Effect of Microplastic Exposure on Earthworms Eisenia Fetida and Eudrilus Eugeniae

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Abstract: ***The present study was conducted to assess the effect of different concentrations of microplastics MPs-110µg, MPs-120µg, MPs-130µg, MPs-140µg on the earthworms, Eisenia fetida and Eudrilus eugeniae for 30 days. It was observed that there was a significant decrease in the body weight of the earthworms when treated with different concentrations of microplastics. There was a significant decrease in the reproduction rate such as decrease in cocoon production as well as decrease in the number of juveniles. Morphological changes like coiling of the body and tail were also observed. Furthermore, the histopathological changes observed were thinning in the muscular layer and epidermis layer. There was damage to the cuticle. In conclusion, the study suggests that microplastics may have a negative effect on the growth and reproductive abilities of earthworms and its survival itself.***

Keywords: Eisenia fetida and Eudrilus eugeniae, microplastics, morphological, histopathological change and reproduction rate

1. Introduction

The soil ecosystem plays a role in providing services like carbon storage, biogeochemical cycling and supporting biodiversity. Recent studies have documented the increasing presence of microplastics in soil, which can significantly impact both the diversity and functioning of soil ecosystems (de Souza Machado et al., 2019; Helmberger et al., 2020; Wang et al., 2019; Zhou et al., 2020). These microplastics can find their way into soil through pathways, such as mulching materials, production processes involving microplastics, breakdown of plastic materials and sludge from wastewater treatment. Until years little attention has been given to sources of microplastics and their effects on terrestrial microorganisms (de Souza Machado et al., 2018; Rillig et al., 2017a).There are two types of MP; primary MP intentionally produced for use in products and secondary MP which results from the breakdown of larger plastic products due to factors like UV radiation, mechanical stress or microbial biodegradation (Meides et al., 2021; Sander, 2019; Zhang et al., 2021). Depending on the properties of the plastic product manufactured by companies these degradation processes lead to the formation of MP, with different polymer types, sizes and shapes.

Nowadays soil contamination caused by MP (Büks and Kaupenjohann 2020; Corradini et al., 2019; Sommer et al., 2018; Weithmann et al., 2018) is widespread. Research suggests that 32% of all plastics produced could potentially remain in ecosystems with agricultural soils harboring even larger amounts of MP compared to the oceanic basins (Nizzetto et al., 2016; de Souza Machado et al., 2018). The gut microbiome is crucial for an organism's wellbeing and nutritional needs. It provides short chain fatty acids through fermentation, which can have effects on the host. However, changes in the gut microbiome caused by MP (microplastics) exposure may have consequences for soil organisms. Earthworms in particular might be highly affected by these alterations.

Earthworms are the invertebrates found in land- based ecosystems making up to 80% of the soil biomass (Wen et al., 2020). They have a role in breaking down matter facilitating microfloral activities and promoting nutrient mineralization (Gao et al., 2015). Due to their skin and digestive system earthworms come into direct contact with environmental chemicals. As a result they exhibit sensitivity, towards substances (Li et al., 2018).

Earthworms play multiple roles in maintaining soil health, serving as bio fertilizers, composting agents, moisture regulators, soil aerators, and biological facilitators. Due to their capacity to accumulate metals, pesticides, and other organic chemicals, they are often used as indicators for assessing soil pollution levels. This study aimed to investigate the impact of microplastics (MPs) on various factors including body weight, reproductive characteristics, mortality rates, and histopathological effects in two earthworm species: *Eisenia fetida* and *Eudrilus eugeniae*.

The comparative study of the effect of Mps of *Eisenia fetida* and *Eudrilus eugeniae.*A study on this aspect has not done so far,and therefore the present work aimed to assess the effect of Mps in different concentration (110 µg, 120 µg, 130 µg, and 140 µg) on finding the changes in body weight, investigating morphological and histopathological changes, and analyzing reproductive parameters such as cocoon production rates and the number of juveniles generated.

