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Research Article

Understanding the harmful effects of polyethylene microplastics on *Eisenia fetida*: A toxicological evaluation

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Abstract
Microplastics, measuring less than 5 mm, are pervasive environmental pollutants raising concerns about their toxic effects on terrestrial ecosystems, especially earthworms.A comprehensive toxicological evaluation of polyethylene microplastics on earthworms will be beneficial for determining the detrimental impacts of these ubiquitous pollutants on soil ecosystem. Therefore, in the present study, the best representative soil organism, earthworms (*Eisenia fetida*), were opted for examining the toxicological effect of polyethylene microplastic. *E. fetida* were subjected to different concentrations of polyethylene microplastic (200, 400, 600, 800, and 1000 mg/kg) in soil and randomly picked out on days 7 to 56. Earthworms exposed to higher concentration of polyethylene (1000 mg/kg of artificial soil) showed a significant reduction in body weight and cocoon formation after 35th days of incubation. A consistent decrease in the concentration of carbohydrates, lipids, and protein was observed when the worms were exposed to the higher concentration of polyethylene. Further, antioxidant enzymes like superoxide dismutase, glutathione S-transferase, peroxidase, catalase, and malondialdehyde were determined for antioxidant stress.Exposure of 200 mg/kg to 1000 mg/kg of artificial soil caused a prominent amplification in the build-up of malonedialdehyde (a biological marker of oxidative stress) by 1.29-fold. It also considerably augmented the activity of the antioxidant enzymes viz., glutathione S-transferase (1.54-fold), superoxide dismutase (1.51-fold), peroxidase (1.25-fold), and catalase (1.87-fold). The present study's findings provide a new understanding of the toxic effect of microplastic on earthworm *E. fetida,* presenting a foundation for its risk evaluation on soil ecosystems and non-target biological toxicity.

Keywords: Antioxidant enzymes, *Eisenia fetida*, Polyethylene, Soil pollution, Toxicity

INTRODUCTION

Global plastic production is predicted to reach 34 billion tons by 2050. The plastic recycling occurring presently is only about 10 %, leading to a large increase of plastic in the environment. The plastic waste accumulation will reach 12 billion by 2050 if the management practices will not change. Eighty percent of the accumulated plastic goes into landfilling or is left in the environment (Muñoz Meneses *et al.*, 2022). This plastic, when left in the open, breaks down into smaller plastic particles due to various chemical and physical weathering processes. Heat, UV irradiation, physico-mechannical processes and microbial degradation lead to the degradation and fragmentation of plastic present in soil and form microplastics (MPs) (Rodriguez-Seijo *et al.*, 2017; Sun *et al.*, 2021). Microplastics (MPs) are plastic particles with a diameter of less than 5 mm. MPs are causing enormous environmental pollution, as reported recently in various literature studies. The small size and difficult disposal have made the MPs a notorious environmental pollutant. The degradation of MPs is challenging due to their chemical nature, making them more harmful than macroplastics. Apart from plastic degradation, microplastics also come into the environment through industrial contaminants and microbeads used in toothpaste and personal care products (Lei *et al.*, 2017). The highest amount of MPs reported in industrially contaminated soil was 2400 mg/kg (Büks and Kaupenjohann, 2020). MPs are easy to diffuse and transfer due to their smaller size and therefore huge quantity of microplastic particles are dispersed in soil, air and sea

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(Wang *et al.*, 2020).

In recent years, the public and scientific community has driven its interest towards MPs remediation. MPs have been reported in deep-sea and soil and they have also been detected in various organisms such as fish, birds and invertebrates (Valavanidis*et al.*, 2013). MPs can enter the terrestrial environment via the disintegration of plastic waste (e.g., plastic mulch films), the appliance of sewage sludge, air deposition, and wastewater irrigation(Sajjad *et al.*, 2022). There are high risks of microplastic entry intothe human body through breathing, skin contact and food chain. Many studies have shown that MPs could cause cytotoxicity, oxidative stress, and continual swelling, thus increase the risk of cancer (Chiu *et al.*, 2015; Dong *et al.*, 2020). MPs also react with other pollutants in the soil and alter the bioavailability and toxicity of microplastics and other pollutants to soil organisms. Therefore, studying microplastics and their effect on the soil ecosystem has become more concerning.

