Ecotoxicology testing of microplastics contaminated sediment by oligochaeta worm, Lumbriculus variegatus

Berte Mekonen Belay

Máster en Calidad de Aguas

MÁSTERES
DE LA UAM
2017 - 2018

Facultad de Ciencias

UAM Universidad Autónoma de Madrid

Trabajo Fin de Máster. Curso 2017-2018

Master in

Inland Water Quality Assessment

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Berte Mekonen Belay

Director: Dr. Olli-Pekka Penttinen
Tutor (si distinto al director): Dr. Antonio Quesada
Lugar de realización: University of Helsinki
Department of Environmental and Biological Science

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1. INTRODUCTION

Since the 50s the plastic industry has been in continuous growth. Moreover, as additional uses for plastic continue to be discovered, plastic production is predicted to steadily increase (PlasticsEurope 2015).The high levels of production and use of this material are causing the accumulation of plastic debris throughout the planet (Bouwmeester et al., 2015). Worldwide, in 2015, about 322 million tons of plastics were produced. Around 30% of this production ends up as waste with the consequent risk of entering the marine and freshwater ecosystems (Ziajahromi et al., 2018).

Plastics have a very long degradation period and remain in the environment hundreds and thousands of years. During their long life they undergo fragmentation due to phenomena of photolysis, hydrolysis and physical forces, leading to the introduction of a large amount of very small plastic particles to the aquatic environment (Ziajahromi et al., 2018). While the presence and toxicity of larger fragments of plastic debris have been well documented in marine ecosystems, and higher trophic level organisms, there is little information readily available on whether smaller fragments of plastic debris pose an ecological and human health threat (Au et al., 2017). During the last years the study of these fragments, called micro-plastics, has gained a great interest for researchers and is an emerging area of environmental science (Lei et al., 2017).

Microplastics are small plastic particles with a size smaller than 5 mm (GESAMP, 2015). Microplastics originating from the most used plastics are PE, PVC, PS, PP and PET; together they form 90% of the world production (Li et al., 2016). Likewise, there are primary microplastics that come from applications in cosmetics, synthetic clothing, and car paints which are introduced to the aquatic environment through the sewerage system (Lei et al., 2017; Ziajahromi et al., 2018). In general, microplastics are a heterogeneous group in terms of size, shape, density and chemical composition (Lei et al., 2017). These properties directly affect and define the sedimentation and resuspension of the particles, therefore, their presence in the water column or sediment (Redondo-Hasselerharm. et al., 2018). It is evident that the densest microplastics sink and end at the bottom of aquatic ecosystems. However, recent studies have shown that low density microplastics can also end up in the bottom sediment (Galloway et al., 2017). To date, the attention was in the marine environment, but recent studies show a large accumulation of microplastics in sediments of freshwater ecosystems. Klein, S. et al., (2015) found values of 1 g kg-1 (dw) in the coastal sediments of the Rhine River (Germany). Likewise, Leslie et al., (2017) found high levels of microplastics in the suspended material of the Rhine and Meuse rivers. Therefore, it is vital to study how microplastics present in sediments and water column may affect freshwater benthic organisms as well as the organism's contribution to microplastic mixing.

In the present study *Lumbriculus variegatus* was used to test the toxicity and ecological impact of polyethylene microplastics. The overall goal of this study was to gain a better understanding of how exposure to microplastics may affect the freshwater oligochaeta, *Lumbriculus variegatus*, by establishing long term endpoints such as survival, feeding activity, growth and reproduction. To achieve this objective, three different experiments were performed.

2. MATERIALS AND METHODS

2.1 Microplastics, culture water and sediment

Irregularly shaped polyethylene microplastics (MPs) with a 10 µm particle size were provided in form of a powder by University of Helsinki, Finland. The special characteristic of these MPs is the ability to glow when illuminated with an intense light, the emitted light can be measured to determine the concentration of MPs in the sediment, inside the worms or in the fecal pellets at the end of the experiment.

