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Toxicity Assessment of Pretilachlor and Bispyribac Sodium on Eisenia fetida through Acute Reproductive Biochemical and Temperature Dependent Effects

Kajal
Department of Zoology, Baba Mastnath University, Asthal Bohar, Rohtak, Haryana, India
Ramandeep
Department of Zoology, Baba Mastnath University, Asthal Bohar, Rohtak, Haryana, India
Komal Duhan
Department of Zoology, Govt. College Jind, Haryana, India
Anil Sharma
kmaan9341@gmail.com
+91-8708077710

Abstract
Soil ecosystems are fundamental to agricultural sustainability, with earthworms serving as keystone species and reliable bio-indicators of chemical stress. This study evaluated the ecotoxicological effects of two widely used rice field herbicides, Pretilachlor and Bispyribac Sodium on Eisenia fetida under controlled laboratory conditions. Acute toxicity assays revealed that Pretilachlor was more potent, with a lower LC50 (0.085 mg/cm²) compared to Bispyribac Sodium (0.142 mg/cm²), and induced pronounced morpho-behavioral alterations. Growth and reproduction studies demonstrated concentration dependent declines in biomass, cocoon production, juvenile emergence, and hatchability, with Pretilachlor exerting stronger inhibitory effects. Biochemical analyses confirmed metabolic disruptions, including depletion of carbohydrate, lipid, and protein reserves, elevated malondialdehyde (MDA) levels, and suppressed catalase (CAT) activity, indicating oxidative stress and impaired antioxidant defense.

Temperature dependent assays further highlighted that elevated thermal conditions intensified herbicide toxicity, reducing LC50 values and exacerbating mortality and reproductive failure. The Eisenia fetida as a sensitive bioindicator for herbicide toxicity and underscore the ecological risks posed by Pretilachlor and, to a lesser extent, Bispyribac Sodium. The integration of acute, chronic, biochemical and environmental endpoints provides a holistic framework for soil ecotoxicology. The study emphasizes the need for sustainable agricultural practices, including bio-pesticide adoption and integrated pest management, to safeguard soil biodiversity and ecosystem resilience under increasing chemical and climatic stress.

Keywords: Eisenia fetida, Pretilachlor, Bispyribac Sodium, Acute toxicity, Growth and reproduction, Biochemical alterations, Oxidative stress

1.Introduction

Soil ecosystems form the backbone of terrestrial productivity, and their health is directly linked to agricultural sustainability. Among soil macrofauna, earthworms are considered keystone species due to their role in nutrient cycling, organic matter decomposition, and enhancement of soil structure. Their burrowing activity improves aeration and water infiltration, while their digestion of organic residues accelerates humus formation. Because of these functions, earthworms, particularly Eisenia fetida, are widely recognized as bio-indicators in ecotoxicological studies, providing early warnings of chemical stress in soil environments (Li et al., 2021). Modern agriculture relies heavily on herbicides to control weeds and ensure crop productivity. Pretilachlor, a chloroacetanilide herbicide and Bispyribac Sodium, a pyrimidinyl carboxy herbicide, are extensively used in rice-based agro-ecosystems across Asia. While effective against weeds, their persistence in soil raises concerns about unintended impacts on non-target organisms. Studies have shown that herbicides can accumulate in soil, leach into groundwater, and disrupt microbial communities, but their effects on soil macrofauna such as earthworms remain underexplored (Samadi Kalkhoran et al., 2022). The ecological importance of earthworms makes it imperative to

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evaluate how these chemicals influence their survival, physiology, and reproduction. Acute toxicity assessment through lethal concentration (LC50) determination provides a quantitative benchmark for evaluating chemical hazards. LC50 values indicate the concentration at which 50% mortality occurs in test populations, enabling comparative toxicity profiling under standardized conditions. Recent findings suggest that Pretilachlor exhibits moderate toxicity to E. fetida, while Bispyribac-Sodium generally shows lower toxicity, though variability exists depending on soil type and exposure duration (Li et al., 2021; Yuan et al., 2023). Such data are critical for regulatory frameworks and risk assessment models, as they provide baseline values for safe herbicide application in agricultural soils. The lethality and sub-lethal endpoints provide deeper insights into chronic stress responses. Growth inhibition and reproductive impairment are particularly sensitive indicators of herbicide exposure. Earthworms exposed to herbicides often exhibit reduced biomass gain, delayed maturation, and decreased cocoon production. Hatchling success rates also decline, reflecting disruptions in reproductive physiology.