Recent research studies have focussed heavily on the effect of microplastics on aquatic systems and terrestrial studies are limited. Some previous studies have reported the effects of microplastic toxicity on earthworms, but still, there is inadequate information onthe toxicological consequences of MPs on reproduction ability and immunity (Huerta Lwanga *et al.*, 2016; Ju *et al.*, 2019; Judy *et al.*, 2019; Lahive*et al.*, 2019; Zhu *et al.*, 2018). MPs, when ingested by terrestrial organisms,accumulate and cause physical destruction of tissues and organs and bring forth a stirring reaction to insidious heterogenic materials and their reproductive toxicity to soil fauna (Boots *et al.*, 2019; Sobhani *et al.*, 2022; Zhou *et al.*, 2020).

Earthworms are essential for the soil food chain and are significant in maintaining the soil ecosystems' fertility, function and structure(Zhang *et al.*, 2022). Also, earthworms have high reproductive ability and well-built adaptableness, although they are also susceptible to noxious and detrimental matter. So, earthworms, particularly *Eisenia fetida,* are extensively used to analyse toxins' toxicity. *Eisenia fetida* is a common species of vermicomposting earthworm that is oftenly used in toxicity tests. These worms are easy to culture and rear and have a short life cycle, making it ideal for studies investigating the effects of toxicants on different life stages. Most of the earlier reports have focused on the toxic effect of contaminants on physical growth, body movement and oxidative stress (Besseling*et al.* 2013; Rodriguez-Seijo *et al.* 2017; Rillig*et al.* 2017; Wang *et al.* 2019; Rodriguez-Seijo *et al.* 2017; Prendergast-Miller *et al.* 2019). Zhou *et al.* (2020) reported the inhibition in growth of the earthworm *Eisenia fetida* after prolonged contact with polypropylene microplastics (Zhou *et al.*, 2020). Similarly, undersized *Aporrectodea rosea* growth was reported inhigh-density polyethylene MPs (Boots *et al.*, 2019).

Therefore, the present study was designed to study the effect of polyethylene (PE) MPs on the growth and reproductive behavior of *E. fetida*. The changes in stress-related antioxidant enzyme (catalase, CAT; peroxidase, POD; superoxide dismutase, SOD) activity, detoxification enzyme (glutathione S-transferase, GST) activity, the content of the lipid peroxidation product malondialdehyde (MDA) were observed to investigate the toxicity mechanism of microplastics in earthworms.

MATERIALS AND METHODS

Microplastic and chemicals

The PE microplastic (125 µm), acid, the substrate for enzymesand sugar standards were acquired from Sigma-Aldrich (US). Calcium carbonate, sodium carbonate, and sodium hydroxide were obtained from Merck (Darmstadt, Germany). All other chemicals used in this work were of the utmost purity grade existing commercially.

Selection and collection of animal model

Earthworms (*E. fetida*) were collected in wooden crates with soil and organic cow manure. Post-collection identification and taxonomy of the earthworms were done using the online taxonomic key and digital library (Thakur *and* Yadav, 2018).

Preparation of bedding material

Tropically enriched soil was utilized to eliminate the chance that the soil might include PE microplastic particles, worms, and cocoons. This is in accordance with the internationally recognized process for evaluating the toxicology of pollutants (OECD Guidelines, 207). An artificial soil was prepared by mixing coconut peat, industrial soil, and kaolinite clay; the components were individually air-dried at room temperature and subsequently mixed in a ratio (w/w) of 7:2.5:0.5. Milli-Q water and $CaCO_3$ (Calcium carbonate) were added to regulate the moisture and pH value (6.0±0.5) of the soil. The water-holding capacity of the artificial soil was maintained at 50 %.

Exposure of earthworms to polyethylene microplastic

Healthy adult worms (ages 2-3 months), with a wet body weight of around 300 mg to 500 mg, and a clitellum were chosen for the experiments. Before the PE microplastic exposure, the earthworms were incubated for one day beneath the identical artificial soil environment to acclimatize to the experimental conditions. PE microplastic toxicological effect was checked at five diverse concentrations (200, 400, 600, 800, and 1000 mg/kg dry soil) in the avoidance test. The experiments were performed in several replicates (4 replicates for

each concentration). Ten adult well-clitellated worms were placed into plastic trays with 1000 g of artificial soil (dry weight) with dissimilar PE microplastic concentrations. The beakers were covered with gauze and incubated at 20 ± 2 °C for 56 days. Feed was given regularly during exposure, and moisture was maintained in the pots. The earthworms were fed weekly (once) by spreading cow manure (5g) on the soil surface. They were collected at a regular interval of 7 days of exposure,then washed with Milli-Q water and depurated for 24 h before further examination. The testing was performed in numerous replicates and variations between the worms' initial and final body weight were noted. The growth rate of earthworms was evaluated by the relative gain and loss of weight.

