



## Assessing the Environmental Safety of Biodegradable Plastics: PLA Microplastic-Induced Stress Responses in *Eisenia Fetida*

Nazima Noor<sup>✉</sup> | Chandan M V | M Keerthana | Srinivas V | Kaveri M

Department of Zoology, Bangalore University, Bangalore, Karnataka, India

| Article Info                                                                                                                                                                                                                                                                                                                                                                              | ABSTRACT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Article type:</b><br>Research Article                                                                                                                                                                                                                                                                                                                                                  | Agricultural soils are among major sinks of microplastics (MPs), which have become an alarming problem in today's environment. While polylactic acid (PLA) is widely advertised as a biodegradable and environmentally friendly material, its persistence in natural soil raises a question whether it is really safe. This study aims to examine the sublethal effects of PLA MPs on <i>Eisenia fetida</i> by measuring alterations in multiple biological characteristics at four different concentrations (0.5, 1.0, 1.5, and 2.0 g/kg soil) across three exposure durations (12, 24, and 36 days). All variables studied were both concentration and time dependent. SOD and CAT activities exhibited irregular changes at different concentrations and time points. At higher concentrations and longer exposure, depletion of antioxidants was observed. Also, the neurotoxicity response to AChE activity presented a complex pattern from low to high concentrations. Higher concentrations reduced the SGR, especially at mid-term exposure time. While the reproduction was greatly decreased with increased concentrations and time. The survival rate remained at 100% until day 12 but exhibited decline during the mid and long-term studies. The tissue damage assessment revealed that the harm to tissues occurred in accordance with both dose levels and duration of exposure. The results show that PLA microplastics, which can decompose in theory, present actual toxicological hazards to earthworms when found in natural soil environments. This study demonstrates that environmental risk assessments for biodegradable plastics, as alternative to conventional plastics, require more detailed and rigorous testing before their widespread commercial implementation. |
| <b>Article history:</b><br>Received: 12 May 2026<br>Revised: 26 Jul 2026<br>Accepted: 16 Sep 2026                                                                                                                                                                                                                                                                                         |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| <b>Keywords:</b><br><i>Microplastics</i><br><i>Polylactic acid (PLA)</i><br><i>Eisenia fetida</i><br><i>Antioxidant</i><br><i>Neurotoxicity and</i><br><i>Histopathology</i>                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| <b>Cite this article:</b> Noor, N., Chandan M V, Keerthana, M., Srinivas, V., & Kaveri, M. (2026). Assessing the Environmental Safety of Biodegradable Plastics: PLA Microplastic-Induced Stress Responses in <i>Eisenia Fetida</i> . <i>Pollution</i> , 12(4), 1026-1044.<br><a href="https://doi.org/10.22059/poll.2026.402229.3332">https://doi.org/10.22059/poll.2026.402229.3332</a> |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |



© The Author(s).

Publisher: The University of Tehran Press.

DOI: <https://doi.org/10.22059/poll.2026.402229.3332>

## INTRODUCTION

Microplastics (MPs), defined as plastic particles, fibres, or fragments smaller than 5 mm, are persistent environmental contaminants due to their chemical stability and resistance to biodegradation (Prendergast-Miller et al., 2019). Originating from primary sources (e.g., microbeads, pellets) and secondary sources (e.g., breakdown of larger plastics), MPs enter terrestrial environments through wastewater irrigation, sewage sludge application, aerial deposition, and fragmentation of plastic waste like mulch films (Hurley and Nizzetto, 2018; de Souza Machado et al., 2018). While oceans have been recognised as significant MP sinks (do Sul and Costa, 2014; Jeong and Choi, 2019), recent estimates suggest agricultural soils may accumulate 23 times more MPs than oceans, primarily via sewage sludge, manure, and plastic mulches (Nizzetto et al., 2016). Approximately 32% of produced plastics remain in continental systems, making soils a major long-term sink (Büks and Kaupenjohann, 2020; Corradini et al., 2019; Sommer et al., 2018; Weithmann et al., 2018). Non-biodegradable MPs, such as

\*Corresponding Author Email: [nazimanoor@yahoo.com](mailto:nazimanoor@yahoo.com)

polyethylene (PE), polypropylene (PP), and polystyrene (PS), persist in soils for decades, potentially altering soil structure, water retention, and microbial communities (de Souza Machado et al., 2018). Biodegradable plastics, such as polylactic acid (PLA) and polyhydroxyalkanoates (PHA), are designed to degrade via microbial activity, but their breakdown is often incomplete under suboptimal conditions, contributing to soil persistence (Haider et al., 2019). Additives or residual monomers in bioplastics may leach into soils, exerting toxic effects (Lambert and Wagner, 2017).

Earthworms, widespread across continents including the Holoarctic region, thrive in nutrient-rich, organic soils with pH 5–8, though some species adapt to extreme conditions like low pH or prolonged frosts (Briones et al., 2009). Their ecological roles—bioindication, composting, and contaminant assessment—stem from their sensitivity to environmental changes and contributions to soil structure, oxygen levels, and microbial activity through burrowing and mucus secretion (Suleiman et al., 2017). *Eisenia fetida*, a detritivore common in compost and agricultural soils, is widely used in ecotoxicology due to its sensitivity to contaminants, including heavy metals, pesticides, and MPs (OECD, 1984; Spurgeon et al., 2003). Standard toxicity endpoints include mortality, weight changes, cocoon production, enzymatic biomarkers, histopathology, and gene expression (Edwards and Bohlen, 1996). Their soil-feeding behaviour makes them prone to ingesting MPs, which accumulate in their gut, disrupt feeding, and cause physiological stress, positioning *E. fetida* as a model species for studying MP impacts (Cole et al., 2011).

PLA MPs have been shown to cause sub-lethal effects in *E. fetida*, including oxidative stress and reduced reproduction Wang et al. (2021), though less severe than non-biodegradable MPs. Qi et al. (2020) compared PLA and PE MPs, finding that biodegradable plastics had lesser but non-negligible effects, impacting cocoon production and enzyme activity.

At significantly greater MP concentrations, PLA has been shown to have detrimental effects on earthworm survival and reproduction comparable to conventional PE Ding et al., (2021). Huerta Lwanga et al. (2021) could not discover any appreciable adverse effects of PLA fibres on the mortality, weight increase, or reproduction of *Lumbricus terrestris*. Bodyweight and reproductive success were influenced by polymer type and additive status; biodegradable polymers had a favourable impact on both weight increase (PCL) and reproductive success (PCL and PLLA). As per Ding et al. (2021), both biodegradable (PLA) and conventional (PE) MPs showed toxicity patterns at higher doses and mortality effects were not significantly different between plastic types. MPs' effect on worms was strongly correlated with their kind, concentration, and particle size. Comparing PLA and polypropylene carbonate (PPC) with PE in standardized exposures, biodegradable plastics were found to be no less toxic than conventional plastics to *E. fetida* (Wang et al., 2021).

The subject of this research pertains to the metabolic effects of microplastics on earthworms, a topic intrinsically linked to diverse pollution concerns. Addressing the global plastic crisis is the most pressing issue of our time, essential for securing a sustainable future. This research endeavor is poised to elucidate the underlying factors contributing to the aberrant growth and metabolic processes observed in *Eisenia fetida*.

## MATERIALS AND METHODS

### *Test substrate*

Experimental soil samples were collected from the Bio Park, Bangalore University campus, Bangalore, Karnataka. Soil samples were collected from subsurface depths of 20–30 cm to prevent soil-surface MP contamination from affecting the experiment. Prior to experimentation, soils were air dried, sieved through a 2 mm screen to remove impurities, and sealed to minimize contamination.

### *Test organism*

Earthworms (*Eisenia fetida*) were obtained from a vermiculture farm—Namma organics vermicompost near kaggalipura, Karnataka. Healthy, active worms with clear clitella, weighing 400–600 mg, as recommended by International guidelines (ISO 1998; OECD, 2004) were selected. Before inoculation, worms were acclimatized for one week to experimental soil under laboratory culture conditions. Earthworms underwent a 12-h fasting period prior to inoculation, during which their intestines emptied; surface impurities were also washed off with distilled water. Worms were then wiped dry and placed into culture dishes. Culture conditions were maintained at  $21 \pm 2$  °C and a light cycle of 12 h D: 12 h L, with adequate ventilation. Moisture and pH were maintained at 40% and 7 respectively (OECD, 2004).

### *Microplastics*

MP used in this experiment was PLA, which we purchased from a plastic material Company called Srushti Global, Sangli, Maharashtra. To remove possible contaminants, MP particles were rinsed once with ethanol (70 %), washed twice with deionized water, and dried at RT.

### *Experimental setup*

PLA MP of particle size less than 2mm at four concentrations (0.5g/kg, 1.0g/kg, 1.5g/kg, 2 g/kg (w/w)) were used. Each treatment was replicated three times, with six additional blank controls (without MP). MP were thoroughly mixed with the soil and packed into culture boxes. Additionally, 300 g of well-decomposed dried cattle manure was added to each culture box as a nutrient source for the earthworms. Worms that had grown actively, appeared healthy, and weighed 400–600 mg after 24 h of purging, were selected for experiments; each culture box contained 50 individuals. The experimental setup is in accordance with that of Lai et al. (2024). To visualize the toxic effects of MPs on earthworms, five earthworms from each culture box were randomly collected on 12th, 24th and 36th days and underwent pretreatment analysis to determine oxidative stress enzyme (superoxide dismutase (SOD), catalase (CAT) and acetylcholinesterase (Ache) activity levels. Specific growth rate (SGR), reproductive capacity, and survival rates (SR) were also analysed during the same time. Histopathological procedures were also carried out simultaneously.

### *Tissue processing*

The whole earthworms were homogenized in ice cold 50 mM phosphate buffer pH (7.0). The resulting homogenate was centrifuged at 1000 rpm for 15 min at 4°C. The supernatant was used for analytical procedures.

SOD activity was determined at room temperature (RT) according to the method of Misra and Fridovich (1972). CAT activity was determined by the method of Aebi (1984). AChE activity was determined by following Ellman et al., (1961).

