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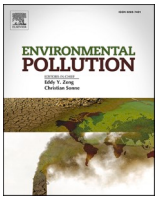

Comparison of the potential toxicity induced by microplastics made of polyethylene terephthalate (PET) and polylactic acid (PLA) on the earthworm *Eisenia foetida*☆

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ABSTRACT

A growing number of studies have demonstrated that microplastic (MP) contamination is widespread in terrestrial ecosystems. A wide array of MPs made of conventional, fossil-based polymers differing in size and shape has been detected in soils worldwide. Recently, also MPs made of bioplastics have been found in soils, but there is a dearth of information concerning their toxicity on soil organisms. This study aimed at exploring the potential toxicity induced by the exposure for 28 days to irregular shaped and differently sized MPs made of a fossil-based (polyethylene terephthalate – PET) and a bioplastic (polylactic acid – PLA) polymer on the earthworm *Eisenia foetida*. Two amounts (1 g and 10 g/kg of soil, corresponding to 0.1% and 1% of soil weight) of both MP types were administered to the earthworms. A multi-level approach was used to investigate the MP-induced effects at sub-individual and individual level. Changes in the activity of antioxidant and detoxifying enzymes, as well as in lipid peroxidation levels, were investigated at specific time-points (i.e., 7, 14, 21 and 28 days) as sub-individual responses. Histological analyses were performed to assess effects at tissue level, while the change in digging activity was considered as a proxy of behavioral effects. Earthworms ingested MPs made of both the polymers. MPs made of PET did not induce any adverse effect at none of the biological levels. In contrast, MPs made of PLA caused the modulation of earthworms' oxidative status as showed by a bell-shaped activity of superoxide dismutase coupled with an increase in glutathione peroxidase activity. However, neither oxidative and tissue damage, nor behavioral alteration occurred. These findings suggest that the exposure to bio-based MPs can cause higher toxicity compared to fossil-based MPs.

1. Introduction

Plastic pollution emerged as one of the most worrisome environmental issues at global level. The increasing demand, production and use of plastics, together with the inappropriate management and disposal of plastic waste at their end-life, have resulted in a massive contamination of both aquatic and terrestrial ecosystems worldwide (e.g., Li et al., 2020; Xu et al., 2020a; Rai et al., 2021). The degradation and/or the fragmentation of plastic objects and large-sized plastic items due to weathering processes led to the formation of microplastics (MPs) (Shen et al., 2019), which have been categorized as any plastic item in the 1 to < 1,000 μm size range (Hartmann et al., 2019). Over the last decade, several studies have investigated the occurrence of MPs in aquatic ecosystems (e.g., Li et al., 2020; Yang et al., 2021a). However, even if the most of plastic waste is generated and discharged on land, and soils are considered as the main long-term sink for MPs, only recently MP contamination has been explored in terrestrial ecosystems (Rillig and Lehmann, 2020; Büks and Kaupenjohann, 2020). MPs enter soils through different pathways, including littering (Akdogan and Guven, 2019), atmospheric deposition (Evangeliou et al., 2020), agricultural practices (e.g., the application of sewage sludge and compost; Huerta Lwanga et al., 2017a; Lv et al., 2019), irrigation (Bläsing and Amelung, 2018) and plastic mulching (Büks and Kaupenjohann, 2020; Crossman et al., 2020), as well as landfills (Geyer et al., 2017). Thus, urban and agricultural soils are prone to suffer MP contamination because they experience diverse anthropic activities and input sources

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(Chae and An, 2018; Mö;ller et al., 2020). MPs have been detected in soils worldwide, with abundances up to tens of thousands items/kg of soil (see Xu et al., 2020b; Sajjad et al., 2022). For instance, large variability in MP levels has been found in different soils, including garden (870 ± 1,900 and 14–895 particles/kg; Huerta Lwanga et al., 2017b; Weithmann et al., 2018, respectively), farmland (0–1.25 particles/kg; Piehl et al., 2018) and paddy soils (12.1 ± 2.5 items/kg in non-rice and 27.6 ± 5.9 items/kg during rice-planting period; Lv et al., 2019).

The pattern of soil contamination was characterized mainly by MPs made of polyolefin polymers, such as polypropylene (PP), polyethylene (PE) and polystyrene (PS), as well as polyester (PL) and polyethylene terephthalate (PET), polyamide (PA), and polyvinyl chloride (PVC) (see Yang et al., 2021b). Moreover, several investigations have pointed out that MP exposure was able to induce a large variety of deleterious effects on diverse soil organisms (Baho et al., 2021 and references therein). For instance, the onset of oxidative stress (e.g., Jiang et al., 2020; Prendergast-Miller et al., 2019), reproductive impairments (Judy et al., 2019; Lahive et al., 2019) and behavioral changes (Huerta Lwanga et al., 2016; Song et al., 2019) have occurred as a consequence of MP administration. These investigations have been mainly addressed on the effects caused by MPs made of fossil-based, non-biodegradable polymers. In contrast, only a limited number of studies has been aimed at assessing the effects related to MPs made of bio-based polymers (also known as bioplastics), which have similar chemical-physical features and the same environmental fate of their fossil-based counterparts (Luo et al., 2020). Indeed, because of the diffusion of plastic (and MPs) contamination, the production and use of alternative and sustainable materials like bioplastics have been identified as one of the potential solutions to the improper disposal and mismanagement of conventional, fossil-based plastics (European Bioplastic, 2021). A bioplastic is a bio-based, biodegradable, or both, plastic material (European Bioplastic, 2018). Bioplastics are mainly used as disposable or non-durable plastic products, including packaging, mulching film and tableware (Gross and Kalra, 2002). In Europe, bioplastics currently account for approximatively 1% of the total plastic demand, although an increase is expected because of regulations and restrictions for the use of conventional plastics (European Bioplastic, 2021). According to this trend, disposable or non-durable bioplastic products have been found in the environment (Merran, 2019). There, bioplastic products can suffer the same weathering processes of their fossil-based counterparts, leading to the generation of bioplastic MPs (hereafter BMPs), but in higher amount and at a faster rate (Yang et al., 2022). Although methods to isolate and to characterize BMPs in different environmental matrices have been recently developed (e.g., Ruggero et al., 2021; Okoffo et al., 2022), the information on their occurrence in natural ecosystems remains very scant. Despite this gap of knowledge, some studies have shed light on the potential adverse effects induced by the exposure to different BMPs towards aquatic organisms. For instance, the exposure to MPs made of polylactic acid (PLA; hereafter PLA-MPs) altered the bioturbation activity of the sand-dwelling lugworm *Arenicola marina* (Green et al., 2016), altered the respiration rates of the European flat oysters (*Ostrea edulis)* and the assemblages of benthic fauna, in terms of decreased species richness, total number and body size of organisms (Green, 2016). Moreover, the administration of PLA-MPs reduced the content of glycerophospholipids (Khalid et al., 2021) and caused the overexpression of a putative heavy metal binding protein and detoxification enzyme (Green et al., 2019) in the blue mussel *Mytilus edulis*. PLA-MPs exposure also reduced the fertilization rate of the ascidian *Microcosmus exasperatus* (Anderson and Shenkar, 2021) and affected the body length, reproductive output and survival in *Daphnia magna* (Zimmermann et al., 2020). Lastly, an oxidative stress condition coupled with changes in anticholinergic activity were observed in the larvae of the dragonfly *Aphylla williamsoni* (Chagas et al., 2021), whereas modulations in acetylcholinesterase activity and swimming behaviour were induced towards *Danio rerio* larvae (de Oliveira et al., 2021). In spite of these findings, only few studies have investigated the toxicity of BMPs on soil organisms. For instance, the exposure to PLA-MPs affected the avoidance behaviour, the biomass and the reproduction of the earthworm *Eisenia foetida* at the same extent of PE-MPs (0.125–500 g/kg of soils; Ding et al., 2021). Moreover, a recent study has investigated the effects induced by MPs made of three bio-based materials, two were PLA-based and one was poly (hydroxybutyrate-*co*-valerate)-based (PHBV-based), on the earthworm *Eisenia andrei* (Liwarska-Bizukojc et al., 2023). No effect on survival and body mass were induced by the exposure to the three MPs types at soil abundances up to 12.5 % soil weight. However, a decrease in reproductive output occurred at the end of 28-days exposure to each of the bio-based MPs administered at 12.5 % soil weight, while the modulation of catalase activity was noted also at lower abundances (Liwarska-Bizukojc et al., 2023). In spite of these findings, the current investigations have limitedly explored and compared the induction and the propagation of adverse effects over different levels of the biological organization induced by the exposure to fossil-based and bioplastic-based MPs in earthworms. Thus, the present study aimed at investigating the potential toxicity induced by the exposure to MPs made of a fossil-based polymer and a bioplastics at sub-individual and individual level in the earthworm *Eisenia foetida*. Polyethylene terephthalate (PET) and the polylactic acid (PLA) were selected as fossil-based and bio-based polymers, respectively. These polymers belong both to the polyester group and are commonly used in different industrial applications. PET is primarily used in the form of fibres, sheets and films in textile and packaging industry. PLA is the most important biodegradable aromatic polyester and it is commonly used for films, extrusion-thermoformed containers and medical applications (Tokiwa and Calabia, 2006), as well as in mulch films for agriculture (Merino et al., 2022). Moreover, PLA is considered as one of the most suitable substitutes of PET on the market of packaging. Because of their large range of applications and demand, differently sized PET and PLA items are commonly detected in soils. For instance, among the more than 20 polymers of MPs found in the soils PET-MPs were the fourth most frequently found (36.4%) after PE-MP (78.8%), PP-MP (78.8%) and PS-MP (45.5%) (Zhang et al., 2021). Moreover, PET was the third most abundant polymer in MPs isolated from non-mulched soils (Long et al., 2023). Concerning PLA, degradation of PLA-based mulch films has been demonstrated to produce 6.7×10^4 items/m^2 of microplastics (Hao et al., 2024). Earthworms were exposed for 28 days to two amounts of MPs (1 g and 10 g/kg of soil, corresponding to 0.1% and 1% of soil weight) made of PET (i.e., PET-MPs) or PLA (i.e., PLA-MPs). At a sub-individual level, a battery of oxidative stress biomarkers was applied to explore the alterations on the activity of antioxidant and detoxifying enzymes and levels of lipid peroxidation. At individual level, histological analyses were performed to assess tissue alterations and/or damage induced by the transit of MPs in the digestive tract of the earthworms. Lastly, changes in digging activity was investigated to assess chances in individual behaviour.