Sediment samples were collected at a 10 m water depth in March 2018 from the mesotrophic Lake Vesijärvi, Lahti, Finland, using an Ekman Bottom Grab Sampler. According to Penttinen et al. (2008), the sediment characteristics were silty (90% <63 µm sediment fraction), with an organic carbon content of 7%, dry: ratio of 0.18, pH 6.5 and a total pore-water ammonium concentration of 1.2 mg/L.

The starter culture was obtained from the Department of Fisheries and Wildlife, Michigan State University, USA and cultured at AlmaLab (Department of Environmental science, University of Helsinki, Lahti, Finland) following the OECD (2007) culture methods for *Lumbriculus variegatus* and artificial fresh water (pH 7.0) was used in all experiments as in the culture.

2.2 Experimental setup

MPs were spiked into sediment or water in three experiments. Survival, feeding activity, growth and reproduction of worms were used as end-points to evaluate the toxicity of MPs. First, worms were exposed to microplastics in different exposure routes water column, pore-water, and whole-sediment to assess the most harmful pathway (Figure 1). Second, different MPs concentrations ranging from 0 to 50 g/kg of MPs were set to analyse how the established endpoints may be altered with the increasing MPs concentration. Third, a layer of 1 mm MPs were placed at different depths, starting at 1, 4 and 7 cm, to study bioturbation effects of *Lumbriculus variegatus*.

Lumbriculus variegatus feeds by introducing the head in the sediment, leaving its tail above, thereby depositing the undigested material (fecal pellets) on top of the sand surface (Leppänen et al., 1998b). Using a glass pipette, the fecal pellets were collected every second day for a period of 14 days. The collected fecal pellets were filtered on pre-weighed glass-fiber filters and dried at room temperature (24ºC approximately) for two days. The dry filters were weighed using a microbalance and all weights were noted. Filters were illuminated for 30 minutes with an intense torch to observe the glowing MPs in the fecal pellets in a dark room. Subsequently, they were observed under a microscope to detect the presence of MPs. At the end of the experiment, the contents of the vials were poured into a Petri dish to recover the worms, which were then transferred using dental tools to other Petri dishes containing culture water to rinse them. The clean worms were transferred to vials containing 8 ml of culture water and left for 24 hours to clean their gut. They were then weighed and their wet weight was recorded. Finally, ethanol was added to kill the worms, they became transparent and were observed under a microscope to detect the MPs inside.

Therefore, feeding activity was expressed as egestion rate mg dry feces per mg dry worm. The weighed fecal pellets represent the ingestion rate of worms with reasonable accuracy because assimilation efficiencies of deposit feeders are relatively low, approximately 5% (Rasmussen et al., 1984). Growth was represented as the difference in wet weight before and after the

experiment. Reproduction was described as the difference between the number of worms before and after the experiment.

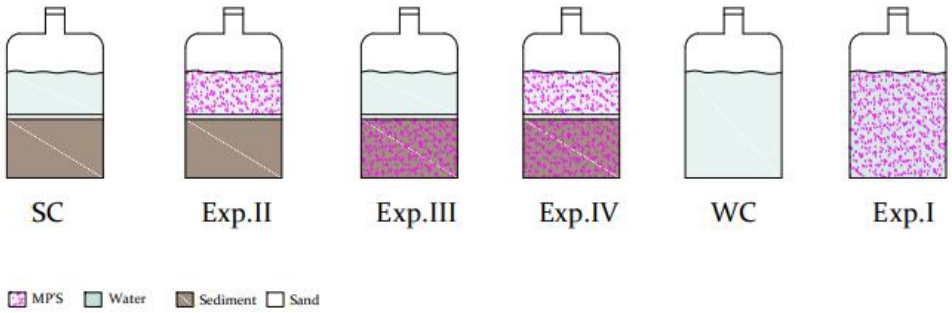

Figure 1: Exposure route experimental setup. First exposure (I), second exposure (II), third exposure (III), fourth exposure (IV), sediment control (SC) and water control (WC).

