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Subcellular Responses and Avoidance Behavior in Earthworm *Eisenia andrei* Exposed to Pesticides in the Artificial Soil

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Abstract: Earthworms are key organisms of the soil ecosystem and bioindicators for soil quality. While pesticides are used for the improvement of crop yields, they also present a burden for soil organisms. To understand the complex effects of pesticides on soil organisms, it is important to test these effects in soil exposures to include influences of the soil matrix on the toxicity. Therefore, the aim of this study was the assessment of the effects pesticides on earthworm *Eisenia andrei*. In an initial screening, active ingredients and commercial preparations were tested for comparison. Since the commercial preparations showed a higher toxicity, all further investigations (biomarkers, multixenobiotic resistance (MXR) activity, and avoidance behavior) were performed using the commercial pesticide formulations only: Sumialfa (esfenvalerate), Calypso (thiacloprid), Frontier (dimethenamid-p), and Filon (prosulfocarb). Significant differences in avoidance behavior were observed for Filon and Frontier. All pesticides inhibited the MXR activity and affected oxidative stress-related markers. Frontier was the only pesticide that did not affect enzymatic biomarkers related to neurotransmission. The results show the potential hazards associated with the usage of the tested pesticides and the importance of evaluating the effects of commercial pesticide preparations for a more realistic insight into the adverse effects on the environment.

Keywords: insecticides; herbicides; commercial formulations; oxidative stress; neurotoxicity; multixenobiotic resistance activity

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1. Introduction

The worldwide usage of synthetic pesticides has been critical in increasing crop yields and controlling vector-borne diseases in the past century [1]. However, contamination of the environment through increased pesticide applications [2,3] has been directly related to vast biodiversity declines [4], which is threatening ecosystems on a global scale. This increase in global contamination and the biodiversity declines have started pushing and exceeding planetary boundaries [5,6]. Concomitantly, these possible adverse outcomes endanger ecosystem services that human populations depend on.

Soil is an essential ecosystem that is being increasingly recognized as an integral part for defining planetary boundaries [7] and for its various ecosystem services, such as providing food and energy, carbon storage, nutrient regulation, flood mitigation, raw materials, water purification, support for biodiversity, and pest control, while soil is at the same time a finite resource [8,9]. Therefore, soil contamination through pesticide usage has been a growing concern in recent years [10], and strengthening soil ecotoxicology has

consistently gained importance. The European Green Deal now gives hope that the impact on soil flora and fauna will gain more attention, especially with the Farm to Fork strategy, Zero Pollution Action Plan for Air, Water, and Soil, as well as the Biodiversity Strategy [11].

Soil organisms live in soil and/or litter and are exposed to xenobiotics through contact and oral uptake routes in the surrounding soil compartment [12]. As ecosystem engineers and keystone species of soil ecosystems, earthworms have been established as model organisms of terrestrial ecotoxicology and have been used as bioindicators for soil quality [13,14]. Besides the traditional endpoints, such as acute toxicity and reproduction tests used in chemical registration and approval processes, more sensitive endpoints such as avoidance behavior [15] and enzymatic biomarkers [14] have gained popularity.

In the present study, two herbicides, dimethenamid-p (Frontier) and prosulfocarb (Filon), as well as two insecticides, thiacloprid (Calypso) and esfenvalerate (Sumialfa), were investigated. The chosen pesticides have been extensively used on European soils but been scarcely studied on their ecotoxicological effects. It is known that both herbicides inhibit long chain fatty acid synthesis [16], while the insecticides stimulate the nicotinic acetylcholine receptor (thiacloprid) and block sodium channels (esfenvalerate) [17,18]. Regarding the effects of these pesticides on earthworms, and soil organisms in general, only several studies were conducted. Inhibition of enzyme activities and DNA damage in *Eisenia fetida* was observed after exposure to thiacloprid [19]. Out of five species of soil invertebrates tested, *Folsomia candida* and *E. andrei* were the most sensitive species to thiacloprid exposure [20]. Thiacloprid also showed reproductive toxicity to *E. andrei* [21]. Toxic effects of esfenvalerate were investigated on aquatic oligochaete *Lumbriculus variegatus* [22] and *E. fetida* [23]. In our previous study, using the filter paper contact test, we established that commercial preparations of these four pesticides exert higher toxicity compared to active ingredient only and affect earthworm *E. andrei* at subcellular level [24].

Due to the low environmental relevance of filter paper tests, it is important to assess pesticide effects also using a soil substrate for exposure. Therefore, the aim of this study was to evaluate the short-term effects after 48 h exposure of the investigated pesticides on the earthworm *Eisenia andrei* in standardized soil using various endpoints. For that purpose, LUFA 2.2 soil was selected due to its wide usage in soil ecotoxicology [25], which enables better comparisons to other studies. The investigated endpoints thus included acute toxicity, avoidance behavior, multixenobiotic resistance (MXR) activity, enzymatic biomarkers, and fluorescence-based markers of oxidative stress. The focus of the study was to determine the potential differences in the toxicity of commercial preparation vs. active ingredient only, and to assess harmful pesticide effects after exposure in the soil matrix. As the respective commercial preparations are used in the environment and showed a higher toxicity during the acute toxicity testing, all sublethal exposures were performed using commercial preparations only. The obtained results emphasize the need for a more integrative and realistic assessment of the effects of commercial pesticide preparations after exposures in soil to better estimate the adverse effects of pesticides on the soil ecosystem.

