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Ecotoxicological Effects of Commercial Microplastics on Earthworm *Eisenia fetida* (Savigny, 1826) (Clitellata; Lumbricidae)

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Abstract: As soil invertebrates with a unique digestive system, earthworms are regularly used as bioindicators and test organisms. Due to their burrowing activity and casting, earthworms are involved in the structuring of the soil. However, this way of life exposes them to different pollutants, including microplastic particles. Although the use of plastics is economically justified, it has a major impact on living organisms. In this study, the influence of different concentrations (2.5%, 5%, and 7% (*w*/*w*)) of commercial glitter as a primary source of microplastics (MPs) on mortality, growth, cocoon production, avoidance behavior, and bioaccumulation ability during a four-week exposure of the earthworm species *Eisenia fetida* was investigated. The mortality was higher at 5% and 7% MPs in the soil than at 2.5% and in the control (0%) after 28 days, and the number of cocoons and growth rate decreased with an increasing MP concentration. However, the earthworms did not avoid the soil with MPs. Furthermore, the dissection of the digestive system enabled the identification of MP distribution. The sections of the digestive system were additionally examined under a fluorescence microscope. The results indicated that non-selective feeding enabled the input of MPs into the earthworm's body and, thus, into food webs.

Keywords: bioindicator; *Eisenia fetida*; environment; glitter; microplastic; soil ecosystem

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1. Introduction

Due to the development of human civilization, the environment has been severely degraded, sometimes without the possibility of recovery. According to the literature, the annual world production of plastic in 2021 was 390.7 million tons, and these numbers are constantly increasing [1]. Every day, plastic is utilized in a wide range of products, such as packaging, films, covers, bags, and containers, as well as in greenhouses, mulches, coatings, and wiring [2]. The problem arises the moment plastics are intentionally or accidentally disposed of in the environment. The decomposition of plastics under the influence of abiotic and biotic factors leads to the formation of many smaller components, such as microplastics (MPs) [3]. Microplastics are generally defined as plastic particles that are smaller than 5 mm in length and tend to accumulate in the environment [4]. According to their origin, we distinguish between primary and secondary sources of MPs [5]. Primary sources include MPs that are already produced in such a size that mostly include personal care products (shower gels, peelings, make-up, etc.). MPs from primary sources can end up in various ecosystems, both through wastewater or direct disposal in the environment [6,7]. Glitter, for example, is a type of primary MP that is produced and used in large quantities but has received less attention from the environmental science community compared to other MPs [8,9]. On the other hand, secondary sources of MPs include all plastics (plastic bags, plastic foil, etc.) that are subjected to the influence of sunlight (photodegradation) as well as mechanical action, resulting in the formation of MP particles [3,10], a process known as weathering.

The use of MPs in various branches of industry contributed to their massive spread and irresponsible disposal, which resulted in their presence in almost all parts of terrestrial and aquatic ecosystems [11–13]. The first studies on the effects of ingested MPs mainly concerned organisms in marine ecosystems [14–16]. However, MPs can be detected not only in aquatic systems but also in terrestrial environments, which are often the first receivers of anthropogenic activities [17]. Soil, a complex and dynamic environment, plays a crucial role in supporting terrestrial life, and the introduction of MPs poses significant challenges to its health and functioning. The migration of MPs into deeper layers of soil as a result of agricultural activities such as plowing followed by dry weather conditions (cracking) greatly contributes to its dispersal. Soil organisms also introduce MPs into deeper layers of soil through their movement, digestion, and subsequent excretion, which results in the pollution of groundwater [18]. The emphasis of research in recent years has been on the ability of MPs to bind toxic compounds in their environment, increasing their concentration on a small surface area. After the ingestion of such particles, the concentration of pollutants is up to a hundred times higher than in the environment [19]. Furthermore, Tourinho et al. [19] also stressed that the combination of pesticides, antibiotics, and other pollutants with MP particles takes place through different hydrophobic and electrostatic interactions, leading to the formation of toxic carriers. In addition, the entry of these particles into the body can lead to the disruption of endocrine functions [20]. Accordingly, a few studies researched the impact of MPs on soil invertebrates: terrestrial snails [21], isopods [22], and earthworms [17,18,23–27]. In most global ecosystems, earthworms make up the majority of animal biomass in the soil [28]. Earthworms have been characterized as typical ecosystem engineers since they have a major influence on soil structure and are not primarily involved in trophic connections [29]. According to some studies [18,30,31], soil quality, which includes physico-chemical properties such as pH and organic content but also soil moisture content and temperature, can influence the health status of earthworms, including their growth and survival. Studies showed that MPs, including glitter, can have adverse effects on the growth and reproduction of earthworms. For example, Sobhani et al. [32] found that polystyrene MPs, even at environmentally relevant concentrations, can reduce earthworm reproduction and cause DNA damage. Cao [33] also reported an inhibition in earthworm growth and increased mortality when exposed to MPs. Kwak [34] further demonstrated that exposure to MPs can damage the spermatogenesis and coelomocyte viability of earthworms. Recent studies showed a positive effect of their intestinal microbes on the biodegradation of low-density polyethylene (LDPE) as well as polyethylene (PE) [18,23], all as a result of a much larger number of microorganisms, up to 4000 times greater than in soil [35]. Therefore, earthworms may be a great model organism to observe the decomposition and fate of plastic particles in the soil environment. *Eisenia andrei* (Bouché 1972), *Eisenia fetida* (Savigny 1826), and *Lumbricus terrestris* Linnaeus 1758 are most frequently used in studies on the decomposition of plastic and MP particles [17,27].

