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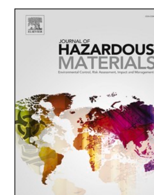

Research Paper

Fragmentation and depolymerization of microplastics in the earthworm gut: A potential for microplastic bioremediation?

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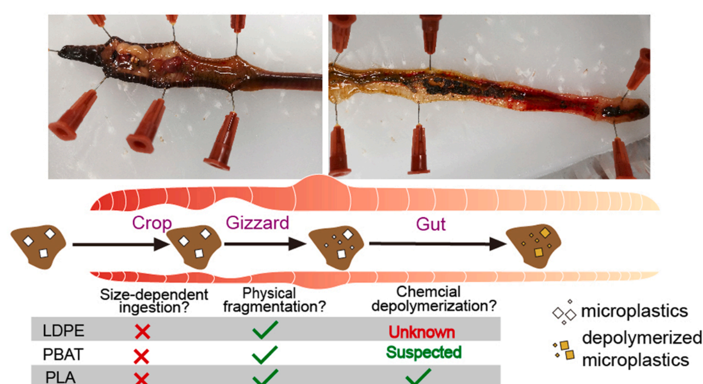
HIGHLIGHTS

- *Lumbricus terrestris* could survive in the soil contaminated with 1% (dw/dw) of LDPE, PLA and PBAT microplastics.
- The ingestion of microplastics by earthworms was not size-dependent.
- Microplastics were fragmented in the earthworm gizzard with/without the help of sand grains, depending on polymer types.
- Depolymerization of PLA and PBAT microplastics did not happen in the soil, but only in the earthworm's gut.
- The gut-related processes inside earthworms provide potential for microplastic bioremediation in the soil.

GRAPHICAL ABSTRACT



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ABSTRACT

The accumulation of microplastics poses potential risks to soil health. Here, we did a preliminary exploration on the potential of *Lumbricus terrestris* (Oligochaeta) to reduce low-density polyethylene (LDPE), polylactic acid (PLA), and polybutylene adipate terephthalate (PBAT) microplastic (20–648 µm) contamination in soils. The ingestion of microplastics-contaminated soil (1% of microplastics, dw/dw) in a mesocosm system and the ingestion of pure microplastics in the Petri Dish by earthworms were studied. Results show that earthworms survived in the microplastics-contaminated soil (0% mortality in 35 days) but barely when exposed solely to microplastics (30–80% mortality in 4 days). Size-dependent ingestion of microplastics was not observed. The fragmentation of LDPE microplastics in the gizzard facilitated by soil was confirmed by the significantly increased ratio of small-sized (20–113 µm) microplastics from the bulk soil to the gut (from 8.4% to 18.8%). PLA and PBAT microplastics were fragmented by gizzard without the facilitation of soil, the ratios of small-sized (20–113 µm) PLA and PBAT microplastics in the gut were 55.5% and 108.2% higher than in respective pristine distributions. Substantial depolymerization of PLA (weight-average molar mass reduced by 17.7% with shift in molecular weight distribution) and suspected depolymerization of PBAT were observed in the worm gut, while

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no change in the molar mass was observed for PLA and PBAT microplastics buried in the soil for 49 days. Our results suggest that ingested microplastics could undergo fragmentation and depolymerization (for certain polymers) in the earthworm gut. Further research is needed to reveal the mechanisms of polymer depolymerization in the earthworm gut and to evaluate the feasibility of microplastic bioremediation with earthworms.

1. Introduction

The global production of conventional plastics has reached 368 million tonnes in 2019 [1], while the bio/biodegradable plastics market has reached 2.1 million tonnes by 2020 and its market share is expected to grow and reach 2.9 million tonnes by 2025 [2]. Plastic products are used in various segments, including agriculture. In 2019, agricultural products accounted for 3.4% and 9% of the total market demand for non-biodegradable and biodegradable plastics, respectively [1,3]. Consequently, plastic debris derived from agricultural products, such as mulch films, could enter the soil, ending up as small fragments (e.g., micro(nano)plastics) due to various physical, chemical, or biological processes.

The increasing use of bio/biodegradable plastics leads to the potential accumulation of bio-microplastics in the soil, as micro(nano) plastics could be released upon their degradation [4]. The degradation performance of biodegradable plastics in the soil has been investigated and concerns have increased over their fate and impacts on the soil environment [5]. Poor biodegradability of some popular bio/-biodegradable polymers and commercial biodegradable plastic products in the soil has been reported under lab conditions and in the field [6–8], which might be explained by that the rate of plastic degradation depends not only on the intrinsic properties (e.g., polymer type, molecular weight, fillers, etc.) and environmental conditions, but also on the extrinsic properties such as the size and shape [9]. For example, the biodegradability of polybutylene adipate terephthalate (PBAT) in the soil has been verified [10], but its degrading performance highly depends on the soil type and the native soil microbial community [11]. The poor biodegradability of polylactic acid (PLA) in the soil at ambient temperature was also reported [12,13], probably due to its critical requirement for temperature. Plastic contamination in the soil has been investigated in different areas with different analytical techniques [14–18], and its impacts on soil biophysical properties, plant growth and their transfer along the terrestrial food chain have been assessed [19–22]. At the nano-scale, plastic particles could even accumulate in edible plants [23].

To disentangle plastic contamination, efforts are underway to develop methods to remediate soils by plastic biodegradation. The biodegradation of polymers in the soil mainly consists of three steps, i.e., colonization of polymer surface by microbes, depolymerization by extracellular enzymes, and utilization of degrading products by soil microorganisms [24]. Attempts have been made by directly screening microbial degraders from environmental samples [25,26], or using the gut microbiome of soil animals to trigger the degradation. For example, waxworms (the larvae of *Plodia interpunctella*), wax moths (*Galleria mellonella*), earthworms (*Lumbricus terrestris*) and the larvae of *Zophobas atratus* were reported to biodegrade polyethylene (PE) with their gut microbiome [27–30], expanded polystyrene could be ingested and biodegraded by dark mealworms (*Tenebrio obscurus*), yellow mealworms (*Tenebrio molitor*), and land snails (*Achatina fulica*) [31,32]. In addition, yellow mealworms were also reported to be able to carry out the biodegradation of PLA and polyvinyl chloride (PVC) [33,34].

Previous studies have proven that soil-dwelling earthworms (e.g., *Lumbricus terrestris*) could transport and ingest plastic debris in the soil. They could drag PE and biodegradable plastic mulch fragments into their burrows when foraging for food [35]. The transportation of micro (nano)plastics from the soil surface to deeper layers by anecic species was also reported [36,37]. Earthworms could ingest polyester microfibers [38] and low-density polyethylene (LDPE) microplastics [39] from the food source and potentially trigger the LDPE degradation with the help of the microbial consortium in the gut [28]. It is therefore interesting to further explore the potential use of earthworms to reduce existing (micro)plastic contamination in soils and prevent the accumulation of bio-based and biodegradable plastics due to their unclear degrading performance under field conditions.

The potential impacts of earthworm activity on plastic biodegradation in the soil include microbial proliferation, physical contact, microbial colonization, enzyme secretion, absorption of hydrolysis products, etc. [40] Among them, we believe the ingestion of microplastics and the sequential exposure to the gut environment (digestion) might play a key role since the worm gut could host up to 4,000 times more microorganisms than the surrounding soil [41], making it an ideal place for the degradation of polymers. In the current research we take three most popular polymers for producing mulch films (LDPE, PLA and PBAT) as test materials and aim at exploring (1) whether earthworms could survive in a microplastic-contaminated soil, (2) whether the ingestion of microplastics by earthworms is size-dependent, and (3) the potential changes, e.g., fragmentation and depolymerization of microplastics, during the ingestion and digestion processes. Given the feeding ecology of earthworms, our hypotheses are as follows: (i) the ingestion of microplastics by earthworms might be size-dependent, (ii) microplastic size distributions in the gut might be different from the pristine and gizzard distribution, (iii) a gradual shift in the size distribution and chemical properties of microplastics may occur during their passage through different sections of the gut.

