Acute Toxicity, Oxidative Stress, Toxicity Mechanism, and Degradation Dynamics of Trifluralin in *Eisenia foetide* (Annelida: Lumbricidae)[1]

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Abstract Trifluralin is a preemergent herbicide that is applied to soil to control annual grasses and broadleaf weeds. It is widely used in cotton, *Gossypium hirsutum* L., production in China; however, the ecological safety of its continued use is a controversial issue. We studied the interaction of trifluralin and earthworms, *Eisenia foetide* Savigny (Annelida: Lumbricidae), to provide additional information for assessing the risk of trifluralin to ecological safety in soils. Contact toxicity assays established median lethal concentrations (LC_{50}) of 726.298 μg/L at 24 h, 418.783 μg/L at 48 h, and 82.007 μg/L at 72 h of exposure to trifluralin. Within 24 to 48 h of exposure to trifluralin, antioxidant activity (e.g., superoxide dismutase, catalase, peroxidase) increased in vivo, but by 72 h of exposure the activity was inhibited and, at high concentrations of trifluralin, death occurred. Based on the activity of glutathione S-transferase (GST) and multifunction oxidase (MFO), it appears that GSTs may be involved in the detoxification of trifluralin in vivo, and that MFOs may be the key detoxification enzymes involved. Earthworm degradation of trifluralin shortened the half-life of trifluralin in soil by as much as 1.78 d. These results provide useful information on the toxicity mechanism of trifluralin in earthworms, the role of earthworms in trifluralin degradation, as well as the ecological safety of trifluralin.

Key Words earthworm, trifluralin, protective enzyme, detoxification enzyme, degradation dynamics

Trifluralin (α,α,α-triflfluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) is a selective preemergent dinitroaniline herbicide (Boutsalis et al. 2012, Saini et al. 2015) that is widely used to control annual grass and broadleaf weeds in field crops (Chen et al. 2018). It is widely used as a broad-spectrum soil-treatment agent in cotton (*Gossypium hirsutum* L.), wheat (*Triticum cereale* Schrank), and oil crops (Bijanzadeh et al. 2010, Chowdhury et al. 2020, Coleman et al. 2020, Zhang 2018), and effectively controls *Echinochloa crus-galli* (L.) Palisot de Beauvois, *Amaranthus retroflexus* L., *Chenopodium glaucum* L., and *Setaria viridis* (L.) Palisot de Beauvois (Qiang et al. 2006). In China, the use of trifluralin is concentrated in the

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main cotton-producing areas of Xinjiang Uygur Autonomous Region, the Yellow River basin, and the Yangtze River basin (Li et al. 2021a). Its mode of action is in the interference with electron transport in α-tubulin and inhibition of mitosis (Blume et al. 2003, Chowdhury et al. 2020, Patrick et al. 2011).

Since trifluralin was registered in 1963, its ecological safety has attracted significant attention (Epp et al. 2018, Fernandes et al. 2013). Studies have shown that trifluralin persists in soil for at least 4.4 h (McFarland et al. 1996) but can remain for up to 18 mo or more (Antonious 2012, Karasali et al. 2017, Malterre et al. 1998). Some of the residual trifluralin in soil is gradually eliminated through microbial degradation and chemical decomposition while the remainder is adsorbed by soil, where it may become a source of pollution in surface water (Karasali et al. 2017, Nguyen et al. 2019, Xavier et al. 2004). Soil and water pollution can directly or indirectly lead to human health risks (Maria et al. 2019).

While biological toxicity evaluation of trifluralin has shown that it presents low toxicity to humans and animals (Ebert et al. 1992, Gaines and Linder 1986), other studies have shown that a small amount of trifluralin exposure to the eyes can lead to corneal ulcers and decreased vision (Li et al. 2003). Furthermore, high doses can have muscarinic effects and lead to death due to depression of respiratory function (Tierney et al. 2006). Trifluralin not only shows cytotoxicity and DNA damage (Ribas et al. 1995, 1996), it also affects mitochondrial function (De Oliveira et al. 2020), induces cancerous growth (Weichenthal et al. 2010), exhibits genotoxicity (Hakala and Chin 2010), and is a persistent biological toxin (Bisceglia et al. 2018). At present, trifluralin is banned in EU countries (European Food Safety Authority 2005), but due to its high-cost performance, its use remains extensive in other countries and regions, including China (Li et al. 2021b, Malterre et al. 1997). Accordingly, the environmental toxicity of trifluralin must be limited as much as possible.

The degradation of pesticides in the environment occurs by both biological and abiotic routes. Abiotic routes include physical degradation (e.g., washing, ultrasonic technology, adsorption, separation, and ionizing radiation) and chemical degradation (e.g., oxidative and photochemical degradation). Biodegradation is the decomposition of macromolecular compounds into small molecules through the action of animals, plants, and microorganisms. These degradation pathways play different roles for different types of pesticides. For example, the photolysis of organophosphates is more important than their biodegradation and hydrolysis (Herrmannr et al. 1999). Carbamate pesticides mainly rely on chemical degradation (Chen et al. 2009), and pyrethroid pesticides can be biodegraded (Lan et al. 2006). Laboratory studies have shown that the main pathways for the elimination of trifluralin from soil are photolysis, volatilization, and leaching (Brian and Allan 1980, Leitis and Crosby 1974, Luo and Guo 1992), with photolysis and volatilization being the most important for trifluralin on or near the soil surface (Konstantinou et al. 2001, Williams et al. 1986). Trifluralin at soil depths exceeding 0.2 m is mainly degraded by microorganisms (Bellinaso et al. 2003, Erguven et al. 2016, Tiryaki et al. 2004). Most microorganisms with degradation functionality are related to earthworm (e.g., *Eisenia foetide* Savigny [Annelida: Lumbricidae]) activity (Contreras-Ramos et al. 2006, Liu et al. 2011). Therefore, earthworms may be an important biological factor in the degradation of trifluralin in soil (Goto and Sudo 2018).