2. Materials and Methods

2.1. Chemicals

Chemicals (analytical grade) used in this study: acetonitrile (C_2H_3N, CAS 75-05-8), β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH) ($C_{21}H_{26}N_7Na_4O_{17}P_3$ xH_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), 1-chloro-2,4-dinitrobenzene (CDNB) ($C_6H_3ClN_2O_4$, CAS 97-00-7), 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), dimethyl sulfoxide (DMSO) $(CH_3)_2SO$, CAS 67-68-5), disodium hydrogen phosphate (NaH_2PO_4, CAS 7558-79-4), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) ($[-SC_6H_3(NO_2)CO_2H]_2$, CAS 69-78-3), ethylenediaminetetraacetic acid disodium salt hydrated ($C_{10}H_{16}N_2O_8$, CAS 6381-92-6), glutathione disulfide (GSSG) ($C_{20}H_{32}N_6O_{12}S_2$, CAS 27025-41-8), glutathione reductase

from baker's yeast (*Saccharomyces cerevisiae*) (ammonium sulfate suspension) EC 1.6.4.2. (CAS 9001-48-3), 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), sodium azide (NaN_3, CAS 26628-22-8). Fluorescence-based oxidative stress-related markers were measured using CellTracker™ Green CMFDA Dye and CM-H2DCFDA and protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (all purchased from ThermoFisher Scientific).

The following analytical standard-grade pesticide active ingredients (a.i.) 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.).

2.2. Test Organism

Adult *E. andrei* (Lumbricidae) were obtained from a local supplier prior to the experiments and acclimatized at the exposure temperature of 20 °C.

The culturing soil was watered daily and additional food (potatoes) was supplied weekly. Prior to all experiments, adult earthworms with well-developed clitellum were selected and rinsed with distilled water. Earthworms were then placed on moist filter paper for at least 12 h to void their gut contents before the exposure experiments started.

2.3. Determination of Acute Toxicity

The following exposures for the determination of acute toxicity were conducted in artificial soil (LUFA 2.2) according to OECD Guideline 207 [26]. LUFA 2.2 is loamy sand soil with the following characteristics: 0.66 ± 0.05% organic carbon, 0.08 ± 0.01% nitrogen, 6.0 ± 0.4 pH, 5.7 ± 0.5 cation exchange capacity. Each exposure was performed in a glass box containing 400 g LUFA 2.2 soil mixed with the respective pesticide. Based on the results from preliminary tests, at least five concentrations were selected for each pesticide. Selected concentrations were in the range from no-effect concentration to concentration causing 100% mortality. Concentrations of the commercial pesticide preparations are henceforward expressed as amounts of active substance in the formulation. Namely, Calypso (thiacloprid) 1, 10, 50, 100, 200, and 250 mg/kg, Sumialfa (esfenvalerate) 5, 7.5, 10, 15, and 20 mg/kg, Frontier (dimethenamid-p) 50, 100, 150, 200, 250, 300, 350, and 400 mg/kg, and Filon (prosulfocarb) 150, 300, 400, 450, 550, 600, and 700 mg/kg were applied. The commercial pesticide preparations were diluted in distilled water, then 40 mL of the solution were added to 400 g soil to receive the respective test concentrations and the soil was thoroughly mixed. Right after mixing, 10 earthworms were added to each box to determine the acute toxicity.

For the active substances, the highest applied concentrations were 850 mg/kg thiacloprid, 300 mg/kg esfenvalerate, 1000 mg/kg dimethenamid-p, and 1200 mg/kg prosulfocarb. As these concentrations were multiple-fold higher than the respective LC_{50}-values of commercial preparations and did not cause 100% mortality, no higher concentrations were tested. The active substances were first prepared in acetone, applied to 80 g soil, and thoroughly mixed. To ensure complete evaporation of the acetone, the soil was left for 24 h. Then, 8 mL of distilled water was added, mixed in, and 5 earthworms were added to the soil.

All exposures consisted of at least 3 independent replicates, and appropriate controls were performed in parallel. The glass boxes were placed in the light at 20 °C. From the number of the earthworms found dead after a 48-h exposure period, the mortality calculations were performed.

2.4. Assessment of Biomarker Responses

As it was not possible to establish LC_{50}-values for the active ingredient, a direct comparison with the commercial preparations was not possible. However, while the highest tested concentrations of the active ingredients did not cause 100% mortality, it was possible to establish LC_{50}-values for the commercial preparations as they showed 100% mortality during exposures to the highest concentrations, overall showing a higher toxicity than the active ingredients. As the commercial preparations are also applied to the field and not the active ingredients, it was decided to conduct all further evaluations only applying the commercial pesticide preparations in sublethal concentrations. The following concentrations that were chosen based on the acute toxicity data were applied: Filon 15, 75, and 150 mg/kg, Frontier 10, 50, and 100 mg/kg, Sumialfa 0.5, 2.5, and 5 mg/kg, and Calypso 1, 5, and 10 mg/kg. Respectively, 10 earthworms per concentration were exposed in soil as previously described. In order to confirm the observed results, the whole experiment was repeated.