Biochemical assays

The whole protein and carbohydrate matters were determined according to the method described De Coen and Janssen (1997) (De Coen *and* Janssen, 1997). Worms exposed to different concentrations of PE microplastic were sacrificed and total protein and carbohydrates content were determined. For protein estimation, worms exposed to PE microplastic for 28 days were rinsed with Milli-Q water, sacrificed, and divided into preclitellar, clitellar and post-clitellar regions. The tissues were homogenized in pre-chilled mortar and pestle potassium phosphate buffer (50 mM; 1:8 w/v; pH -7.0). The centrifugation of the homogenate was carried out at 4 °C and 9,600g for 10 min and the fraction in the supernatant was collected for protein assays. As explained previously, protein concentration was estimated using the spectrophotometric method (Lowry *et al.*, 1951). The quantification of total carbohydrate content was done by the addition of phenol 5 % (v/v) and concentrated H_2SO_4 to the supernatant fraction in a proportion of 1:1:4. The absorbance was measured at 540 nm after incubating the mixture for 30 min at room temperature. Glucose was taken as the standard for measurement (Miller, 1959).

For estimation of lipids, the method developed by Bligh and Dyer method (1959) was followed (Bligh *and* Dyer, 1959). Earthworms were homogenized in 0.2 ml of Milli -Q. After homogeneity, 0.5 ml of chloroform, methanol, and 0.25 ml of DDW were added (all HPLC grade). The reaction mixture was vortexed amid each step. The supernatant was recovered after centrifugation (6000 rpm, 8 min). To this supernatant of the remaining lipid extract, 0.5 ml of H_2SO_4 was added and then incubated at 200 °C for 15 min. The total lipid matter was measured by taking the absorbance at 400 nm using Tripalmitine as standard.

Total antioxidant capacity

Superoxide dismutase (SOD), glutathione S-transferase (GST), peroxidase (POD), catalase (CAT), and malondialdehyde (MDA) levels were quantified to evaluate oxidative injury in earthworms subjected to PE microplastic treatment. In brief, after 28 days of treatment, worms from the control faction and those exposed to PE microplastic at concentrations of 200 mg/kg to 1000 mg/kg were utilized for further study. Worms (3 individuals per exposure) were cleaned with Milli-Q water, then quickly cut into pieces, and homogenized using pre-chilled mortar and pestle with chilled potassium phosphate buffer (50 mM; 1:8 w/v; pH-7.0). The centrifugation of the homogenate was carried out at 4 ° C and 9,600 rpm for 10 min and the supernatant was collected for enzyme activity assays.

SOD was quantified using the Spectrophotometric method as illustrated previously (Song *et al.*, 2009). Peroxidase activity (EC 1.11.1.7) was determined by the guaiacol method, as described elsewhere (Kochba *et al.*, 1977). Catalase activity (EC 1.11.1.6) was determined using hydrogen peroxide method developed by Claiborne (1985) (Claiborne, 2018). GST activity was estimated following the method explained in other studies (Hans *et al.*, 1993). MDA content was quantified using the Spectrophotometric method developed by Gao *et al.*, (2016) with slight modification (Gao *et al.*, 2016).

Statistical analysis

All the experiments were conducted in 3 or multiple sets, and the data was presented as a mean ± SD. Significant differences (p value < 0.05) between the means were calculated following t-test using SigmaPlot -10.

