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Isolation and Characterization of Microplastics from Soil Samples and its Toxicological Consequences on *Eudrilus eugeniae*: A Comprehensive Study

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ABSTRACT:
Soil is an important sink of microplastics (MPs), but their effect on soil organisms needs to be better studied. The presence of MPs in soil has an adverse impact on the environment. In the present study, we characterized the MPs present in the soil using scanning electron microscopy and Fourier-transform infrared spectroscopy. After characterization, the earthworm *Eudrilus eugeniae* was exposed to different concentrations of polythene MPs ranging from 200 mg/kg to 1000 mg/kg. The toxic effects of MPs on earthworm were determined by studying the biochemical enzymes, mortality and growth of earthworms. It was observed that there was an increase in mortality and a decrease in the weight of earthworms after exposure to higher concentrations of MPs and for a longer duration (35-56 days). There was also an alteration in metabolic profile also, leading to alteration in carbohydrate, lipid and protein content. This study aims to provide information on the toxicological effect of MPs on *E. eugeniae*, a commonly less studied earthworm species but of vast importance in vermicomposting.

Keywords:
Eudrilus eugeniae; Microplastics; Earthworms; Toxicity; FTIR; SEM

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INTRODUCTION

The durability, lightweight, and high plasticity have made the extensive use of plastics in different fields. However, the problem arises when the proper recycling of plastic waste doesn't occur, which leads to the accumulation of large amounts of environmental pollutants (Wong, Lee, Tang, & Yap, 2020). The natural degradation of plastic by UV radiation, physical abrasion, oxidation and biodegradation leads to smaller plastic fragments of <5 mm in size, which are known as microplastics (MPs) (Chen et al., 2022). The small size, large surface area and superior hydrophobicity of MPs make them suitable vectors for other contaminants as well. MPs' chemical stability and non-biodegradation lead to their accumulation in the environment (Li et al., 2021).

There are multiple pathways through which the MPs can enter the environment, viz., aerial deposition, soil conditioners, wastewater irrigation, plastic mulch and application of sewage sludge. MPs alter the chemical and physical properties of soil, which affect the soil microorganisms and terrestrial ecosystem (Wang, Ge, Yu, & Li, 2020). The MPs are transported to the soil profile due to burrowing, adhesion, excretion and death of earthworms (Rillig, Ziersch, & Hempel, 2017). The gut of earthworms can fragment MPs, but the higher concentration of MPs becomes toxic. The increased concentration of MPs in the soil can affect earthworms by reducing their body growth, decreasing enzyme activity, oxidative stress and reducing cocoon production (Sobhani, Panneerselvan, Fang, Naidu, & Megharaj, 2022).

Studies related to the determination of MPs toxicity are mostly investigated in earthworm species of *Eisenia* and *Lumbricus* with little or no emphasis on *Eudrilus*. *Eudrilus* plays a vital role in cellulose decomposition and therefore its role is essential for sustainable agriculture. They have shown promising results for the decomposition of kitchen waste along with microorganisms (Miglani & Bisht, 2019). Das and Deka in 2021 showed that a combination of *Eudrilus eugeniae* and microorganisms led to faster and more efficient decomposition of kitchen waste than individual treatments (Das & Deka, 2021). The presence of *E. eugeniae* during vermicomposting increases fungal growth, which leads to enhanced cellulolytic activity. Also, the vermicast contains a higher amount of bacterial population than the surrounding soil, which makes the soil rich in nutrients (Khobragade & More, 2016). Balachandar and colleagues in 2020 carried out the decomposition of cow dung and green manure using *E. eugeniae*. They observed enhanced growth and reproduction of earthworms and nutrient-enriched vermicompost production (Balachandar et al., 2020).

Similarly, Pandit and co-workers used paddy straw, vegetable waste, leaf litter, maize stover, and temple waste flowers for vermicomposting. They also observed microbial richness and higher enzyme activities in vermicompost (Pandit, Sethi, Pattanayak, & Nayak, 2020). Therefore, *E. eugeniae* in agriculture and waste valorization make their usage imperative.