After 48 h, earthworms were removed from the soil, rinsed with distilled water, patted dry, and the weight was determined. Each earthworm was then placed in a 2 mL tube and homogenized in cold sodium phosphate buffer (0.1 M, pH 7.2, in ratio 1:5 w:v, e.g., 200 mg earthworm in 1 mL buffer)on ice with an Ultra-Turrax T18 homogenizer. After centrifugation for 30 min (9000× *g*, 4 °C), the supernatant (post-mitochondrial fraction, S9) was transferred to a set of fresh tubes in 3 aliquots per sample and stored at −80 °C until further usage.

Protein concentrations were measured in 96-well plates using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) as described in a previous study [24].

Spectrophotometric measurements of glutathione S-transferase (GST), glutathione reductase (GR), carboxylesterase (CES), and acetylcholinesterase (AChE) activity were conducted as described in previous study [24]. Specific GST activity [27] is expressed as nmol of conjugated glutathione (GSH) in one min per mg of proteins and specific GR activity [27] is expressed as nmol of reduced glutathione (GSSG) in one min per mg of proteins. Specific AChE activity [28] is expressed as nmol of acetylthiocholine iodide hydrolyzed in one min per mg of proteins, and specific CES activity [29] is expressed as nmol of 4-nitrophenol produced per one min per mg of protein.

For the spectrophotometric measurements of catalase (CAT) activity [30], 3 µL of the sample (S9), 100 µL of sodium phosphate buffer (0.1 M, pH 7.2), and 100 µL of H_2O_2 (0.019 M) were added per well in 96-well UV plates in triplicates. Kinetic absorbance measurements were performed using a Tecan Spark microplate reader at 240 nm for 3 min (measuring every 15 s) at 20 °C. The specific enzyme activity is expressed as µmol of degraded H_2O_2 in one min per mg of proteins.

Fluorescence-based oxidative stress measurements were performed according to [24] and results are given in relative fluorescence.

For the assessment of MXR activity, we applied the same concentrations as for the other sublethal exposures, but a separate set of exposures was performed. Fluorescence-based measurements were performed based on [31] with changes as described in [24], and MXR activity was expressed as nmol RB per mg proteins.

2.5. Assessment of Avoidance Behavior

The assessment of avoidance behavior (ISO 17512-1:2008) was performed as described in detail in [32].

Briefly, glass containers were divided in two separate chambers by using a tray that was placed in the middle of the container. One side of the container was filled with 200 g of the negative control (soil with distilled water) and the other side with 200 g of the treated soil (respective pesticide and concentration). After the container was filled with both soils, the separating tray was removed and 10 earthworms per container were placed on the middle line, the container covered to avoid any earthworms escaping and then incubated

at 20 °C. The experiment ends after 48 h when the soil is again separated in the middle and the number of earthworms on each side determined by hand sorting.

2.6. Data Analysis

The statistical software R version 3.3.2. and logit procedure of the drc package within the R software environment was used for the determination of lethal concentrations (LC_{10}, LC_{50}, LC_{90}). Four-parameter logistic curve was used for fitting the mortality concentration–response curves. Subcellular markers were analyzed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). In order to determine the appropriate statistical tests, data were checked for homoscedasticity (Bartlett test) and normality (Shapiro–Wilk test). For normally distributed data, a one-way ANOVA analysis followed by the Dunnett's multiple comparison test to determine the significance levels reached in comparison to the control was performed. If the data were not normally distributed, the Kruskal–Wallis one-way analysis of variance by ranks followed by Dunn's multiple comparison test was performed. For the avoidance behavior, significant differences in the preference of the earthworms for control or exposed soil were determined by means of the *t*-test. The level of significance was set to $p < 0.05$ throughout the study.

3. Results

3.1. Acute Toxicity of Investigated Pesticides

The lethal concentrations (LC) after exposures to a range of concentrations of the respective commercial preparations are presented in Table 1. The LC_{50}-values were 102.035 mg/kg Calypso, 10.149 mg/kg Sumialfa, 249.804 mg/kg Frontier and 558.376 mg/kg Filon. As 100% mortality could not be achieved for any of the active ingredients, the highest tested concentration and the corresponding achieved mortality is shown in Table 1. Therefore, no statistical comparison between the acute toxicity of active ingredients and commercial preparations could be performed. However, from the obtained results it is well visible that substantially higher concentrations of active ingredients (compared to the respective commercial pesticide preparation) did not cause mortality, indicating their lower toxicity. The concentration–response curves for the commercial pesticide preparations are shown in Figure S1.

Table 1. Acute toxicity results after 48 h pesticide exposures to *E. andrei* in soil.

Active Ingredient	Mortality [1] in %	Commercial Preparation		Concentration in mg/kg
thiacloprid	53% at 850 mg/kg	Calypso	LC_{10} LC_{50} LC_{90}	29.720 102.035 350.312
esfenvalerate	80% at 300 mg/kg	Sumialfa	LC_{10} LC_{50} LC_{90}	5.723 10.149 17.800
dimethenamid-p	80% at 1000 mg/kg	Frontier	LC_{10} LC_{50} LC_{90}	108.468 249.804 575.302
prosulfocarb	0% at 1200 mg/kg	Filon	LC_{10} LC_{50} LC_{90}	312.452 558.376 982.145

[1] Highest achieved mortality percentages of active ingredients and lethal concentrations (LC_{10}, LC_{50}, and LC_{90}) of the investigated pesticides to earthworm *E. andrei* for 48 h in LUFA 2.2 soil; $n = 4$.

