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Effect thresholds for the earthworm *Eisenia fetida*: Toxicity comparison between conventional and biodegradable microplastics

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HIGHLIGHTS

- Response of earthworms exposed to PE, PLA and PPC microplastics was studied.
- Avoidance, survival, biomass and reproduction of earthworms were tested.
- Earthworms clearly avoided microplastic concentrations > 40 g kg^{-1}.
- Number of cocoons during reproduction was significantly reduced at 53 g kg^{-1}.
- PLA and PPC microplastics showed no less toxicity compared to PE.

ABSTRACT

Biodegradable plastics have been developed to eliminate the progressive accumulation and ever-growing threat posed by conventional fossil fuel-derived plastics. The impact of these bioplastics, particularly in an agricultural context (e.g. biopolymer mulch films), however, remains poorly understood. In this study, we compared the biotoxicity of biodegradable (polylactic acid, PLA; polypropylene carbonate, PPC) and non-degradable (polyethylene, PE) microplastics using a series of standardized bioassays using the earthworm *Eisenia fetida*. The responses studied included: avoidance behavior, mortality, biomass, and reproduction responses. We incubated earthworms in artificial soils amended with different concentrations of microplastic (0, 0.125, 1.25, 12.5, 125, 250, and 500 g kg^{-1}) under laboratory conditions. This wide range allowed linear regression modeling and estimation of microplastic effect thresholds. Our results showed that microplastic concentration rather than plastic type was more important in regulating earthworm responses to soil contamination. The critical threshold for microplastic contamination was 40 g kg^{-1}, after which earthworms exhibit microplastic avoidance behavior. A significant reduction (EC$_{10}$) in number of cocoons and juvenile earthworms occurred at a concentration of 53 g kg^{-1} and 97 g kg^{-1}, respectively; while no significant effect was found for survival of earthworm until levels of 500 g kg^{-1}. Overall, the two biodegradable materials (PLA and PPC), appeared to be no more biofriendly than PE. Based on reported levels of plastic contamination in soil of up to 67 g kg^{-1}, we conclude that microplastics are now starting to pose a threat to earthworm population. To better evaluate the risk posed by biodegradable and nondegradable plastics, further mechanistic studies on how microplastics affect earthworm behaviour and the potential long-term impacts of this on soil functioning are required.

Keywords**:** Plastic mulching film, Microplastic safety, Earthworm response, Biotoxicity, Effect threshold

1. Introduction

Due to the action of heat, UV irradiation, mechanical forces and microbial degradation, large plastic debris in soil progressively deteriorates, leading to its fragmentation and the formation of microplastics (Li et al., 2019; Steinmetz et al 2016; Ammala et al., 2011; Laycock et al., 2017). Plastic debris less that 5 mm in size (i.e. microplastics) are generally thought to be more harmful when they enter the environment in comparison to macroplastics (Steinmetz et al., 2016; Rillig et al., 2017). A growing number of studies have reported ingestion of microplastics by different organisms, causing inflammation and damage to tissues and organs, and which also leads to further transport and accumulation in the food chain when these organisms are consumed (Lwanga et al., 2017). In addition, microplastics have the ability to bind xenobiotics and undergo long distance migration which further adds to their hazardous effect and the spread of pollution (Qi et al., 2020; Qian et al., 2020). In recent years, microplastics have been increasingly recognized as one of the most important environmental pollutants that threaten organismal health and the sustainability of ecosystem food webs (Gall and Thompson, 2015; Horton et al., 2017; Hurley and Nizzetto, 2018). A recent literature survey on the behaviour and fate of microplastics in the environment indicated that most studies have focused on aquatic ecosystems, especially oceans (71% of the total) or on sediments from aquatic environments, or beaches and sludges (24% of the total) (Qi et al., 2020). There is therefore a paucity of knowledge regarding microplastic pollution in agricultural soils and terrestrial ecosystems (Bakir et al., 2016; Qi et al., 2020)

