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Effects of triclopyr on the earthworm (*Eisenia fetida*) under laboratory conditions: assessment of growth inhibition and oxidative stress

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Triclopyr is a widely used systemic herbicide in forestry and agricultural management. However, its potential sublethal effects on non-target soil organisms remain insufficiently understood. In this study, the chronic toxicity of triclopyr to the earthworm *Eisenia fetida* was systematically evaluated under laboratory conditions, with a focus on growth inhibition and oxidative stress responses. Earthworms were exposed to triclopyr-amended artificial soil at concentrations of 0–10 mg kg^{-1} for 28 days. Growth performance was assessed using growth inhibition rate (GIR) and specific growth rate (SGR), while oxidative stress was evaluated through a suite of biomarkers, including reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), and glutathione *S*-transferase (GST). An integrated biomarker response index (IBRv2) was further applied to quantify cumulative physiological stress. Results showed that triclopyr exposure induced significant, time- and concentration-dependent effects. Growth inhibition was evident during early and mid-exposure periods, while delayed negative SGR values indicated cumulative growth impairment under prolonged exposure. ROS and MDA levels increased markedly, with MDA reaching up to approximately fourfold higher than the control at 10 mg kg^{-1} after 7 days, demonstrating pronounced lipid peroxidation. Antioxidant and detoxification enzymes exhibited an initial induction followed by partial suppression during chronic exposure, suggesting a transition from compensatory defense to oxidative dysfunction. IBRv2 values peaked at day 7 and increased again at day 28, with maximum values observed in the highest concentration group, indicating substantial integrated biological stress. This study provides a comprehensive assessment of triclopyr-induced growth inhibition and oxidative stress in *E. fetida* using a multi-biomarker and IBR-based approach, offering valuable insights for ecological risk assessment of triclopyr in terrestrial environments.

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1. Introduction

Herbicides play a positive role in modern agriculture by effectively controlling unwanted weeds and ensuring crop productivity, and they have made an important contribution to the protection of food security.[1,2] However, excessive application of herbicides has raised growing concerns regarding potential non-target effects on soil biota, which are fundamental to ecosystem functioning and soil fertility.[3,4] Among various herbicides, the 3,5,6-trichloro-2-pyridinyloxyacetic acid (TPA), also known as triclopyr, is a systemic, selective herbicide extensively applied in forestry, pastures, and non-crop areas for the control of broadleaf weeds and woody plants.[5] Owing to its relatively high mobility and persistence in soil, triclopyr and its metabolites may pose ecological risks to soil-dwelling organisms, particularly earthworms, which are key indicators of soil health.

Triclopyr was first reported in 1975, as a postemergence herbicide, it acts primarily as a synthetic auxin that disrupts plant hormonal balance, leading to uncontrolled growth and eventual plant death.[6] Although its herbicidal mechanism is plant-specific, studies have indicated that triclopyr residues may accumulate in soil matrices, with reported half-lives ranging from 6 to 90 days in the soil, depending on environmental conditions such as pH, organic matter content, and microbial activity; field application rates typically range from 0.56 to 6.7 kg acid equivalent (ae) per ha, with soil residues reported at 0.05–5.2 mg kg^{-1} in the top 15 cm layer during the first 30 days post-application.[7,8] As triclopyr can leach into the soil and persist for extended periods, understanding its impact on non-target soil fauna is essential for a comprehensive ecological risk assessment.

Earthworms, especially *Eisenia fetida*, are key functional organisms in soil ecosystems, contributing substantially to organic matter turnover, nutrient transformation, and soil structural stability.[9,10] Owing to their ecological relevance and pronounced sensitivity to environmental pollutants, *E. fetida* has been widely adopted as a model species for terrestrial

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ecotoxicological assessments by both the OECD (Organization for Economic Cooperation and Development) and the ISO (International Organization for Standardization). Previous research has shown that a broad range of pesticides, such as insecticides, fungicides, and herbicides, can exert detrimental effects on earthworm viability, growth performance, reproductive capacity, and physiological and biochemical processes.[11,12] Nevertheless, although triclopyr is extensively used in agricultural and forestry practices, information regarding its sublethal and long-term toxic impacts on *E. fetida* under controlled laboratory conditions remains scarce.

Previous investigations have primarily focused on triclopyr's effects on aquatic organisms, plants, and microbial communities, revealing potential risks associated with oxidative stress and growth inhibition. For example, triclopyr-containing herbicide formulations have been reported to exhibit moderate toxicity toward certain sensitive aquatic organisms, including amphibian larvae and fish, with 96-h LC_{50} values falling within the range of 0.36–2.7 mg acid equivalents per L.[13,14] For juvenile salmonids, lower LC_{50}-96 h values ranging from 5.3 to 9.7 mg L^{-1} have been documented, indicating a higher sensitivity among these species.[15] In addition, triclopyr has been shown to delay egg hatchability and alter swimming activity of zebrafish after exposure to triclopyr.[16] Nevertheless, information regarding its impact on terrestrial invertebrates, especially earthworms, remains scarce. Integrated biomarker approaches combining growth, oxidative stress, and IBRv2 analysis have been successfully applied to other herbicides in earthworm ecotoxicology, including glyphosate, atrazine, and 2,4-D;[17,18] however, no prior study has systematically combined these endpoints specifically for triclopyr in *E. fetida*. Understanding both lethal and sublethal endpoints, such as mortality, biomass loss, and growth inhibition, is critical to elucidating the overall toxicity profile of triclopyr in soil ecosystems.

Given the ecological importance of earthworms and the increasing application of triclopyr, there is an urgent need to evaluate its potential risk to soil fauna. Therefore, this study aimed to systematically assess chronic toxicity of triclopyr to *E. fetida* under controlled laboratory conditions. Specifically, we use the growth inhibition and the IBR version 2 (IBRv2) method based on six biomarkers (reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), glutathione *S*-transferase (GST), guaiacol peroxidase (POD) and malondialdehyde (MDA)) to evaluate the sublethal effects. The novelty of this work lies in providing the integrated assessment of triclopyr-induced chronic stress in *E. fetida*, combining organism-level growth endpoints with biochemical biomarkers and IBRv2 analysis to elucidate sublethal toxicity mechanisms. The results of this study will provide valuable data for ecological risk assessments of triclopyr in terrestrial environments and contribute to the establishment of safer herbicide management practices.