2. Materials and methods

2.1. Preparation of microplastics

Fossil-based non-biodegradable low-density polyethylene (LDPE, Dow™ LDPE 310E), bio-based compostable PLA (NatureWorks® Ingeo™ Biopolymer 2003D, $M_N/M_W/M_Z$: 73/164/257 kg mol^{-1}), and fossil-based biodegradable (in soil) PBAT (Ecoflex® F Blend C1200, $M_N/M_W/M_Z$: 23/76/183 kg mol^{-1}) were used in our experiment. Thoroughly cleaned additive-free plastic polymers were used to produce microplastics. Pellets were fed into an ultra-centrifugal mill (ZM200, Retsch GmbH) with liquid nitrogen at 14000 rpm. A ring sieve with a trapezoid hole size of 1.5 mm was used for the cryogenic fragmentation. Fragmented polymers were collected and sieved with 212 μm and 420 μm metal sieves. Due to different material properties, microplastics with different average diameters and size distributions were produced. The sizes of our artificially prepared microplastics were determined by Laser Direct Infrared (LDIR) chemical imaging system, under the 'particle analysis' mode (see Section 2.5). The area of each particle was measured by the software and the diameter was calculated from the area based on a round shape by the software. The average diameters are 362 ± 119 μm (n = 1720), 300 ± 167 μm (n = 1421), and 234 ± 139 μm (n = 1743) for LDPE, PLA, and PBAT, respectively.

2.2. Earthworms and soil

Lumbricus terrestris, a widespread anecic species, was selected for the experiments due to its wide food preferences, including pure soil, soil-litter mixture, and soil-cow dung mixture [42], and its aptitude for survival under different microplastic concentrations (0–60% of microplastics in food sources) [39]. *Lumbricus terrestris* was purchased from Star Food Company (Barneveld, The Netherlands). Adult worms with a

clear clitellum and similar body weights were selected. Clean soil was prepared with the following composition, 26% loamy sand, 24% quartz sand, and 50% loamy silt, as described by Huerta Lwanga et al.,[39] and sieved through a 2 mm mesh. The loamy sand and loamy silt (containing organic matter) were collected from clean fields in Unifarm (Wageningen University & Research), and the quartz sand (free of organic matter) was ordered. The final measured soil texture was 1.75% clay, 50.36% silt, 16.42% very fine sand, 10.53% fine sand, 20.87% medium sand, and 0.07% coarse sand (volume-based) (Table S1). The final soil had a pH of 6.4 and contained 0.2% organic matter.

2.3. Experimental set-up

2.3.1. Experiment 1: changes in microplastics during the passage through the earthworm gut in a mesocosm system containing a microplastics-contaminated soil

A 35-day mesocosm experiment was conducted to study the changes of microplastics during the passage through the earthworm gut. The experiment was carried out in a 40 × 30 × 3 cm glass box (Photo S1A, containing 1500 g of dry clean soil or microplastics-contaminated soil) as described by Huerta Lwanga et al. [36]. Four treatments were set up, namely Control (free of microplastics), LDPE (1% of LDPE microplastics, dw/dw), PBAT (1% of PBAT microplastics, dw/dw), and PLA (1% of PLA microplastics, dw/dw).

To prepare a mesocosm containing 1500 g of microplastics-contaminated soil, 495 g of dry clean soil were thoroughly mixed with 5 g of respective microplastics, then transferred into the glass box. This step was repeated twice to fill 1500 g into the mesocosm and to achieve a homogeneous distribution of microplastics. For Control treatment, 1500 g of dry clean soil were filled in the glass box. The soil moisture was adjusted to 25% by adding distilled water, and the mesocosms were pre-incubated for 2 weeks. After 48 h of gut purging in the dark, four worms with clean guts were rinsed with cold distilled water, dried with a paper towel, weighed on an electronic balance, and placed on the soil surface in the mesocosm. Four replicates were prepared for each treatment, and the mesocosms were kept in the dark at 16 °C for 35 days. Distilled water was added to the mesocosm weekly to maintain the soil moisture at 25% (based on gravimetric measurements). The average fresh body weight of all worms before the experiment was 4.47 ± 0.53 g (n = 64), and there was no difference in initial weights between different treatments. Worms that escaped during the experiment were collected and removed from further analyses (Table S3).

After 35 days, mesocosms were opened for sampling (Photo S1C). Worms that survived were immediately rinsed with ice-cold distilled water, dried with a paper towel, weighed, and kept at − 20 °C. After 1 h, frozen worms were defrosted and dissected as follows [43] (Fig S1, a-d): the worm was first divided into three equivalent portions, the anterior section consisting of the pharynx, esophagus, crop, gizzard, and the foregut. The middle and posterior sections are midgut and hindgut. The opening was made carefully with a sterile surgical scissor from the dorsal side of the body. The gut content of each section was collected separately with sterile spatulas and preserved in microcentrifuge tubes (1.5 mL). The length of galleries created by worms in each mesocosm was measured to estimate the ingestion rate of worms. Bulk soil, which was not processed by earthworms, was visually identified as per Photo S1C and sampled on day 35 (total incubation time 49 days). All samples were stored at 4 °C for further analysis.

2.3.2. Experiment 2: changes in microplastics during the ingestion and digestion processes by *Lumbricus terrestris* in Petri Dishes containing only microplastics

After Experiment 1, another experiment was performed to assess whether there is a size-dependent selection of microplastics during the ingestion (by checking microplastic distributions in the crop and gizzard) and if fragmentation happens in the gizzard (by comparing size distributions in the gizzard and the gut). Briefly, two grams of microplastics (LDPE, PLA, and PBAT) were added to each Petri Dish, and the moisture was adjusted to 25% with distilled water. Two worms with clean gut and known weight were then placed in the Petri Dish (Photo S1B). The experiment was carried out in the dark at 16 °C for 4 days, and five replicates were prepared for each polymer type (ten worms in total for each polymer type). The initial fresh body weight of earthworms was 4.15 ± 0.39 g (n = 30), and there was no difference in the fresh weight between different treatments before the experiment. A microplastic-free treatment was also prepared to check the mortality of *Lumbricus terrestris* when no food was provided.

After 4 days, worms that survived were collected, rinsed with ice-cold distilled water, dried with a paper towel, and kept in the − 20 °C freezer immediately. After 1 h, frozen worms were defrosted at room temperature and dissected with sterile tools. In this experiment, the worm dissection was conducted differently (Fig S1, e-g). Crop, gizzard, and gut contents were collected separately and subjected to the extraction of microplastics right after the dissection. Due to the crop and gizzard's limited contents, replicates of these two sections were pooled as one sample for extraction.