2. Materials and Methods

For this study, earthworms belonging to the species *Eisenia fetida* and *Eudrilus eugeniae* were selected. They were sourced from vermibeds located at the Vermicomposting unit of Patna Women's College, Patna, and then cultured for a period of 30 days in three separate pots. The microplastic material used in the experiment was obtained from the Central Research Laboratory (CRL) of Patna Women's College. Various chemicals were employed in the study, including Formalin N/10, alcohol, xylene, eosin, DPX, and paraffin wax. The experimental procedures were carried out at the CRL lab of Patna Women's College. A total of twelve earthen pots were allocated for the experiment, divided evenly between two distinct experimental setups, with six pots dedicated to each setup. The soil used in the experiment was carefully sieved to ensure a fine consistency, and the cow dung utilized was sun-dried to eliminate any urine content. A standardized mixture comprising dry soil, cow dung, and vegetable peels in a ratio of 2:1:1 was prepared and distributed among all twelve experimental pots. These setups were specifically arranged to evaluate the impact of micro plastics on the behavior and physiology of earthworms. Two sets of pots were established for this purpose, allowing for detailed observation and analysis throughout the duration of the experiment.

Set -1 (*Eisenia fetida*)

a) Control 1 and 2 (100g soil + 50g cow dung + 50g kitchen waste +earthworm (10 each).
b) Treated – 1- 4 (100g Soil+ 50g cow dung + 50g kitchen waste + microplastic (110 µg,120 µg 130 µg, 14 µg) + earthworm (10 each)

Set – 2 - (*Eudrilus eugeniae*)

a) Control 1 and 2 (100g soil + 50g cow dung + 50g kitchen waste +earthworm 10 each
b) Treated – 1- 4 (100g Soil+ 50g cow dung + 50g kitchen waste + microplastic (110µg,120 µg, 130 µg , 140 µg) earthworm.

Throughout the experimental duration, diligent efforts were made to sustain consistent moisture levels within each setup, ensuring optimal conditions for the earthworms' habitat and behavior. Evaluation of the impact of microplastics on the earthworms occurred at two specific intervals, on the 15th and 30th day of the experiment. This assessment involved meticulous manual counting through hand sorting to determine factors such as mortality rates, reproductive changes, and the quantification of cocoons and juvenile earthworms.

Subsequently, following a 30-day exposure period to microplastics, histopathological slides were prepared, comparing samples from control (untreated) and treated (microplastic-exposed) earthworms. This step aimed to expose any histological alterations or abnormalities resulting from the earthworms' exposure to microplastics, providing crucial insights into potential morphological changes or pathological effects induced by the microplastic substances.

Histopathological test:

After completion of the treatment, earthworms from each pot of MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg concentrations were taken and were washed with distilled water and kept to remove the gut content. Then they were cut into small pieces and the tissue was fixed using a suitable fixative to preserve its structure and to prevent degradation. The tissue was then dehydrated through a series of alcohol solutions, cleared in a suitable clearing agent and infiltrated with the help of supporting medium such as paraffin wax. The tissue was then embedded in a mold filled with molten paraffin wax and was allowed to solidify and hold tissue in its proper place. The embedded tissue blocks were sectioned into 5 micrometers thick by using microtome, the sections were then carefully transferred to a water bath ro remove wrinkles and picked up onto glass slides and allowed to dry, the dried sections were stained using the various histological stains to highlight the structures. A cover slip was placed over the stained sections using the mounting medium to protect the tissue and to preserve the stain. It was examined under the research microscope and photographed. Initial and final body weights of earthworms were compared with Student's t-test. The results are presented as Mean ± S.E. Calculations were performed with the Graph using Microsoft Excel. The criterion for statistical significance was set as $P<0.05$

3. Results and Discussion

The present study was carried out to determine the body weight loss, reproductive aspects, mortality rate, morphological and histopathological changes of *Eisenia fetida* and *Eudrilus eugeniae* which were exposed to different concentrations of MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg.

After 30 days of exposure to microplastic in different concentration MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg the worms from each pot were separately, washed with distilled water and, counted the number of dead and living worms and weighed the live worms. Worms were considered dead when they were either missing or did not move any longer when handled.

There was a weight loss in earthworms in each concentration MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs- 140 µg of Microplastic when the mean body weight of earthworms after 30 days was compared with mean body weight of worms at day 1. There was an increase in the body weight of the control group. (Table-1&2). It has been reported to be more sensitive to body weight changes in earthworms than mortality in toxicity assessments of pesticides (Zhou et al., 2006). There was a significant decrease in the body weight of *Eisenia fetida* treated 1- 6%, treated 2- 35%, treated 3-45% and treated 4-55% (Graph-1). In *Eudrilus eugeniae* treated 1-15%, treated 2-22%, treated 3 -27% and treated 4-51% (Graph-2). The weight loss of *Eisenia fetida* was comparatively decreased that of *Eudrilus eugeniae* (Table-7).