2. Materials and methods

2.1 Test chemical reagents and earthworms

Triclopyr (CAS: 55335-06-3, analytically pure) was obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd. The enzyme activity assay kits were all purchased from Nanjing Jiancheng Bioengineering Institute (Nangjing, China). The other chemicals are all of analytical grade.

The experimental earthworms (*Eisenia fetida*) were obtained from the laboratory and have been continuously raised for more than one year. Sexually mature and physiologically intact earthworms exhibiting clearly developed clitella, with individual body masses ranging from 300 to 600 mg and relatively consistent body size, were selected for the study. Before experimental exposure, the organisms were acclimated for 7 days in clean artificial soil under controlled environmental conditions (20 ± 1 °C; relative humidity 75 ± 2%).

2.2 Soil and exposure design

Artificial soil was prepared by blending 10% sieved sphagnum peat, 20% kaolin clay, 69% industrial-grade quartz sand, and 1% calcium carbonate. The homogenized soil was apportioned into 750 g subsamples. For sub-chronic toxicity assessment, triclopyr was applied at nominal concentrations of 0, 0.1, 0.5, 1, 5, and 10 mg kg^{-1}, with three independent replicates established for each treatment level. The concentration range was selected based on environmentally relevant soil residues (0.05–5.2 mg kg^{-1}),[7,8] with 0.1 mg kg^{-1} approximating near-background levels and 10 mg kg^{-1} representing a high but plausible scenario following direct spillage or repeated applications. The test compound was first dissolved in acetone and then evenly incorporated into the soil matrix to achieve uniform distribution across concentrations. Soil moisture was adjusted to 35%, followed by a 24 h equilibration period prior to organism exposure.

Fifteen earthworms of comparable body length and mass were randomly introduced into each beaker containing triclopyr-amended artificial soil. The containers were covered with plastic film and maintained in a controlled-environment chamber at 20 ± 1 °C and 75 ± 2% relative humidity. A 12 h light/12 h dark photoperiod was applied to approximate natural environmental conditions. Soil moisture was maintained at 35% of dry weight throughout the 28-day experiment by weighing containers twice weekly and replenishing evaporated water with deionized water to maintain constant mass. Gravimetric monitoring at each sampling point confirmed deviations <2% from target moisture.

2.3 Growth inhibition of earthworms

To assess the effect of TRI on earthworm biomass over the course of the 28-day exposure experiment, the Growth Inhibition Rate (GIR) was computed using the relative change in average body mass across different sampling intervals. The equation is adapted from previously reported approaches:[19,20]

$$\mathrm{GIR}_n = \left(\frac{w_0 - w_t}{w_0}\right) \times 100\%$$

Here, GIR_n refers to the growth suppression percentage for treatment group n, W_t is the mean weight of earthworms at a given time point (*e.g.*, day 7, 14, 21, or 28), and W_0 represents the average initial weight measured prior to exposure to the contaminated soil medium.

To further evaluate the dynamic growth trend over shorter intervals, the Specific growth rate (SGR) over a fixed 7-day period was calculated using the natural logarithmic difference in body mass:[21]

$$\mathrm{SGR}_n = \frac{\ln W_{i+7} - \ln W_i}{7}$$

In this formula, SGR_n denotes the relative growth rate of earthworms within a 7-day interval starting from day i, where W_i and W_{i+7} are the mean weights of earthworms on day i and day i + 7, respectively. This index facilitates the comparison of biomass change rates across treatment levels.

2.4 Biomarker assay

2.4.1 ROS levels. Intracellular reactive oxygen species (ROS) levels in *Eisenia fetida* were quantified using the 2,7-dichlorofluorescein diacetate (DCFH-DA) assay under different exposure conditions.[22] After 7, 14, 21, and 28 days of treatment, three individuals were randomly selected from each group, homogenized in 0.1 mol per L phosphate-buffered saline (PBS) (1 : 10), and processed for subcellular fractionation. The homogenates were initially centrifuged at 1000×g for 6 min at 4 °C, and the resulting supernatants were further centrifuged for 16 min. Subsequently, the supernatant was aliquoted into three 2 mL centrifuge tubes and centrifuged at 21 000×g for 18 min at 4 °C. After discarding the supernatant, the pellets were resuspended in 1 mL PBS and sequentially transferred among three tubes within the same treatment group to ensure thorough homogenization. This procedure yielded 1 mL of mitochondrial suspension. A reaction mixture containing 190 μL of the suspension and 10 μL of DCFH-DA probe (10 μmol L^{-1}) was incubated at 37 °C for 20 min, after which the reaction was terminated by adding 10 μL of 1 mol per L HCl. Using a Microplate System (Molecular Devices, M2), the excitation wavelength was 485 nm, and the fluorescence intensity was measured at 538 nm.

2.4.2 Determination of biomarkers. All biomarker measurements were conducted with standardized commercial kits according to the instructions supplied by the manufacturer (Nanjing Jiancheng Bioengineering Institute, China). Protein content was quantified using the Bradford method,[23] with bovine serum albumin as the calibration standard and absorbance measured at 595 nm. CAT activity was assayed according to Xu *et al.*[24] by monitoring the decomposition of H_2O_2 at 250 nm every 5 s over 60 s, where one unit (U) represented the enzyme quantity required to decompose 50% of H_2O_2 within 100 s at 25 °C. SOD activity was determined based on its inhibition of nitroblue tetrazolium (NBT) photoreduction following Giannopolitis and Ries,[25] with absorbance recorded at 560 nm; one unit (U) of SOD activity was defined as the enzyme amount causing 50% inhibition of NBT reduction. GST activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate following Habig *et al.*,[26] with absorbance monitored at 340 nm every 30 s for 3 min at 25 °C, one unit of enzyme activity (U) is defined as the amount of enzyme per milligram of tissue protein that, at 37 °C, results in a decrease of 1 μmol per L GSH concentration per minute in the reaction system, corrected for the non-enzymatic reaction. Malondialdehyde (MDA) levels were assessed according to Bony *et al.* (2008).[27] POD activity was measured using the guaiacol method as described by Kochba *et al.*,[28] with absorbance at 470 nm recorded at 30 s intervals for 3 min.