2.4. Extraction of microplastics

A sequential density-based extraction method, modified from Corradini et al. [44] and Zhang et al. [45], was established to recover target microplastics (LDPE/PLA/PBAT) from the soil, gut contents, and worm casts. Briefly, for LDPE (density ~0.94 g cm^{-3}), dried samples were extracted with two solutions: firstly with 70% ethanol solution (density 0.88 g cm^{-3}) to remove light impurities and then with distilled water (density 1.0 g cm^{-3}) to recover microplastics. For PLA (density 1.24 g cm^{-3}) and PBAT (density 1.26 g cm^{-3}), dried samples were firstly extracted with distilled water to remove light impurities and then with sodium dihydrogen phosphate (NaH_2PO_4) solution (density 1.28 g cm^{-3}) to recover microplastics. The main procedures are depicted in Fig. S2, and the detailed extraction protocol is provided in Text S1. A weight-based recovery test for this extraction protocol was conducted (Table S2), and the recovery rates of LDPE, PBAT, and PLA microplastics were 103.9 ± 4.6%, 104.0 ± 2.9%, and 102.2 ± 2.1%, respectively.

2.5. Identification of microplastics with LDIR and generation of microplastic size distributions

Dried particles preserved in glass scintillation vials were re-suspended by adding 0.5 mL of 96% ethanol solution and treated with an ultrasonic bath for up to 30 s. The suspension was then transferred with a Pasteur pipet onto an infrared-reflective glass slide (7.5 ×2.5 cm; MirrIR, Kevley Technologies). The slide was covered with a glass lid and left to air dry. Sample slides were subjected to the microplastics identification on the Agilent 8700 LDIR using the Clarity software with a customized library. Settings for identification and library information are provided in Text S2. Besides polymer identification, LDIR also measures the size of detected microplastics. Detected microplastics were sorted in the order of particle area (μm^2) since the particle area was the direct measurement from LDIR. Microplastic size distributions were calculated based on the ratio (%) of microplastics in each size fraction (the number of microplastics per size fraction divided by total number of microplastics). As the smallest detectable particle size with current LDIR settings was 325 μm^2 (equivalent to 20 μm), and the biggest particle detected across all samples was 329,425 μm^2 (equivalent to 648 μm), 33 size fractions (i.e., from 325 to 10,000 μm^2, 10,000–20,000 μm^2 to 320,000–330,000 μm^2, bin size 10,000 μm^2) were defined. For the ease of understanding, size distributions were alternatively displayed based on calculated diameter (20–113 μm, 113–226 μm, 226–339 μm, 339–451 μm and 451–648 μm). Size distributions were calculated for pristine microplastics (PristineMPs), microplastics extracted from bulk soils (Experiment1-BulkSoil), and worm guts (Experiment1-Gut) in Experiment 1, microplastics extracted from crops (Experiment2-Crop),

gizzards (Experiment2-Gizzard) and worm guts (Experiment2-Gut) in Experiment 2.

2.6. Characterization of microplastics recovered from bulk soil and the worm gut

Pristine microplastics, microplastics extracted from bulk soils and worm guts in Experiment 1, and microplastics extracted from worm guts in Experiment 2 were subjected to a cleaning procedure (Text S3) to remove residual biomass and soil organic matters on the particle surface. Cleaned particles were measured with Gel Permeation Chromatography (GPC) to determine molar mass. Detailed procedures for GPC analysis are provided in Text S4. Weight-average molecular weight (M_W), Number-average molecular weight (M_N), Z-average molecular weight (M_Z), and polydispersity index ($PDI = M_W/M_N$) were generated from the measured molecular weight distributions (MWDs). Cleaned pristine microplastics, microplastics extracted from bulk soils and worm guts in Experiment 1 were also characterized by Fourier transform infrared spectroscopy with attenuated total reflectance accessory (FTIR-ATR). Samples were measured in duplicate to generate an average spectrum.

2.7. Calculations

Mortality, gross growth rate (*gGR*), and ingestion rate (*IR*) were calculated to profile the physiological conditions of earthworms:

(1) $Mortality : M = \frac{N0-Nt}{N0} \times 100\%$(for Experiment 1 and 2).

where *N0* and *Nt* represent the numbers of worms that survived at the beginning and by the end of the experiment, respectively.

(2) $Gross\ growth\ rate : gGR = \frac{gMt-M0}{M0} \times 100\%$ (for Experiment 1 only).

as gut purging was not conducted by the end of Experiment 1 (immediate dissection instead), the *gGR* was calculated to profile the growth of worms with the assumption that adult worms with similar body weights contain similar amounts of gut contents and similar gut content to body weight ratios. *M0* is the initial weight (without gut contents) before the experiment, *gMt* is the final weight (with gut contents) at the end of the experiment,

(3) $Ingestion\ rate : IR = \frac{Vg}{\sum gMt}$ (cm^3 soil g^{-1} worm) (for Experiment 1 only).

since no additional food was added to the mesocosms, earthworms could only ingest soil, leading to the forming of galleries in the mesocosm. *Vg* is the volume (cm^3) of galleries estimated based on the total length of galleries (galleries were treated as cylinders with a diameter of 1 cm) in each mesocosm, and $\sum gMt$ is the total final body weight of survival worms in each mesocosm on day 35. The occurrence of microplastics in the bulk soil and different gut sections were measured and reported in mass concentration (*Cmpm*, %, w/w) and number-based concentration (*Cmpn*, p g^{-1}). The equations are as follows:

(4) $Cmpm(in\ soil/gut) : Cmpm = \frac{Mmp}{Ms} \times 100\%$(for Experiment 1 only).

(5) $Cmpn(in\ soil/gut) : Cmpn = \frac{Nmp}{Ms}$(p g^{-1}) (for Experiment 1 only).

where *Mmp* and *Nmp* represent the weight and number of microplastics extracted from the sample, and *Ms* is the dry weight of samples used for microplastic extraction.

2.8. Statistics and data analysis

One-way analysis of variance (ANOVA) and student's t-test were used to test significant differences between values of different treatments. Levene's test was used to test the homogeneity of variance. Duncan's test (for equal variance) and Games-Howell test (for unequal variance) were utilized to conduct the post hoc test. Kolmogorov-Smirnov test was used to test whether normal distribution occurred and whether two microplastic size distributions were different. The significant level was set as 0.05.

3. Results and discussion

3.1. Impacts of microplastics on basic physiological indicators of *Lumbricus terrestris*

No mortality was found in Experiment 1 (35 days), except for one removed replicate from LDPE treatment. Replicate LDPE-D was removed at the early stage because the worms were abnormally inactive from the beginning and all four worms died within 2 weeks. Based on our previous experience with commercially purchased earthworms, this could be a result of using unhealthy worms (e.g., worms carrying pathogens or disease). The information on individual worms in the mesocosms is provided in Table S3. In Experiment 2 (4 days), the mortality was 30% (7 survivors), 40% (6 survivors), and 80% (2 survivors) for worms fed with solely PBAT, PLA, and LDPE microplastics, respectively, and 0% for worms without food. The zero mortality for Experiment 1 was expected since the concentration of microplastics in the soil was 1% (dw/dw), and the exposure time was relatively short. While the mortalities in Experiment 2 indicate that microplastics could cause lethal damage to earthworms if they are ingested in large quantities at exceedingly high concentrations, despite the polymer types. We speculate that the death of worms fed with solely microplastics might be resulted from gut damage caused by plastic particles, which was confirmed for another species *Eisenia andrei* by histopathological analysis [46].

In Experiment 1, there was no significant difference in initial body weights between different treatments and no significant difference in final body weights between different treatments (Fig. 1A). The *gGR* was 7.7% for Control, 7.8% for LDPE, 15.6% for PLA, and 17.7% for PBAT. Worms fed with PBAT-contaminated soil showed the highest *IR* (9.55 $\pm$ 2.50 cm^3 g^{-1} worm), while those fed with PLA-contaminated soil showed the lowest (6.04 $\pm$ 0.97 cm^3 g^{-1} worm). The *IR* in the PBAT treatment was significantly higher than that in the PLA treatment ($p < 0.05$). However, no significant difference was found between Control and microplastic-addition treatments (Fig. 1B). This could suggest that the presence of microplastics (1%, dw/dw) in the soil did not affect the activity of earthworms in 35 days of incubation period.

