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Chronic effects of commercial pesticide preparations on biomarkers and reproductive success in earthworm *Eisenia andrei*

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Abstract

Chemical pollution resulting from pesticide usage has been a continuous issue since the 1960s, despite comprehensive European Union legislation designed to safeguard human health and the environment from the adverse effects of pesticides. While regulatory risk assessments primarily focus on the active ingredients, recent research indicates ecotoxicological impacts of commercial preparations on non-target organisms, particularly within the soil ecosystem where key species such as earthworms play a vital role in maintaining soil quality and fertility. Therefore, the aim of this study was the assessment of the long-term effects of the following respective commercial preparations: the insecticides Sumialfa (esfenvalerate) and Calypso (thiacloprid), as well as the herbicides Frontier (dimethenamid-*p*) and Filon (prosulfocarb) on the earthworm *Eisenia andrei* in standardized soil during long-term exposures of 7, 14, and 28 days. To study the possible effects on different levels of biological organization, enzymatic biomarkers: acetylcholinesterase (AChE), carboxylesterase (CES) glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx); non-enzymatic biomarkers: multixenobiotic resistance activity (MXR), levels of glutathione (GSH), and reactive oxygen species (ROS) as well as reproductive success were investigated. While Calypso appeared to be the least toxic substance, all pesticides showed significant effect on multiobiomarker response in *E. fetida*. That being said, the response of MXR activity was significantly altered by all tested pesticides indicating MXR being the most sensitive endpoint of the present research. Recovery of MXR was observed after 28 days, however, only in case of exposure to Filon, while the recovery of CAT activity was recorded after 28 days as well, subsequent to Sumialfa exposure. Reproductive success was negatively impacted regarding the Frontier and Sumialfa exposure at the highest concentration (100 mg/kg) reflected in reduced number of cocoons, while only the exposure to Frontier (100 mg/kg) reduced the number of juveniles. Based on the results, it is important to include commercial pesticide formulations in pesticide risk assessments. The toxicity classifications of the studied pesticides suggest the potential detrimental consequences to the key soil species in terrestrial ecosystems at various concentrations. Future studies should include other soil species as well as investigation of higher levels of biological organization, i.e., behavioral endpoints, to determine the potential risks to terrestrial ecosystems.

Keywords Earthworms, Non-target organisms, Soil, Insecticides, Herbicides

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Background

Pesticides, essential tools in modern agriculture for controlling pests and ensuring crop yields, have garnered increasing attention due to their potential environmental impacts. While they play a pivotal role in securing food production, their widespread application raises questions about unintended consequences. Despite the ongoing increase in awareness regarding sustainable and organic farming practices, the global usage of pesticides is consistently rising [35]. Pesticides are chemical or biological agents used to control pests and vectors of disease. They can be classified according to their target organism or their mode of action. Global data of pesticide usage show that herbicides are by far the most used type of pesticide, followed by insecticides [13]. Insecticides are designed to target various stages of an insect's life cycle, disrupting their development or causing direct harm, and are known to influence various beneficial insects and other non-target organisms. Herbicides usually target plant-specific mechanisms and they are often considered as safe for other non-target animals. However, various research has shown that is not always true [16, 17, 23].

For this study, four pesticides available on the market were chosen: the herbicides dimethenamid-*p* (Frontier ®) and prosulfocarb (Filon ®); and the insecticides esfenvalerate (Sumialfa ®) and thiacloprid (Calypso ®). Frontier, with active ingredient dimethenamid-*p* belongs to the acetamide herbicide that are commonly used on soybeans and corns [41]. It exhibits significant water solubility and moderate soil absorption, and resistance to volatilization and photolysis [6]. Prosulfocarb is an active ingredient in Filon, a selective pre- and early post-emergence herbicide [33], and its main mode of action is inhibition of synthesis of long-chain fatty acids [3]. Sumialfa, containing the active ingredient esfenvalerate, functions as a pyrethroid insecticide. Its mechanism of action involves the modulation of voltage-gated sodium channels, leading to prolonged channel opening. This, in turn, triggers a continuous firing of action potentials, resulting in the impairment of animal locomotor behavior. Subsequent effects include lethargy, paralysis, and ultimately, mortality [2]. Calypso, with thiacloprid as active ingredient, is a neonicotinoid insecticide that stimulates the nicotinic acetylcholine receptor causing its abnormal excitation, thus leading to insect death through convulsive paralysis [4].

All of the abovementioned pesticides are still in usage, so the main aim was to investigate its effects on non-target soil organisms. Earthworm *Eisenia andrei* was chosen as the test organism, due to its widespread usage in ecotoxicology. An earlier study has demonstrated that the commercial formulations of these four pesticides exhibit increased toxicity relative to their active ingredients [21]. Furthermore, these formulations impact the earthworm species *E. andrei* at the subcellular level following a 48-h filter paper test [21]. The main goal was, thus, to investigate the long-term effects of the selected pesticides on a set of biomarkers as well as on reproduction success of *E. fetida*.

Methods

Chemicals

The following chemicals were used: acetonitrile (C_2H_3N, CAS 75-05-8), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH) ($C_{12}H_{26}N_7Na_4O_{17}P_3 \times H_2O$, CAS 2646-71-1 (anhydrous)), 9-(2-carboxyphenyl)-6-diethylamino-3-xanthenylidene-diethylammonium chloride (rhodamine B) ($C_{28}H_{31}ClN_2O_3$, CAS 81-88-9), CellTracker™ Green CMFDA Dye ($C_{25}H_{17}ClO_7$, CAS 136832-63-8) (ThermoFisher Scientific), 1-chloro-2,4-dinitrobenzene (CDNB) ($C_6H_3ClN_2O_4$, CAS 97-00-7), CM-H_2DCFDA ($C_{27}H_{19}Cl_3O_8$, CAS 1219794-09-8) (ThermoFisher Scientific), hydrogen peroxide (H_2O_2, CAS 7722-84-1), (2-mercaptoethyl)trimethylammonium iodide acetate (acetylthiocholine iodide) ($CH_3COSCH_2CH_2N(CH_3)_3I$, CAS 1866-15-5), disodium hydrogen phosphate (Na_2HPO_4, CAS 7558-79-4), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) ($[-SC_6H_3(NO_2)CO_2H]_2$, CAS 69-78-3), glutathione disulfide (GSSG) ($C_{20}H_{32}N_6O_{12}S_2$, CAS 27025-41-8), 4-nitrophenyl acetate ($C_8H_7NO_4$, CAS 830-03-5), (2S)-2-amino-4-{[(1R)-1-[(carboxymethyl)carbamoyl]-2-sulfanylethyl]carbamoyl}butanoic acid (glutathione (GSH)) ($C_{10}H_{17}N_3O_6S$, CAS 70-18-8), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \times 2H_2O$, CAS 13472-35-0), ethylenediaminetetraacetic acid disodium salt hydrated ($C_{10}H_{16}N_2O_8$, CAS 6381-92-6), dimethyl sulfoxide (DMSO) ($(CH_3)_2SO$, CAS 67-68-5), glutathione reductase from baker's yeast (*Saccharomyces cerevisiae*) (ammonium sulfate suspension) EC 1.6.4.2. (CAS 9001-48-3), sodium azide (NaN_3, CAS 26628-22-8. Protein concentrations were measured using the PierceTM BCA Protein Assay Kit.

Following analytical standard grade pesticide active ingredients and their respective commercial preparations were used: dimethenamid-p ($C_{12}H_{18}ClNO_2S$, CAS 163515-14-8) (Frontier, BASF, 720 g/L a.i.), esfenvalerate ($C_{25}H_{22}ClNO_3$, CAS 66230-04-4) (Sumialfa, Arysta LifeScience, 50 g/L a.i.), prosulfocarb ($C_{14}H_{21}NOS$, CAS 52888-80-9) (Filon, SYNGENTA, 800 g/L a.i.), thiacloprid ($C_{10}H_9ClN_4S$, CAS 111988-49-9) (Calypso, Bayer Crop Science, 480 g/L a.i.).

Test organism

Adult earthworms (*E. andrei*) with a well-developed clitellum were used for the experiments with an average

weight of 0.3 g. They were supplied from a local earthworm farm in Croatia (OPG Škrljak, Sv. Ivan Zelina-Biškupec, 45° 57′ 27.6″ N, 16° 14′ 03.8″ E) and acclimatized at 20 °C prior to all experiments. 24 h before the experiment, earthworms were washed with distilled water, placed on damp filter paper in petri dishes, and covered with aluminum foil containing aeration holes and left to empty their gut contents.