2.5 Integrated biomarker response (IBR)

The Integrated Biomarker Response version 2 (IBRv2) index was applied to integrate multiple biomarker responses derived from the bioassays, providing an overall assessment of pollutant-induced ecological risk.[19,20] IBRv2 values were calculated following the procedure described by Sanchez *et al.*[29] with modifications:

Individual biomarker data (X_i) are compared to a mean reference data (X_0), and a log transformation is applied to reduce variance.

$$Y_i = \log(X_i/X_0)$$

To standardize the Y_i, the general mean (μ) and standard deviation (σ) of Y_i.

$$Z_i = (Y_i - \mu)/\sigma$$

To create a basal line centered on zero and to represent biomarker variation according to this basal line, the mean of standardized biomarker response (Z_i) and mean of reference biomarker data (Z_0) are used to define a biomarker deviation index (A_i).

$$A_i = Z_i - Z_0$$

Summed the absolute value of A_i of each biomarker and we get the integrated multi-biomarker response named IBR.

$$\mathrm{IBR} = \sum |A_i|.$$

2.6 Statistical analysis

All experimental data were statistically processed using SPSS software (version 20.0 for Windows), while figures were generated with Origin 2022. Differences among exposure concentrations for each biomarker at individual sampling times were assessed by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* test. Statistical significance was denoted by lowercase letters on all figures ($p < 0.05$). The influences of exposure concentration, exposure duration, and their interaction on biomarker responses were further examined using two-way ANOVA with LSD multiple comparisons. Full two-way ANOVA results (F-values, degrees of freedom, p-values) are provided in Table S1 (SI).

3. Result and discussion

3.1 Effect of triclopyr on earthworm growth

Growth responses of *Eisenia fetida* to triclopyr exposure are shown in Fig. 1A and B. No mortality was observed in any treatment group during the 28-day exposure, confirming the sublethal nature of the tested concentrations. However, dose-dependent behavioral changes were noted: earthworms in high-concentration groups (5 and 10 mg kg^{-1}) exhibited reduced burrowing activity during the first 7 days, with more individuals remaining at the soil surface compared to controls. By day 14, burrowing behavior appeared normalized in most groups except 10 mg kg^{-1}.

The growth inhibition rate (GIR) revealed clear time- and concentration-dependent effects. During the early exposure period (7–14 days), negative GIR values were observed in most treatment groups, indicating inhibited biomass accumulation relative to the control. At 21 days, low- and medium-dose treatments (≤1 mg kg^{-1}) still exhibited growth inhibition, whereas higher concentrations (5 and 10 mg kg^{-1}) resulted in positive GIR values. By day 28, a marked increase in GIR was observed in the high-dose groups, suggesting a compensatory growth response following prolonged exposure. Analysis of the instantaneous growth rate (IGR) further demonstrated delayed growth impairment under chronic triclopyr exposure. Positive or near-zero IGR values were observed during the initial exposure intervals (0–14 days), while pronounced negative IGR values occurred during the 14–21 days and 21–28 days intervals, particularly at higher concentrations.

Negative impact of pesticides on earthworm growth has been reported by various researchers, more and more reports have reach a consensus that the impact of pesticides on earthworm weight was a more sensitive index compared to the mortality in indicating toxic effects.[30–32] Furthermore, loss of body mass has been widely recognized as a sensitive indicator of physiological stress in earthworms, closely associated with both the intensity of toxic exposure and its duration.[33,34] These results in the present study indicate that triclopyr exerts limited short-term effects on earthworm growth but causes cumulative inhibitory impacts with increasing exposure duration. The transient growth stimulation observed at later stages may reflect adaptive or hormetic responses; however, the sustained decline in IGR suggests an overall adverse effect on growth dynamics under chronic exposure. Together, these findings highlight the importance of long-term growth endpoints for evaluating the ecological risks of triclopyr to soil invertebrates.

3.2 Effect of triclopyr on ROS contents

The effects of triclopyr exposure on reactive oxygen species (ROS) levels in *Eisenia fetida* are shown in Fig. 2. Compared with the control, triclopyr exposure resulted in a consistent increase in ROS content across all sampling times, demonstrating a clear concentration-dependent pattern. At day 7, ROS levels increased progressively with increasing triclopyr concentrations, indicating an early induction of oxidative stress. Similar dose-related elevations were observed at 14 and 21 days, with higher concentrations (5.0 and 10.0 mg kg^{-1}) inducing the most pronounced ROS accumulation.