The result of the experiment showed that there was no mortality in the control group throughout the study period. MPs are destructive for earthworms because they cause intestinal disruption or obstruction and oxidative stress, as well as affect food accessibility and availability (Rodriguez-Seijo et al., 2017; Boots et al., 2019; Chen et al., 2020). The mortality in the treated groups differed significantly at each interval (Table 3&4). The percentage of mortality in *Eisenia fetida* treated with MPs-110 µg and MPs- 120 µg did not vary after 15 days and MPs-130 µg and MPs- 140 µg 10% mortality rate increased. The percentage of mortality in case of MPs-130 µg and MPs- 140 µg increased 20% after 30 days (Graph-3).

The percentage of mortality in *Eudrilus eugeniae* treated with MPs-110 µg, MPs-120 µg and MPs- 130 µg did not vary after 15 days and MPs- 140 µg 10% mortality rate increased. The percentage of mortality in cases of MPs-130 µg, 10% and MPs- 140 µg 20% increased after 30 days (Graph-4). The mortality rate of *Eisenia fetida* was comparatively increased that of *Eudrilus eugeniae* (Table-8).

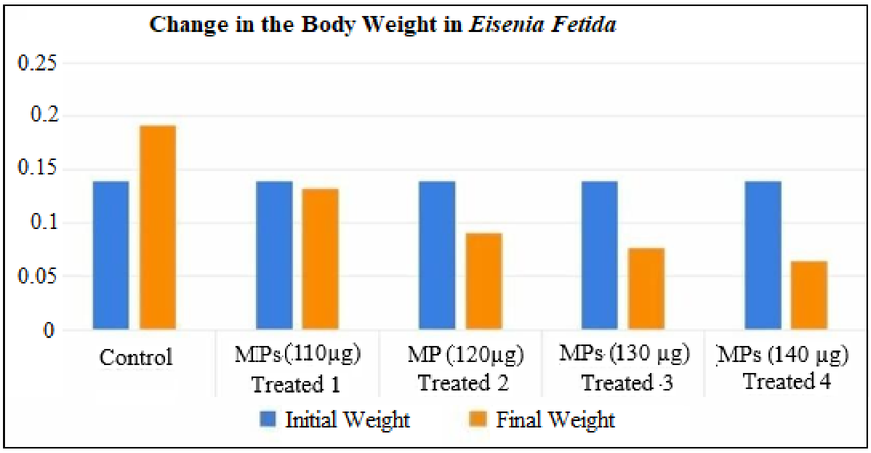
A significant effect of Microplastic on reproductive output was obtained in both *Eisenia fetida* and *Eudrilus eugeniae* (Zhou et al., 2006). It was observed there is no production of cocoons in the treated with different concentrations of MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg of microplastic compared to control on the 15th and 30th day. Same with the juveniles. (Table 5 & 6).

Studies show that the morphological and histological changes were outstanding when earthworms were exposed to different deadly metals and microplastics (Amaral et al., 2006). After 30 days of exposure, it was observed that there were morphological changes such as coiling of the body and tail (fig 1). Fragmentation at the posterior region was observed (fig 2). These changes were clearly observed in the earthworms treated with 130 µg of Microplastics for 30 days. Venkateswara Rao et al (2008) suggested that copious amounts of mucous secretion and fragmentation occurs due to tissue damage in the body wall by migration of pesticide to the contact surface.

Table 1: Change in the Body weight of the species *Eisenia fetida* when treated with microplastic in different concentrations of MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 30 days

	Control	Treated group-1 (MPs-110µg)	Treated group-2 (MPs-120µg)	Treated group-3 (MPs- 130 µg)	Treated group-4 (MPs-140 µg)
The initial weight of the earthworm (g)	0.1394±0.14	0.1393±0.14	0.1392±0.14	0.1390±0.14	0.1391±0.14
The Final weight of the earthworm (g)	0.1912±0.16	0.1320±0.13	0.09070±0.11	0.0768±0.10	0.0639±0.09