RESULTS AND DISCUSSION

Growth and mortality of earthworms

The results on body weight measured after exposing the earthworms(*E.fetida)* to the soil amended with PE microplasticsare (Fig. 1) showed no significant decrease in the body weight at lower concentrations of PE microplastics and up to 14 days of exposure. But the body weight decreased significantly (p value < 0.05) at high concentrations,i.e. 800-1000 mg/kg from 35^{th} to 56^{th} day. Similar results were observed by Cao *et al.* (2017), where a high concentration of microplastics inhibited the growth of *E. fetida*, leading to a decrease of 29.8 % in body weight (Cao *et al.*, 2017). Zhang *et al.*(2022) also reported asignificant decrease in body weight of *E. fetida* by application of PE and ZnO particles. Thepresent study also favoured the earlier published data,indicating earthworms' weight loss during microplastic exposure. This could be due to insufficient nutrient uptake and damage to the digestive tract of earthworms. The absorption of nutrients is also limited due to damage tothe digestive tract, leading to decreased weight (Ding *et al.*, 2021). After long expo-

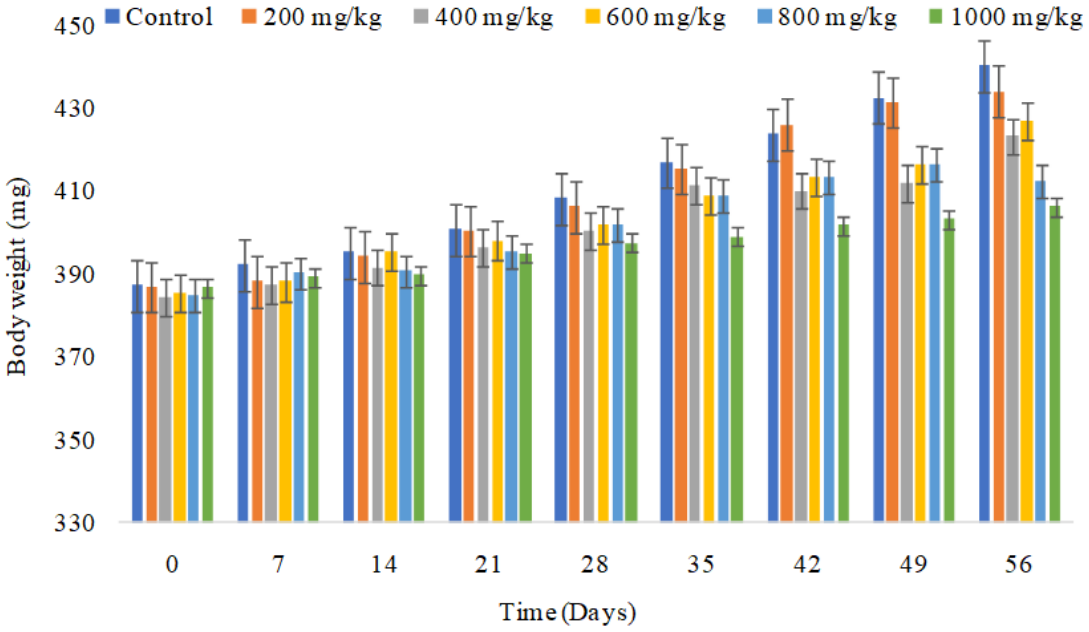

Fig. 1.*Body weight of Eisenia fetida after exposure to a control soil and soil amended with of PE microplastics at different concentrations (200-1000 mg/kg of artificial soil) for 56 days*