Interestingly, the majority of the research has focused on MPs from secondary sources, while MPs from primary sources have been neglected, even though their surface-to-volume ratio can potentially lead to enhanced mobility and higher toxicity and sorption [35]. One typical form of primary MP that can easily end up in the environment is glitter. Glitters are polyphase materials that have a polymer core and a shiny aluminum layer coated with acrylic lattice [36]. The composition of glitters is complex and can include PET, PBT, PCV, SBC, PS, epoxy, aluminum, and Fe_2O_3 [37]. According to some estimates, only 1 g of glitter can provide about 50,000 MP particles in soil.

The aim of this paper is to obtain a more comprehensive understanding of the influence of different concentrations of primary MPs in the form of glitter on the behavior, reproduction, and ingestion of the experimental model organism *E. fetida*, as well as to provide the necessary information to be used in ecological risk assessment in soil ecosystems.

2. Materials and Methods

2.1. Earthworm Cultivation and Artificial Soil Medium

The organisms *E. fetida* were obtained from the Botanical Garden in Kragujevac, Serbia. *Eisenia fetida* is an epigeic earthworm usually found in compost heaps and used as a test organism. Individuals of *E. fetida* were cultured in the Laboratory of Zoology, Institute of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia, in a medium as recommended by OECD [37]. The experimental medium (artificial soil) was also used according to the OECD [37] recommendations (70% quartz sand, 20% kaolin clay, and 10% sphagnum peat and calcium carbonate for a pH of 6.0 ± 0.5). To prepare this medium, the dry components of the artificial soil are mixed thoroughly, and then distilled water is added in the volume needed to achieve a 35% dry weight moisture content. The natural condition of the soil was maintained, and, respectively, soil was not sterilized to avoid additional stress in the bioassay. Therefore, these optimal conditions allowed for the observation of MPs as the only source of stress.

2.2. Microplastics and Experimental Design

A commercial glitter, readily available in shops, was used in the study. A unique type of MPs known as glitter typically has three layers: a plastic core made of a stretched polyester PET film called BoPET (biaxially oriented polyethylene terephthalate), which is frequently coated in aluminum to give it a reflective appearance and covered with another thin plastic layer, such as styrene acrylate [9]. The radius of glitter particles in hexagons, squares, and rectangles was measured as small as 0.075 mm (75 µm) up to 0.2 mm (200 µm) using ImageJ software 1.54f.