Earthworms are the most commonly used indicator species in soil ecological safety assessment (Katagi and Ose 2015, Pelosi et al. 2013) as they have high

tolerance to many organic and inorganic chemical pollutants in soil and can often bioaccumulate them in tissues without deleterious effects (Contreras-Ramos et al. 2006). Therefore, the biodegradation of organic pollutants by earthworms is emerging as an environmentally friendly bioremediation method (Liu et al. 2011, Tejada and Masciandaro 2011).

Studies have shown that the activities of earthworms have important effects on the degradation and transformation of organic pollutants in soil (Hickman and Reid 2008). Pollutants are metabolized and immobilized by the earthworm digestive system during the ingestion and excretion of soil (Drake and Horn 2006, Horn et al. 2003, Phillips et al. 2000). Furthermore, the activities of earthworms change the permeability of soil and the structure of soil aggregates, affecting the redox state of soil and affecting the bioavailability of organic pollutants (Alekseeva et al. 2006, Binet et al. 2006, Bolan and Baskaran 1996). It also has been reported that earthworms exert different degradation effects on organic soil pollutants, such as atrazine, polychlorinated biphenyls, methamidophos, acetochlor, and pentachlorophenol (Hao et al. 2018; Lin et al. 2016; Liu et al. 2008a, 2008b; Schreck et al. 2008). However, the role of earthworms in the degradation of trifluralin has not been reported.

Herein, we defined the toxicity of trifluralin to earthworms using the filter paper–contact bioassay method as recommended by the Organization for Economic Cooperation and Development (OECD 1984). We also quantified the activity of the protective enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), as well as the glutathione S-transferase (GST), multifunction oxidase (MFO), and cellulase in earthworms upon exposure to trifluralin. Knowledge of the roles of these enzymes in oxidative stress response and the detoxification and metabolism of trifluralin will provide information on the involvement of earthworms in the trifluralin-degradation dynamics in soil. Thus, our study should provide additional guidance for the safe and scientific use of trifluralin, provide a reference for assessing the risk of trifluralin on soil ecological safety, and offers new insights for the sustainable biodegradation of trifluralin.

Materials and Methods

Earthworms and chemicals. Earthworms used in this study were *E. foetide* that were reared in the Laboratory of Insect Ecological Resources, College of Agriculture, Shihezi University. Before the beginning of the study, they were precultured in the dark for 24 h, and healthy adult worms with obvious bands and weighing approximately 300 mg were selected for the study.

Trifluralin standard: 1 ml/branch (effective component 100 μg/ml; No. GSB05-2313-2008, Ministry of Agriculture Environmental Protection Research Monitoring Institute of China). Trifluralin EC (48%) was provided by Jiangsu Lianyungang Second Pesticide Factory (Lianyungang City, Jiangsu Province, China).

Bioassay. Trifluralin EC (48%) was used to prepare solutions with concentrations of 0 (blank control), 1.152, 11.52, 46.08, 115.2, 460.8, and 576.0 μg/L. Each solution (10 ml) was evenly sprayed into individual glass petri dishes containing a double layer of 15-cm-diameter filter paper. Earthworms that had been starved for 24 h were washed in water and dried on filter paper. Ten earthworms were placed in

each petri dish, which was then sealed with cling film perforated with a needle to ensure ventilation and relatively constant humidity. These petri dishes were maintained in an incubator at 20°C for 72 h without light. Mortality and toxic symptoms of the earthworms were checked and recorded at 24, 48, and 72 h after initial exposure. An earthworm was considered dead when being gently touched with a capillary resulted in no reaction or movement.

Enzyme activity determination. Earthworms surviving the toxicity bioassay were collected at 24, 48, and 72 h after initiation of exposure. Earthworms that had been washed and dried were homogenized using a high-speed homogenizer at 0°C. The homogenate was centrifuged at 2,500 rpm at 0°C. The supernatant was centrifuged at 3,000 rpm under the same conditions. That supernatant was used for the determination of enzymatic activity.

Protein content and the activities of the enzymes SOD, POD, CAT, GST, MFO, and cellulase were determined in triplicate. Protein content was determined by the Coomassie brilliant blue G-250 staining method (Leong and Ho 1995). Enzyme activity was measured as follows: POD activity by the guaiacol oxidation method (Simon et al. 1974); SOD activity by the nitrotetrazolium blue reduction method (Saint-Denis et al. 1999); CAT activity by the ultraviolet absorption method (Xu et al. 1997); GST activity by methods of Oppenoorth et al. (1979); MFO activity by methods of Yu and Nguyen (1992); and cellulase activity by the carboxymethyl cellulose method (Stellmach 1992).