3.2. Avoidance Behavior

The results sublethal exposures of *E. andrei* to commercial pesticide preparations for the determination of avoidance behavior is shown in Table 2. Both insecticides Calypso and Sumialfa did not show a reduced habitat function according to the ISO toxicity evaluation (ISO 17512-1, 2008). However, an increased net response to the highest concentrations of both pesticides was observed. In the case of the herbicides, 100 mg/kg Frontier and 75 and 150 mg/kg Filon showed a reduced habitat function. None of the performed control exposures showed a reduced habitat function (results from control exposures not shown).

Table 2. Effect of commercial pesticide preparations on the avoidance behavior of *E. andrei* in soil—48 h exposures to the respective commercial pesticide preparations and controls in LUFA 2.2 soil for the determination of avoidance behavior. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Pesticide	Concentration (mg/kg)	Distribution (%)		Net Response (%)	Toxicity Evaluation [1]
		Control	Treated		
Calypso	1	60	40	20	NRHF
	5	50	50	0	NRHF
	10	68	32	36	NRHF
Sumialfa	0.5	48	52	−4	NRHF
	2.5 *	70	30	40	NRHF
	5 ***	76	24	52	NRHF
Frontier	10	48	52	−4	NRHF
	50 *	70	30	40	NRHF
	100 * **	**100**	**0**	**100**	**RHF**
Filon	15	70	30	40	NRHF
	75 **	**87**	**13**	**73.33**	**RHF**
	150 * **	**94**	**6**	**87.5**	**RHF**

[1] NRHF: no reduced habitat function is considered when >20% of earthworms in treated soil, RHF: reduced habitat function is considered when ≤20% of earthworms in treated soil (marked in bold letters), $n = 3$.

3.3. Biomarker Responses

The results of the measured specific enzyme activities after 48-h exposures of earthworms to sublethal concentrations of the commercial insecticides Calypso and Sumialfa in soil are presented in Figure 1 (significant differences only) and Figure S2 (no significant differences). For Calypso, four out of five biomarkers showed significant differences to the control. The highest concentration of 10 mg/kg Calypso caused a decrease of GST, CAT, and AChE activity. Regarding the AChE activity, although 5 mg/kg did not cause a significant change, 1 mg/kg also caused a decrease. On the other hand, CES activity was increased only for 5 mg/kg. In the case of Sumialfa, a decrease of AChE and CAT activity was observed for the highest concentration of 5 mg/kg. The lowest tested concentration of 0.5 mg/kg caused a significant decrease of GR activity.

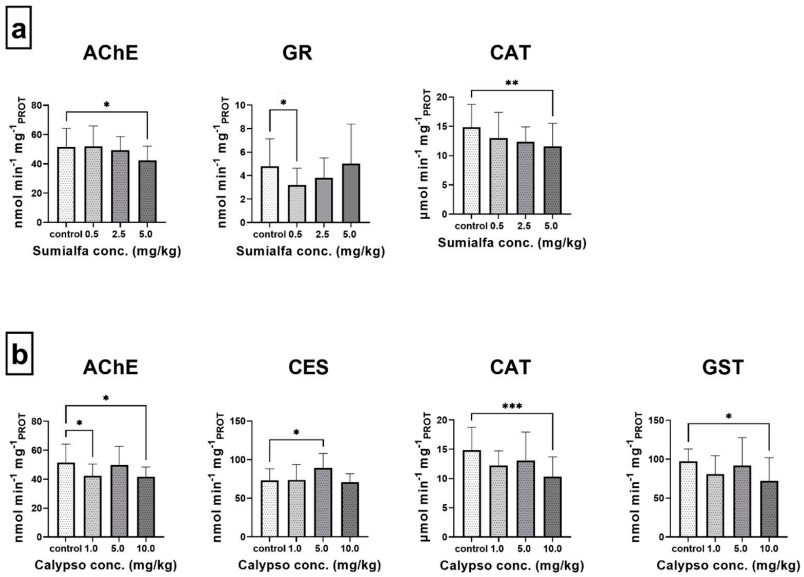

Figure 1. Biomarker responses after 48-h exposures to insecticides Calypso and Sumialfa in soil. Acetylcholinesterase (AChE), carboxylesterase (CES), glutathione S-transferase (GST), glutathione reductase (GR), and catalase (CAT) activities measured in *E. andrei* earthworms exposed to the commercial pesticide preparations (**a**) Sumialfa (active ingredient esfenvalerate) and (**b**) Calypso (active ingredient thiacloprid) for 48 h in LUFA 2.2 soil. Results are presented as mean ± standard deviation ($n = 20$) and only significant differences (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) between control and pesticide treatments are shown (ANOVA followed by Dunnett's multiple comparison test).

The results of the measured specific enzyme activities after 48-h exposures of earthworms to sublethal concentrations of the commercial herbicides Frontier and Filon in soil are presented in Figure 2 (only significant differences) and Figure S3 (no significant differences). Significant changes in enzyme activity after exposure to Frontier could only be observed for CAT at a concentration of 50 mg/kg. The concentration of 75 mg/kg Filon caused a significant decrease of CES and GR activity, while 15 mg/kg caused a significant increase of GST activity.