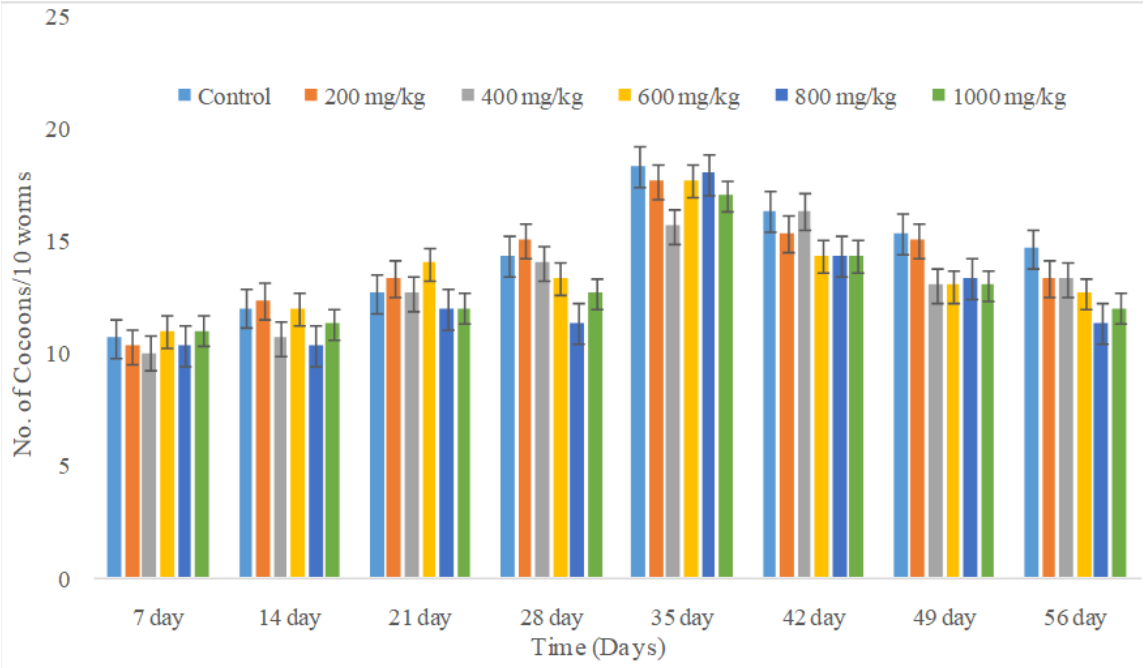

Fig. 2. *Number of cocoons by adult Eisenia fetida after exposure to a control soil and soil amended with of PE microplastics at different concentrations (200-1000 mg/kg of artificial soil) for 56 days*

sure to 56 days, the weight loss was more significant, indicating the persistent inhibition by microplastics in the growth of earthworms.

At harvest on day 35, the number of cocoons of earthworms started decreasing with increasing concentration of PE microplastics (Fig. 2). The highest decrease in cocoon numbers was observed at 800 mg/kg concentration. Similar result was observed by Ding*et al.*(2021) when they used different concentrations of microplastics. They also observed that at lower concentrations of microplastics, there was no significant decrease in the amount of cocoons but there was a decrease at higher concentrations. The present study also reports similar results at higher concentrations of PE microplastic.

Effect of PE microplastic on carbohydrate, lipid, and protein concentration

Further, the effects of PE microplastics on the carbohydrate, lipid, and protein concentration in *E. fetida* are depicted in Fig.3 and Fig.4. It was observed that the

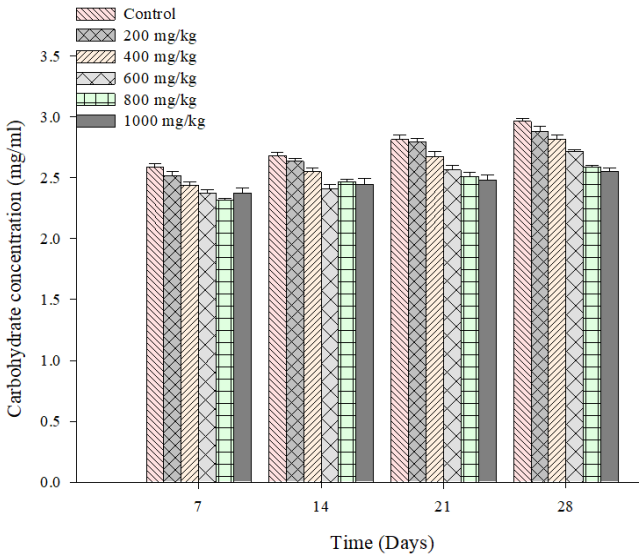

Fig. 3. *Carbohydrates concentration in Eisenia fetida after 28 days of PE microplastics exposure at different concentrations (200-1000 mg/kg of artificial soil)*