Specific growth rate (SGR%) was determined by following (Zhu et al., 2014) where weight, log of weight, time was considered. The reproductive capability was determined by counting the total number of hatchlings (Angmo et al., 2023). Survival rate (%) was determined by following (Zhu et al., 2014) where number of organisms were counted.

### *Histopathology*

The transverse section of earthworm was studied adopting the routine paraffin method (Humason, 1979). Agar serves as a good medium for emptying the gut contents. 50ml jars were filled with 30ml of 1.5% agar gel prepared with deionized water (Pokarznerskii et al., 2000). After cooling and solidifying, this gel in the jars was taken out and cut into small pieces. The earthworms were then transferred into the agar pieces containing jars and kept for 24 hrs. At the end of the depuration period, all the earthworms were placed in a 4% formaldehyde (=10%

buffered formalin solution) ( $\text{pH} = 7.0 \pm 0.2$ ) and kept there for at least 24 h. Afterwards, the animals were removed and preserved as described above for histopathological analysis. All slides were examined by light microscopy using Optika microscope with an inbuilt camera.

### Statistics

The data are expressed as mean  $\pm$  SD and were analysed using Graph Pad Prism 10. Statistical differences between treatments were assessed using one-way Anova and differences were considered statistically significant when  $p < 0.0001$ , 0.001, 0.01 and 0.05 respectively.

## RESULTS AND DISCUSSION

This study investigated the toxicological impacts of polylactic acid (PLA) microplastics on the earthworm *Eisenia fetida* across short-term (12 days), mid-term (24 days), and long-term (36 days) exposure durations at concentrations of 0.5, 1.0, 1.5, and 2.0 g/kg. Although PLA is widely perceived as an environmentally benign alternative to conventional plastics owing to its biodegradable character, the present findings establish that PLA microplastics exert measurable toxicological effects on soil organisms. The complete biodegradation of PLA in natural soil environments requires specific conditions of temperature, pH, and microbial community composition that are rarely met simultaneously, resulting in the persistence of PLA fragments as biodegradable microplastics (BMPs) capable of inducing measurable ecotoxic effects (Baihetiyaer et al., 2023; Teng et al., 2025). Furthermore, PLA releases chemicals including monomers and associated additives during partial degradation that may enter soil animals and disrupt their normal physiology (Baihetiyaer et al., 2023). The following sections synthesise and discuss the PLA-specific findings across all measured endpoints, corroborated by published literature on biodegradable microplastic ecotoxicology.

### Superoxide Dismutase (SOD) Activity

#### Short-term Exposure (12 days)

*Eisenia fetida* maintained a baseline SOD activity (Table 1) of ( $8.32 \pm 0.60$ ) in control, representing normal antioxidant status in uncontaminated soil. SOD activity levels in PLA-exposed *Eisenia fetida* was lowest ( $3.31 \pm 0.58$ ) at 1.5 g/kg and highest ( $7.30 \pm 2.77$ ) at 2.0 g/kg during the 12-day exposure period. This dose-dependent upregulation at the highest concentration demonstrates that PLA microplastics trigger an oxidative stress response in earthworms even within the short term, likely through concurrent physical abrasion of the gut epithelium by ingested particles and the early release of PLA degradation monomers into worm tissues. SOD represents the organism's primary enzymatic defence against superoxide radicals, and its upregulation at 2.0 g/kg indicates that PLA-induced ROS generation at this concentration exceeds the baseline antioxidant capacity. Parolini et al. (2024), studying the toxicity of PLA microplastics on *Eisenia fetida* over 28 days, observed progressive alterations in antioxidant enzyme activities and noted that even at relatively low concentrations, PLA

**Table 1.** SOD activity levels in *E. fetida* induced by exposure to MP – PLA for 12, 24 and 36 days respectively.

| S. No. | Concentration of MP - PLA<br>(g/kg) | Short term exposure<br>(12 days) | Midterm exposure<br>(24 days) | Long term exposure<br>(36 days) |
|--------|-------------------------------------|----------------------------------|-------------------------------|---------------------------------|
| 1      | Control                             | $8.32 \pm 0.60$                  | $7.56 \pm 2.56$               | $8.79 \pm 4.06$                 |
| 2      | 0.5                                 | $3.56 \pm 1.91$                  | $7.09 \pm 1.25$               | $12.39 \pm 6.07$                |
| 3      | 1.0                                 | $5.10 \pm 2.01$                  | $5.62 \pm 0.63$               | $13.31 \pm 3.73$                |
| 4      | 1.5                                 | $3.31 \pm 0.58$                  | $4.77 \pm 0.72$               | $13.27 \pm 1.94$                |
| 5      | 2.0                                 | $7.30 \pm 2.77$                  | $1.58 \pm 2.17^d$             | $7.53 \pm 2.65$                 |

Values are expressed in u/ml enzyme and are displayed as mean  $\pm$  SD. The superscripts a, b, c and d indicate statistical mean differences at  $p < 0.0001$ , 0.001, 0.01 and 0.05 respectively.

elicits early biochemical stress responses. The dip in SOD at 1.5 g/kg may reflect a transient enzymatic suppression at intermediate concentrations where the rate of ROS generation temporarily outpaces antioxidant induction — a biphasic dose-response pattern that has been documented in ecotoxicological studies of soil invertebrates exposed to microplastics (Parolini et al., 2024). Overall, the short-term SOD response confirms that PLA microplastics generate oxidative challenge in *E. fetida* in a concentration-dependent but non-linear manner even within the initial phase of exposure.

#### *Mid-term Exposure (24 days)*

At 24 days, SOD activity levels (Table 1) in PLA-treated worms peaked ( $7.09 \pm 1.25$ ) at 0.5 g/kg and was lowest ( $1.58 \pm 2.17$ ) at 2.0 g/kg, representing a reversal of the short-term dose-response pattern while as the control exhibited a value of  $7.56 \pm 2.56$ . This shift likely reflects the onset of antioxidant system suppression at the highest concentration during mid-term

exposure, a phenomenon whereby sustained oxidative overload depletes or oxidatively damages the SOD enzyme itself, compromising upregulation capacity. Baihetyaer et al. (2023) reported that PLA BMP exposure caused a substantially decreased activity of SOD in *Eisenia fetida* over time, attributing this to ROS-induced damage to the antioxidant defence system under conditions of chronic oxidative burden. The higher SOD response at 0.5 g/kg at this stage suggests that at low concentrations, the oxidative challenge of PLA is sustained but manageable, maintaining enzymatic upregulation, while at 2.0 g/kg the defence system becomes progressively overwhelmed. This temporal inversion in the dose-response relationship underscores that the antioxidant capacity of *E. fetida* is both concentration-dependent and time-sensitive under PLA exposure.

#### *Long-term Exposure (36 days)*

At 36 days, SOD activity levels (Table 1) peaked ( $13.31 \pm 3.73$ ) at 1.0 g/kg and was lowest ( $7.53 \pm 2.15$ ) at 2.0 g/kg while as control exhibited a value of  $8.79 \pm 4.06$ . The persistent elevation at 1.0 g/kg through the long-term exposure period suggests that this concentration sustains a level of oxidative challenge that continues to stimulate SOD production without overwhelming the enzyme. At 2.0 g/kg, the continued suppression of SOD at the long-term stage indicates chronic antioxidant exhaustion, wherein the cumulative oxidative burden of sustained PLA particle–tissue interaction depletes enzymatic reserves beyond the worm’s capacity for replenishment. Qi et al. (2020) noted that while the degradation products of PLA such as lactic acid are less acutely toxic than those of non-biodegradable plastics, they still contribute to a sustained oxidative microenvironment within worm tissues over extended exposures, consistent with the residual SOD disruption observed at 36 days. Han et al. (2023) similarly reported that PLA induces progressive, dose-dependent oxidative stress in *E. fetida* that evolves with exposure duration, with antioxidant enzyme activities reflecting the cumulative tissue burden of PLA degradation intermediates.

#### *Catalase (CAT) Activity*

##### *Short-term Exposure (12 days)*

*Eisenia fetida* showed a CAT activity (Table 2) of ( $0.849 \pm 0.498$ ) in control, reflecting normal peroxide turnover in clean soil. CAT activity levels in PLA-treated *E. fetida* were lowest ( $0.002 \pm 0.001$ ) at 1.5 g/kg and highest ( $0.049 \pm 0.015$ ) at 1.0 g/kg during the 12-day exposure. CAT is the primary enzymatic scavenger of hydrogen peroxide ( $H_2O_2$ ) generated downstream of SOD-mediated superoxide dismutation, and its upregulation at 1.0 g/kg signals that this concentration induces sufficient peroxidative stress to require active enzymatic detoxification. The lower CAT activity at 1.5 g/kg during this early phase — in parallel with the SOD dip at the

**Table 2.** Catalase activity levels in *E. fetida* induced by exposure to MP- PLA for 12, 24 and 36 days respectively.

| S. No. | Concentration of MP - PLA (g/kg) | Short term exposure (12 days) | Midterm exposure (24 days) | Long term exposure (36 days) |
|--------|----------------------------------|-------------------------------|----------------------------|------------------------------|
| 1      | Control                          | 0.849 ± 0.498                 | 0.013 ± 0.018              | 0.014 ± 0.007                |
| 2      | 0.5                              | 0.025 ± 0.014 <sup>a</sup>    | 0.001 ± 0.002              | 0.006 ± 0.002                |
| 3      | 1.0                              | 0.049 ± 0.015 <sup>a</sup>    | 0.001 ± 0.001              | 0.027 ± 0.017                |
| 4      | 1.5                              | 0.002 ± 0.001 <sup>a</sup>    | 0.001 ± 0.000              | 0.018 ± 0.016                |
| 5      | 2.0                              | 0.023 ± 0.014 <sup>a</sup>    | 0.007 ± 0.004              | 0.007 ± 0.000                |

Values are expressed in  $\mu\text{moles of H}_2\text{O}_2$  degraded/min/mg protein and are displayed as mean  $\pm$  SD. The superscripts a, b, c and d indicate statistical mean differences at  $p < 0.0001$ , 0.001, 0.01 and 0.05 respectively.

same dose — is consistent with a non-monotonic, hormetic-type enzymatic response that has been documented in earthworms exposed to biodegradable microplastics (Liwarska-Bizukojc, 2025). Parolini et al. (2024) noted that antioxidant and detoxifying enzyme activities in *Eisenia fetida* exposed to PLA microplastics showed time-point-specific variability across their 28-day study, consistent with the non-linear CAT pattern observed here at 12 days. The variable early CAT response across concentrations indicates that the peroxidative stress profile of PLA is shaped by complex particle-tissue interaction kinetics during the initial phase of exposure, and should not be interpreted as a simple monotonic dose relationship.