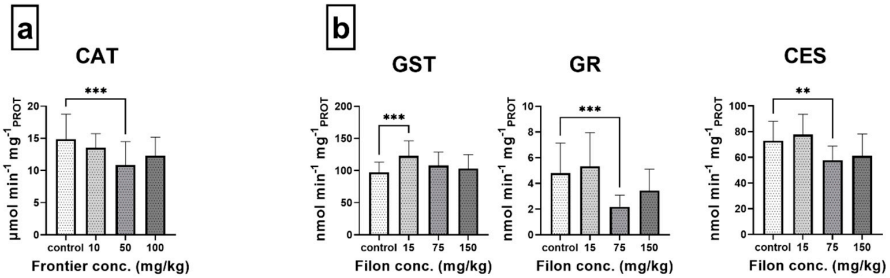

Figure 2. Biomarker responses after 48-h exposures to herbicides Frontier and Filon in soil. Carboxylesterase (CES), glutathione S-transferase (GST), glutathione reductase (GR), and catalase (CAT) activities measured in *E. andrei* earthworms exposed to the commercial pesticide preparations (**a**) Frontier (active ingredient dimethenamid-p) and (**b**) Filon (active ingredient prosulfocarb) for 48 h in LUFA 2.2 soil. Results are presented as mean ± standard deviation (n = 20) and only significant differences (** $p < 0.01$ and *** $p < 0.001$) between control and pesticide treatments are shown (ANOVA followed by Dunnett's multiple comparison test).

3.4. Fluorescence-Based ROS and GSH Determination

The fluorescent probe CM-H_2DCFDA was used for the detection of reactive oxygen species (ROS) after a 48-h exposure to the investigated insecticides and herbicides in soil, and the results of the relative fluorescence are presented in Figure S4. No significant changes in relative fluorescence could be detected for any of the pesticide exposures.

The fluorescent probe Celltracker Green CMFDA was used for the detection of glutathione (GSH) after a 48-h exposure to the investigated insecticides and herbicides in soil, and the results of the relative fluorescence are presented in Figure 3. Significant changes in relative fluorescence could be detected after exposures to two out of four commercial pesticide preparations, namely, 5 mg/kg of the insecticide Calypso caused an increase of relative fluorescence while concentrations of 75 and 150 mg/kg of the herbicide Filon caused a decrease of relative fluorescence. For both Sumialfa and Frontier, no significant differences could be detected.

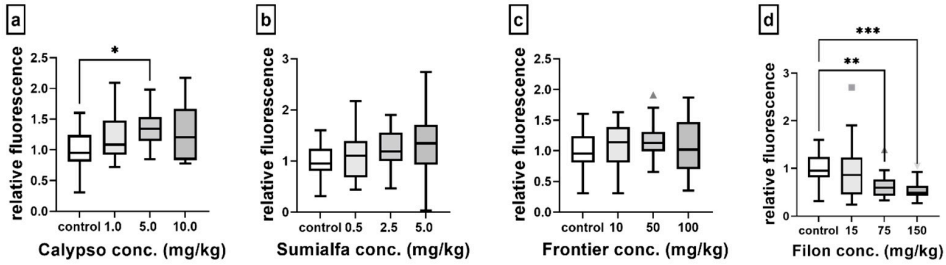

Figure 3. Results of GSH measurements after 48-h pesticide exposures in soil. Relative fluorescence for glutathione (GSH) measurements in *E. andrei* exposed to the investigated pesticide preparations (**a**) Calypso (active ingredient thiacloprid), (**b**) Sumialfa (active ingredient esfenvalerate), (**c**) Frontier (active ingredient dimethenamid-p), and (**d**) Filon (active ingredient prosulfocarb) for 48 h in LUFA 2.2 soil. Data is presented using Tukey's boxplot (n = 20). Significant differences (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) between control and pesticide treatments are presented (ANOVA followed by Dunnett's multiple comparison test).

3.5. MXR Activity

Results of the relative rhodamine B (RB) concentrations in *E. andrei* after 48-h exposures to the investigated commercial pesticide preparations in soil are presented in Figure 4. A significant decrease in the relative RB concentration compared to the control was detected for all pesticides, namely 5 and 10 mg/kg Calypso, 5 mg/kg Sumialfa, 50 and 100 mg/kg Frontier, and 75 and 100 mg/kg Filon. The induction of the MXR activity is reflected in observed decrease in relative RB concentration.

