual input of MPs from sewage sludge is up to 430 000 tons in Europe. This is higher than MP loads in the oceans (Nizzetto *et al.,* 2016).

There is a vast scarcity on the quantities and distributions of plastic debris in landfills and freshwaters. Thus, plastic contamination issues needs more attention and profound researches on the quantity and distribution of plastic debris in freshwaters and terrestrial environments.

### 1.3 Impacts of Microplastics on biota

The first attention to plastic debris as a threat to biota started after the evidences of plastic ingestions by seabirds worldwide (Day *et al.,* 1980; Baltz and Morejohn, 1976; Gray *et al.,* 2012). One of the first investigations was done in Alaska where Day *et al.* (1980) revealed that 15 species from 37, in total 448 birds from 1968 individuals had some amounts of plastic debris in their stomachs.

Until now the adverse effects of microplastics to biota is well documented. There are many studies confirming negative effects of MPs to various aquatic (Derraik, 2002) and terrestrial organisms (Huerta Lwanga *et al.,* 2016), including inhibited growth, bioaccumulation, lowered feeding and reproduction (Wright *et al.,* 2013; Farrell and Nelson, 2013; Setälä *et al.,* 2014; Gregory, 1991; Anbumani and Kakkar, 2018). For example, Sussarellu *et al.* (2016) revealed adverse effects of polystyrene (PS) (2-6 μm) at concentration 0.023 mg per L on oysters after 30 days of exposure. There were the negative effects, including 38% lowered oocyte number, 23 % decreased sperm velocity, also the offspring of the exposed parents showed decrease in D-larval yield and larval development (Sussarellu *et al.,* 2016). Also, negative impacts of PS (30 nm) on mussels (*Mytilus edulis*) after 8 hours exposure at concentrations up to 0.3 g per L showed reduced filtering activities (Wegner *et al.,* 2012).

Due to small size and resemblance to prey, microplastics get ingested easily by variety of organisms (Moore *et al.,* 2001). Many studies confirm that MPs are mistaken for food by variety of animals, including fish, birds, turtles, mammals and invertebrates (Bergmann *et al.,* 2015).

According to the study, at least 267 species worldwide, with 86 % of all sea turtle species, 44 % of all seabird species, and 43 % of all marine mammal species are affected by plastic contamination (Laist, 1997).

## 1.4 Aims of the study

The main goal of this study is to evaluate the potential risk of two of the most abundant microplastics (polyethylene, polystyrene) on soil and freshwater organisms. The general objective is to evaluate the effects on standard organisms through the measurement of several endpoints in biological tests with different terrestrial and aquatic invertebrate species in laboratory conditions.

Despite the aim of evaluating possible impacts of microplastics on terrestrial and aquatic organisms, results obtained from ecotoxicological tests will be discussed separately for soil and freshwater organisms due to physiological and habitat differences of the tested organisms.

# CHAPTER II
# IMPACT OF MICROPLASTICS ON SOIL ORGANISMS

# 2 CHAPTER II

## Introduction

## 2.1 Microplastic contamination in soil environments

There is scarce information on the abundance of microplastics in soil. Significant amount of MPs in soils is originated through the application of residues from wastewater treatment plants as soil organic improvements. According to Brawne *et al.* (2011) one single use of a washing machine could release 1900 fibres to waste water treatment plants. A study in USA has estimated that 808 trillion MPs might be washed every day from household to waste water treatment plants. From this 8 trillion MPs are estimated to enter water surfaces, while 800 trillion of MPs are likely to settle into the sludge (Rochman *et al.*, 2015). The behavior of MPs in wastewater treatment plants depends on their density. While heavier MPs are retained within the sludge, the lighter MPs float to the surface waters. It has been identified that 90 % of MPs are retained in sewage sludge (Carr *et al.*, 2016).

The sewage sludge is often used as a fertilizer in agriculture due to economic benefits. Nizzetto *et al.* (2016) reported that Europe and North America apply 50 % of sewage sludge for agricultural purposes. Also the same research estimated that annual input of MPs from sewage sludge is between 63 000 and 430 000 tons in Europe. This is higher than MP loads in the oceans (Nizzetto *et al.* 2016). Moreover, MPs resulting from sludge applications can accumulate in soil for a long time. A study developed by Zubris and Richards (2005) showed that microplastics were detectable in soil after 15 years of sludge application. Applying sewage sludge in long term might cause huge amounts of MPs due to accumulation.

Another significant source of MPs in soil is fragmentation of plastic materials used for agricultural purposes (Horton *et al.*, 2017). Plastic materials might be used with different purposes in agriculture like to keep soil moisture or to control soil temperature. Due to abiotic factors such as high temperature and humidity, plastic materials tend to fragmentize and sink to soil with time (Horton *et al.*, 2017). This reality has been shown in agricultural lands in China practicing full plastic coverage (e.g. Huerta Lwanga *et al.* 2016; **Fig. 2**).

Thus, the effect of MP on soil biota should be an issue of concern.


**Fig. 2. a) Soil surface covered by plastic in an agricultural land in Shanghai province, China, b) Fragmented plastics. Credits: Huerta Lwanga *et al.*, 2016.**

## 2.2 Impact of microplastics on soil biota

Impacts of microplastics were mainly studied on aquatic organisms and there is a scarcity of information on soil biota. However, soil biota might be vulnerable to MPs contamination as well (Rillig, 2012). One of the first works on the impact of MPs to soil biota, Huerta Lwanga *et al.* (2016) observed up to 25 % mortality after 60 days of exposure to <150 μm polyethylene (PE) at 60% (w/w). Also, Cao *et al*, (2017) found adverse effects of 58 μm polystyrene (PS) at concentrations 1 % and 2 % on survival and growth of earthworms from the species *E. fetida* in an agricultural soil.

However, another study on earthworms (*E. andrei)* reported no significant impacts of PE (250-1000 μm) at concentrations up to 0.1 % (w/w) in OECD artificial soil to survival, growth and reproduction after 28 days of exposure (Rodriguez-Seijo *et al.*, 2017). Although, that study discovered some damages in gut tissue and immune system responses at concentrations ≥ 0.01 % (w/w).

Beyond earthworms, other soil organisms, such as mites, collembolans, or enchytraeids might be impacted by MPs contamination (Rillig, 2012). A recent study reported decreased reproduction and altered gut microbiota in the collembolans *Folsomia candida* after exposure to

polyvinyl chloride (80–250 μm) at a concentration 0.1 % (w/w) after 28 and 56 days respectively. (Zhu *et al.,* 2018).

The impact of MPs to soil organisms seems to be not straightforward. Without any visible effects, MPs could impact organisms at molecular level, causing stress or changing bacterial diversity in organisms (Rodriguez-Seijo *et al.,* 2017;.Zhu *et al.,* 2018). These adverse impacts of MPs on soil biota are far from well understood, both in short and long term exposures. Therefore, further researches are needed in this area.

## 2.3 Standard species

The standard species, *Eisenia andrei, Enchytraeus crypticus,* and *Folsomia candida* are some of the most species used in soil ecotoxicological tests. Because of that, these species may be also adequate to evaluate the impact of MPs in soil. These soil invertebrates are important model species for assessing the effects of pollutants on soil environment because they play important functional role in ecosystem, including litter decomposition, nutrients cycling, improving soil structure and water infiltrations.

### *Eisenia andrei*

Earthworms are important members of the soil community; they are considered ecosystem engineers due to their ability to improve soil properties (Jones, Lawton, & Shachak, 2012).

The species *Eisenia andrei* (**Fig 3**) has a mean length between 60 and 120 mm and a diameter of 3 to 6 mm (Jänsch *et al.,* 2005). The tolerance to a wide range of temperature and moisture and its easiness to be handle and kept under laboratory conditions allowed *Eisenia andrei* to be a model organism in ecotoxicology (Domínguez *et al.,* 2005).

**Fig. 3. Earthworms *Eisenia andrei*; Credit:B.Pohl, ECT**

Moreover, another reasons to use earthworms species in laboratory bioassays are: breeding of some species in laboratory is relatively easy; standardized guidelines for tests with earthworms have been developed by OECD and International Organization for Standardization (ISO); earthworm stress reactions against contaminants presence are measurable, earthworm species are usually tolerant to low levels of contamination (Römbke *et al.,* 2005).

### *Enchytraeus crypticus*

Enchytraeids are soil-dwelling invertebrates, widespread in many soil types with 950 species described worldwide (Jänsch *et al.,* 2005). On average the body size of Enchytraeus crypticus (**Fig. 4.**) is about 7 mm. They are well known to reproduce very fast with an embryological development time of 9.1 days in average (Jänsch *et al.,* 2005).

**Fig. 4. *Enchytraeus crypticus* ; Credit: Ecotox 2016-10;**

Enchytraeids are generally important in organic matter decomposition and soil bioturbation (Castro-Ferreira *et al.,* 2012). Also, Enchytraeids are known to improve plants growing by aerating soils, and improving the small scale water (Jänsch *et al.,* 2005).

*E. crypticus* is used in ecotoxicological tests due to the advantages such as easy handling in laboratory conditions, high reproductive rates, short life-cycle (which allow short period tests), and high tolerance range to soil pH, texture, and organic matter content (Castro-Ferreira *et al.,* 2012).

### *Folsomia candida*

Known as springtails, Collembolans are widely distributed soil arthropods. They are part of soil decomposers, well known for breaking down and recycling organic wastes (Jänsch *et al.,* 2005).

The species *F. candida* (**Fig. 5**) is 1.5 to 3.0 mm and the population consists of only parthenogenetic females. The species reproduces fast and has the highest abundances in comparison to other collembolan species. The eggs of *F. candida* hatch in 7 to 10 days. The

lifespan of the organism depends on temperature, at 15◦C they can live about 240 days, while at 24◦C they live only 111 days (Fountain & Hopkin, 2005).

**Fig. 5. *Folsomia candida*, Credit:Enfo.agt.bme.hu**

*F.candida* has been used as a model organism to assess the impact of pesticides and other pollutants for more than 40 years (Fountain & Hopkin, 2005). The species is easy to maintain and reproduce in laboratory conditions.

## 2.1 Objectives

The main goal of this study is to evaluate the impact of polyethylene (PE) to soil invertebrates, namely to the earthworms *Eisenia andrei*, the potworms *Enchytraeus crypticus*, and the springtails *Folsomia candida.*

More specific objectives of the study are as following:

1. To study survival, weight change, histopathological analysis of gut tissues of surviving adult earthworms and reproduction after 28 and 56 days respectively.
2. To assess the mortality and reproduction of collembolans after 28 days of exposure.
3. To study reproduction and oxygen consumption of enchytraeids after 28 days of exposure.

## 2.4 Materials and Methods

### 2.1.1 Materials

**Soil**

For the laboratory ecotoxicological tests artificial soil was prepared and used as substrate. The preparation of artificial soil followed requirements described by ISO 16387 (ISO, 2012). The artificial soil was composed by 5 % of *Sphagnum* peat, (previously air-dried and sieved at 5 mm), 20% of kaolinite clay, 74 % of industrial quartz sand (oven-dried and with more than 50 % mass fraction having particle size 0.05 mm to 0.2 mm), and up to 1.0% of calcium carbonate ($CaCO_3$, pulverized, analytical grade) to obtain a pH of 6.0 ± 0.5.

**Test substance**

The microplastic used in the present study was the Polyethylene 40-48 μm in dry powder (Sigma-Aldrich, Lisbon, Portugal, 2018). These MPs were non-colored and ultra-high molecular weight with a density of 0.94 g/mL at 25 °C.

**Test Organisms**

*Eisenia andrei*, *Enchytraeus crypticus*, and *Folsomia candida* were the standard species used in the laboratory tests. These organisms were maintained in a culture room in the University of Coimbra. The species were kept at 20 ± 2$^{o}$C with a photoperiod of 16:8h, light:dark. The organisms were maintained as described by (Renaud *et al.*, 2017).

*Eisenia andrei* Bouché (Annelida, Clitellata, Oligochaeta, Lumbricidae) cultures were maintained in plastic boxes with a substrate composed of cow manure (previously defaunated by two freeze and thaw cycles) and *Sphagnum* sp. peat (1:1, w:w) and a small amount of $CaCO_3$ to raise the pH. Cultures were fed with cow manure and moisture was adjusted weekly. Earthworms with a well-developed clitellum, more than one month old and with an average weight between 250 and 600 mg were used for testing.

*Enchytraeus crypticus* Westheide & Graefe (Annelida, Clitellata, Oligochaeta, Enchytraeidae) cultures were maintained in petri dishes with agar medium. Cultures were fed weekly with finely ground rolled oats. For testing, organisms with a visible well-developed clitellum were selected

*Folsomia candida* Willem (Arthropoda, Hexapoda, Collembola, Isotomidae) cultures were maintained in plastic boxes with the bottom filled with a mixture of plaster of Paris and activated charcoal in a proportion of 11:1 (w:w). Springtails were fed weekly with granulated dry

yeast and moisture was adjusted weekly. For laboratory tests, 10–12 day old individuals from synchronized cultures were used for testing.

### 2.1.2 Experimental design

The gradients of increasing concentrations of MPs used for laboratory ecotoxicological tests are presented in **Table 1**. Soil-polyethylene mixtures were prepared by manually mixing (with the help of a spoon) both components (polyethylene and artificial soil) in the right proportions in order to obtain the desired concentrations in a large container over several minutes until obtain a visually homogeneous mixture. The water-holding capacity of the mixtures was determined following procedures described in ISO (2012). The procedures adopted in the reproduction tests followed standard ISO guidelines and are summarized in **Table 2**. Laboratory tests were conducted under the same conditions of temperature and photoperiod used for culture maintenance. All test vessels were covered with a lid and weighted at the beginning of the test to allow the reestablishment of water losses over the test period. Soil pH and water content of test mixtures were measured at the beginning and at end of each test.