As microplastics of all polymer types were found in the worm gut in both experiments, it is possible to point out that *Lumbricus terrestris* could ingest LDPE, PLA, and PBAT microplastics within the given size ranges when they are either provided as sole food or mixed with soil. Our findings also indicate that the presence of 1% (dw/dw) of LDPE, PLA, and PBAT microplastics in the soil did not significantly impact the earthworms' health after 35 days, reverberating existing findings on the same or other species [38,39,46]. Although the approach-avoidance behavior of earthworms to different microplastic- contaminated soils was not studied in the current research, some studies reported that *Lumbricus terrestris* did not actively avoid polyester fibers at the concentration of 1% (dw/dw) [38], and *Eisenia fetida* avoided the food source when the microplastic concentration exceeded 4% (dw/dw) [47]. A recent research even observed that *Eisenia fetida* preferred soils contaminated with certain polymer types, e.g., PET and PLA, rather than clean soil [48]. The robustness of earthworms against microplastic-contaminated soil provides the potential for bioremediation with earthworms.

3.2. Microplastics in different gut sections

One of our hypotheses was that microplastics might undergo a gradual change in the size distribution during the passage through different gut sections, which was explored in Experiment 1. Microplastics were separately extracted from different gut sections, and their concentrations were measured in Experiment 1 (Table 1). The average *Cmpm* in the bulk soil on day 35 was 0.98% for the LDPE treatment, 1.14% for the PBAT treatment, and 1.26% for the PLA treatment, while the corresponding average *Cmpn* was 1419 p g^{-1}, 2837 p $g^{-1}$and 946 p g^{-1}, respectively (Table 1). From the bulk soil to the hindgut, no clear

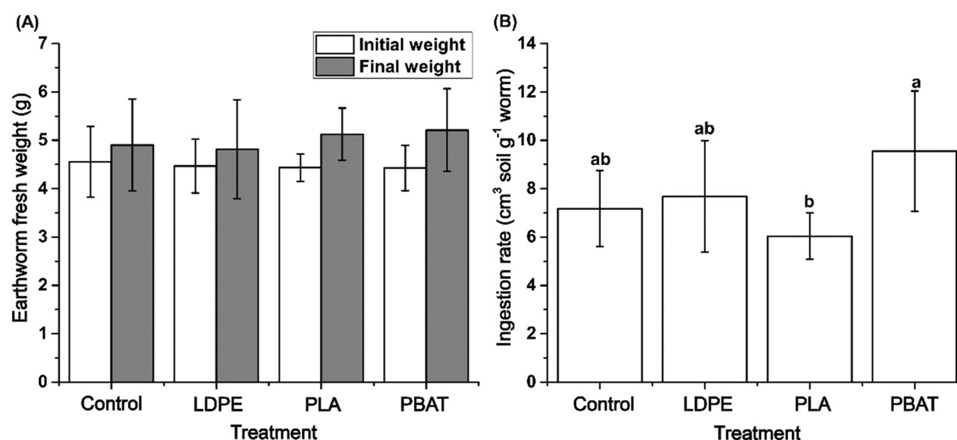

Fig. 1. (A) Fresh body weights of *Lumbricus terrestris* before and after Experiment 1. The initial weight is the net weight without gut contents, while the final weight is with gut contents. (B) Ingestion rate of *Lumbricus terrestris* in different treatments in Experiment 1. Error bars represent standard deviations and significant difference between different treatments is labeled with different letters.

Table 1
Concentrations of microplastics in the bulk soil and different gut sections (foregut, midgut, hindgut, and whole gut) in Experiment 1. *Cmpm* represents the concentration of microplastics in weight percentage (%), and *Cmpn* represents the number-based concentration of microplastics (p g^{-1}). Data was presented as mean±SD, for *Cmpm*, N = 3–4; For *Cmpn*, N = 3. One-way ANOVA was tested for bulk soil, foregut, midgut, and hindgut (significant difference labeled with lowercase letters). Student's *t*-test was tested between bulk soil and whole gut (significant difference labeled with uppercase letters).

Sections	LDPE		PLA		PBAT	
	Cmpm (%)	*Cmpn* (p g^{-1})	*Cmpm* (%)	*Cmpn* (p g^{-1})	*Cmpm* (%)	*Cmpn* (p g^{-1})
Bulk Soil	$0.98 \pm 0.25^{ab,A}$	$1419 \pm 501^{a,A}$	$1.26 \pm 0.20^{ab,A}$	$946 \pm 258^{a,A}$	$1.14 \pm 0.14^{a,A}$	$2837 \pm 839^{a,A}$
Foregut	1.34 ± 0.37^{a}	1628 ± 267^{a}	1.15 ± 0.54^{ab}	421 ± 203^{a}	1.26 ± 0.51^{a}	1861 ± 1172^{ab}
Midgut	0.78 ± 0.30^{ab}	1254 ± 414^{a}	0.80 ± 0.53^{b}	468 ± 424^{a}	0.94 ± 0.29^{a}	957 ± 286^{b}
Hindgut	0.74 ± 0.41^{b}	819 ± 608^{a}	2.16 ± 1.37^{a}	618 ± 277^{a}	1.13 ± 0.18^{a}	1630 ± 484^{ab}
Whole Gut	0.83 ± 0.23^{A}	1143 ± 265^{A}	1.15 ± 0.38^{A}	523 ± 283^{A}	1.08 ± 0.18^{A}	1407 ± 416^{A}

trend in *Cmpm* or *Cmpn* could be observed for any polymer type. Both *Cmpm* and *Cmpn* were calculated in the whole gut by pooling different gut sections, and the average concentrations of microplastics in the entire gut were 0.83% (1143 p g^{-1}) for LDPE, 1.08% (1407 p g^{-1}) for PBAT and 1.15% (523 p g^{-1}) for PLA.

Several studies have reported the ingestion of microplastics by soil invertebrates; however, quantitative studies on the microplastic concentrations in the digestive tract or excreta are scarce. Existing information was derived from different polymer types, particle sizes, feeding strategies, extraction methodologies, and identification methods. For example, Huerta Lwanga et al. [39] reported that when fed with an LDPE-litter mixture, the microplastic concentration in the cast of *Lumbricus terrestris* varied about 0.20–2.0 times compared with those in the LDPE-litter mixture, depending on the ratio of microplastics in the food. Up to 1.6 p mg^{-1} of particles were found in the cast of *Eisenia fetida* exposed to soil mixed with 0.15% of LDPE microplastics [49]. Small-sized PS microplastics (0.1 and 1.3 μm) were found to accumulate heavily in the intestine of *Eisenia fetida*, and microplastic concentration in the intestine can be up to 800 times that in the soil [50]. In Experiment 1, the *Cmpm* was not different between the bulk soil and the gut. While a noteworthy reduction in the *Cmpn* was observed in the gut, especially for PLA and PBAT, the *Cmpns* of the respective polymers in the gut were 44.7% ($p = 0.128$) and 50.4% ($p = 0.057$) lower than corresponding values in the bulk soil. The inconsistency between the *Cmpm* and *Cmpn* in our research might be caused by the limitations to extract and identify microplastics. The *Cmpm* was measured for all particles larger than 5 μm (due to the use of a filter membrane with a pore size of 5 μm), while the *Cmpn* calculations included all detectable microplastics under LDIR (>20 μm). The contribution of small-sized microplastics to the *Cmpm* is minimal, but their contribution to the *Cmpn* is noticeable. The observed lower *Cmpns* in the worm gut in Experiment 1 could be resulted from the fast degradation of small-sized microplastics in the gut due to their substantial surface area. Another possible explanation is that some processes, which occurred during ingestion and digestion, led to particles below the detection limit of LDIR (20 μm). To our knowledge, except for a recent work by Wang et al., [48] which reported the mass concentration and number-based concentration of PET and PLA microplastics in the cast of *Eisenia fetida*, there is a lack of studies quantifying microplastic concentrations both mass-wise and number-wise.