Exposures

The exposures for the determination of acute toxicity were conducted in the artificial soil LUFA 2.2 (LUFA Speyer, Speyer, Germany). Information by the supplier on the soil characteristics: 1.72 ± 0.54% organic carbon, 0.20 ± 0.06% nitrogen and pH of 5.5 ± 0.10. Exposures were conducted according to OECD Guideline 207 Artificial soil test [27] and OECD Guideline 222 Earthworm Reproduction Test (*Eisenia fetida*/*Eisenia andrei*) [28]. Exposure concentrations were chosen based on previous research [22]. All pesticide concentrations are expressed as amount (mg) of the active ingredient per kg of dry weight of soil. Following concentrations were chosen: Calypso 1, 5, and 10 mg/kg; Filon 15, 75, and 150 mg/kg; Frontier 10, 50, and 100 mg/kg; and Sumialfa 0.5, 2.5, and 5 mg/kg. To receive the respective end concentrations, the necessary amount of commercial pesticide preparations was diluted in distilled water and then 40 mL of the respective solution was added to 400 g of soil. After the soil was thoroughly mixed, ten randomly selected earthworms were added per box and covered with cling wrap (with aeration holes). The boxes were placed in the light at 20 °C for 7, 14 and 28 days. All exposures were performed in three independent replicates and controls were performed in parallel. Controls contained distilled water only.

Subcellular markers sample preparation

After the respective exposure periods, the earthworms were removed from the soil (hand collected), rinsed with distilled water, patted dry and the weight determined. Sample preparation and measurements were conducted according to Lackmann et al. [21]. Shortly, each earthworm was placed in a 2 mL tube and homogenized in cold sodium phosphate buffer on ice. After centrifugation for 30 min (9000*g*, 4 °C) the supernatant (post-mitochondrial fraction, S9) was stored at − 80 °C until further usage. Protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

Reproduction

Reproductive success was assessed according to OECD Guideline 222 [28] with some modifications. Earthworms were fed weekly with 3.5 g of cooked potatoes. After 28 days, adult earthworms were removed from the soil. Then cocoons and juveniles were manually accounted for and additionally removed through wet sieving. Juveniles and cocoons were counted and placed on damp filter paper. Hatching of the cocoons was monitored daily for additional 28 days or more, and the filter paper was regularly moistened. Reproduction test was performed in three independent replicates. Appropriate negative controls containing only distilled water were run in parallel to each exposure.

Measurements of subcellular markers

Measurements were conducted as described in detail in Lackmann et al. [21] and Lackmann et al. [22]. Specific GST activity [14] is given in nmol of conjugated GSH in one min per mg of proteins and specific GR activity [14] is given in nmol of reduced GSSG in one min per mg of proteins. Specific AChE activity [7] is given in nmol of acetylthiocholine iodide hydrolyzed in one min per mg of proteins and specific CES activity [18] is given in nmol of 4-nitrophenol produced per one min per mg of protein. Specific CAT activity [5] is given in µmol of degraded H_2O_2 in one min per mg of proteins. GPx activity [39] was measured according to [22]. The specific enzymatic activity was expressed as nmol of oxidized NADPH per mg of proteins.

Fluorescence-based oxidative stress measurements were performed according to Lackmann et al. [21] and results given in relative fluorescence.

For the assessment of MXR activity, the same concentrations were used as for the other sublethal exposures, but a separate set of exposures was performed due to the addition of rhodamine B (RB). Measurements were performed based on Hackenberger et al. [15] with changes as described in Lackmann et al. [21] and MXR activity was expressed as nmol RB per mg proteins.

Data analysis

Data analysis was performed using the statistical software R version 4.2.2 [31] and R Studio Team [32]. Prior to analysis, data were tested for normality using Shapiro–Wilk test, and homogeneity of variance was tested using Levene test. As no significant deviations from normality and variance homogeneity were detected, data were analyzed using two-way ANOVA, with factors being exposure time (ET) and exposure concentration (EC). If significance was obtained after two-way ANOVA, pairwise contrasts were evaluated to detect which groups differ significantly (*post-hoc* test) using R package emmeans [24].

Results of the reproduction test were analyzed using one-way ANOVA. If significant result was obtained,

Dunnett's test was used to detect which groups differ significantly from the control treatment. Three levels of significance are reported: $p < 0.05$; $p < 0.01$; $p < 0.001$.

Biomarker responses were first normalized to the protein content and then expressed relative to their respective controls, allowing for comparisons across multiple exposure times.

Results

Reproduction success

Significant effects on reproduction are observed only at the highest pesticide concentrations. Frontier significantly reduced cocoon production at 100 mg/kg with 92.6% reduction in number of cocoons compared to the control (Fig. 1). Sumialfa inhibited cocoon production at concentration of 5 mg/kg by 98.2%. Calypso and Filon had no effects on cocoon production (Fig. 1). Significant changes in the number of juveniles were observed after exposure also to both Frontier and Sumialfa at highest tested concentrations, where number of juveniles was reduced by 91.9% compared to the control due to exposure to Frontier (Fig. 2). On the highest Sumialfa concentration, there were no juveniles present.

Calypso

The effects of the insecticide Calypso on measured biomarkers are presented in Fig. 3. Although different concentrations of Calypso did not affect AChE activity, results of the two-way ANOVA showed significant effect of exposure time (ET, $p < 0.001$) and significant two-way interaction of concentration and exposure time (C x ET, $p < 0.001$) (Table 1). At all tested concentrations, significantly higher AChE activity was observed after 28 days of exposure to Calypso (Fig. 3). CES response followed a similar pattern as the AChE activity (Table 1, Fig. 3), with significantly higher activities obtained after 28 days of exposure. Two-way ANOVA also showed significant effect of exposure time (ET, $p < 0.001$) and significant two-way interaction (C x ET, $p < 0.001$) (Table 1). There was no difference with respect to different exposure concentration. CAT and GST activities were not affected by different concentration of Calypso, and the two-way ANOVA showed only significant effect of exposure time (Table 1) (ET, $p < 0.001$). Both enzyme activities increased with exposure time, and they were significantly higher after 28 days of exposure. GR activity showed different temporal responses with concentration (Fig. 3). Two-way ANOVA showed significant effect of exposure time (ET, $p < 0.01$) and significant two-way interaction (EC x ET, $p < 0.001$). At the lowest tested concentration, GR activity decreased after 28 days of exposure. At 5 mg/kg, the highest activity was obtained after 14 days of exposure, while no difference in temporal response was obtained at 10 mg/kg. Two-way ANOVA showed that both concentration (C, $p < 0.05$) and exposure time (ET, $p < 0.001$) affected GPx activity, and interaction of both factors was also significant (C x ET, $p < 0.01$). Concentrations 5 and 10 mg/kg significantly induced GPx after 14 days of exposure, compared to its respective controls. When examining the effects of exposure time it is evident that GPx

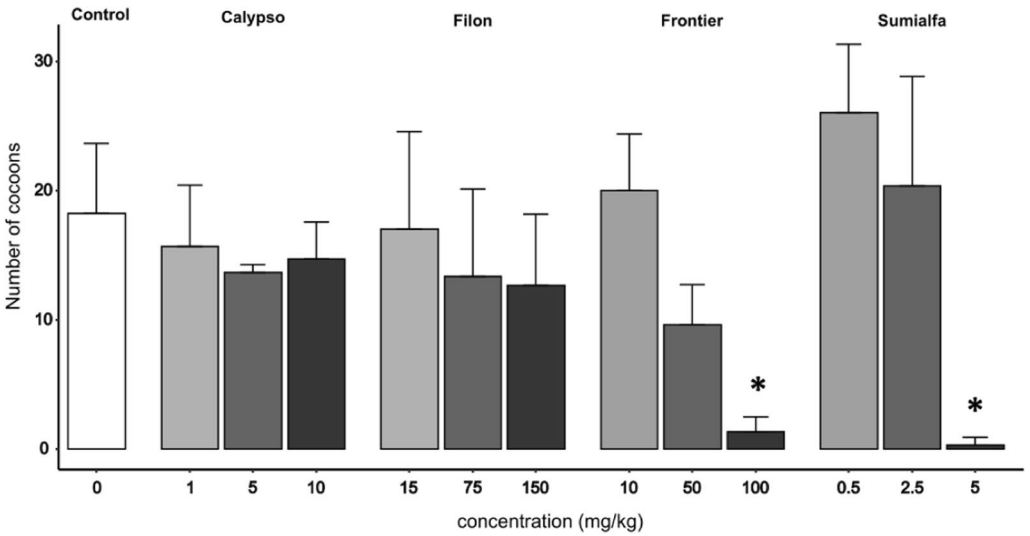

Fig. 1 Results of the earthworm reproduction test after exposure to commercial pesticide preparations—numbers of cocoons after 28-day exposures of earthworm *E. andrei* to Filon, Frontier, Sumialfa, and Calypso in standardized LUFA 2.2 soil. Results are expressed as mean ± standard deviation. Asterisk (*) represents statistically significant difference compared to the control (one-way ANOVA, followed by Dunnett post hoc; $p < 0.05$)