**Table 1. Concentrations used in the reproduction tests with Eisenia andrei, Enchytraeus crypticus and Folsomia candida.**

| | *E. andrei* | *E. crypticus* | *F. candida* |
|---|---|---|---|
| Concentrations (%, w/w) | 0.5; 1; 2.5; 5; 10; | 0.5; 1; 2.5; 5; 10; | 0.5; 1; 2.5; 5; 10; |
| Concentrations (g) | 2.5; 5; 12.5; 25; 50; | 0.13; 0.26; 0.65; 1.3; 2.6; | 0.13; 0.26; 0.65; 1.3; 2.6; |

**Table 2. Procedures adopted in reproduction tests with *Eisenia andrei, Enchytraeus crypticus* and *Folsomia candida.***

| | *E. andrei* | *E. crypticus* | *F. candida* |
|---|---|---|---|
| Guideline considered | ISO 11268-2 (ISO, 1998). | ISO 16387 (ISO, 2012). | ISO 11267 (ISO, 1999). |
| Test period (day) | 56 | 28 | 28 |
| Test containers (cm) | 11 × 12 (D × H) | 5 × 9 (D × H) | 6 × 7 (D × H) |
| Number of replicates per treatment | 4 | 5+1[a] | 5+1[a] |
| Number of organisms per replicate | 10 | 10 | 10 |
| Food source | Cow manure | Rolled oats | Dry yeast |
| Food per test container (g FW) | 15 | 0.001 | 0.002 |
| Days of food supply | 7th, 14th, 21th;28th; | 7th, 14th, 21th | 7th, 14th, 21th |
| Days of aeration and moisture reestablishment | 7th, 14th, 21th;28th; | 7th, 14th, 21th | 7th, 14th, 21th |
| Soil per test container (g DW) | 500 | 26 | 26 |

D – diameter, H – height. [a] Additional replicate without organisms to control soil pH and moisture content at the end of the test.

At the 28th day of *E. andrei* reproduction tests, surviving adults were removed from test vessels, counted, washed and weighted to determine changes in body mass. After removing adults, the test vessels containing the cocoons remained in the incubation chamber for an additional four week period, after which the test vessels were placed in a water bath between 50 and 60°C to extract and count juveniles

For reproduction tests with *E. crypticus*, at the end of the test (after 28 days), the content of each test vessel was preserved with a 70% ethanol solution and 200–300 µL of Bengal red (1% solution in ethanol) were added to stain the test organisms. The total number of individuals in each replicate was determined following the procedures described by Chelinho *et al.* (2014). Briefly, samples were wet sieved to remove small particulates and increase clarity. After sieving, stained organisms were transferred to petri dishes and counted using a binocular microscope (**Fig. 6**).

**Fig. 6. Method for counting adults and juveniles of *Enchytraeus crypticus* at the end of the reproduction test.**

In reproduction tests with *F. candida*, at the end of the test period, the content of each replicate was transferred to a plastic vessel and filled with water (see **Fig. 7**). In order to increase contrast between the water surface and floating springtails a few drops of blue ink were added. Floating adults were counted to determine adults survival and photographs were taken through which the total number of juveniles was determined using the open source software Image J.

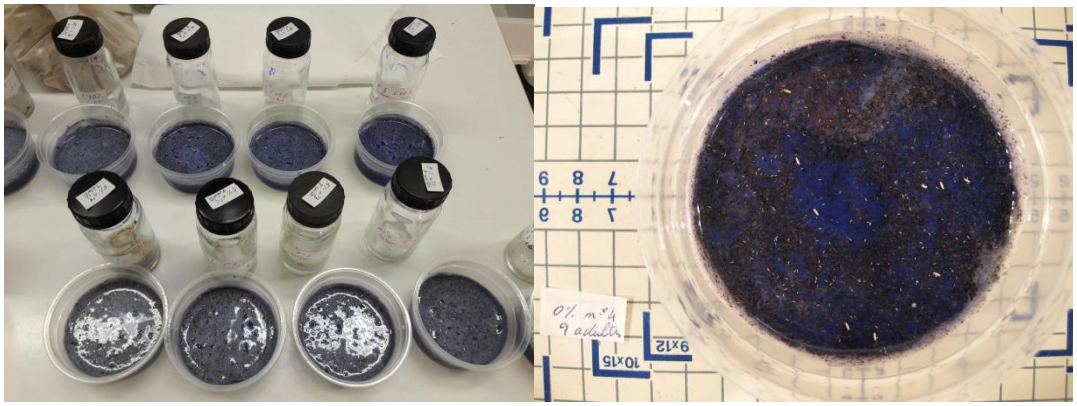

**Fig. 7. Method for counting juveniles of *Folsomia candida* at the end of the reproduction test**

## Histopathological analysis of gut tissues of adult surviving earthworms *E. andrei*

### Tissue preparation

Three earthworms were sampled from each treatment for gut tissue analysis. The gut tissue analysis followed all the procedures described by Briones and Álvarez-Otero (2018). The earthworms were washed in distilled water and left on wet tissue paper for gut depuration for 24 hours. After that period, each individual was anesthetised with a 0.03% tricaine methanesulfonate solution (MS-222, Sigma) before being killed and dissected. Three sections (thick slices) from the pre-clitellar region (regions immediately before the clitellum) and 6 from the post-clitellar region (region immediately after the clitellum) were excised. This procedure was adopted to allow the examination of the gut along its whole length. Then the tissue materials were fixed in an aqueous Bouiṅs solution, dehydrated and finally embedded in paraffin. Histological slices (7–10 μm thickness) were cut by means of a Leica Rotary Microtome (Model RM 2145, Leica, Germany). Slides were deparaffinised and rehydrated prior to the haematoxylin eosin staining process, following the routine histochemical procedures of periodic acid-Schiff's reagent (PAS), Alcian Blue at pH 2.5 (AB) and PAS-AB (pH 2.5).

### Morphometric analyses of the gut tissues

The quantitative analysis of the gut tissues was performed under a light microscope (Olympus BX51, Japan) equipped with a DP71 digital camera (Olympus, Japan) and using a micrometre eyepiece with medium-high power magnification. The parameters measured were intestinal epithelium thickness (InE), typhlosole length (TyL) and typhlosole epithelium thickness (TyE) using a 40x objective (see **Fig. 8**). In order to consider variability among and within treatments three individuals from each treatment were studied and 10 measurements were performed in each individual for each parameter (InE, TyL and TyE).

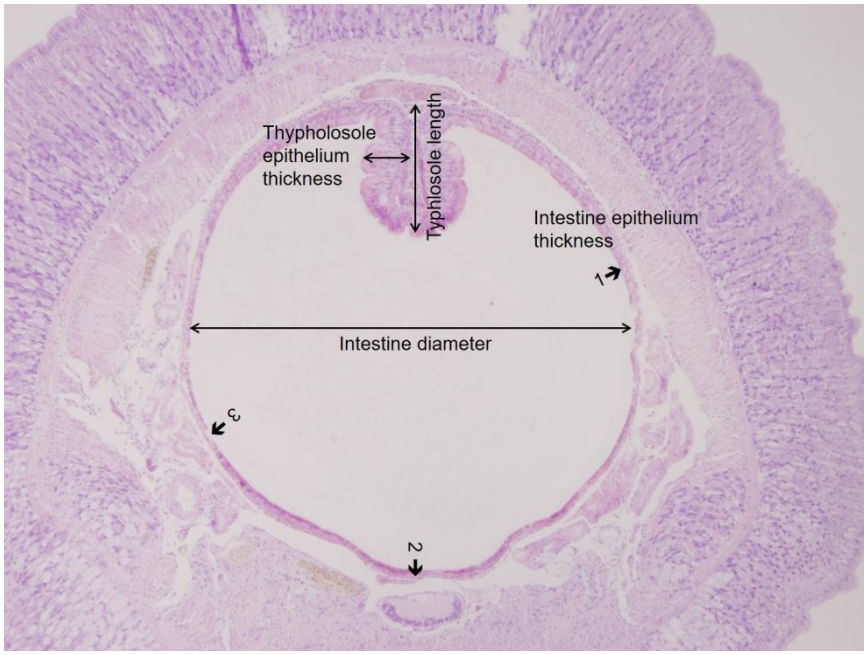


**Fig. 8. Measured endpoints of gut tissues of earthworms *Eisenia andrei*, Intestinal epithelium thickness (InE), Typhlosole length (TyL), and Typhlosole epithelium thickness (TyE).**

### Measuring oxygen consumption by surviving *E. crypticus*

Oxygen consumption by *E. crypticus* was measured according to the protocol described by Palikaras and Tavernarakis (2016) using a Clarke-type oxygen electrode (Oxygraph plus, Hansatech Instruments, UK). Ten potworms (either surviving adults or juveniles) were randomly sampled from each treatment and resuspended in 1 mL of 25 mM $K_2HPO_4$, 25 mM $KH_2PO_4$, and 100 mM NaCl, pH 6.8 and transferred into the chamber. Respiration was measured at 20°C, and the oxygen consumption rates were automatically calculated by using the "Rate Cursors" function in the Oxygraph program (Hansatech).

### Statistical analysis

The significant differences in reproduction of test organisms (number of juveniles), percentage of initial earthworm biomass and oxygen consumption rates between treatments (soil-PE mixtures) and control were evaluated by one-way ANOVA analysis. When differences were detected a Dunnett's post hoc test was used to identify treatments where significant differences compared to control were found. Differences of measurements of the different parts of the earthworm gut tissue were tested by nested ANOVA using individual and treatment as factors, being the first nested in the second one.

Normality and homogeneity of data was checked before statistical analysis using Shapiro-Wilks and Bartlett tests, respectively. All statistical analysis was performed using the software R 3.3.1.

## 2.5 Results

The reproduction test with the soil invertebrate specie *Eisenia andrei*, *Enchytraeus crypticus*, and *Folsomia candida* fulfilled all the validity criteria described in the ISO guidelines (ISO 11268-2, 1998; ISO 16387, 2012; ISO 11267, 1999), respectively.

For earthworms, at the end of 28 days of exposure to the gradient of soils with polyethylene (PE) 100 % of adult survival was found in control replicates and in average 98% of survival was recor ded in replicates of treatments with PE.

The earthworms from both from control and test treatments reached the end of the test without significant differences in body mass (ANOVA, $F_{5,18} = 0.541$, $P = 0.743$). There were no differences of biomass changes (or % of initial weight) (ANOVA, $F_{5,18} = 0.226$, $P = 0.946$) at the end of the experiment between treatments and control (**Fig. 9**).

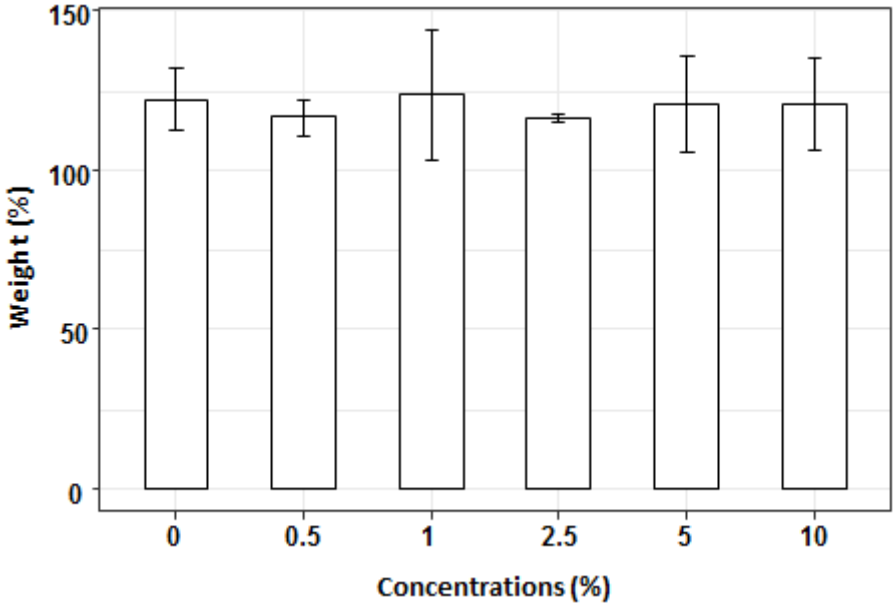


**Fig. 9. % of initial weight of the earthworms *Eisenia andrei* (± standard deviation, n = 4) at different concentrations of Polyethylene after a 28-days test period**

The earthworms reproduction test showed significant differences (ANOVA, $F_{5,17} = 6.699$, $P < 0.01$) in the number of juveniles between control and test treatments after the test period (see **Fig.10**). More precisely, there were significantly less juveniles at 0.5 % (P< 0.001), at 1 % (P < 0.001), at 2.5 % (P<0.01), at 5% (P < 0.01) and 10 % (P < 0.001) of PE in comparison to control replicates (Dunnett post-hoc test).

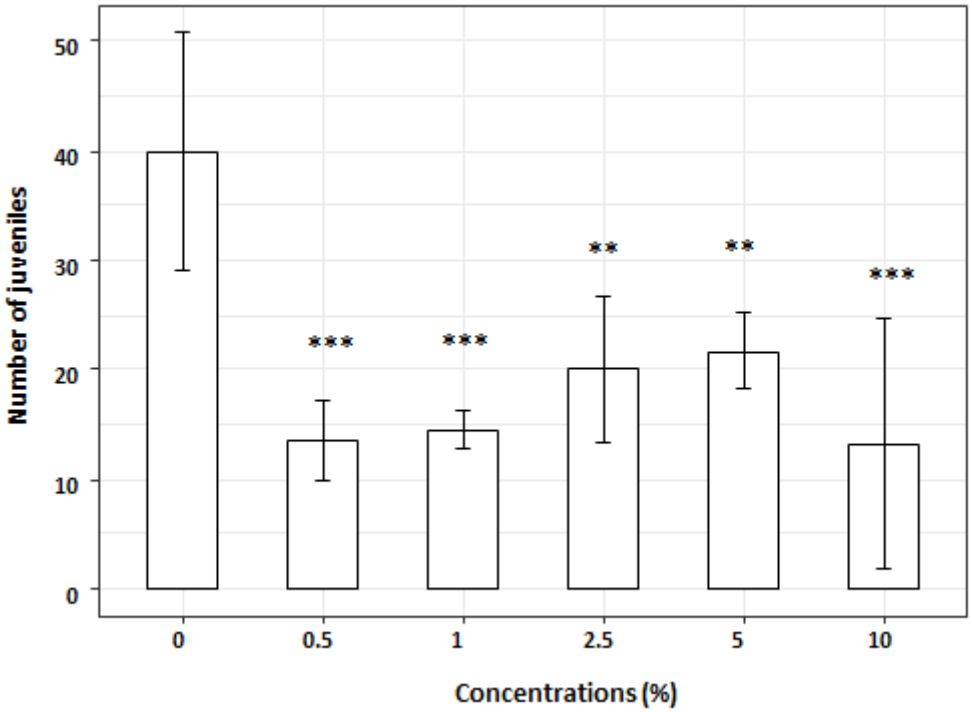


**Fig. 10. Average number of juveniles (± standard deviation, n = 4) produced by *Eisenia andrei* at different concentrations of Polyethylene after a 56-days test period. ** and *** indicates statistical differences compared to control with P < 0.01 and P < 0.001, respectively, after after one-way ANOVA followed by Dunnett post-hoc test**

According to the observations of gut tissues of the surviving adult *E. andrei* selected, there were no damages in the gut tissues of the earthworms from any of the test treatments. The thickness of intestinal epithelium was not significantly different (Nested ANOVA, $F_{5,12} = 0.419$, $P > 0.05$) between control and test replicates (see **Fig. 11**).