*The data are represented as Mean ±S.E, n = 10 Significant at $P < 0.05$

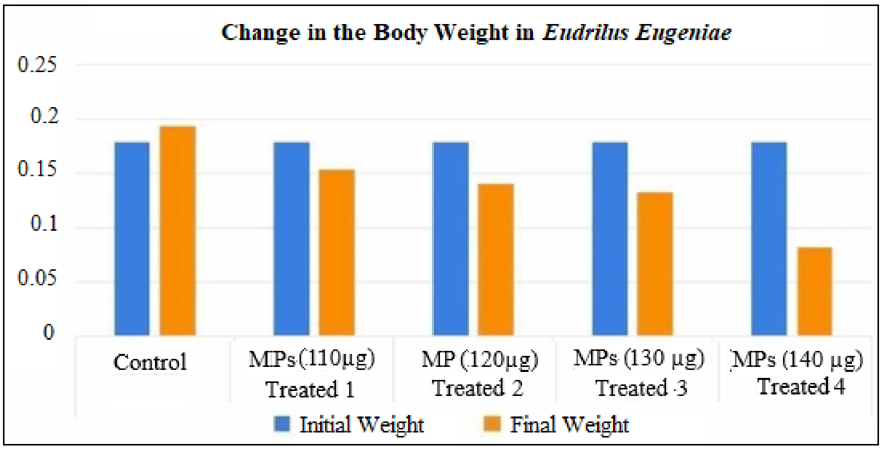


Graph 1: Effect on the weight of *Eisenia fetida* when treated with microplastic in different concentrations: MPs- 110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 30 days

Table 2: Change in the body weight of the species *Eudrilus eugeniae* when treated with microplastic in different concentrations MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 30 days

	Control	Treated group-1 (MPs-110µg)	Treated group-2 (MPs-120µg)	Treated group-3 (MPs-130µg)	Treated group-4 (MPs-140µg)
The initial weight of the earthworm (g)	0.1793±0.15	0.1792±0.15	0.1792±0.15	0.1791±0.15	0.1790±0.15
The Final weight of the earthworm (g)	0.1935±0.16	0.1537±0.14	0.1409±0.13	0.1325±0.13	0.08196±0.10

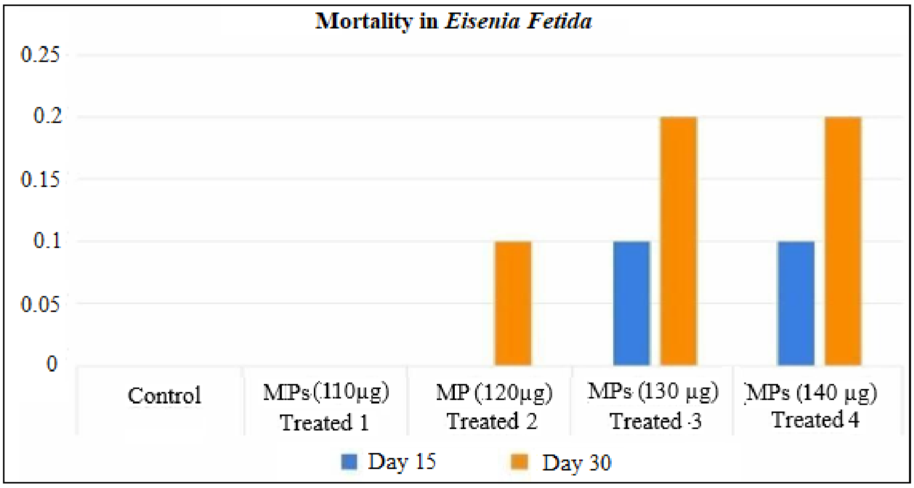
*The data are represented as Mean ±S.E, n = 10 Significant at $P < 0.05$



Graph 2: Effect on the weight of *Eudrilus eugeniae* when treated with microplastic in different concentrations: MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 30 days.

Table 3: Mortality of the species *Eisenia fetida* when treated with microplastic in different concentrations: MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 40 days

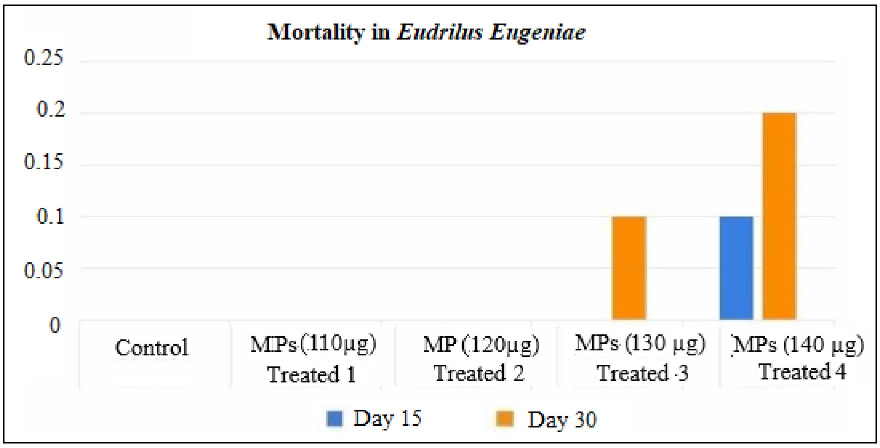
	Control	Treated group-1 (MPs-110µg)	Treated group-2 (MPs-120µg)	Treated group-3 (MPs-130µg)	Treated group-4 (MPs-140µg)
Duration of Treatment (15 days)	-	-	-	1	1
Duration of Treatment (30 days)	-	-	1	2	2



Graph 3: Effect on the Mortality of *Eisenia fetida* when treated with microplastic in different concentrations: MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 30 days.