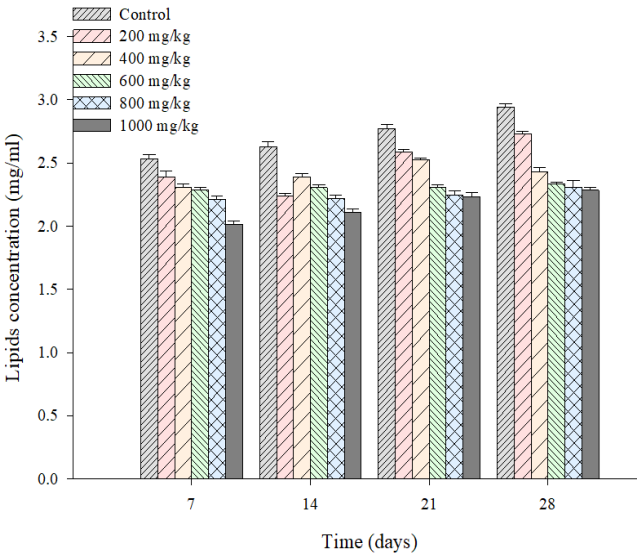

Fig. 4. *Lipids concentration in Eisenia fetida after 28 days of PE microplastics exposure at different concentrations*

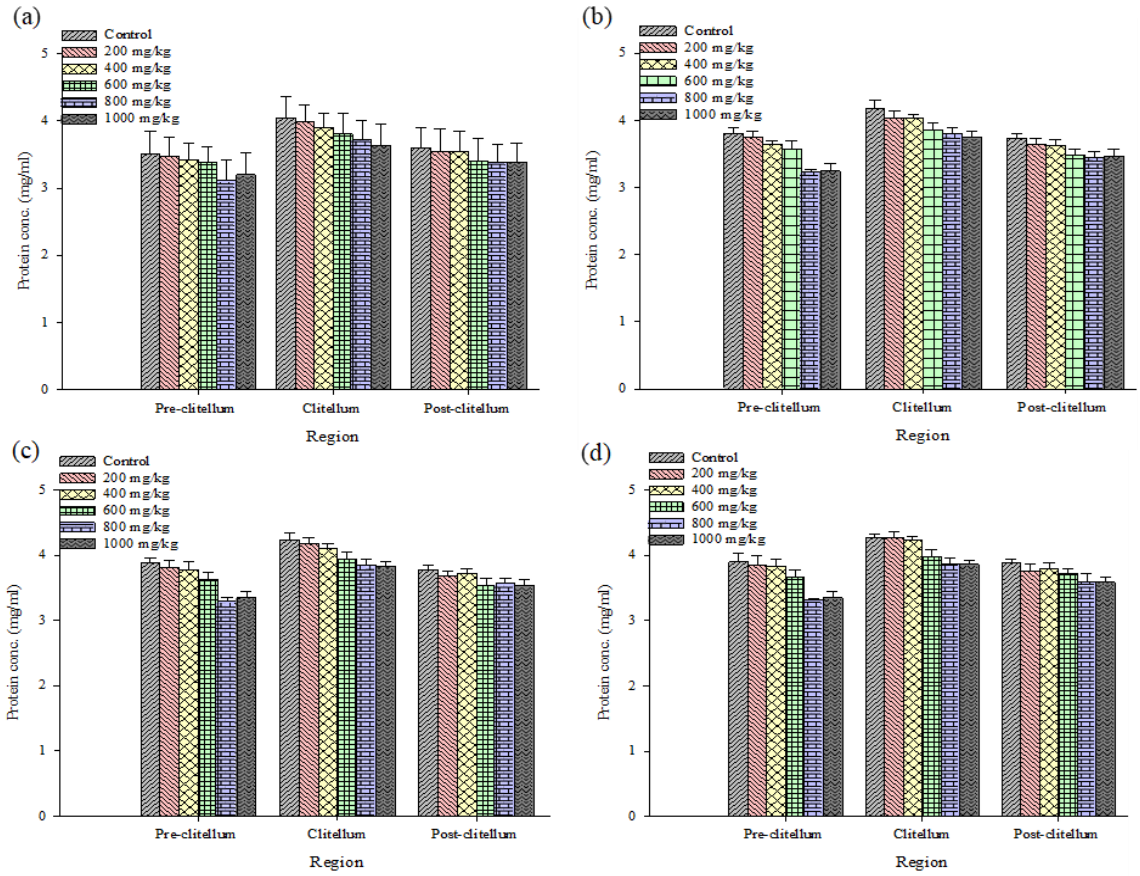

Fig. 5. *Effect of PE microplastic on protein concentration in Eisenia fetida after 28 days of exposure. (a) Protein concentration Eisenia fetida after 7th day, (b) 14th day, (c) 21st day, and 28th day*

carbohydrates and lipid concentrations decreased at harvesting days 7, 14, 21, and 28. There was a steady decrease in the carbohydrates and lipids content when the PE microplastic concentration was increased from 200 mg/kg to 1000 mg/kg of artificial soil. The maximum reduction in the carbohydrate content, i.e. 13.1 %, was observed after 28 days of PE microplastic exposure at 1000 mg/kg of artificial soil (Fig. 3). Likewise, the maximum reduction in the lipids content, i.e. 22.44 % was observed after 28 days of PE microplastic exposure at 1000 mg/kg of artificial soil (Fig. 4).