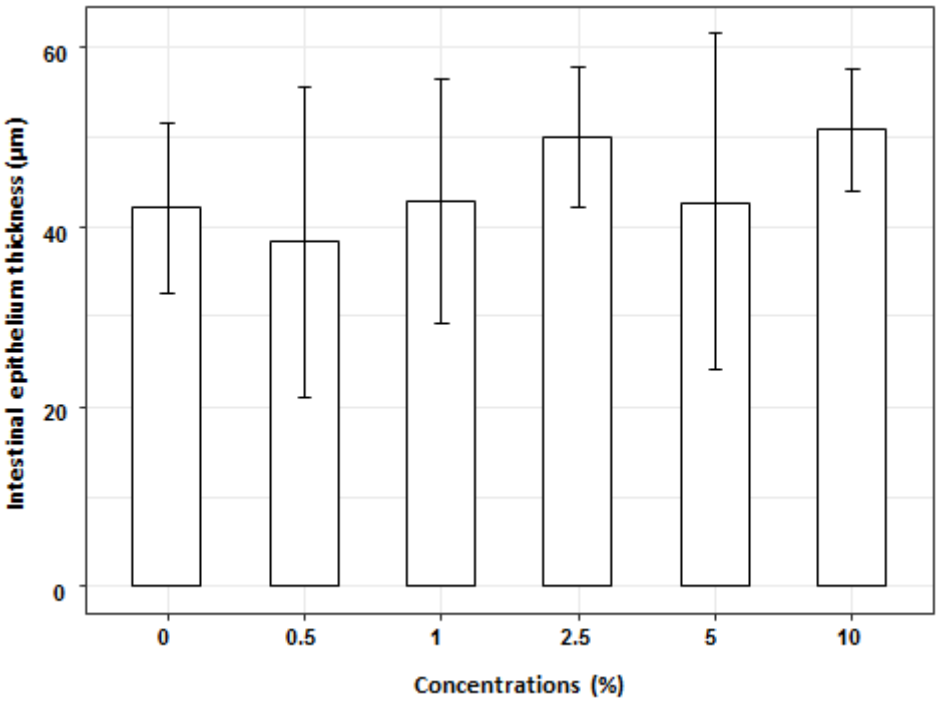


**Fig. 11. Average thickness of intestinal epithelium (± standard deviation, n = 10 ) of surviving adults of the species *Eisenia andrei* selected at different concentrations of Polyethylene after 28 days of exposure.**

Furthermore, no significant differences (Nested ANOVA, $F_{5,12}$ = 1.813, $P$ > 0.05) between control and test replicates were detected in the thickness of typhlosole epithelium (see **Fig. 12**).

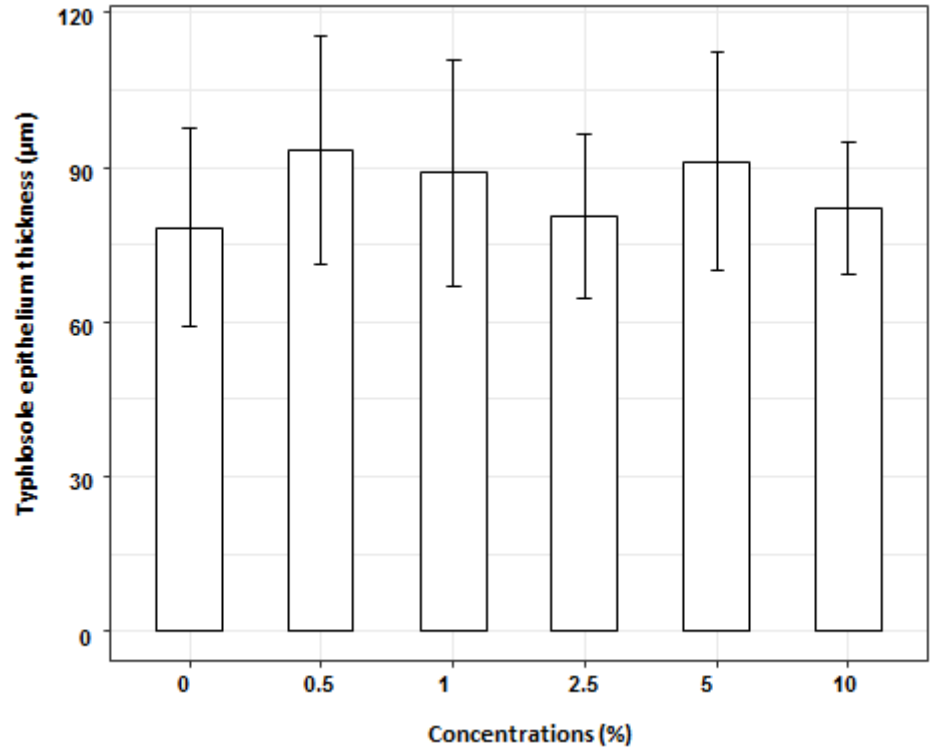


**Fig. 12. Average thickness of typhlosole epithelium (± standard deviation, n = 10) of surviving adults of the species *Eisenia andrei* selected at different concentrations of Polyethylene after 28 days of exposure.**

Also, no significant differences (Nested ANOVA, $F_{5,12} = 2.35$, $P > 0.05$) were found in longitude of typhlosole between control and test replicates (see **Fig. 13**).

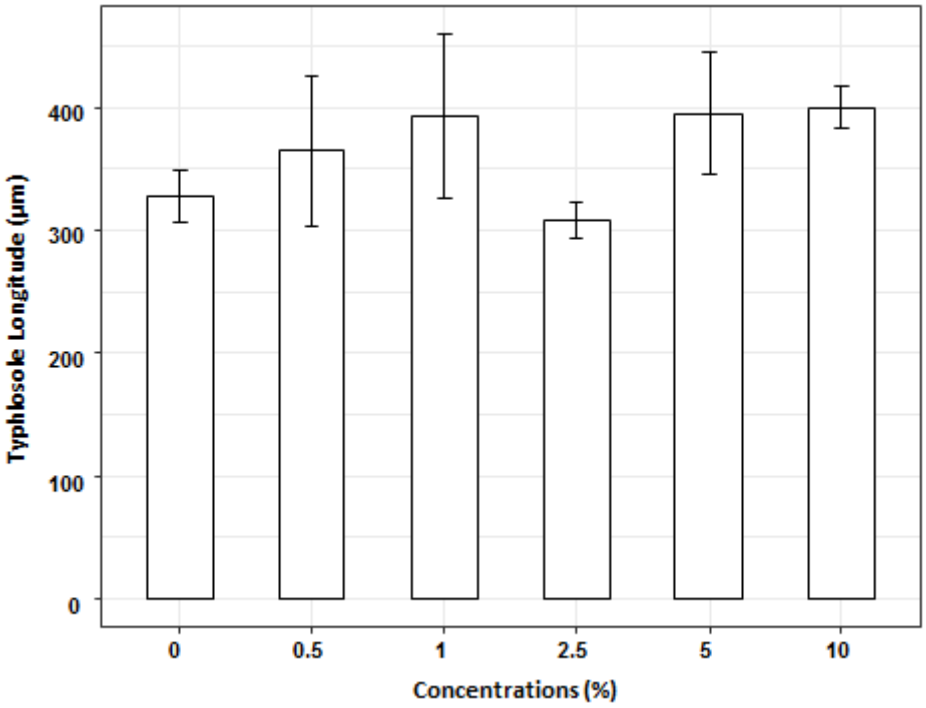


**Fig. 13. Average longitude of typhlosole (± standard deviation, n = 10) of surviving adults of the species *Eisenia andrei* selected at different concentrations of Polyethylene after 28 days of exposure.**

In the Enchytraeids reproduction test, the number of juveniles produced by *E. crypticus* was significantly different (ANOVA, $F_{5,27} = 9.895$, $P < 0.001$) between control and test replicates after 28 days of exposure to soils with PE (see **Fig.14**). More precisely, there were significantly less juveniles at concentration 0.5 % ($P < 0.05$), and at concentrations 2.5 %, 5%, and 10 % ($P < 0.001$) of PE in comparison to control replicates and 1 % of PE the only test concentrations that dis not chow significant difference compared to control ($P = 0.154$) (Dunnett post-hoc test).

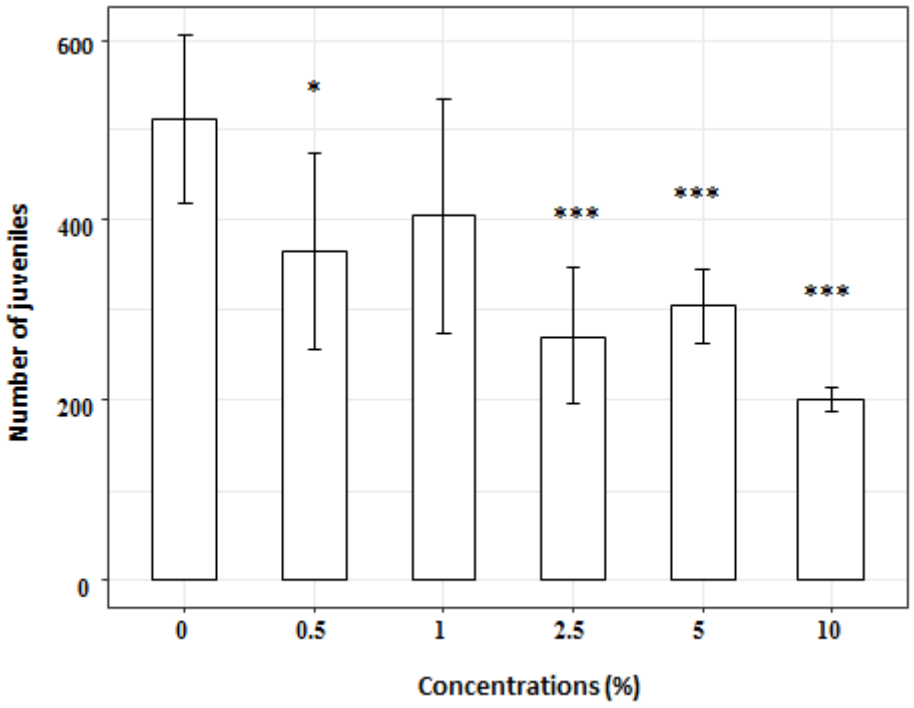


**Fig. 14. Average number of juveniles (± standard deviation, n = 5) produced by *Enchytraeus crypticus* at different concentrations of Polyethylene after 28 days of exposure. * and *** indicates statistical differences compared to control with P < 0.05 and P < 0.001, , respectively, after one-way ANOVA followed by Dunnett post-hoc test.**

Oxygen consumption rates of surviving *E. crypticus* selected from test replicates were not significantly different (ANOVA, $F_{5,11}$ = 1.634, $P$ = 0.231) between control and test treatments after 28 days of exposure to soils with PE. Although, a negligible decrease of 36.31% was observed at the highest concentration of PE (10%) in comparison to control (see **Fig. 15**).

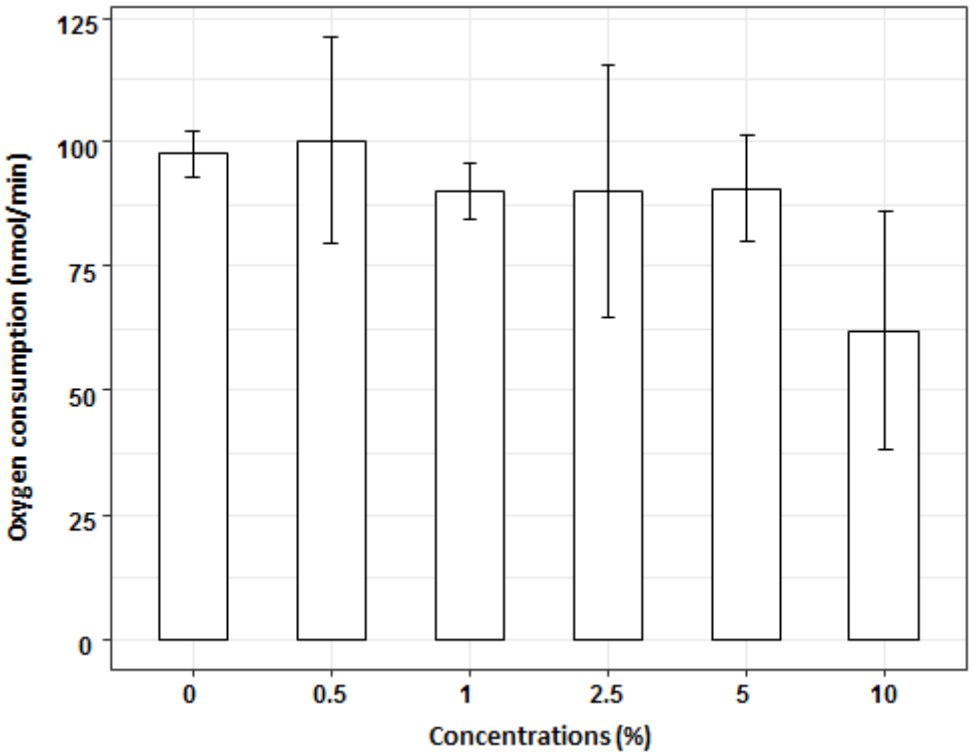


**Fig. 15. Average oxygen consumption (± standard deviation, n = 3 ) by E*nchytraeus crypticus* at different concentrations of Polyethylene after 28 days of exposure**

In collembolans reproduction test, on average, 86% of adults survival was observed in replicates both from control and test treatments with no significant differences (ANOVA, $F_{5,24}$ = 1.263, $P$ = 0.312) after 28 days of exposure. Unlike to reproduction rates of *E. andrei* and *E. crypticus*, the number of juveniles produced by *F. candida* was not significantly different (ANOVA, $F_{5,24}$ = 0.41, $P$ = 0.837) between control and test treatments at the end of 28 days (see **Fig. 16**).

