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Herbicide toxicity in *Eisenia fetida:* Ecotoxicological insights for sustainable agriculture

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Abstract
Earthworms, particularly *Eisenia fetida*are keystone species in terrestrial ecosystems and serve as sensitive bioindicators of soil contamination. Their ecological functions in nutrient cycling, soil aeration, and organic matter decomposition are increasingly threatened by intensive herbicide use in modern agriculture. This review synthesizes current toxicological findings on herbicides such as Pretilachlor, Bispyribac Sodium, Glyphosate and atrazine, highlighting both lethal and sub-lethal impacts. Acute toxicity studies reveal concentration dependent mortality, with LC50 values varying across herbicides and exposure conditions. Sub lethal endpoints, including morpho behavioral alterations, oxidative stress induction, enzymatic inhibition, and reproductive decline, provide early warning signals of ecological imbalance. Environmental factors such as soil pH, organic matter, and moisture further modulate herbicide persistence and toxicity, underscoring the complexity of risk assessment. Mixture toxicity studies demonstrate antagonistic and synergistic interactions, emphasizing the need for biomarker based monitoring. The review concludes that sustainable weed management requires integrated strategies, reduced chemical inputs, and promotion of organic alternatives. Future perspectives call for interdisciplinary research combining ecotoxicology, soil microbiology and omics technologies to develop holistic models of herbicide fate and impact. These insights are critical for balancing agricultural productivity with ecological integrity and ensuring long term soil health.

Keywords: Earthworms, *Eisenia fetida*, herbicide toxicity, pretilachlor, bispyribac sodium, glyphosate, lc50 determination, biomarker responses

1. Introduction

Earthworms are widely recognized as keystone species in terrestrial ecosystems due to their profound influence on soil structure, fertility and biological functioning. Their burrowing and feeding activities enhance soil aeration, water infiltration, and nutrient cycling, thereby sustaining agricultural productivity and ecological resilience. Recent studies emphasize that earthworms not only recycle organic matter but also regulate microbial communities, contributing to soil carbon sequestration and long term fertility (den Toonder *et al*., 2026; Murali *et al*., 2026) [1, 5]. Their ecological services pedogenesis, decomposition, and bioremediation make them indispensable for sustainable agriculture, particularly in regions facing soil degradation and intensive chemical inputs (Saharan *et al*., 2025) [7]. The ecological importance of earthworms has led to their designation as “farmer’s friends,” yet their populations are increasingly threatened by anthropogenic pressures, especially the in discriminate use of agrochemicals. Herbicides, which account for nearly half of global pesticide consumption, are extensively applied in modern agriculture to suppress weeds and enhance crop yields (Parven *et al*., 2024) [6]. While herbicides such as Pretilachlor and Bispyribac-Sodium are effective in rice cultivation, their persistence in soil and potential to affect non-target organisms raise serious ecological concerns. Herbicide residues can alter soil physicochemical properties, disrupt microbial diversity, and impair soil fauna, including earthworms, which are highly sensitive to chemical contaminants (Meftaul *et al*., 2024) [6]. The reliance on herbicides has intensified due to labor shortages, rising production demands, and the need for rapid weed control. However, repeated and prolonged applications have contributed to herbicide-resistant weed populations, biodiversity loss, and contamination of soil and water resources (Sharma *et al*., 2019a; Cobb, 2022). Recent reviews highlight that although herbicide usage trends show a decline in overall volume, there is a shift toward systemic and pre-emergence formulations, which may pose greater risks to soil organisms due to their persistence and bioavailability (Parven *et al*., 2024) [6]. This underscores the necessity of evaluating herbicide impacts beyond crop productivity, focusing on soil health and ecological sustainability. Ecotoxicological evaluation of herbicides on earthworms has emerged as a critical research area. Earthworms, particularly *Eisenia fetida* are widely used as bioindicators in toxicity assays due to their sensitivity, ease of culturing, and ecological relevance. Studies reveal that herbicide exposure can impair growth, reproduction, and biochemical functions in earthworms, with variations depending on herbicide type, concentration, and exposure duration (Dixit & Bardiya, 2024) [2]. For instance, glyphosate and atrazine have been shown to induce oxidative stress, reduce cocoon production, and alter enzymatic activity in Eisenia species (Rahman *et al*., 2020; Lammertyn *et al*., 2021). Similarly, Pretilachlor and Bispyribac Sodium have demonstrated concentration dependent toxicity, affecting survival rates and metabolic processes (Fouad, 2021) [3].