Table 4: Mortality of the species *Eudrilus eugeniae* when treated with microplastic in different concentrations MP- 110 µg, MP-120 µg, MP-130 µg, MP-140 µg for 30 days

	Control	Treated group-1 (MPs-110µg)	Treated group-2 (MPs-120µg)	Treated group-3 (MPs-130µg)	Treated group-4 (MPs-140µg)
Duration (15 of Treatment days)	-	-	-	-	1
Duration (30 of Treatment days)	-	-	-	1	2



Graph 4: Effect on the mortality of *Eudrilus eugeniae* when treated with microplastic in different concentrations: MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 30 days

Table 5: Reproduction rate of the species *Eisenia fetida* when treated with microplastic in different concentrations: MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 30 days

Indications	Control	Treated group -1 (MPs-110 µg)	Treated group-2 (MPs-120 µg)	Treated group -3 (MPs-130 µg)	Treated group-4 (MPs-140 µg)
Reproduction					
Day – 1	-	-	-	-	-
Day -15	-	-	-	-	-
Day - 30	3	-	-	-	-
Cocoon – (30th day)	9	-	-	-	-
Juveniles (30th day)	3	-	-	-	-

Paper ID: SR26722123805 DOI: https://dx.doi.org/10.21275/SR26722123805

Table 6: Reproduction rate of the species *Eudrilus eugeniae* when treated with microplastic in different concentrations, MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 30 days

Indications	Control	Treated group-1 (MPs- 110µg)	Treated group-2 (MPs-120µg)	Treated group-4 (MPs-130µg)	Treated group-4 (MPs-140 µg)
Reproduction					
Day – 1	-	-	-	-	-
Day -15	-	-	-	-	-
Day - 30	4	-	-	-	-
Cocoon – (30th day)	12	-	-	-	-
Juveniles (30th day)	4	-	-	-	-

Table 7: Comparative study on Change in the Body weight of the species *Eisenia fetida* and *Eudrilus eugeniae* when treated with microplastic MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 30 days

		Control	Treated group-1 (MPs-110 µg)	Treated group- 2 (MPs-120 µg)	Treated group-3 (MPs-130 µg)	Treated group-4 (MPs-140 µg)
Initial	*Eisenia fetida*	0.1394±0.14	0.1393±0.14	0.1392±0.14	0.1390±0.14	0.1391±0.14
	Eudrilus eugeniae	0.1793±0.15	0.1792±0.15	0.1792±0.15	0.1791±0.15	0.1790±0.15
Final	*Eisenia fetida*	0.1912±0.16	0.1320±0.13	0.9070±0.11	0.0768±0.10	0.0639±0.09
	Eudrilus eugeniae	0.1935±0.16	0.1537±0.14	0.1409±0.13	0.1325±0.13	0.08196±0.10

*The data are represented as Mean ±S.E, n = 10 Significant at $P < 0.05$

Table 8: Comparative study on Reproduction and mortality rate in the species *Eisenia fetida* and *Eudrilus eugeniae* when treated with microplastic concentrations MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg for 30 days

		Control	Treated group-1 (MPs-110 µg)	Treated group- 2 (MPs-120 µg)	Treated group-3 (MPs-130 µg)	Treated group-4 (MPs-140 µg)
Cocoons	*Eisenia fetida*	12	-	-	-	-
	Eudrilus eugeniae	9	-	-	-	-
Juvenile	*Eisenia fetida*	4	-	-	-	-
	Eudrilus eugeniae	3	-	-	-	-
Mortality	*Eisenia fetida*	-	-	1	2	2
	Eudrilus eugeniae	-	-	-	1	2

Pictures showing morphological changes in earthworms treated with MP

Figure 1: Effect of MP (140 µg) on morphology of the earthworm *Eisenia fetida* after 30 days of exposure. Coiling of body (CB) and Tail (CT)