#### Mid-term Exposure (24 days)

At 24 days, CAT activity level (Table 2) was suppressed at 0.5, 1.0, and 1.5 g/kg and elevated ( $0.007 \pm 0.004$ ) at 2.0 g/kg in PLA-exposed worms where the value, while still below control ( $0.013 \pm 0.018$ ), was the closest among PLA groups to the control baseline at this time point. The marked CAT peak at 2.0 g/kg at the mid-term stage suggests that hydrogen peroxide production intensifies with prolonged PLA exposure at the highest dose as particle accumulation in the gut and progressive release of degradation byproducts increase the peroxidative burden. Lai et al. (2024) reported that biodegradable plastics like PLA elicit CAT activation that varies by dose and duration, with higher concentrations sustaining more pronounced peroxidative responses into the mid-term exposure period. The suppressed CAT response at lower PLA concentrations during mid-term exposure may indicate partial enzymatic adaptation — wherein alternative antioxidant pathways such as peroxidase (POD) are recruited to manage  $\text{H}_2\text{O}_2$  — or reflect a transient enzymatic downregulation as oxidative stress remains at a sub-threshold level at those doses. The systematic 2025 study by Liang et al. reassessing biodegradable microplastic toxicity confirmed that earthworms activate detoxifying enzymes in the mid-term phase to repair oxidative damage, consistent with the concentration-specific CAT patterns observed here.

#### Long-term Exposure (36 days)

At 36 days, CAT activity (Table 2) was highest ( $0.027 \pm 0.017$ ) at 1.0 g/kg and lowest ( $0.006 \pm 0.002$ ) at 0.5 g/kg in PLA-treated worms when compared to control ( $0.014 \pm 0.007$ ). The persistent peak at 1.0 g/kg through the long-term exposure period indicates that this concentration sustains the greatest peroxidative burden over time, maintaining an active CAT response even after 36 days. Yang et al. (2021) confirmed that biodegradable plastics including PLA elicit measurable CAT activation over extended exposure periods, with intermediate concentrations often sustaining more prolonged enzymatic responses than the highest doses, likely because high-dose enzyme depletion overrides upregulation capacity. The suppressed CAT at 0.5 g/kg at this time point may reflect a reduction in peroxidative challenge at low PLA concentrations over time as degradation products are metabolised and dispersed within the gut microenvironment. Taken together, the long-term CAT data indicate that 1.0 g/kg PLA represents the most biochemically demanding exposure scenario with respect to peroxidative stress in *E.*

**Table 3.** AChE activity levels in *E. fetida* induced by exposure to MP – PLA for 12, 24 and 36 days respectively

| S. No. | Concentration of MP - PLA (g/kg) | Short term exposure<br>(12 days) | Midterm exposure<br>(24 days) | Long term exposure<br>(36 days) |
|--------|----------------------------------|----------------------------------|-------------------------------|---------------------------------|
| 1      | Control                          | 0.002 ± 0.001                    | 0.003 ± 0.001                 | 0.002 ± 0.001                   |
| 2      | 0.5                              | 0.004 ± 0.001                    | 0.008 ± 0.000 <sup>a</sup>    | 0.003 ± 0.001 <sup>d</sup>      |
| 3      | 1.0                              | 0.002 ± 0.001                    | 0.005 ± 0.001 <sup>a</sup>    | 0.004 ± 0.001                   |
| 4      | 1.5                              | 0.003 ± 0.002                    | 0.006 ± 0.001                 | 0.003 ± 0.001                   |
| 5      | 2.0                              | 0.003 ± 0.001                    | 0.005 ± 0.001                 | 0.004 ± 0.001                   |

Values are expressed in u/ml enzyme and are displayed as mean ± SD. The superscripts a, b, c and d indicate statistical mean differences at  $p < 0.0001$ , 0.001, 0.01 and 0.05 respectively.

*fetida*, and that the earthworm's antioxidant capacity operates within a finite enzymatic window across concentration and time.

### Acetylcholinesterase (AChE) Activity

#### Short-term Exposure (12 days)

AChE activity levels in PLA-exposed *E. fetida* (Table 3) was highest ( $0.004 \pm 0.001$ ) at 0.5 g/kg and lowest ( $0.002 \pm 0.001$ ) at 1.0 g/kg during the 12-day exposure. AChE catalyses the hydrolysis of acetylcholine at cholinergic synapses and is a classical biomarker of neurotoxicity in soil invertebrates. *E. fetida* maintained a baseline AChE activity ( $0.002 \pm 0.001$ ) in control reflecting normal cholinergic neurotransmission in uncontaminated conditions. The elevated AChE at 0.5 g/kg in the short term suggests an initial neurostimulatory response at low PLA concentrations, possibly driven by the early release of lactic acid monomers or other PLA degradation intermediates into the worm's neural tissue environment. Earthworms have been reported to activate detoxifying enzymes in the mid-term phase to repair oxidative damage, consistent with the concentration-specific CAT patterns observed here (Liang et al., 2025). The suppression of AChE at 1.0 g/kg at this stage may indicate dose-dependent inhibitory effects of PLA-associated chemical stressors on the enzyme at that concentration. This is consistent with the complex, dose- and time-dependent neurotoxic responses documented in earthworms exposed to PLA microplastics (Baihetiyaer et al., 2023).

#### Mid-term Exposure (24 days)

At 24 days, AChE activity (Table 3) remained highest ( $0.008 \pm 0.00$ ) at 0.5 g/kg and was lowest ( $0.005 \pm 0.001$ ) at 1.0 g/kg and 2.0 g/kg. The persistence of elevated AChE at 0.5 g/kg into the mid-term exposure period indicates a sustained neurostimulatory effect of low-dose PLA, possibly because low concentrations of lactic acid degradation products continue to modulate cholinergic signalling without causing outright neural tissue damage. Baihetiyaer et al. (2023) demonstrated that PLA BMP exposure significantly altered AChE activity in *Eisenia fetida*, with the direction of change varying by concentration and time point, consistent with the dose-dependent divergence in AChE response observed here at 24 days. The suppression of AChE at higher doses during mid-term exposure suggests that at 1.0 and 2.0 g/kg, sustained PLA stress begins to inhibit AChE through oxidative damage to the enzyme or progressive disruption of neural tissue integrity, which is consistent with the histological evidence of nerve fibre abnormalities observed at mid-term in higher-concentration PLA treatments (Han et al., 2023).

#### Long-term Exposure (36 days)

AChE activity levels (Table 3) of control at 36 days exhibited lesser values ( $0.002 \pm 0.001$ ) than treated ones. Over 36 days, AChE activity was lowest ( $0.003 \pm 0.001$ ) at 0.5 g/kg and 1.5 g/kg and highest ( $0.004 \pm 0.001$ ) at 1.0 g/kg and 2.0 g/kg in PLA-exposed worms. The dual elevation of AChE at 1.0 and 2.0 g/kg during long-term exposure indicates that chronic PLA

**Table 4.** Specific growth rate levels in *E. fetida* induced by exposure to MP– PLA for 12, 24 and 36 days respectively.

| S. No. | Concentration of MP - PLA<br>(g/kg) | Short term exposure<br>(12 days) | Midterm exposure<br>(24 days) | Long term exposure<br>(36 days) |
|--------|-------------------------------------|----------------------------------|-------------------------------|---------------------------------|
| 1      | Control                             | $0.40 \pm 0.001$                 | $0.01 \pm 0$                  | $0.04 \pm 0.004$                |
| 2      | 0.5                                 | $0.79 \pm 0.001$                 | $-0.48 \pm 0$                 | $-1.45 \pm 0.004^a$             |
| 3      | 1.0                                 | $1.32 \pm 0.002$                 | $1.11 \pm 0$                  | $-1.51 \pm 0^a$                 |
| 4      | 1.5                                 | $0.48 \pm 0.001$                 | $-0.31 \pm 0$                 | $-0.83 \pm 0^a$                 |
| 5      | 2.0                                 | $0.83 \pm 0.000$                 | $-0.64 \pm 0$                 | $-0.75 \pm 0^a$                 |

Values are expressed in % and are displayed as mean  $\pm$  SD. The superscripts a, b, c and d indicate statistical mean differences at  $p < 0.0001$ , 0.001, 0.01 and 0.05 respectively.

stress at these concentrations maintains the neural system in a state of sustained activation, possibly as a compensatory response to ongoing perturbation of cholinergic signalling by accumulated PLA particles and their degradation byproducts. Liang et al. (2025) confirmed that PLA microplastics can induce neurotoxicity through AChE modulation across multiple time points, with earthworms maintaining physiological balance through metabolic regulation and immune enhancement during later stages of exposure. The divergent AChE pattern across concentrations at 36 days — with suppression at 0.5 and 1.5 g/kg and elevation at 1.0 and 2.0 g/kg — reflects the complexity of PLA's neurotoxic action, which does not follow a simple linear trajectory and is likely governed by simultaneous neural stimulation, oxidative inhibition, and tissue-level adaptation at different concentration thresholds.