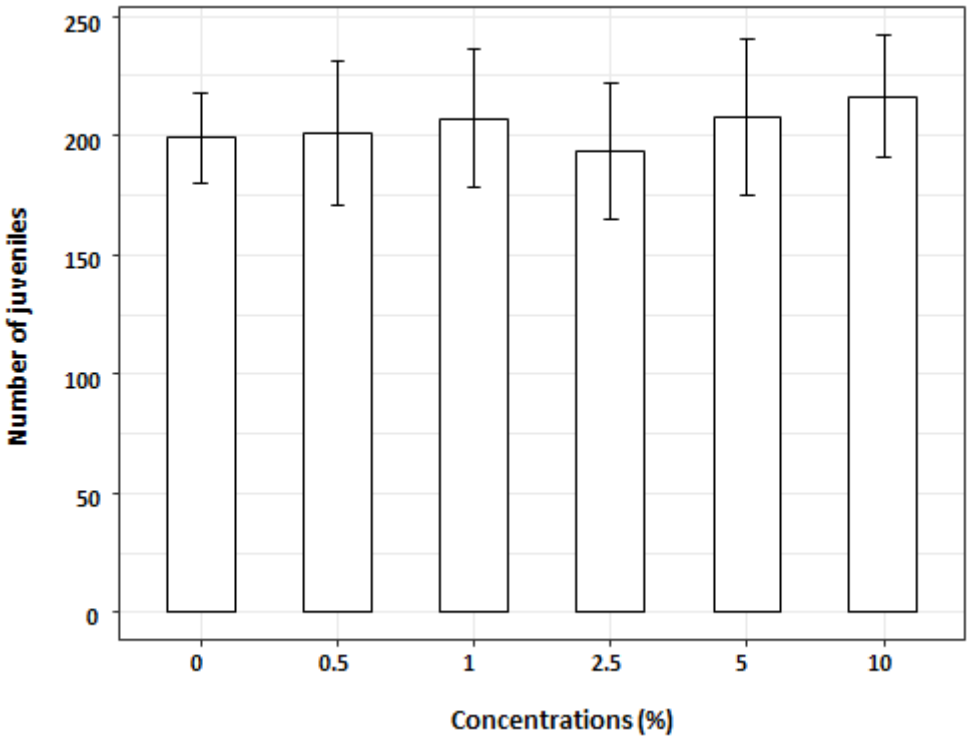


**Fig. 16. Average number of juveniles (± standard deviation, n = 5) produced by *Folsomia candida* at different concentrations of Polyethylene after 28 days of exposure.**

## 2.6 Discussion

### Impact of Polyethylene on survival, reproduction and growth of soil invertebrates

Significant decrease in reproduction of the exposed earthworms *E. andrei* was observed after 56 days. However, no mortality or weight loss was found among the earthworms after 28 days of exposure to 40-48 µm polyethylene (PE) at concentrations up to 10% in OECD soil.

Under stressful conditions trade-off mechanism might arise between survival and reproduction in iteroparous animals such as the earthworms *Eisenia* sp. (Aira *et al.*, 2007; Gomes *et al,* 2015). Especially, simultaneous hermaphrodites such as *E. andrei* might have higher cost since they should adjust their resource allocation to each sex function, including the investment in bodymass growth. (Schärer *et al.*, 2005; Hughes *et al.*, 2002). Due to this, two distinct strategies of energy expenditure may exist, one favoring organism survival and other favoring the total number of offspring produced. Aira *et al.*, (2007) studied energy allocation in the earthworms *Eisenia* sp. under stressful conditions where the earthworms showed energy allocation into bodymass by compromising it with lowered reproduction (Aira *et al.*, 2007). Therefore, when exposed to toxic environments organisms might maintaine their weight in order to survive (Daniel *et al.*, 1996) decreasing reproduction, which could happened in the present

study. Indeed in the present work, earthworms were not affected on mortality and growth, but significantly lower reproduction was observed at concentrations ≥ 0.5% of PE. Previous studies have reported that reproduction in earthworms *Eisenia* sp. is more sensitive than mortality after exposure to several contaminants (Spurgeon *et al.*, 1994; Kuperman *et al.*, 2004; Van Gestel *et al.*, 1992; Simini *et al.*, 2003; Žaltauskaitė and Sodienė, 2010).

On the other hand, Lwanga *et al.*, (2016) reported no effects in reproduction of earthworms *Lumbricus terrestris*, but significant biomass reductions with increasing percentages of PE and 25% mortality after 60 days of exposure to PE (<150 μm) at concentrations > 28% and 60 % (w/w), respectively. Most probably, large earthworms like *L. terrestris* might allocate energy into reproduction, and by that way weight loss might be comprimised under microplastic exposure (Huerta Lwanga *et al.*, 2016). Despite that possibility, it should be taken into account that the period of exposure that caused mortality and growth reduction in *L. terrestris* was twice longer that the exposure period considered in the present study (28 days of exposure to the adult earthworms) and the maximum concentration of PE (60%) was six times higher comparing to maximum concentration tested in the present study (10%).

On the other hand, Cao *et al.* (2017) found that at considerably lower concentrations of a MP, 1-2% of 58 μm Polystyrene (PS), mortality up to 40% and biomass decreases up to 29.8% were observed in earthworms *E. fetida* after 30 days of exposure. Despite the fact that earthworms *E. fetida* have similar morphology and in life cycles to the earthworm species used in the present study (*E.andrei*) (Domínguez *et al.*, 2005) the difference in type of tested microplastics (PS and PE) does not allow to make comparisons between these two studies, since PS and PE contain different physical and chemical properties (*Lithner et al.*, 2011), which could differently affect organisms. Also, no reproduction was measured to understand and confirm the strategy of energy allocation adopted by the test organisms over its exposure to microplastics in the study conducted by Cao *et al.* (2017).

Unlike previous works assessing the impact of microplastics on earthworms, no negative effects of PE (250-1000 μm) were found on mortality, biomass change and reproduction of earthworms *E. andrei* after 28 and 56 days respectively at concentrations up to 0.1 % (w/w) in OECD artificial soil in a study conducted by Rodriguez-Seijo *et al.* (2017). However, in that study the size of microplastics was considerably bigger and the concentrations of PE were considerably lower comparing to the previous works (Huerta Lwanga *et al.*, 2016; Cao *et al.*, 2017) including the present study. Therefore, the absence of effects of PE observed in that study might be related to the particular particle sizes and concentrations of PE tested. The importance of particle sizes was highlited by a study conducted by Eisenhauer *et al.* (2009) who found a negative corellation between ingestion rates of seeds by endogeic earthworms and the size of

seeds. The authors found that earhworms prefer smaller seeds over bigger seeds. This preference was particularly noted in smaller species of earthworms (*Aporrectodea rosea, Allolobophora chlorotica*) that showed strong preference to small seeds (Eisenhauer *et al.*, 2009). Despite that, Rodriguez-Seijo *et al.* (2017) reported that the earthworms ingested PE particles in sizes between 250 and 1000 μm and that gut tissues of the exposed earthworms contained microplastics. Therefore, the absence of negative effects of microplastics on survival, growth and reproduction in that study could be related to the extremely low concentrations used.

As occurred to earthworms, reproduction of potworms *E. crypticus* significantly decreased after 28 days of exposure to PE at almost all test treatments. According to the available literature, the present study was the first and until date the only one that evaluated the impact of microplastics on potworms. Therefore, there are no other data available in the literature to compare these results. However, *E. crypticus* has been used in ecotoxocology for a long time (Castro-Ferreira *et al.,* 2012), and its reproduction is one of the highly sensitive endpoints to evaluate risk associated to the presence of contaminants (Dhawan *et al.,* 1999). Similar to that discussed above for earthworms *E. andrei* in this study, potworms also might allocate energy into survival under microplastic exposure. It is known that reproduction or growth of organisms is depended on cellular energy allocation (CEA) (Gomes *et al,* 2015). For example, a strong depletion of the energy reserves (lipids and proteins) and decreased oxygen consumption in *E. crypticus* possibly resulted in decreased reproduction after exposure to Ag (EC50) (Gomes *et al.*, 2015).

Reproduction of earthworms and enchytraeids has been reported to be more sensitive than survival of adults (Kuperman *et al.,* 2004). However, the survival of *E. crypticus* is not an always measurable endpoint, since at the end of the test it is often hard to distinguish between survived adults and juveniles. Therefore, in this case no information was obtained regarding mortality among potworms. However, reproduction of *E. crypticus* has been reported as more sensitive than mortality for several contaminants. For example, reproduction of *E. crypticus* was significantly affected after exposure to manganese at 99 mg/kg, while at concentrations up to 267 mg/kg no mortality was recorded (Kuperman *et al.,* 2004). Also, no mortality was found among *E. crypticus* after 21 days of exposure to Tetrabromobisphenol A at concentrations up to 1000 mg/kg, while negative impacts on reproduction was found at 10 mg/kg (Sverdrup *et al.,* 2006).

Neither mortality nor reproduction were affected in collembolans *F.candida* after 28 days of exposure at concentrations up to 10% of PE. Since, collembolans do not pass soil through their body (Rusek, 1998) unlike to *E. andrei* and *E. crypticus*, it was expectable that collembolans could be less sensitive to microplastic exposure than the soil-dwelling annelids. Moreover, *F.candida* is known to have preference for grazing on fungi rather than on soil

particles (Fountain & Hopkin, 2005). Another study observed that collembolans did not feed on urea-formaldehyde microplastic (100-200 μm and <100 μm) in laboratory experiment (Maaß *et al.*, 2017). Most probably, the route of exposure represented by collembolans makes them less prone to be affected by PE comparing to *E. andrei* and *E. crypticus*.

There is no available literature evaluating impacts of microplastics on mortality of collembolans. However, a recent study detected decreases in reproduction of *F.candida* at 0.1 % of 80-250 μm polyvinyl chloride (PVC) after 28 days (Zhu *et al.*, 2018). In comparison, even at higher concentrations of PE (up to 10%) no reproduction descreases were observed in the present study. However, impacts of microplastics might dependent on type of plastic and its additive chemicals. According to hazard ranking of plastic materials, PVC is considered one of the most hazardous plastics due to the presence of vinyl chloride monomers (V=most hazardous) (Lithner *et al.*, 2011), which might leach into the environment (Rochman *et al.*, 2013), while in this ranking polyethylene is classified among the least hazardous plastics (II=hazard level) (Lithner *et al.*, 2011). Therefore, the highest toxicity reported by Zhu *et al.*, (2018) is most probably related to the particular properties of the MP used in that study, which is not comparable to those of PE.

**Impact of Polyethylene of oxygen consumption of *Enchytraeus crypticus* and on gut tissues of *Eisenia andrei***

In ecotoxicological assessments of pollutants on soil biota mainly traditional endpoints such as reproduction, growth rate and mortality are considered. However, it is suggested that molecular endpoints could serve as earlier warnings to predict potential hazards of pollutants (Chen *et al.*, 2011; Straalen and Roelofs, 2008). For example, in a study conducted by Rodriguez-Seijo *et al.* (2017) who exposed earthworms *E. andrei* to soils with PE no effects on growth, reproduction and mortality were found at concentrations up 0.1%, but immune system responses and gut tissue damages were reported starting from the lowest tested concentration (0.006%).

In the present study, histopathological analysis of gut tissues of adult surviving earthworms of the species *E. andrei* were investigated after 28 days of exposure to 40-48 μm PE. The measurements recorded were thickness of intestinal epithelium, thickness of typhlosole epithelium and longitude of typhlosole. No damages were detected in gut tissues of exposed earthworms and no alterations were found due to microplastic exposure in all these measurements. In the previous study of Rodriguez-Seijo *et al.* (2017), gut tissue damages starting were reported at the lowest concentration (0.006%) of PE (250-1000 μm) and followed with more severe damages with the development of fibrosis and congestion at concentrations ≥

0.01 % (w/w). Contrarily, in the present study, even in much higher concentrations of PE (10%) no negative effects were found. However, it should be taken into account that while in the present study PE composed by particles of 40-48 μm was used, in the study of Rodriguez-Seijo *et al.* (2017) a PE composed by particles of 250-1000 μm was investigated. Gut tissues damages could occur depending on size of microplastic, and probably bigger particles of PE might damage gut tissues of earthworms. Since no more studies are known on this topic further investigation is needed to evaluate physical effects of microplastics on gut tissues of earthworms.

The rate of oxygen consumption was chosen as an endpoint to evaluate effects of PE on molecular level in *E. crypticus,* since oxygen consumption rate could give a dose-response for many pollutants at lower concentrations (Handyu and Depledge, 1999; McKim and Erickson, 1991). Oxygen consumption rate is often used to evaluate changes in metabolism under environmental changes (Dube & Hosetti, 2010). Moreover, potworms are known to be sensitive to hypoxia due to the absence of vascularization of their body wall. This results to the fact that Enchytraeids are usually confined to latitudes where oxygen availability rarely becomes a limiting factor (Rota & de Jong, 2015). These facts lead to assume that oxygen consumption rate could be a sensitive endpoint to assess effects of microplastics on potworms.

In the present study, no differences were found in oxygen consumption rates of *E. crypticus* after 28 days of exposure to PE at concentrations up to 10%. Apparently, this agrees to a previous study developed by Senga *et al.* (2016) who reported that oxygen consumption rates of lugworms (*Arenicola marina*) were not affected after 31 days of exposure to PE (2.5-316 μm). In the same study, significant increases in oxygen consumption rates was detected after exposure of lugworms to polyvinyl chloride (PVC) in sizes 8.7-478 μm at 2 % by wet weight (Senga *et al.,* 2016). In the present study decreases of 36.31% in relation to control were observed at the highest concentration of PE (10%) but even these decreases were not significant. Oxygen consumption changes might depend on physical and chemical properties of MPs used. PE and PVC contain different physical and chemical properties and, as detailed above, PVC is known to belong to the class of the most hazardous plastics (Lithner *et al.*, 2011), which may justify the higher toxicity found in soils contaminated with PVC.