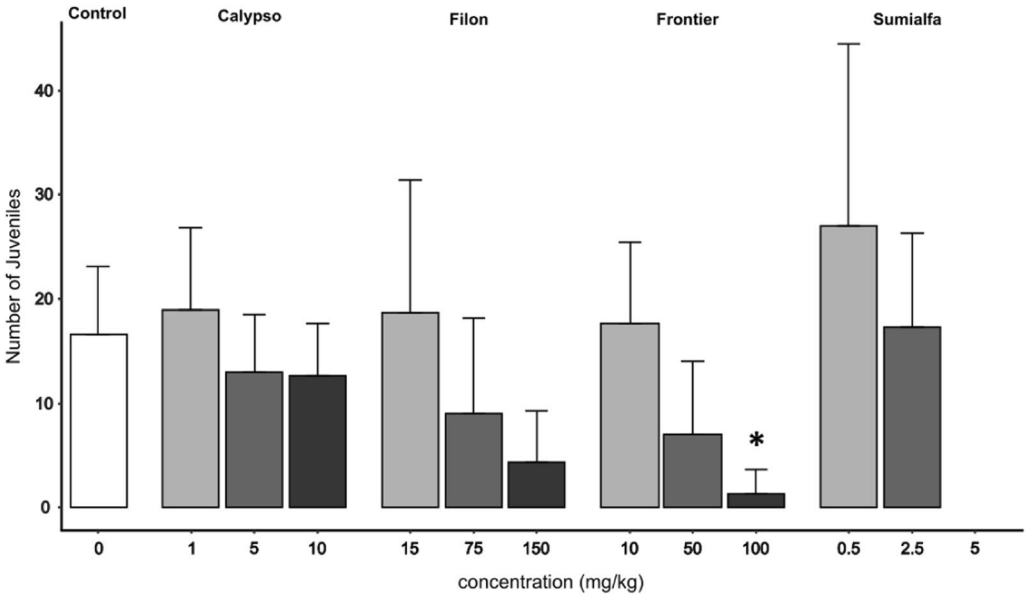

Fig. 2 Results of the earthworm reproduction test after exposure to commercial pesticide preparations—numbers of juveniles after 28 days exposures of earthworm *E. andrei* to Filon, Frontier, Sumialfa, and Calypso in standardized LUFA 2.2 soil. Results are expressed as mean ± standard deviation. Asterisk (*) represents statistically significant difference compared to the control (one-way ANOVA, followed by Dunnett post-hoc; $p < 0.05$)

activity was significantly lower after 28 days of exposure, compared to other time points. MXR activity was not significantly affected by different concentrations, however, exposure time had significant effect ($p < 0.01$) and two-way interaction was also significant (C x ET, $p < 0.01$). After initial reduction of MXR activity, it started to increase with exposure time. An induction of MXR activity, indicates a decrease in RB content, and vice versa, i.e., an inhibition of MXR activity indicates an increase in RB accumulation. Levels of GSH and ROS were not affected by different Calypso concentrations, but exposure time was significant as well as two-way interaction (Table 1). GSH concentration was significantly higher after 28 days of exposure, except at 10 mg/kg. ROS levels significantly increased after 28 days of exposure, but only at lowest concentration of 1 mg/kg while no difference with respect to exposure time could be observed at 5 and 10 mg/kg.

Filon

The biomarker results after exposures of *E. andrei* to the herbicide Filon for 7, 14, and 28 days are shown in Fig. 4. AChE activity was not affected by Filon at any of the tested concentrations. However, two-way ANOVA showed significant effects of both tested factors to CES activity as well as significant two-way interaction (C x ET, $p < 0.01$) (Table 1). CES activity was significantly inhibited at all tested concentrations after 28 days of exposure, compared to its respective controls. CAT activity was not affected by any Filon concentration, but there were significant effects of exposure time (ET, $p < 0.001$) and significant two-way interaction (C x ET, $p < 0.01$) (Table 1). CAT activity was lowest after 14 days of exposure, while it was similar after 7 and 28 days of exposure. Filon significantly induced GST activity at all tested concentrations compared to the control treatment (C, $p < 0.01$), except at 75 and 150 mg/kg, after 14 days of exposure. Interestingly, although both factors were significant, no significant two-way interaction was present (Table 1). GR and GPx activities were not affected by different Filon concentrations when comparing them to the control treatment, but there were significant effects of exposure time and significant two-way interactions. GPx activity was lowest after 28 days of exposure, compared to other exposure times. The relative RB concentration significantly decreased after exposure to 75 and 150 mg/kg after 7 and 14 days of exposure, while after 28 days values returned to the control levels. GSH levels were significantly decreased at all tested concentrations and at all tested exposure durations. Interestingly, no significant interaction is observed. For ROS concentration, two-way ANOVA showed significance of both concentration (C, $p < 0.05$) and exposure time (ET, $p < 0.001$) with no interaction. ROS levels were significantly increased after exposure to 75 and 150 mg/kg but only after 7 days of exposure (Fig. 4). When examining response with respect to exposure time, it is evident that at both concentrations after initial increase, ROS levels decrease steadily with exposure time.