Size distributions of microplastics in different gut sections were measured (Fig. 2). In general, a minimal difference was observed in size distributions from foregut to hindgut for all polymer types. For LDPE, ratios in the foregut and midgut exceeded the ones in the hindgut ($p < 0.05$), standing out as the only significant difference in the fraction 160,000–170,000 μm^2. For PBAT, the only significant difference was found in the fraction 60,000–70,000 μm^2, where the ratio in the foregut was higher than in the midgut ($p < 0.05$). For PLA, the only significant difference was found in the fraction 170,000–180,000 μm^2, where the ratio in the midgut was higher than in the hindgut ($p < 0.05$). In addition, the comparison using distributions pooled from three replicates of each gut section showed that the distributions of microplastics in the foregut and the hindgut were always the same for all polymer types (Fig S3), which indicates that the gradual shift in the microplastic size distribution did not occur during the passage through the gut.

Indeed, the earthworm gut is a complex environment where different biological processes occur in several sections, and the foregut, midgut, and hindgut take on different tasks during digestion [41]. However, combining the results of microplastic concentrations and size distributions in different gut sections led to an unclear gradual shift in these indicators during the passage through the gut (foregut-midgut-hindgut). Therefore, the earthworm gut was treated as a single environment and

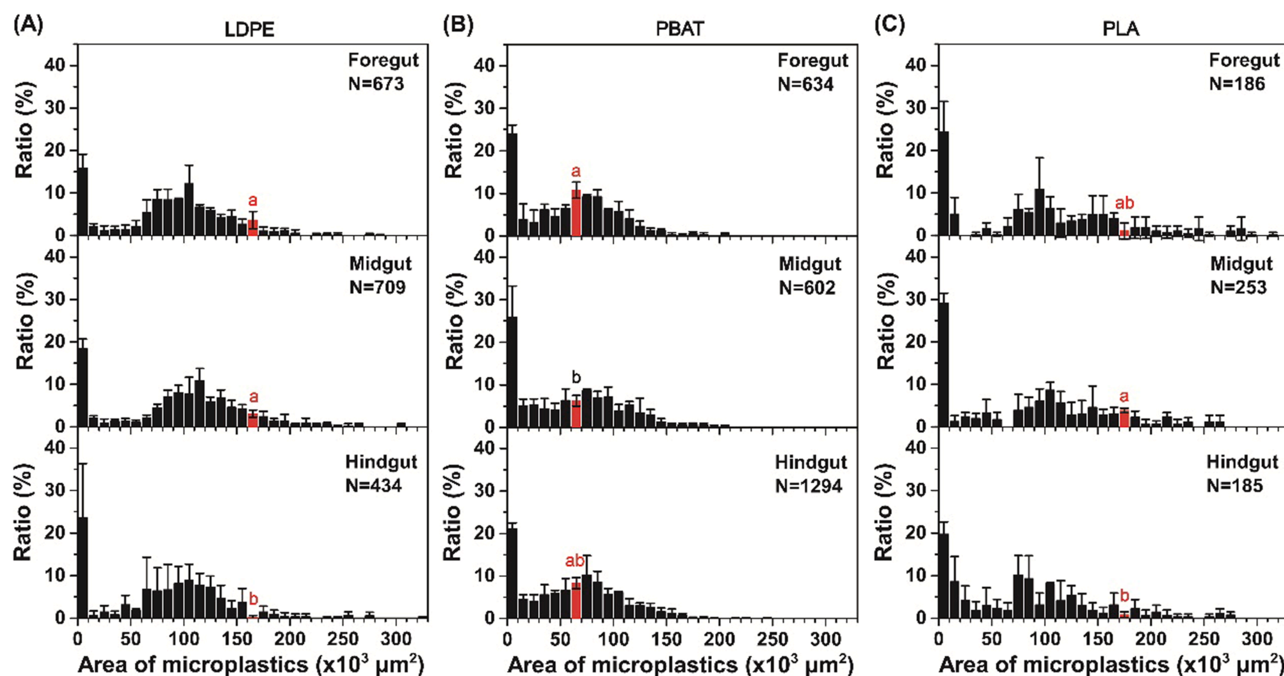

Fig. 2. Size distributions of (A) LDPE, (B) PBAT, and (C) PLA microplastics in different sections of the gut in Experiment 1. Microplastic sizes were calculated from three replicates for each section. N is the total number of microplastic particles recovered from the triplicate samples. One-way ANOVA was conducted to test whether the ratios of certain size fraction in different gut sections were different. Error bars represent standard deviations and significant difference was labeled vertically across different distributions with letters. Size fractions with significant difference between gut sections were highlighted in red. Calculated diameters (based on a circle) were provided as a reference, e.g., 10×10^3 μm^2 ~ 113 μm, 50×10^3 μm^2 ~ 252 μm, 100×10^3 μm^2 ~ 357 μm, 10×10^3 μm^2 ~ 437 μm.

studied in our research.

3.3. Microplastics after different ingestion and digestion processes

The size distributions and cumulative size distributions of microplastics in different samples in both experiments are displayed in Fig. 3 (A-C) and Fig. S4. As only a few microplastics could be extracted from the crops of worms in Experiment 2 (14 LDPE particles, 66 PLA particles, and 190 PBAT particles recovered), microplastic size distributions from the worm crop were disregarded.

In Experiment 1, microplastic size distributions were traced from the pristine microplastics (PristineMPs) to both the bulk soil (Experiment1-BulkSoil, buried in the soil for 49 days), and the worm gut (Experiment1-Gut, by the end of the experiment). The size distributions of PristineMPs and Experiment1-BulkSoil were about the same for all polymer types. Only 1–2 out of the 33 size fractions showed a significant ($p < 0.05$) but slight difference in the ratio (Table S4 to S6), which indicates that natural fragmentation of LDPE, PBAT, and PLA microplastics did not happen in the soil in 49 days. Interestingly, a distinctly different LDPE microplastic size distribution was observed in the worm gut (Fig. 3A and inset), where the ratio of the smallest size fraction 325–10,000 μm^2 (18.8 ± 1.7%) was significantly higher than that in the pristine distribution (8.6 ± 0.2%) and in the bulk soil (8.4 ± 0.8%) ($p < 0.05$). For PBAT and PLA, size distributions in the gut were not perceptibly different from those in the bulk soil and the pristine distribution (Fig. 3, B-C).