3. RESULTS

3.1 Exposure route: feeding activity, growth and reproduction

In the 16-day exposure route experiment with blue MPs, all worms in the first exposure (I) survived as well as in the WC. Figure 2 shows an increase of egestion rate during the experiment. Worms in all scenarios produced below 20 mg of dry fecal pellets/mg of dry worm at day 4. However, by day 16 all average fecal weights were above 120. This relationship with time was found to be significant (ANOVA, day: F=188.86, p< 2e-16, n=80).

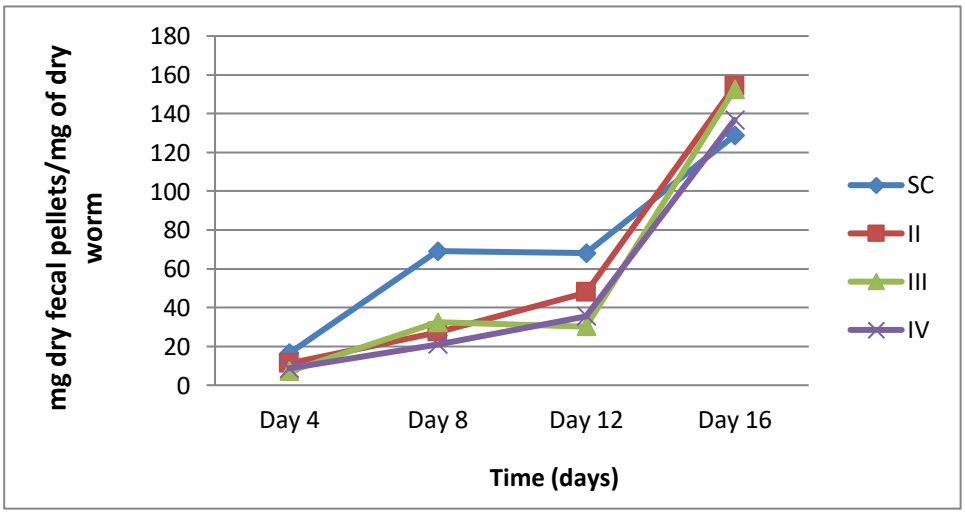

Figure 2: Mean daily sediment egestion rate of the worm L. variegatus in sediment blue microplastics exposures during the 16-d test. Second exposure (II), third exposure (III), fourth exposure (IV) and sediment control (SC).

The egestion rate in SC was remarkably superior to the other experimental conditions until day 12, when it was overtaken by the other exposures. The highest egestion rate at the end of the experiment was observed in II, followed by III, IV and SC (154.11, 152.61, 136.77, and 128.71 mg/dry fecal pellets/mg of dry worm). This variation in egestion rate between the different exposure routes was significant (ANOVA, treatment: F=4.326, p=0.0077, n=80).

According to growth, the worms in the SC increased their biomass an average of 0.94 mg. Nonetheless, in the other treatments, the biomass decreased by 0.85 mg in the II exposure, 0.72 in the III exposure and remarkably, decreased by 1.77 mg in the IV exposure. The growth of worms differed significantly between the treatments (one way-ANOVA, treatment: F=3.156, p=0.0537), but Tukey pairwise comparisons showed that the only statistically significant difference between pairs was between SC-IV (p=0.037). Growth in water exposure decreased by an average weight of 6.95 mg in WC and 7.91 mg in I exposure. However, this difference in growth between the two treatments was not statistically significant (one way-ANOVA, treatment: F=0.946, p=0.344).

With regards to reproduction, there was no pattern in the reproduction nor statistically relevant difference between treatments; only 1 out of 10 worms reproduced during water exposure in WC and I, with more worms reproducing in the sediment exposures in SC (2 out of 5), II (4 out of 5), III (5 out of 5) and IV (4 out of 5).