This result was similar to that of Qiao *et al.* who focused on the effect of flupyradifurone on oxidative stress of earthworms.[35] With prolonged exposure to 28 days, ROS levels in the high concentration treatment remained elevated in all treated groups relative to the control, although the magnitude of increase was less pronounced than at earlier sampling points. This suggests that while chronic triclopyr exposure persistently induces oxidative stress, earthworms may activate antioxidant defense mechanisms over time to partially counterbalance excessive ROS production.[35]

ROS collectively refer to a group of oxygen-derived reactive molecules that are naturally present in organisms and maintained under tightly regulated redox homeostasis.[37,38] However, exposure to external stressors can disrupt this balance, leading to excessive ROS generation and subsequent oxidative stress.[39] The sustained elevation of ROS under triclopyr exposure indicates that oxidative stress is a key component of its sublethal toxicity in earthworms.[40] While triclopyr acts as a synthetic

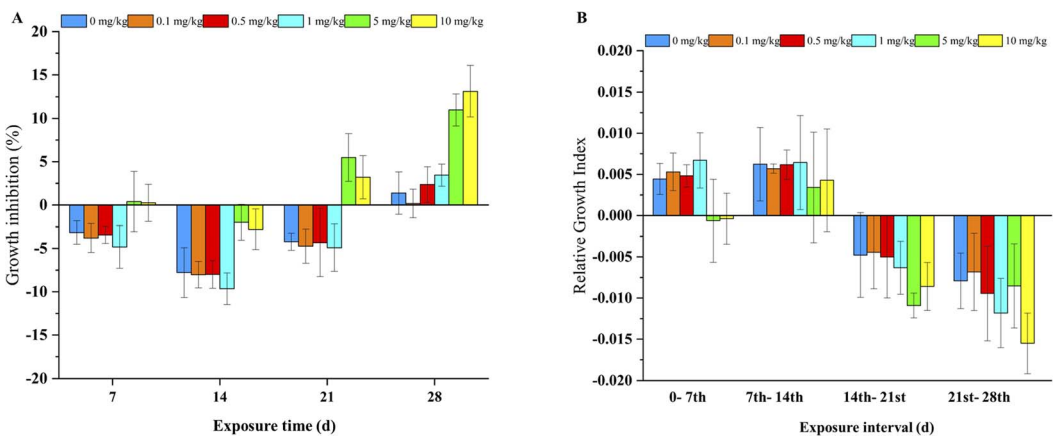

Fig. 1 Growth inhibition rate (A) and specific growth rate (B) of earthworm exposed to triclopyr amended soil. Data are presented as mean ± standard deviation (M ± SD, $n = 3$). Different lowercase letters above bars indicate significant differences between treatments at the same time point ($p < 0.05$).

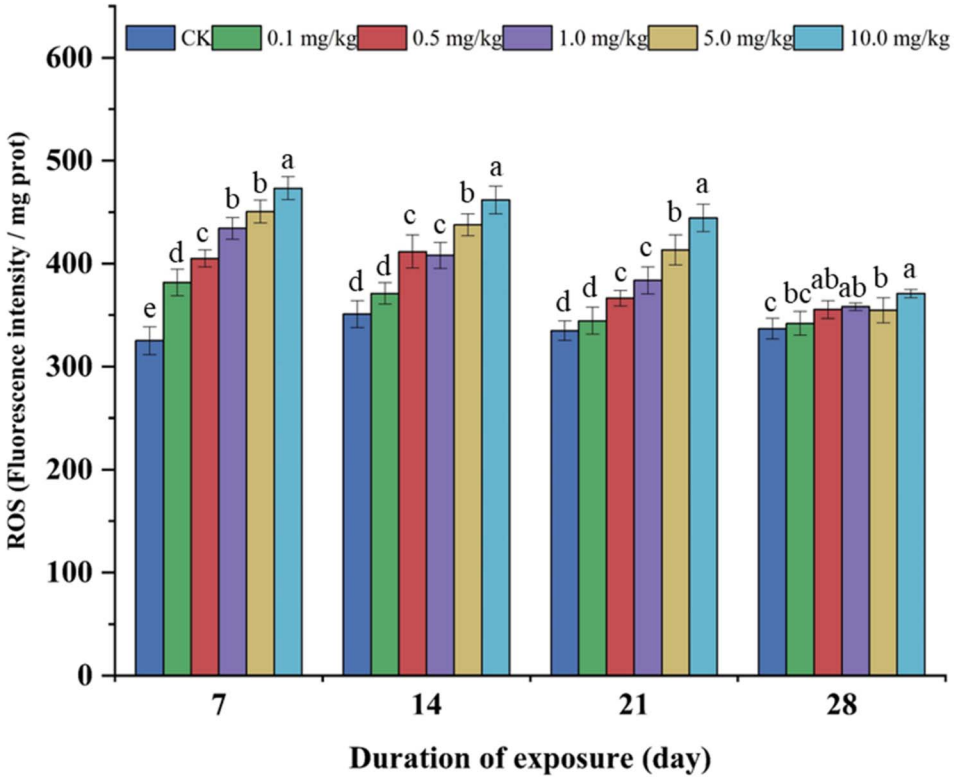

Fig. 2 ROS level of earthworms exposed to triclopyr on days 7, 14, 21, and 28. Bars are means ± SD ($n = 3$). Different letters indicate significant differences between the treatments of the same day ($p < 0.05$).

auxin in plants (disrupting hormonal balance *via* TIR1/AFB auxin receptors), its toxicity in animals likely involves non-target interactions with mitochondrial respiration and oxidative phosphorylation, rather than auxin signaling. Phenoxy herbicides are known to uncouple mitochondrial electron transport and disrupt the proton gradient, leading to electron leakage and superoxide formation.[36,41] Additionally, cytochrome P450 (CYP450) enzyme induction during phase I metabolism of triclopyr may generate ROS as byproducts through redox cycling.[40] Excessive ROS generation can disrupt cellular redox homeostasis, leading to oxidative damage to lipids, proteins, and nucleic acids, and may ultimately impair growth and physiological performance. When considered alongside growth inhibition and other biochemical responses, the ROS results highlight the potential of triclopyr to induce long-term oxidative stress in non-target soil organisms, underscoring the importance of incorporating oxidative biomarkers into ecological risk assessments of herbicides in terrestrial environments.

3.3 Effects of triclopyr on antioxidant in earthworm

Alterations in ROS levels and antioxidant enzyme activities (SOD, CAT, POD, and GST) represent key protective responses of organisms to contaminant-induced stress.[42] Monitoring these biomarkers in earthworms is therefore a reliable approach for assessing the ecological toxicity of triclopyr. ROS consist of several oxygen-derived reactive species, including H_2O_2, O_2^-, and $^{\bullet}OH$, which are normally regulated by antioxidant enzymes to maintain redox homeostasis.[43] Excessive ROS production may disturb this balance, resulting in modulation of antioxidant enzyme activities, with SOD serving as a primary defense by converting superoxide radicals into hydrogen peroxide.