Interestingly, in the present study, the lowest rate of oxygen consumption was coincident with the lowest reproduction at 10% of PE. This suggests that under exposure to microplastics, potworms might consume less oxygen, which could be possible if the energy was allocated only to survive by lowering reproduction. Previous studies give strength to this assumption. In fact, oxygen consumption rate has been found to rise considerably during reproduction in several invertebrates (Calow, 1978; Berg and Ockelmann, 1959; Phillipson, 1963).

In most cases, the research works developed until date on the impact of microplastics on biota (including the present study) were performed in laboratory conditions using pure and calibrated microplastics. However, in the environment, microplastics are irregularly shaped, have different sizes and might contain many/different chemical additives. Moreover, microplastics are prone to adsorb pollutants from the environment and, by that way, to interfere on the availability of those pollutants to biota (Rochman *et al.*, 2013; Antunes *et al.*, 2013; Koelmans *et al.*, 2016; Browne *et al.*, 2013). From that point of view, negative effects found in laboratory studies like the present one where pure microplastics were used, could be even more severe in environment, which increase the relevance of these findings. For instance, the study conducted by Browne *et al.* (2013) revealed that polyvinyl chloride (PVC) has the ability to adsorb several pollutants and chemicals from sand and to transfer them into gut tissues of lugworms (*Arenicola marina*). The authors stressed that this fact may contribute to increase mortality and decrease feeding activity among the lugworms in comparison to virgin PVC. Another recent study also highlighted that polluted PVC causes more severe histopathological alterations in intestinal tissues of the European sea bass *Dicentrarchus labrax* than virgin PVC (Peda *et al.*, 2016). Therefore, the use of microplastics from the environment instead of calibrated and pure microplastics in laboratory assessments could be more appropriate to increase realism of research studies in the future.

## 2.7 Conclusion

Polyethylene do not affect survival of earthworms *E. andrei* and collembolans *F. candida* after 28 days when present in artificial soil until a concentration of 10%. None effects were observed in gut tissues of *E. andrei* neither on oxygen consumption rates of potworms *E. cripticus*.

The most sensitive endpoint towards microplastic exposure was reproduction. Significant reproduction decreases were found among earthworms and potworms in all soils mixed with PE (except for *E. crypticus* in 1%). These data suggest that earthworms and enchytraeids might allocate energy into keeping biomass in order to improve resistance against MPs, compromising by this way its reproduction. Polyethylene did not affect reproduction of collembolans *F.candida*. Apparently, the route of exposure represented by collembolans makes them less prone to be affected by PE comparing to *E. andrei* and *E. crypticus*.

# CHAPTER III
# IMPACT OF MICROPLASTICS ON FRESHWATER ORGANISMS

# 3 CHAPTER III

## Introduction

### 3.1 Microplastic contamination in fresh waters

The attention to MP contamination in freshwater environments is increasing rapidly. Worldwide, lakes and rivers have been reported to contain different amounts of MPs. According to the existing literature on the abundance of MPs, the Chinese Lake Taihu was found to be the most MP contaminated freshwater lake in the world. The lake contained from 3.4 to 25.8 MP particles (< 1 mm) per L (Su *et al.*, 2016). The studied area is densely populated and well known for the developed industry, agriculture, fishing and tourism.

The concentration of MPs was also measured in the Laurentian Great Lakes. Eriksen *et al.* (2013) found on average 43,000 MP particles per $km^2$ in size <1 mm. The highest MP concentration in this study was found in the downstream of Lake Erie, which is located near densely populated cities. The samples from Lake Erie contains up to 466,000 particles per $km^2$, while less populated areas showed significantly lower amounts of MPs (Eriksen *et al.*, 2013).

Several studies have found correlations between higher plastic loads and high population density. Mani *et al*. (2015) studied the abundance of MPs in one of the largest rivers of Europe, in the Rhine River. All the samples were found to contain MPs (<1 mm) with an average of 892,777 particles per $km^2$. The higher concentration of 3.9 million particles per $km^2$ was measured in the highly populated Rhine-Ruhr metropolitan area (Mani *et al.,* 2015). However, the population density is not the only reason for MP contamination. According to Mani *et al.* (2015) large industries of plastic, textile and metal plants are located along the river. The manufacturing also could contribute to higher abundance of MPs. For example, in Portugal, Matosinhos, Vieira de Leiria and Sines, near industrial and harbor areas, were the most contaminated beaches with plastics (Antunes *et al.,* 2013).

It is hard to identify sources of plastic debris, since factors such as wind, runoffs and waste management facilities could also influence the plastic loads. For instance, Free *et al*. (2014) investigated the abundance of MPs in the rarely populated remote lake in Mongolia. The Lake Hovsgol is located in a protected national park without any industry, agriculture and waste water treatment plants. Although, the results show that all the samples contained plastic debris with an average of 20,264 particles per $km^2$ (Free *et al.,* 2014). Despite the low population, the absence of industry and urbanization, the Lake Hovsgol appeared to be highly contaminated with plastic debris. The authors noted that the plastic debris found in this lake did not include industrial pellets or MPs from personal care products. Instead, they were mainly composed by

fragments of bags, bottles and fishing gears. The authors stressed that the lack of waste management seems to be the reason for the MP contamination found.

These studies suggest that freshwaters are not only a pathway for MP loads to the oceans, but a major sink for MPs accumulation.

### 3.2 Microplastics accumulation in benthic sediments of lakes and rivers

Besides the contamination in pelagic zones, benthic sediments of lakes and rivers were also found to hold MPs. Benthic zones provide important ecosystem functions, regulates flow of energy, the cycling of nutrients and constitute ecological habitat for benthic invertebrate community (Covich *et al.*, 2004).

The benthic sediment of Lake Ontario in Canada was studied for the abundance of microplastics. Ballent *et al.* (2016) reported 980 MP particles per kg of dry sediment with a maximum of 27830 particles per kg near shore sediment on average (Ballent *et al.*, 2016). Fragmented plastic debris and fibres of size < 2 mm were dominated in this area. High concentrations of MPs in this lake might pose serious environmental threats. The trophic chain of the lake is closely connected to benthic sediment habitats, thus, MPs contamination could affect the normal functioning of the ecosystem (Ballent *et al.*, 2016). Another study also reported MP contamination in the littoral zone of Beijiang River, where the concentrations of MPs ranged from 178 to 544 particles per kg of sediment (Wang *et al.*, 2017). The MPs found in the sediments were mainly composed by fragmented polyethylene (PE), polypropylene (PP) and copolymer.

In Europe, some works also reported high concentrations of MPs in sediments. For instance, up to 660 MP particles (1mm-4mm) per kg of sediment was reported in the benthic sediment of River Thames, UK (Horton *et al.* , 2016). In Italy, remote alpine lake, Lake Garda contained on average 1108 MP particles (9 μm-5 mm) per $m^2$ in north shores and 108 MP particles per $m^2$ in south shores (Imhof *et al.*, 2013). The largest contamination of PE and PP was found also in the Rhine-Main, Germany. In this study averages between 228 and 3763 MP particles (<5 mm) per kg of sediment were measured (Klein *et al.*, 2015). The high MP concentrations in this study may be due to the high population density and urbanization and due to the presence of large industries in the surroundings of the river.

## 3.3 The impact of microplastics on freshwater biota

### 3.3.1 The impact of microplastics on freshwater producers

The studies on the impact of MPs to freshwater biota have increased recently but only few works have assessed the effects of MPs on producers. For the first time, Bhattacharya *et al.* (2010) studied freshwater algal (*Chlorella sp.)* and freshwater/saltwater algal *(Scenedesmus sp.*) to evaluate the impacts of 20 nm PS after 24 hours of exposure. The result showed that the PS at concentrations 0.08-0.8 mg/mL provoke negative effects on photosynthesis of both algal species due to the physical blockage of light and air by the nanoparticles (Bhattacharya, Lin, Turner, & Ke, 2010). However, another study reported no significant effects on photosynthetic performance of freshwater algal (*Chlorella vulgaris*) and other marine species (*Dunaliella tertiolecta, Thalassiosira pseudonana*) under exposure to PS (0.05 and 6 μm) at concentration 0.250 mg/ml (Sjollema *et al.*, 2016).

In a recent study, Kalčíková *et al.*( 2017) investigated the impact of PE (4-12 μm) from cosmetic products at concentrations 0, 10, 50, and 100 mg/L on freshwater duckweed (*Lemna minor*) after 7 days of exposure. There was no significant reduction of photosynthetic pigment concentration as well as no significant decrease in growth rate. (Kalčíková *et al.*, 2017).

In comparison to short term experiments, Lagarde *et al* .(2016) studied the impact of polypropylene (PP) and high density polyethylene (HDPE) of size > 400 μm on freshwater microalgae, *Chlamydomas reinhardtii* after 80 days exposure. After 78 days of exposure to 100 mg of PP only a negligible decrease of 18% in growth was observed in comparison to control.

### 3.3.2 Impacts of microplastics on freshwater consumers

The impact of MPs on freshwater consumers has been tested mainly under laboratory conditions. The main issue of study has been the ingestion of plastic particles. Animals often confound plastic debris with food and ingest it. Thus, the uptake of plastic particles is highly influenced by feeding needs of individuals and food availability. Scherer *et al.* (2017) studied the uptake of PS (1, 10, 90 μm) at concentrations 3–3 000 Particles per mL by fresh water invertebrates with different type of feeding strategies. The highest uptake was observed for the filter feeder *Daphnia magna* (6180 particles $h^{-1}$), followed by the collector-gatherer *Chironomus riparius* (226 particles $h^{-1}$), scraper and surface grazer *Physella acuta* (118 particles $h^{-1}$), shredder *Gammarus pulex* (10 particles $h^{-1}$) and deposit feeder *Lumbriculus variegatus* (8 particles $h^{-1}$) (Scherer *et al.*, 2017).

Apart from ingestion, microplastics could cause biological adverse effects, for example 21 days of exposure to PS (5 µm and 70 nm) at a concentration of 2000 µg/L caused negative effects such as necrosis, inflammation, and lipid accumulation in the exposed livers of zebrafish (*Danio rerio*) in comparison to the controls (Lu *et al.*, 2016). Another freshwater species, the African catfish (*Clarias gariepinus*), was also found to be vulnerable to MPs exposure. PE (> 60 µm) at concentration of 500 µm/L, caused epithelial lifting, hyperplasia, necrosis, extensive cell sloughing, and other abnormalities in cells (Karami, Romano, Galloway, & Hamzah, 2016). Also, Rehse *et al.* (2016) reported that 1 µm PE at a concentration of 200 mg/L caused up to 75% of immobilization in *D. magna*.

Another issue of concern is that microplastics might be transferred through food chain. Indeed, this was previously observed in marine species (Farrell & Nelson, 2013). In the freshwater species, only Zebrafish (*Danio rerio*) was studied for possible uptake of MPs of size 1-20 µm via exposed brine shrimp *Artemia nauplii* at the concentration of 2.5 mg of 10 µm to 20 µm or 0.5 mg of 1 µm to5 µm microparticles particles per 20 000 nauplii (Batel *et al.*, 2016). Although the most particles were excreted after 5-6 hours without causing any adverse impact to the zebrafish (Batel *et al.*, 2016).

On the other hand, many other works found no significant adverse effects of MPs in fresh water consumers. For example, Weber *et al.* (2018) reported that survival, development, metabolism and feeding activity of *G. Pulex* was not impacted at concentrations 0.8 - 4,000 particles per mL of polyethylene terephthalate fragments (10-150 µm). Also, freshwater snail *Potamopyrgus antipodarum* showed no morphological changes after 56 days of exposure at 0, 30 and 70% (w/w) of MPs mixture (polyamide, polyethylene terephthalate, polycarbonate, polystyrene, polyvinylchloride) of average size 118 ± 105 µm (Imhof & Laforsch, 2016).

### 3.4 Standard species

There are standard guidelines describing methods to evaluate the impact of substances on freshwater species belonging to producers and consumer organisms under laboratory conditions. *Lemna minor* and *Gammarus pulex* are standard species representing the group of producers and consumers, respectively, widely used in freshwater ecotoxicological tests. Therefore, these species are adequate key-organisms to be used in the evaluation of the impact of microplastics on freshwater systems.

### *Lemna minor*

*Lemna minor* is a floating freshwater duckweed of the family *Lemnaceae* (**Fig. 17**). It has a worldwide population that may be found in any lakes and slow streams. Duckweeds are important freshwater plants. They are food and habitat for invertebrates, fishes and are often used for agricultural purposes (Les *et al.,* 2016).

**Fig. 17. Duckweed *Lemna minor***

Certain physiological properties such as small size and fast multiplication rates made the duckweeds ideal model species in aquatic ecotoxicology (Naumann *et al.,* 2007). *Lemna minor* has been used for a long time to assess the toxicity of pesticides, heavy metals (Radić *et al.*, 2011; Geoffroy *et al.,* 2004; Samardakiewicz and Wo, 2000) and, more recently, microplastics (Kalčíková *et al.,* 2017).

### *Gammarus pulex*

*Gammarus pulex* is a freshwater amphipod crustacean found nearly all over the Europe, North Africa and most part of Asia (Pinkster, 1970). The species *G. pulex* represents the most dominant macroinvertebrate in freshwater ecosystems in terms of abundance and biomass (MacNeil *et al.,* 1999). The color of *G. pulex* (**Fig. 18**) is greyish with some parts of the body dark brown and green. The males of this specie grow until 21 mm, while the females grow until 14 mm in maximum (Pinkster, 1970).

**Fig. 18. *Gammarus pulex*( Linnaeus, 1758 )**

*Gammarus pulex* is known as an important fish food, being available to fish predators all year around unlike many insects (MacNeil *et al.*, 1999). These organisms also play an important role in organic matter decomposition (Lange *et al.*, 2006).

This amphipod has been used in ecotoxicological studies for a long time. The species is known as a good indicator of stress in both laboratory and field studies (Maltby & Naylor, 1990) and has been used to assess the toxicity of many pollutants, such as pharmaceuticals *(Lange et al.*, 2006), heavy metals (Alonso *et al.*, 2010) and pesticides (Adam *et al.*, 2009) and has also been used to assess the water quality in biomonitoring schemes (Lorraine *et al.*, 2002).

## 3.5 Objectives

The main objective of this work is to evaluate the impact of microplastics on freshwater duckweed *Lemna minor* and freshwater amphipod *Gammarus pulex*. More specifically the objectives of the present study are the following:

1) To understand the impact of 1 µm Polystyrene and 45 µm and 48 Polyethylene MPs on photosynthetic performance and on growth rate of *L. minor* after 7 days of exposure.