3.2 Concentration effect: feeding activity, growth and reproduction

In general, the fecal egestion rate increased during the 14-day experiment in all concentrations as shown in Figure 3. On the third day of the experiment (day 2), concentration B had the highest mean feeding rate (20.93 mg of dry fecal pellets/mg of dry worm) followed by C (12.19), A (9.15), E (5.11) and D (4.02). It was observed that the feeding activity was clearly dependent on the time and treatments. The results of ANOVA showed that the variation between the different concentrations (ANOVA, treatment: F=144.215, p<2e-16), time (ANOVA, day: F=35.256, p=0.000) and their interaction (ANOVA, treatment: day F=9.223, p<2e-16) were statistically significant.

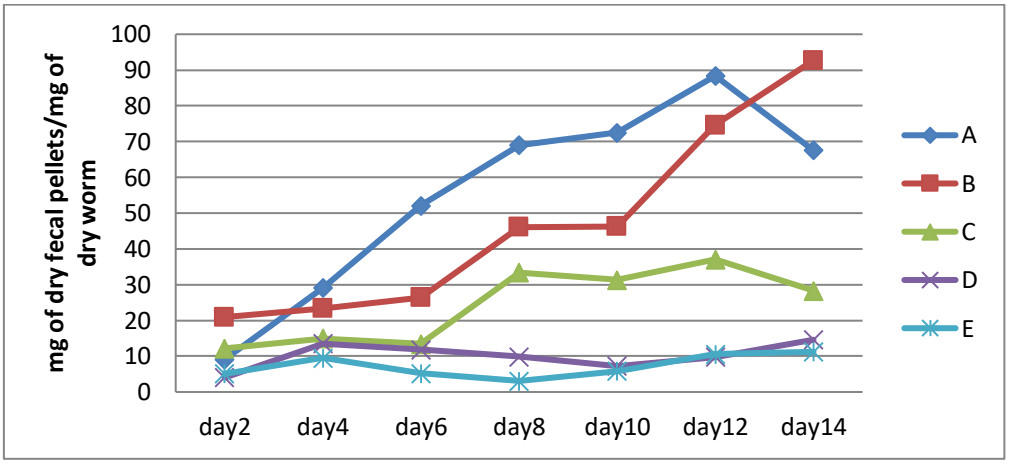

Figure 3: Mean daily fecal egestion rate of L. variegatus in different microplastics concentration exposure during 14 days. A (0 mg/kg, control), B (1.78 g/kg), C (5 g/kg), D (20 g/kg) and E (50 g/kg).

However, Tukey multiple comparisons of means found that there was no significant difference between treatments D and E (p=0.788). The difference between concentration A and B was not high compared to the other pairs, although it was not reach significance (p value=0.014). The difference between the means of the remaining pairs compared was highly significant, with the p value practically zero in all cases. Feeding activity has shown a clear dose-response correlation with increasing MPs concentration for most days EC_{50} and EC_{10} values were possible to obtain. However, only for day 10 was obtained an EC_{50} statistically significant and reliable (EC_{50} = 3.29 g/kg, r^2 = 0.968, p value= 0.016).

Worms in the control (A) and concentration B increased their biomass an average of 1.09 mg and 0.23 mg, respectively. By contrast, in the higher MP concentrations C, D and E, the biomass of worms at the end of the experiment decreased by an average of 1.58 mg, 1.74 mg and 2.82 mg, respectively. This difference between treatments was found to be statistically significant (ANOVA, treatment: $F=8.627$, $p=0.0003$). However, Tukey multiple pairwise comparisons found the following pairs to be statistically different: A-C ($p=0.017$), A-D ($p=0.011$), A-E ($p=0.0004$) and B-E ($p=0.006$). Therefore, there was significant variation between the control and higher MP exposure (C, D, and E).

With regards to reproduction, worms in the control A divided in 4 out of 10, B 8 out of 10, C 7 out of 10, D 5 out of 10 and E 6 out of 10, these variations between treatments was not statistically significant (one-way-ANOVA, treatment: $F=1$, $p=0.431$).