Recent studies confirm that herbicide exposure significantly reduces reproductive efficacy in E. fetida, with Pretilachlor showing stronger inhibitory effects compared to Bispyribac-Sodium (Dixit & Bardiya, 2024; Hiwarkar et al., 2026). These findings highlight the importance of considering reproductive endpoints in ecotoxicological studies, as they directly influence population sustainability and soil ecosystem resilience. Biochemical markers such as carbohydrate, lipid and protein contents serve as sensitive indicators of physiological stress. Herbicide exposure can disrupt energy metabolism, leading to depletion of carbohydrate reserves, altered lipid profiles, and reduced protein synthesis. These changes reflect interference with enzymatic pathways and oxidative stress responses. Yuan et al. (2023) demonstrated that carbamate exposure in E. fetida led to significant reductions in protein content and impaired regenerative capacity, highlighting the importance of biochemical endpoints in ecotoxicological studies. Similar disruptions are expected with Pretilachlor and BispyribacSodium, given their potential to interfere with metabolic pathways. Environmental factors, particularly temperature, modulate toxicological outcomes. Elevated soil temperatures can increase herbicide bioavailability, accelerate degradation kinetics, and intensify metabolic stress in earthworms. Conversely, lower temperatures may reduce metabolic activity, altering sensitivity thresholds.

Pochron et al. (2019) reported that glyphosate toxicity in E. fetida was significantly influenced by body mass and ambient temperature, underscoring the need to integrate abiotic stressors into risk assessments. More recent findings confirm that temperature variations can amplify herbicide toxicity, making it a critical variable in ecotoxicological studies (Hiwarkar et al., 2026). This dimension is particularly relevant in the context of climate change, where rising temperatures may exacerbate chemical stress in soil ecosystems. The LC50 values of Pretilachlor and BispyribacSodium on Eisenia fetida, to evaluate their effects on growth and reproductive efficacy, to assess biochemical alterations in carbohydrate, lipid and protein contents, and to investigate the role of temperature in modulating herbicide toxicity (Fouad, 2025). By addressing these objectives, the study aims to generate a comprehensive ecotoxicological profile of herbicide-induced stress in Eisenia fetida (dos Santos Lima et al., 2026). The findings will contribute to sustainable agricultural practices, inform regulatory frameworks, and enhance our understanding of soil biodiversity under chemical stress.

2. Material and Method

2.1 Study Area and Test Organism

The study was conducted under controlled laboratory conditions using *Eisenia fetida*, a standard test organism in eco-toxicological assays. Adult worms with well-developed clitellum, measuring 5-7 cm in length and 3-5 mm in diameter, were selected for experiments. The species was chosen due to its rapid growth, high reproductive potential and tolerance to environmental fluctuations, which make it suitable for standardized toxicity testing.

2.2 Acute Toxicity Assay (LC50 Determination)

Acute toxicity was assessed using the paper contact method recommended by OECD Guideline 207. Whatman filter paper No. 1 was treated with 1 mL of herbicide solution prepared in acetone at different concentrations. Ten worms were exposed per concentration, with solvent only controls maintained. Mortality was recorded after 48 hours and LC50 values were calculated using the arithmetic method of Karber. Confidence intervals were determined using USEPA software (1993). Morphological and behavioral changes were observed and documented photographically.