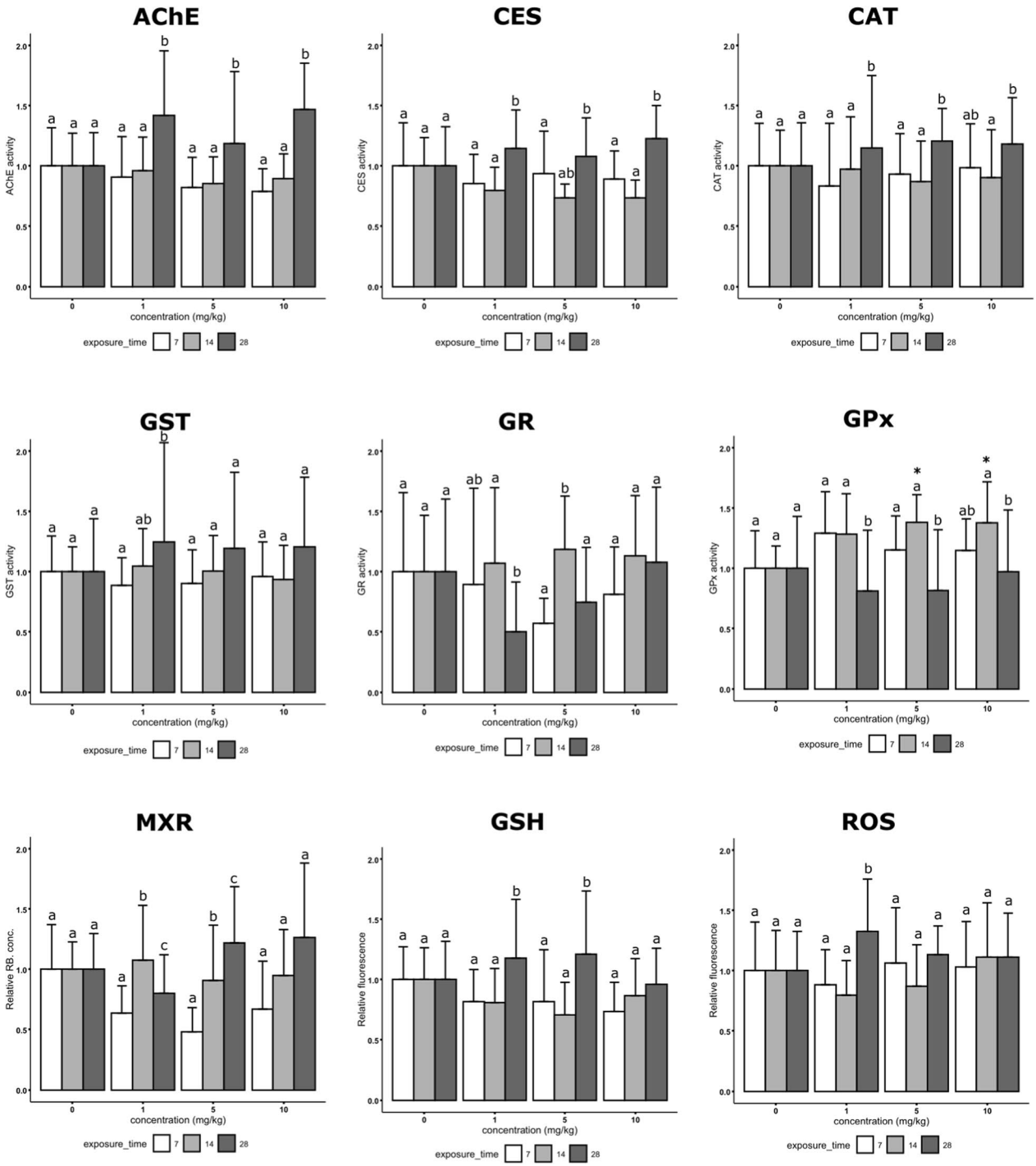

Fig. 3 Subcellular responses after 7, 14, and 28 days of exposures of earthworm *E. andrei* to the insecticide Calypso. Specific activities of: acetylcholinesterase (AChE), carboxylesterase (CES), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx); and concentrations of: rhodamine B (MXR), reduced glutathione (GSH), reactive oxygen species (ROS). All activities and concentrations are expressed as relative values and the results are presented as mean ± standard deviation, $N = 30$. Significant differences at different concentrations within the same exposure time compared to the control treatment are presented with asterisk (*). Different letters indicate statistically significant differences within the same concentration and between different exposure times (Tukey post hoc, $p < 0.05$). The different letters indicate significant differences at $p < 0.05$ level among different exposure times

Table 1 Results of the two-way ANOVA on the effects of pesticides Calypso, Filon, Frontier, and Sumialfa on biomarkers in earthworm *E. andrei*

		df	AChE	CES	CAT	GST	GR	GPx	MXR	GSH	ROS
Calypso	Concentration C	3	1.93	1.071	0.081	0.202	1.909	2.988*	2.361	1.962	0.680
	Exposure time ET	2	29.95***	23.973***	6.438**	5.830**	6.849**	17.741***	20.567***	12.919***	6.322**
	C x ET	6	4.12***	3.376**	1.070	0.828	2.634*	3.020**	5.359***	2.725*	2.915**
Filon	concentration C	3	1.87	4.069**	2.175	17.621***	5.721***	4.884**	28.554***	45.825***	2.803*
	Exposure time ET	2	2.44	9.873***	17.083***	5.329**	0.130	16.958***	13.630***	4.457*	7.929***
	C x ET	6	1.35	2.279*	3.293**	1.169	2.482*	4.196***	4.003***	1.845	1.919
Frontier	concentration C	3	5.96***	7.099***	3.198*	0.948	1.857	3.065*	109.639***	2.095	1.569
	Exposure time ET	2	3.12*	4.520*	8.021***	5.067**	22.134***	6.138**	25.566***	1.171	5.288**
	C x ET	6	2.26*	1.200	5.168***	1.584	3.315**	1.591	6.734***	1.249	3.320**
Sumialfa	concentration C	3	11.74***	10.704***	4.657**	8.553***	2.853*	1.061	38.502***	1.505	0.728
	Exposure time ET	2	32.07***	3.857*	4.885**	1.695	25.740***	40.949***	11.106***	18.496***	8.276***
	C x ET	6	5.87***	0.742	4.878***	4.561***	3.836**	6.397***	2.232*	2.870*	2.434*

F values for the two-way ANOVA on the effects of pesticide concentration (C) and exposure time (ET) on the acetylcholinesterase (AChE), carboxylesterase (CES), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) and rhodamine B (MXR), reduced glutathione (GSH) and relative fluorescence of reactive oxygen species (ROS). Df—degrees of freedom. Statistically significant differences are marked with asterisk (*) with three levels of significance reported: ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$

Frontier

The biomarker response after exposures of *E. andrei* to the herbicide Frontier for 7, 14, and 28 days are shown in Fig. 5. Two-way AOVA showed that AChE activity was significantly affected by concentration (C, $p < 0.001$) and exposure time (ET, $p < 0.05$) and that significant interaction of both factors is present (C x ET, $p < 0.05$) (Table 1). When examining activity compared to control treatment, AChE activity is significantly inhibited after exposure to the highest concentration after 14 days of exposure; while at other time points, there is no difference compared (Fig. 5). CES activity was significantly inhibited compared to the control at 50 and 100 mg/kg, but only after 7 days of exposure (Fig. 5). CAT activity is significantly inhibited at 10 and 50 mg/kg, but only after 7 days of exposure, while with increase in exposure time activities steadily increase and return to the control levels. GST, GR, and GPx activities were not affected by different Frontier concentrations, however, significant effect of exposure time is present (Table 1). The effects of exposure time are most pronounced GR activity, where its activity is lowest after 7 and 28 days of exposure, while after 14 days it is similar to the control levels. The relative RB concentration significantly decreased after at 50 and 100 mg/kg at all exposure time, indicating strong induction of MXR response (Fig. 5). At lowest concentration, MXR activity is induced only after 7 days, while it afterward returns to the control levels. Frontier did not affect levels of GSH at any concentration or any exposure time (Table 1). Levels of ROS was not affected by any concentration, but there was a significant effect of exposure time, which is observed only at the highest tested concentration, where ROS concentration is elevated after 28 days compared to other exposure times.

Sumialfa

The biomarker response after exposures of *E. andrei* to the insecticide Sumialfa for 7, 14 and 28 days are shown in Fig. 6. AChE activity was significantly affected by both concentration (C, $p < 0.001$) and exposure time ($p < 0.001$), with their interaction present (C x ET, $p < 0.001$) (Table 1). Activity of AChE was significantly inhibited by Sumialfa at highest tested concentration after 7 and 14 days (Fig. 6). Interestingly at 2.5 mg/kg AChE activity was inhibited after 7 days, returned to the control level after 14 days, and was significantly induced after 28 days of exposure. When examining temporal response of AChE activity, it is evident that its activity is highest after 28 days. CES activity was inhibited at 5 mg/kg but only after 7 and 14 days of exposure (Fig. 6). For activity of CAT, two-way ANOVA showed significance of both factors (C, $p < 0.05$; ET, $p < 0.001$) as well as their interaction (C x ET, $p < 0.001$) (Table 1). Although different Sumialfa concentrations did not affect GST response, exposure time was significant (Table 1) but the effects were inconsistent. At the highest concentration, GST activity decreases with exposure time, while at the lowest concentration the effects is opposite (Fig. 6). For GR activity, both concentration and exposure time are significant as well as their interaction (Table 1). Compared to the control treatment, GR activity was significantly inhibited at 2.5 mg/kg after 7 and 28 days, while at 5 mg/kg it is induced after 14 days of exposure (Fig. 6). GR activity is highest after 14 days of exposure at all tested