But the use of plastic mulch in agriculture and other biotic and abiotic factors have increased MPs concentration in soil (Salama & Geyer, 2023). The dump sites in Asian countries contain 2600-3500 pieces/kg of dry soil (Tun et al., 2022). This total amount of MPs in agricultural soil of Europe and North America varies from 44,000-430,000 tons (Dissanayake et al., 2022). Therefore, the assessment of the impact of MPs on growth and reproduction is necessary for understanding their behaviour under high MPs toxicity. In the present study, the isolation and characterization of MPs present in the soil is done and the toxicity effect of MPs on the earthworm *E. eugeniae* is evaluated to understand their behaviour under such conditions. This study serves as the foundation for in-depth studies of metabolic and physiological shifts in earthworms during exposure to MPs.

MATERIALS AND METHODS

Microplastic and chemicals

The study obtained microplastic polyethylene (MPE) with a particle size of 125 µm from Sigma-Aldrich in the United States. Additionally, Sigma-Aldrich provided the acid and sugar standards, as well as the enzyme substrate. Sodium hydroxide, calcium carbonate, and sodium carbonate were procured from Merck in Darmstadt, Germany. All chemicals utilized in this investigation were of the highest commercially available purity. The MPs from various agricultural and soil samples were extracted by following the density separation procedure, as described elsewhere (D. He et al., 2018).

Characterization of extracted MPs using FTIR and SEM

The surface architecture and functional groups of the microplastic were studied using SEM (scanning electron microscopy) and ATR-FTIR (Attenuated total reflection-Fourier transform infrared spectroscopy), respectively. Morphological characterization of the particles was performed using a scanning electron microscope (Zeiss EVO-50) at 15 kV. Samples were fixed on double-sided adhesive carbon tabs on metal stubs (Saini, Kuhad, & Sharma, 2023). Further, samples were sputter-coated with a thin film in an MCM-100P gold coating unit with an average 10 nm coating thickness and dried under vacuum conditions. Samples were photographed using an EmCrafts virtuoso v1.1 operated and at a working distance of 10 mm. The SEM photographs were taken at various magnifications.

The IR spectra of microplastic were recorded between 500 cm^{-1} to 4000 cm^{-1} with a spectral resolution of 4 cm^{-1} and averaged over 50 scan. ATR-FTIR was conducted using a Bruker-Alpha ATR system, Germany. The microplastic were characterized and identified by comparing FTIR absorbance spectra of the particles to those in a polymer reference library (Tiwari, Rathod, Ajmal, Bhangare, & Sahu, 2019).

Selection and collection of animal model

E. eugeniae, a commonly chosen earthworm species for vermicomposting and toxicity studies, is favoured due to its ease of cultivation, low maintenance requirements, and short life cycle, which proves beneficial for researching the effects of toxins on different life stages. The worms were gathered from a wooden container filled with soil and organic cow manure. To accurately identify and classify the worms, the team employed online taxonomic resources and a digital library, occasionally resorting to dissection as needed (Thakur & Yadav, 2018).

Preparation of bedding material

Tropical artificial soil was employed to prevent the potential presence of MPs particles, *E. eugeniae*, and their cocoons in the soil. This internationally accredited method for evaluating pollutant toxicology follows OECD guidelines (207). The artificial soil was created by blending coconut peat, industrial soil, and kaolinite clay. Each component was individually air-dried at room temperature and then combined in a weight-to-weight ratio of 7:2.5:0.5. Milli-Q water and calcium carbonate were introduced to control soil moisture and maintain a pH level of 6.0 ± 0.5. The artificial soil was designed to have a water-holding capacity of 50 %.

Exposure of earthworms to extracted MPs

For the experiments, healthy adult earthworms aged 2-3 months, weighing between 250 mg to 350 mg, and displaying a clitellum were carefully selected. To acclimatize them to the experimental conditions, the earthworms were incubated in the same artificial soil for one day before exposure to MPs.