In Experiment 2, microplastic size distributions were traced from the pristine distribution (PristineMPs) to the gizzard (Experiment2-Gizzard) and the worm gut (Experiment2-Gut). The aim of measuring microplastic size distributions in the gizzard was to check whether the potentially observed size changes in the gut could result from size-dependent selection during ingestion. For LDPE, there seemed to be a slight shift in the size distribution and cumulative size distribution (Fig. 3A, Fig S4) from PristineMPs to Experiment2-Gizzard. However, we cannot conclude that there was a size-dependent selection during ingestion as the gizzard samples were pooled as one for the microplastic extraction and the observed difference was minimal. The same happened for PBAT and PLA since no size-dependent selection occurred during the ingestion of microplastics by the worm. However, after passing through the gizzard, some changes in the size distribution occurred depending on polymer types. For LDPE, no distinctly different size distribution stood out between Experiment2-Gut and Experiment2-Gizzard (Fig. 3A and inset). Despite the high variance, the ratio of PBAT microplastics in the smallest fraction, i.e., 325–10,000 μm,^{2} was 55.5% higher in the gut than in the gizzard (Fig. 3B and inset). While for PLA, Experiment2-Gut was noticeably different from PristineMPs and Experiment2-Gizzard. The ratio of the size fraction 325–10,000 μm^2 was 48.3 ± 5.6% in the gut, significantly higher than 23.2 ± 6.7% in the pristine distribution ($p < 0.05$) (Fig. 3C and inset). In addition, significantly lower ratios were also found for size fractions of 80,000–90,000 μm^2, 100,000–110,000 μm^2, 120,000–130,000 μm^2, 150,000–170,000 μm^2, 180,000–205,000 μm^2 in the gut ($p < 0.05$).

In Experiment 1, worms were incubated in mesocosms with the microplastics-contaminated soil for 35 days, which means microplastics were ingested together with the soil. While in Experiment 2, worms were incubated in glass Petri Dishes with moist microplastics for 4 days, where microplastics acted as the only food source. By comparing the size distributions of microplastics in two different gut environments (Experiment1-Gut and Experiment2-Gut) (Fig. 3, D-F), we found more considerably small-sized LDPE microplastics (325–10,000 μm^2) in Experiment1-Gut, and the ratios of larger microplastics were, in general, lower in Experiment1-Gut. On the contrary, more small-sized PBAT and PLA microplastics (325–10,000 μm^2) were present in Experiment2-Gut, and the ratios of larger microplastics were generally lower in Experiment1-Gut.

PLA and PBAT microplastics recovered in Experiment 1 and 2 were subjected to GPC analysis to study the potential changes in their molecular weight distribution (MWD). The cleaning efficiency for recovered microplastics with SDS solution has been validated and the presence of SDS residuals on microplastics has been ruled out (Text S3).

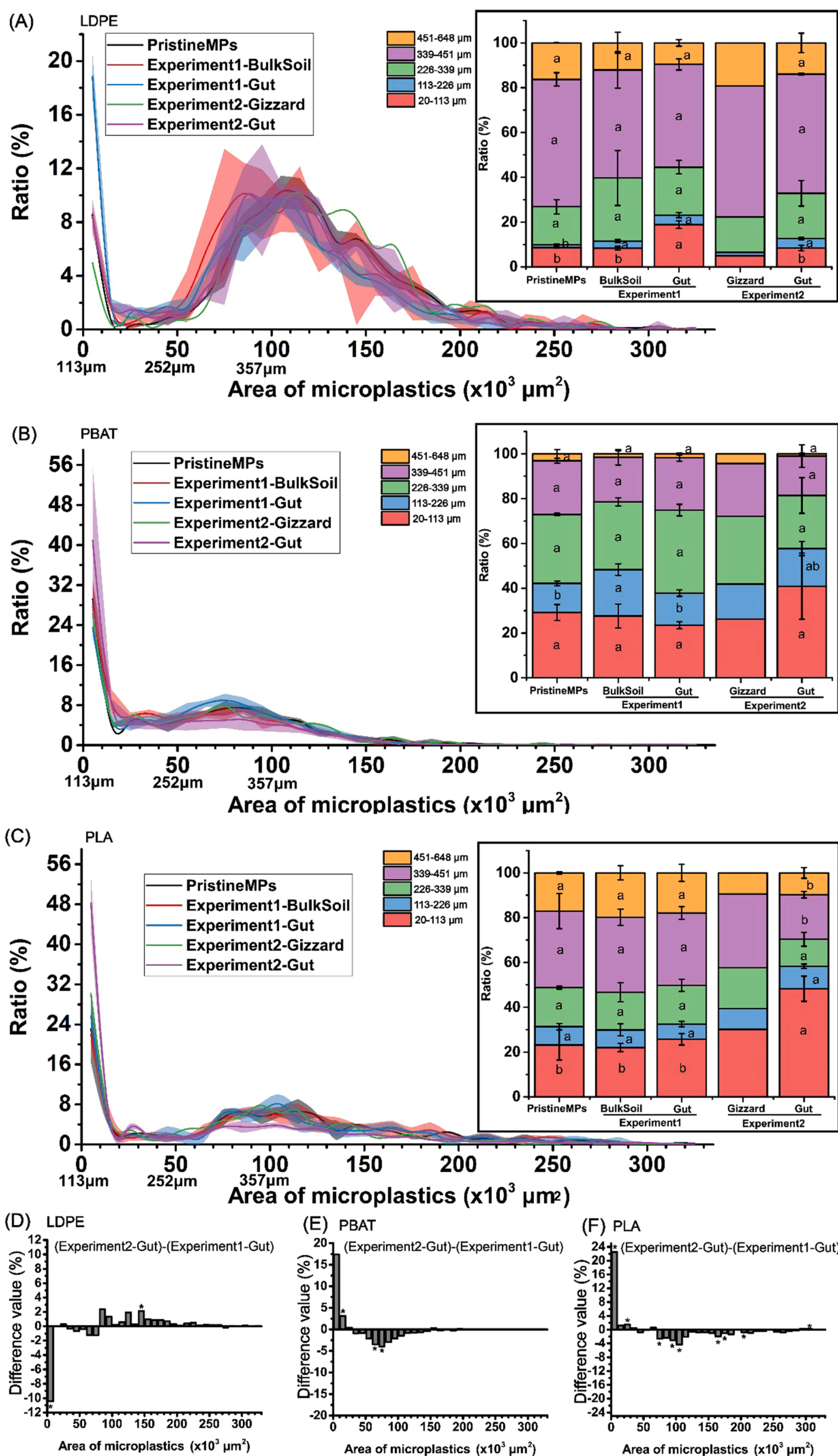

Fig. 3. (A-C) Size distributions (sorted by particle area) of LDPE, PBAT, and PLA microplastics in different samples in Experiment 1 and 2. The shadow area above and under the curve represents standard deviations. Inset bar charts in (A-C) provide an overview of size distributions sorted by calculated diameters (significant difference tested with One-way ANOVA, except for Experiment2-Gizzard). All PristineMPs distributions and Experiment2-Gut distribution in the LDPE treatment were generated from duplicate measurements. All Experiment2-Gizzard distributions were generated from one pooled sample (one measurement). Other distributions were generated from triplicate measurements. Error bars in the inset bar charts represent standard deviations. (D-F) Comparison (per size fraction) between Experiment2-Gut and Experiment1-Gut distributions (significant increase/decrease tested with Student's t-test). Calculated diameters were provided as a reference, e.g., 10×10^3 μm^2 ~ 113 µm, 50×10^3 μm^2 ~ 252 µm, 100×10^3 μm^2 ~ 357 µm, 10×10^3 μm^2 ~ 437 µm. Detailed size distributions can be found in Table S4 to S6.