In this study, two experiments were carried out: one with the assessment of avoidance behavior and the other with mortality and reproduction as the endpoints following the OECD [38] and ISO [39] standards. In both experiments, the same test box design was used (10.5 cm × 9.6 cm × 7 cm) containing 300 g of the appropriate substrate (Table 1). In this study, different concentrations of commercial glitter MPs were added to test boxes, including a control box without MPs (Table 1). The concentrations of MPs used were 2.5%, 5%, and 7%; i.e., it is the percentage of the MP weight added to the weight of the dry artificial soil (w/w), following the protocol by [17]. The concentrations were chosen according to the same literature data [17]. The earthworms selected for the test were acclimatized to the test soil under test conditions for at least 48 h before use. The earthworms used in the experiments were adults with well-developed clitellum and weighed between 300 mg and 400 mg [38]. Ten earthworms were added to each test box, and all experiments were performed in triplicate. During the experiments, test boxes were covered with perforated plastic lids and were kept at a temperature of 20 ± 2 °C. The test was carried out under light–dark cycles (16:8) as described by Gavina et al. [40].

Table 1. Test boxes with respective concentrations of glitter microplastics (MPs) compared to the total substrate in avoidance experiment (test box divided into two zones) and reproduction experiment (whole substrate was prepared as the right zone in the table).

Test Boxes	Left Zone (LZ)	Right Zone (RZ)
Control	300 g of soil	300 g of soil
2.5%	300 g of soil	7.5 g of MPs, 292.5 g of soil
5%	300 g of soil	15 g of MPs, 285 g of soil
7%	300 g of soil	21 g of MPs, 279 g of soil

The first experiment was carried out for 28 days, with a mortality and growth rate assessment after 7, 14, and 28 days and cocoon production only after 28 days. The individuals were fed with 5 g of horse manure once a week per box, and water was added, if necessary, once a week by replenishing the loss estimated by weighting.

In the first experiment, the mortality rate after 28 days was assessed by recording the number of dead earthworms. Earthworms were considered dead if they did not respond to the probing stimulus or were missing. The earthworms were weighed after 7, 14, and 28 days of exposure to assess their growth during the experiment. The growth was evaluated through weight loss or gain. Preparation for weighing involved searching for the organisms within the substrate, followed by rinsing in the distilled water to remove any traces of soil and MPs that could affect the assessment of their progress. Excess moisture was absorbed using filter paper. After 28 days, the number of earthworm cocoons was counted according to ISO 11268-2 [41]. In the second experiment, the avoidance behavior bioassay was used [39]. The earthworm avoidance behavior bioassay serves as a tool for assessing the early detection of potential impacts of exposure to certain pollutants (Figure 1).

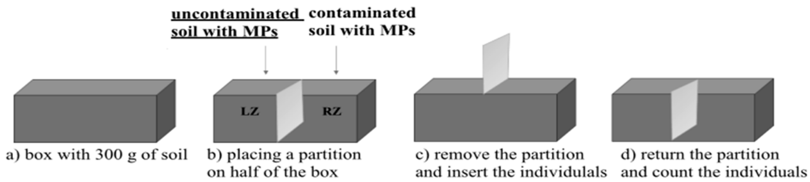

Figure 1. Schema of the bioassay procedure (avoidance behavior) of earthworms [38] for assessing the effects of different concentrations of glitter MP particles.

2.3. Microplastic Ingestion

Additionally, to observe whether MPs were ingested and visible to the naked eye after 28 days of exposure in the first experiment, randomly selected individuals from each box were dissected. The dissection was performed along the mid-dorsal line, from the prostomium to the pigidium, using a binocular microscope (Portable LCD Microscope G1200, China) with a magnification of 1200× to avoid damaging organs during the incision. This enabled a view of the whole digestion system. However, to determine the distribution of MPs, it was necessary to make cross-sections, which were then analyzed using fluorescence microscope in a dark chamber. The filter used for green fluorescence during the study was a Green Fluorescent Protein (GFP) (wavelength range from 380 to 440 nm). To prepare cross-sections, the earthworms were treated with formaldehyde to remove excess water, which increased the sample's firmness and facilitated processing. Subsequently, the samples were exposed to low temperatures (−80 °C), which further improved the quality of the cross-sections.

2.4. Statistical Analysis

Data are presented as the mean ± standard deviation (SD). The normality of the data and equality of variance were tested by the Shapiro–Wilk test and one-way analysis of variance (ANOVA) followed by a post hoc (Tukey's test) using SPSS v20.0 [42].