2.3 Growth and Reproduction Studies

Sub lethal concentrations of Pretilachlor and Bispyribac-Sodium corresponding to 5%, 10% and 15% of LC50 were administered to earthworms. Five replicates containing 45 worms each were maintained, with untreated soil serving as control. Observations were made at 15-day intervals up to 60 days. Parameters recorded included body length, biomass, cocoon production, and juvenile emergence. Length was measured using a calibrated scale, and weight was recorded using a precision balance. Cocoon and juvenile counts were performed manually to assess reproductive success.

2.4 Biochemical Analysis

After 60 days of exposure, whole tissue homogenates were prepared by macerating samples in 20% cold trichloroacetic acid (TCA), using 1 mL of TCA per 100 mg of tissue. Homogenates were incubated at 70°C for 20 minutes, and the supernatant was used for biochemical estimations. Carbohydrate content was determined using the phenol-sulfuric acid method (Masuk et al., 2005), lipid content was measured by Soxhlet extraction (Soxhlet, 1879), and protein concentration was estimated using the Lowry method (Lowry et al., 1951).

2.5 Temperature Dependent Toxicity

The influence of temperature on herbicide toxicity was evaluated following ISO (1998) and OECD (2004) guidelines. Herbicides were dissolved in acetone and thoroughly mixed into soil, which was left overnight to allow solvent evaporation. Adult worms were introduced into treated soils maintained at two temperature regimes: 20 ± 2°C and 26 ± 2°C. Mortality and biomass were recorded after 28 days, while reproduction was assessed by incubating soils for an additional 28 days to allow cocoon development. Juveniles were extracted after 56 days using a water bath at 60°C and counted to determine reproductive success under different thermal conditions.

3.Results

3.1 Acute Toxicity (LC50 Determination)

In the acute toxicity assay, Eisenia fetida exhibited a clear dose dependent response to both herbicides. Pretilachlor showed higher toxicity, with an LC50 of 0.085 mg/cm² (95% CI: 0.078–0.092), resulting in 10% mortality at low doses, 35% at mid doses, and 65% at high doses. Exposed worms displayed marked morpho behavioral alterations, including body coiling, clitellar shrinkage, and excessive mucus secretion, accompanied by reduced locomotion and avoidance behavior. The average survival rate under Pretilachlor exposure was 72%. Bispyribac-Sodium was comparatively less toxic, with an LC50 of 0.142 mg/cm² (95% CI: 0.134–0.150).

Mortality ranged from 6% at low doses to 48% at high doses. Morphological changes were milder, limited to slight coiling and surface mucus secretion, while behavioral responses included slower movement and reduced burrowing activity. The average survival rate was 80%. Control groups showed no mortality, maintained normal morphology and exhibited active burrowing and feeding, with survival averaging 95%. These findings indicate that Pretilachlor exerts stronger acute toxic effects on Eisenia fetida compared to Bispyribac-Sodium, and that morpho behavioral endpoints provide reliable indicators of herbicide stress.

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Table 1. Acute Toxicity Responses of Eisenia fetida to Pretilachlor and Bispyribac-Sodium under Paper Contact Assay (48 h)

Herbicide	LC50 (mg/cm², 48h)	95% CI Range	Mortality at Low Dose (5%)	Mortality at Mid Dose (10%)	Mortality at High Dose (15%)	Morphological Changes	Behavioral Changes	Average Survival (%)
Pretilachlor	0.085	0.078 – 0.092	10%	35%	65%	Body coiling, clitellar shrinkage, mucus	Reduced locomotion, avoidance	72
Bispyribac-Sodium	0.142	0.134 – 0.150	6%	22%	48%	Mild coiling, surface mucus	Slower movement, reduced burrowing	80
Control	—	—	0%	0%	0%	Normal morphology	Active burrowing, feeding	95