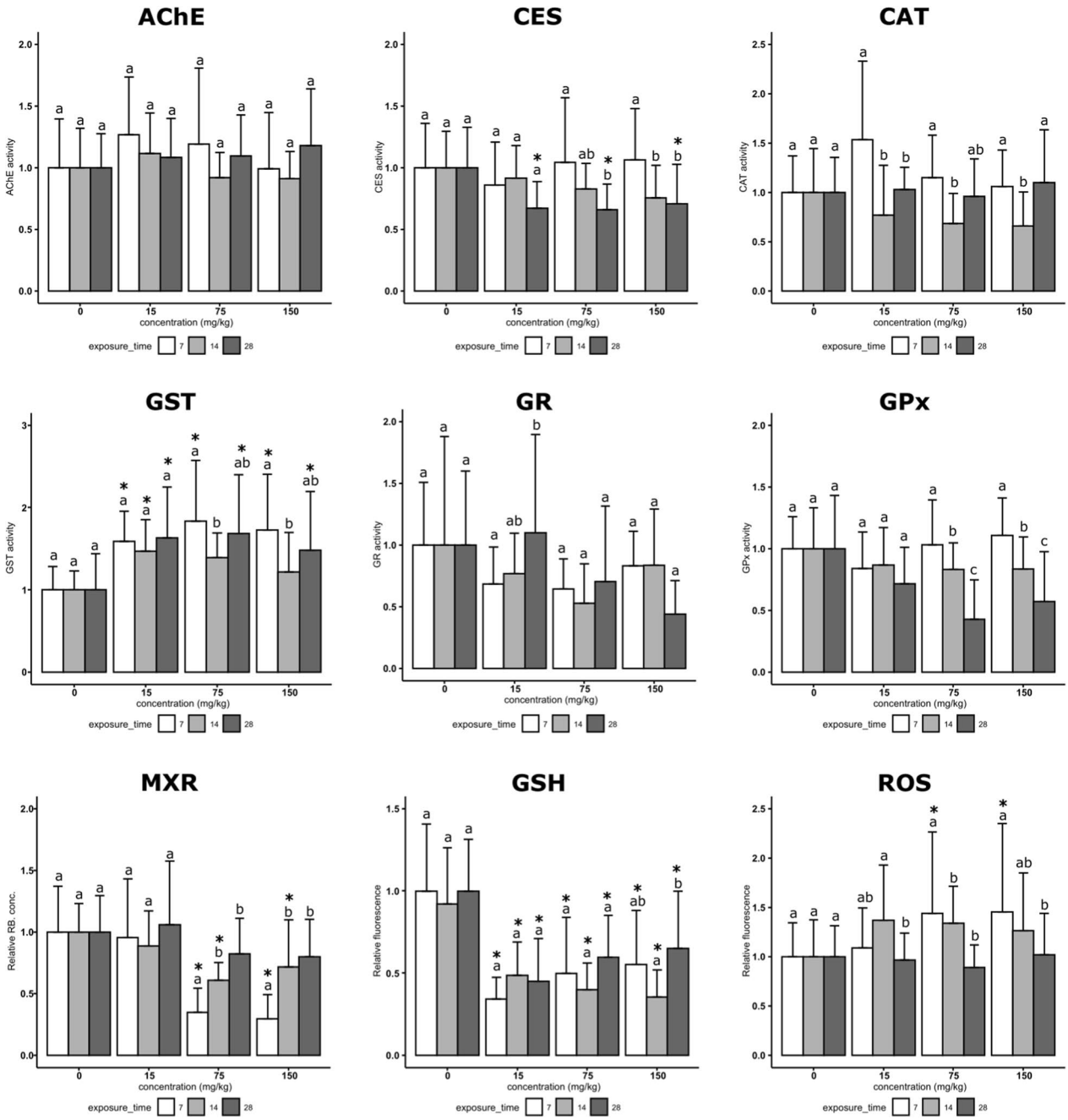

Fig. 4 Subcellular responses after 7, 14, and 28 day of exposures of earthworm *E. andrei* to the herbicide Filon. Specific activities of: acetylcholinesterase (AChE), carboxylesterase (CES), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx); and concentrations of: rhodamine B (MXR), reduced glutathione (GSH), reactive oxygen species (ROS). All activities and concentrations are expressed as relative values and the results are presented as mean ± standard deviation, $N = 30$. Significant differences at different concentrations within the same exposure time compared to the control treatment are presented with asterisk (*). Different letters indicate statistically significant differences within the same concentration and between different exposure times (Tukey post-hoc, $p < 0.05$). The different letters indicate significant differences at $p < 0.05$ level among different exposure times

concentrations compared to other exposure times. GPx activity was not affected by different concentrations, however, effects of exposure time were significant (Table 1). When examining temporal response at different concentrations it is evident that GPx response is highest after 14 days, and then it declines significantly

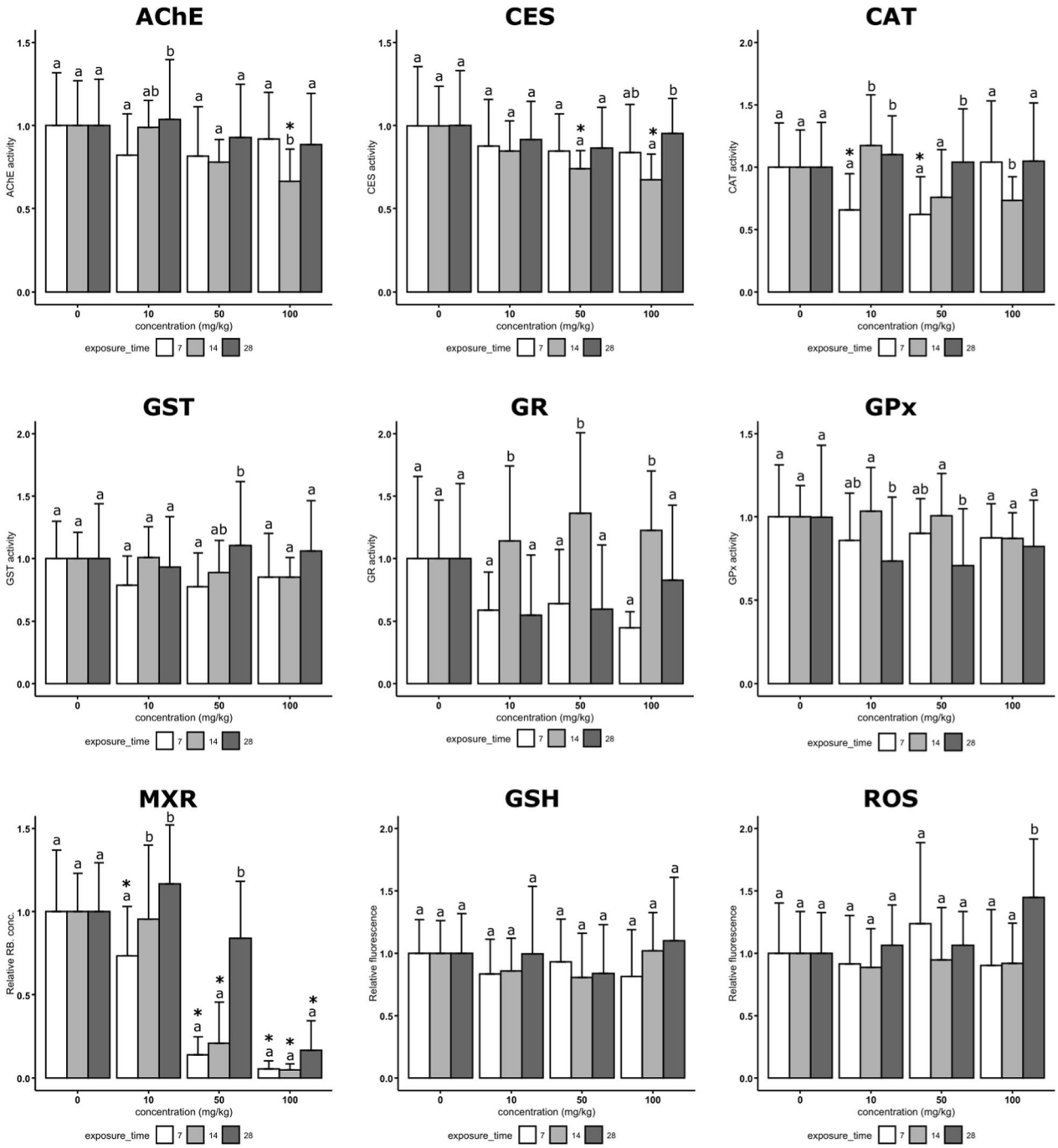

Fig. 5 Subcellular responses after 7, 14, and 28 day of exposures of earthworm *E. andrei* to the herbicide Frontier. Specific activities of: acetylcholinesterase (AChE), carboxylesterase (CES), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx); and concentrations of: rhodamine B (MXR), reduced glutathione (GSH), reactive oxygen species (ROS). All activities and concentrations are expressed as relative values and the results are presented as mean ± standard deviation, $N = 30$. Significant differences at different concentrations within the same exposure time compared to the control treatment are presented with asterisk (*). Different letters indicate statistically significant differences within the same concentration and between different exposure times (Tukey post-hoc, $p < 0.05$). The different letters indicate significant differences at $p < 0.05$ level among different exposure times