To assess the toxicological effects of MPs, the avoidance test was conducted with five different concentrations: 200, 400, 600, 800, and 1000 mg/kg of dry soil. The experiments were carried out in multiple replicates, with 4 replicates for each concentration. In each replicate, ten well-clitellated adult worms were placed into a plastic tray containing 1000 g of artificial soil (dry weight) with varying MPs concentrations. These trays were covered with gauze and incubated at a controlled temperature of 20 ± 2 °C for 56 days. The earthworms were regularly provided with feed throughout the exposure period, and soil moisture was carefully maintained. The earthworms were fed with 5.0 g of cow manure distributed on the soil surface once a week.

At regular intervals of 7 days during the exposure period, earthworms were collected, rinsed with Milli-Q water, and depurated for 24 hours before undergoing further analyses. These experiments were conducted with multiple replicates, and the differences between the initial and final body weights of the worms were recorded. The growth rate of the earthworms was evaluated by measuring the relative gain and loss of weight.

Biochemical assays

E. eugeniae exposed to varying concentrations of MPs were subjected to analysis to determine their total protein, carbohydrate, and lipid content. To estimate protein levels, earthworms exposed to MPs for 28 days were rinsed with Milli-Q water and subsequently divided into three segments: preclitellar, clitellar, and post-clitellar regions. These tissue segments were homogenized in a pre-chilled mortar and pestle using a potassium phosphate buffer (50 mM; in a ratio of 1:8 w/v; pH-7.0). The resulting homogenate was then centrifugated at 4 °C and 9,600g for 10 minutes, and the supernatant was collected for protein assays. The protein concentration was determined using a spectrophotometric method following the procedure previously described (Lowry, Rosebrough, Farr, & Randall, 1951).

The quantification of total carbohydrate content was done by the addition of phenol 5.0 % (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 & 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 (6,000 rpm, 8 min). To this supernatant of the remaining lipid extract, 0.5 ml of H_2SO_4 was added and then incubated at 20 °C for 15 min. The total lipid matter was measured by taking the absorbance at 400 nm using Tripalmitine as standard.

RESULT AND DISCUSSION

FTIR and surface analysis of extracted MPs

The composition of sample was confirmed by using the FTIR analysis. There was a strong absorption band at 2848 and 2915 cm^{-1}, which shows symmetric and asymmetric stretching vibrations of C-H. Also, the CH_2 bending and CH_2 rocking were confirmed by peaks at 1465 cm^{-1} and 718 cm^{-1}, respectively (Tiwari et al., 2019). The spectral observations confirmed the presence of polyethylene microplastic in the sample (Fig. 1). The results were in agreement to the results as observed by Rozman and his colleagues for Polyethylene (Rozman, Filker, & Kalčíková, 2023; Rozman et al., 2021). Further the surface analysis of MPs was done using the SEM analysis which showed the scattered and irregularly shaped particles. The MPs particles were flat (Fig. 2).