Plastic mulching has been widely adopted in many regions of the world to promote agricultural production due to its proven ability to improve water and nutrient use efficiency and suppress weed growth (Kader et al., 2017; Li et al.,2020; Tang et al., 2020). However, incomplete removal of plastic mulch films from soil at the end of the growing season has led to a progressive accumulation of macroplastic fragments in soil. This has been reported to cause deterioration in soil health by negatively affecting the soil’s water holding capacity, damaging soil structure, slowing nutrient cycling and adversely affecting soil organisms (Liu et al., 2014; Steinmetz et al., 2016; Yan et al., 2014; Li et al., 2020). To overcome the problem of soil contamination by conventional film fragments, the agricultural industry is rapidly adopting the use of biodegradable mulch films which are designed to degrade in soil within 18 to 24 months (Feng, et al., 2019; Rodrigues et al., 2021). Typical materials used in biodegradable mulch films include biobased polymers such as polylactic acid (PLA), and chemo-synthetic polymers for instance polypropylene carbonate (PPC). Despite the growing market for biodegradable mulch films, and many studies looking at improving their tensile strength and functional properties (temperature and moisture conservation) (Rodrigues et al.,2021; Deng et al.,2019), it is still unclear whether biodegradable mulch films and their constituents are truly environmentally benign (Qi et al., 2018). This is of particular importance given the large amount of microplastic particles that may be produced by biodegradable mulch films in a concentrated time period before ultimate degradation (Qi et al., 2021; Sintim et al., 2019; Steinmetz et al., 2016; Ammala et al., 2011; Laycock et al., 2017). There is therefore a critical need to investigate and compare the effect of biodegradable and nondegradable plastics on agroecosystem health.

Due to their presence in upper trophic levels in soil food webs, earthworms (e.g. *Eisenia fetida* and *Lumbricus terrestris*) are often used as bioindicators for assessing critical thresholds for pollutant loading in soil (Calisi et al., 2013). As earthworms are also central to the delivery of a wide range of soil-based ecosystem services, these thresholds can also be used to predict when a loss of soil functioning will occur (Pérès et al., 2011). Studying the response of earthworms to non-degradable and biodegradable microplastics therefore represents an important measure to evaluate how these contaminants affect soil quality (Spurgeon et al., 2003; Zhang et al., 2018). Many studies have indicated that non-degradable plastic can adversely affect earthworm fitness by causing intestinal damage (*E. andrei*) under the exposure conditions of 125 mg kg^{-1}, producing an immune stress response (Rodriguez-Seijo et al., 2017), and reducing the growth rate of earthworms (*Lumbricus terrestris*) at high exposure levels (> 280 g kg^{-1}) in soil litter (Huerta Lwanga et al., 2016). Cao et al. (2017) also highlighted that polystyrene particles (58 μm) at a loading rate of 10-20 g kg^{-1} significantly inhibited the growth and increased the mortality of *E. fetida*, while Jiang et al. (2020) also reported that exposure to polystyrene microplastics damaged the intestinal cells and DNA of *E. fetida*. However, there are few studies on the effect of degradable plastic particles on earthworms, and none have compared biodegradable materials with non-biodegradable materials.

The objectives of this study were therefore to: (1) ascertain the acute and chronic effect of microplastics on *Eisenia fetida*; (2) determine the effect thresholds of microplastics to different toxicity endpoint traits; and (3) determine the toxicity of biodegradable and nondegradable microplastic particles.

2. Materials and Methods

2.1. Artificial soil media

An artificial soil was used to exclude the possibility that the soil may contain plastic particles, earthworms and their cocoons and is an internationally accredited method for evaluating the biotoxicity of pollutants (OECD, 2006). The artificial soil was prepared according to ISO 11268-1 and ISO 11268-2 (ISO, 2012). Peat was bought from the Beijing Guangda Hengyi Technology Co., Ltd., China, and kaolinite clay and quartz sand were bought from the Shanghai Macklin Biochemical Technology Co., Ltd., China. The different constituents were separately air-dried at room temperature, and then mixed in a ratio (w/w) of 1:2:7. Subsequently, deionized water and calcium carbonate were added to adjust the water content and pH value of the soil. After thorough mixing, the soil was stored at room temperature for 48 h to equilibrate. The pH value was determined using standard electrodes (Mettler Toledo, Switzerland) using a soil: distilled water ratio of 1:2.5 (w/w). Soil water holding capacity was measured according to ISO11268-1 Annex C (ISO11268-1, 2012). The pH of the artificial soil was 6.5 ± 0.5 and the final water content was 30% (i.e. 50% of the maximum water holding capacity).