2) To investigate the potential of trophic transfers of 1 µm Polystyrene and 45 µm Polyethylene from the exposed duckweed to the amphipod *G. pulex* over 24 and 48 hours of exposure.

## 3.6 Materials and Methods

### Test organisms

#### *Lemna minor*

The duckweed *Lemna minor* (**Fig. 19**) was cultured at the school of Biological, Earth and Environmental Sciences of the University of College Cork, Ireland. These stocks were originated from a pond in Blarney area, Co. Cork, Ireland. The *L. minor* strain used is registered in the Rutgers Duckweed Stock Cooperative (RDSC) database as strain number 5500 "Blarney".


**Fig. 19. Cultures of *Lemna minor***

The cultures were maintained according to the protocol described by Brain and Solomon, 2007. The cultures were kept on 100 ml half-strength Hutner's nutrient media in 300 ml magentas under a temperature of 22 ± 2°C, a photoperiod of 16:8h, light:dark and with an average light intensity of 50 µmol.$m^{-2}s^{-1}$. The relative humidity used in the growth room was set to 50 %.

#### *Gammarus pulex*

The amphipods *Gammarus pulex* were sampled in June of 2018, from a tributary of the Shournagh river in Bridgetown, Co. Cork, Ireland (Coordinates: 51.918636, -8.630122). The amphipods were kept in aerated tanks with freshwater at 20 ± 2 °C with a photoperiod of 16:8h, light:dark, for 3 days before the experiment. Since the animals were sampled with plants from their natural habitat, no additional food was given during the culturing time.

Before the experiments, individuals with similar size were chosen and each individual was acclimatized in a glass vessel filled with 60 ml of freshwater for 24 hours before the experiments. During acclimatization period no food was given to determine equal starvation among the amphipods. Each vessel was covered by a glass lid.

**Test substances**

**Microplastics**

1 µm polystyrene (PS) was ordered from Phosphorex Inc (South St., Hopkinton, US, 2018). The PS was non-colored, and fluorescence labeled (orange). The density of PS was 1.05 g/cm$^3$. This PS was purchased in a 1 ml suspension, and it contained 0.01 mg of PS per 1 µl of the suspension.

Red fluorescence 45 µm polyethylene (PE) was ordered from Cospheric (Santa Barbara, US, 2018). The PE was provided in a dry powder (10 grams), with a density of 0.995 g/cm$^3$. Since, 45 µm PE was insoluble and hydrophobic it was previously suspended with surfactant 0.1 % Tween 20. The 200 µl suspension contained 20 % (v/v) of PE. To know a precise mass of PE in 200 µl of the solution, 200 µl was pipetted on a filter paper, and dried at 40ºC for 24 hours, then weighed (**Fig. 20**). The average dry weight of PE was 36.8 mg.

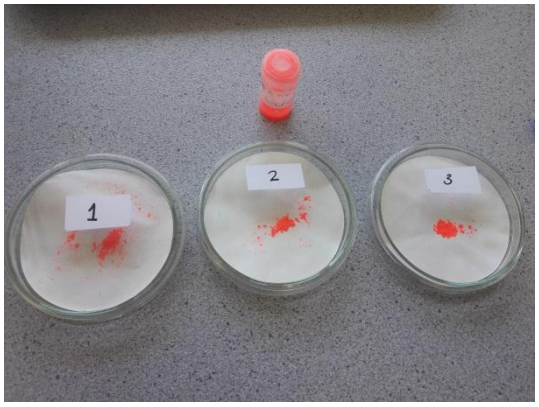


**Fig. 20. Dried microplastic particles (PE)**

48 µm PE was purchased from Sigma Aldrich Ireland Ltd (Wicklow, Ireland, 2018). These MPs were non-colored and non-fluorescent and were provided in a 100 g portion of a dry powder with a density of 0.987 g/cm$^3$. Since, 48 µm PE is also insoluble and hydrophobic, a suspension with 0.1 % Tween 20 was prepared. The 200 µl suspension contained 20 % (v/v) of PE. The exact concentration in mg was identified as described above for 45 µm PE. The average dry weight of PE was 36.8 mg.

Since, the 45 and 48 µm PE solutions contain mixture of Tween 20 and PE, the possible negative effects of Tween 20 was considered with 6 extra control replicates with 0.1 % of Tween 20.

**Surfactant Tween 20**

A 0.1 % Tween 20 solution was prepared by mixing 0.1 g of Tween 20 in 100 ml of deionized water. The volumetric concentration of the solution was 0.09 % ≈ 0.1%.

## Experimental design

### Growth inhibition test

Glass vessels with 740 ml of capacity were filled with 100 ml of half-strength Hutner's nutrient medium as described by Brain and Solomon, (2007). Then all vessels were autoclaved to ensure sterilization and homogeneity.

Procedures adopted in the growth inhibition tests followed standard OECD guideline (2002) and are summarized in **Table 3**.

Into each replicate previously weighed colonies of *L. minor* were gently added and MPs were added according to the desired concentrations (see **Table 4**). Control replicates (without MPs) followed all the same procedures. All test vessels were covered with a glass lid. Laboratory tests were conducted under the same conditions used for culture maintenance.

**Table 3. Procedures adopted in Growth inhibition test with *L. minor*.**

| | ***Lemna minor*** |
|---|---|
| Guideline considered | OECD guideline 221 (OECD, 2002) |
| Tested MPs | 45 and 48 μm PE; 1 μm PS; |
| Number of replicates per treatment | 6+3[a] |
| Number of plants per replicate | 3 |
| Number of fronds in each plant | 3 |
| Test period | 7 days; |

[a] additional replicates to measure Chlorophyll a fluorescence

**Table 4. Concentrations used in Growth inhibition test with *L. minor* are given mg per ml and particle number per ml**

| | Concentration (mg/ ml) | Concentration (particles/ ml) |
|---|---|---|
| 45 μm Polyethylene | 0.37 | 9,825 |
| 48 μm Polyethylene | 0.37 | 5,973 |
| 1 μm Polystyrene | 0.0005 | $1{,}110\times10^{3}$ |

### Relative growth rates based on biomass and frond number

At the end of 7 days, relative growth rates (RGR) of *L. minor* was measured by the logarithmic increase in biomass or frond number. The weight of colonies on a mass balance was measured by collecting all colonies from each vessel, and by gently removing water with absorbent paper and then weighting colonies. The number of fronds was determined by counting visible fronds.

The RGR based on biomass and number of fronds was calculated individually for each replicate following the formula by Connolly and Wayne (1996)

$$\text{RGR} = \frac{\ln Yf/Yi}{t}$$

Where, *ln* is the natural logarithm, *Yf* and *Yi* are the final and initial biomass or frond number, respectively, and *t* is the time of exposure (7 days).

### Chlorophyll a fluorescence

After the end of 7 days, 3 replicates from control and 3 replicates from test replicate were acclimatized to dark for 15 minutes before measurements. After that period, three random colonies were chosen from each replicate and analyzed individually. The colonies from test replicates were first washed with 10 ml of 0.1 % Tween 20 solution, and then cleaned from adhered MPs by the addition of 10 ml of distilled water.

Chlorophyll a fluorescence was measured using an imaging fluorometer (IMAGING-PAM M-Series, MAXI version) equipped with ImagingWin software (Heinz Walz GmbH PAM, Effeltrich, Germany). The settings used in the equipment were: measuring light ML (<1 µmolm-$^{2}$s-$^{1}$) = 2, actinic light AL (30-40 µmol/m$^{2}$/s) = 3, gain amplification = 2, damping = 2 and saturation pulse = 10. In each colony analyzed, three areas of interest (AOIs) were randomly drawn as polygons. Then the results of maximal quantum yield of PSII photochemistry for the dark-adapted state Fv/Fm ratio were obtained.

### Trophic transfer of microplastics from *Lemna minor* to *Gammarus pulex*

Several colonies of *Lemna minor* were pre-exposed as described for the growth inhibition test above (OECD, 2002) to fluorescent labeled microplastics, 45 µm polyethylene (**Fig. 21**) and 1 µm polystyrene at the concentrations given in Table 5 for 3 days.

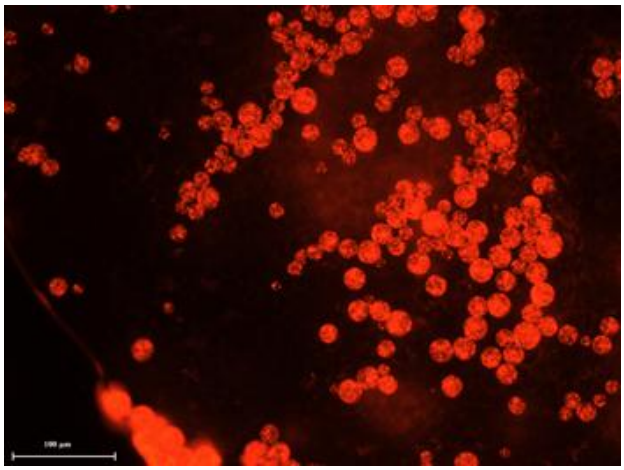

**Fig. 21. Fluorescence microscope image (amplification 10×) a frond of *L.minor* pre-exposed to 45 µm polyethylene (PE ) for 3 days. The bright red circles are PE particles.**

Then the exposed colonies were dried at 40ºC for 24 hours. Then, the colonies were weighed in mass balance to obtain the mass of colonies before the trophic transfer experiment.

**Table 5. Concentrations used to expose L. minor are given in mg per ml and in particle number per ml**

| | Concentration (mg/ ml) | Concentration (particles/ ml) |
|---|---|---|
| 45 µm Polyethylene | 0.37 | 9,825 |
| 1 µm Polystyrene | 0.0005 | $1{,}110\times10^{3}$ |

For the trophic transfer experiment 45 amphipods in similar size were chosen and individually transferred to a glass vessel with 60 ml of freshwater. The procedures adopted in the trophic transfer experiment are summarized in **Table 6**.

**Table 6. Procedures adopted in the trophic transfer test with *G. pulex*.**

| | ***Gammarus pulex*** |
|---|---|
| Tested MPs | 45 µm PE; 1 µm PS; |
| Number of replicates per time periods | 7 for 24 hours; 8 for 48 hours; |
| Number of amphipod per replicate | 1 |
| Test period | 24 hours; 48 hours; |
| Acclimatization time | 24 hours |
| Depuration time | 24 hours |
| Capacity of glass vessel | 100 ml |

Each individual from test replicates was given prior weighed and exposed to microplastics colonies of *L. minor*, each individual from control replicates was given prior weighed, clean (without microplastics) colonies of *L. minor*.

The two time periods of exposure tested were 24 and 48 hours. After these periods, all amount of food ingested in each replicate was measured in order to check differences in food uptake of organisms from control and test replicates. Then, each amphipod was changed into a clean vessel with 60 ml of clean freshwater and food *(L. minor)* for 24 hours for gut depuration. After this period, the amphipods were washed to remove adhered MPs on their body and frozen at 80ºC for two days and then individually dissected to check for possible microplastic accumulation in gut tissues. The gut tissues were observed with a microscope of fluorescent (Leica DFC490, Germany) and the number of MPs observed in the tissues was recorded.

### Statistical analysis

The significant differences in relative growth rate of *L.minor* or mass of consumed food between test and control treatments were evaluated by one-way ANOVA analysis. Normality and homogeneity of data was checked before statistical analysis using Shapiro-Wilks test and Bartlett tests, respectively. All statistical analysis was performed using R 3.3.1. software.

## 3.7 Results

### Relative growth rate (RGR) of *Lemna minor* after 7 days of exposure to polyethylene (PE) and polystyrene (PS)

The growth inhibition tests with *L.minor* fulfilled all the validity criteria described in the OECD 221 guideline (OECD, 2002). No toxic effects of the surfactant Tween 20 on growth or photosynthetic performance of *L.minor* were found.

RGR based on biomass (ANOVA, $F_{1,10} = 4.375$, $P = 0.063$) or RGR based on the number of fronds (ANOVA, $F_{1,10} = 1.109$, $P = 0.317$) in test treatment with 48 µm PE was not significantly different from control after 7 days of exposure (**Fig.22; Fig.23**).