Similarly, there was a decrease in protein concentration

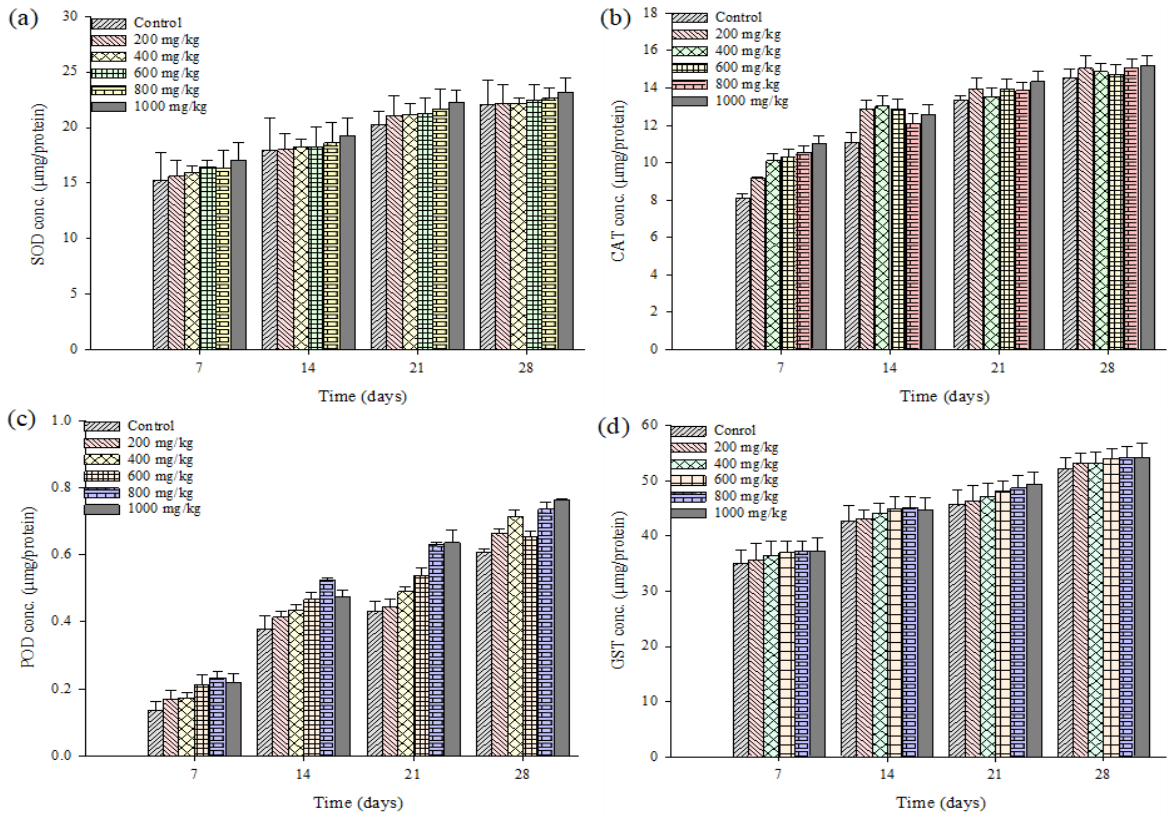

Fig. 6. *Effects of PE microplastic on the activities of different antioxidant-related enzymes in Eisenia fetida after 28 days exposure. (a) SOD, (b) CAT, (c) POD, and (d) GST*