2. Materials and methods

2.1. Preparation of PET- and PLA-MPs

Microplastics made of PET and PLA were obtained by mechanical grinding of PET and PLA bottles, in order to obtain items with irregular shape and variable size mimicking those that can be found in the environment. The polymer of PET and PLA plastic bottles was confirmed through a Fourier Transformed Infrared Spectroscope (FT-IR; PerkinElmer Spectrum 100). Bottles were cut in small pieces, which underwent a series of freezing (in liquid nitrogen) and grinding cycles (see Parolini et al., 2020a for procedure details). According to a procedure previously applied on different micronized plastic materials (Parolini et al., 2020a), obtained items were sorted using a series of stainless steel sieves with different meshes (5 mm–2 mm and 1 mm) to select only particles in the range of MPs (1 μm–1 mm; Hartmann et al., 2019). Items were all white/transparent and owned irregular shape and variable size

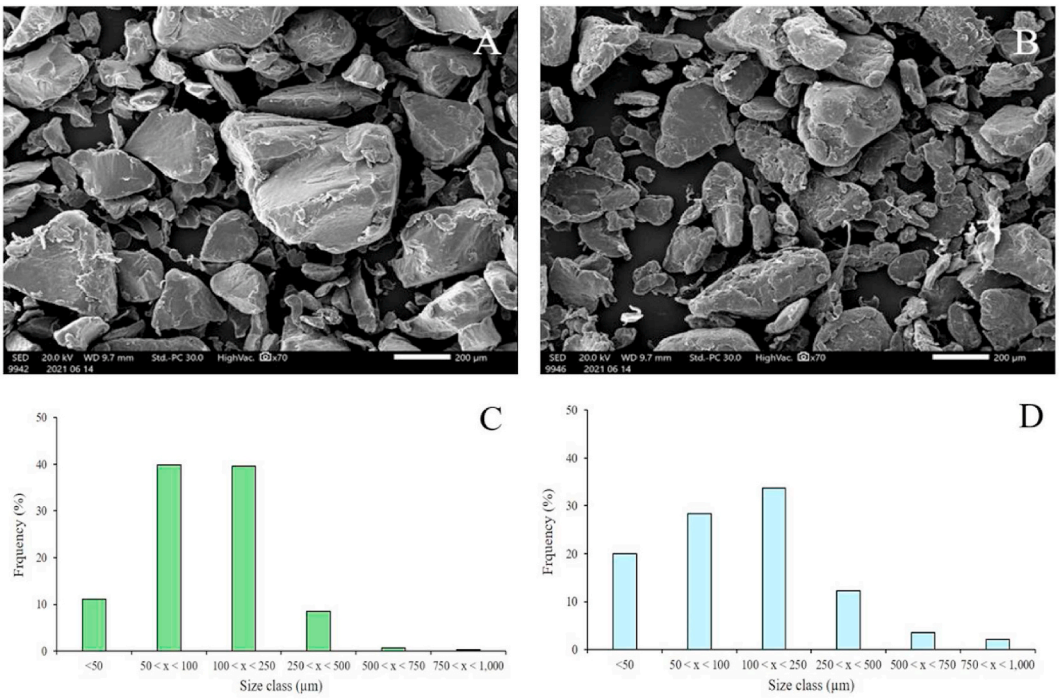

Fig. 1. Scanning Electron Microscopy (SEM) image of PET-MPs (A) and PLA-MPs (B), and distribution frequency of PET-MPs (C) and PLA-MPs (D) for each size class.

(Fig. 1 A and B). Images of PET- and PLA-MPs were captured under a scanning electron microscope (LEO1430, Zeiss, Oberkochen, Germany). Perimeter, area, diameter and circularity ($4\pi \times \text{area/perimeter}^2$) of 500 MPs for each polymer were measured using the ImageJ freeware software (Schneider et al., 2012). Considering the high irregularity of the particles, their size was calculated as the diameter of a spherical particle having the same area. The morphometric features of PET- and PLA-MPs used in these experiments are reported in Table 1. Finally, the MPs were grouped into size classes based on the calculated diameter: <50 μm; 50 < x < 100 μm; 100 < x < 250 μm; 250 < x < 500 μm; 500 < x < 750 μm; 750 < x < 1,000 μm (Fig. 1 C and D).