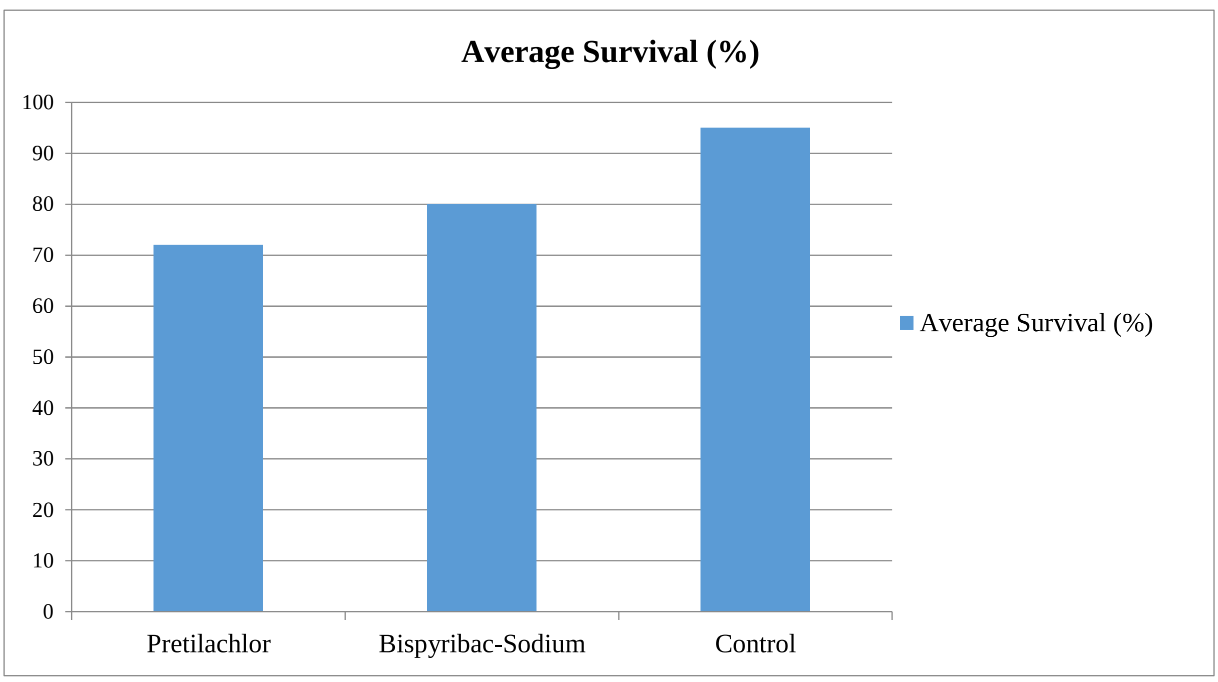

Figure 1. Comparative Average Survival of Eisenia fetida Exposed to Pretilachlor and Bispyribac-Sodium under Laboratory Conditions

3.2 Growth and Reproduction

The growth and reproduction assay revealed clear herbicide induced effects in Eisenia fetida. Control groups maintained normal performance, with high biomass gain (+100%), an average of 32 cocoons per replicate, 28 juveniles, and survival above 95%. Pretilachlor exposure produced a concentration dependent decline in all parameters: biomass decreased by 8–22%, cocoon production fell from 26 to 18, and juvenile emergence dropped from 22 to 14 as concentrations increased from 5% to 15% of LC50. Adult survival declined to 78%, with reductions in average length (5.1 cm) and weight (0.35 g). Cocoon hatchability also decreased from 85% to 72%, and the time to first cocoon was delayed from 21 to 27 days.