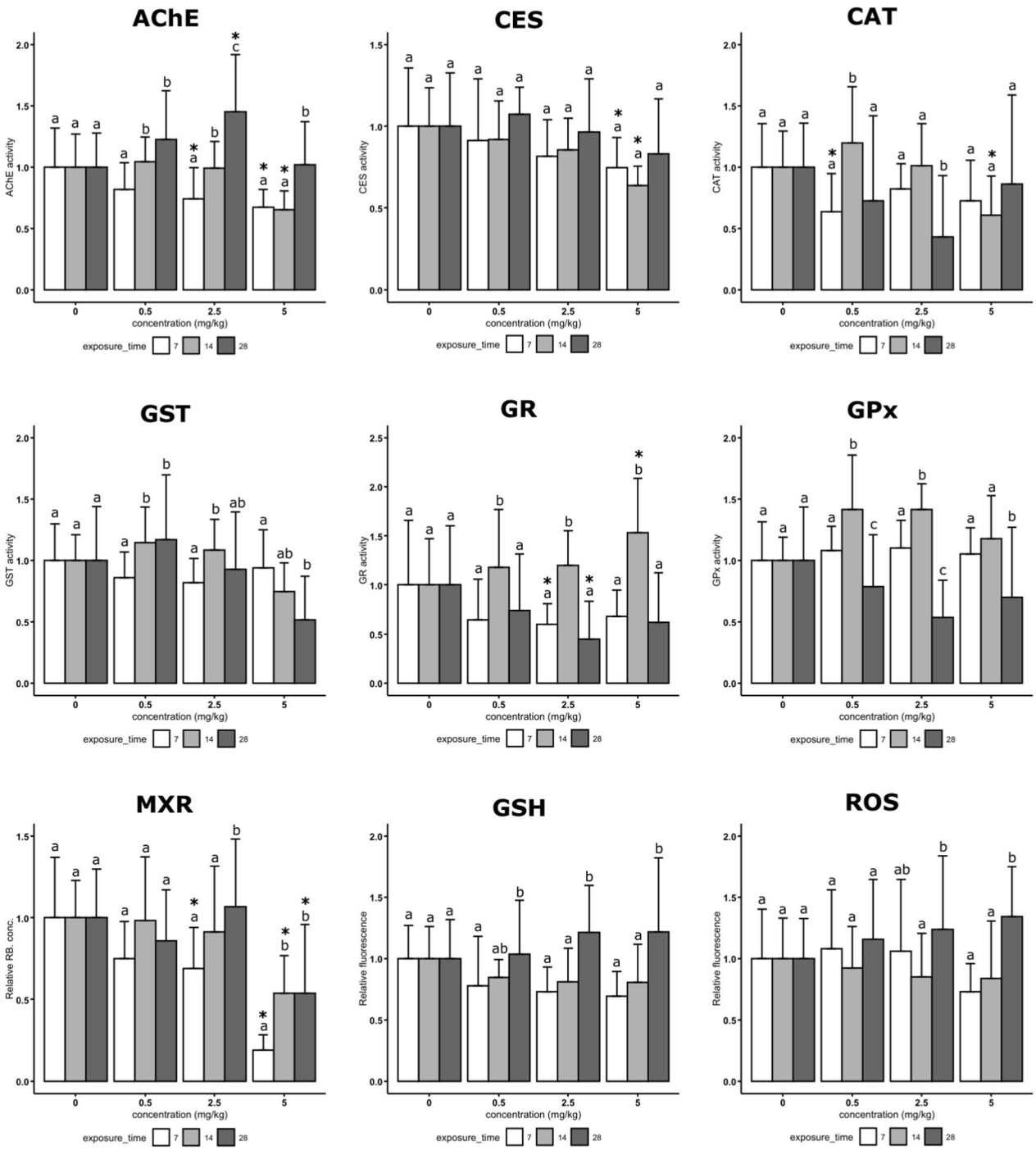

Fig. 6 Subcellular responses after 7, 14 and 28 day of exposures of earthworm *E. andrei* to the insecticide Sumialfa. Specific activities of: acetylcholinesterase (AChE), carboxylesterase (CES), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx); and concentrations of: rhodamine B (MXR), reduced glutathione (GSH), reactive oxygen species (ROS). All activities and concentrations are expressed as relative values and the results are presented as mean ± standard deviation, $N = 30$. Significant differences at different concentrations within the same exposure time compared to the control treatment are presented with asterisk (*). Different letters indicate statistically significant differences within the same concentration and between different exposure times (Tukey post-hoc, $p < 0.05$). The different letters indicate significant differences at $p < 0.05$ level among different exposure times

after 28 days. MXR activity was significantly induced at highest tested concentration at all exposure times (Fig. 6), and at 2.5 mg/kg after 7 days of exposure (Fig. 4). Temporal response is similar at all concentrations, with initial decrease in activity (7 days) and slow increase with exposure duration. Concentrations of GSH and ROS were not affected by varying concentrations, but the exposure time was significant (Table 1). Their temporal response is also similar, with initial, more or less pronounced decrease, followed by increase with exposure duration (Fig. 6).

Discussion

Previous research has showed the differences in acute toxicity of active substances and their respective commercial preparations to the earthworm *E. andrei* after short-term exposures in standardized soil [20]. After observing a higher toxicity of the commercial preparations and significant changes in biomarker responses [20], the present study aimed to investigate the long-term effects of sublethal concentrations and potential time-dependent changes on a subcellular level. A biomarker is a measurable response on molecular, biochemical, cellular, or physiological level in an organism as a response to a toxicant, i.e., a disturbance of the normal functioning of an organism, that are often regarded as "early-warning signals" before irreversible damage occurs [26]. A single biomarker is not able to provide the necessary information to evaluate the effects of xenobiotics as the exposure time is highly important and depends on the individual biomarker. Therefore, a set of biomarkers should be evaluated simultaneously. Furthermore, they rarely follow a concentration–response relationship, and the response times differ for each biomarker. Thus they should be evaluated at different exposure times [26]. These differences in response times of different biomarkers can be seen in the present study, where both enzymatic and non-enzymatic biomarkers were used. Biomarker responses after three different exposure periods (7, 14, and 28 days) were evaluated after earthworms were exposed to four commercial pesticide preparations. Furthermore, reproductive success was investigated as an endpoint as it is considered to be more sensitive test than classic mortality assessments and due to its high relevance on a population level.

The standardized earthworm reproduction test is viewed as a more sensitive test than most apical endpoints, e.g., mortality. However, it is rather labor-intensive and, thus, time and money consuming compared to the fast and cheap biomarker measurements. However, biomarkers have a low ecological relevance compared to observed effects on the reproduction of organisms. Therefore, in the present study, exposures with the same sublethal concentrations were conducted to be able to observe effects on different levels of biological organization. It was previously shown that exposure of *E. andrei* to Calypso causes reduction of cocoons and survival [30]; however, in the present study, only the insecticide Sumialfa (esfenvalerate) and the herbicide Frontier (dimethenamid-*p*) showed a significant decrease of cocoon production. Previous research observing the reproductive toxicity as an effect of pesticides to earthworms are limited; however, neonicotinoids are known to exhibit adverse effects and high toxicity to earthworms [29]. For example, the neonicotinoid imidacloprid showed a high toxicity to *E. andrei* with an EC_{50} of 4.07 mg/kg. [1]. Furthermore, previous research with thiacloprid and imidacloprid using the natural Lufa 2.2 artificial soil also found a similar pattern of sensitivity, i.e., imidacloprid was shown to be more toxic than thiacloprid for the most sensitive species—*E. andrei* and *Folsomia candida* [25]. Unfortunately, due to the time- and cost-intensive nature of the reproduction test only three biological replicates could be performed. While this number of replicates was enough to observe statistical differences in cocoon numbers after exposures to the herbicide Frontier and the insecticide Sumialfa, no statistical differences could be observed for the number of juveniles, likely due to the low number of replicates and high variability of the data.

The results of the biomarker responses after exposure of *E. andrei* to the insecticide Calypso (thiacloprid) supports the observations of the other pesticide exposures. Initially, only a response in MXR activity, GPx activity, and GSH levels was observed. Interestingly, compared to the other pesticides, Calypso showed the lowest responses of the MXR activity, as it already recovered after 14 days of exposure. Also, while after 14 days, a decrease in CES activity could be observed. Feng et al. [12] assessed the chronic effects of the active ingredient thiacloprid on *E. fetida* in artificial soil and observed a significant decrease in CES activity following a 7 day exposure. AChE activity remained stable and only showed significant changes after 28 days of exposure. As a neonicotinoid, it affects the nervous system of insects through stimulating the nicotinic acetylcholine receptors, thus only showing an effect on the increase of AChE activity was unexpected. The lack of significant response in AChE activity in the 7 and 14 days of exposure is corroborated by the results in Lackmann et al. [21] who also did not observe significant response in AChE activity 48 h after exposure. However, as this was the only biomarker related to neurotoxicity, it shows that the used biomarker set had a stronger focus on oxidative stress and xenobiotic metabolism and might miss effects on neurotransmission, if only a shorter exposure period

had been investigated. Thus, future investigations using a multiobiomarker approach should include a more diverse set of biomarkers.