2.2. Experimental plan

Individuals of *Eisenia foetida* belonging to the same age class were obtained from the Consorzio Italiano Allevamento Lombrichi (Con.It.A. Lo.). Earthworms were stocked in large glass vessels filled with 3 kg of natural soil for 7-days acclimation under the following laboratory conditions: temperature 20 ± 1 °C, humidity close to 80%, pH = 6.5. Soil was preliminarily sieved (<1 mm) to remove clays and large vegetable residues. The soil was collected in a small, private vegetal garden (coordinates: 45.794755; 8.255989) in a small hamlet (<100 inhabitants; 604 m a.s.l.) of Varallo, a municipality located in Northern Piedmont (Italy). A preliminary analysis of soil biodiversity confirmed the lack of contamination, the presence of earthworms and, consequently, its suitability for maintenance and exposure of earthworms under laboratory conditions. Soil texture (clay: <0.002 mm = 9.38%; silt: 0.002–0.05 mm = 30.74%; sand: 0.05–2 mm = 59.68% and gravel: >2 mm = 0.2%), pH (=6.4), carbonate content (<1 g/kg dry soil), organic carbon (75.8 g/kg dry soil) and total organic matter content (131 g/kg dry soil) were certified by the Minoprio Analisi e Certificazioni S.r.l. laboratory. At the end of the acclimation period, 15 earthworms were randomly transferred to smaller glass vessels (26 × 21 × 10 cm) filled with 1 kg of soil and exposed to two amounts of PET- or PLA-MPs (1 g and 10 g/kg of soil, corresponding to 0.1% and 1% of soil weight). The soil used for the exposures came from the same batch used for acclimation to laboratory conditions and, consequently, had the same features texture and composition. The selected MP amounts were similar to the lowest ones administered to earthworms in previous studies (e.g., Chen et al., 2020a; Jiang et al., 2020; Prendergast-Miller et al., 2019) and might represent the exposure levels experienced by these organisms in real soils. Five experimental groups were created (i.e., control, 0.1 and 1% of PET-MPs on soil weight, and or 0.1 and 1% of PLA-MPs on soil weight). Three independent replicates per experimental group were performed (n = 45 individuals per group). The mean (± standard deviation; n = 3 replicates or glass vessels) body weight of the 15 earthworms assigned to each experimental group was summarized in Table S1.

Table 1
Perimeter, area, diameter and circularity (mean ± standard deviation) of PET- and PLA-MPs administered to *Eisenia foetida* individuals.

	Perimeter (μm)	Area (mm^2)	Diameter (μm)	Circularity
PET-MPs	512.91 ± 39.51	19.08 ± 1.63	125.79 ± 4.11	0.73 ± 0.02
PLA-MPs	516.29 ± 30.02	7.06 ± 1.00	164.34 ± 3.01	0.31 ± 0.05

Body weight did not significantly differ between experimental groups (Student's t-test, P > 0.05 for all the pairwise comparisons). The exposure lasted 28 days and it was performed under static, non-renewal conditions in a thermostatic chamber (temperature = 20 °C) under a 16 h light: 8 h dark photoperiod. The humidity was maintained close to the 80% through nebulization of bi-distilled water (filtered on 0.45 μm cellulose filters) every single day. To obtain 0.1 and 1% MPs on soil weight, 1 g or 10 g of PET- or PLA-MPs were added to the soil, respectively. Then, soil was mixed using a stainless steel shovel to obtain a homogeneous distribution of MPs. The number of administered MPs was counted in 1 g of soil (three replicates) after the homogenization procedure. The mean (± standard deviation) of PET-MPs in 0.1 and 1% experimental groups was 18.98 ± 10.34 and 211.86 ± 52.67 MP/g of soil weight, respectively. The mean (± standard deviation) of PLA-MPs in 0.1 and 1% experimental groups was 24.97 ± 11.34 and 223.74 ± 47.90 MP/g of soil weight, respectively. Each tank (i.e., replicate) was covered with a tinfoil to avoid airborne MP contamination and the escape of earthworms. According to OECD guidelines (OECD n° 222, Earthworm Reproduction Test (*Eisenia fetida/Eisenia andrei*), 5 g of oatmeal was spread on the soil surface and moistened before adding earthworms. Food was the provided once a week during the 28-days

exposure period. Every 7 days up to the end of the exposure tests (i.e., after 7, 14, 21 and 28 days of exposure) the survival of the earthworms was checked. Then, a behavioral test aimed at evaluating the digging activity after the exposure to a light stimulus was performed on three individuals from each tank per experimental group (see 2.5 *Digging activity* section). At the end of the behavioral test, the individuals were sacrificed by immersion in liquid nitrogen and maintained at −80 °C until the biochemical analyses (see 2.3 *Oxidative stress biomarkers* section*)*. Only at the end of the exposure, three individuals were processed for histological analyses (see 2.4 *Histological analyses* section), while other three individuals were fixed in 96% ethanol for 24 hrs and dissected to check for the presence of MPs in their digestive tract using optical microscopy.

2.3. Oxidative stress biomarkers

The anterior part (up to the clitellum) of three earthworms (~0.3 g per individual) collected at selected time-points (i.e., 7, 14, 21 and 28 days) from each replicate (i.e., exposure vessel) was pooled and homogenized in potassium phosphate buffer (100 mM; pH = 7.4) added with KCl (100 mM), ethylenediaminetetraacetic acid (EDTA; 1 mM), dithiothreitol (DTT; 1 mM) and 1:100 vol/vol protease inhibitors (Protease Inhibitor Cocktail containing six individual components for inhibiting serine, cysteine, and acid proteases, and aminopeptidases; P8340 – Sigma-Aldrich®). Samples were homogenized using a pellet motor pestle (Sigma-Aldrich®). Three pools per experimental group were prepared for each time-point. The homogenates were centrifuged at 17,000 × *g* for 30 min at 4 °C and the supernatant was processed to assess the protein content, enzyme activities and lipid peroxidation. As oxidative stress biomarkers, the activity of antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), detoxifying enzyme glutathione S-transferase (GST) and lipid peroxidation levels were measured. Protein content and oxidative stress biomarkers were assessed through the application of spectrophotometric methods described in detail elsewhere (Parolini et al., 2020a,b) and adapted for the model species. A detailed description of biomarker methods was reported in Supporting Information. Biomarker analyses were performed using reagents purchased from Merck (Darmstadt, Germany) and a Genova Bio spectrophotometer (Jenway).

2.4. Histological analyses

Histological analyses were performed using reagents purchased from Merck (Darmstadt, Germany). The anterior part of earthworms was immersed in Bouin's fixative for 24 hrs at 4 °C. Each sample was washed several times in tap water, dehydrated in an increasing series of ethanol (70 %, 90 %, 95 % and 100%) and finally cleared with xylene. After overnight inclusion in a xylene:paraffin mixture (1:1 vol/vol) and embedment in pure paraffin (56−58 °C), samples were sectioned with a Leitz 1512 rotary microtome (ca. 8 μm in thickness of each transverse section). Sections were cut approximatively at the pharyngeal and esophagus level of the earthworm to investigate the potential effects of MPs in different parts of the digestive tract. Sections were then stained following the Milligan's trichrome technique (Milligan, 1946). All the sections were observed and photographed under a Jenaval light microscope provided with a Leica EC3 Camera, which was managed by the Leica Application Suite LAS EZ Software (Version 1.8.0). At least 3 glass slides, each one containing 5–7 histological sections cut at the pharyngeal or esophagus level, were prepared and observed, for a total of about 15–21 sections per each level and 30–42 per single individual.