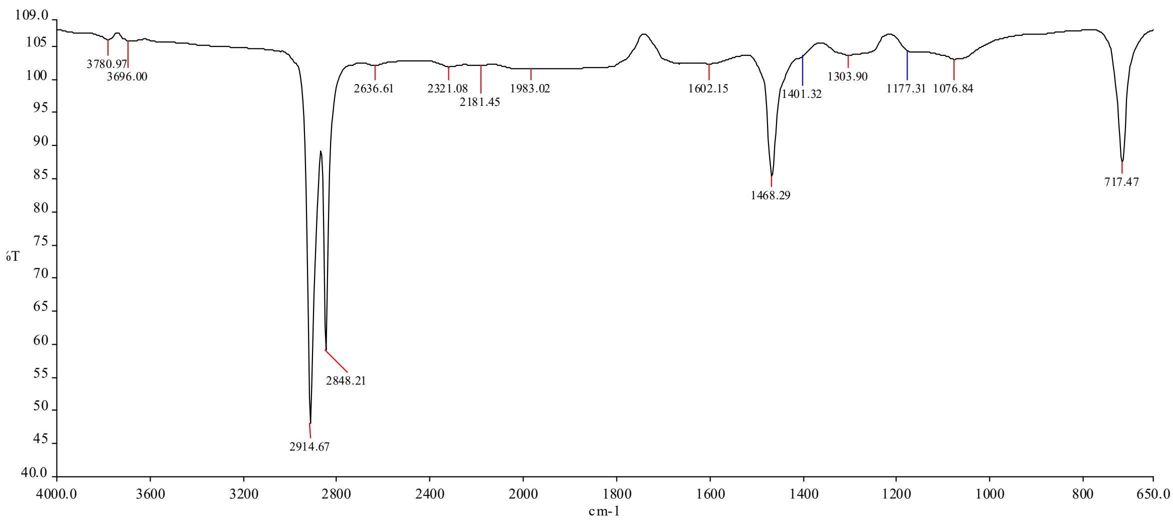

Figure 1: Physico-chemical characterization of microplastic using ATR-FTIR analysis. IR spectra of microplastic particles

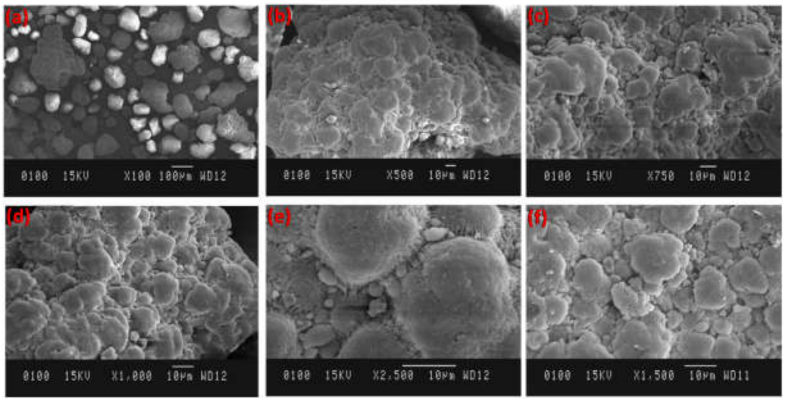

Figure 2: SEM microphotographs of microplastic (a-f) taken at different magnification at 15 kV and 10 mm working distance

Growth and mortality of *E. eugeniae*

The effect on body weight was measured after exposing the earthworms to the soil amended with MPs (Fig. 3). There was no significant decrease in the body weight at lower concentrations of MPs and up to 14 days of exposure. The results were similar to the results obtained by Chan and co-workers in 2023. They also observed an insignificant change in weight with a p-value of 0.712 (Chan, Medriano, & Bae, 2023). But the body weight decreased significantly (p value<0.05) athigh concentration i.e. 800-1000 mg/kg from 35th to 56th day. Cao and co-workers observed similar results in 2017, where a high concentration of MPE inhibited the growth of earthworms, leading to a decrease of 29.8 % in body weight (Cao, Wang, Luo, Liu, & Zheng, 2017). Zhang and colleagues in 2022 also reported a significant decrease in body weight by application of PE and ZnO particles (Zhang et al., 2022).