Paper ID: SR26722123805 DOI: https://dx.doi.org/10.21275/SR26722123805

Figure 2: Effect of MP (140 µg) on morphology of the earthworm *Eisenia fetida* after 30 days of exposure. Fragmentation at posterior region (FP)

Microphotographs of cross sections of earthworm *Eudrilus eugeniae* treated with different concentrations MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg of microplastics for 30 days

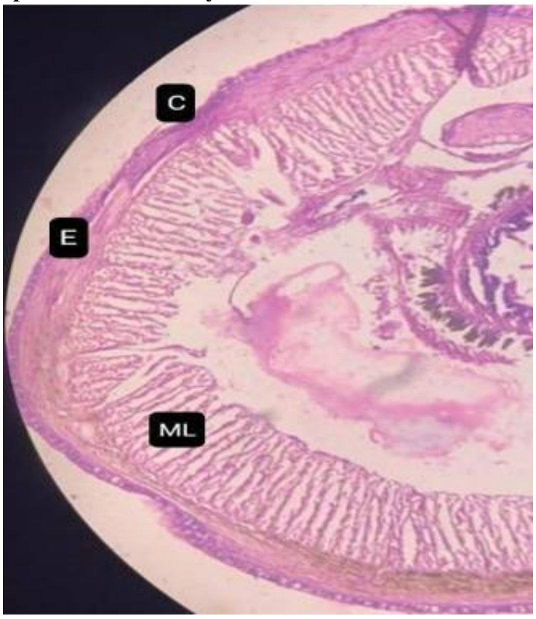


Figure 3: Microphotograph of intestinal region of *Eudrilus eugeniae* control group. It shows normal epidermis (E), cuticle (C) and muscular layer (ML). (H &E x 400)

Figure 4: Microphotograph of intestinal region of *Eudrilus eugeniae* treated group I (MPs- 110 µg). It shows change in the muscular layer and thinning of the epidermis layer. E- Epidermis, C- Cuticle, and ML- Muscular layer. (H &E x 400)

International Journal of Science and Research (IJSR)
ISSN: 2319-7064
Impact Factor 2025: 7.089

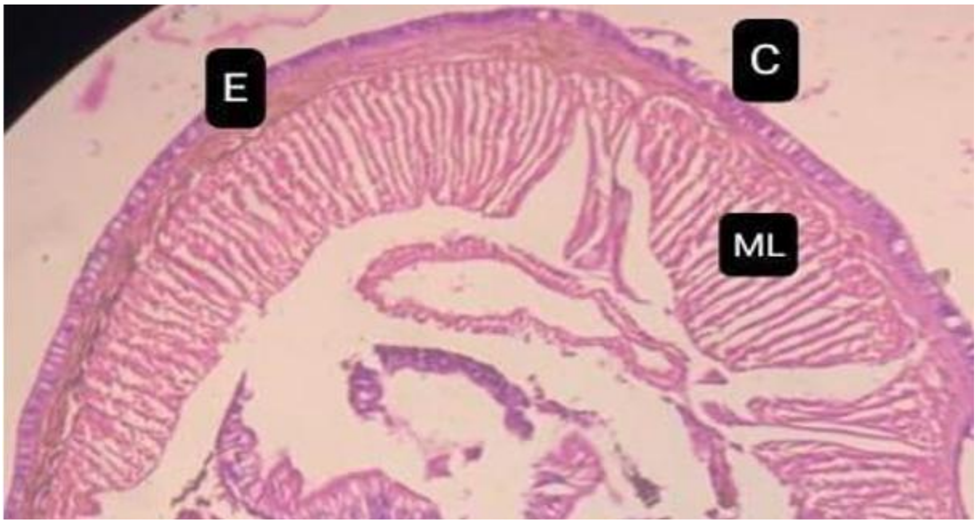


Figure 5: Microphotograph of intestinal region of *Eudrilus eugeniae* treated group II (MPs- 120 µg). It shows change in the muscular layer and thinning of the epidermis layer. The areas of inflammation are seen. E-Epidermis, C- Cuticle, and ML-Muscular layer. (H &E x 400)

Figure 6: Microphotograph of intestinal region of *Eudrilus eugeniae* treated group III (MPs- 130 µg). It shows thinning of the epidermis layer and distortion of the muscular layer. E-Epidermis, C- Cuticle, and ML- Muscular layer. (H &E x 400)