In the present study, as it shown in Fig. 3A, SOD activity exhibited a clear concentration-dependent increase during the early exposure period (7 and 14 days), with significantly higher activities observed in medium- and high-dose treatments compared with the control. This elevation suggests an enhanced conversion of superoxide radicals into hydrogen peroxide as an early protective response to triclopyr-induced oxidative stress. However, at 21 and 28 days, SOD activity declined in most treatment groups, particularly at higher concentrations, indicating a potential exhaustion or inhibition of enzymatic defense capacity under prolonged exposure.

As another key anti-oxidant enzyme, CAT scavenges H_2O_2 to water and oxygen.[44] Noticeably, a similar trend was observed for CAT activity in Fig. 3B. CAT activity increased significantly with increasing triclopyr concentration during the initial exposure stages, reflecting an accelerated decomposition of H_2O_2 generated by SOD activity.[35] In turn, enhanced SOD activity in this stage promotes the accumulation of H_2O_2, thereby triggering CAT-mediated scavenging of H_2O_2 to maintain cellular redox balance.[45] With prolonged exposure, CAT activity decreased, especially at higher concentrations, suggesting an impaired

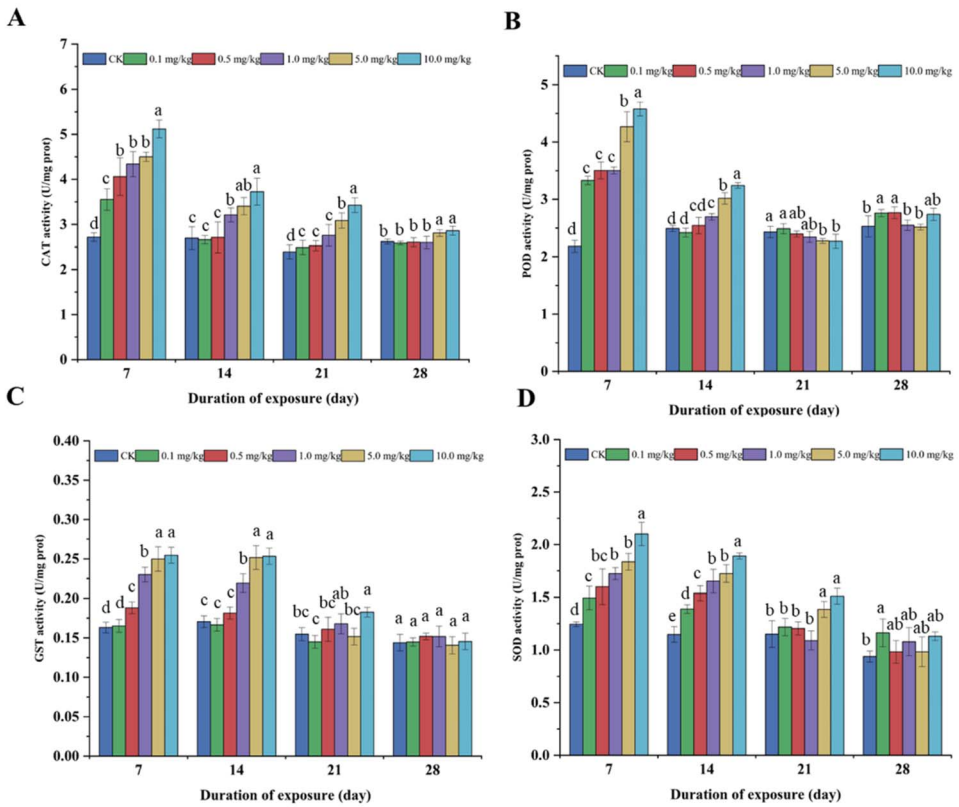

Fig. 3 Impact of triclopyr on SOD (A), CAT (B), POD (C) and GST (D) activity. The same letter indicates no significant difference ($P > 0.05$). The difference letter indicates marked difference ($P < 0.05$). Data were expressed as mean ± standard error ($n = 3$).

ability to maintain redox homeostasis during chronic stress conditions.[46]

Peroxidase (POD) plays a key role in eliminating hydrogen peroxide and related free radicals.[47] Under pollutant-induced stress, excessive ROS production may impair CAT and POD activities, thereby weaken the antioxidant defenses and promote oxidative injury in earthworms.[48] As shown in Fig. 3C, POD activity showed marked stimulation at 7 days, particularly in high-dose treatments, indicating its important role in the early-stage detoxification of reactive oxygen species. At 14 days, POD activity remained elevated but to a lesser extent, while a noticeable reduction occurred at 21 days. At 28 days, POD activity exhibited partial recovery in some treatment groups, implying a dynamic adjustment of antioxidant pathways during prolonged exposure.

Glutathione *S*-transferase (GST) plays central roles in xenobiotic detoxification, enabling organisms to metabolize and sequester foreign chemicals and thereby limit cellular damage.[49,50] Accordingly, variations in their activities are commonly used as biomarkers to assess pollutant-induced impairment of detoxification pathways.[51] Changes in GST activity in *Eisenia fetida* following triclopyr exposure are shown in Fig. 3D. Compared with the control, GST activity was significantly elevated in most triclopyr-treated groups during the early exposure period (7 and 14 days), exhibiting a clear concentration-dependent pattern. The highest GST activities were generally observed at medium- and high-dose treatments, indicating an enhanced phase II detoxification response at the initial stage of exposure. At 21 days, GST activity showed a marked decline in all treatment groups relative to earlier sampling times, with values approaching or falling below those of the control in some concentrations. By day 28, GST activity remained at relatively low levels, with only slight differences observed among treatments, suggesting that prolonged triclopyr exposure may overwhelm detoxification capacity or disrupt glutathione-dependent defense mechanisms.