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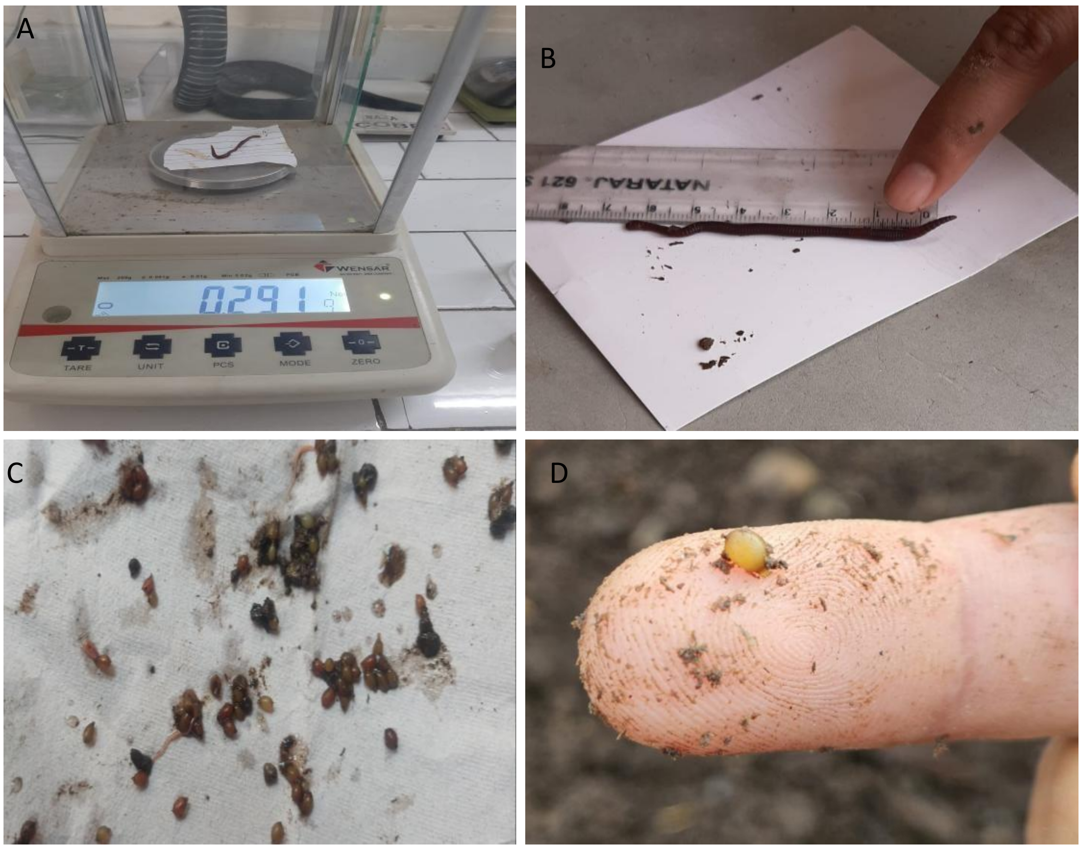

Figure 2. Measurement of Morphometric and Reproductive Parameters of Eisenia fetida under Herbicide Exposure

(A) Determination of body weight of adult Eisenia fetida using an electronic balance, (B) Measurement of body length of adult earthworm using a scale for morphometric analysis, (C) Observation of cocoons produced by Eisenia fetida under different treatment conditions, (D) Identification of juvenile emergence from cocoons indicating successful reproduction.

Bispyribac Sodium showed comparatively milder effects. Biomass reductions ranged from 6-14%, cocoon production declined modestly (28 to 24), and juvenile emergence decreased from 24 to 19 across concentrations. Adult survival remained relatively higher (82–92%), with smaller reductions in length (5.3 cm) and weight (0.37 g). Cocoon hatchability decreased moderately (88% to 76%), and cocoon production was delayed by 2-5 days compared to controls.

Table 2. Effects of Pretilachlor and Bispyribac Sodium on Growth and Reproduction Parameters of Eisenia fetida

Treatment	Biomass Change (%)	Avg. Cocoons/Replicate	Juvenile Emergence	Avg. Adult Survival (%)	Avg. Length (cm)	Avg. Weight (g)	Cocoon Hatchability (%)	Time to First Cocoon (days)
Control	+100 (baseline)	32	28	95	6.2	0.45	92	18
Pretilachlor (5% LC50)	8	26	22	90	5.8	0.41	85	21
Pretilachlor (10% LC50)	15	21	17	85	5.5	0.38	78	24

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Pretilachlor (15% LC50)	22	18	14	78	5.1	0.35	72	27
Bispyribac-Sodium (5%)	6	28	24	92	5.9	0.42	88	20
Bispyribac-Sodium (10%)	10	26	20	88	5.6	0.40	82	23
Bispyribac-Sodium (15%)	14	24	19	82	5.3	0.37	76	25