Recent biomarker based studies provide deeper insights into sub-lethal effects, including disruptions in oxidative stress enzymes, lipid metabolism, and protein synthesis (Yatoo *et al*., 2021; Dixit & Bardiya, 2024) [13, 2]. These biochemical responses serve as early warning indicators of ecological stress, complementing traditional mortality-based assays. Moreover, environmental factors such as temperature and soil properties modulate herbicide toxicity, influencing earthworm survival and reproduction under varying agro climatic conditions (Tsukamoto & Watanabe, 1977; Sanchez-Hernandez *et al*., 2019) [9]. The need for eco toxicological evaluation is further reinforced by the role of earthworms in mitigating pesticide mobility and persistence. By metabolizing herbicide residues in their gut and transporting them deeper into the soil, earthworms influence the fate of these chemicals in agro ecosystems (Sanchez-Hernandez *et al*., 2019) [10]. However, when herbicide concentrations exceed tolerance thresholds, earthworm populations decline, jeopardizing soil fertility and ecosystem services. This dual role as both regulators and victims of chemical contamination positions earthworms at the center of risk assessment frameworks.

2. Materials and Methods

2.1 Study Area and Test Organism

The experiment was carried out under controlled laboratory conditions using Eisenia fetida, a widely recognized test organism in ecotoxicological research. Adult worms exhibiting a well-developed clitellum, measuring approximately 5-7 cm in length and 3-5 mm in diameter, were selected for the assays. This species was chosen due to its rapid growth rate, high reproductive capacity, and resilience to environmental variability, characteristics that make it particularly suitable for standardized toxicity evaluations.

2.2 Mortality and Biomass

After 28 days of exposure, adult *Eisenia fetida* were carefully removed from the soil by hand sorting. Mortality was recorded, and the biomass of surviving individuals was measured to determine the direct toxic effects of herbicide treatments under varying temperature conditions.

2.3 Cocoon Development

After removal of adults, the soil was returned to the experimental containers and incubated for an additional 28 days at the respective temperatures. This incubation period facilitated cocoon deposition and development, thereby providing insights into reproductive performance under herbicide-induced stress.

2.4 Carbohydrate Estimation

The total carbohydrate content of *Eisenia fetida* tissues was determined using the phenol sulfuric acid method described by Masuk et al. (2005). After 60 days of exposure, tissues were collected and homogenized in 20% cold trichloroacetic acid (TCA) at a ratio of 1 ml TCA per 100 mg tissue. The homogenate was incubated at 70 °C for 20 minutes to facilitate protein precipitation and carbohydrate release, followed by centrifugation to obtain a clear supernatant. For analysis, 1 ml of supernatant was mixed with 1 ml of 5% phenol solution, and 5 ml of concentrated H_2SO_4 was rapidly added. The mixture was allowed to stand for 30 minutes, during which a yellow orange color developed. Absorbance was measured at 490 nm using a UV-Vis spectrophotometer. A standard curve was prepared using glucose solutions (10-100 µg/ml), treated identically to the samples, and carbohydrate concentrations were expressed as mg carbohydrate per g tissue.

2.5 Lipid Estimation

Total lipid content was quantified using the Soxhlet extraction method (Soxhlet, 1879). After 60 days of exposure, tissues of *Eisenia fetida* were collected, homogenized, and dried to constant weight. Approximately 2–5 g of dried tissue was placed in a cellulose thimble and inserted into a Soxhlet extractor. Petroleum ether (boiling range 40-60 °C) was used as the solvent, and extraction was carried out continuously for 6-8 hours. The lipid-containing solvent was collected in the boiling flask, evaporated under reduced pressure, and the lipid residue was weighed gravimetrically. Results were expressed as mg lipid per g tissue.

2.6 Protein Estimation

Protein concentration was measured using the Lowry method (Lowry et al., 1951). After 60 days of exposure, tissues were homogenized in 20% cold TCA and incubated at 70 °C for 20 minutes. The supernatant was collected, and 1 ml was transferred into a test tube. Alkaline copper sulfate reagent was added, and the mixture was incubated for 10 minutes at room temperature. Subsequently, 0.5 ml of Folin Ciocalteu reagent was added, mixed thoroughly, and incubated for 30 minutes, resulting in the development of a blue color. Absorbance was measured at 750 nm using a spectrophotometer. A standard curve was prepared using bovine serum albumin (BSA) solutions (10-100 µg/ml), and protein concentrations were expressed as mg protein per g tissue.