2.5. Digging activity

At each selected time point, earthworms were individually transferred in a beaker filled with 250 g of natural soil uncontaminated from MPs. Each individual was placed on the surface of the soil and left in dark for 3 min for acclimation. Then, a light stimulus was administered using a warm-light lamp for a maximum of 5 min. The time (expressed in seconds) taken by the earthworm to dig in the soil was considered as endpoint.

2.6. Differential Scanning Calorimetry (DSC)

DSC analyses were conducted using a Mettler Toledo DSC1. Melting and crystallization temperatures were measured using the following temperature cycles (1) heating from 0 °C to 200 °C at 10 °C/min; (2) cooling from 200 °C to 25 °C at 10 °C/min; (3) heating from 25 °C to 200 °C at 10 °C/min.

2.7. Statistical analysis

The effects of PET-MPs or PLA-MPs treatments, time of exposure and their interactions on oxidative stress biomarker endpoints were investigated by a two-way analysis of variance (ANOVA), after checking for normality and homoscedasticity of data through the application of Shapiro–Wilk and Levene's tests, respectively. Significant differences among groups were checked through the Tukey's *post-hoc* test ($*P < 0.05$ and $**P < 0.01$). Linear mixed models (LMMs), including the treatment and the time of exposure as fixed effect factors, while the identity of the exposure tank as a random factor, were applied to assess the MPs-induced effects on digging activity of *E. foetida*. Fisher' LSD *post-hoc* test was applied to assess significant differences among groups ($*P < 0.05$ and $**P < 0.01$). All the statistical analyses were run in R 4.03 (R Core Team 2020).

3. Results

Although the method to obtain PET- and PLA-MPs was the same, the morphometric features of MPs were different, with the exception of the perimeter, which did not differ between the two groups of MPs (paired Student's *t*-test; $t = 1.523$; $P > 0.05$). In contrast, the area and circularity of PLA-MPs were significantly lower compared to PET-MPs, while PET-MPs had a larger diameter than PLA-MPs (paired Student's *t*-test; $t > 163.516$; $P < 0.05$ in all the cases).

No mortality occurred over the 28 days of exposure to both the amounts of PLA-MPs and PET-MPs. Hence, all the 225 individuals were processed for biochemical, histological and behavioral analyses.

3.1. Ingestion of MPs

Overall, no item referring to PET- or PLA-MPs was observed in the digestive tract of earthworms from the control groups (data not shown). Dissection of earthworms did not allow confirming the ingestion of PLA-MPs. Indeed, no PLA-MPs were visualized in the digestive tract of individuals exposed to both the amounts of MPs (Fig. 2b). However, histological analyses confirmed the presence of PLA-MPs in the lumen of the digestive tract of earthworms exposed to both the treatments (Fig. 2d). In contrast, the dissection (Fig. 1a) and the histological analyses (Fig. 1c) confirmed the ingestion and the presence of PET-MPs in the digestive tract of earthworms exposed to both the treatments.

3.2. Differential Scanning Calorimetry

As we were not able to collect a sufficient amount of PLA-MPs after the transit in the earthworms' gut to check for their degradation, we extracted them directly from the soil to check for any sign of degradation. All the collected MPs were opaque and showed a notable change in crystallinity. The thermal properties of the extracted PLA-MPs were evaluated through Differential Scanning Calorimetry (DSC; Fig. 3).

The first heating scan was considered to evaluate the direct effects of the exposure on the thermal properties of the material. The glass transition temperature (Tg) kept constant between the two samples, hinting

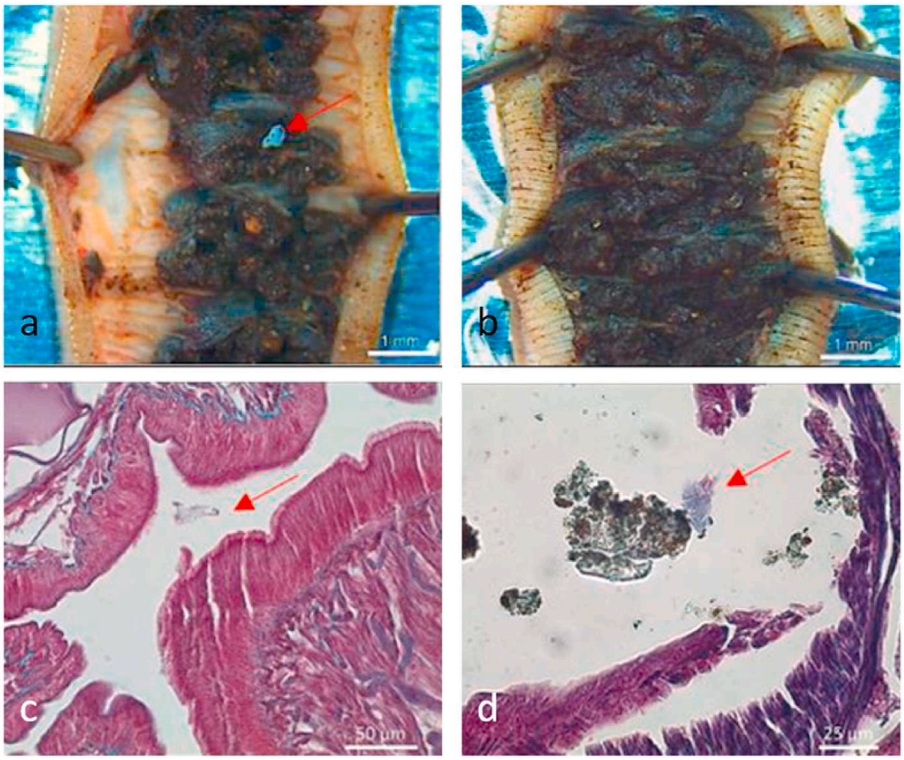

Fig. 2. Presence of PET- and PLA-MPs in the digestive tract of earthworms after dissection (panel a and b) and histological (panel c and d) analyses. Panel a) shows the presence of PET-MPs (red arrow) in earthworm digestive tract, while no PLA-MPs were observed (panel b). Red arrows in panel c) and d) show the presence of PET- and PLA-MPs, respectively, in histological (paraffin) sections of the digestive tract of earthworms exposed to 1% MPs of soil weight. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

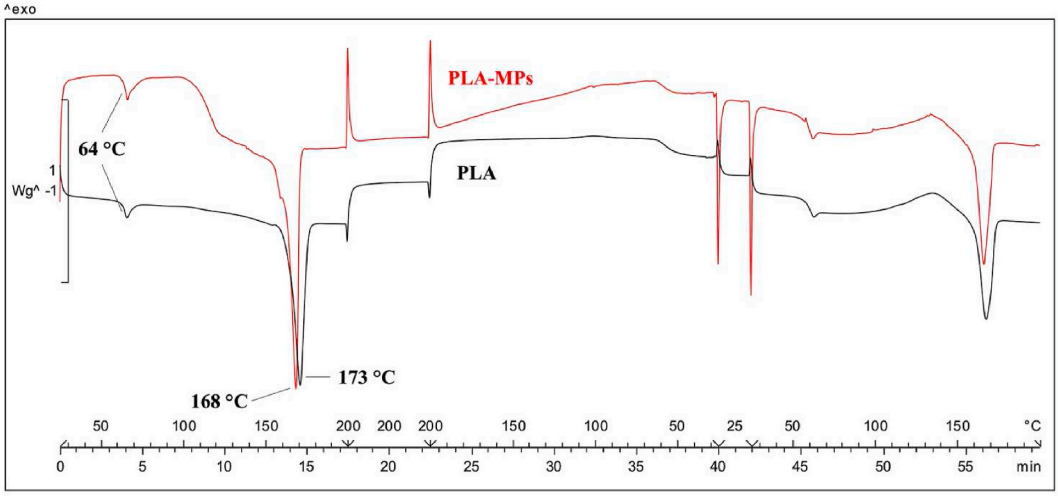

Fig. 3. DSC thermogram of extracted PLA-MPs after the exposure compared to the thermogram of the standard PLA before the exposure.