#### Specific Growth Rate (SGR)

##### Short-term Exposure (12 days)

SGR level (Table 4) was minimum ( $0.48 \pm 0.001$ ) at 1.5 g/kg and maximum ( $1.32 \pm 0.002$ ) at 1.0 g/kg in PLA-treated worms during the 12-day exposure. While as control worms recorded an SGR of ( $0.40 \pm 0.001$ ), reflecting stable, normal growth in uncontaminated soil. The elevated growth rate at 1.0 g/kg in the early phase may reflect a stress-induced metabolic stimulation, wherein *E. fetida* transiently increases metabolic activity in response to the novel chemical and physical challenge introduced by PLA particles. This type of short-term stimulatory response, sometimes described in the ecotoxicological literature as a hormetic growth effect, has been attributed to the energetic mobilisation that accompanies initial stress responses in soil invertebrates (Sibly & Calow, 1989). The decline in SGR at 1.5 g/kg indicates that beyond a certain threshold, even short-term PLA exposure begins to impair metabolic efficiency and nutrient assimilation, likely through the onset of gut epithelial damage that reduces the functional absorptive surface. Zhang et al. (2023) reported a significantly increased

growth inhibition rate in *Eisenia fetida* upon exposure to PLA BMPs alone, confirming that PLA microplastics are capable of suppressing earthworm growth even in the absence of co-contaminants.

##### Mid-term Exposure (24 days)

SGR (Table 4) was highest ( $1.11 \pm 0$ ) at 1.0 g/kg and lowest ( $-0.64 \pm 0$ ) at 2.0 g/kg during mid-term PLA exposure. The continued positive growth at 1.0 g/kg into the 24-day period suggests that at this concentration, the worm maintains a positive energy balance despite ongoing PLA exposure, whereas growth inhibition becomes pronounced at 2.0 g/kg as particle accumulation within the gut physically interferes with digestion and nutrient absorption. SGR in control worms indicated a near-zero net growth ( $0.01 \pm 0$ ) at this time point. Gutiérrez-Rial et al. (2025), studying the effects of PLA microplastics on the earthworm *Eisenia andrei* in short- and medium-term assays, reported decreased growth parameters during medium-term exposure to PLA, consistent with the progressive growth inhibition observed at higher concentrations in the present study. Parolini et al. (2024) similarly noted that PLA microplastic ingestion disrupts

**Table 5.** Reproductive capacity levels in *E. fetida* exposed to MP – PLA for 12, 24 and 36 days respectively.

| S. No. | Concentration of MP - PLA (g/kg) | Short term exposure (12 days) | Midterm exposure (24 days) | Long term exposure (36 days) |
|--------|----------------------------------|-------------------------------|----------------------------|------------------------------|
| 1      | Control                          | 0 ± 0                         | 34.5 ± 0.71                | 110 ± 0.00                   |
| 2      | 0.5                              | 0 ± 0                         | 4 ± 0.00 <sup>a</sup>      | 120.5 ± 0.71 <sup>a</sup>    |
| 3      | 1.0                              | 0 ± 0                         | 4 ± 0.00 <sup>a</sup>      | 128 ± 2.83 <sup>a</sup>      |
| 4      | 1.5                              | 0 ± 0                         | 7 ± 1.41 <sup>a</sup>      | 60 ± 0.00 <sup>a</sup>       |
| 5      | 2.0                              | 0 ± 0                         | 3 ± 2.83 <sup>a</sup>      | 68 ± 0.00 <sup>a</sup>       |

Values are expressed in number of hatchlings and are displayed as mean ± SD. The superscripts a, b, c and d indicate statistical mean differences at  $p < 0.0001$ , 0.001, 0.01 and 0.05 respectively.

gut function in *E. fetida* in a dose- and time-dependent manner, with higher concentrations producing more pronounced physiological impairments during sustained exposure.

#### *Long-term Exposure (36 days)*

At 36 days, SGR (Table 4) was highest ( $-0.75 \pm 0$ ) at 2.0 g/kg and lowest ( $-1.51 \pm 0$ ) at 1.0 g/kg in exposed worms while as control displayed a small ( $0.04 \pm 0.004$ ) but positive growth value in uncontaminated worms. The counterintuitive recovery of growth at the highest concentration during long-term exposure likely reflects a form of physiological or behavioural adaptation, wherein *E. fetida* reduces PLA particle ingestion through avoidance behaviour or enhances metabolic compensation mechanisms in response to chronic exposure. Liwarska-Bizukojc (2025) documented clear avoidance behaviour in earthworms exposed to PLA microparticles in soil, with the majority of worms moving downward away from PLA-contaminated zones, suggesting that behavioural modification significantly shapes the physiological outcome of long-term PLA exposure. Qi et al. (2020) noted that as PLA degradation products such as lactic acid are progressively metabolised and dispersed within the gut environment, the chemical toxicological burden may partially diminish over time, which — combined with reduced ingestion — may allow some recovery of growth capacity at the highest concentration during the 36-day period.

#### *Reproductive Capability*

##### *Mid-term Exposure (24 days)*

Reproductive Capability or hatchling production (Table 5) was minimal ( $3 \pm 2.83$ ) at 2.0 g/kg and maximal ( $7 \pm 1.41$ ) at 1.5 g/kg in PLA-exposed worms at 24 days when compared to control ( $34.5 \pm 0.71$ ), reflecting normal reproductive output in uncontaminated conditions. The elevated reproductive output at 1.5 g/kg during mid-term exposure suggests that *E. fetida* may mount a compensatory reproductive effort under moderate PLA stress — a well-documented strategy in invertebrate stress ecology, wherein organisms prioritise reproduction under sub-lethal toxicant conditions to ensure population continuity (Sibly & Calow, 1989). This compensatory response implies that 1.5 g/kg PLA represents a concentration at which the worm experiences sufficient stress to trigger reproductive effort upregulation but not sufficient systemic toxicity to suppress reproductive capacity entirely. The sharp suppression of reproduction at 2.0 g/kg indicates that beyond this concentration threshold, the cumulative toxicological burden of PLA — including oxidative tissue damage, gut wall disruption, and neural stress — becomes incompatible with sustained reproductive effort, as the worm redirects available energy toward survival and detoxification.

##### *Long-term Exposure (36 days)*

At 36 days, hatchling production (Table 5) was highest ( $128 \pm 2.83$ ) at 1.0 g/kg and lowest ( $60 \pm 0.00$ ) at 1.5 g/kg when compared to control ( $110 \pm 0.00$ ). The marked reproductive suppression across the higher concentration range during long-term PLA exposure is consistent

**Table 6.** Survival rate levels in *E. fetida* exposed to MP – PLA for 12, 24 and 36 days respectively.

| S. No. | Concentration of MP - PLA (g/kg) | Short term exposure (12 days) | Midterm exposure (24 days) | Long term exposure (36 days) |
|--------|----------------------------------|-------------------------------|----------------------------|------------------------------|
| 1      | Control                          | 100 ± 0                       | 83.5 ± 0.71                | 77.5 ± 2.12                  |
| 2      | 0.5                              | 100 ± 0                       | 90.5 ± 0.71 <sup>b</sup>   | 54 ± 0.00 <sup>a</sup>       |
| 3      | 1.0                              | 100 ± 0                       | 92 ± 0.00 <sup>b</sup>     | 53 ± 1.41 <sup>a</sup>       |
| 4      | 1.5                              | 100 ± 0                       | 90 ± 0.00 <sup>c</sup>     | 79 ± 1.41                    |
| 5      | 2.0                              | 100 ± 0                       | 93 ± 1.41 <sup>a</sup>     | 84 ± 0.00 <sup>c</sup>       |

Values are expressed in % and are displayed as mean ± SD. The superscripts a, b, c and d indicate statistical mean differences at  $p < 0.0001$ , 0.001, 0.01 and 0.05 respectively.

with the chronic accumulation of PLA particles and their degradation products in worm tissues, which progressively impairs reproductive organ function, energy allocation, and overall physiological condition. Gutiérrez-Rial et al. (2025) confirmed that PLA bioplastic exposure produces reproductive effects comparable to conventional plastics in medium-term earthworm studies, with decreased cocoon production and reduced juvenile numbers observed, validating the reproductive toxicity of PLA over sustained exposure. Zhang et al. (2023) similarly reported that PLA BMP exposure alone had significant negative effects on *E. fetida* offspring numbers, confirming that reproductive suppression is a primary long-term consequence of PLA microplastic exposure in earthworms. Furthermore, Ding et al. (2021) noted that the reproductive toxicity of PLA microplastics at high concentrations in soil invertebrates is attributable to the combined effects of physical gut obstruction and chemical disruption of endocrine and metabolic processes involved in gametogenesis and cocoon production.

### Survival Rate

#### Short-term Exposure (12 days)

Survival rate (Table 6) was 100% across all PLA concentrations during the 12-day exposure period matching with control, confirming that PLA microplastics do not induce acute lethality in *E. fetida* at the tested concentrations. This finding is consistent with the broader ecotoxicological literature on PLA, which generally characterises its toxicity as sub-lethal and chronic rather than acutely lethal. Liwarska-Bizukojc (2025) reported that the mortality and body mass of *Eisenia andrei* were unaffected even at PLA MP concentrations as high as 12.5% (w/w) in short-term studies, further confirming the low acute lethality of PLA to earthworms. However, the full survival at 12 days should not be interpreted as an absence of toxicity, as the biochemical disruptions in SOD, CAT, and AChE already documented at this time point indicate that sub-lethal oxidative and neurotoxic stress is active from the earliest stages of PLA exposure.

#### Mid-term Exposure (24 days)

Survival rate (Table 6) was lowest ( $90 \pm 0.00$ ) at 1.5 g/kg and highest ( $93 \pm 1.41$ ) at 2.0 g/kg during mid-term PLA exposure. The control survival rate was lesser ( $83.5 \pm 0.71$ ) compared to the treated ones might be because of no triggering by any other external stresses as opposite to PLA treated ones. The onset of mortality at 1.5 g/kg at 24 days indicates that this concentration represents a threshold of cumulative toxicological significance during the mid-term period, where the combined effects of oxidative stress, gut damage, and neural disruption begin to compromise the worm's capacity for survival. Zhang et al. (2023) confirmed that PLA BMP exposure alone produced a sustained increase in *E. fetida* mortality reaching 16.7% over the exposure period, demonstrating that PLA microplastics, without co-contaminants, are capable of inducing mortality in earthworms under laboratory conditions. The higher survival at 2.0 g/kg compared to 1.5 g/kg at this time point may reflect an adaptive response at the highest dose, possibly involving reduced particle ingestion or an initial physiological tolerance response, which paradoxically provides some protection against lethality at the highest concentration at this stage.