The coordinated changes in SOD, CAT, POD, and GST activities indicate that triclopyr induces oxidative stress in *E. fetida*, triggering an integrated antioxidant defense response.[40,47] The early upregulation of these enzymes reflects an adaptive mechanism aimed at neutralizing excess reactive oxygen species and mitigating cellular damage. However, the subsequent decline in enzyme activities under prolonged exposure suggests that sustained oxidative pressure may impair antioxidant systems, leading to redox imbalance. When considered together with elevated ROS levels and growth inhibition, the observed enzymatic patterns suggest that oxidative stress is a central mechanism underlying triclopyr toxicity in earthworms. We propose the following mechanistic model: triclopyr exposure, then mitochondrial dysfunction/CYP450

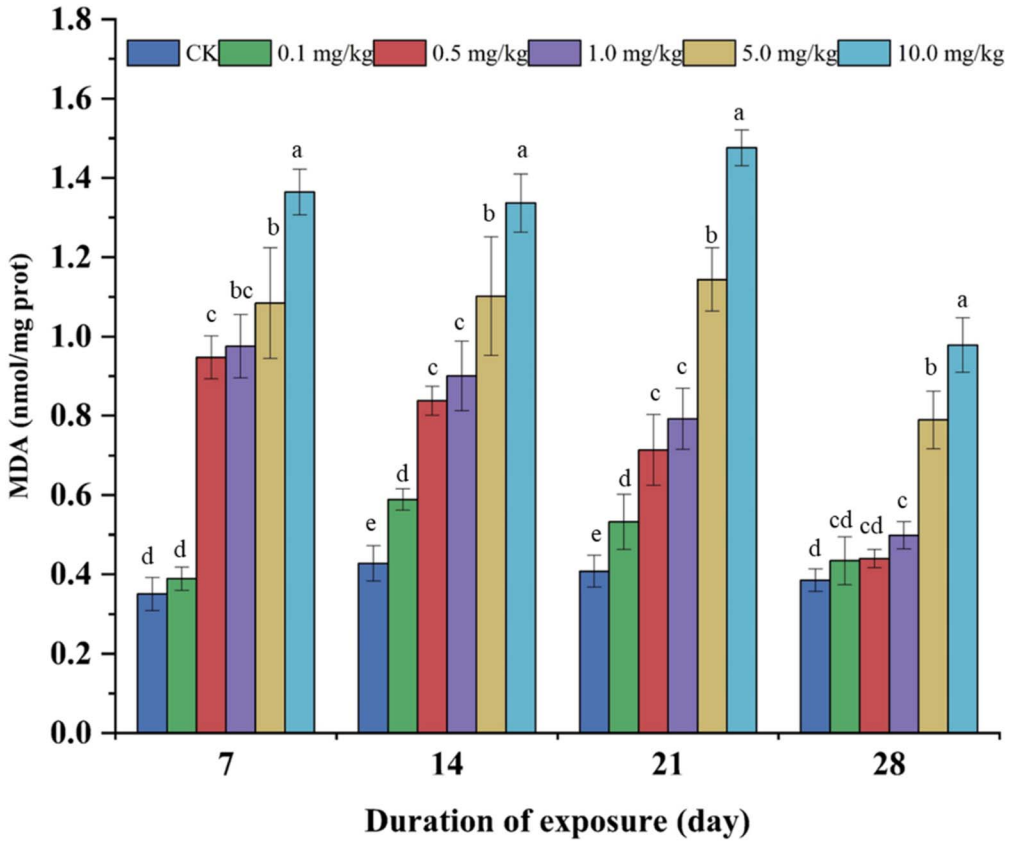

Fig. 4 MDA content changes in earthworm that were exposed to triclopyr. Data are presented as means ± standard deviations ($n = 3$). Different letters above bars indicate significant differences at the $p < 0.05$ level between treatments of the same day.

induction, and ROS overproduction, inducing lipid peroxidation (MDA), antioxidant enzyme induction (SOD/CAT/POD) enzymatic exhaustion under chronic exposure, finally lead to growth inhibition due to energy reallocation and cellular damage. Such disruption of antioxidant homeostasis may contribute to impaired metabolism, reduced growth performance, and increased susceptibility to cellular injury.

These findings demonstrate that antioxidant enzyme responses are sensitive and effective biomarkers for assessing sublethal and chronic toxicity of triclopyr in soil organisms. Incorporating biochemical endpoints with growth and integrated biomarker indices provides a comprehensive evaluation of the ecological risks posed by triclopyr to non-target terrestrial invertebrates.

3.4 Effects of triclopyr on lipid peroxidation in earthworm

Lipid peroxidation induced by free radical attack results in the generation of malondialdehyde (MDA), which contributes to cytotoxicity and reflects the severity of oxidative stress.[52] Therefore, MDA levels are commonly used as an indirect measure of membrane lipid peroxidation and oxidative tissue damage.[53] The malondialdehyde (MDA) content in *Eisenia fetida* exposed to triclopyr-contaminated soil exhibited a clear time- and concentration-dependent response (Fig. 4). Throughout the 28-day exposure period, MDA levels in the control groups (CK) remained stable, fluctuating within a narrow range (0.35–0.42 nmol per mg prot), indicating minimal background oxidative lipid damage. Upon triclopyr exposure, a significant induction of lipid peroxidation was observed. After 7 days, MDA content in all treatment groups (≥0.5 mg kg^{-1}) was markedly elevated compared to the control, with the highest concentration (10.0 mg kg^{-1}) causing an approximately 4-fold increase. This dose-dependent pattern persisted through days 14 and 21. Notably, by day 28, MDA levels in all exposed groups showed a distinct decline from their earlier peaks, particularly evident in the low to medium concentration groups (0.5 and 1.0 mg kg^{-1}). However, even at day 28, MDA content in the 5.0 and 10.0 mg kg^{-1} groups remained significantly elevated (2.4- and 2.7-fold higher than the control, respectively).