2.2. Earthworm cultivation

Adult earthworms of the species *Eisenia fetida* were purchased from Dilongli Group (Tianjin, China) and incubated for several generations in the laboratory. Before the experiment, the worms were incubated for a week in the artificial soil to adapt to the experimental conditions during which time they were regularly fed with cow dung. Subsequently, they were transferred into artificial soils without cow dung for 24 h to clean up the intestines before use in experiments. Adult worms at age of 2-3 months, with wet mass of 0.4 ± 0.05 g, and a clitellum that represents their maturity were chosen for the subsequent incubation experiments. This is the growth period when most earthworms become mature and are ready to produce offspring (Guo et al., 1981). A population of ten earthworms was assigned to each mesocosm in the biotoxicity assays according to ISO 11268-1 and ISO 11268-2 (ISO, 2012).

2.3. Microplastics

Three types of microplastic particles were purchased from Zoomlion Plasticizing Ltd. (Changsha, China). The properties of the three plastics, namely polyethylene (PE), polylactic acid (PLA), and polypropylene carbonate (PPC) are shown in Table 1. A gradient concentration of microplastics (PE, PLA or PPC) were used for the biotoxicity assays, that begins from an under environmentally relevant exposure to 50% soil dry weight (0, 0.125, 1.25, 12.5, 125, 250 and 500 g kg^{-1}). This doses were chosen to provide a sufficient microplastic range for the linear regression modeling and calculation of effect thresholds.

2.4. Mesocosm design

The mesocosms consisted of polypropylene plastic boxes with dimensions 19 cm × 12.5 cm × 10 cm (length × width × height) (Fig. S1). Aeration holes (*n* =15) were placed along the two longer sides while a further 4 holes were placed in the top cap (Fig. S1). The mesocosms were filled with 450 g (dry weight) of artificial soil. The mesocosms were placed in a RDN-800D-4 climate chamber (Ningbo Southeast Instrument Co. Ltd., China) with temperature of 20°C, light intensity of 400~800 lux, a 12 h photoperiod and relative humidity of 70% (ISO11268-2, 2011). The moisture content of the soil was maintained at 30% by periodically weighing the mesocosms and replacing any water which had been lost by evaporation.

2.5. Earthworm avoidance in response to microplastic exposure

Microplastics (PE, PLA or PPC) were added to soil at six different concentrations in the avoidance test: 0.125, 1.25, 12.5, 125, 250 and 500 g kg^{-1} dry soil. In this experiment, a split mesocosm approach was used whereby artificial soil was placed in one half of the container (450 g) and plastic-contaminated soil (450 g) placed in the other half. A baffle plate was initially used to separate the two compartments. At the start of the experiment, the baffle plate was removed and ten earthworms placed on the soil surface at the boundary of the two compartments. Fresh cow dung (5 g) was placed on the soil surface in the center of each compartment. Each treatment had four independent replicates. After incubation for 48 h in the climate-controlled chambers, the numbers of worms on each side of the test container were recorded alongside the mass of cow dung remaining.

2.6. Earthworm biomass, reproduction and mortality in response to microplastic exposure

Bio-toxicity assays were performed according to the international standard procedures ISO 11268-1 and ISO 11268-2 (ISO, 2012). Boric acid was used as a reference substance to validate the condition of laboratory testing. As expected, the survival rate of earthworms to H_3BO_3, and the mean 50% lethal concentration (LD_{50}) to H_3BO_3 (Supplementary materials A) were highly consistent to the reference values presented in ISO 11268 (ISO, 2012).