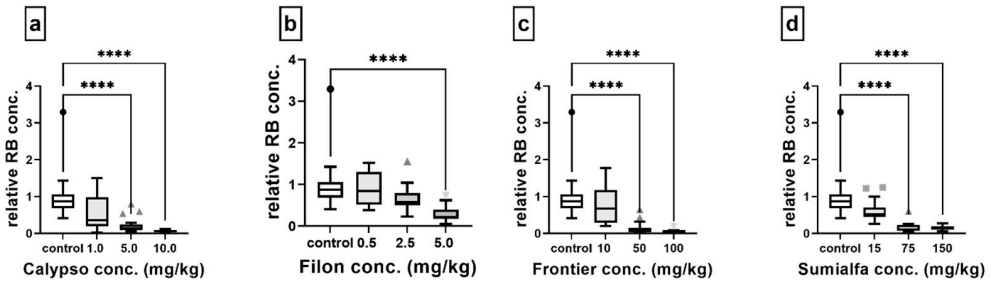

Figure 4. Results of MXR activity determination in *E. andrei* after 48-h pesticide exposures in soil. Relative rhodamine B (RB) concentrations for the determination of MXR activity after 48 h exposures to the investigated pesticide preparations (**a**) Calypso (active ingredient thiacloprid), (**b**) Sumialfa (active ingredient esfenvalerate), (**c**) Frontier (active ingredient dimethenamid-p), and (**d**) Filon (active ingredient prosulfocarb). Results are presented as mean ± standard deviation (n = 20). Significant differences (**** $p < 0.0001$) between control and pesticide treatments are presented (ANOVA followed by Dunnett's multiple comparison test).

4. Discussion

Pesticide effects have been known to span across different environmental compartments, i.e., both aquatic and terrestrial organisms have reportedly been negatively impacted by pesticides [2,33–35]. According to [14], pesticides can impact terrestrial organisms such as earthworms on all levels of biological organization such as disruption of enzymatic activities, individual mortality, or a change in behavior. Our results support these observations after even short-term exposures. In the present study, the short-term toxicity of four pesticides in standardized soil has been investigated through traditional acute toxicity tests, avoidance behavior tests, and means of subcellular markers, e.g., enzymatic biomarkers, oxidative stress-related responses, and MXR activity. As shown in our previous study after toxicity assessments on filter paper, all four commercial pesticide preparations showed a higher toxicity than their respective active ingredients. Furthermore, effects on a subcellular level (oxidative stress-related markers, MXR activity) could be observed as well [24]. As these results obtained using filter paper exposures can however only be seen as an initial screening of toxicity and determining other modes of action, the effects after exposures in standardized artificial soil were assessed in the present study.

4.1. Acute Toxicity

Determinations of toxicity by means of mortality is usually the first investigated endpoint in ecotoxicological research and an important endpoint used in regulatory decision-making. However, for some substances, as shown in this study, it might not be possible to obtain a dose–response and establish lethal values, making it difficult to decide on a further test strategy. In the case of the pesticides investigated here, the acute toxicity of both the active ingredients and their respective commercially available counterparts, meaning the mixture that is eventually applied on the field and in the environment, were evaluated. No clear concentration–response relationships or 100% mortality could be established for any of the active ingredients. The relatively low toxicity compared to the commercial preparations (Table 1)—for which lethal values could be established using the same procedures—could be due to the different distribution, uptake, and fate in the soil caused by the adjuvants present in the commercial preparations [36]. However, some studies were

able to establish lethal concentrations of the active ingredient thiacloprid to the earthworm *Eisenia fetida* [20,37]. This huge discrepancy compared to our experiments (53% mortality at 850 mg/kg) might be explained by differences in species sensitivity or a difference in the used soil. This shows how the exposure protocols for active ingredients and different chemical classes need to be challenged in the future and improved accordingly.

Compared to common earthworm toxicity classifications on filter paper [38], there are no previously established toxicity classifications after exposures in soil. However, our results show a difference in order of magnitude in lethal values between the investigated herbicides (558.367 mg/kg Filon, 249.804 mg/kg Frontier) and insecticides (10.149 mg/kg Sumialfa, 102.035 mg/kg Calypso), with relatively higher LC_{50}-values and thus lower acute toxicity of the herbicides. There is a relatively small amount of data on the effects of the investigated pesticides in soil and their respective commercial preparations, which makes a comparison of the obtained data difficult but also shows the need to fill this knowledge gap. According to the EFSA report on esfenvalerate [39], the overall risk to soil organisms has been described as low with a reported acute LC_{50} of 10.6 mg/kg using a commercial formulation, which fits very well with the LC_{50}-value (10.15 mg/kg) determined in this study. For thiacloprid, a high risk of the active substance and varying risk of a respective commercial preparation to earthworms depending on the respective usage (oilseed rape vs. maize) was concluded [40], which would fit with the relatively low acute toxicity of Calypso that has been observed in this study. For both herbicides, the risk to earthworms according to EFSA reports is low, with a reported LC_{50} value of 54.5–545 mg/kg (commercial preparation of prosulfocarb) while no LC_{50} value was reported for dimethenamid-p or any of its commercial preparations [41,42].

It is also important to note that the established lethal values and chosen sublethal concentrations for all further investigations cannot be easily compared to environmental concentrations. These depend on the application rates, which might vary based on the chosen application and the number of applications per year. The predicted environmental concentrations for investigated pesticide active ingredients are much lower according to the EFSA evaluations, e.g., 0.031 mg/kg for thiacloprid (multiple application, time-weighted average, 2 days) [40], 0.0271 mg/kg esfenvalerate (multiple application, time-weighted average, 2 days) [39], 5.06 mg/kg prosulfocarb (single application, time-weighted average, 2 days) [41], and 1.15 mg/kg dimethenamid-p (multiple application, actual) [42] are much lower than the here-investigated concentrations. Therefore, for a risk assessment of the investigated pesticides, these need to be taken into account.