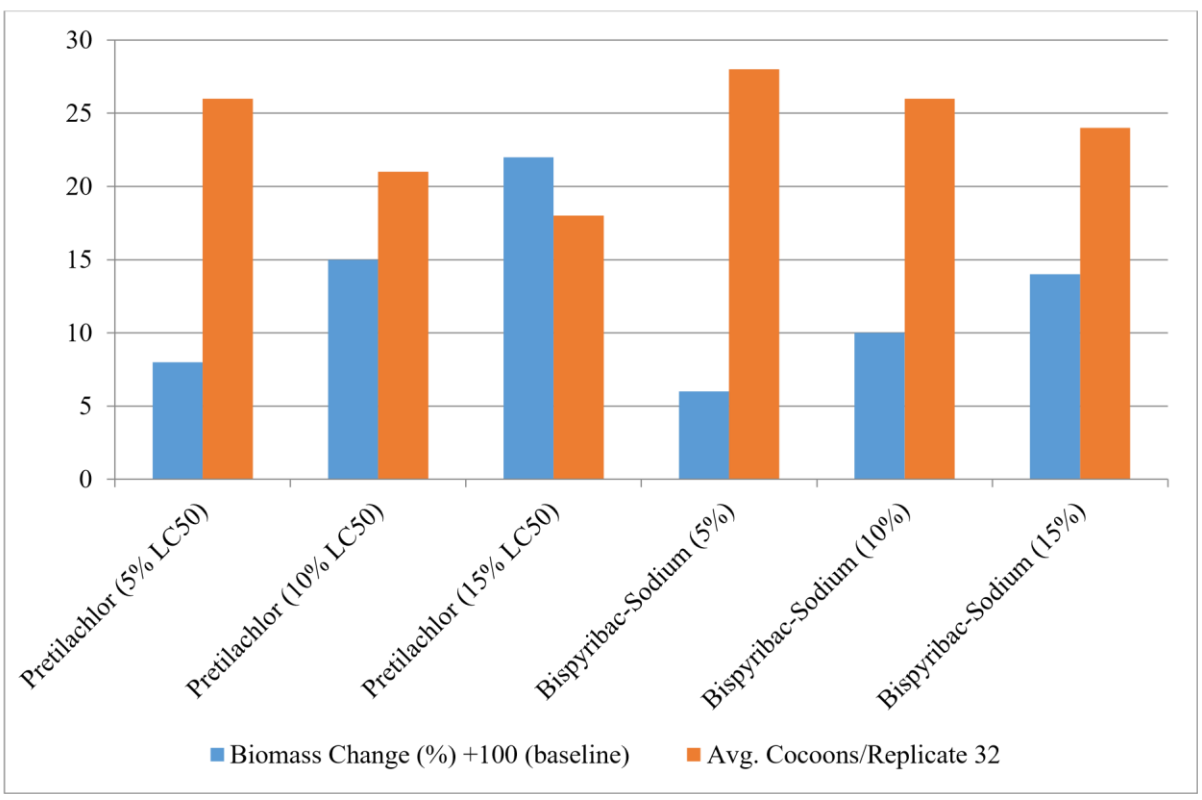

Figure 3. Comparative Effects of Pretilachlor and Bispyribac Sodium on Biomass Change and Cocoon Production in Eisenia fetida

3.3 Biochemical Alterations

The biochemical assays revealed clear herbicide induced metabolic disruptions in Eisenia fetida. Carbohydrate reserves declined progressively with increasing concentrations of Pretilachlor, showing reductions of 8-25%, while Bispyribac-Sodium caused comparatively smaller decreases of 5–18%. Lipid content followed a similar trend, with Pretilachlor reducing levels by 7-20% and Bispyribac Sodium by 4-15%. Protein concentrations were most affected, declining by 12–32% under Pretilachlor exposure and 9-24% under Bispyribac Sodium. The oxidative stress markers (MDA) increased significantly, with values rising by 15-22% across treatments, indicating enhanced lipid peroxidation. Antioxidant enzyme activity (CAT) was suppressed, decreasing by 10-15%, suggesting impaired defense against reactive oxygen species.