in pre-clitellum, clitellum, and post-clitellum regions. A positive relation was observed between the PE microplastic and total protein concentration. With the increase in the PE microplastic up to 1000 mg/kg the release of reduction in the concentration of protein was observed. During the time course study, similar trends of protein reduction were achieved (Fig. 5). He *et al.* (2021) observed with the help of lipidomics analysis that there was perturbation in lipid metabolism after exposure to microplastics in *E. fetida*. In a similar study, Griffith *et al.*(2019) observed a decrease in levels of maltose and lactate, indicatingadisruption in energy metabolism. These studies indicate earthworms' reduced carbohydrate and lipid levels due to exposure to microplastics. The decrease in protein levels is associated with the disturbance of osmoregulation (Tang *et al.*, 2023). In several studies, transcriptomic analysis showed that the protein concentration was disturbed in earthworms due to oxidative stress damage, and arachidonic acid metabolism and steroid biosynthesis werealso disturbed. The muscle proteins are also broken down to protect from reactive oxygen species (Chen*et al.*, 2022).

Oxidative stress enzymes in *Eisenia fetida*

The results on the effect of different concentrations of PE microplastic on activities of antioxidants SOD, CAT, GST, and POD determined from 7 to 28 days are depicted in Fig. 6. These enzymes were significantly higher in treatment, with all the concentrations of PE microplastics with the highest activity showing at a concentration of 1000 mg/kg of artificial soil.

Antioxidant enzymes are used as effective indicators of pollution as they play an important role in the removal of excess amounts of reactive oxygen species (ROS) in organisms (Nel *et al.*, 2006). SOD catalyzes the conversion of O_2^- into H_2O_2 and O_2(Li *et al.*, 2023). CAT is a heme-containing tetrameric enzyme that can convert H_2O_2 into water and oxygen (Chen *et al.*,2020). GST has the prospective of scavenging lipid peroxidation metabolites and reducingDNA damage. The buildup of ROS can direct lipid peroxidation and reduction of cell membrane permeability,causingharm to biological tissue and cell structure. In the present study, SOD, CAT, POD and GST activity was increased, indicating the successful elimination of ROS. Similar results were obtained by Zhang *et al.* (2022) by coexposure of *E. fetida* from zinc oxide nanoparticles and PE microplastic. Further, the MDA level analyzed to determine the oxidative damage of membrane lipids was almost similar to control till 7th day but increased significantly on a longer exposure period, indicating toxicity by PE microplastics (Fig. 7). Previosly, Chen *et al.*(2020) reported that CAT activity and MDA content increased significantly at the concentration of 1.0 g/kg low-density polyethylene after exposure 28 days, from *E. fetida*.

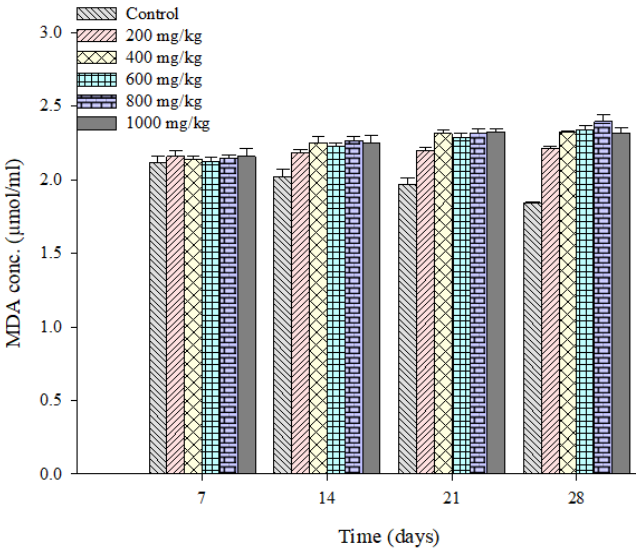

Fig. 7. *MDA level in Eisenia fetida after 28 days of PE microplastics exposure at different concentrations.*

Conclusion

Earthworms participate in the essential activity of increasing soil fertility, and their growth is affected due to the use of polyethylene microplastics in daily life.The present study concluded that the increased use of polythene microplastic led to decreased reproductive capacity and metabolic activity of *Eisenia fetida.*This indicated an evident decline in fertility of earthworms and an increase in stress-related enzymes, which could disrupt the normal metabolism, indicating the toxicological effects of polythene microplastic on *E. fetida*. Further, histological studies, along with proteomic and transcriptomic analysis are suggested, which can provide complete insight into the harmful effects of microplastics on different organs of earthworms.

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Conflict of interest

The authors declare that they have no conflict of interest.

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