For PLA (Fig. 4A), microplastics in the bulk soil (BulkSoil) did not show any significant difference in the weight average molecular weight (M_W) and Z-average molecular weight (M_Z) compared with pristine ones (Pristine) after burial in the soil for 49 days. However, the M_W and M_Z of PLA microplastics in the worm gut (Gut) was 17.7% and 12.3% lower than BulkSoil ($p < 0.05$), indicating a substantial depolymerization given the relatively short gut transit time of 11.6 h for *Lumbricus terrestris* [51]. The PDIs of PLA microplastics in Pristine, BulkSoil and Gut were 2.28, 1.92 and 1.94 respectively. In addition, the entire MWD curve of PLA-Gut shifted clearly to low molecular weight area compared with Pristine and BulkSoil (Fig. 4C). However, in Experiment 2, no significant change in the M_W, M_Z, PDI, and the MWD was observed for PLA microplastics after entering the worm gut (Fig S5A). Characterization with FTIR showed that a broad peak around 3340 cm^{-1} occurred in the BulkSoil and Gut but not in the Pristine, and the absorbance was higher in the Gut than BulkSoil (Fig S6). This peak could possibly be caused by the stretching of -OH groups in the alcohol end and the carboxylic end of depolymerized PLA, which were generated during chain scission at the ester bond.

In Experiment 1, the M_W of PBAT in the Gut was 5.3% lower than Pristine ($p < 0.05$) and the M_Z of BulkSoil and Gut were 9.9% and 9.2% lower than Pristine ($p < 0.05$) (Fig. 4B). The PDIs of Pristine, Bulksoil and Gut were 3.30, 2.84 and 2.56, respectively. Nevertheless, the MWD curves of PBAT microplastics showed minimal difference between Pristine, BulkSoil and Gut (Fig. 4D). No significant difference was found for M_W between Pristine and Gut in Experiment 2 but a slight reduction of M_Z in Gut was observed (−9.2%, $p < 0.05$). It is necessary to point out that the observed changes in M_W and M_Z in the earthworm gut may not necessarily lead to the conclusion that depolymerization of PBAT happened in the gut, as no synchronous shift in the MWD curves was observed (Fig. 4D, Fig S5B). However, it is possible that the PBAT depolymerization in the gut followed a surface erosion mechanism [52] that could have triggered the peeling of surface materials by worm gut enzymes, and the remaining part stayed the same. This is also supported by the FTIR spectra (Fig S6). Therefore, we suspect that PBAT depolymerization could have happened in the worm gut in our experiment.

Considering the feeding ecology of earthworms [53], we propose three biological processes where earthworms could interact with microplastics and potentially lead to some changes in microplastic properties. The first is the size-dependent selection during the ingestion of microplastics, which would determine the initial size distribution of microplastics entering the earthworm's digestive system. A second process is the fragmentation of microplastics due to the grinding action in the gizzard, which could lead to the generation of smaller particles and the reduction of larger particles. A third process is the biodegradation of microplastics that takes place simultaneously with the assimilation of nutrients in the gut.

In Experiment 2, we ruled out the size-dependent ingestion of

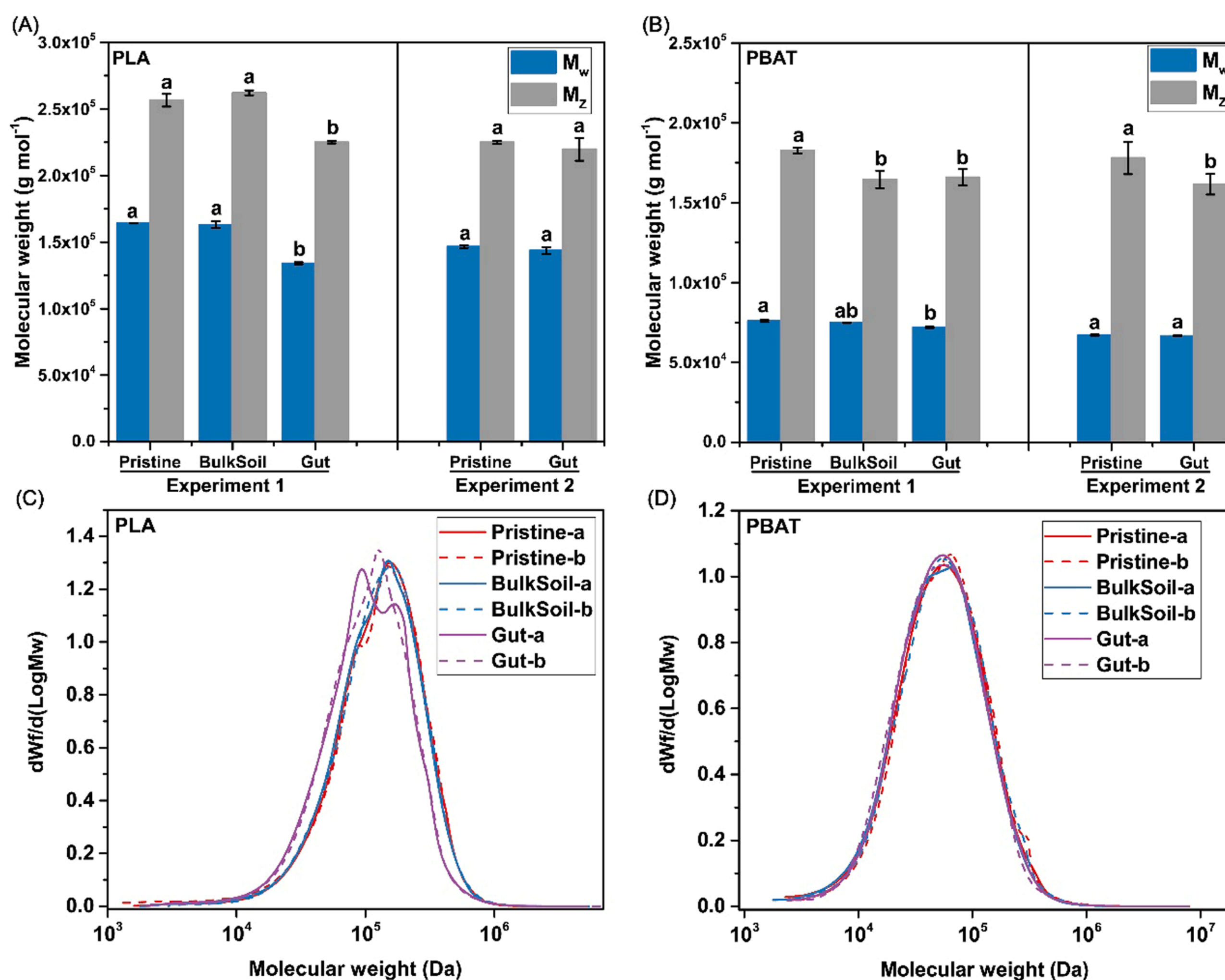

Fig. 4. (A-B) Weight average molecular weight (M_W) and Z-average molecular weight (M_Z) of PLA and PBAT microplastics in Experiment 1 and 2. Error bars represent standard deviations and significant difference was labeled with different letters. (C-D) GPC molecular weight distribution (MWD) curves of PLA and PBAT microplastics in Experiment 1. 'Pristine' represents pristine microplastics used for the experiment, 'BulkSoil' represents microplastics recovered from the bulk soil in Experiment 1, 'Gut' represents microplastics recovered from the worm gut. Duplicate analyses (-a and -b) were performed for each sample.

microplastics by checking the size distribution in the gizzard. Some studies have reported that *Lumbricus terrestris* actively select small-sized sands/seeds over big-sized sands/seeds when they forage for food [54–56]. However, these studies were usually conducted with larger particles (0.5–3.0 mm in diameter), and the observed size selection possibly reflected the limitation of ingestible particle sizes by the diameter of the intestinal tract [56]. It has already been reported that earthworms could ingest and excrete PE and polypropylene (PP) microplastics with a diameter up to 1400–1660 μm [57,58]. However, the largest microplastic in our study was 329,425 μm^2 (equivalent to a circle with a diameter of 648 μm), i.e., not large enough to cause any difficulty for the ingestion by *Lumbricus terrestris*.