Determination of trifluralin dynamics in soil by earthworms. After screening and sterilization, the physicochemical properties of soil without trifluralin were determined as a loamy soil with pH = 7.29, organic matter = 20.43 g/kg, total nitrogen = 1.38 g/kg, alkaline hydrolysis nitrogen = 51.3 mg/kg, rapidly available phosphorus = 23.03 mg/kg, and rapidly available potassium = 519.8 g/kg. The treated soil was placed into a plastic container (30 × 25 cm) and compacted. Then, trifluralin EC (48%) was sprayed on the surface of the soil according to the recommended field dosage (2.25 L/hm^2), and the soil was mixed to a depth of approximately 5 cm. Fifty earthworms were then added to each container, the soil was sprinkled with water, and the earthworms were kept in the containers overnight. The earthworms were removed, and the containers were maintained in a shaded facility at approximately 25°C. Drip irrigation was applied at 200 m^3/667 m^2 each week. At day 1, 7, 14, and 42, soil samples at depths of 0, 5, 10, 15, 20, and 25 cm were taken from each container.

Trifluralin content was determined at each of those depths on those days for each of three replicates. Methanol (25 ml) was added to each 10-g sample. After an ultrasonic extraction for 1 h, 10 ml of filtrate was obtained and centrifuged. The supernatant was transferred to a separating funnel, and 50 ml 5% Na_2SO_4 was added. The mixture was extracted sequentially with 25, 15, and 10 ml of petroleum ether. The extracts were combined and dehydrated through an anhydrous Na_2SO_4 funnel and concentrated by rotary evaporation. Then, 2 cm anhydrous Na_2SO_4, 6 g Florisil® (US Silica, Katy, TX), and 2 cm anhydrous Na_2SO_4 were layered sequentially into a column and washed with 10 ml of petroleum ether. The extract was then loaded onto the column and eluted with 40 ml petroleum ether. The eluent was concentrated with a rotary evaporator, brought to 5 ml with methanol, and passed through a 0.45-μm filter membrane pending liquid chromatography analysis.

Table 1. Earthworm mortality in response to trifluralin concentration.

	Duration of Exposure (h)		
Concentration (μg/L)	**24**	**48**	**72**
0	0.0	0.0	0.0
1.152	0.0	0.0	20.0
11.52	0.0	0.0	20.0
46.08	0.0	0.0	43.3
115.2	0.0	13.3	60.0
460.8	30.0	43.3	100.0
576.0	36.7	80.0	100.0

The high-performance liquid chromatography (HPLC) was performed with a LC1200 (Agilent, Santa Clara, CA) as follows: the detection wavelength was 274 nm; separation was performed on a C18 column (15 cm × 4.6 mm ID, 5 μm); column temperature: 38°C; mobile phase: methanol/water (8:2), flow rate: 0.5 ml/min; injection volume: 5 μl. The results were quantified by the external standard method. The detection time of trifluralin standard was 2.977 min, and that of fluralin in soil samples was 2.978 min.

Statistical analysis. The data were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL). Concentration–mortality response of earthworms to trifluralin was calculated by probit analysis to yield median lethal concentrations (LC_{50}) and associated parameters. One-way analysis of variance was used for multiple comparative analysis, and treatment means were separated by the least significant differences (LSD) method, when applicable. Statistical differences were considered significant at $P < 0.05$. The first-order kinetic equation of trifluralin was $C = C_0 e^{-Kt}$, where C = pesticide residues at time t, μg/kg; C_0 = original deposition after application, μg/kg. The half-life formula is $T_{1/2} = \ln 2/K$, where K = degradation coefficient; T = days after application.

Results

Toxicity of trifluralin to earthworms. The filter paper–contact bioassay showed that there was a concentration-dependent and time-dependent relationship between trifluralin and *E. foetide* mortality (Table 1). Mortality ranged from 0% to 100% with increasing trifluralin concentration and increased time of exposure with none of the earthworms exposed for 72 h to the two highest trifluralin concentrations (460.8 and 576.0 μg/L) surviving (Table 2). The LC_{50} of trifluralin in *E. foetide* also significantly decreased with the duration of exposure (Table 1).

Oxidative enzyme activity. The activity of the oxidative enzymes in response to the concentrations of trifluralin tested were compared within each interval after exposure. At 24 h of exposure, POD activity was significantly higher (11.52, 115.2,

Table 2. Concentration–mortality response of earthworms to trifluralin.

Time (h)	Regression Equation	Slope of the Regression Line	95% Confidence Limit	LC_{50} (μg/L)*	R^2
24	$Y = -7.878 + 2.748X$	2.748	551.131–2,016.273	736.705	0.995
48	$Y = -6.577 + 2.555X$	2.555	290.073–501.063	375.465	0.935
72	$Y = -1.560 + 1.054X$	1.054	0.582–285.728	30.251	0.944

* LC_{50}, median lethal concentration.

576.0 μg/L) and significantly lower (11.52, 115.2 μg/L) than the control group ($F = 346.705$; df = 6, 20; $P < 0.05$; Fig. 1A). After 48 h of exposure, POD activity at the lowest (1.152 μg/L) and highest (576 μg/L) trifluralin concentrations were significantly higher than that of the control ($F = 8.061$; df = 6, 20; $P = 0.01 < 0.05$), while the remaining concentrations did not differ from the control (Fig. 1A). In comparison to the control, POD activity at 72 h was significantly increased with the 11.52-μg/L treatment and significantly decreased with the 46.08-, 115.2-, and 460.8-μg/L treatments ($F = 342.008$; df = 4, 14; $P < 0.05$; Fig. 1A).