the absence of any contamination occurring during the exposure. On one hand, the absorption of water or small organic molecules by the material during the exposure may result in plasticization effects, which, in turn, end up in a lowering of the Tg. On the other hand, the appearance of the melting transition changed, denouncing possible differences in the crystallization behaviour of the material. After the exposure, the melting peak shifted from 173 °C of the starting PLA to 168 °C. In addition, the melting peak of PLA-MPs appeared to be sharper, pointing out a more defined transition, which is usually related to lower molecular weight molecules. These findings suggest a partial degradation of PLA-MPs under our exposure conditions.

3.3. Oxidative stress

The exposure to PLA-MPs induced the modulation of the oxidative status in *E. foetida* individuals compared to the control group (Table 2). Despite no significant effect of the treatment, a significant time × treatment interaction on SOD activity was observed (Fig. 4a). A significant activation of SOD was noted after 21 days of exposure in earthworms exposed to 0.1% ($P < 0.001$) and 1% ($P = 0.033$) PLA-MPs of soil weight compared to individuals from the corresponding control group. Moreover, a significant inhibition of SOD activity was found at the end of the exposure in individuals from both the experimental groups

Table 2
Effect of treatment, time of exposure and their interaction on oxidative stress biomarkers (i.e., SOD, CAT, GPx, GST and lipid peroxidation) measured in *Eisenia foetida* specimens exposed for 28 days to two amounts (0.1% and 1% of MPs on weight of soil) of PLA-MPs and PET-MPs. Significant effects are reported in bold. df = degrees of freedom.

	F	df	*P*
PLA-MPs			
SOD			
Treatment	0.305	2,24	0.740
Time of exposure	9.637	3,24	**<0.001**
Treatment × time of exposure	3.247	6,26	**0.017**
CAT			
Treatment	0.236	2,24	0.791
Time of exposure	3.427	3,24	**0.033**
Treatment × time of exposure	1.533	6,26	0.210
GPx			
Treatment	8.993	2,24	**0.001**
Time of exposure	36.979	3,24	**<0.001**
Treatment × time of exposure	14.349	6,26	**<0.001**
GST			
Treatment	0.033	2,24	0.967
Time of exposure	0.972	3,24	0.422
Treatment × time of exposure	1.010	6,26	0.441
Lipid peroxidation			
Treatment	0.900	2,24	0.419
Time of exposure	0.236	3,24	0.869
Treatment × time of exposure	0.502	6,26	0.800
PET-MPs			
SOD			
Treatment	0.493	2,24	0.616
Time of exposure	2.183	3,24	0.116
Treatment × time of exposure	1.192	6,26	0.343
CAT			
Treatment	2.225	2,24	0.129
Time of exposure	46.385	3,24	**<0.001**
Treatment × time of exposure	1.760	6,26	0.150
GPx			
Treatment	0.049	2,24	0.951
Time of exposure	1.608	3,24	0.213
Treatment × time of exposure	2.648	6,26	**0.041**
GST			
Treatment	1.459	2,24	0.252
Time of exposure	10.308	3,24	**<0.001**
Treatment × time of exposure	1.962	6,26	0.111
Lipid peroxidation			
Treatment	2.244	2,24	0.127
Time of exposure	2.379	3,24	0.094
Treatment × time of exposure	0.443	6,26	0.842

compared to the corresponding control ($P < 0.001$ for 0.1% PLA-MPs of soil weight and $P = 0.005$ for 1% PLA-MPs of soil weight). A significant effect of the treatment was noted for GPx, with an overall increase of enzyme activity observed in individuals from both the 0.1% ($P = 0.001$) and 1% PLA-MPs of soil weight ($P = 0.026$) compared to the control group. Moreover, according to a significant time × treatment interaction, the GPx activity of individuals from both the experimental groups significantly increased at the end of the exposure with respect to conspecifics from the control group ($P < 0.001$ for both the experimental groups; Fig. 4b). No effect of treatment, time and their interaction of CAT (Fig. 4c) and GST (Fig. 4d) activities occurred.

Overall, no effect of PET-MPs exposure on the oxidative status was found when comparing treated earthworms and conspecifics from the control group (Fig. 5 a–d). A large variability of antioxidant enzymes and GST activity was observed in individuals from the control group. Such condition could depend on different inter-individual health status of earthworms, as also suggested by minor alterations pointed out by histological analyses (see *3.4. Histology* section), and could preclude highlighting potential effects induced by PET-MPs. However, significant effects of the exposure time were observed on CAT and GST activity only (Table 2). Whilst a significant increase of CAT activity was noted in earthworms at the end of the exposure (i.e., 28 days) compared to previous time-points, no clear temporal trends were found for GST. No effect of treatment and time × treatment interaction was highlighted on all the investigated endpoint, except a significant effect of the interaction of the two predictors on GPx activity. However, no biologically relevant effects emerged from the pairwise comparisons.

Overall, a slight decreasing trend was noted in treated earthworms over the exposure to PLA-MPs, while an opposite trend occurred as consequence of PET-MPs. However, no significant effect of the treatment, the time of exposure and their interaction was observed on lipid peroxidation levels in earthworms exposed to both the amounts of PLA- and PET-MPs compared to the control group (Table 2 and Fig. 6).

3.4. Histology

Exposure to PLA- and PET-MPs did not induce any notable adverse effect on tissues of *Eisenia foetida* treated individuals compared to the conspecifics from the control group. Indeed, no histological alteration was observed in pharyngeal and esophageal tissues comparing sections from treated and control individuals (Figs. S1 and S2). The pharynx of both PLA- and PET-MPs treated groups, as well as control one, showed a regular and uniform shape characterized by the columnar epithelial layer formed by the juxtaposition of elongated cells containing elliptical nuclei. The cells rest on a thin basement membrane that separated them from the thick surrounding pharyngeal musculature, while on the apical portion of the cells microvilli and thin cilia were visible that extended towards the lumen. The different layers of the esophagus, as well as the epithelial architecture, shape and distribution of the cells/nuclei and cilia were comparable between the different experimental treatments. No sign of inflammation (e.g., edema, hypertrophy or accumulation of immune cells) was observed in the pharyngeal and esophageal tissues. Some spaces between the epithelial cells were occasionally noted, suggesting a slight alteration in the arrangement of the cells (black arrows in Figs. S1 and S2). However, these effects could not be referred to MPs of both the polymers because a similar condition was observed also in individuals from the control group.

3.5. Behavioral response

No effect of treatment ($F_{2,96} = 0.188$; $P = 0.828$), time of exposure ($F_{3,96} = 2.440$; $P = 0.069$) and their interaction ($F_{6,96} = 0.422$; $P = 0.862$) was observed on the digging activity of earthworms exposed to both the amounts of PLA-MPs compared to the control group (Fig. 7A). Similarly, no effect of treatment ($F_{2,96} = 0.203$; $P = 0.821$), time of exposure ($F_{3,96} = 2.690$; $P = 0.051$) and their interaction ($F_{6,96} = 1.495$; $P = 0.188$) was observed on the digging activity of earthworms exposed to both the amounts of PET-MPs compared to the control group (Fig. 7B).