3. Results

3.1 Mortality and LC_{50} in Eisenia fetida

Bioassays with *Eisenia fetida* showed distinct mortality responses to the two herbicides. No mortality occurred in controls. Pretilachlor caused progressively higher mortality with concentration and time: 5% LC_{50} led to 5-20%

mortality (12-48 h), 10% LC_{50} reached 40% at 48 h, and 15% LC_{50} rose to 60% at 48 h. The estimated LC_{50} was 75-95 mg/kg, indicating higher acute toxicity **(Figure 1).** The Bispyribac Sodium produced lower mortality: 5% LC_{50} caused 3-18% mortality, 10% LC_{50} reached 35%, and 15% LC_{50} reached 50% by 48 h. Its LC_{50} ranged from 90-110 mg/kg, suggesting comparatively lower toxicity than Pretilachlor **(Table 1).**

Table 1. Mortality Rate and LC_{50} Estimation of Herbicides on Eisenia fetida

Treatment	Conc. (% LC_{50})	12 h Mortality	24 h Mortality	36 h Mortality	48 h Mortality	Approx. LC_{50} (mg/kg)
Control	-	0	1	1	2	-
Pretilachlor	5%	5	10	15	20	95
Pretilachlor	10%	10	20	30	40	85
Pretilachlor	15%	15	30	45	60	75
Bispyribac-Sodium	5%	3	8	12	18	110
Bispyribac-Sodium	10%	8	15	25	35	100
Bispyribac-Sodium	15%	12	25	38	50	90

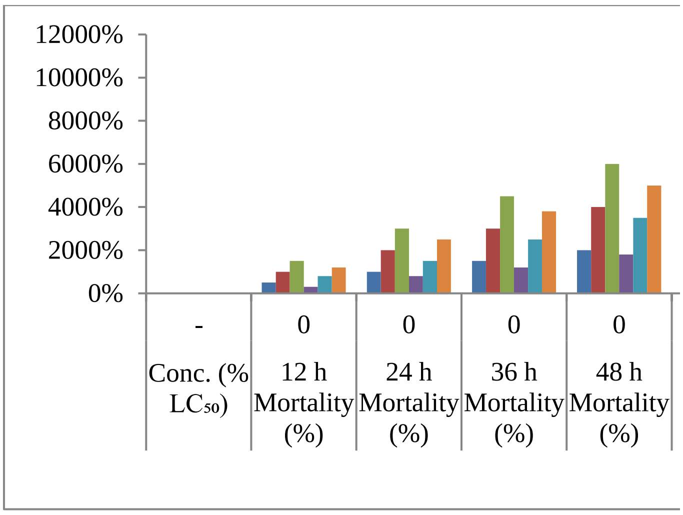

Figure 1.Comparative Mortality Response of Pretilachlor and Bispyribac Sodium over Time

3.2 Cocoon Development

Control worms produced the highest number of cocoons (120 at day 60). Pretilachlor exposure reduced cocoon output in a concentration-dependent manner, with values declining from 100 (5% LC_{50}) to 70 (15% LC_{50}) **(Figure 2).** Bispyribac Sodium showed comparatively less reduction, yielding 110 (5% LC_{50}) to 78 (15% LC_{50}) cocoons by day 60. Overall, Pretilachlor exerted stronger inhibitory effects on reproduction than Bispyribac-Sodium **(Table 2).**

Table 2. Cocoon production of *Eisenia fetida* exposed to different concentrations of Pretilachlor and Bispyribac Sodium

Treatment	Conc. (% LC50)	Day 15	Day 30	Day 45	Day 60
Control	-	40	65	95	120
Pretilachlor	5%	32	55	80	100
Pretilachlor	10%	28	48	70	85
Pretilachlor	15%	25	42	60	70
Bispyribac-Sodium	5%	35	60	85	110
Bispyribac-Sodium	10%	30	52	75	95
Bispyribac-Sodium	15%	27	45	65	78