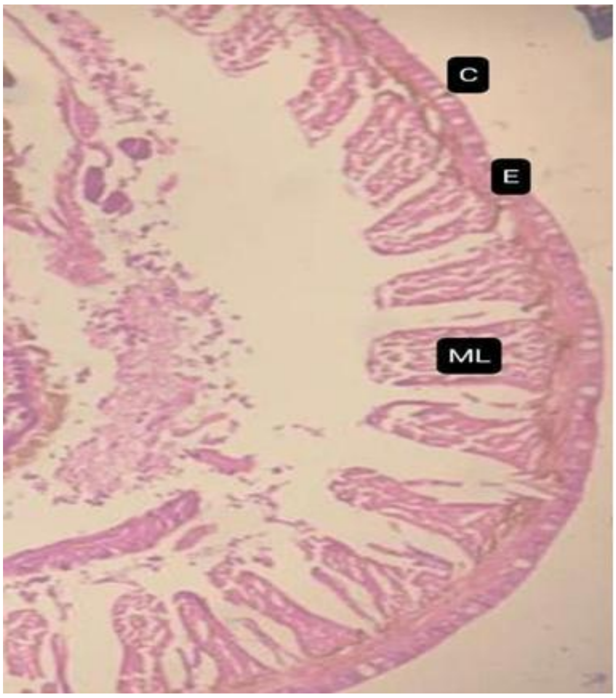


Figure 7: Microphotograph of intestinal region of *Eudrilus eugeniae* treated group IV (MPs- 140 µg). It shows change in the muscular layer, damage of cuticle and thinning of epidermis layer. E-Epidermis, C- Cuticle, and ML-Muscular layer. (H &E x 400)

Microphotographs of cross sections of earthworm *Eisenia fetida* treated with different concentrations MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg of microplastics for 30 days

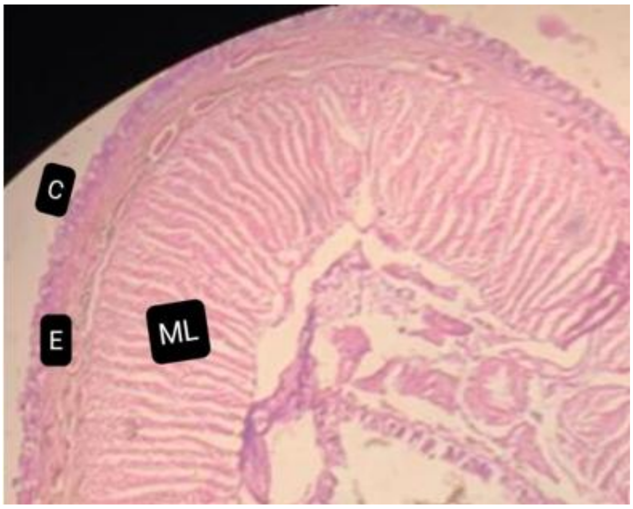


Figure 8: Microphotograph of intestinal region of *Eisenia fetida* control group. It shows normal epidermis (E), cuticle (C) and muscular layer (ML). (H &E x 400)

Figure 9: Microphotograph of intestinal region of *Eisenia fetida* treated group I (MPs-110 µg). It shows change in the muscular layer and thinning of the epidermis layer. E-Epidermis, L-Lumen, C- Cuticle, and ML- Muscular layer. (H &E x 400)

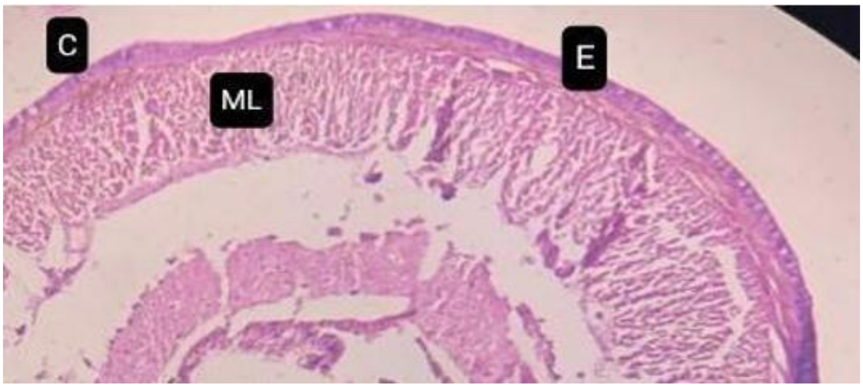


Figure 10: Microphotograph of intestinal region of *Eisenia fetida* treated group II (MPs-120 µg). It shows change in the muscular layer and thinning of the epidermis layer. E-Epidermis, C- Cuticle, and ML- Muscular layer. (H &E x 400)