3.3 Bioturbation

At the end of the 15-day experiment, MPs located at 1 cm depth were found to be completely mixed, with occurring in the upper layers, from 1 cm and beyond. The worms reached the microplastics located 4 cm deep, with multiple grooves observed in each replicate, extending up to 4 cm deep. However, the worms did not penetrate below that layer. As observed previously, there was a slight mixing of the microplastics toward the upper layers due to the worms' movement. The furrows present in each replicate reached a depth of approximately 4 cm, therefore, the microplastics layer at a depth of 7 cm remained intact.

4. DISCUSSION

4.1 Microplastics exposure routes

Bioassays with benthic macroinvertebrates are widely used to estimate the potential risk of contaminated sediments. Although whole-sediment testing seems the most realistic approach to evaluate the bioavailability of contaminants in sediments, pore-waters and aqueous extracts are frequently used (Liß et al., 1997). In this study, four exposure routes (I, II, II, IV) were considered, which represent all possible locations of MPs in the aquatic environment. It has been shown that when *Lumbriculus variegatus* were exposed to1 µm, 10 µm and 90 µm polystyrene beads in water, they did not feed on microplastics. When the worms were examined, only 5, 24 and 2 beads, respectively, were found inside (Scherer et al., 2017). This was in accordance with growth results, which did not show significant reduction in the worm biomass in exposure I compared to WC.

However, the blue MPs results reveal that the different exposure routes have an impact on the feeding activity of the worms. The main route of ingestion of microplastics is through the sediment. *Lumbriculus variegatus* ingests a mixture of organic and inorganic material, with particle sizes smaller than 100 µm (Scherer et al., 2017). Microplastics in pore-water are less available than in spiked sediments, accounting for the higher feeding activity in exposure II than in exposure III. MPs in the whole-sediment and pore-water (exposure IV) resulted in a more harmful, statistically significant reduction in feeding activity compared to SC.

Similarly, the different exposure routes also produced a reduction in growth, in line with the feeding activity results. Once again, the most toxic route was exposure IV, which was logical as it contained a higher concentration of MPs in the whole system. However, while the principal exposure route was through the ingestion of spiked sediment, sinking MPs from the water column

affected the feeding and growth of *Lumbriculus variegatus*. This finding was in accordance with a study by Lu et al. (2004). They used the deposit-feeding oligochaeta, *Ilyodrilus templetoni* and found that benzopyrene and phenanthrene uptake from sediment ingestion accounted for almost all the total uptake and estimated absorption from pore-water accounted for only 5%. The toxicity of microplastics towards *Lumbriculus variegatus* was though ingestion of contaminated sediment, while exposure to MPs in the water did not affect the worm. Nonetheless, for the evaluation of the potential impact of sediment contaminated with MPs, all exposure routes should be examined by aqueous extract, pore-water, and whole-sediment testing.

4.2 Microplastics concentration effects

Polyethylene MPs did not affect the survival of *Lumbriculus variegatus*, even at a very high concentration (50 g/kg). This is in accordance with other studies which found that MPs did not significantly affect the survival of freshwater invertebrates such as *Gammarus pulex, Hyalella azteca, Asellus aquaticus, Sphaerium corneum,* and *Tubifex spp* (Redondo-Hasselerharm et al., 2018; Weber et al., 2017).

The feeding rate was greatly affected by MPs, reducing to a minimum at the higher concentrations (20 and 50 g/kg). Similarly, *Chironomus tepperi* worms exposed to 500 MPs particles/kg in the size ranges 10–27 µm and 100–126 µm significantlyaffected the number of emerged adults, with the smaller MPs causing higher mortality (Ziajahromi et al., 2018). It is of note that MPs size is an important factor as smaller MPs are more likely to aggregate together in the worm's gut or attach to the bowel wall. This phenomenon can negatively affect the egestion mechanism of *Lumbriculus variegatus*, consequently blocking the digestive tract, thereby impacting on the ability to uptake sufficient amounts of sediment (Ziajahromi et al., 2018). Exposure of the zebrafish *Danio rerio* and nematode *Caenorhabditis elegans* to different sized MPs polymers (0.1, 1.0 µm and 5.0 µm) resulted in intestinal damage. MPs caused cracking of the villi and splitting of enterocytes in zebrafish, as well as oxidative stress and changes in intestinal calcium levels in the nematode (Lei et al., 2017). This may be another logical and likely explanation for the reduction in the feeding rate observed in *Lumbriculus variegatus*, since the worm and nematode have similar gut physiology.