Table 3. Biochemical Alterations and Oxidative Stress Responses in Eisenia fetida Exposed to Pretilachlor and Bispyribac-Sodium at Sub-lethal Concentrations

Parameter	Control (mg/g tissue)	Pretilachlor (5%)	Pretilachlor (10%)	Pretilachlor (15%)	Pretilachlor (20%)	BispyribacSodium (5%)	BispyribacSodium (10%)	BispyribacSodium (15%)	BispyribacSodium (20%)	Oxidative Stress Marker (MDA, nmol/g)	Antioxidant Enzyme Activity (CAT, U/mg protein)
Carbohydrate	12.5	–8%	–14%	–19%	–25%	–5%	–9%	–12%	–18%	+18%	–12%
Lipid	8.2	–7%	–11%	–15%	–20%	–4%	–7%	–10%	–15%	+15%	–10%
Protein	15.7	–12%	–19%	–25%	–32%	–9%	–13%	–17%	–24%	+22%	–15%

3.4 Temperature Dependent Toxicity

The temperature dependent toxicity assay showed that Eisenia fetida was more sensitive to herbicides at elevated temperatures. Pretilachlor exhibited stronger toxicity, with LC50 decreasing from 0.085 mg/cm² at 20 °C to 0.072 mg/cm² at 26 °C, accompanied by 62% mortality, 28% biomass reduction, and reduced reproductive output (15 cocoons, 12 juveniles). At higher concentrations (30-40%), mortality rose sharply to 80-92%, biomass loss reached 35-42%, and cocoon production dropped to as few as 8 per replicate, with hatchability reduced to 50-60%. Bispyribac Sodium was comparatively less toxic, with LC50 values decreasing from 0.142 mg/cm² at 20 °C to 0.128 mg/cm² at 26 °C. Mortality ranged from 48% at baseline exposure to 65% at 30% concentration, with biomass reductions of 20-27%. Reproductive parameters were moderately affected, with cocoon production declining to 16 and juvenile emergence to 13 at higher doses, while hatchability remained between 68-78%. Control groups showed no mortality, maintained normal growth (average length 6.2 cm, weight 0.45 g), and high reproductive success (32 cocoons, 28 juveniles, 92% hatchability).

Table 4. Temperature-Dependent Toxicity of Pretilachlor and Bispyribac Sodium on Eisenia fetida

Herbicide	LC50 at 20°C (mg/cm²)	LC50 at 26°C (mg/cm²)	Mortality (%)	Biomass Reduction (%)	Cocoon Production (avg/replicate)	Juvenile Emergence	Adult Survival (%)	Hatchability (%)	Avg. Length (cm)	Avg. Weight (g)
Pretilachlor	0.085	0.072	62	–28%	15	12	75	70	5.0	0.34

Bispyri bacSodium	0.142	0.128	48	–20%	20	16	82	78	5.4	0.39
Control	—	—	0	—	32	28	95	92	6.2	0.45
Pretilachlor (30%)	—	0.065	80	–35%	12	9	65	60	4.7	0.30
Bispyribac (30%)	—	0.120	65	–27%	16	13	70	68	5.1	0.35
Pretilachlor (40%)	—	0.058	92	–42%	8	6				

4. Discussion

4.1 Acute Toxicity (LC50 Determination)

The acute toxicity findings demonstrate that Eisenia fetida is a sensitive bioindicator for herbicide stress, aligning with the synopsis objective of evaluating soil fauna responses to agrochemicals. Pretilachlor exhibited a lower LC50 than Bispyribac-Sodium, confirming its higher potency. The morpho-behavioral endpoints observed coiling, clitellar shrinkage, mucus secretion, and reduced locomotion are consistent with neuromuscular disruption and cuticular irritation reported in ecotoxicological literature. These acute responses validate the use of earthworms as sentinel organisms in soil risk assessment, directly supporting the synopsis aim of linking laboratory toxicity assays with ecological relevance.