3. Results and Discussion

The basis of the study was to assess the effects of the stress conditions induced by different concentrations of primary source MPs—commercial glitter on the earthworm species *E. fetida*.

3.1. Mortality, Growth, and Reproduction

Mortality was determined by the percentage of dead (non-responsive to a stimulus or missing) *E. fetida* individuals per treatment after 7, 14, and 28 days of exposure. No mortality occurred in any treatment after 7 and 14 days of exposure (Table 2). However, after 28 days, mortality was significantly higher in the 5% and 7% MP treatments in comparison to the control treatment ($p > 0.05$). These results are similar to those reported by Huerta Lwanga et al. [18], according to which both the duration of exposure and concentration of MPs are related to earthworm mortality. It is also important to mention that different types of MPs affect earthworm survival differently. For example, polyester-derived microfibers were found to be non-lethal to earthworms but still alter their behavior and biomarker expression [26]. A recent meta-analysis showed that concentrations of both MPs and nanoplastics (NPs) greater than 1 g kg^{-1} are most likely to affect the growth and survival of earthworms [43]. The size of the MPs may also play a role, as smaller particles are more likely to be ingested and cause adverse effects [18]. This could be the additional factor causing mortality in our study besides concentration.

Table 2. Growth rate, mortality, and cocoon production (Mean ± SD) of *Eisenia fetida* in the different treatments with glitter MPs after 28 days of exposure.

	Treatment			
MPs	0%	2.50%	5%	7%
Growth rate	3.8 ± 0.10	2.93 ± 0.06	2.70 ± 0.05 *	21.70 ± 0.08 *
Cocoon production	3.32 ± 7.42	2.26 ± 5.05	1.92 ± 0.89 *	1.85 ± 0.58 *
Mortality	0 ± 0	0 ± 0	0.2 ± 0.44	0.6 ± 1.34

* Indicate significant differences among treatments ($p < 0.05$).

To determine growth, each earthworm was weighed individually during the experiment, and the average final weight was compared with the initial weight values. When the individuals were removed from the test boxes for the growth measurements (on the 7th but also on the 14th day of exposure), it was observed that at two higher concentrations (5% and 7%), the individuals were in a state of lethargy or rest, which can be attributed to the MPs irritating them during movement and causing physical discomfort both on the outside (dermal damage) and on the inside (in the digestive system) of the body. Based on the results, it is evident that the growth rate of the individuals in the control treatment is consistent, while a clear inhibitory effect can be observed in the treatments with MPs. The measurements on the seventh day show that at concentrations of 2.5% and 5% of MPs, the individuals have a slight decrease in growth, whereas, at the concentration of 7% MPs, there is an increase in body mass ($p < 0.05$). On the 14th and 28th day, the growth decrease in treatments with MPs persisted, likely due to histopathological changes. However, a significant decrease is observed in the treatments with 5% and 7% MP ($p < 0.05$) (Figure 2, Table 2). The reduction in the growth rate of earthworms is a consequence of the interaction between food and MPs, which leads to a decrease in nutrient resources and reduced ingestion. In addition, Rodriguez-Seijo et al. [25] explain in their paper the reduction in growth with histological damage and changes in gene transcription. On the other hand, mucus is produced in the gut, which serves to protect the digestive system from undigested materials, including MPs. This results in increased metabolic activity and the formation of a greater amount of humus [44]. In a study in which weight gain was noted, Ding et al. [45] found that the final weight of earthworms in the experiment with MPs may also include the weight of MPs that were ingested but not excreted and does not necessarily indicate growth.

Similar to the effect on growth, the number of cocoons decreased after exposure to 5% and 7% MPs ($p > 0.05$) but not after exposure to the lowest concentration (2.5%). This can be explained both by the reduced fitness of the individuals in treatments with MPs and by damaged spermatogenesis. Namely, after exposing earthworms to two different sizes of polyethylene MPs for 21 days, Kwak and An [34] came to the conclusion that MPs had a negative impact on the viability of coelomocytes and damaged male reproductive organs while having little to no impact on female reproductive organs. This may have an impact on the reproduction of earthworms. The aforementioned effects were not observed in the reproduction of the anecic species *L. terrestris,* which invests energy in a few heavy cocoons [18,46]. As with mortality and growth, the findings of previous studies suggest that while MPs may have adverse effects on earthworms, the specific impact may vary depending on the type and concentration of MPs.