SOD activity at the 24-h interval was significantly depressed at the 46.08-, 115.2-, 460.8-, and 576.0-μg/L trifluralin treatments ($F = 36.385$; df = 6, 20; $P < 0.05$); there were no significant differences among the lowest concentrations and the control (Fig. 1B). At 48 h, SOD activity at the 1.152-, 11.52-, and 46.08-μg/L concentrations was significantly higher ($F = 76.468$; df = 6, 20; $P < 0.05$) than the control, while activity in response to the higher concentrations did not differ from the control (Fig. 1B). SOD activity at all concentrations was significantly lower than the control at 72 h ($F = 11.190$; df = 4, 14; $P = 0.001 < 0.05$; Fig. 1B).

CAT activity in response to the trifluralin concentrations tested did not differ from the control at 24 h ($F = 0.300$; df = 6, 20; $P = 0.927 > 0.05$) and 72 h ($F = 0.753$; df = 4, 14; $P = 0.578 > 0.05$) (Fig. 1C). At 48 h, CAT activity at the 11.52-, 115.2-, 460.8-, and 576.0-μg/L treatments was significantly higher than that of the control ($F = 15.664$; df = 6, 20; $P < 0.05$), while activity at 1.152 and 46.08 μg/L did not differ from that of the control (Fig. 1C).

Detoxifying enzyme activity. Statistical analysis showed no significant increases or decreases in GST activity at 24, 48, and 72 h of exposure to all trifluralin concentrations tested (Fig. 2A). MFO activity generally increased with increased trifluralin concentration (Fig. 2B). The only decrease in MFO activity was observed with the 1.152-μg/L concentration at 24 h, with the activity in response to the remaining concentrations being significantly higher than the control ($F = 41.757$; df = 6, 20; $P < 0.05$). At 48 h, MFO activity was significantly higher with the 460.8- and 576.0-μg/L treatments ($F = 17.099$; df = 6, 20; $P < 0.05$); exposure to the remaining concentrations yielded no significant differences from the control (Fig. 2B). At 72 h, MFO activity was higher than the control with all trifluralin concentrations tested ($F = 20.804$; df = 4, 14; $P < 0.05$) (Fig. 2B).

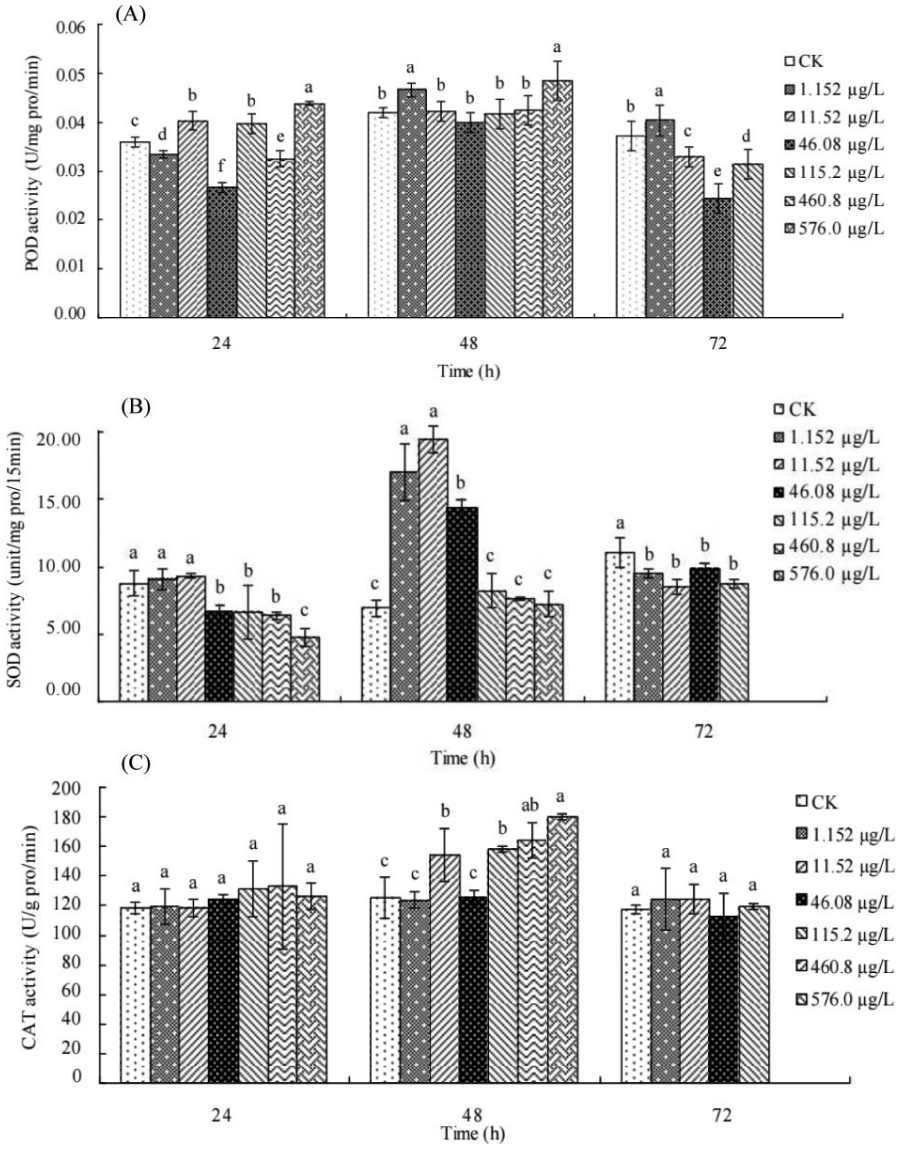

Fig. 1. Oxidative enzyme activity in earthworms 24, 48, and 72 h after exposure to different concentrations of trifluralin: peroxidase (POD) (A), superoxide dismutase (SOD) (B), and catalase (CAT) (C). Means within the same enzyme and time interval followed by the same lowercase letter are not significantly different (LSD, $P < 0.05$).