### *Long-term Exposure (36 days)*

Survival rate (Table 6) was lowest ( $53 \pm 1.41$ ) at 1.0 g/kg and highest ( $84 \pm 0$ ) at 2.0 g/kg in PLA-treated worms at 36 days. The control worms recorded a survival rate of  $77.5 \pm 2.12$  which seems a normal rate as it is under no stress and no physiological adaptations. The pattern of lower survival at intermediate concentrations during long-term exposure may reflect the metabolic cost of sustained antioxidant and detoxification responses — at 1.0 g/kg, the worm expends significant energy maintaining biochemical defence without the behavioural compensation that appears to emerge at 2.0 g/kg. Wang et al. (2022) reported that earthworms show physical and physiological adaptation to microplastic exposure during long-term exposure, including modified ingestion behaviour, consistent with the survival recovery observed at 2.0 g/kg at 36 days. Qi et al. (2020) additionally noted that the progressive metabolisation of PLA degradation products over time may partially reduce the chemical toxicological burden at higher concentrations, contributing to the relative maintenance of survival in the long-term high-dose group.

### *Histopathology*

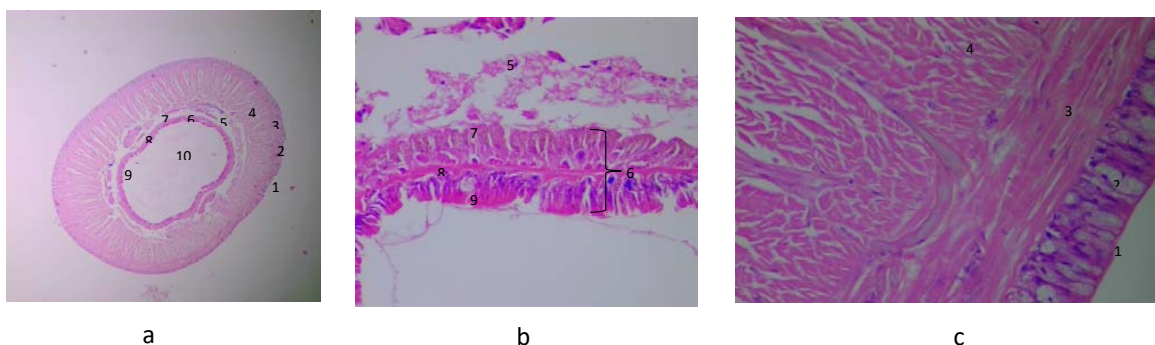
#### *Short-term Exposure (12 days)*

Histological analysis (Plate I & II) of PLA-treated *E. fetida* at 2.0 g/kg at 12 days revealed slight cuticle thinning and moderate epithelial cell injury when compared to control. Although mild, these structural alterations confirm that PLA particle ingestion and contact with gut tissue produce measurable tissue-level changes even within the first 12 days of exposure. Han et al. (2023) reported that PLA induces less severe histological changes in *E. fetida* compared to non-biodegradable plastics because of its biodegradable nature and the relatively lower toxicity of its primary degradation product, lactic acid, yet histological disruption was still clearly evident even in PLA-only exposures. The epithelial damage observed at 12 days is mechanistically consistent with the elevated SOD and CAT activities at this time point, as ROS-mediated oxidative damage is a well-established driver of cellular membrane disruption and gut epithelial injury in earthworms exposed to microplastics (Parolini et al., 2024). These early structural changes are biologically significant because disruption of the gut epithelium compromises nutrient absorption and gastrointestinal barrier integrity, laying the physiological foundation for the growth and reproductive impairments that emerge progressively with continued PLA exposure.

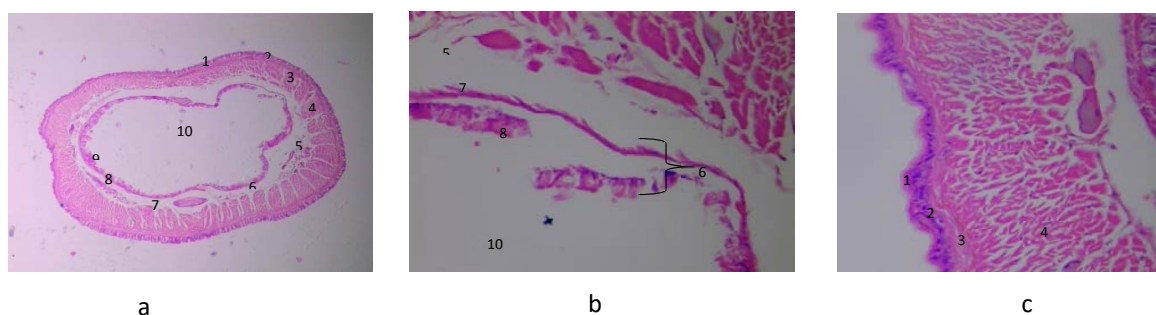
#### *Mid-term Exposure (24 days)*

When compared to control, at 24 days (Plates III & IV), PLA-treated worms at 0.5 g/kg exhibited mild changes in the muscle layer and nerve fibres, consistent with their elevated AChE activity at this concentration and confirming that even low-dose PLA produces neural and muscular stress at the tissue level during mid-term exposure. Han et al. (2023) attributed PLA-induced transient neural stress in *E. fetida* to the entry of lactic acid and other early-stage degradation products into neural tissue, consistent with the mild nerve fibre changes documented here at low concentrations. At 2.0 g/kg, mid-term PLA exposure produced minimal epithelial thinning, mild gut wall inflammation, and moderate changes in chloragogen cells. The involvement of chloragogen tissue at this stage is toxicologically significant: as the primary site of metabolic regulation, detoxification, and lipid storage in earthworms, disruption of chloragogen cells directly impairs the worm's capacity to manage oxidative stress and maintain energy balance. Zhang et al. (2023) reported intestinal damage and oxidative tissue damage in *E. fetida* exposed to PLA BMPs alone, with histological changes becoming progressively more evident across the exposure period, consistent with the mid-term chloragogen and epithelial alterations observed in the present study.

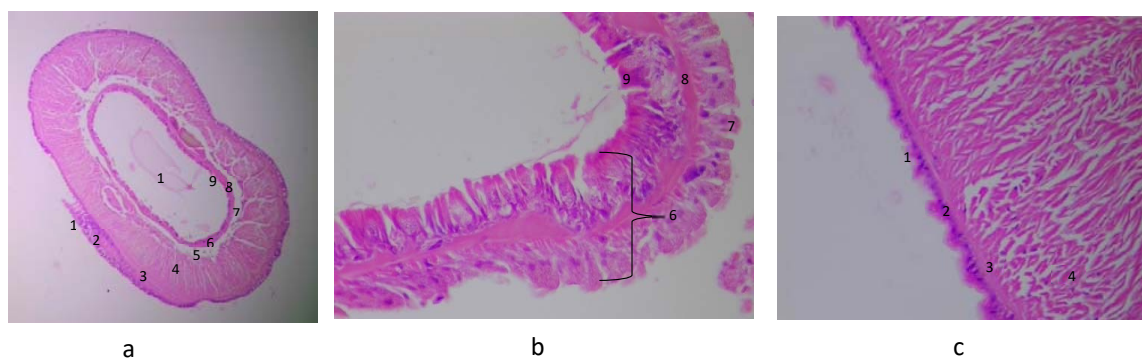
## PLATE -I



Images of transverse section of control *E. fetida* - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification



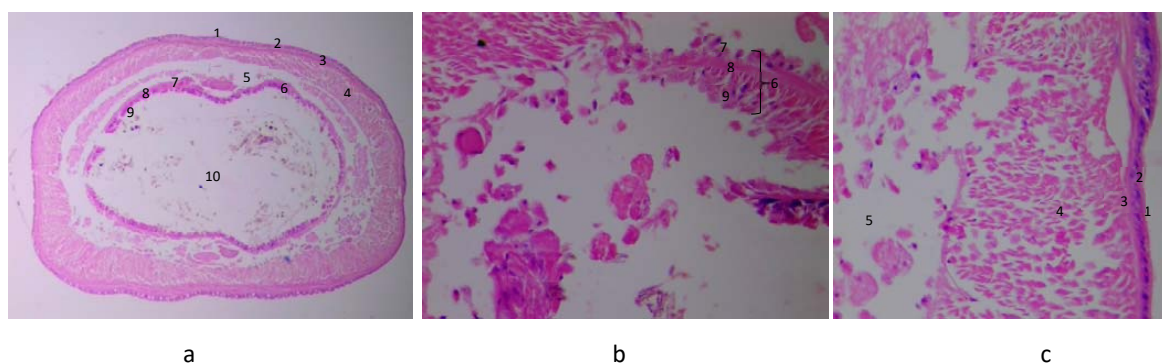
Images of transverse section of *E. fetida* exposed to MP – PLA at 0.5g/kg concentration for 12 days - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification.