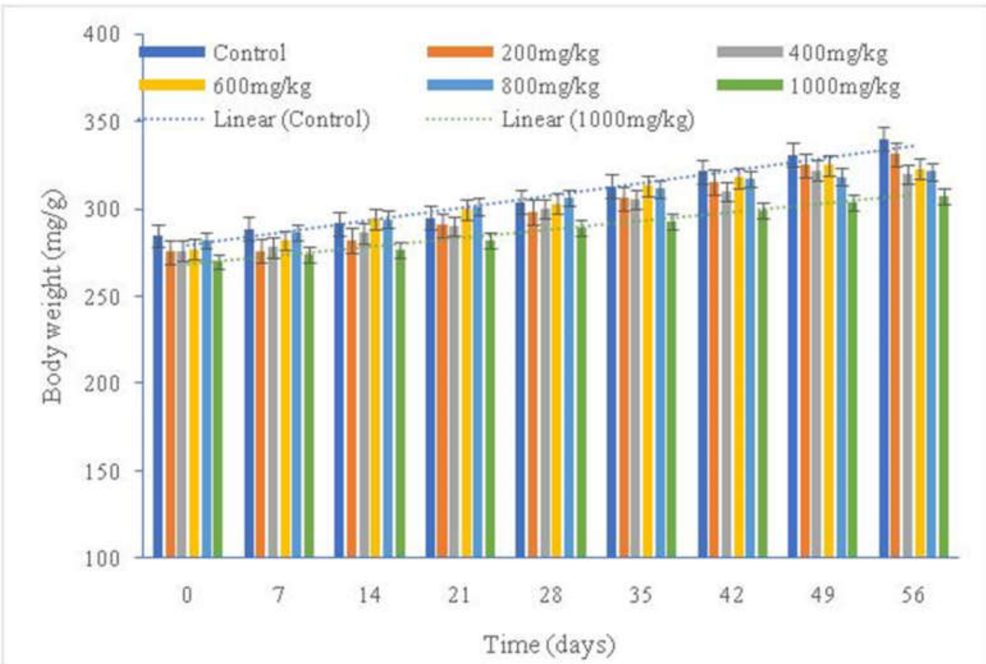

Figure 3: Body weight of *E. eugeniae* after exposure to a control soil and soil amended with of MPs at different concentrations (200-1000 mg/kg of artificial soil) for 56 days

The result of the present study also favours the earlier published data, which indicates the weight loss of earthworms during exposure to MPE. 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 to the digestive tract, which further leads to a decrease in weight (Ding et al., 2021). After a long exposure for 56 days, the weight loss was more significant, indicating the persistent inhibition by MPs in the growth of *E. eugeniae*.

At harvest on day 35, the number of cocoons of earthworms started decreasing with increasing concentration of MPs (Fig. 4). The highest decrease in cocoon numbers was observed at 400 mg/kg concentration on the 35th day and at 800 mg/kg on the 56th day.

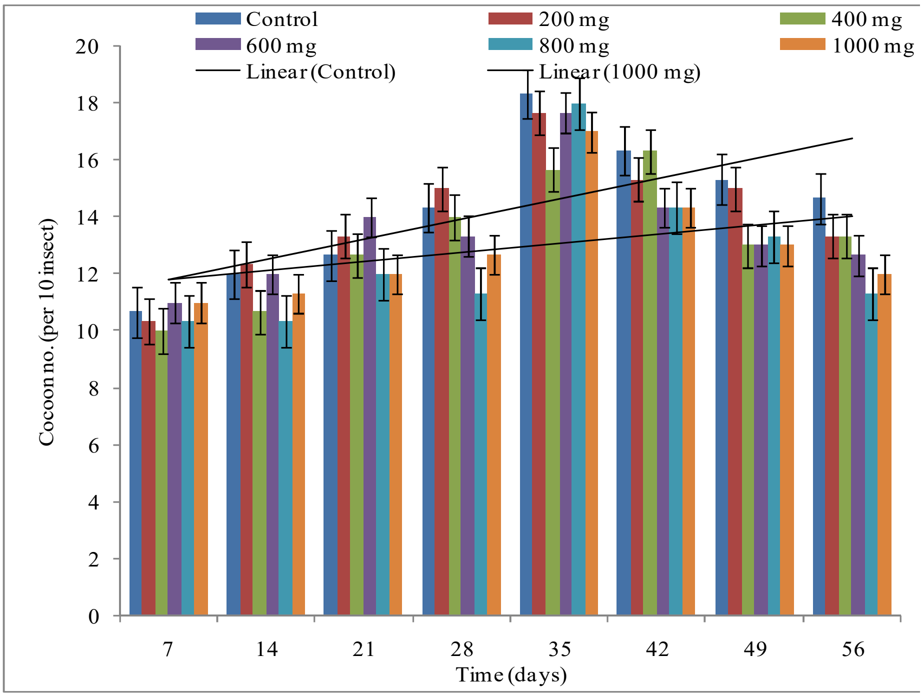

Figure 4: Number of cocoons by adult *E. eugeniae* after exposure to a control soil and soil amended with of MPs at different concentrations (200-1000 mg/kg of artificial soil) for 56 days