The incubation conditions for toxic bioassays of PE, PLA and PPC microplastics were identical to those used in the H_3BO_3 test. In detail, ten earthworms of uniform age and weight were incubated in replicate mesocosms (n = 3) containing artificial soil (450 g) and various concentrations of either PE, PLA or PPC (0, 125, 250, and 500 g kg^{-1}). The mortality of earthworms was assessed by recording the percentage of dead individuals after either 7 or 14 d.

In a parallel experiment, the earthworms were initially washed, dried with paper towels and weighed (±0.0001 g) before being placed in the mesocosms. The earthworms were recovered at day 7, 14, 21 and 28 and reweighed. Mechanical handling and the time out of soil was kept to a minimum. After 28 d, we removed the adult worms and then counted the number of earthworm cocoons according to ISO 11268-2 (ISO, 2012). The cocoons were then returned back to the original plastic container so that the offspring number and biomass could be recorded at day 56.

2.7. Data analysis

All statistical analysis was undertaken in the R platform (R-Core-Team, 2019) and lme4 package (Bates et al., 2014). The cut-off for statistical significance was considered to be $p < 0.001$. Linear regression analyses were conducted using SPSS v22.0 (IBM Inc, Armonk, NY).

For the earthworm avoidance assays we recorded the number of live earthworms (Fig. S2) and calculated the rate of avoidance (R) as follows:

$$R = (N\mathrm{c} - N\mathrm{p}) / N\mathrm{t} \qquad \text{(Eqn. 1)}$$

where Nc, Np and Nt were the number of earthworms in the control compartment, in the plastic-amended compartment and in total, respectively (Martinez Morcillo et al., 2013). This gives a proportion-like variable ranging from -1 to 1, and $(R+1)/2$ values ranging from 0 to 1. We used the transformation arcsine square root of $(R+1)/2$ to normalize the data (denoted hereafter as TR). Then, we analyzed this dataset in two ways. Firstly, we included all the data. We considered the combination of plastic material and a concentration as a treatment and the contrast as a separate treatment. Therefore, there were nineteen treatments in total. We built a linear model taking the transformed rate of avoidance (TR) as a response variable and treatment as an independent variable. For the second approach, we excluded the contrast. We built another linear model taking TR as the response variable and type of plastic and concentration as independent variables.

For the biomass, reproduction and mortality assays, we excluded the contrast. We built linear mixed models by taking the logarithmically transformed biomass (or reproduction or mortality) as response variables, material, concentration and days as independent variables and experiment box as a random effect. The calculation was implemented with the stats package on the R platform (R Core Team 2019).

In addition, the effect concentrations of EC_{10} and EC_{50} for behavior and development of earthworms were calculated by solving the linear regression models for 10% and 50% effect doses compared to C_0 control ($0\ \mathrm{g\ kg^{-1}}$).

3. Results

3.1. Earthworm avoidance test

Statistical analysis revealed that rates of earthworm avoidance sharply increased with PE, PLA, and PPC microplastic concentration. However, interestingly, the avoidance behavior of earthworms was relatively less sensitive to PLA in comparison to PE and PPC (Fig. S3). As shown in Figure 1, the avoidance behavior of earthworms to PLA started at a concentration of 50 g kg^{-1}, behind that of PE and PPC. Overall, however, that avoidance behavior of earthworms was not shown to be significantly affected by the different types of plastic ($p = 0.894$), but was highly responsive to soil microplastic concentration ($p < 0.001$). The interaction between plastic type and concentration was not significant. In addition, the residual amounts of cow dung remaining on the soil surface on the plastic contaminated side of the mesocosm after 48 h increased with increasing microplastic concentration (Fig. S4). This reflected the avoidance behavior of the earthworms in the plastic contaminated compartment. The disappearance of cow dung also showed no significant difference between the three types of plastic tested. The changes in the abundance of cow dung on the surface of the soil at each of the six concentration levels (i.e. 0.125, 1.25, 12.5, 125, 250, and 500 g kg^{-1}) and microplastics (i.e. PE, PLA, and PPC) is shown in Figure S5.