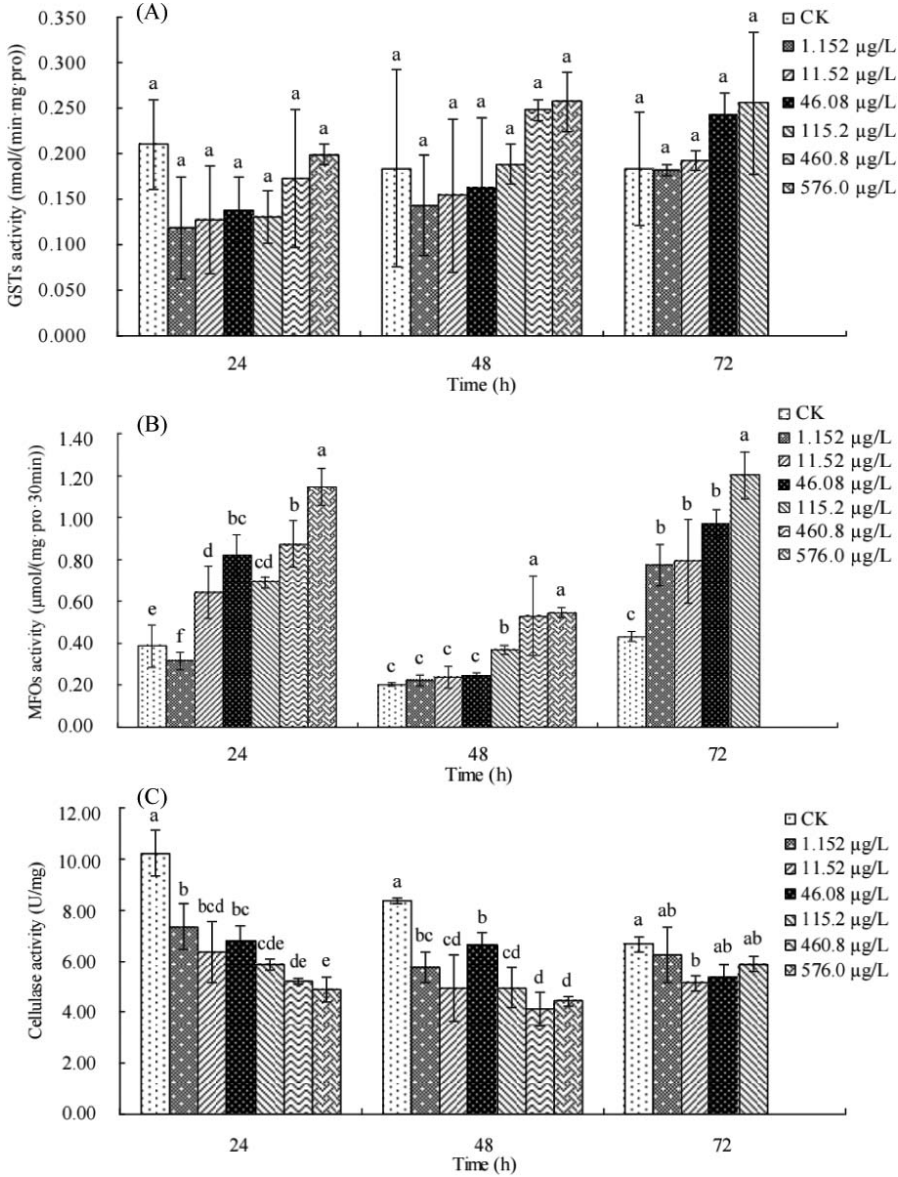

Fig. 2. Detoxifying enzyme activity in earthworms 24, 48, and 72 h after exposure to different concentrations of trifluralin: glutathione S-transferase (GST) (A), multifunction oxidase (MFO) (B), and cellulase (C). Means within the same enzyme and time interval followed by the same lowercase letter are not significantly different (LSD, $P < 0.05$).

Cellulase activity, in general, decreased with increasing levels of trifluralin (Fig. 2C). At 24 and 48 h, cellulase activity in each treatment group was significantly lower than that of the control (24 h: $F = 17.437$; df = 6, 20; $P < 0.05$; 48 h: $F = 18.554$; df = 6, 20; $P < 0.05$) (Fig. 2C). At 72 h, only the treatment with 11.52 μg/L significantly decreased cellulase activity in direct comparison to the control ($F = 1.036$; df = 6, 20; $P < 0.05$) (Fig. 2C).

Trifluralin dynamics in soil with and without earthworms. We determined trifluralin quantity in soils based upon the standard curve for increasing concentration of trifluralin (Fig. 3A), detection of trifluralin by HPLC (Fig. 3B), and detection of trifluralin in soil by HPLC (Fig. 3C). In the soil without earthworms, we found that 1 d after application, trifluralin content at the 0- to 5-cm depth was 10.6× that in the 5- to 10-cm depth, and we were unable to detect trifluralin at greater depths (Table 3). At the 7-d interval, we detected trifluralin into the 10- to 15-cm and 10- to 20-cm depths, although the quantity of trifluralin was significantly highest in the 0- to 5-cm depth ($F = 259.688$; df = 3, 11; $P < 0.05$). After 14 d, trifluralin was detected in each level of the soil profile with a significantly higher quantity in the 0- to 5-cm depth than in the lower layers ($F = 65.369$; df = 4,14; $P < 0.05$). No significant differences in trifluralin content were detected in the 0- to 5-cm, 5- to 10-cm, and 10- to 5-cm soil depths at 42 d, but the content in these depths were significantly higher than detected in the 15- to 20-cm and 20- to 25-cm depths ($F = 14.866$; df = 4, 14; $P < 0.05$) (Table 3).