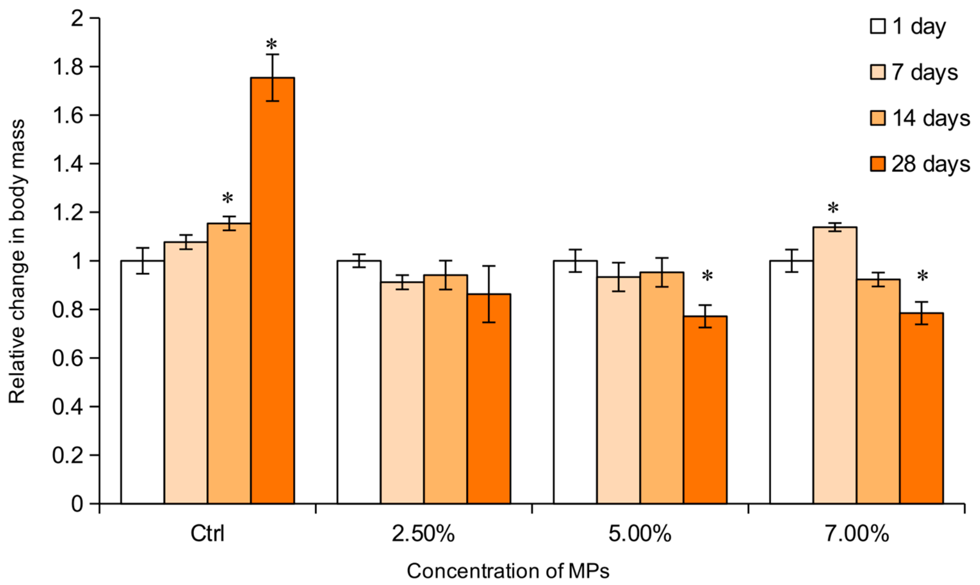

Figure 2. Results of the influence of glitter microplastic (MPs) particles in different concentrations (2.5%, 5%, and 7%) on growth and development of earthworm *E. fetida* expressed by a relative change to the 1st day in the body mass. Significant differences are marked with * ($p < 0.05$).

3.2. Avoidance Behavior

The possibility of avoiding areas with higher concentrations of MPs was not observed, as the earthworms were found to be evenly distributed in both areas. In fact, the results showed that 60% of the individuals were found on the side containing a mixture of soil and 2.5% and 5% MPs, while the individuals in the mixture with 7% did not exhibit a preference for either soil, as 50% were found in each section (Figure 3). However, some authors pointed out the potential of avoiding MPs, which can be linked with the use of higher concentrations of these particles [23,27,40]. On the other hand, Baeza et al. [17] and Lackmann et al. [47] did not confirm the mentioned results in their tests. Ding et al. [45] identified a critical threshold of 40 g kg^{-1} for avoidance behavior and stated that the type of plastic, whether conventional or biodegradable, had no significant effect on this behavior. In our study, avoidance behavior was not a good indicator. However, further research is required to understand the mechanisms and factors that influence the avoidance behavior of earthworms.