3.2. Mortality of earthworms exposed to microplastics

In the unamended soil (control) and 125 g kg^{-1} PE treatment, no earthworm mortality was recorded. In contrast, earthworm mortality was recorded in all other treatments. It is worth mentioning that while PE microplastics showed a more moderate effect than PLA and PPC at relatively low concentrations, it caused more severe mortality of earthworms compared to PLA and PPC at high concentrations. When the microplastic increased from 125 to 500 g kg^{-1}, the mortality of worms increased from 0% to 12.5% under the PE treatment, 2.5% to 5% under the PLA, but stabilized at 6% for PPC. Exposure time ($p = 0.264$) and the interaction of the type and concentration of plastic ($p = 0.075$) had no significant influence on earthworm mortality rate. Regardless of time and concentration, the type of plastic (PE, PLA, and PPC) had no significant influence on earthworm death rate ($p = 0.256$) (Fig. 2).

3.3. Earthworm biomass and reproduction changes in response to microplastic exposure

Earthworm biomass significantly increased with exposure time in the PE, PLA, and PPC microplastic treatments ($p < 0.001$, Fig. 3); although the increase in biomass generally decreased with microplastic concentration. Specifically, earthworm growth rate in all the PE treatments and higher concentrations (250 and 500 g kg^{-1}) of thePPC treatment were lower in comparison to the control treatment. In contrast, at all PLA doses and the 125 g kg^{-1} of PPC treatment, earthworm growth rates were higher than in the control treatment. Overall, the type and concentration of microplastic and their interaction had no significant influence on earthworm biomass ($p > 0.1$).

At harvest on day 28, the number of earthworm cocoons in soil was found to decrease with increasing microplastic concentration. The amount of cocoons were similar between the control treatment (C_0) and at lower levels of plastic contamination (PE, PLA, and PPC at 125 g kg^{-1}). This contrasts with the lower cocoon counts recorded in the PE, PLA, and PPC treatments at doses of 250 and 500 g kg^{-1} (Fig. 4 and 5). Again, compared to the biodegradable microplastics (PLA and PPC), PE displayed less severe impact at lower doses, but more severe damage at higher contamination levels.

After returning the cocoons to the soil and incubation for a further 28 d, juvenile earthworms were collected and their number and biomass recorded. The results showed that the number of offspring in the PE_{125} treatment was the highest, followed by that in the C_0, PLA_{125} and PPC_{125} treatments, while the lowest numbers were reported in the PE_{500} and PLA_{500} treatments (Fig. 6). Regardless of plastic type (PE, PLA, and PPC), the number of offspring decreased as microplastic concentration increased ($p < 0.001$).

The total biomass of all the juvenile earthworms in each test container was determined on day 56. The total biomass of offspring in the PE treatment showed no difference to the control at PE_{125} (10.2 g), but was lowest in the PE_{500} treatment (7.4 g), decreasing significantly by 27%. In comparison, the total biomass of offspring only decreased by 7% for PLA from 9.10 g in PLA_{125} to 8.5 g in PLA_{500}, and from 15% for PPC from 10.7 g to 9.02 g (Table S6).

3.4. RDA analysis and biotoxicity thresholds

RDA was used to explain the biomass and reproduction of earthworms (response variables) using concentration and plastic type (explanatory variables) after incubation (Fig. 7, $p = 0.002$). We found that the biomass and reproduction of earthworms were negatively correlated with microplastic concentration, but not significantly with the type of plastic material.

Therefore, we calculated the EC_{10} and EC_{50} effect concentrations of the microplastics using generalized linear regression models (Fig. 8), which instead of distinguishing between the types of microplastics reflected them all as a whole. This was used to determine EC_{10} and EC_{50} values for the effect of microplastic on the survival, development, behavior and reproduction of earthworms. Our results showed that plastic avoidance is a very sensitive response of earthworms to soil microplastic contamination, with EC_{10} and EC_{50} values of 40 g kg^{-1} and 207 g kg^{-1}, respectively. Reproduction of earthworms were also significantly affected by microplastic exposure, as with number of cocoons and juvenile earthworms sharply reduced by10% at 53 g kg^{-1}, 97 g kg^{-1}, and 50% at 347 g kg^{-1}, 500 g kg^{-1}, respectively (Table 2). However, microplastics caused no significant effect on survival of earthworms until they were present at extremely high concentrations (500 g kg^{-1}).