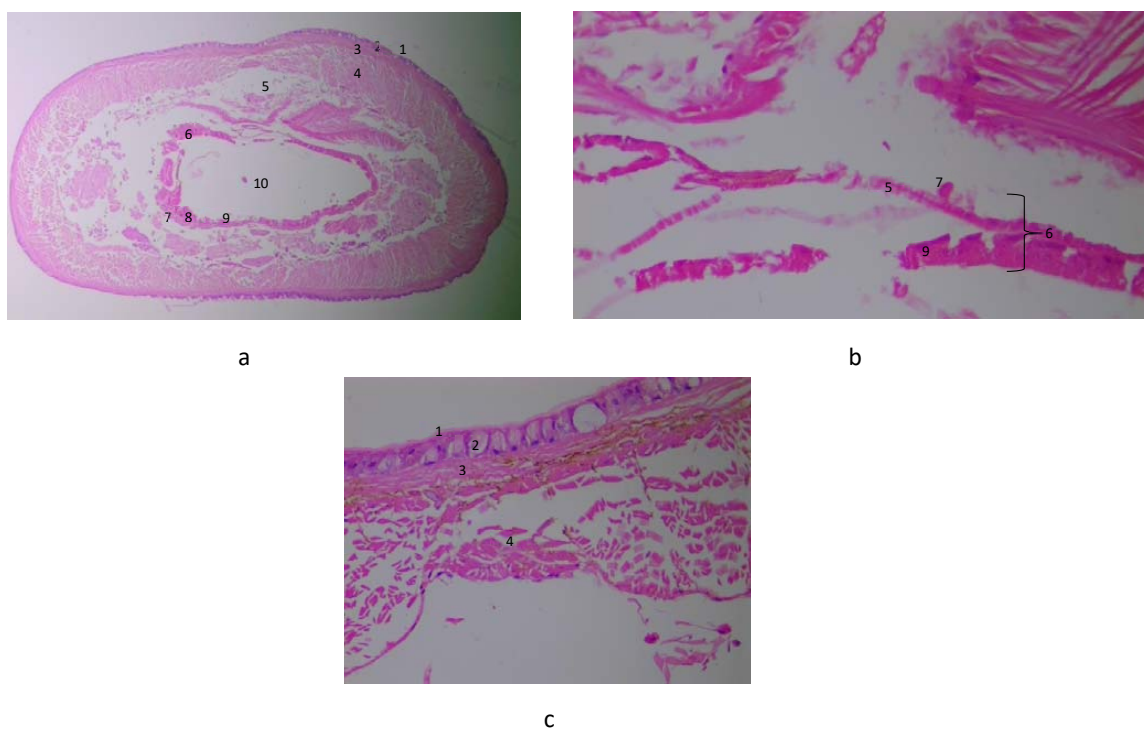
Images of transverse section of *E. fetida* exposed to MP – PLA at 1.0g/kg concentration for 12 days - (a) represents image at 4x magnification. (b) and (c) represent different views at 40X magnification.

**Note:** 1 - cuticle, 2 – Epithelial Layer, 3- circular muscle layer, 4 – Longitudinal muscle layer, 5 – coelomic cavity, 6 – Gastrointestinal tract, 7 – Chloragogen tissue, 8 – Muscle layer, 9 – Intestinal villi and 10 - Lumen

## PLATE - II



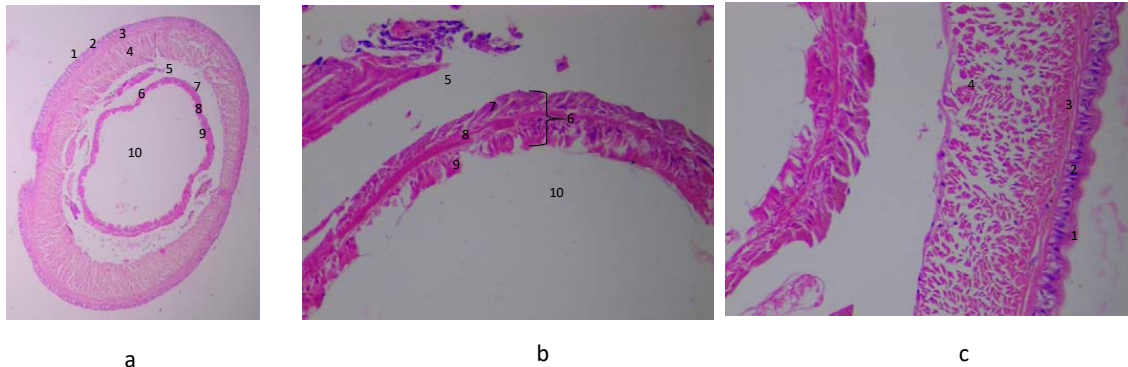
Images of transverse section of *E. fetida* exposed to MP – PLA at 1.5g/kg concentration for 12 days - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification.



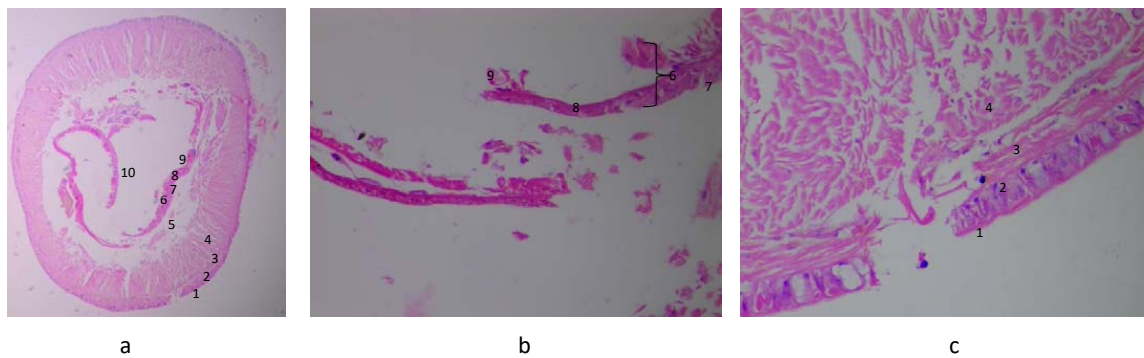
Images of transverse section of *E. fetida* exposed to MP – PLA at 2.0g/kg concentration for 12 days - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification.

**Note:** 1 - cuticle, 2 – Epithelial Layer, 3- circular muscle layer, 4 – Longitudinal muscle layer, 5 – coelomic cavity, 6 – Gastrointestinal tract, 7 – Chloragogen tissue, 8 – Muscle layer, 9 – Intestinal villi and 10 - Lumen

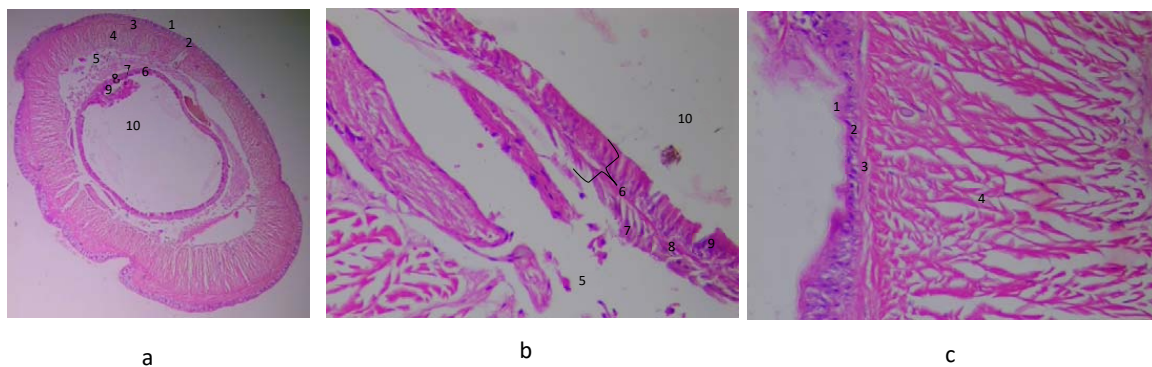
## PLATE - III



Images of transverse section of *E. fetida* exposed to MP – PLA at 0.5g/kg concentration for 24 days - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification.



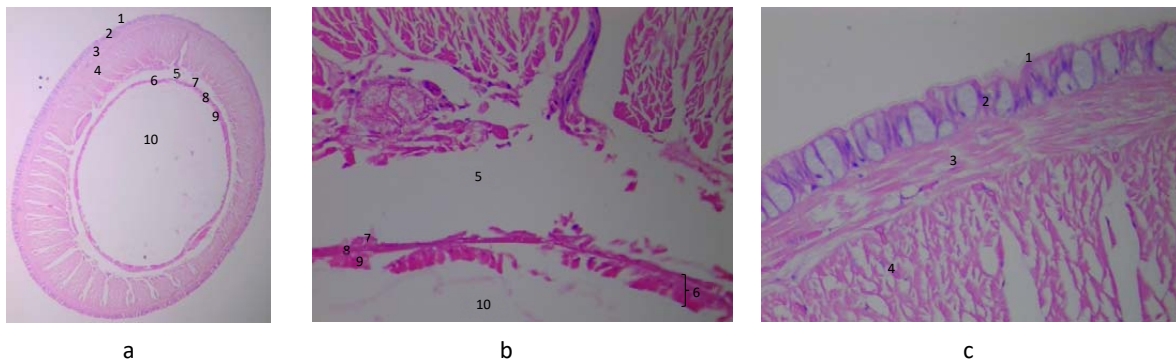
Images of transverse section of *E. fetida* exposed to MP – PLA at 1.0g/kg concentration for 24 days - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification.



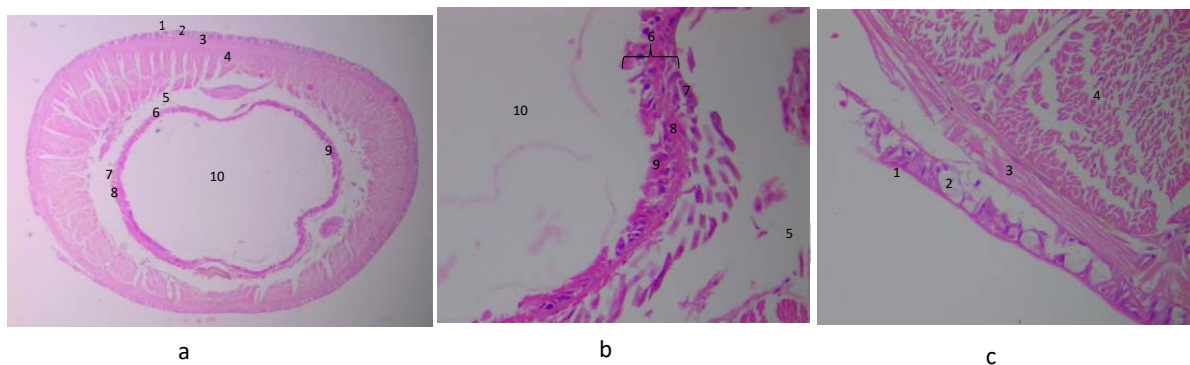
Images of transverse section of *E. fetida* exposed to MP – PLA at 1.5g/kg concentration for 24 days - (a) represents image at 4x magnification. (b) and (c) represent different views at 40X magnification.