The results demonstrate that subchronic exposure to the herbicide triclopyr induces significant oxidative stress in the earthworm *Eisenia fetida*, as evidenced by the substantial increase in tissue MDA content, a robust biomarker of lipid peroxidation.[54,55] The pronounced, dose-dependent elevation of MDA levels, particularly during the initial and mid-phase exposure periods (7–21 days), strongly suggests a rapid overproduction of reactive oxygen species (ROS) that overwhelmed the organism's constitutive antioxidant defenses, leading to cellular membrane damage. The magnitude of MDA induction aligns with the known mode of action of phenolic herbicides,

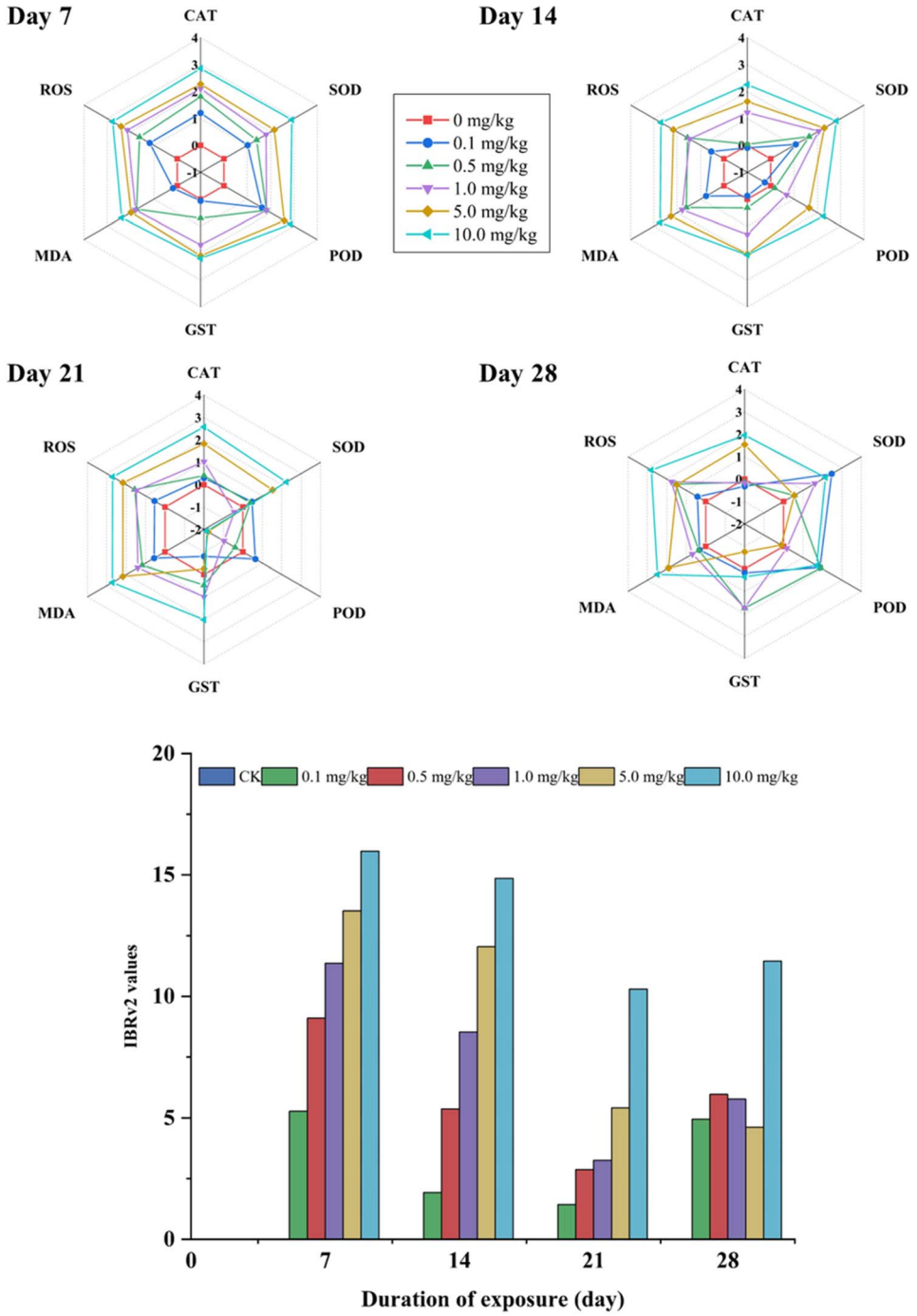

Fig. 5 Time-dependent integrated biomarker response (IBRv2) profiles of *Eisenia fetida* exposed to triclopyr, showing radar plots of biomarker deviations and corresponding IBRv2 values.

which can disrupt electron transport chains, leading to ROS generation.[41,56] Comparatively, triclopyr appears to induce more pronounced lipid peroxidation than structurally related phenoxy herbicides such as 2,4-D at comparable concentrations, possibly due to its pyridinyloxyacetic acid structure enhancing redox cycling.[56] The initial sharp rise in MDA (by day 7) indicates an immediate oxidative insult following triclopyr exposure. The sustained high levels through day 21 in the higher concentration groups imply a persistent oxidative challenge, potentially leading to cumulative membrane damage that could compromise cellular integrity and organismal health. This progressive damage at higher concentrations correlates

with the potential for reduced survival, growth, or reproduction reported for earthworms under herbicide stress.[57,58] From an ecological risk perspective, the significant increase in MDA even at the relatively low concentration of 0.5 mg kg^{-1} after just one week is concerning. It suggests that environmentally relevant levels of triclopyr residue can trigger measurable oxidative stress in soil detritivores. While the apparent physiological adaptation observed at 28 days might suggest a capacity for acclimation, the initial period of significant membrane damage could have sublethal consequences for burrowing efficiency, nutrient assimilation, or energy allocation.