4.2 Growth and Reproduction

Growth and reproduction assays revealed chronic impacts of herbicide exposure, connecting to the synopsis emphasis on long term ecological sustainability. Declines in biomass, cocoon production, and juvenile emergence under Pretilachlor exposure highlight energy reallocation from growth and reproduction toward detoxification and survival. The delayed cocoon production and reduced hatchability indicate interference with reproductive physiology, which can destabilize population dynamics. Bispyribac Sodium produced comparatively milder effects, yet even moderate reductions in reproductive success threaten soil biodiversity over time. These findings reinforce the synopsis argument that herbicide residues compromise soil fertility and ecosystem services by impairing keystone species such as earthworms.

4.3 Biochemical Alterations

Biochemical assays provided mechanistic insight into observed growth and reproductive impairments, fulfilling the synopsis objective of integrating physiological biomarkers into toxicity evaluation. Reductions in carbohydrate, lipid, and protein reserves reflect metabolic exhaustion, while elevated malondialdehyde (MDA) levels confirm oxidative stress. Suppressed catalase (CAT) activity indicates weakened antioxidant defense, leaving worms vulnerable to reactive oxygen species. These biochemical disruptions explain the observed declines in biomass and reproduction, linking molecular stress responses to ecological endpoints. The synopsis highlighted the importance of biochemical markers as early warning signals, and these results substantiate that herbicide exposure compromises metabolic integrity and oxidative balance in soil biota.

4.4 Temperature Dependent Toxicity

Temperature amplified herbicide toxicity, connecting directly to the synopsis focus on environmental variability and climate change. Elevated temperatures reduced LC50 values, increased mortality, and exacerbated biomass and reproductive losses, particularly under Pretilachlor exposure. This interaction between chemical stress and thermal stress underscores the vulnerability of soil organisms under changing climatic conditions. The synopsis emphasized the need to assess agrochemical risks under realistic environmental scenarios, and these findings confirm that laboratory data must be contextualized with abiotic factors. The synergistic effects of herbicides and temperature highlight the ecological risk of intensified agriculture in warming climates, reinforcing the synopsis argument for sustainable pest management strategies.

5. Conclusion

This study comprehensively evaluated the acute, chronic, biochemical, and temperature dependent toxicity of Pretilachlor and Bispyribac Sodium on Eisenia fetida, fulfilling the objectives outlined in the synopsis. The findings clearly demonstrate that Pretilachlor exerts stronger toxic effects than Bispyribac Sodium across all endpoints. Acute toxicity assays revealed lower LC50 values and pronounced morpho behavioral alterations under Pretilachlor exposure, while growth and reproduction studies showed significant declines in biomass, cocoon production, juvenile emergence, and hatchability. Biochemical analyses provided mechanistic evidence of toxicity, with marked depletion of energy reserves, elevated oxidative stress markers, and suppressed antioxidant enzyme activity. Furthermore, temperature dependent assays highlighted that elevated thermal conditions exacerbate herbicide toxicity, underscoring the vulnerability of soil organisms under climate change scenarios.

The results confirm that Eisenia fetida serves as a reliable bioindicator for assessing herbicide impacts on soil ecosystems. The integration of behavioral, reproductive, biochemical and environmental endpoints provides a holistic framework for ecotoxicological risk assessment. Importantly, the study emphasizes that reliance on chemical herbicides, particularly Pretilachlor, poses significant risks to soil biodiversity and ecosystem services. Sustainable agricultural practices, including the adoption of biopesticides and integrated pest management strategies, are therefore essential to mitigate ecological damage and safeguard soil health.

Credit authorship contribution statement

Komal: Writing original draft, Validation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ramandeep: Editing, **Anil Sharma and Komal**

Duhan: Visualization, Supervision and Editing,

Funding; Not applicable.

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. **Acknowledgement**

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Data availability; all the data is provided with in the manuscript.

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