**Note:** 1 - cuticle, 2 – Epithelial Layer, 3- circular muscle layer, 4 – Longitudinal muscle layer, 5 – coelomic cavity, 6 – Gastrointestinal tract, 7 – Chloragogen tissue, 8 – Muscle layer, 9 – Intestinal villi and 10 - Lumen

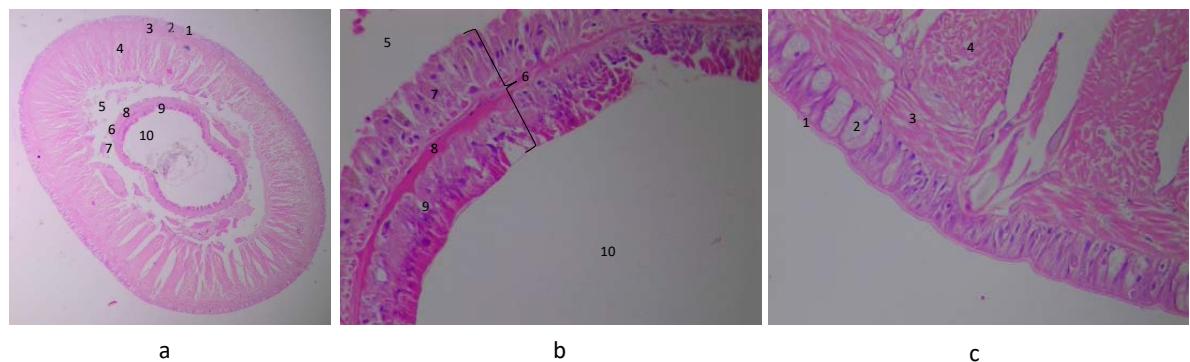
## PLATE - IV



Images of transverse section of *E. fetida* exposed to MP – PLA at 2.0g/kg concentration for 24 days - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification.



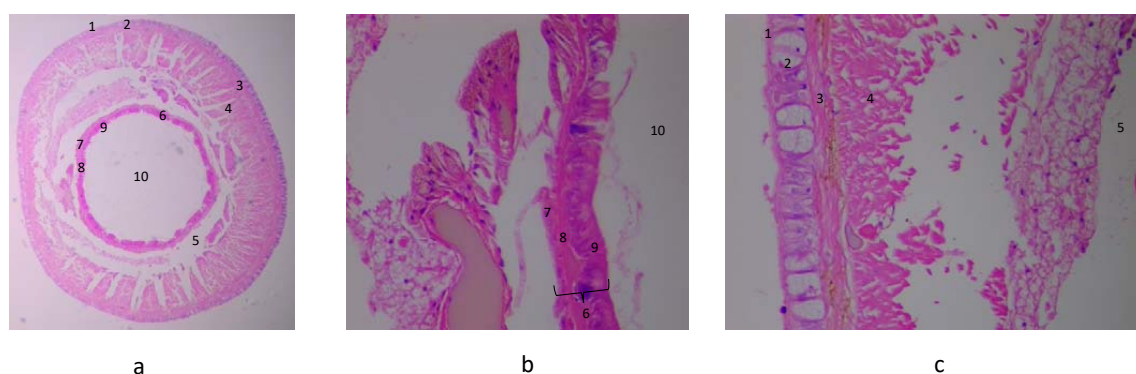
Images of transverse section of *E. fetida* exposed to MP – PLA at 0.5g/kg concentration for 36 days - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification.



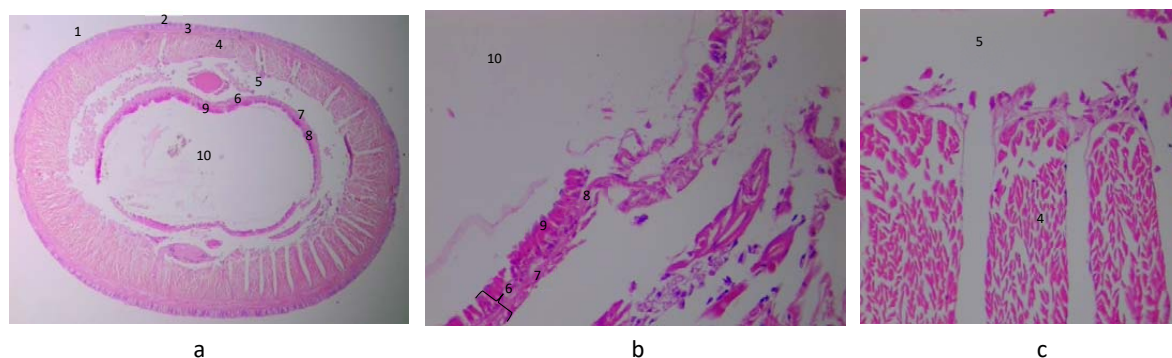
Images of transverse section of *E. fetida* exposed to MP – PLA at 1.0g/kg concentration for 36 days - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification.

**Note:** 1 - cuticle, 2 – Epithelial Layer, 3- circular muscle layer, 4 – Longitudinal muscle layer, 5 – coelomic cavity, 6 – Gastrointestinal tract, 7 – Chloragogen tissue, 8 – Muscle layer, 9 – Intestinal villi and 10 - Lumen

## PLATE - V



Images of transverse section of *E. fetida* exposed to MP – PLA at 1.5g/kg concentration for 36 days - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification.



Images of transverse section of *E. fetida* exposed to MP – PLA at 2.0g/kg concentration for 36 days - (a) represents image at 4x magnification, (b) and (c) represent different views at 40X magnification.

**Note:** 1 - cuticle, 2 – Epithelial Layer, 3- circular muscle layer, 4 – Longitudinal muscle layer, 5 – coelomic cavity, 6 – Gastrointestinal tract, 7 – Chloragogen tissue, 8 – Muscle layer, 9 – Intestinal villi and 10 - Lumen

### Long-term Exposure (36 days)

At 36 days as compared to control (Plate IV-VI), PLA-treated worms at 1.0 g/kg exhibited moderate epithelial disruption and partial loss of chloragogen cells, mechanistically consistent with the sustained elevated SOD response and intermediate CAT activity at this concentration, reflecting ongoing oxidative damage to metabolically active tissues. Han et al. (2023) confirmed that PLA induces progressive dose-dependent histological changes in *E. fetida* across extended exposures, with chloragogen and epithelial tissue remaining primary targets of PLA-associated oxidative damage. At 2.0 g/kg, PLA-exposed worms at 36 days showed moderate muscle damage, yet survival was maintained — a finding that reflects the adaptive capacity of *E. fetida* under chronic microplastic exposure. Han et al. (2023) reported that earthworms exposed to PLA microplastics in combination with other contaminants develop adaptive physiological responses over long-term exposure, including compensatory cellular repair mechanisms, which may explain the maintenance of survival despite measurable histological damage at the highest PLA concentration. The collective histological evidence across all three time points confirms that PLA microplastics produce a progressive, dose- and time-dependent pattern of tissue injury in *E. fetida*, targeting the gut epithelium, chloragogen cells, muscle layer, and nerve fibres in a manner that is biologically coherent with the biochemical and physiological endpoints observed

throughout this study. These structural impairments underpin the reduced reproductive output, altered growth trajectories, and modified survival patterns documented across the exposure period, collectively supporting the conclusion that PLA microplastics pose a genuine sub-lethal ecotoxicological risk to soil-dwelling earthworms and warrant careful consideration in environmental risk assessments of biodegradable plastic alternatives.

## CONCLUSION

The findings of the present study clearly show that despite being biodegradable and environmentally friendly plastic material, PLA microplastic exert some negative effects on soil biota. Various biochemical and physiological changes were induced in *Eisenia fetida* worms exposed to the different concentration of PLA microplastics for 12, 24 and 36 days. The alterations in the activity of the SOD and CAT enzymes were indicative of oxidative stress, confirming the occurrence of oxidative stress in worms exposed to PLA. Change in the activity of another enzyme – AChE – indicates possible neurotoxicity of PLA on earthworms. In addition, growth retardation was observed at some concentrations of the tested substance. Furthermore, the reduced reproductive capacity of the worms suggests that a continued presence of PLA in field soils would likely have a negative impact on worm populations in those soils. Worms were alive at all concentrations used in the short-term test. However, survival decline was observed after prolonged exposure to some concentrations. Tissue damage was observed as alterations in gut wall, muscle layers and chloragogen cells. Concentration-dependent and dose-related effects were also observed here. The data we have derived from this study challenge the assumption that biodegradable plastics are inherently safe for soil ecosystems, and that PLA fragments can persist in soils long enough to cause measurable sub-lethal harm to ecologically important organisms like earthworms. Their actual impact in soil environments deserves far more attention before advocating biodegradable plastic as green alternatives in large scale.

## GRANT SUPPORT DETAILS

The present research did not receive any financial support.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

## LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

## REFERENCES

- Aebi, H. (1984). Catalase. *Methods in Enzymology*, 105, 121–126.
- Angmo, D., Dutta, R., Singh, J., Chowdhary, A. B., Quadar, J., & Vig, A. P. (2023). Biochemical responses, growth and reproduction of earthworm in low density polyethylene (LDPE). *Environmental Quality Management*, 33(2), 223–237.
- Baihetiyaer, B., Jiang, N., Li, X., He, B., Wang, J., Fan, X., Sun, H., & Yin, X. (2023). Oxidative stress and gene expression induced by biodegradable microplastics and imidacloprid in earthworms (*Eisenia fetida*) at environmentally relevant concentrations. *Environmental Pollution*, 323, 121285.