Microplastic fragmentation in the gizzard was verified by the observed differences between microplastic size distributions before ingestion, in the gizzard, and in the gut. LDPE microplastics were not broken up by the gizzard when ingested solely (without soil) but fragmented into smaller particles when uptake together with soil (Fig. 3A). Previous studies on *Lumbricus terrestris* have reported that sand ingestion facilitates assimilation by enhancing the grinding action in the gizzard [59]. Moreover, the enrichment of smaller PE microplastics in the worm cast has been reported by Huerta Lwanga et al. [39] and Chen et al. [49] Recently, the presence of PE nanoplastics (potentially fragmented from PE microbeads) in the worm cast has also been confirmed [60]. Some have also observed that plastic particles at submicron and nanocron scale can be excreted from *Eisenia fetida* at a slower rate than microplastics [48], which potentially provides alternative explanation to the higher ratio of smaller microplastics in the gut. However, this is unlikely an explanation to our findings because size distributions were compared section by section from bulk soil to hindgut in our experiment. Together with already published studies, our results indicate that LDPE microplastics could be physically fragmented into smaller particles with the assistance of sand grains in the soil, but the gizzard itself may not be strong enough to break up LDPE microplastics. The generation of smaller LDPE microplastics or even nanoplastics suggests that LDPE microplastics are biodegraded in the earthworm gut at extremely slow rate or not biodegraded at all, although the presence of LDPE-degrading gut microbes cannot be ruled out. Nevertheless, PE in other forms, e.g., films and expanded foams have been reported to be biodegraded by other macroinvertebrates (waxworms and mealworms) with their saliva or synergistic enzymatic reactions of the host and gut microbiome [61–63]. More evidence is needed to confirm the biodegradability of LDPE microplastics in the earthworm' gut.

Interestingly, opposing phenomena were observed for PLA and PBAT microplastics. When ingested solely (without soil), a larger ratio of small-sized PLA and PBAT microplastics were found in the gut. In contrast, no significant change in the size distribution was found in the earthworm gut compared with the bulk soil when ingested with soil (Fig. 3, B-C). In addition, GPC results showed that depolymerization only happened to PLA microplastics (potentially also to PBAT microplastics) when exposed to the gut environment with the presence of soil (Fig. 4). It has been reported that a wide range of enzymes, such as lipase, chitinase, cellulase, protease, and carboxylesterase, are secreted into the gut by ingested microbes and the worm itself [41,64]. Furthermore, several studies indicate that the microbial composition of the earthworm gut reflects that of the ingested soil [53]. Since both PBAT and PLA are polyesters, it is highly possible that the unique gut environment of *Lumbricus terrestris*, where microbial activity and enzymatic activity are much higher than the surrounding soil, triggered the hydrolysis of ester bonds, accelerating their biodegradation. Therefore, we propose a possible explanation for these results: PLA and PBAT microplastics could be fragmented by the grinding action in the gizzard even without the presence of soil (Fig. 3, B-C) due to their material properties (e.g., strength and ductility), which could be corroborated by the size distributions and average diameters of different pristine microplastics produced by the same cryogenic grinding process. In Experiment 2, PLA and PBAT microplastics were physically fragmentized into smaller particles. However, the worm gut could not trigger the hydrolysis of these polyesters with its indigenous microbial community and enzymatic activity (Fig. 4, A-B). In Experiment 1, alternatively, PLA and PBAT microplastics went through both physical fragmentation and depolymerization during their passage through the digestive tract due to the high microbial and enzymatic activity in the gut environment boosted after soil ingestion. The freshly generated small particles were either too small to be detected by the LDIR or assimilated by the gut in a short time.

3.4. Environmental implications and limits

The presence of microplastics in the soil and their impacts on the soil properties, soil microorganisms, plants, and soil animals have been reviewed [65]. Challenges arise when tackling microplastic contamination in the soil due to their strenuous recovery from the soil at a large scale and limited contamination levels in this environment, which highlights that conducting a large-scale cleaning for contaminated sites is inessential and not feasible. Therefore, we propose the approach of in-situ bioremediation, seeking help from earthworms inhabiting the soil and carrying out the remediation in the long term. Sanchez-Hernandez et al. [40] also proposed that the decaying of biodegradable plastics could be enhanced by earthworm activities in the soil, e.g., the formation of middens and burrows, the excretion of casts, and the passage through the gastrointestinal lumen of earthworms. In the current research, we mainly focused on ingestion and digestion processes of earthworms. Given the results of our study, we foresee earthworm gut as a potential 'factory' for the bioremediation of microplastic-contaminated soil, especially for polymers with a relatively easy-to-degrade structure (e.g., PLA and PBAT).

The current research focused on the processes inside the worm. However, it is also pivotal to study the fate of microplastics after excretion back into the soil together with the worm cast. For PLA and PBAT microplastics, the depolymerization may continue in the cast, or other worms could ingest them again due to the coprophagy, leading to further fragmentation and depolymerization in the worm gut. Due to technical limitations, we could not assess the MWDs of LDPE microplastics and the FTIR spectra alone is not sufficient to lead to any solid conclusion. Therefore, we can only conclude that LDPE microplastics were physically fragmented by the gizzard. In this case, LDPE microplastics in the worm cast could be another source of even smaller and nano-scaled particles. Further studies are needed to provide a holistic picture of the interactions between microplastics and earthworms in the soil. Finally, the current study was conducted at mesocosm scale in the lab that mimicked natural conditions. In future work, field experiments are needed to evaluate the feasibility of this bioremediation approach.

4. Conclusions

In this study, the potential of earthworms to reduce microplastic contamination in the soil was explored, focused on the ingestion and digestion processes. No mortality was recorded for *Lumbricus terrestris* in a microplastics-contaminated (LDPE, PBAT and PLA) soil (1%, dw/dw), and their ingestion rates and growth rates were not affected. The ingestion of microplastics by earthworms were not size-dependent. Fragmentation of microplastics in the gizzard was confirmed by comparing microplastic size distributions in different gut sections. Substantial depolymerization of PLA and suspected depolymerization of PBAT were observed in the gut, while no sign of biodegradation was found for PLA and PBAT microplastics in the soil after 49 days incubation. In general, the results of the current study suggest that ingested microplastics could undergo fragmentation and depolymerization (depending on polymer type) in the earthworm gut. No significant evidence supported biodegradation of LDPE in earthworms although the presence of PE-degrading gut bacteria, which perform degradation at extremely slow rate, cannot be ruled out. Further research is needed to

reveal the mechanisms of polymer depolymerization in the earthworm gut and to evaluate the feasibility of bioremediation.

Environmental implication

Microplastics derived from mulch films (PE films and biodegradable plastic films) have been reported to accumulate in soils or possess the potential. Microplastic accumulation in soils has potential negative impacts on soil properties and soil organisms, and the transfer of microplastics along the food chain may possess risk to the food safety. Therefore, it is necessary to explore techniques to mitigate microplastic contaminations in soils. Our work explored the interactions between microplastics and earthworms during the gut processes in the soil. Based on our findings, we propose the potential of using earthworms to carry out the in-situ bioremediation of microplastics in soils.

CRediT authorship contribution statement

Ke Meng: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Esperanza Huerta Lwanga:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Maarten van der Zee:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Davi Renato Munhoz:** Writing – original draft, Writing – review & editing. **Violette Geissen**: Conceptualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.130765.

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