A similar result was observed by Ding and colleagues in 2021 when they used different concentrations of MPs. They also observed that at lower concentrations of MPs, there was no significant decrease in the amount of cocoons, but there was a decrease at higher concentrations (Ding et al., 2021). The present study also reports similar results at higher concentrations of MPE.

Effect of MPs on carbohydrate, lipid, and protein concentration

Further, the effect of MPs on the carbohydrate, lipid, and protein concentrations in *E. eugeniae* was studied. It was observed that the carbohydrates and lipid concentration was decreased at harvesting days 7, 14, 21, and 28. The results depicted in Fig. 5 and Fig. 6 revealed a steady decrease in the carbohydrates and lipids content when the MPs concentration was increased from 200 mg/kg to 1000 mg/kg of artificial soil. The maximum reduction in the carbohydrates content i.e., 14.1 % was observed after 28th days of MPs exposure at 1000 mg/kg of artificial soil (Fig. 5). Likewise, the maximum reduction in the lipids content i.e., 25.44 % was observed after 28th days of MPs exposure at 1000 mg/kg of artificial soil (Fig. 6).

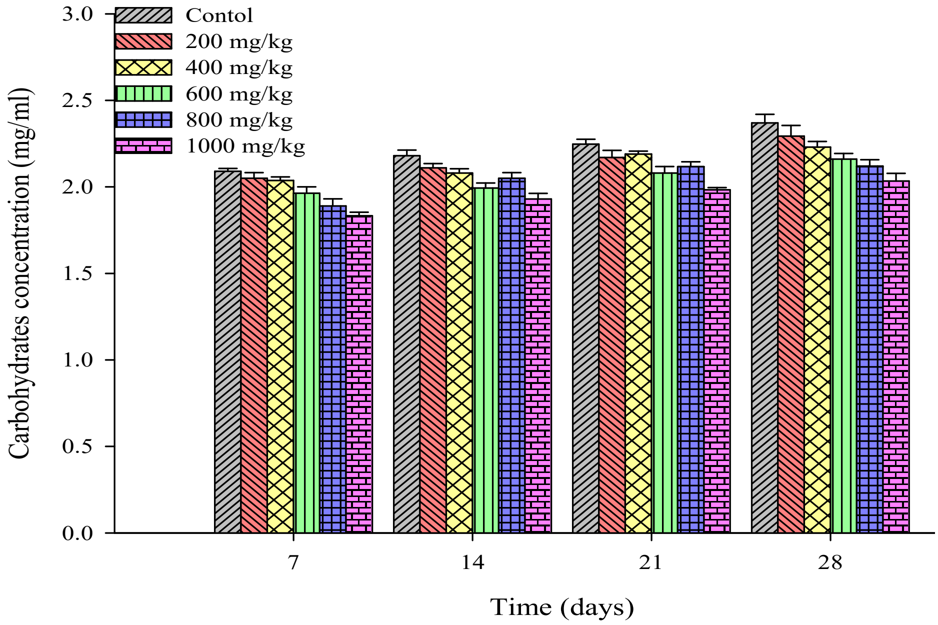

Figure 5: **Carbohydrates concentration in *E. eugeniae* after 28 days of MPs exposure at different concentrations (200-1000 mg/kg of artificial soil)**