However, the present assessments of acute toxicity have supplied important information to start filling current knowledge gaps. The reported results both in this study and in [24] furthermore emphasize the importance of including the commercial formulations—which are mixtures of active ingredients, solvents, dispersants, and adjuvant chemicals [43]—in the assessment of pesticide toxicity as it might otherwise result in a misinterpretation and underestimation of the toxicological profile of the investigated pesticides [44].

4.2. Avoidance Behavior

When comparing endpoints on different levels of biological organization, behavioral changes are considered to have a high ecological relevance while simultaneously being highly sensitive, as these organism-scale sublethal effects can be observed already after exposures to low concentrations [45]. As explained by [14], the range in behavioral responses in earthworms is rather limited; however, due to their role in the soil ecosystem, their avoidance behavior might have important consequences for the soil structure and functioning. Besides subcellular responses, behavioral changes are often a first response to altered conditions such as chemical pollution [46] and should thus be integrated into ecotoxicological test batteries [47].

The results of the present study showed that both herbicides caused a reduced habitat function, Frontier at 100 mg/kg and Filon at 75 and 150 mg/kg, respectively (Table 2).

It has been shown before that pesticides can cause a significant response of avoidance behavior in earthworms, therefore making avoidance behavior a suitable endpoint in test batteries for pesticide assessments [15]. Data on avoidance behavior of earthworms after herbicide exposures, however, is relatively scarce. The difference in avoidance behavior after exposures in the standardized LUFA 2.2 soil to the active ingredients sulcotrione and penoxsulam as well as their respective commercial formulations Mikado and Viper has been reported [15]. They observed a higher toxicity of the commercial formulations and stronger avoidance responses in similar concentration ranges (1012.8–2025.7 mg/kg Mikado; 52.7–177.8 mg/kg Viper) as the herbicide exposures in this present study; however, only Viper exposures resulted in a reduced habitat function. In a previous study, we investigated the herbicides diuron and fluazifop-p-butyl, which affected the avoidance response at concentrations as low as 5 mg/kg and 10 mg/kg, respectively, but only diuron caused a reduced habitat function at 50 and 100 mg/kg [32]. This shows that, while herbicides can cause a shift in earthworm behavior, it is important to differentiate the results obtained by the avoidance test. While the avoidance of contaminated soil might be significant compared to the control, it does not necessarily mean a loss of soil structure or function as the soil could potentially still be used as a habitat even if some individuals prefer to leave the contaminated soil.

As can be seen in the results of the insecticide exposures, Calypso caused no significant changes, but an increase in avoidance behavior could be observed after 2.5 and 5 mg/kg Sumialfa were applied; however, no reduced habitat function was observed (Table 2). This again supports the observation from before that not all observed behavioral changes result in a reduced habitat function of the contaminated soil. Another aspect that needs to be considered in the case of insecticides is the applicability of the test to gain insight into the effects of these pesticides. Past studies have reported on the avoidance behavior of earthworms after insecticide exposures, e.g., the avoidance behavior of earthworms exposed to the pyrethroid insecticide cypermethrin in three different tropical soils [48]. However, the sensitivity of the avoidance test for the assessment of insecticides has been controversial as earthworms seem to not avoid soil after organosphosphate or neonicotinoid exposures [49]. Thus, it was suggested [50] that the avoidance test might not be feasible for pesticides that show neurotoxic pathways as their mode of action will likely interfere with the avoidance behavior, hence suggesting that the assessment of Calypso and Sumialfa by means of the avoidance test might not be feasible even though avoidance tendencies were observed.

While the avoidance test might not be applicable in the testing of certain substances, it is one of the few standardized behavioral assays and thus an important addition to test batteries for the effect on soil organisms. According to [47], there is a need for a better integration of behavioral ecotoxicology into environmental protection as so far, and the acceptance of behavioral data for regulatory purposes has been very low. Therefore, the integration of this already-standardized behavioral assay and highly sensitive endpoint in future test batteries can potentially help to raise the acceptance of behavioral data in risk assessments.

4.3. Enzymatic Biomarker Responses

Changes in enzymatic activity are often considered as early-warning signals, thus measurements of enzymatic biomarkers have become a popular endpoint in ecotoxicology [51–54]. As the results in this study showed, all pesticides affected at least one the chosen biomarkers significantly. The insecticides Calypso and Sumialfa both affected the acetylcholinesterase, a marker for neurotoxicity, but they also affected markers for oxidative stress and xenobiotic detoxification (Figure 1). Namely, Calypso caused an increase in CES activity, caused a decrease in CAT activity, and caused a decrease in GST activity. Overall, these enzymatic responses show that already after short-term exposures, the insecticide can significantly impact important enzymes related to neuronal pathways, oxidative stress, and xenobiotic detoxification. Comparing these results to our previous study with filter paper

exposures [24] shows the importance of also performing the exposures in soil. While the filter paper exposures only showed a significant increase in CES activity, the soil exposures showed a significant impact on all enzyme activities except for GR activity. Similar observations could be made for the insecticide Sumialfa, where besides AChE activity, GR activity and CAT activity were also significantly decreased in the soil exposures. The previous filter paper exposures, however, only showed a decrease of CAT activity. The general decrease in activity could be observed in both exposure scenarios confirming its mode of action. This stronger effect on enzyme activities in soil might be explained by the different uptake route, as the soil is ingested orally and not just taken up through the outer dermal tissue. The effect of Sumialfa on AChE activity can be explained through a mechanism of synthetic pyrethroids that show hydrophobicity in vivo and interact at the aromatic surface of Ache, which leads to the AChE binding space to be decreased and simultaneously results in the inhibition of AChE activity [55].