In contrast to present study, Weber et al. (2017) found that irregular polyethylene terephthalate did not affect the feeding activity of *Gammarus pulex,* even at high MPs concentration. Furthermore, Redondo-Hasselerharm et al. (2018) reported irregular polystyrene fragments in sediment exposure did not impact on the feeding activity of *Lumbriculus variegatus* and *Tubifex spp,* even at very high MPs concentration (40% plastic weight). In these studies, MPs ranging in size 10–150 µm and 20–500 µm were used, respectively. This further supports the hypothesis that the size of the MPs size is an important factor in their toxicity, since *Lumbriculus variegatus* ingests particles smaller than 100 µm (Rillig et al., 2017).

Microplastics at higher concentrations (5, 20, and 50 g/kg) had a statistically significant effect on *Lumbriculus variegatus* growth. The worms exposed to higher concentrations experienced a decrease in their biomass, while the biomass increased in the controls and low concentrations. This finding is in contrast to that of Redondo-Hasselerharm et al. (2018), who found no relationship between the concentration of MPs in sediment and growth of *Lumbriculus variegatus* and *Tubifex spp* among others. Growth is clearly related with feeding activity, the less they feed, the more weight they lose. In addition, the worm's slow excretion may lead to a depletion of energy reserves, as found for marine worms as a result of microplastic ingestion (Wright et al., 2013). With regard to reproduction, the concentration of MPs did not have any clear effect as the

worms divided randomly and did not follow any pattern. However, they divided less in the controls, which may suggest MPs act as possible stressors towards the worms. Indeed, the irregular MPs used had a remarkable concentration effect which correlated with the analysed endpoints.

4.3 Bioturbation

It is well known that most particles entering the freshwater and marine ecosystem sink to the bottom sediment and to some extent, interact with the benthic community before their permanent burial (Wheatcroft, 1992). The results of the bioturbation experiment showed the ability of *Lumbriculus variegatus* to mix MPs in the upper layer of freshwater sediments. The worms were only able to mix MPs in the first 1 cm and slightly at 4 cm deep. However, mixing occurred to the upper layers, which is opposite to other studies which reported that MPs are transported to deepest part of the sediment or soil by the action of different animals (Näkki et al., 2017; Rilling et al., 2017). This study demonstrated that *Lumbriculus variegatus* bioturbation action was up to 4 cm deep, suggesting that the worm could be affected by MPs present in the upper layers, with the highest MPs exposure to the worm in the 1 cm layer.

The size of MPs is also related with vertical transportation. According to Näkki et al. (2017), the Baltic clam *Macoma balthica* increased the concentration of MPs from the surface to a depth of 1.7–5.1 cm. They found more small size microplastics (50 μm) concentrated in the upper layer, with the larger size (150 μm and 300 μm) in the deeper part. In another study using the earthworm, *Lumbricus terrestris* and larger polyethylene MPs sizes (710–850 μm, 1180–1400 μm, 1700–2000 μm, 2360–2800 μm), the worm was able to transport MPs from the surface to 10 cm deep. By contrast, they found the smallest particles in the deepest layer (Rilling et al. 2017). In the present study, very small MP particles (10 μm) were used compared to the literature, which may explain why the mixing occurred to the upper layers instead of downwards.

The worm density in the sediment surface is also an important factor for bioturbation. In the present study, a density of 25,000 worms/m^2 was used, which is a fair approximation of the real density in the environment. Leppänen et al. (1998b) used a density of 24,900 to 49,750 worms/$m^2$and did not find any changes in feeding activity. However, the feeding depth of worms increased with increasing density. Therefore, it is difficult to affirm that *Lumbriculus variegatus* are not affected by MPs at deeper layers. As shown in this experiment, *Lumbriculus variegatus* did not burrow further than 4 cm, but over time, the worms may reach the MPs located at 7 cm deep.