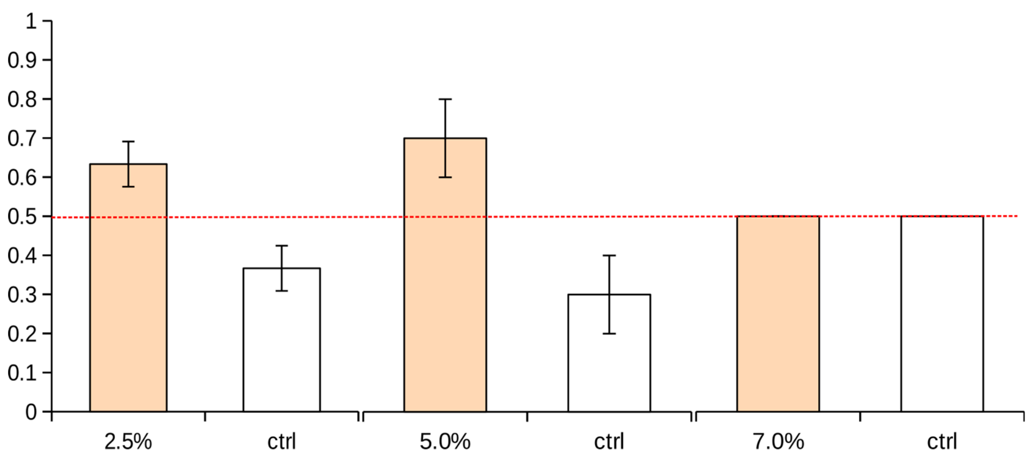

Figure 3. Results of the earthworm avoidance test after exposure to different glitter microplastic (MPs) concentrations (2.5%, 5%, and 7% w/w). Red dotted line represents 50%.

3.3. MPs in the Digestive System

The digestive system of the earthworm consists of a pharynx, esophagus, crop, gizzard, anterior intestine that secretes enzymes, and a posterior intestine that absorbs nutrients [48]. The earthworm's active muscles and the actions of the digestive system together participate in the connection process of swallowed materials with the liquid that is rich in enzymes. When the MP enter the earthworm, they are first exposed to acidic mucus in the anterior part of the body, followed by the gizzard, where the ingested material is comminuted and mixed, followed by the intestines. In the intestines, the MPs pass through a peritrophic membrane that encloses the undigested material and covers the casts when they are expelled. Kiyasudeen et al. [49] consider that the earthworm's waste is full of vital minerals that draw bacteria and cause additional nutritional mineralization. To determine whether larger MP particles, visible to the naked eye, could be ingested, a dissection was performed, clearly confirming the presence of MP particles in the region of the esophagus, gizzard, and intestines (Figures 4 and 5). We came to the following measurements of 0.0016 $\pm$ 0.002 g MPs/earthworm. The highest recorded amount of MPs detected in the digestive system of the surviving individuals was at a concentration of 7%, which is consistent with the amount of MPs that were in the boxes. Such a dose–response manner of ingested MPs was reported by Chen et al. [50]. Since MPs can not be readily digested, they remain in the digestive tract longer and hinder normal function. This was reported in *Lumbricus terrestris* after exposure to synthetic fibers [51]. In the digestive system, they can decrease food assimilation and fecal excretion [26] but also cause physical damage to tissues during passage [21]. The effects of ingested MPs depend on their size, with nano-sized ones being able to cross biological barriers and penetrate tissues [51] or larger ones with sharp edges (such as the glitters we investigated) that mainly cause physical damage and obstruction in the digestive system.

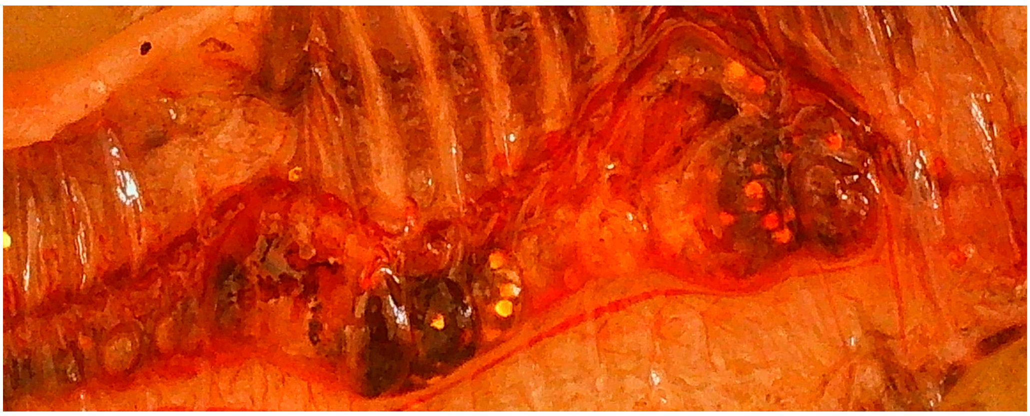

Figure 4. Ingested glitter microplastics (MPs) are visible in different gut sections of the earthworm *Eisenia fetida*.