4. Discussion

4.1. Acute response of earthworms upon exposure to microplastics

Earthworms are commonly used as model organisms to assess the potential toxicity of soil contaminants (ISO11268, 2012; Rombke et al., 2007). In our artificial mesocosms we showed that earthworms exhibited clear avoidance behavior when the concentration of microplastics in soil reached 40 g kg^{-1}. This supports the previous study of Huerta Lwanga et al. (2017) who reported that earthworms migrated to deeper soil layers when polyethylene concentrations in soil litter layers reached up to 70 g kg^{-1}. A key finding from this study was that all microplastic types induced avoidance behavior, irrespective of chemical formulation, suggesting that the avoidance behavior of earthworms was mainly related to the physical properties of the microplastic or its chemical properties. Some previous studies have reported that high concentrations of microplastic can adversely affect soil structure (e.g. soil bulk density, water holding capacity, and soil aggregates), unfavorable for earthworm movement and soil ingestion (de Souza Machado et al., 2018). In addition, microplastics have been shown to cause burns and lesions on the surface of earthworms (Baeza et al., 2020), leading to avoidance behavior. It is worth mentioning that the avoidance behavior of earthworms to PLA was always relatively lower than to PE and PPC (Fig. S3). A possible reason might be that PLA is a biopolymer material obtained by polymerization of lactic acid that might represent a supply of available carbon for the earthworms at relatively low concentrations. However, the mechanistic basis and factors influencing earthworm avoidance behavior needs to be investigated further.

In our experiment, exposure time (7 or 14 days) had no significant influence on the mortality of earthworms. It is possible that the earthworms had adapted to the presence of microplastics, especially low levels, after incubation for one week. Mortality was, however, significantly higher in the high PE treatment (250 g kg^{-1}) in comparison to the control treatment. This result is similar to that reported by Huerta Lwanga et al. (2016), however, it should be noted that these concentrations represent extreme addition rates to soil which are typically only seen in waste contaminated urban soils (He et al., 2019;

Yang et al., 2018). It is also likely that these urban soils would contain a range of other co-contaminants (e.g. metals, PAHs) which may also compound the effect of the plastics (Browne et al., 2013; Gomiero et al., 2018). It is worth mentioning that, PE, PLA, and PPC had a different influence on the death rate of worms, with PE being particularly toxic at high concentrations, while less harmful than PPC and PLA at lower contamination levels. The underlying reason may be related to selective uptake of different materials and degradable degrees of microplastics in the earthworm intestine (Zhang et a., 2018). Biodegradable microplastics such as PLA and PPC might also be ingested by earthworms at higher proportions due to their higher degradability and thus greater associated biofilm and microbial load (Zhang et a., 2018). The greater biofilm and nutritional content may also reduce their toxicity. In contrast, PE might accumulate more in the earthworm intestines/typhlosole inducing blockages (Chen et al., 2020; Huerta Lwanga et al., 2016). Huerta Lwanga et al. (2018) indicated that low-density polyethylene (LDPE) microplastics could be degraded by bacteria isolated from the *Lumbricus terrestris* gut, however, this breakdown process is expected to be very slow relative to the transit time through the gut. Zhang et al. (2018) also reported that earthworms did not ingest PE, but foraged partial field-weathering biodegradable microplastics with smaller particle sizes for food. Currently, there is no consistent evidence on the adverse effect of nondegradable and biodegradable microplastics on earthworm intestines. In spite of this, the discussion above indicates the potential risk of microplastic particles to the survival of earthworms.