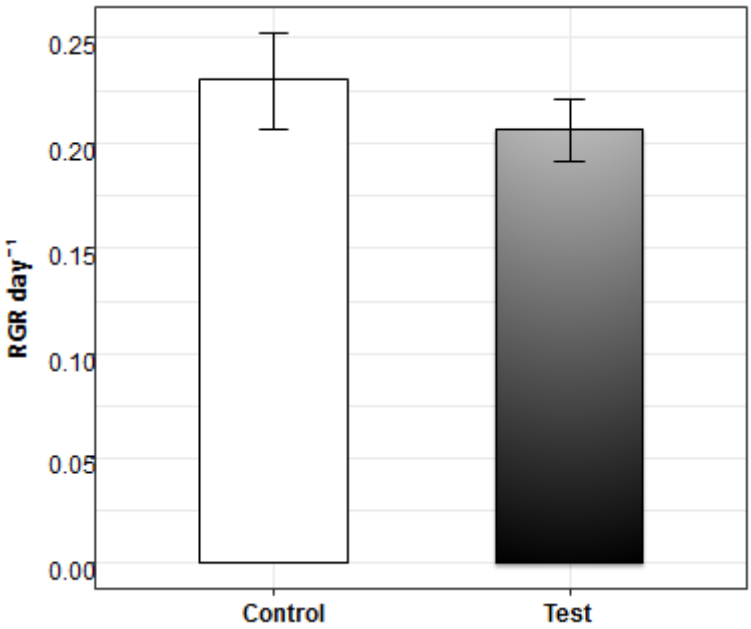


**Fig. 22. Average relative growth rate (RGR; ± standard deviation, n=6) of *Lemna minor* based on biomass after 7 days of exposure to without PE (Control) or to a solution with 48 µm PE at concentration 0.37 mg/ml (Test).**

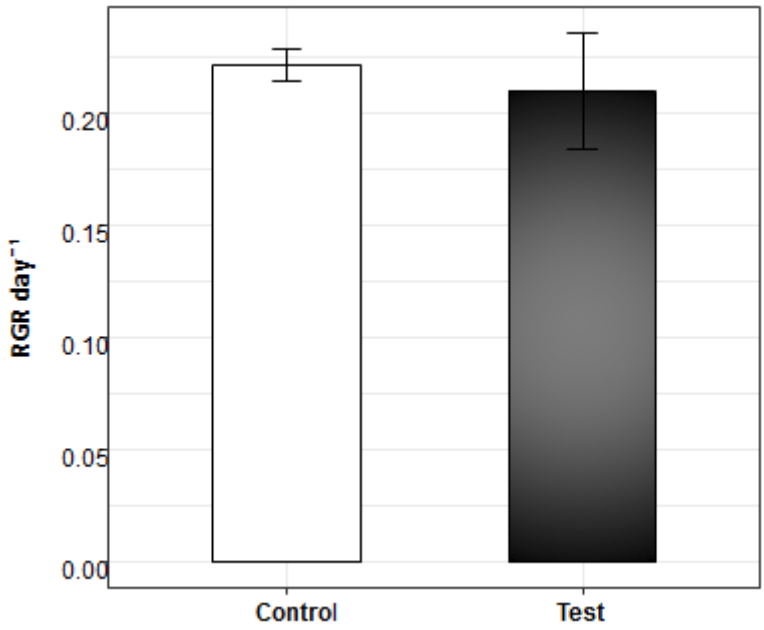


**Fig. 23. Average relative growth rate (RGR; ± standard deviation, n=6) of *Lemna minor* based on number of fronds after 7 days of exposure to without PE (Control) or to a solution with 48 µm PE at concentration 0.37 mg/ml (Test).**

No significant differences were detected between control and replicates with 45 µm PE in RGR based on biomass (ANOVA, $F_{1,10}$ = 0.529, $P$ = 0.484) or on number of fronds (ANOVA, $F_{1,10}$ = 1.8, $P$ = 0.209) after 7 days exposure (**Fig.24; Fig.25**)

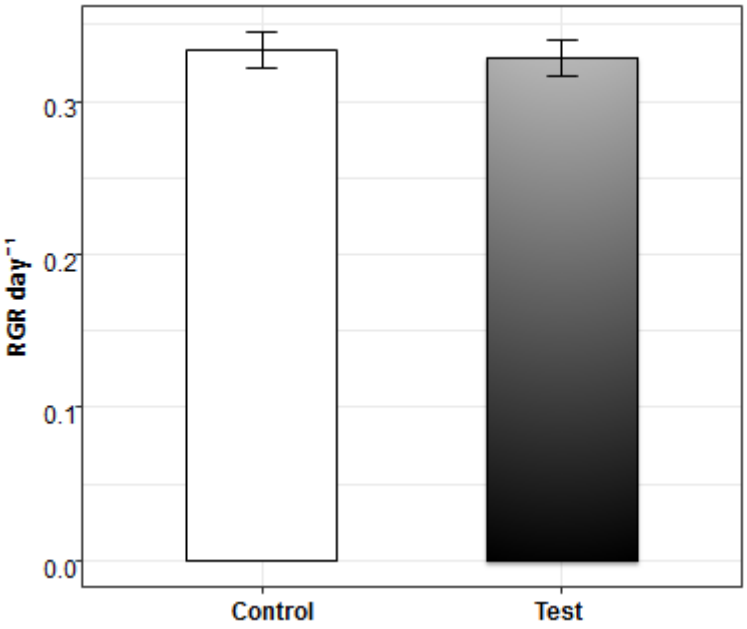


**Fig. 24. Average relative growth rate (RGR; ± standard deviation, n=6) of *Lemna minor* based on biomass after 7 days of exposure to without PE (Control) or to a solution with 45 µm PE at concentration 0.37 mg/ml (Test).**

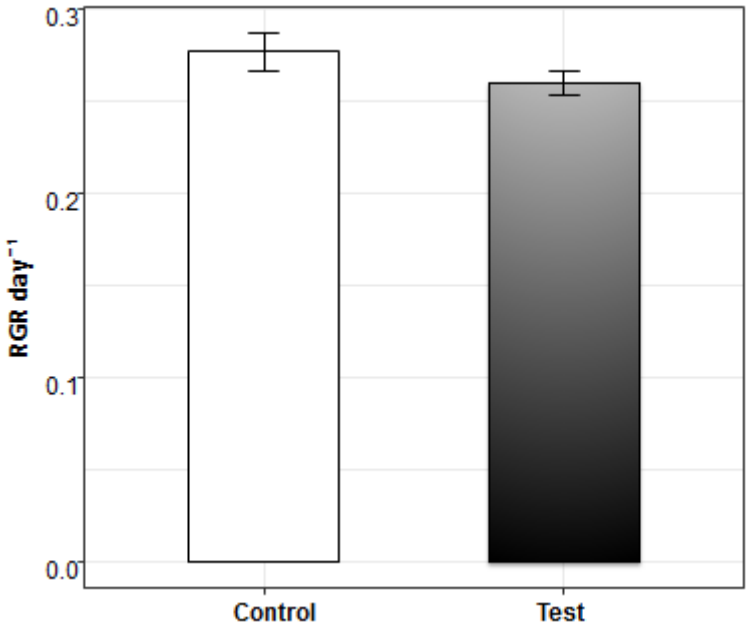


**Fig. 25. Average relative growth rate (RGR; ± standard deviation, n=6) of *Lemna minor* based on number of fronds after 7 days of exposure to without PE (Control) or to a solution with 45 µm PE at concentration 0.37 mg/ml (Test).**

Also, no significant differences were detected in RGR based on biomass (ANOVA, $F_{1,6}$ =0.128, $P$ = 0.733) and on number of fronds (ANOVA, $F_{1,6}$ = 0.133, $P$ = 0.728) after 7 days of exposure to 1 µm PS (**Fig.26; Fig.27**).

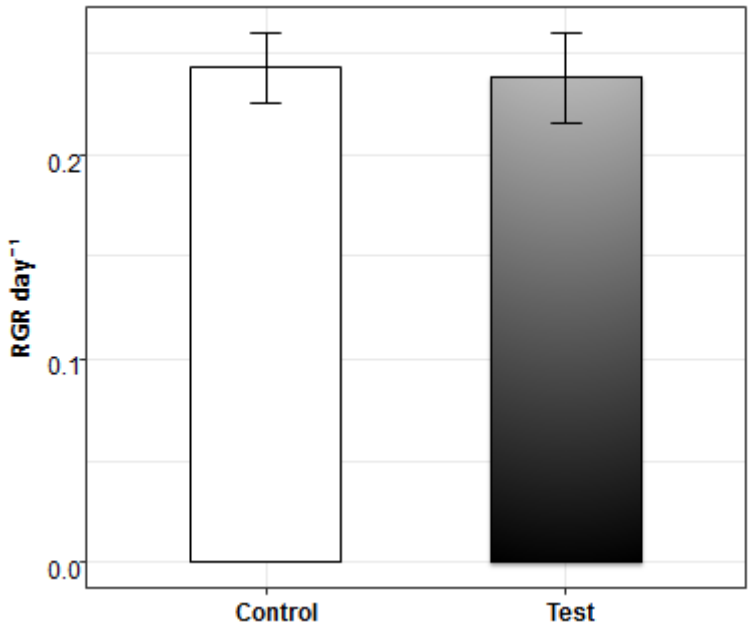


**Fig. 26. Average relative growth rate (RGR; ± standard deviation, n=5) of *Lemna minor* based on biomass after 7 days of exposure to without PE (Control) or to a solution with 1 µm PS at concentration 0.0005 mg/ml (Test).**

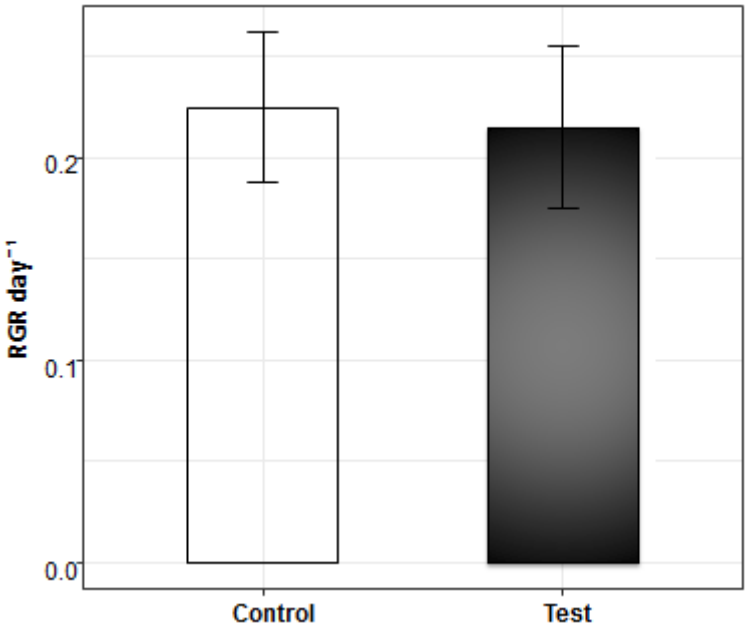


**Fig. 27. Average relative growth rate (RGR; ± standard deviation, n=6) of *Lemna minor* based on number of fronds after 7 days of exposure to without PE (Control) or to a solution with 1 µm PS at concentration 0.0005 mg/ml (Test).**

### Photosynthetic efficiency of PSII (Fv/Fm) after exposure to PE and PS

There were no significant differences (ANOVA, $F_{1,4} = 0$, $P = 0.05$) in the maximum photosynthetic efficiency of PSII (Fv/Fm) between control and replicates with 48 µm PE after 7 days of exposure (**Fig.28**).

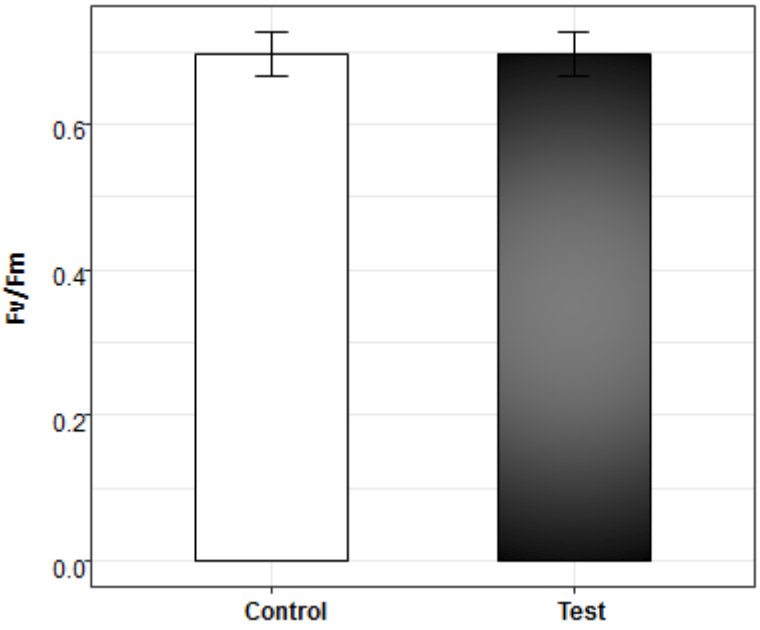


**Fig. 28. Average of the maximum photosynthetic efficiency of PSII (Fv/Fm; ± standard deviation, n=3 of *Lemna minor* after 7 days of exposure to without PE (Control ) or to a solution with 48 µm PE at concentration 0.37 mg/ml (Test).**

No significant differences (ANOVA, $F_{1,4}$= 0.006, P= 0.943) were detected in the Fv/Fm ratio between control and replicates with 45 µm PE after 7 days of exposure (**Fig. 29**).

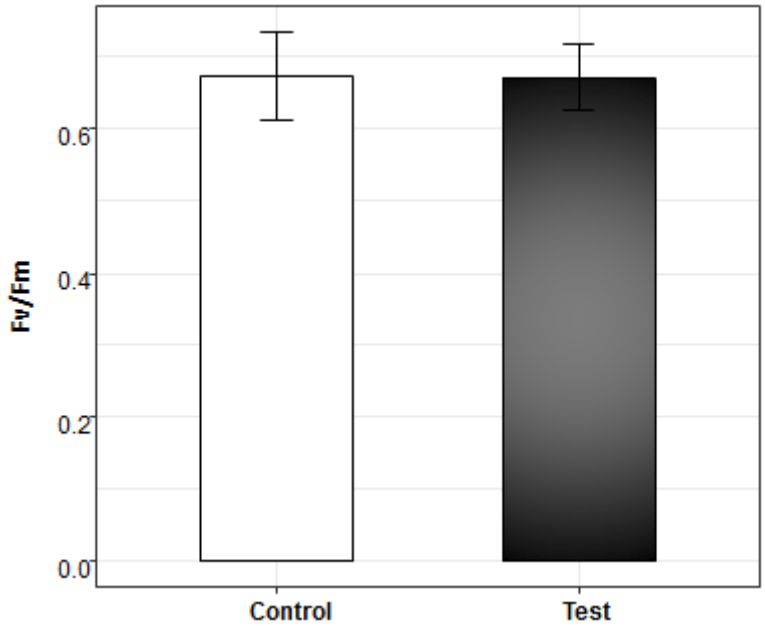


**Fig. 29. Average of the maximum photosynthetic efficiency of PSII (Fv/Fm; ± standard deviation, n=3) of *Lemna minor* after 7 days of exposure to (Control) or to a solution with 45 µm PE at concentration 0.37 mg/ml (Test).**

Furthermore, there were no significant differences (ANOVA, $F_{1,6}$ = 1.271, $P$ = 0.303) in Fv/Fm ratio between control and replicates with 1 µm PS after 7 days of exposure (**Fig. 30**).