4.4 General discussion

The present study assessed the toxic effects and ecological impact of MPs towards the oligochaeta, *Lumbriculus variegatus* by setting relevant long-term endpoints (feeding activity, growth and reproduction). MPs are present in all aquatic environments at all levels (water column, sediment surface and mixed into sediment). Therefore, it is important to use the appropriate organism to test the toxicity of MPs in each section of the aquatic media. Here, *Lumbriculus variegatus* did not feed on MPs present in the water, only feeding on MPs contained in the ingested sediment (Scherer et al., 2017). However, pore-water or surface microplastics are a source of toxicity for the worms, while the toxicity of pore-water or whole-sediment substances such as pyrene, benzopyrene, phenanthrene or heavy metal is dependent on their location (Leppänen et al., 1998; Liß &Ahlf, 1997; Lu et al., 2004). MPs in pore-water or sediment surface do not increase their toxicity but start mixing with the sediment after sinking from the water column, so are bioavailable for *Lumbriculus variegatus,* subsequently affecting feeding activity

and growth. Moreover, the benthic community has a key role in MPs mixing into sediment. This study demonstrated the ability of *Lumbriculus variegatus* to mix MPs in the upper layer (1 cm). Bioturbation is important as it causes MPs to become bioavailable for deposit feeders such as *Lumbriculus variegatus*, *Ilyodrilus templetoni* and *Macoma balthica* (Jumars &Wheatcroft, 1989; Näkki et al., 2017).

In this experiment, it was demonstrated that the feeding activity endpoint is particularly important as the other endpoints were related to the feeding process. Furthermore, high MPs concentrations significantly altered the feeding activity of the oligochaeta *Lumbriculus variegatus*. The literature and current experiment suggest that the toxicity of MPs depends on size, rather than the type of microplastic polymers (Lei et al., 2017; Weber et al., 2017; Redondo-Hasselerharm et al., 2018; Ziajahromi et al., 2018). *Lumbriculus variegatus* was affected by smaller sized MPs, preferring 10 µm sized MPs. However, their mechanism of action is still unclear, intestinal damage being one possible explanation. The current concentration of MPs in freshwater ecosystems is approximately 1 g/kg (Klein et al., 2015). The obtained EC_{50} value (3.29 g/kg, d-10, p value = 0.016) for feeding activity suggests there is not an immediate high risk for *Lumbriculus variegatus.* However, the concentrations of microplastics are expected to increase (Rochman et al., 2013), therefore *Lumbriculus variegatus* and other more sensitive species may be in danger in the near future. This study provides an insight into the negative effects of microplastics at an environmentally relevant concentration (5 g/kg) and higher on the feeding activity and growth of the oligochaeta worm, *Lumbriculus variegatus*.

5. CONCLUSION

Microplastics water exposure neither affects *Lumbriculus variegatus* survival nor growth. Therefore, water column MPs may not affect the worm until they sink and become bioavailabel for ingestion. MPs located in overlaying water (exposure II) sink to the sediment surface and have a low impact in the worms feeding activity and growth. However, the main exposure route towards Lumbriculus variegatus is through ingestion of MPs contaminated sediment. Consequently, the most hazardous exposure pathway is when MPs are present into sediment and overlaying water (exposure IV).

Environmentally relevant microplastics concentrations affect considerably feeding activity and growth of *Lumbriculus variegatus*. Nevertheless, the worm's reproduction does not show correlation with increasing MPs concentrations. MPs toxicity depends on size rather than polymer type, being the smaller (around 10 µm) particles the most hazardous for *Lumbriculus variegatus*.

Lumbriculus variegatus bioturbation effects are limited. The worm is able to mix MPs in the upper layer very close to sediment surface, but cannot burrow further than 4 cm deep. Indeed, it has shown very low MPs transport capacity. The worm may not be the most appropriate organism to study MPs mixing into sediment.

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