4.2. Chronic response of earthworms to microplastics

The growth rate and biomass of earthworms decreased with the increasing concentration of PE in this study, a finding also demonstrated by Huerta Lwanga et al. (2016), who indicated that the growth rate of *L. terrestris* decreased with a higher percentage of PE microplastics (280 g kg^{-1}, 450 g kg^{-1}, and 600 g kg^{-1}, size < 150 μm) in the soil litter layer. A similar dose-dependent decrease of growth rate was also reported by Redondo-Hasselerharm et al. (2018) in a study of freshwater benthic macroinvertebrates with polystyrene microplastics ranging from 0 g kg^{-1} to 400 g kg^{-1} in sediment. In addition, the number and mass of microplastics inside the body of *G. pulex* showed a positive relationship to sediment exposure (Redondo-Hasselerharm et al., 2018). The adverse effects of microplastics would be mainly caused by the significant accumulation of microplastics in the gut and stomach of organisms, which can damage their immune systems and affect their feeding behavior and development (Eltemsah et al., 2019; Liu et al., 2019; Rist et al., 2017; Rodriguez-Seijo et al., 2017;

Yin et al., 2019). Moreover, it is important to stress that biomass is not strictly a reliable indicator on the growth of earthworms, as it may also include the weight of microplastics that have not been egested.

For the eggs and reproduction (including offspring number and biomass) of earthworms, there was no obvious distinction between the low concentration treatment (125 g kg^{-1} of PE, PLA, and PPC) and the control (C0) which decreased with increasing concentration level. Kwak and An (2021) exposed earthworms to two different sizes of polyethylene microplastic for 21 days, and concluded that microplastics affected coelomocyte viability and caused damage to male reproductive organs, while having negligible effects on female reproductive organs, which may affect the reproduction of earthworms.

4.3. Effect thresholds of microplastics on development and behavior of earthworms

Nanoplastics generated from ingested microplastics can be introduced into soils through cast excretion and these may pose an additional risk to soil organism and environment (Rillig, 2012; Rodriguez-Seijo et al., 2017). Our results showed clear avoidance of earthworms to microplastic exposure > 40 g kg^{-1}, which is the most sensitive response of earthworms observed in our study, suggesting an instinct capability for self-preservation. In addition, microplastics also caused a significant inhibition on the reproduction of earthworms as the number of cocoons sharply decreased by 10% at concentrations of 53 g kg^{-1}. On the basis of reported levels of plastic contamination in terrestrial soils as high as 67 g kg^{-1} (Fuller et al., 2016), it is possible that microplastics are already starting to pose a threat to earthworm populations. However, no significant effect of microplastics was observed on the survival of earthworms, which may suggest some autoregulation and physiological protective effects within the adult earthworm population. Further studies should seek to gain a deeper mechanistic understanding of the effects of different microplastics on earthworms. In our study, microplastic concentration was the dominant factor affecting earthworm biomass and reproduction, while material type had a much lesser effect. PLA and PPC, as two biodegradable materials, were no more benign than PE. The biosafety of biodegradable plastic film remains to be verified (Qi et al., 2018; Sintim et al., 2019; Zhang et al., 2018). Additionally, this study mainly compared the effects of three types of microplastics (PE, PLA, and PPC) on earthworm behavior and survival, and did not explore their ecotoxicological mode of action. The ingestion of microplastics in earthworm bodies and its effect on the pathological tissue of earthworms should therefore be further studied.

5. Conclusions

Here we evaluated the response of *Eisenia fetida* in soils amended with different concentrations and types of microplastic. We found that the biomass and reproduction of the earthworms was negatively affected at microplastic concentrations greater than 40 g kg^{-1}. With microplastic concentrations as high as 67 g kg^{-1} being reported in terrestrial environments, this suggests that microplastics may already be adversely affecting native earthworm populations and thus negatively impacting on soil functioning. Concentration proved to be the dominant factor affecting earthworm biomass and reproduction, rather than type of plastic material. The two biodegradable microplastics (PLA and PPC) did not appear to be more environmentally benign than PE. To improve our understanding of microplastic behavior in agricultural soil, further work is needed to identify the production rate of microplastics from biodegradable and nondegradable films, and their distribution in the natural environment. Additionally, further studies are needed to gain a better mechanistic understanding of how biodegradable microplastics affect earthworms and the potential long-term impacts of these effects on soil functioning. Together, these will allow a more holistic evaluation of the safety of biodegradable plastic use in agriculture.

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