In the soil without earthworms, we also noted that trifluralin content in the 0- to 5-cm soil depth significantly decreases over time ($F = 727.835$: df = 3,11; $P < 0.05$) (Table 3). In contrast, trifluralin quantity in the 5- to 10-cm depth significantly increased from day 1 to day 7 ($F = 7.898$; df = 3, 11; $P = 0.009 < 0.05$) (Table 3), due to an apparent leaching of the chemical from the upper layer.

In the soil containing earthworms, we detected trifluralin to a depth of 15 cm 1 d after application (Table 3) with a significantly higher amount in the 0- to 5-cm depth than in either the 5- to 10-cm and 10- to 15-cm depths ($F = 21{,}656.435$; df = 2, 8; $P < 0.05$). By 7 d, we detected trifluralin throughout the soil profile with the significantly greatest amount detected in the 0- to 5-cm layer ($F = 109.613$; df = 4, 14; $P < 0.05$).

Over time, trifluralin content in the 0- to 5-cm soil layer significantly decreased at each sampling interval ($F = 3{,}321.730$; df = 3, 11; $P < 0.05$) (Table 3). Trifluralin content in the 5- to 10-cm and 10- to 15-cm soil layers first increased and then decreased, reaching their maximum peaks at 7 d (5–10 cm: $F = 20.606$; df = 3, 11; $P < 0.05$; 10–15 cm: $F = 55.667$; df = 3, 11; $P < 0.05$) (Table 3). Trifluralin content in the 15- to 20-cm depth decreased gradually after initially appearing in that layer at 7 d (Table 3). Trifluralin content in the 20- to 25-cm layer did not differ significantly over time after its initial appearance in that layer at 7 d.

The dynamics of trifluralin in soil conformed to the first-order kinetic equation $C = C_0 e^{-0.0326t}$, with a calculated half-life of 21.29 d. The first-order kinetics model of trifluralin $C = C_0 e^{-0.0355t}$ was employed to determine a half-life of trifluralin in soil containing earthworms as 19.51 d. Thus, the presence of earthworms in the trifluralin-treated soil shortened the trifluralin half-life by 1.78 d.

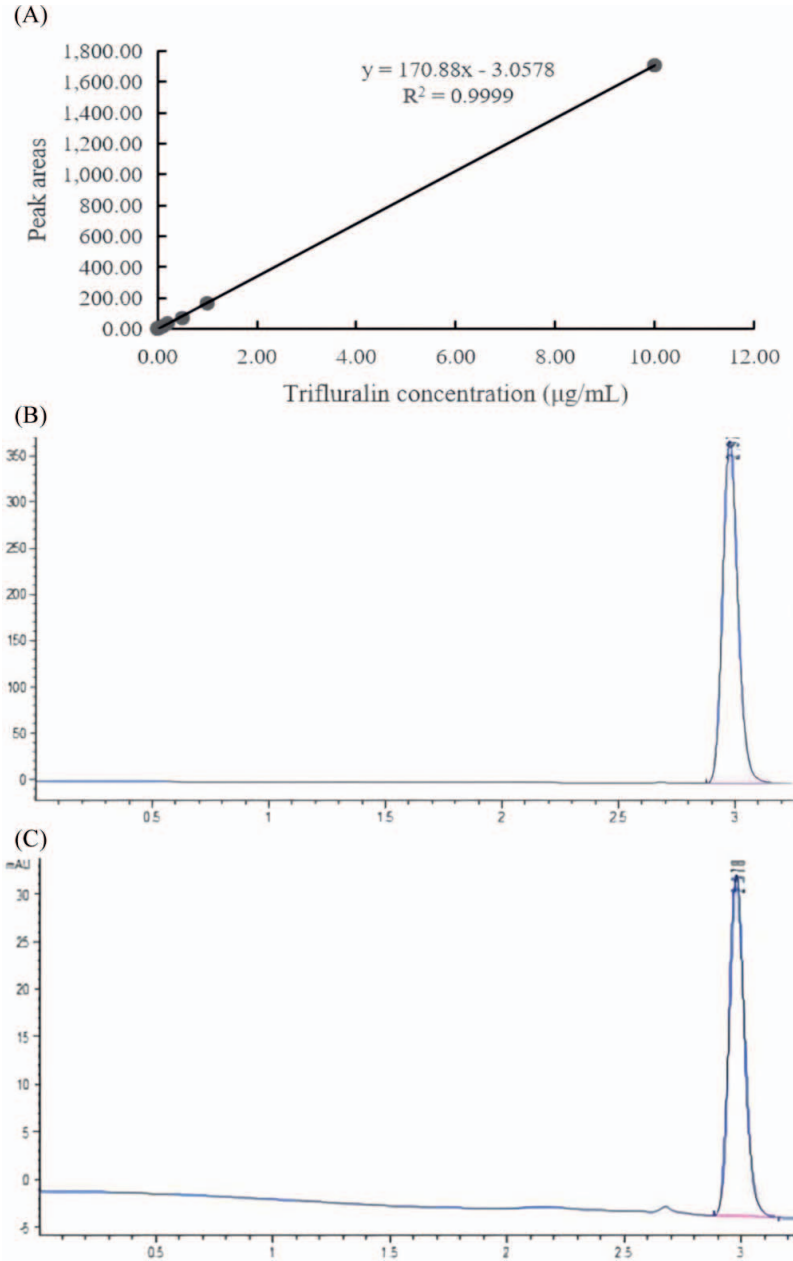

Fig. 3. High-performance liquid chromatography chromatograms of trifluralin: standard curve (A), standard content in soil (B), and test samples of soil (C).