Similar observations could be made for the herbicide Filon (prosulfocarb), which showed initial responses of an increased MXR activity and an influence on oxidative stress-related biomarkers (GR, GSH) and GST, an enzyme involved in phase II of xenobiotic metabolism. When assessing MXR activity, which acts as a primary defence mechanism against toxic compounds, earthworms exposed to the pesticide Filon (prosulfocarb) showed notable changes in the accumulation of RB and after 28 days, a recovery of MXR activity was observed. The RB concentration decreased significantly in *E. fetida* indicating an activation of MXR activity. This MXR induction suggests that as an initial defence mechanism, the activity is heightened to expel the parent compound as well as their metabolites from the cells. Previous research on earthworms [21], (Velki et al. 2018) and on zebrafish, *Danio rerio* [36–38] showed commercial preparations of pesticides, Filon included, affect MXR activity. Pesticide induced-oxidative stress has been well documented by previous research. For example, Schreck et al. [34] observed the increase in activities of GST and CAT followed by exposure to several pesticides. While these responses remained after 14 days of exposure, CES activity was also decreased, another indication for a change in the different phases of the xenobiotic metabolism. Another important observation were the changes in CES activity over the different exposure times. Initially, after 7 days there was no impact on CES activity, after 14 days only the highest concentration caused a significant decrease in CES activity and eventually after 28 days all exposure concentrations including the lowest of 15 mg/kg. This not only shows the time-dependent differences, but also the potential long-term effects of lower pesticide concentrations on soil organisms. Higher sensitivity of CES activity compared to AChE activity was observed following Filon exposure in the present study. A study by Keizer et al. [19] showed the potential of AChE activity as a useful biomarker, however, in future assessment of the potential pesticide effects, a combination of measuring both the activities of AChE and CES should be taken into account [36, 38].

After 7 days of exposure, the herbicide Frontier (dimethenamid-*p*) showed significant changes of MXR activity and oxidative stress-related markers (CAT and GR activity). These initial responses slightly changed after 14 days, the exposure time where the strongest biomarker responses were observed when comparing the different time points after Frontier exposure. Both AChE activity, an enzyme associated with neurotransmission, and CES activity, an enzyme of the phase I of xenobiotic metabolism, were significantly decreased. The inhibition, in both AChE and CES, is previously documented following an exposure to a mixture of six pesticides on the earthworm *A. caliginosa*, after a few days of insecticide and/or fungicide exposure, which is a response indicative of a neurotoxicity in earthworms [34]. However, after 28 days of exposure only a significant change of MXR activity and ROS levels after exposure to the highest exposure concentration were observed. These results underline the importance of using a diverse set of biomarkers, as well as different exposure times to effectively screen for 'early-warning signals' that might be of importance for higher levels of biological organization.

Similar observations could be made for the insecticide Sumialfa (esfenvalerate) where significant changes in MXR activity and oxidative stress (CAT, GSH) xenobiotic metabolism (CES) and neurotransmission-related (AChE) biomarkers were observed after 7 days. This effect on neurotransmission is not surprising, as esfenvalerate affects sodium channels. After 14 days, slight changes in the responses could be observed with a more diverse response of enzymatic biomarker for oxidative stress could be observed, namely GR and GPx activity showed significant changes, while CAT activity only showed a significant decrease after exposure to the highest concentration. Additionally, several studies have investigated the impacts of oxidative stress-related enzyme reactions in *E. fetida*. These studies demonstrate effects on CAT, GST, GPx, superoxide dismutase (SOD), as well as malonaldehyde (MDA) levels [21, 34, 40]. Interestingly, the non-enzymatic oxidative-related marker (GSH) completely recovered. The responses after 28 days were rather similar, with only CAT activity showing a recovery compared to the previous exposure period. While a previous study showed a higher sensitivity of the fluorescence-based non-enzymatic oxidative stress biomarkers [21], the present study underlines the importance of using a diverse set of biomarkers as xenobiotics can have a variety of toxic mechanisms to affect organisms, and thus, e.g., effects on oxidative stress might remain hidden if only a single biomarker is used, as the sensitivity of the chosen biomarker might differ between different organisms or xenobiotics.

Overall, looking at the biomarker responses of the different pesticides, it is also important to note that the exposures were simple applications of the pesticides at the beginning of the experiment, which might explain the partial recoveries observed after the 28-day exposure period. The reported half-lives of the four investigated pesticides, namely 14–18 days for prosulfocarb [10], 11.1 days for dimethenamid-*p* [9], 6–17 days for thiacloprid [8] and 9.4–36.5 days for esfenvalerate [11] (all data from field studies) could support these claims. However,

as no chemical analysis of the exposed soils were performed, it is not possible to see how the changing exposure concentrations could have affected the biomarker responses. While single applications are more realistic for pesticide exposures in the field, future studies should include a detailed chemical analysis of the exposure concentrations to gain more insight into the effects of these commercial pesticide preparations.

Conclusions

The results of the present study showed the potential long-term effects commercial pesticide preparations might have on key organisms of the soil ecosystem. Furthermore, it showed the importance of investigating subcellular responses at different exposure times and the necessity of using a diverse set of biomarkers to be able to use these 'early-warning signals' as tools for biomonitoring. Namely, sensitivity of applied assays was different for different pesticides. In case of the Calypso exposure, the most sensitive biomarkers appear to be activities of CES, GPx, CAT, and MXR. However, all the biomarkers were affected, with ROS the least. The overall effect is reflected in metabolism disruption, oxidative stress and detoxification mechanism; therefore, it is beneficial to measure a battery of biomarkers. Multiobiomarker assessments in case of Calypso exposure would provide a comprehensive picture of mode of action and overall effect on the organism. In case of Filon exposure, the most sensitive biomarkers are activities of GSH, MXR, and levels of GSH and ROS, and in case of Frontier exposure the most sensitive endpoints are activities of CAT and MXR. The observed effect on the aforementioned biomarkers is an indication of a disruption in cellular detoxification mechanisms and impaired antioxidant defences. In case of the Sumialfa exposure, the most sensitive endpoints were AChE, glutathione-dependent enzymes (GST, GR, GPx), and MXR, indicating the potential involvement of neurotoxicity. To enhance the interpretability of results, future investigations should incorporate chemical analyses of the exposed soils, enabling a correlation between time-dependent biomarker responses and alterations in exposure concentrations. While the reproductive test holds high ecological relevance, the data reveal considerable variability. Consequently, future studies would benefit from an increased number of replicates in comparison to biomarker measurements, ensuring a more robust ability to discern and validate significant effects.

Author contributions

Conceptualization: ŽL, CL, MV, TBS and HH. Investigations: CL, DB, AŠ and SE. Data analysis: ŽL, CL, DB and MV. Writing: ŽL, CL and MV. Review and editing: all.

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Availability of data and materials

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

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. Alves PRL, Cardoso EJBN, Martines AM, Sousa JP, Pasini A (2013) Earthworm ecotoxicological assessments of pesticides used to treat seeds under tropical conditions. Chemosphere 90:2674–2682. https://doi.org/10.1016/j.chemosphere.2012.11.046
2. Awoyemi OM, Kumar N, Schmitt C, Subbiah S, Crago J (2019) Behavioral, molecular and physiological responses of embryo-larval zebrafish exposed to types I and II pyrethroids. Chemosphere 219:526–537. https://doi.org/10.1016/j.chemosphere.2018.12.026
3. Barba V, Marín-Benito JM, García-Delgado C, Sánchez-Martín MJ, Rodríguez-Cruz MS (2019) Assessment of 14C-prosulfocarb dissipation mechanism in soil after amendment and its impact on the microbial community. Ecotoxicol Environ Saf 182:109395. https://doi.org/10.1016/j.ecoenv.2019.109395
4. Brown LA, Ihara M, Buckingham SD, Matsuda K, Sattelle DB (2006) Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors. J Neurochem 99:608–615. https://doi.org/10.1111/j.1471-4159.2006.04084.x
5. Claiborne, A.I. (1985). Catalase activity. In Handbook of methods in oxygen radical research, 283–284.
6. Crawford JJ, Sims GK, Simmons FW, Wax LM, Freedman DL (2002) Dissipation of the herbicide [14C] dimethenamid under anaerobic conditions in flooded soil microcosms. J Agric Food Chem 50(6):1483–1491. https://doi.org/10.1021/jf010612i
7. Ellman GL, Courtney KD, Andres V Jr, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95. https://doi.org/10.1016/0006-2952(61)90145-9
8. European Food Safety Authority (EFSA), Abdourahime H, Anastassiadou M, Arena M, Auteri D, Barmaz S, Brancato A, Brocca D, Bura L, Carrasco Cabrera L, Chiusolo A, Civitella C, Court Marques D, Crivellente F,