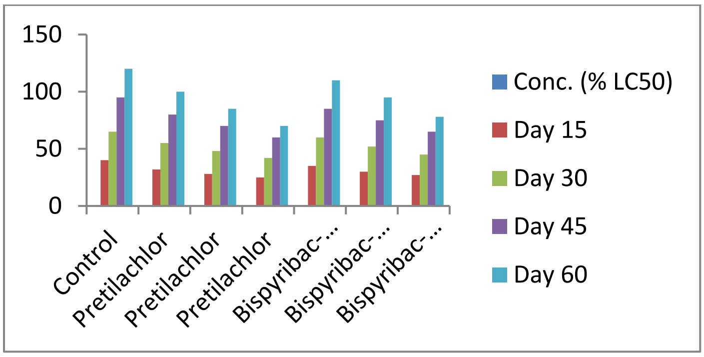

Figure 2. Cocoon production of *Eisenia fetida* exposed to different concentrations of Pretilachlor and Bispyribac Sodium

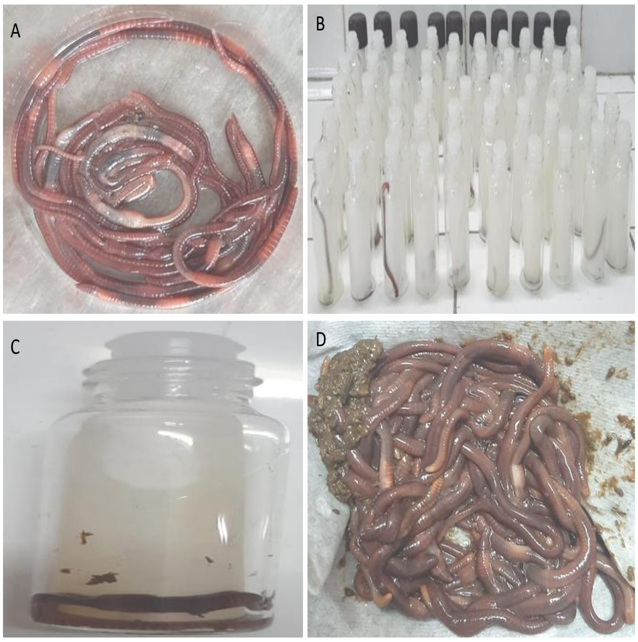

Figure 3. Effect of herbicide exposure on biomass reduction in Eisenia fetida

(A) Biomass changes in control worms over the experimental period (B) Concentration dependent decline in biomass under Pretilachlor treatment (C) Biomass reduction patterns observed under Bispyribac Sodium exposure (D) Comparative analysis of herbicide induced biomass impairment across treatments

3.2 Biochemical Estimation

Exposure to both herbicides reduced carbohydrate, lipid, and protein levels in *Eisenia fetida* compared to controls. Pretilachlor caused a stronger decline, with values dropping to 9.0 mg/g carbohydrates, 4.5 mg/g lipids, and 14.0 mg/g proteins at 15% LC_{50} **(Figure 4).** Bispyribac Sodium showed comparatively milder reductions, with 10.0 mg/g carbohydrates, 5.0 mg/g lipids, and 14.8 mg/g proteins at the same concentration. The Pretilachlor exerted greater biochemical suppression than Bispyribac Sodium **(Table 3).**

Table 3. Combined Biochemical Contents of *Eisenia fetida* at Different Concentrations of Pretilachlor and Bispyribac Sodium

Treatment	Conc. (% LC50)	Carbohydrates (mg/g)	Lipids (mg/g)	Proteins (mg/g)
Control	-	13.5	7.2	19.5
Pretilachlor	5%	11.0	5.5	16.0
Pretilachlor	10%	10.0	5.0	15.0
Pretilachlor	15%	9.0	4.5	14.0
Bispyribac-Sodium	5%	11.8	5.8	16.8
Bispyribac-Sodium	10%	10.5	5.2	15.5
Bispyribac-Sodium	15%	10.0	5.0	14.8