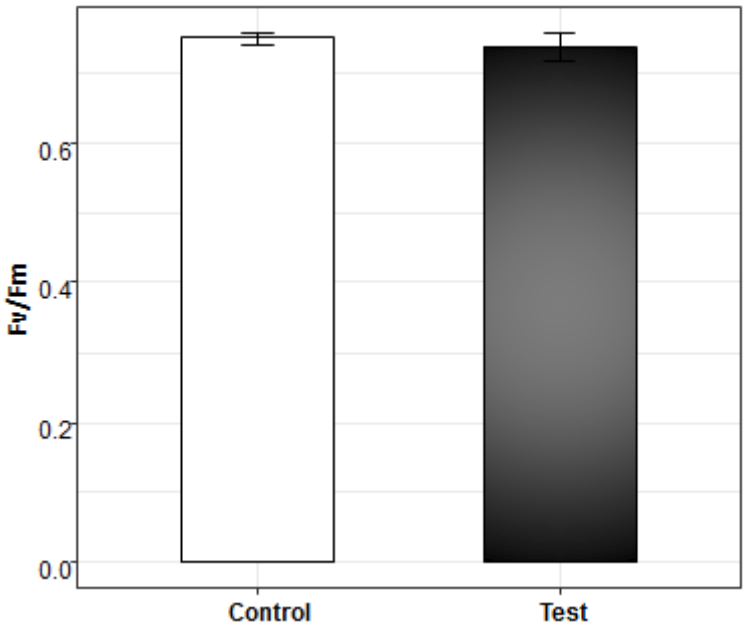


**Fig. 30. Average of the maximum photosynthetic efficiency of PSII (Fv/Fm; ± standard deviation, n=3) of *Lemna minor* after 7 days of exposure to (Control) or to a solution with 1 µm PS at concentration 0.0005 mg/ml (Test).**

**Trophic transfer of microplastics via *L.minor* to amphipod *G.pulex***

After 24 hours of exposure to 1 μm PS and 45 μm PE via *L.minor,* no mortality was detected in control replicates neither in test replicates. After 48 hours of exposure to 1 μm PS and 45 μm PE, no mortality was observed in control replicates, while one amphipod from replicates with 1 μm PS and also another one from replicates with 45 μm PE were found dead at the end of 48 hours of exposure via *L.minor*.

In total, 25 % of amphipods from the exposed individuals (n= 28) had microplastics in their gut tissues after 24 hours of depuration. More specifically, 28.57 % of amphipods from the individuals (n=7) exposed to 1 μm PS (**Fig. 31**) and 28.57 % of amphipods from the individuals (n=7) exposed to 45 μm PE for 24 hours were found to contain up to 2 particles of PE in their gut tissues after 24 hours of depuration.

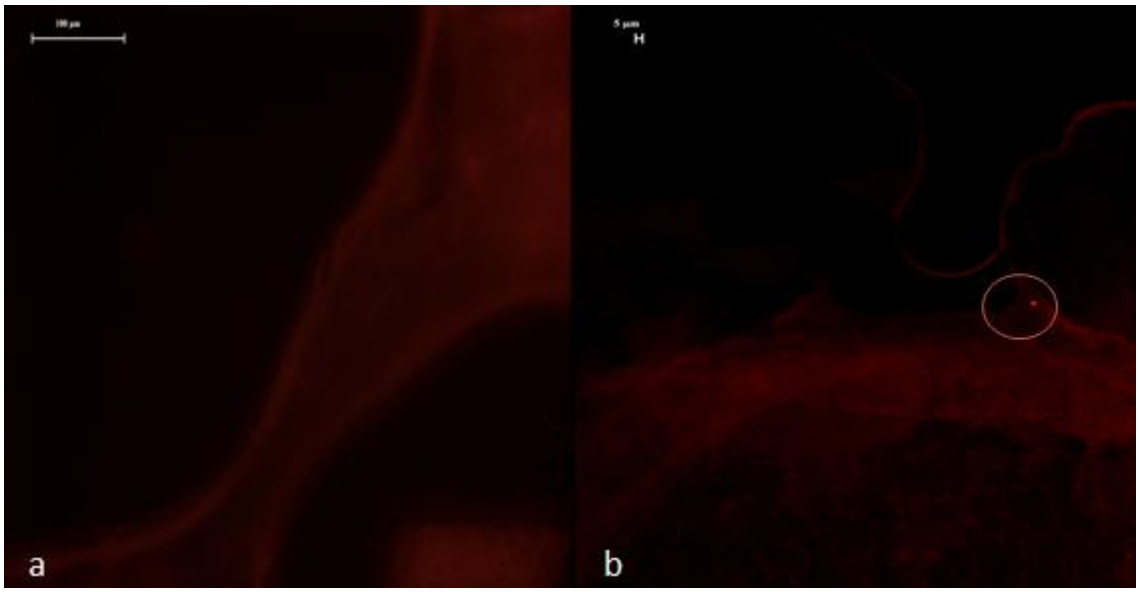


**Fig. 31. Fluorescence microscope image (amplification 10×) of the gut of amphipod from a control replicate (a) and the gut of amphipod from a replicate with 1 μm PS after 24 hours of exposure (b). The identified MP is highlighted by a red circle.**

Furthermore, 14.28 % of amphipods from the individuals (n=7) exposed to 1 μm PS and 28.57 % of amphipods from the individuals (n=7) exposed to 45 μm PE (**Fig. 32**) for 48 hours were found to have up to 2 particles of MPs in their gut tissues after 24 hours of depuration.

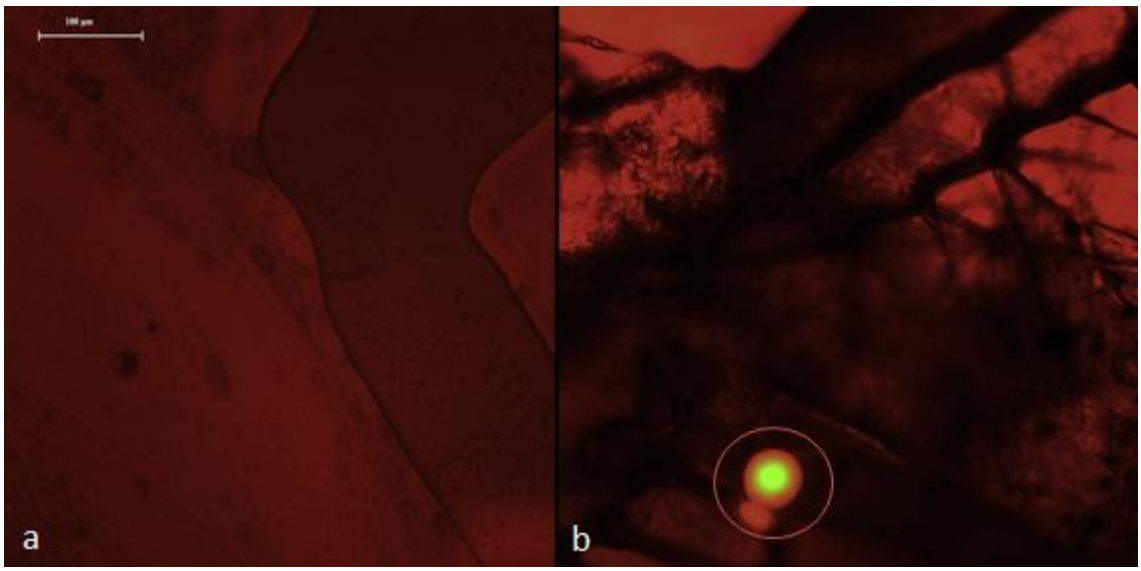


**Fig. 32. Fluorescence microscope image (amplification 10×) of the gut of amphipod from a control replicate (a) and the gut of amphipod from a replicate with 45 µm PE after 48 hours of exposure (b). The identified MP is highlighted by a red circle.**

### *L.minor* pre-exposed to 45 µm polyethylene (PE) and 1 µm polystyrene (PS) consumed by the amphipods *G.pulex* after 24 and 48 hours

There were no significant differences in food consumption between control and treatments with *L. minor* pre-exposed to 45 µm PE and 1 µm PS after 24 hours (ANOVA, $F_{1,11}$= 2.457, $P$= 0.145; ANOVA, $F_{1,11}$= 4.458, $P$= 0.0584, respectively).

Furthermore, no significant differences were found in food consumption between control and replicates with *L. minor* pre-exposed to 45 µm PE and 1 µm PS after 48 hours (ANOVA, $F_{1,14}$ = 0, $P$ = 1; ANOVA, $F_{1,14}$ = 0.134, $P$ = 0.72, respectively).

## 3.8 Discussion

### Relative growth rate (RGR) and Photosynthetic efficiency of PSII (Fv/Fm) of *L. minor* after 7 days of exposure to 45 and 48 µm polyethylene and 1 µm polystyrene

After 7 days of exposure to 1 µm polystyrene (PS) and 45 and 48 µm polyethylene (PE) particles, no negative effects on growth or photosynthetic performance of *L.minor* were found. Before this study, the effect of microplastics on *L.minor* was studied only once by Kalčíková *et al.* (2017). These authors reported no negative effects of PE (4-12 µm) on growth and photosynthetic pigments after 7 days of exposure, which agrees to the absence of effects also found in the present study.

In other previews studies, growth inhibition or lowered photosynthetic performance in *L.minor* have been reported due to the presence of soluble chemicals (Khellaf and Zerdaoui, 2009; Drost *et al.*, 2007; Frankart *et al.*, 2003), which was not the case in the present study. The PE and PS particles were insoluble; therefore they cannot be adsorbed by the duckweed unlike soluble chemicals. On the other hand a physical impact through a strong binding of PE and PS particles to the duckweed's fronds was observed. Although, binding of microplastics on fronds of the duckweed does not seem to affect significantly health of the duckweed. Apparently, microplastic particles were not sufficient to block air or light passage neither to enable nutrients uptake by the duckweed since these functions were not conditioned in the organisms and PE and PS particles were not able to penetrate into cells of the duckweed. This could be due to the sieving properties of the plant cells, determined by pore diameter between 5 and 20 nm, that acts as a barrier against external agents including particles from the environment (Nair *et al.*, 2010; *Fleischer et al.*, 1999; Li *et al.*, 2013). Therefore, only particles smaller than the pore diameter of the plant pores (5 to 20 nm) could penetrate into the cells, which was not expectable since the PE and PS particles tested in this study had sizes of 1, 45, and 48 µm.

Possibly, the impact of smaller microplastics (nanosized plastics) could be more severe effects on plants. This assumption has been supported by data reported in the available literature. For instance, Bhattacharya *et al.* (2010) reported negative effects on freshwater algal (*Chlorella sp.)* and freshwater/saltwater algal *(Scenedesmus sp.*) after 24 hours of exposure to 20 nm polystyrene (PS) on photosynthesis due to the physical blockage of light and air by the nanoparticles. In comparison, in another study conducted by Lagarde *et al.* (2016) bigger microplastics like polypropylene (PP) and high density polyethylene (HDPE) (> 400 µm) did not cause negative effects on growth of freshwater microalgae, *Chlamydomas reinhardtii* after 80 days of exposure. Despite these evidences, data from literature also reveal that the effect of MPs is not only dependent on particle sizes, but also on the material type. Previous studies have

shown that growth of *L.minor* is not inhibited under exposure to 21 nm titanium dioxide nanoparticles and 70 nm copper oxide nanoparticles after 14 and 7 days of exposure, respectively (Li *et al.*, 2013; Lalau *et al.*, 2014;). Moreover, 14 days of exposure to 21 nm titanium dioxide nanoparticles showed strong adherence of the nanoparticles on the fronds of *L.minor*, without penetrating into the cells of duckweed (Li *et al.*, 2013) as it was observed in the present study.

Although, microplastics did not affect negatively health of the duckweed in this study, strong adherence of microplastics on the duckweed could be a greater concern in the aquatic food web, since *L. minor* is food and habitat for many freshwater invertebrates and fishes. Therefore, possible trophic transfer of microplastics via the duckweed to amphipod *G.pulex* should be further investigated.

**Trophic transfer of microplastics via *L.minor* to amphipod *G.pulex***

The trophic transfer of microplastic has been showed in many organisms mainly at consumer level (Batel *et al.*, 2016; Tosetto *et al.*, 2017; Farrell and Nelson, 2013). However, there are no studies evaluating possible trophic transfer of microplastics from plants to animals. In this study, strong adherence of microplastics on *L.minor* showed that plants also could contribute to food transfer of microplastics. Duckweeds as a floating plant prone to accumulate certain pollutants (Radić *et al.*, 2011), and being food and habitat for many freshwater invertebrates and fishes (Les *et al.*, 2016), are potential organisms to serve as a pathway for microplastics enter in aquatic food chains.

In this study, freshwater amphipod *G. pulix* was fed with duckweed *L.minor* prior exposed to 1 μm PS and 45 μm PE at concentrations 0.37 mg/ml and 0.0005 mg/ml respectively, over 24 and 48 hours.

After 24 hours of depuration, 25 % of amphipods had up to 2 particles of microplastics in their gut tissues. These data are not sufficient to allow concluding with high degree of certainty that microplastics have potential to accumulate in gut of the amphipods. Moreover, the reduced number of retained particles observed in amphipods gut could be excreted in subsequent feeding periods. This assumption is supported by the study developed by Farrell and Nelson (2013), who exposed crabs *(Carcinus maenas)* to 0.5 μm PS via feeding on mussels *(Mytilus edulis)*. The authors found that while a high number of microplastics were found in tissues of crabs after 24 hours, this number decreased over time and after 21 days microplastics were almost gone. Another study showed that zebrafish (*Danio rerio*) exposed to 1–20 μm microplastics via feeding on brine shrimp (*Artemia nauplii)* and the zebrafishes excreted majority of microplastics after 5-6 hours after the exposure (Batel *et al.*, 2016).

Previous studies suggested that apart from ingestion, microplastics might cause false satiation which might result in decreased food uptake (Moore, 2008; Besseling *et al.*, 2012). However, no negative effect of microplastics was observed in food uptake of the amphipods in this case. False satiation might happened if microplastic particles take volume in stomach of exposed animals (Welden & Cowie, 2016), since microplastics did not accumulate in gut of the amphipods and most probably were excreted, false satiation was not observed in this study.

These experiments did not show evident accumulation of microplastics in gut of amphipods, although the small amount of microplastic particles were transferred from the duckweed to the amphipods suggest that microplastics can be transferred from plants to animals even at low level.

## 3.9 Conclusion

Microplastics are growing concern in the environment including freshwater systems and should be further investigated to understand their potential threat to the environment and biota. In this study, no negative effects of microplastics on growth and photosynthetic performance of duckweed *L.minor* was found most probably due to the size of microplastics that were not able to penetrate into cells of the duckweed. May be the impact of nanosized plastics could be more severe than microplastics on plants including duckweeds.

Strong adherence of microplastics on the duckweed suggested that plants might contribute to microplastic transfer through aquatic food chain. Despite that, a small number of microplastics were found to be transferred from the duckweed to the amphipod.

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