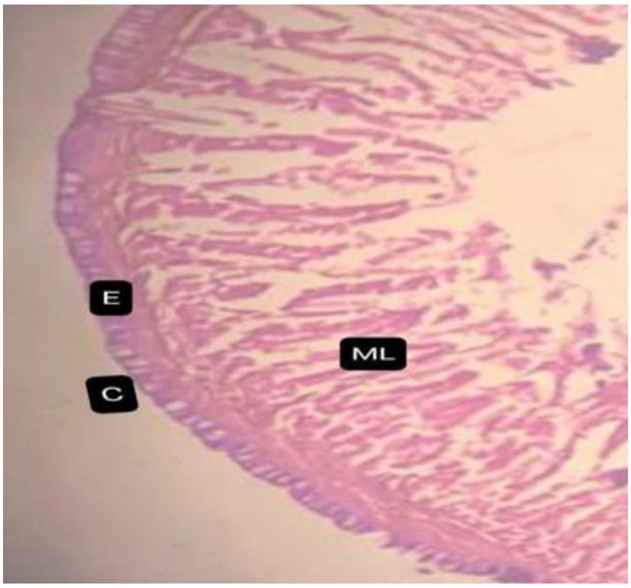


Figure 11: Microphotograph of intestinal region of *Eisenia fetida* treated group III (MPs-130 µg). It shows change in the muscular layer and thinning of the epidermis layer. E-Epidermis, C- Cuticle, and ML- Muscular layer. (H &E x 400)

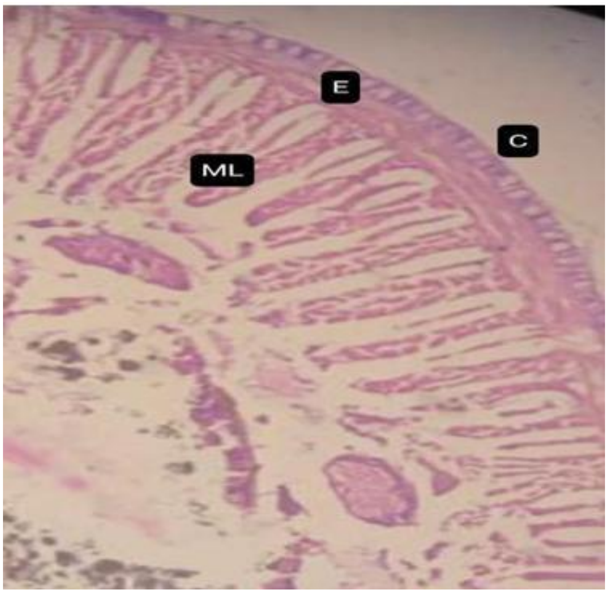


Figure 12: Microphotograph of intestinal region of *Eisenia fetida* treated group IV (MPs-140 µg). It shows loss of structural integrity of the muscle layer, and thinning of the epidermis layer. E-Epidermis, C- Cuticle, and ML-Muscular layer. (H &E x 400)

At the end of the experiment, histopathological changes were observed as important markers of toxicity. *Eisenia fetida* and *Eudrilus eugeniae* which were exposed to different microplastic concentrations: MPs-110 µg, MPs-120 µg, MPs-130 µg, MPs-140 µg. Observations under compound microscope with magnification 40X indicated that as the concentration of microplastic goes higher it causes thinning of muscular and epidermis layers (fig 3,4,5,9 & 10) and distortion of the shapes muscular layer (fig 6). The areas of inflammation are seen and thinning of the epidermis layer. There is also damage to the cuticle and loss of structural integrity of the muscle layer (Fig 7 & 12). Similar reporting was done by (Lourenco et al., 2011). Qi et al. (2018) found that microplastic induced more damages to the earthworm epidermis and atrophy of the longitudinal muscle layer. Therefore, environmental exposure to microplastics may present a significant risk for this species and other soil organisms. The study concludes that the exposure to MP is detrimental to the morphology, histology and reproduction of earthworms.

4. Conclusion

The present study reveals that when earthworms *Eisenia fetida* and *Eudrilus eugeniae* are exposed to different concentrations of microplastics causes changes in body weight, mortality and morphology. It effects the reproductive rate by decreasing the number of cocoons and juvenile production. Further it also causes histopathological changes. The microplastics disrupt the normal metamorphic function of soil organisms. Therefore, it suggested that single use plastics like carry bags should not be used or should be banned.

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