Ctverackova L, de Lentdecker C, Egsmose M, Fait G, Ferreira L, Villamar-Bouza L (2019) Peer review of the pesticide risk assessment of the active substance thiacloprid. EFSA J 17(2):32. https://doi.org/10.2903/j.efsa.2019.5595
9. European Food Safety Authority (EFSA), Arena M, Auteri S, Barmaz S, Bellisai G, Brancato A, Brocca D, Bura L, Byers H, Chiusolo A, Marques DC, Crivellente F, De Lentdecker C, Egsmose M, Erdos Z, Fait G, Lucien SJ, Kardassi D, Leuschner R, Villamar-Bouza L (2018) Peer review of the pesticide risk assessment of the active substance dimethenamid-P. EFSA J 16(4):5211. https://doi.org/10.2903/j.efsa.2018.5211
10. European Food Safety Authority (EFSA) (2007) Conclusion regarding the peer review of the pesticide risk assessment of the active substance prosulfocarb. EFSA J 5(8):111r. https://doi.org/10.2903/j.efsa.2007.111r
11. European Food Safety Authority (EFSA) (2014) Conclusion on the peer review of the pesticide risk assessment of the active substance esfenvalerate. EFSA J 12(11):3873. https://doi.org/10.2903/j.efsa.2014.3873
12. Feng L, Zhang L, Zhang Y, Zhang P, Jiang H (2015) Inhibition and recovery of biomarkers of earthworm *Eisenia fetida* after exposure to thiacloprid. Environ Sci Pollut Res 22:9475–9482. https://doi.org/10.1007/s11356-015-4122-6
13. Food and Agriculture Organization of the United Nations (2020) Pesticides use. http://www.fao.org/faostat/en/?#data/RP/visualize
14 Habig WH, Jakoby WB (1981) Assays for differentiation of glutathione S-transferases. Methods Enzymol 77:398–405. https://doi.org/10.1016/S0076-6879(81)77053-8
15. Hackenberger BK, Velki M, Stepić S, Hackenberger DK (2012) First evidence for the presence of efflux pump in the earthworm *Eisenia andrei*. Ecotoxicol Environ Saf 75:40–45. https://doi.org/10.1016/j.ecoenv.2011.06.024
16. Hackenberger DK, Stjepanović N, Lončarić Ž, Hackenberger BK (2018) Acute and subchronic effects of three herbicides on biomarkers and reproduction in earthworm *Dendrobaena veneta*. Chemosphere 208:722–730. https://doi.org/10.1016/j.chemosphere.2018.06.047
17. Hasenbein S, Peralta J, Lawler SP, Connon RE (2017) Environmentally relevant concentrations of herbicides impact non-target species at multiple sublethal endpoints. Sci Total Environ 607:733–743. https://doi.org/10.1016/j.scitotenv.2017.06.270
18 Hosokawa M, Satoh T (2001) Measurement of carboxylesterase (CES). Curr Protocols Toxicol 10:4.7.1-4.7.14. https://doi.org/10.1002/0471140856.tx0407s10
19. Keizer J, D'Agostino G, Nagel R, Volpe T, Gnemi P, Vittozzi L (1995) Enzymological differences of AChE and diazinon hepatic metabolism: correlation of in vitro data with the selective toxicity of diazinon to fish species. Sci Total Environ 171:213–220. https://doi.org/10.1016/0048-9697(95)04687-0
20. Lackmann C, Šimić A, Ečimović S, Mikuška A, Seiler TB, Hollert H, Velki M (2023) Subcellular responses and avoidance behavior in earthworm *Eisenia andrei* exposed to pesticides in the artificial soil. Agriculture 13:271. https://doi.org/10.3390/agriculture13020271
21. Lackmann C, Velki M, Bjedov D, Ečimović S, Seiler T-B, Hollert H (2021) Commercial preparations of pesticides exert higher toxicity and cause changes at subcellular level in earthworm *Eisenia andrei*. Environ Sci Eur 33:12. https://doi.org/10.1186/s12302-021-00455-5
22. Lackmann C, Velki M, Šimić A, Müller A, Braun U, Ečimović S, Hollert H (2022) Two types of microplastics (polystyrene-HBCD and car tire abrasion) affect oxidative stress-related biomarkers in earthworm *Eisenia andrei* in a time-dependent manner. Environ Int 163:107190. https://doi.org/10.1016/j.envint.2022.107190
23. Lackmann C, Velki M, Seiler TB, Hollert H (2018) Herbicides diuron and fluazifop-p-butyl affect avoidance response and multixenobiotic resistance activity in earthworm *Eisenia andrei*. Chemosphere 210:110–119. https://doi.org/10.1016/j.chemosphere.2018.07.008
24 Lenth RV (2016) Least-Squares means: the R package lsmeans. J Stat Softw 69(1):1–33. https://doi.org/10.18637/jss.v069.i01
25. de Limae Silva C, Brennan N, Brouwer JM, Commandeur D, Verweij RA, van Gestel CAM (2017) Comparative toxicity of imidacloprid and thiacloprid to different species of soil invertebrates. Ecotoxicology 26:555–564. https://doi.org/10.1007/s10646-017-1790-7
26 Nikinmaa M (2014) An introduction to aquatic toxicology. Academic Press, Cambridge. https://doi.org/10.1016/C2012-0-07948-3
27. OECD (1984) OECD Guideline 207: earthworm, acute toxicity tests. OECD Publishing, Paris. https://doi.org/10.1787/9789264070042-en
28. OECD (2016) OECD guideline 222: Earthworm reproduction test (Eisenia fetida/ Eisenia andrei) (Issue July). OECD Publishing, Paris. https://doi.org/10.1787/9789264264496-en
29. Pelosi C, Barot S, Capowiez Y, Hedde M, Vandenbulcke F (2014) Pesticides and earthworms. A review. Agron Sustain Dev 34:199–228. https://doi.org/10.1007/s13593-013-0151-z
30. Renaud M, Akeju T, Natal-da-Luz T, Leston S, Rosa J, Ramos F, Azevedo-Pereira HMVS (2018) Effects of the neonicotinoids acetamiprid and thiacloprid in their commercial formulations on soil fauna. Chemosphere 194:85–93. https://doi.org/10.1016/j.chemosphere.2017.11
31. R Core Team (2022) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-projec-t.org/
32 R Studio Team (2022) RStudio. Integrated Development for R. RStudio Inc., Boston
33. Scherner A, Schreiber F, Fomsgaard IS, Melander B, Avila LA, Kudsk P (2018) Effect of tillage systems on the dissipation of prosulfocarb herbicide. Weed Technol 32(2):195–204. https://doi.org/10.1017/wet.2017.103
34. Schreck E, Geret F, Gontier L, Treilhou M (2008) Neurotoxic effect and metabolic responses induced by a mixture of six pesticides on the earthworm *Aporrectodea caliginosa nocturna*. Chemosphere 17(10):1832–1839. https://doi.org/10.1016/j.chemosphere.2008.02.003
35. Sharma A, Kumar V, Shahzad B, Tanveer M, Sidhu GPS, Handa N, Kohli SK, Yadav P, Bali AS, Parihar RD, Dar OI (2019) Worldwide pesticide usage and its impacts on ecosystem. SN Appl Sci 1:1446. https://doi.org/10.1007/s42452-019-1485-1
36. Velki M, Lackmann C, Barranco A, EreñoArtabe A, Rainieri S, Hollert H, Seiler T-B (2019) Pesticides diazinon and diuron increase glutathione levels and affect multixenobiotic resistance activity and biomarker responses in zebrafish (*Danio rerio*) embryos and larvae. Environ Sci Eur 31:4. https://doi.org/10.1186/s12302-019-0186-0
37. Velki M, Meyer-Alert H, Seiler T-B, Hollert H (2017) Enzymatic activity and gene expression changes in zebrafish embryos and larvae exposed to pesticides diazinon and diuron. Aquat Toxicol 193:187–200. https://doi.org/10.1016/j.aquatox.2017.10.019
38. Velki M, Weltmeyer A, Seiler T-B, Hollert H (2019) Acute toxicities and effects on multixenobiotic resistance activity of eight pesticides to the earthworm *Eisenia andrei*. Environ Sci Pollut Res 26:4821–4832. https://doi.org/10.1007/s11356-018-3959-x
39 Wendel A (1980) Glutathione peroxidase. In: Jakoby WB (ed) Enzymatic basis of detoxication. Academic Press, Cambridge
40. Zhang YN, Zhang L, Feng L, Mao LG, Jiang HY (2017) Oxidative stress of imidaclothiz on earthworm *Eisenia fetida*. Comp Biochem Physiol C Toxicol Pharmacol 191:1–6. https://doi.org/10.1016/j.cbpc.2016.09.001
41. Zimmerman LR, Schneider RJ, Thurman EM (2002) Analysis and detection of the herbicides dimethenamid and flufenacet and their sulfonic and oxanilic acid degradates in natural water. J Agric Food Chem 50(5):1045–1052. https://doi.org/10.1021/jf010779b

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