Table 3. Quantity of trifluralin detected at selected soil depths at 1, 7, 14, and 42 d in soil with and without earthworm activity.*

Soil Depth (cm)	1 d	7 d	14 d	42 d
Soil without earthworms				
0–5	23.696 ± 0.666A	12.752 ± 0.333aB	8.071 ± 0.858aC	2.726 ± 0.155aD
5–10	2.238 ± 0.089B	2.843 ± 0.176bA	2.921 ± 0.089bA	2.726 ± 0.310aA
10–15	ND**	2.277 ± 0.089bcA	2.316 ± 0.101bA	2.531 ± 0.244aA
15–20	ND	1.251 ± 1.084cA	1.887 ± 0.034bA	1.946 ± 0.068bA
20–25	ND	ND	0.615 ± 1.067c	1.850 ± 0.004b
Mean value	5.186 ± 10.392A	3.824 ± 5.107A	3.162 ± 2.871A	2.356 ± 0.426A
Soil with earthworms				
0–5	21.745 ± 0.117aA	9.144 ± 0.147aB	5.905 ± 0.390aC	3.53 ± 0.234aD
5–10	2.433 ± 0.176bB	3.038 ± 0.206bcB	2.960 ± 0.155bA	2.141 ± 0.101bC
10–15	2.121 ± 0.089bB	3.018 ± 0.176bA	2.414 ± 0.089cB	1.906 ± 0.059cC
15–20	ND	2.316 ± 0.089cA	2.239 ± 0.270dAB	1.868 ± 0.034cB
20–25	ND	1.252 ± 1.067dA	1.965 ± 0.059dA	1.926 ± 0.034bcA
Mean value	5.260 ± 9.286A	3.754 ± 3.100A	3.096 ± 1.612A	2.219 ± 0.588A

* Means within the same row followed by the same uppercase letter are not significantly different (LSD, $P < 0.05$). Means within the same column and soil treatment (with and without earthworms) followed by the same lowercase letter are not significantly different (LSD, $P < 0.05$).
** ND, not detected.

Discussion

Toxic mechanism of trifluralin on earthworms. Ecotoxicological tests based on earthworms have been widely used in the ecological evaluation and risk prediction of contaminated soil (Paoletti 1999, Rodriguez-Campos et al. 2014). We observed a significant correlation of trifluralin concentration with earthworm mortality. Upon exposure to trifluralin for 72 h, the mean mortality of earthworms at the lowest concentration tested (1.152 μg/L) was 20%, which is much higher than that reported by Goto and Sudo (2018) (2% for *Eisenia* spp.). This may be due to the different toxicity-determination methods used (Gu et al. 2021, Katagi and Ose 2015, Wang et al. 2014). We adopted the filter paper–contact method, while those studies used the soil-treatment method, perhaps accounting for the significant differences in results. The actual concentration of residual pesticides in soil is often lower than the lethal concentration for soil organisms, so the determination of the toxic effects of pesticides on earthworms at lower concentrations is of greater significance for evaluating the effect of pesticides on the soil environment (Capolupo et al. 2016, Zhang et al. 2013).

Enzymatic activities are regarded as biomarkers of environmental pollution (Liang et al. 2013). In previous studies, SOD, CAT, POD, GST, MFO, and cellulase were selected for their sensitive responses to various environmental pollutants (Yang et al. 2018, Zhang et al. 2013, Zheng et al. 2013). In our study, with the passage of time, a low concentration (1.152 μg/L) of trifluralin induced an increase of POD activity, indicating a possible response to attack by hydroxyl radicals (Zhang et al. 2013). SOD and CAT activity in earthworms is activated after 48 h of exposure to trifluralin, which aids in adapting to the effect of pollutants. However, after 72 h of exposure to trifluralin, the activity of the antioxidant enzymes in earthworms was inhibited, and death occurs at high concentrations in some cases. This may be because long-term exposure to trifluralin leads to excessive production of reactive oxygen species (Nordberg and Arner 2001, Sun et al. 2007, Zhang et al. 2013), intestinal injury, and toxic reactions (Li et al. 2020, Yang et al. 2018), which inhibits or inactivates these enzymes. These mechanisms need to be verified further.

GSTs and MFOs are important detoxification metabolic enzymes in insects and mollusks (Pedersen et al. 2019, Rane et al. 2019). These enzymes reduce pesticide stress by enhancing metabolic activity (Ishaaya 1993). Our results showed that trifluralin has a certain time-dependent effect on the induction of GSTs in earthworms. After exposure for 24 h, the GST activities were lower than that of the control group, and the GST activities of the treatment groups were higher than those of the control group after 48 and 72 h. Therefore, GSTs may be involved in the detoxification of trifluralin. However, during the entire exposure process, MFO activity in earthworms was significantly induced, indicating that MFOs are key detoxification enzymes that mediate the toxic effects of trifluralin on earthworms. This result differs from the previously reported toxicity mechanisms of glyphosate, sulfentrazone, carfentrazone-ethyl, atrazine, and other herbicides in earthworms (Lammertyn et al. 2021; Li et al. 2020, 2021b; Zaller et al. 2021), thus indicating that the toxicity mechanism of herbicides in earthworms may be related to the type of herbicide. Furthermore, this phenomenon indicates that we should consider whether trifluralin in the soil has a synergistic effect with other pesticides and

fungicides, further aggravating its toxicological effect on earthworms (Lydy and Linck 2003, Sánchez-Bayo 2021).