- Baihetiyaer, B., Jiang, N., Li, X., Song, J., Wang, J., Fan, X., Zuo, Y., & Yin, X. (2023). Exploring the toxicity of biodegradable microplastics and imidacloprid to earthworms (*Eisenia fetida*) from morphological and gut microbial perspectives. *Environmental Pollution*, 337, 122547.
- Briones, M. J. I., Ostle, N. J., McNamara, N. P., & Poskitt, J. (2009). Functional shifts of grassland soil communities in response to soil warming. *Soil Biology & Biochemistry*, 41(2), 315–322.
- Büks, F., & Kaupenjohann, M. (2020). Global concentrations of microplastics in soils – a review. *Soil*, 6(2), 649–662.
- Cole, M., Lindeque, P., Halsband, C., & Galloway, T. S. (2011). Microplastics as contaminants in the marine environment: A review. *Marine Pollution Bulletin*, 62(12), 2588–2597.
- Corradini, F., Meza, P., Eguiluz, R., Casado, F., Huerta-Lwanga, E., & Geissen, V. (2019). Evidence of microplastic accumulation in agricultural soils from sewage sludge disposal. *Science of the Total Environment*, 671, 411–420.
- de Souza Machado, A. A., Hempel, S., Rillig, M. C., Kloas, W., & Zarfl, C. (2018). Microplastics as an emerging threat to terrestrial ecosystems. *Global Change Biology*, 24(4), 1405–1416.
- de Souza Machado, A. A., Lau, C. W., Till, J., Kloas, W., Lehmann, A., Becker, R., & Rillig, M. C. (2018). Impacts of microplastics on the soil biophysical environment. *Environmental Science & Technology*, 52(17), 9656–9665.
- Ding, L., Luo, T., Wang, X., Ouyang, Z., Lu, G., Zhang, W., Yao, H., & Zhu, L. (2021). Effects of thresholds for plant-derived microplastics on earthworm health and sludge properties during sewage sludge treatment. *Science of the Total Environment*, 802, 149962.
- Ding, W., Li, Z., Qi, R., Jones, D. L., Liu, Q., Liu, Q., & Yan, C. (2021). Effect thresholds for the earthworm *Eisenia fetida*: Toxicity comparison between conventional and biodegradable microplastics. *Science of the Total Environment*, 781, 146884.
- Edwards, C. A., & Bohlen, P. J. (1996). *Biology and ecology of earthworms* (Vol. 3). Springer Science & Business Media.
- Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, 7(2), 88–95.
- Gutiérrez-Rial, D., Represas, D., Carral, E. V., & Rodríguez, T. (2025). Short- and medium-term effects of biodegradable microplastics (PLA and PHB) on earthworm development and reproduction. *Ecotoxicology*. Advance online publication.
- Haider, T. P., Volker, C., Kramm, J., Landfester, K., & Wurm, F. R. (2019). Plastics of the future? The impact of biodegradable polymers on the environment and on society. *Angewandte Chemie International Edition*, 58(1), 50–62.
- Han, Y., Fu, M., Wu, J., Zhou, S., Qiao, Z., Peng, C., Zhang, W., Liu, F., Ye, C., & Yang, J. (2023). Polylactic acid microplastics induce higher biotoxicity of decabromodiphenyl ethane on earthworms (*Eisenia fetida*) compared to polyethylene and polypropylene microplastics. *Science of the Total Environment*, 862, 160909.
- Huerta-Lwanga, E., Mendoza-Vega, J., Ribeiro, O., Gertsen, H., Peters, P., & Geissen, V. (2021). Is the polylactic acid fiber in green compost a risk for *Lumbricus terrestris* and *Triticum aestivum*? *Polymers*, 13(5), Article 703.
- Humason, G. L. (1979). *Animal tissue techniques* (4th ed.). W. H. Freeman.
- Hurley, R., & Nizzetto, L. (2018). Fate and occurrence of micro(nano)plastics in soils: Knowledge gaps and possible risks. *Current Opinion in Environmental Science & Health*, 1, 6–11.
- International Organization for Standardization. (1998). *Soil quality – Effects of pollutants on earthworms – Part 2: Determination of effects on reproduction* (ISO 11268-2).
- Ivar do Sul, J. A., & Costa, M. F. (2014). The present and future of microplastic pollution in the marine environment. *Environmental Pollution*, 185, 352–364.
- Jeong, J., & Choi, J. (2019). Adverse outcome pathways potentially related to hazard identification of microplastics based on toxicity mechanisms. *Chemosphere*, 231, 249–255.
- Lai, H., Li, X., Zhang, M., Liu, Y., Wang, H., & Chen, J. (2024). The comparison effect on earthworms between conventional (polyethylene) and biodegradable (polylactic acid) microplastics. *Heliyon*, 10(24), e41339.
- Lambert, S., & Wagner, M. (2017). Microplastics are contaminants of emerging concern in freshwater environments: An overview. In M. Wagner & S. Lambert (Eds.), *Freshwater microplastics* (pp. 1–23). Springer.
- Liang, Y., He, D., Pan, Y., Wang, Y., Zhang, T., Tao, S., & Peng, J. (2025). Reassessing whether biodegradable microplastics are environmentally friendly: Differences in earthworm physiological responses and soil carbon function impacts. *Antioxidants*, 14(10), 1197.
- Liwarska-Bizukojc, E. (2025). Evaluation of the impact of polylactide microparticles on soil biota. *Environmental Science and Pollution Research*, 32, 21187–21197.

- Misra, H. P., & Fridovich, I. (1972). The role of superoxide anion in the auto-oxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry*, 247, 3170–3175.
- Nizzetto, L., Futter, M., & Langaas, S. (2016). Are agricultural soils dumps for microplastics of urban origin? *Environmental Science & Technology*, 50(20), 10777–10779.
- Organisation for Economic Co-operation and Development. (1984). *Test No. 207: Earthworm, acute toxicity tests*. OECD Publishing.
- Organisation for Economic Co-operation and Development. (2004). *Test No. 222: Earthworm reproduction test (Eisenia fetida/Eisenia andrei)* (OECD Guidelines for the Testing of Chemicals, Section 2). OECD Publishing.
- Parolini, M., De Felice, B., Gazzotti, S., Sugni, M., & Ortenzi, M. A. (2024). Comparison of the potential toxicity induced by microplastics made of polyethylene terephthalate (PET) and polylactic acid (PLA) on the earthworm *Eisenia foetida*. *Environmental Pollution*, 348, 123868.
- Pokarzhevskii, A. D., Van Straalen, N. M., & Semenov, A. M. (2000). Agar as a medium for removing soil from earthworm guts. *Soil Biology and Biochemistry*, 32, 1315–1317.
- Prendergast-Miller, M. T., Katsiamides, A., Abbass, M., Sturzenbaum, S. R., Thorpe, K. L., & Hodson, M. E. (2019). Polyester-derived microfibre impacts on the soil-dwelling earthworm *Lumbricus terrestris*. *Environmental Pollution*, 251, 453–459.
- Qi, R., Jones, D. L., Li, Z., Liu, Q., & Yan, C. (2020). Behavior of microplastics and plastic film residues in the soil environment: A critical review. *Science of the Total Environment*, 703, 134722.
- Sibly, R. M., & Calow, P. (1989). A life-history theory of responses to stress. *Biological Journal of the Linnean Society*, 37(1–2), 101–116.
- Sommer, F., Dietze, V., Baum, A., Sauer, J., Gilge, S., Maschowski, C., & Gieré, R. (2018). Tire abrasion as a major source of microplastics in the environment. *Aerosol and Air Quality Research*, 18(8), 2014–2028.
- Spurgeon, D. J., Svendsen, C., Hankard, P. K., & Weeks, J. M. (2003). Review of sublethal endpoints for earthworm ecotoxicological assessment. *European Journal of Soil Biology*, 39(2), 201–210.
- Suleiman, H., Rorat, A., Grobelak, A., Grosser, A., Milczarek, M., Plytycz, B., Kacprzak, M., & Vandenbulcke, F. (2017). Determination of the performance of vermicomposting process applied to sewage sludge by monitoring of the compost quality and immune responses in three earthworm species. *Bioresource Technology*, 241, 103–112.
- Teng, Y., Zhang, X., & Zhou, W. (2025). Environmental impacts of polylactic acid microplastics on soil and aquatic ecosystems: A comprehensive review. *Journal of Environmental Sciences*. Advance online publication.
- Wang, F., Wang, Q., Adams, C. A., Sun, Y., & Zhang, S. (2021). Polylactic acid and polypropylene carbonate microplastics affect growth and reproduction in earthworm *Eisenia fetida*. *Science of the Total Environment*, 791, 148206.
- Wang, H., Zhang, L., Guo, X., & Ren, J. (2022). Histopathological effects and oxidative stress induced by mixed microplastics in earthworm *Eisenia fetida*. *Ecotoxicology and Environmental Safety*, 239, 113657.
- Weithmann, N., Möller, J. N., Löder, M. G. J., Piehl, S., Laforsch, C., & Freitag, R. (2018). Organic fertilizer as a vehicle for the entry of microplastic into the environment. *Science Advances*, 4(4), eaap8060.
- Yang, Y., Liu, W., Zhang, Z., Grossart, H.-P., & Gadd, G. M. (2021). Comparative toxicity of biodegradable and conventional plastics to earthworms. *Journal of Hazardous Materials*, 401, 123429.
- Zhang, S., Ren, S., Pei, L., Sun, Y., & Wang, F. (2023). Toxicological effects of polyethylene and polylactic acid microplastics on earthworms. *Chinese Journal of Applied and Environmental Biology*, 29(2), 322–327.
- Zhu, D., Yang, K., Gul, Y., Song, W., Zhang, X., & Wang, W. (2014). Effect of photoperiod on growth and gonadal development of juvenile topmouth gudgeon (*Pseudorasbora parva*). *Environmental Biology of Fishes*, 97, 147–156.