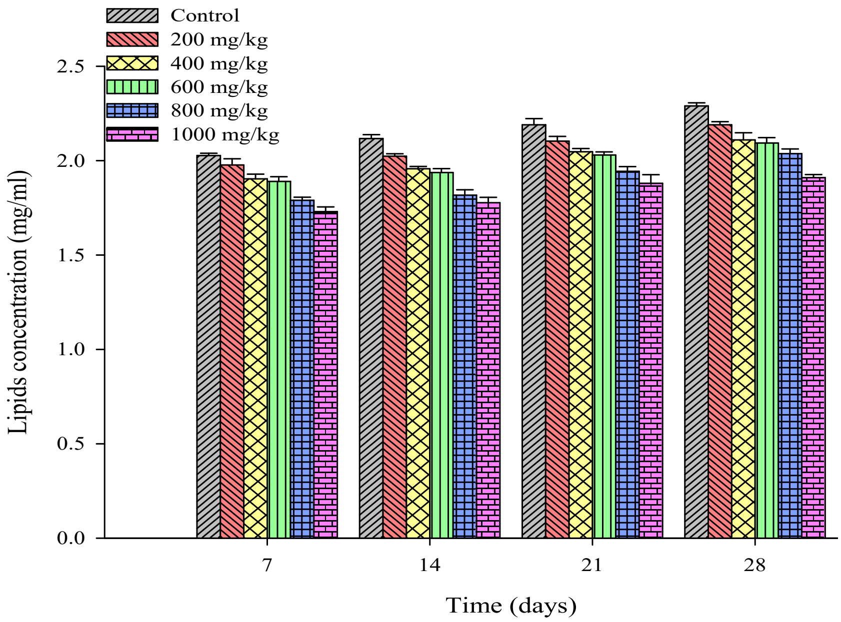

Figure 6: Lipids concentration in *E. eugeniae e*after 28 days of microplastics exposure at different concentrations

Similarly, there was a decrease in protein concentration in pre-clitellum, clitellum, and post-clitellum regions. A positive relation was observed between the MPs and total protein concentration (Fig. 7). With the increase in the MPs up to 1000 mg/kg, a reduction in the concentration of protein was observed. During the time course study, similar trends of protein reduction were achieved (Fig. 7a-d). He and colleagues 2021 observed, with the help of lipidomics analysis, that there was perturbation in lipid metabolism after exposure to microplastics in *Eisenia fetida* (He et al., 2021). In a similar study, Griffith and coworkers i019 observed da ecrease in levels of maltose and lactate ,which indicates tadisruption in energy metabolism (Griffith, Thai, & Larive, 2019). Chan and colleagues studied the metabolomics of *E. eugeniae* and observed that there was a change in the energy metabolism. They observed dysregulation in lipid metabolism and protein integrity. The transport proteins were altered after exposure to MPE (Chan et al., 2023). These studies indicate earthworms' reduced carbohydrate and lipid levels due to exposure to microplastics. The decrease in protein levels is associated with a disturbance of osmoregulation (Tang et al., 2023). Transcriptomic and metabolomics analysis in several studies has shown the protein concentration was disturbed in earthworms due to oxidative stress damage, and arachidonic acid metabolism and steroid biosynthesis were also disturbed. The muscle proteins are also broken down to protect from reactive oxygen species (Chen et al., 2022).