4. Discussion

The exposure to PLA-MPs induced an alteration of the oxidative status in the earthworm *E. foetida*, but this condition did not result neither in histological nor in behavioral effects. In contrast, the exposure to PET-MPs did not induce adverse effects at any level of biological organization we investigated.

Some previous studies compared the ingestion and the potential toxicity of MPs made of fossil-based polymers (i.e., conventional plastics) and bio-based ones (i.e., bioplastics) towards different earthworm species (e.g., Meng et al., 2023; Ding et al., 2021). Independently of the model species, earthworms were able to efficiently ingest and egest MPs made of diverse polymers and displaying a wide range of shapes and sizes. For instance, the ingestion of polyester microfibers (Prendergast-Miller et al., 2019) and low-density PE (LDPE; Huerta Lwanga et al., 2016; Meng et al., 2023), PLA, polypropylene carbonate (PCC; Ding et al., 2021) and polybutylene adipate co-terephthalate (PBAT; Meng et al., 2023) items was demonstrated. Our microscopy analyses

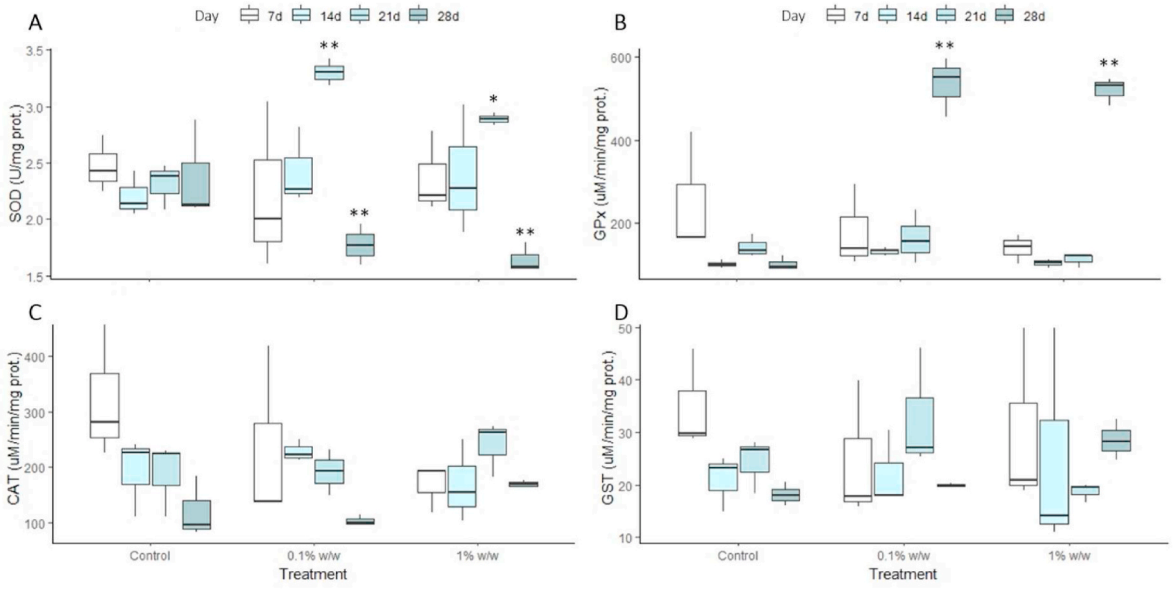

Fig. 4. Box-plots of the activity of superoxide dismutase (SOD, a), glutathione peroxidase (GPx, b), catalase (CAT, c) and glutathione S-transferase (GST, d) measured in pools of *Eisenia foetida* individuals (n = 3 pools of three individuals each) at different time-points of exposure to two amounts (0.1% and 1% of MPs of soil weight) of PLA-MPs (two-way ANOVA, Tukey's *post-hoc* test: *P < 0.05; **P < 0.01 compared to the correspondent control).

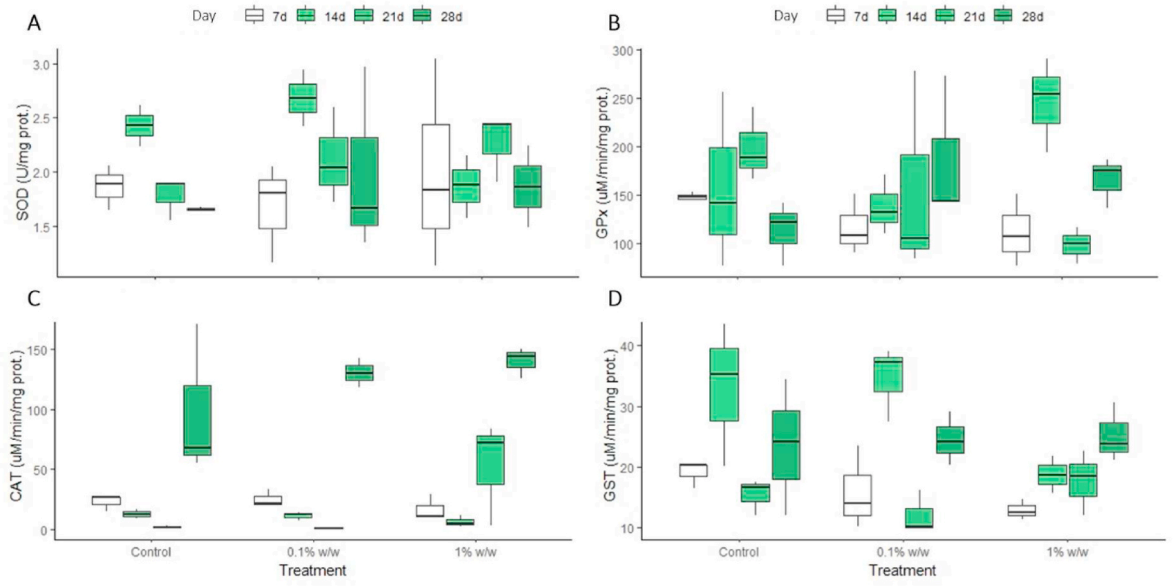

Fig. 5. Box-plots of the activity of superoxide dismutase (SOD, a), glutathione peroxidase (GPx, b), catalase (CAT, c) and glutathione S-transferase (GST, d) measured in pools of *Eisenia foetida* individuals (n = 3 pools of three individuals each) at different time-points of exposure to two amounts (0.1% and 1% of MPs of soil weight) of PET-MPs.