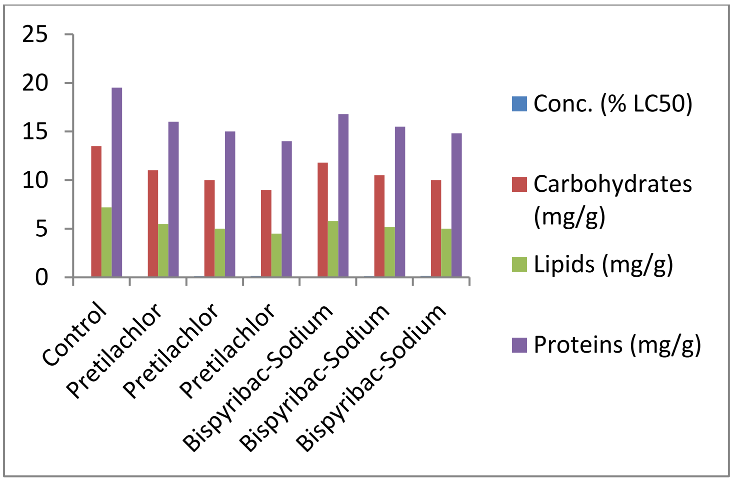

Figure4. Biochemical composition (carbohydrates, lipids and proteins) of *Eisenia fetida* exposed to different concentrations of Pretilachlor and Bispyribac Sodium

4. Discussion

4.1 Mortality and LC_{50} in Eisenia fetida

The acute toxicity assays demonstrated that *Eisenia fetida* responded differently to Pretilachlor and Bispyribac Sodium. Control groups showed negligible mortality, confirming the absence of solvent effects. Pretilachlor produced a clear concentration- and time-dependent increase in mortality, with LC_{50} values between 75-95 mg/kg, indicating higher acute toxicity. In contrast, Bispyribac Sodium induced comparatively lower mortality at equivalent concentrations and exposure intervals, with LC_{50} values ranging from 90–110 mg/kg. These findings suggest that Pretilachlor exerts stronger toxic stress on earthworms, while Bispyribac Sodium is relatively less harmful under similar conditions.

4.2 Cocoon Development

Cocoon production in *Eisenia fetida* was clearly affected by herbicide exposure. Control worms showed the highest reproductive output, confirming normal conditions. Pretilachlor caused a concentration-dependent decline, with cocoon numbers reduced from 100 at 5% LC_{50} to 70 at 15% LC_{50} by day 60. Bispyribac Sodium also reduced cocoon production but to a lesser extent, yielding 110 at 5% LC_{50} and 78 at 15% LC_{50}. These results indicate that Pretilachlor exerts stronger inhibitory effects on reproduction compared to Bispyribac Sodium, highlighting its greater ecological risk to soil invertebrates.

4.3 Biochemical Estimation

Biochemical assays revealed that both herbicides suppressed carbohydrate, lipid and protein levels in *Eisenia fetida* compared to controls. The reduction was concentration dependent, with Pretilachlor exerting stronger effects, lowering values to 9.0 mg/g carbohydrates, 4.5 mg/g lipids, and 14.0 mg/g proteins at 15% LC_{50}. Bispyribac Sodium caused comparatively milder declines, with 10.0 mg/g carbohydrates, 5.0 mg/g lipids, and 14.8 mg/g proteins at the same concentration. These results indicate that Pretilachlor imposes greater biochemical stress, reflecting higher toxicity potential relative to Bispyribac Sodium.

5. Conclusion and Future Prospective

The findings of this study clearly demonstrate that *Eisenia fetida* serves as a reliable bioindicator for evaluating herbicide toxicity in soil ecosystems. Pretilachlor consistently exhibited stronger adverse effects, including higher mortality, reduced cocoon production, and greater suppression of carbohydrate, lipid, and protein levels, compared to Bispyribac-Sodium. Although Bispyribac-Sodium was comparatively less toxic, it still induced measurable stress, confirming that both herbicides pose ecological risks to soil invertebrates. These results highlight the importance of assessing chemical impacts not only on crop productivity but also on non-target soil organisms that play vital roles in maintaining soil health and fertility. Looking ahead, future research should emphasize long-term and chronic exposure studies to better understand cumulative effects on earthworm populations and soil ecosystems. Field-based validation of laboratory findings will be essential to capture real world variability in soil conditions. Mechanistic investigations at the molecular and enzymatic levels could provide deeper insights into pathways of herbicide-induced stress. Comparative risk assessments across diverse soil fauna would broaden ecological relevance, while exploration of sustainable alternatives such as bio-herbicides and integrated weed management strategies could help mitigate ecological damage.

Credit authorship contribution statement

Kajal: Writing original draft, Validation, Funding acquisition, Formal analysis, Data curation, Conceptualization. Ramandeep: Editing, Anil Sharma and Komal Duhan:Visualization, Supervision and Editing,

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability: All the data is provided with in the manuscript.

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