The cellulase level in the earthworm gut reflects its ability to decompose plant detritus and other cellulosic materials (Shi et al. 2007). Many herbicides (e.g., acetochlor and fomesafen) have been found to inhibit cellulase activity in earthworms (Xiao et al. 2006, Zhang et al. 2014). We found that cellulase activity in earthworms is also inhibited upon exposure to trifluralin, and this is consistent with previous reports (Xiao et al. 2006, Zhang et al. 2014). However, with the passage of time, this inhibitory effect weakens, which is consistent with the results of Zhang et al. (2014). This change may be due to the degradation of trifluralin in soil, adaptation through enhanced metabolism, and/or absorption by hydrolytic fermentation in the earthworm (Xiao et al. 2006).

Effect of earthworms on the dynamics of trifluralin in soil. In general, the physicochemical properties of pesticides determine their residual dynamics in soil environments (Pandit et al. 1995, Pinto et al. 2011). Studies have shown that volatility, photolysis, and water-solubility affect the residual dynamics of trifluralin in soil (Bedos et al. 2006, Fenoll et al. 2010). We found that the content of trifluralin in the 0- to 5-cm soil layer decreases significantly with time. This may reflect that trifluralin in surface soil is more likely to volatilize and photolyze (Konstantinou et al. 2001, Williams et al. 1986). Furthermore, trifluralin is leached into the soil with irrigation or rainfall (Malterre et al. 1997, 1998). Trifluralin was detected in the 20- to 25-cm soil layer until the 14th day, which may be due to the adsorption of organic matter on the soil, resulting in the low mobility of trifluralin in soil (Senesi and Testini 1984, Wang et al. 2011).

We determined that the dynamics of trifluralin in soil conformed to the first-order kinetic equation $C = C_0 e^{-0.0326t}$, and the half-life was 21.29 d. This approximates the measured half-life values of trifluralin in Canada, Texas (21 d), and South Australia (27–30 d) (Jensen and Kimball 1980, Ying and Williams 2000), but differs from those obtained in soils in Nova Scotia, Canada (126 d), and western Greece (67.9–71.4 d) (Jensen and Kimball 1980, Triantafyllidis et al. 2010). These differences among these studies could be due to differences in soil organic matter content, pH, and climatic conditions in the different regions that affect the degradation rate of trifluralin in soil (Chowdhury et al. 2021, Farenhorst 2007, Kanburoglu et al. 2017).

The biological effects of earthworms play an important role in pesticide degradation (Givaudan et al. 2014). Studies on the degradation of pesticides, such as metolachlor (Sun et al. 2019), glyphosate (Lescano et al. 2020), epoxiconazole and tebuconazole (Bošković et al. 2021), and pyraclostrobin (Zhang et al. 2021) by earthworms have been reported. We detected trifluralin to a depth of 20–25 cm in soil with earthworms on the 7th day after application of trifluralin. This was 1 wk earlier than our detection at that depth of soil with no earthworms, thus indicating that earthworm activity in the soil has a significant effect on the movement of trifluralin. Furthermore, we observed an interesting phenomenon, whereby on the 7th and 14th days of earthworm degradation, the trifluralin content in the 5- to 25-cm soil layer was higher than that in the control, while on the 42nd day, the trifluralin content in each worm-treated soil layer was lower than those in the soil without earthworms. We postulate that earthworm activity transports trifluralin to other soil layers, and the degradation of trifluralin by earthworms is less than the amount transported, resulting in trifluralin content in the soil with earthworms on the 7th and

14th days as higher than that detected in soil without earthworms. However, with the passage of time, the content of trifluralin transported reaches saturation, the content of trifluralin degraded by earthworms is higher than that transported, and finally the content of trifluralin in the soil on the 42nd day is significantly lower than that of the control. We also calculated, based on the first-order kinetics model of trifluralin $C = C_0e^{-0.0355t}$, that the half-life trifluralin in soil containing earthworms was 19.51 d, which was 1.78 d shorter than that observed in soil without earthworms, thus further indicating that earthworms play a role in accelerating the dynamics of trifluralin in soil.

Conclusion. The results of our study show that trifluralin has a toxic effect on earthworms and that there is a significant correlation of earthworm mortality with trifluralin concentration. When stressed by trifluralin, the activities of SOD, CAT, and POD are increased in vivo and alleviate the oxidative stress of trifluralin in earthworms. Furthermore, GSTs and MFOs, which are detoxification enzymes, are involved in the metabolism of trifluralin in earthworms (especially MFOs). However, with extended exposure time and increasing trifluralin concentration, enzyme activity in earthworms is inhibited, leading to death in some cases. Cellulase activity is inhibited at all concentrations of trifluralin. We also found that earthworm activity in the soil shortens the half-life of trifluralin in soil. Moreover, earthworm activity has a positive effect on the movement of trifluralin in soil. Accordingly, we suggest that trifluralin should be used in the field according to the recommended dose, reducing its impact on soil ecological security.

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