partially confirmed these findings. Indeed, whilst at the end of the 28 days of exposure PET-MPs were clearly visible in the gut of treated earthworms, no PLA-MPs were observed. These results should suggest a preference of earthworms towards PET rather than PLA, as suggested by a previous approach–avoidance behaviour experiment (Wang et al., 2022). The different mechanical strength and flexibility or the hardness of PET particles, which can help earthworms to grind food in the gizzard (Wang et al., 2022), might explain the differences in earthworms' ingestion behaviour. However, histological analyses confirmed the presence of both PET- and PLA-MPs in the digestive tract of earthworms (Fig. 2). The illusory absence of PLA-MPs in the digestive tract of the earthworms might be related to their partial degradation and/or changes in their chemical-physical features (and consequently in colour/appearance) preventing their visual observation after dissection. In fact, a recent study performed on the terrestrial earthworm *Lumbricus terrestris* showed that PLA-MPs (20–648 μm in size) suffered notable modifications after ingestion and transit into the digestive tract. Fragmentation occurred because of the grinding activity in the gizzard, which is facilitated by the ingestion of hard soil particles (Mahran et al., 2005; Meng et al., 2023). In addition, depolymerisation (i.e., the reduction in the molecular weight of the polymer) occurred in the gut (Meng et al., 2023), probably mediated by a diverse enzymes, including carboxylesterase, chitinase, cellulase, lipase, and protease, which are all secreted into the gut by ingested soil microbes (Drake and Horn, 2007; Sanchez-Hernandez et al., 2009). These processes could act in the gut of *E. foetida*, reducing the size and/or the appearance of PLA-MPs,

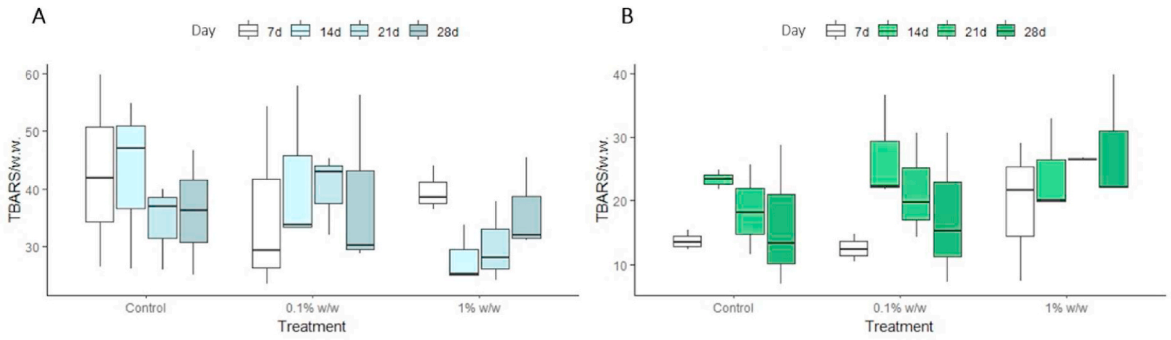

Fig. 6. Box-plots of lipid peroxidation levels measured in pools of *Eisenia foetida* individuals (n = 3 pools of three individuals each) at different time-points of exposure to two amounts (0.1% and 1% of MPs of soil weight) of PLA-MPs (panel A) and PET-MPs (panel B).

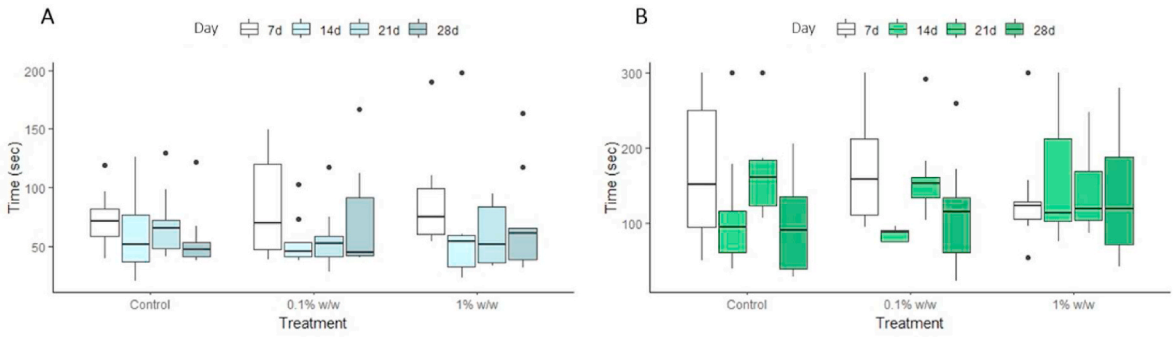

Fig. 7. Box-plots of digging time measured in pools of *Eisenia foetida* individuals (n = 15 individuals per each experimental group) at different time-points of exposure to two amounts (0.1% and 1% of MPs of soil weight) of PLA-MPs (panel A) and PET-MPs (panel B). Black dots represent the outliers.

precluding our visual identification. Alternatively, degradation of PLA-MPs might occur into the soil before earthworm ingestion, because of microbial activity. Several studies have demonstrated the role of different microbial communities in the degradation of PLA into the soil (e.g., Karamanlioglu et al., 2014; Boonluksiri et al., 2021). The slight de-polymerization of PLA-MPs highlighted by lower molecular weight and changes in crystallinity could be responsible of the observed differences. Our findings confirmed the results from studies reporting that PLA degrades in the soil and suffers changes in physical, mechanical and chemical properties (Teixeira et al., 2021; Rudnik and Briassoulis, 2011; Vasile et al., 2018). The degradation of PLA-MPs could cause the release of by-products into the soil, including the lactic acid or low molecular weight species such as dimers and/or trimers of the lactic acid (Torres et al., 1996; Karamanlioglu and Robson, 2013), or the formation of small-sized MPs and nanoplastics (González-Pleiter et al., 2019). The exposure to degradation by-products and/or the ingestion of small-sized items might affect the health status of earthworms inducing stressful conditions.

Despite the ingestion of both PET- and PLA-MPs, no acute effects (i.e., mortality) occurred over the 28 days of exposure in earthworms treated with MPs made of both the polymers. These results confirmed previous findings on the same or similar species exposed to MPs of different polymeric composition, including PLA and other bioplastics (Huerta Lwanga et al., 2016; Rodriguez-Seijo et al., 2017; Prendergast-Miller et al., 2019; Meng et al., 2023). However, the ingestion of PLA-MPs, but not of PET-MPs, induced the modulation of the activity of antioxidant and detoxifying enzymes in treated earthworms, suggesting an alteration in the oxidative status. Several studies have highlighted that the activity of antioxidant enzymes in earthworms can be increased, decreased, or unchanged following the exposure to MPs depending on polymer composition, particle size, concentration/abundance and duration of the exposure (Chen et al., 2020a,b; Lackmann et al., 2022; Li et al., 2021; Rodríguez-Seijo et al., 2018; Yu et al., 2022). In our study, significant changes in SOD, and GPx activities occurred exclusively after 21 days of exposure to PLA-MPs, exhibiting peculiar trends. In detail, the significant increase of SOD activity observed after 21 days of exposure to both the amount of PLA-MPs followed by the significant inhibition observed after 28 days of exposure (Fig. 4a) suggested an overproduction of superoxide anion (Verlecar et al., 2008). Whilst the activation of SOD confirmed the reaction triggering the dismutation of superoxide anions into hydrogen peroxide, its further inhibition could suggest an excess and the potential accumulation of radicals that the organism could not counterbalance. The bell-shaped trend of SOD follows the typical response of enzymes after the exposure of the organism to a xenobiotic over the time and/or in response to increasing exposure concentrations/abundances. The increase in SOD activity observed after 21 days of exposure could be due to the activation its synthesis, while the further decrease suggested the enhanced catabolic rate and/or a direct inhibitory action of the xenobiotic on the enzyme (Viarengo et al., 2007). Thus, the reduced SOD activity observed at the end of the exposure might be due to the inhibition effect and/or negative feedback due to the production of hydrogen peroxide (Vlahogianni and Valavanidis, 2007). Thus, this effect may be associated with excessive pro-oxidant species affecting the normal functions of treated earthworms, inhibiting the synthesis and/or promoting the inactivation of antioxidant enzymes (Verma and Dubey, 2003). The significant increase of GPx activity observed after 28 days of exposure to both the PLA-MPs amounts confirmed that exposed earthworms experienced an overproduction of H_2O_2 (Fig. 4b). The lack of modulation in CAT activity, which is usually activated only in response to high concentrations of hydrogen peroxide (Pereira et al., 2013), suggests that GPx was able to counteract the toxicity of this pro-oxidant molecule. Lastly, despite its crucial role in the elimination of lipid hydroperoxides (Chen et al., 2020b), the activity of the detoxification enzyme GST was not modulated by the exposure to PLA-MPs. Our findings on antioxidant and detoxifying enzymes confirmed that the