Furthermore, the monitoring of fluorescence intensity clearly showed an increased intensity in samples b, c, and d (MP treatment samples) compared to the control (Figure 6). Fluorescence was observed in cross-sections at the level of longitudinal muscles, except in the control sample, which clearly indicates the presence of MPs (Figure 6). When comparing the intensity of the fluorescent signal in preparations with different concentrations of MPs, it can be concluded that as the concentration of MPs in the soil increases, a stronger signal appears, attributable to a higher quantity of relevant particles being introduced.

Overall, some authors considered the adverse effects of MPs would be mainly caused by the significant accumulation of MPs in the gizzards and intestines of organisms, which can damage their immune systems and affect their feeding behavior and development [25,52,53].

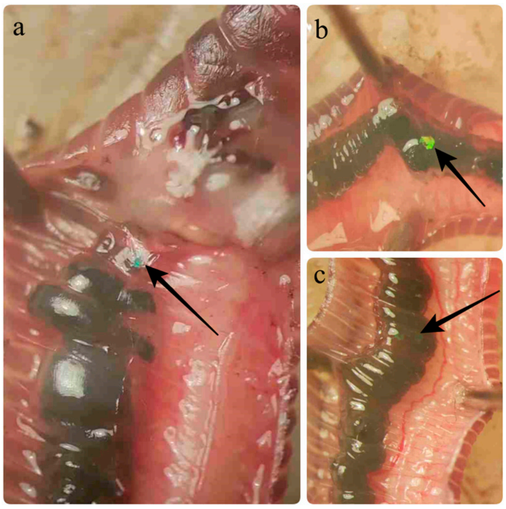

Figure 5. After exposure to glitter, the ingested microplastics (MPs) are visible to the naked eye in the region of the esophagus (**a**), gizzard (**b**), and intestine (**c**). Arrows in the figure show ingested MPs.

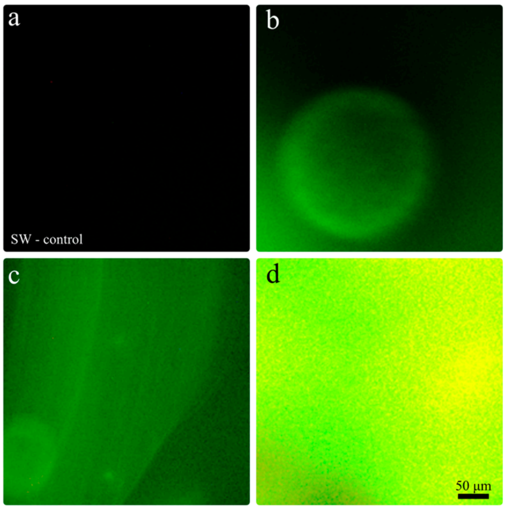

Figure 6. Overview of native preparations of cross-sections of *Eisenia fetida* species under a fluorescent microscope: control (no fluorescence) (**a**); 2.5% MPs (**b**); 5% MPs (**c**); 7% MPs (**d**). A GFP filter was used.

4. Conclusions

The pollution of the environment by MPs is a contemporary eco-societal (ecological and societal) problem that is increasing in severity due to the accelerated pace of life, as well as the improper and insufficient management of the waste generated by humanity's unrestrained and pointless consumption, as we have developed a habit of using disposable goods. When the benefits of using glitter are weighed against the potential hazards, it becomes clear that glitter is used for makeup and decoration and only provides a sense of satisfaction. Even though glitter is a profitable material, its environmental impacts should not be neglected, particularly as it has the potential to accumulate as a pollutant in the environment. A positive step towards the regulation of loose microplastics such as glitter was made by the European Commission [54] at the end of last year.

Overall, our study provides further evidence of the significance of *E. fetida* in assessing the impact of MP particles. This can be correlated with other organisms, both through the food chain and through active transmission within the entire substrate. Essentially, research focusing on the potential use of species from the family Lumbricidae for MP degradation is crucial and warrants further attention. Such studies would provide valuable insights into the ability of these organisms to serve as a first line of defense in anthropogenically disturbed ecosystems. Further research on the impacts and increased engagement of the scientific community are necessary to acquire timely knowledge regarding the negative effects of these pollutants.

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