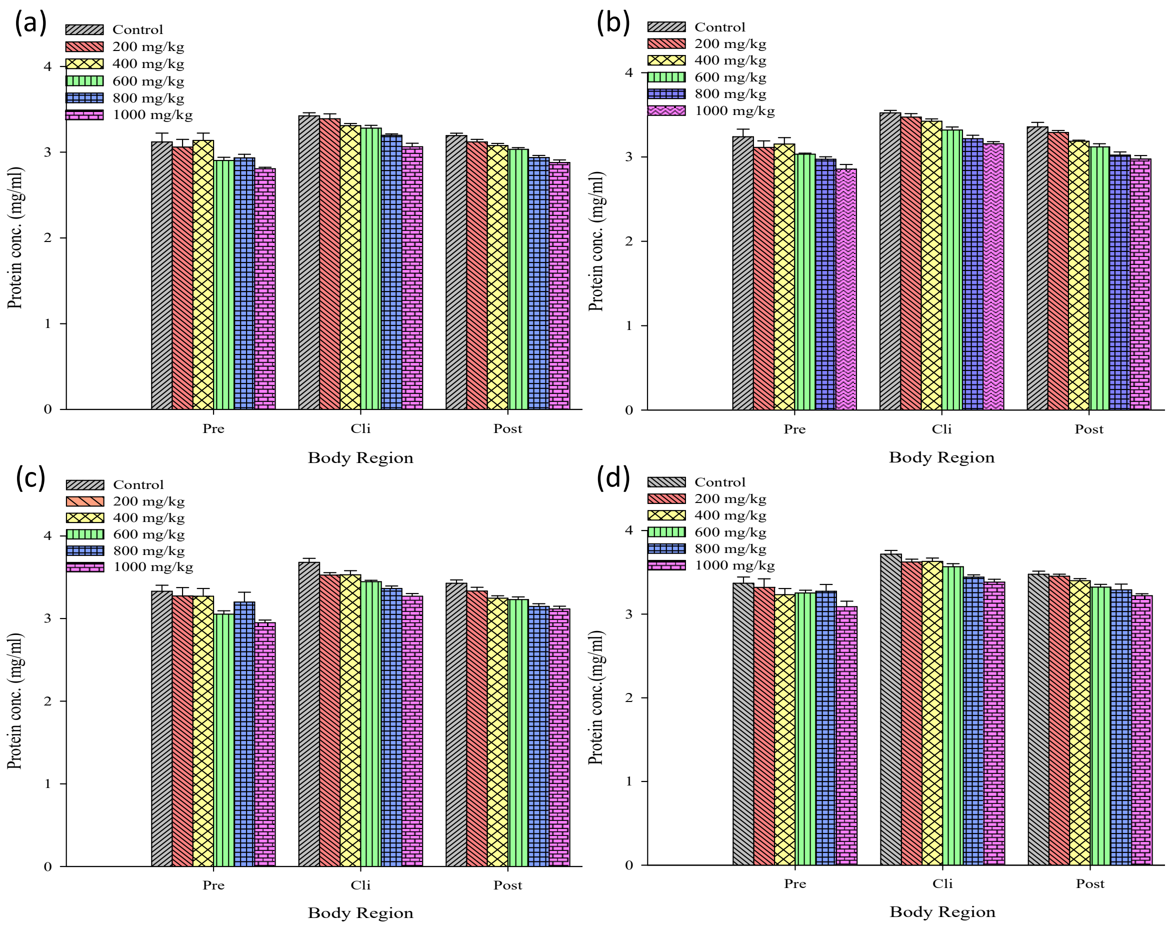

Figure 7: Effect of microplastic on protein concentration in *E. eugeniae* after 28 days of exposure. (a) Protein concentration *E. eugeniae* after 7th day, (b) 14th day, (c) 21st day, and 28th day

CONCLUSIONS

Earthworms participate in the essential activity of increasing soil fertility. However, the increased use of polythene microplastic has led to a decrease in the reproductive capacity and metabolic activity of earthworms. The present analysis indicated that there was an evident decline in the fertility of earthworms along with disruption of lipid and carbohydrate metabolism. There was also an alteration in transport-related proteins, which could disrupt normal metabolism. This study indicates the toxicological effects of MPs on *E. eugeniae*. Further histological studies, along with proteomic and transcriptomic analysis, canprovide complete insight into the harmful effects caused by different organs of earthworms.

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Credit author statement:
Garima Sheoran: Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualization; Writing - original draft.
Vineeta Shukla: Conceptualization; Formal analysis; Funding acquisition; Investigation; Project administration; Resources; Supervision; Validation; Visualization; Writing - review & editing.

Data availability:
All data generated or analyzed during this study are included in this published article

Conflict of interest:
The authors declare that they have no conflict of interest with contents of this article

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