exposure to PLA-MPs could affect the oxidative status of earthworms, as previously demonstrated by an experiment performed on the same earthworm species that investigated oxidative stress-related responses after 14 and 28 days of exposure (Yu et al., 2022). Indeed, after 14 days of exposure to PLA-MPs the activities of SOD, CAT, GPx and GST decreased, while levels of lipid peroxidation (MDA) increased, showing a "decrease–increase" trend with increasing MP exposure doses. In contrast, opposite trends of all the responses were noted after 28 days of exposure, confirming the increase or decrease in responses with increasing PET-MPs abundances. Although the exposure to a very low abundance (i.e., 0.1 % PLA-MPs/kg soil) of PLA-MPs did not trigger significant responses in treated earthworms over 28 days of exposure, the exposure to PLA-MPs higher than 0.5% items/kg soil induced significant modulations of oxidative stress related biomarkers, with different responses depending on the duration of the exposure and MP abundance (Yu et al., 2022). Our results supported the hypothesis that the amount of MPs and the duration of the exposure (Yu et al., 2022), can influence biochemical responses in earthworms. Moreover, also the polymer composition of MPs can affect earthworm responses. In fact, our study showed that the toxicity of MPs made of a biodegradable polymer (i.e., PLA) could be higher compared to that caused by MPs made of a conventional polymer (i.e., PET). Such findings were previously observed also by Yu et al. (2022), who pointed out that the exposure to PLA-MPs did not cause lower toxicity compared to MPs made of a fossil-based, conventional polymer, such as the PE. Overall, the modulation of antioxidant machinery observed after 28 days of exposure to PLA-MPs suggests, on one hand, the increase of pro-oxidant molecules but, on the other hand, the effective scavenging activity of antioxidant enzymes to prevent oxidative damage. Indeed, treated earthworms did not suffer an oxidative stress situation because no oxidative damage to lipids occurred (Fig. 5a). In addition, no signs of inflammation was noted neither in the esophagus nor in the pharynx of treated earthworms (Fig. S1). These results confirmed the effectiveness of antioxidant machinery to counteract the toxicity of pro-oxidants. In contrast to effects observed in earthworms exposed to PLA-MPs, no biochemical nor histological effects were induced by the same amount of PET-MPs. This discrepancy was not unexpected, as some recent studies comparing the potential toxicity of MPs made of fossil-based, traditional polymers and bio-based ones such as bioplastics, showed that bioplastics could be more reactive (Ding et al., 2021).

Despite the modulation of the oxidative status suggests that earthworms treated with PLA-MPs suffered a stressful condition, no behavioral effects were noted. No difference in digging activity occurred between earthworms treated with PLA-MPs (and PET-MPs) compared to conspecifics from the control group. These results disagreed previous studies suggesting that the exposure to MPs of different polymers could affect the behaviour of earthworms. For instance, a clear avoidance behaviour was observed in *E. foetida* individuals exposed to high abundances of MPs made of PLA, polypropylene carbonate (PPC) and polyethylene (PE) (higher than 40 g/kg soil weight). Similarly, a previous study by Huerta Lwanga et al. (2017a) showed that earthworms migrated to deeper soil layers when the abundance of PE-MPs in soil litter layers was 70 g/kg soil weight. The exposure to MPs was demonstrated to alter the burrowing activity of earthworms. For instance, an increased in the number of *L. terrestris* burrows, which were also heavier and denser compared to control group, occurred after a 14-days exposure to 7% LDPE-MPs on soil weight (Huerta Lwanga et al., 2017a). Yang et al. (2019) obtained contrasting results on the same model species experiencing similar experimental setup, with gallery volume decreased and the weight increased after the exposure to 7% LDPE-MPs on soil weight. In the present study, no alteration of digging behaviour of earthworms was noted after the exposure to both 1 and 10 g PET- and PLA-MPs/kg soil weight (0.1% and 1% MPs on soil weight). Although behavioral responses induced by MPs exposure can vary depending on the considered behavioral task, our results should support previous findings proving that the concentration/abundance of MPs represent the main determinant factor affecting earthworm behaviour, rather than the polymer composition of MPs (i.e., the type of plastic material; Ding et al., 2021).

5. Conclusions

The results of the present study pointed out that, also at low amounts, MPs made of a bio-based, biodegradable polymer such as the PLA can affect the health status of earthworms more than MPs made of a fossil-based polymer, such as the PET. In detail, the exposure to PLA-MPs modulated the activity of SOD and GPx, suggesting an overproduction of pro-oxidant molecules that was efficiently counteracted by antioxidant machinery, preventing the onset of oxidative stress condition. Indeed, neither oxidative damage nor histological injuries and behavioral alterations occurred. In contrast, no effects was caused by PET-MPs exposure under the same experimental conditions. The higher biochemical effects induced by PLA-MPs compared to PET-MPs might be due to the biodegradation of PLA, which might occur both into the soil through the activity of microbiota and/or into earthworm digestive tract because of mechanical grinding or enzymatic digestion. Biodegradation of PLA could release byproducts and/or small-sized MPs and nanoplastics, which could cause or exacerbate the toxicity of the polymer compared to its fossil-based counterpart. These results support the findings of some previous studies comparing the toxicity of MPs form bio-based and fossil-based polymers, suggesting that the exposure to MPs originated from bioplastics can induce higher effects towards soil organisms and cannot be considered as more 'bio-safe' than those from fossil-based ones. However, considering that biodegradable MPs might remain into the soil for a shorter period of time than conventional, fossil-based ones because of their biodegradation, their toxic effects could appear early but gradually disappear. For this reason, despite the growing information regarding the degradation and biodegradation of PLA in the soil and mediated by soil organisms, further studies should be necessary to confirm the release of byproducts, their accumulation in the organisms and the onset of adverse effects. Moreover, it should be interesting to assess the fate of PLA-made objects into the soil under natural conditions, to estimate the (bio)degradation rate and the interaction with organisms over short- and long-term exposures. Although our findings enlarge the understanding of the potential ecological risk of biodegradable MPs in soil organisms, there is a need of mechanistic studies assessing the influence of PLA-MPs on the health status of earthworms and other soil organisms at different life stages to shed light on the potential long-term impact on soil functioning. Thus, considering the role of bioplastics in our daily life and agriculture, as well as their growing demand and subsequent release into the environment, multi-level and multi-species experiments should be a priority for a proper environmental risk assessment of bioplastics and related biodegradation byproducts.

CRediT authorship contribution statement

Marco Parolini: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Beatrice De Felice:** Writing – review & editing, Investigation. **Stefano Gazzotti:** Writing – review & editing, Investigation. **Michela Sugni:** Writing – review & editing, Investigation. **Marco Aldo Ortenzi:** Writing – review & editing, Investigation.

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. Supplementary data

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