3.5 Comprehensive biomarker response assessed by IBRv2

The integrated biomarker response version 2 (IBRv2) was applied to synthesize changes in oxidative stress indicators (ROS, MDA), antioxidant enzymes (SOD, CAT, POD), and detoxification-related enzymes (GST) in *Eisenia fetida* exposed to triclopyr (Fig. 5). The radar plots clearly demonstrate that triclopyr induced distinct and time-dependent alterations in the overall biomarker response profile, while the corresponding IBRv2 values provided a quantitative measure of cumulative physiological stress.[59]

At day 7, IBRv2 values increased markedly with rising triclopyr concentrations, indicating a strong early biological response even at relatively low exposure levels. Radar plots showed a coordinated activation of antioxidant and detoxification biomarkers, accompanied by elevated ROS and MDA signals. This pattern suggests that earthworms rapidly mobilized defense mechanisms to counteract triclopyr-induced oxidative stress during the initial exposure stage.[60] The highest IBRv2 values were observed in the high-dose treatments, reflecting a substantial deviation from the physiological baseline. By day 14, although the overall response pattern remained concentration-dependent, IBRv2 values were lower than those observed at day 7 for most treatments. This reduction implies a partial physiological adjustment or acclimation, whereby antioxidant and detoxification systems temporarily mitigated the oxidative challenge imposed by triclopyr. Nevertheless, biomarker deviations remained pronounced at higher concentrations, indicating that compensatory responses were insufficient to fully restore homeostasis.

At day 21, IBRv2 values reached their lowest levels across exposure concentrations, suggesting an apparent attenuation of the integrated biomarker response. Radar plots revealed reduced contributions from several antioxidant enzymes, despite sustained ROS and MDA signals. This response pattern may reflect a transitional stage in which prolonged exposure suppressed enzymatic activity, potentially due to energetic constraints or enzyme inhibition, rather than a genuine recovery of physiological function.[46,61] By day 28, IBRv2 values increased again, particularly in medium- and high-dose treatments. The renewed elevation of IBRv2 was mainly driven by persistent oxidative stress indicators and altered antioxidant enzyme activities, indicating that chronic triclopyr exposure ultimately disrupted physiological regulation. This late-stage increase suggests that long-term exposure imposes cumulative stress on earthworms, overriding earlier compensatory mechanisms.

Overall, the IBRv2 analysis highlights a non-linear temporal response of *E. fetida* to triclopyr exposure, characterized by an early activation phase, a transient adjustment period, and a subsequent re-escalation of stress under prolonged exposure. These findings demonstrate that integrated biomarker indices provide a more comprehensive evaluation of ecological risk than single biomarkers alone. The elevated IBRv2 values under chronic exposure underscore the potential long-term ecological risks of triclopyr to non-target soil organisms, even at sublethal concentrations, and emphasize the importance of incorporating multi-biomarker integration approaches in soil ecotoxicological assessments.

3.6 Study limitations

We acknowledge several limitations of this study. First, the exposure concentrations used in this study were nominal values; analytical verification of triclopyr concentrations in soil was not performed. While analytical-grade triclopyr was used and uniformly mixed into freshly prepared artificial soil under controlled conditions, we recognize that adsorption to soil organic matter and potential degradation over the 28-day exposure period could result in discrepancies between nominal and actual concentrations. Future studies should incorporate chemical analysis (*e.g.*, HPLC or LC-MS) to confirm exposure levels, particularly when using natural soils or longer exposure durations. Second, this study was conducted under standardized laboratory conditions using artificial soil; the findings may not fully capture the complexity of natural soil ecosystems, including microbial degradation, varying organic matter content, and interactions with other contaminants. Third, while we evaluated a comprehensive suite of biomarkers, molecular-level endpoints (*e.g.*, gene expression of antioxidant and detoxification pathways) were not assessed. Integrating transcriptomic or proteomic analyses in future work would provide deeper mechanistic insights into triclopyr toxicity in earthworms.

4. Conclusion

This study demonstrates that subchronic exposure to triclopyr induces significant physiological disturbances in *Eisenia fetida*, even at sublethal concentrations. Triclopyr exposure resulted in pronounced growth inhibition, elevated ROS production, enhanced lipid peroxidation, and marked alterations in antioxidant and detoxification enzyme activities. The observed pattern of early enzymatic activation followed by suppression under prolonged exposure indicates that antioxidant defense systems may become compromised during chronic stress.

The integrated biomarker response analysis (IBRv2) effectively captured the cumulative impact of triclopyr, revealing a non-linear temporal response characterized by early activation, transient adjustment, and eventual re-escalation of physiological stress. These findings highlight oxidative stress as a central mechanism underlying triclopyr toxicity in

earthworms and demonstrate the advantage of combining growth endpoints with biochemical biomarkers and integrated indices for ecological risk evaluation.

From an environmental perspective, the significant biomarker responses observed at relatively low triclopyr concentrations raise concerns regarding its potential long-term effects on soil invertebrates. Based on the sustained oxidative stress and growth impairment observed, triclopyr concentrations exceeding 1 mg kg^{-1} in soil may pose significant chronic risks to earthworm populations. We recommend this threshold for consideration in soil quality guidelines, pending field validation. Future research should focus on linking these biochemical responses to molecular and reproductive endpoints, as well as validating laboratory findings under field conditions. Such efforts will contribute to a more comprehensive understanding of triclopyr's ecological risks and support the development of safer herbicide application and soil management strategies.

Author contributions

Likun Wang: writing – original draft, software, investigation, resources. Zhuzhu Gu: writing – review & editing, software. Jiayao Luo: software, investigation. Zixuan Qiu: writing – review & editing, supervision, conceptualization.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Supplementary information (SI): the summary of growth parameters and biomarker responses in *Eisenia fetida* exposed to triclopyr. See DOI: https://doi.org/10.1039/d6ra00908e.

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