As can be seen in both herbicide exposures (Figure 2), AChE activity has not been affected as was to be expected due to their main mode of action not involving neurotoxic pathways. However, both pesticides affected enzyme activities, with Filon significantly decreasing GR and CES activity and causing an increase in GST activity. This again shows the impact different uptake routes could potentially have on the obtained results as the previous filter paper exposures showed no effect on any of the tested enzymatic biomarkers, thus emphasizing the need for additional soil exposures as more realistic experimental setups. However, the results obtained after exposures with the herbicide Frontier show that the filter paper exposures can be useful as an initial screening, as both the filter paper and soil exposures showed an impact on CAT activity and no other enzymes. Therefore, it can indeed be a fast and easy first step in toxicity testing to evaluate the modes of action without implications of the soil matrix but by no means a complete evaluation of toxicity where soil exposures become necessary.

4.4. Non-Enzymatic Oxidative Stress-Related Responses

When the naturally occurring redox balance of reactive oxygen species and antioxidants, such as glutathione, is compromised, all components of a cell can potentially be damaged. This disturbance of the pro-oxidant–antioxidant balance is also known as oxidative stress. Oxidative stress may occur due to an increase in ROS production or the failure of their removal because of a decrease in ROS scavengers in the system [56]. Pesticides are a class of chemicals that have been shown in the past to affect this redox balance, thus oxidative stress is one of the often-reported toxicity mechanisms of pesticides [57,58].

In our previous study, we established a method for fluorescence-based assessment of oxidative stress-related markers [24] and have applied this method in this research. As the occurrence of oxidative stress involves a rather complex redox balance involving various molecules summarized in the general term of ROS and a diverse antioxidant system, the used method to measure ROS and GSH can only be seen as a general indication whether oxidative stress-related markers have been affected in any way. As shown in Figure 3 and Figure S4, using these fluorescence-based methods for the determination of oxidative stress showed no significant difference in ROS levels for any of the investigated pesticides. However, a significant difference in GSH levels could be observed, namely, 5 mg/kg Calypso caused an increase in GSH levels and 75 and 100 mg/kg Filon resulted in a decrease in GSH levels. As shown in our previous study with pesticide exposures using the filter paper method, the same tendencies could be observed with Calypso resulting in a significant increase in GSH levels and Filon exposures causing a slight decline in GSH levels, although not significant [24]. Overall, either an increase or decrease in GSH levels show an impact on the antioxidant system and can indicate the potential occurrence of oxidative stress. As no increased ROS levels were observed, this might indicate that the antioxidant system is still able to uphold its defense mechanisms and in the case of Filon, this can be seen in the increased GSH production.

A difference in response after filter paper and soil exposures could be observed for the herbicide Frontier. After filter paper exposures, earthworms exposed to Frontier showed significantly decreased GSH levels, while the same could not be observed after exposures in soil. This shows that, while information on the mode of action can be gathered after filter paper exposures, it is important to then continue with soil exposures for an insight into the effects on soil organisms under more realistic conditions as previously stated by [59].

In a study by [60], a similar method based on fluorescence has been used to assess ROS levels after long-term exposures of the earthworm *E. fetida* to the neonicotinoid insecticide imidacloprid. However, in order to obtain a closer look into the mechanisms behind oxidative stress related responses, it is important to also assess the antioxidant response as shown by the results in the present study. Overall, there seems to be a lack in observations of short-term exposures, which could be important in regard to potential adverse effects of point sources directly after pesticide application. The fluorescence-based protocol used in this study could therefore be a simple, easy, and sensitive tool to get more insight into oxidative stress-related responses even after short-term exposures of pesticides.

4.5. MXR Activity

The multixenobiotic resistance activity is often, similarly to the avoidance behavior, considered as a first line of defense to xenobiotics, but in this case a cellular defense mechanism. The results of the present study show that all investigated pesticides caused a dose-dependent decrease in relative rhodamine B content and thus an increase in MXR activity (Figure 4). As a first line of defense, an increased activity indicates that the toxic pesticides are pumped out of the cells to alleviate toxic effects. While not all concentrations caused a significant decrease in relative RB concentrations, a slight dose-dependent decrease was observed for all pesticides and their respective exposure concentrations. Again, comparisons can be made with our previous study [24] where exposures with the same pesticide preparations were performed using the filter paper method. In the case of the filter paper exposures, only Calypso and Filon caused a significant difference in MXR activity. Interestingly, while Calypso also caused an increased MXR activity, the lowest concentration of Filon caused a decreased MXR activity. We hypothesized before that this could be explained through cell defense mechanisms being activated at higher concentrations preventing an inhibition of MXR activity. Similarly, as a significant increase in MXR activity could be observed after Filon exposures in soil, the concentrations might be comparatively high, where the efflux pumps were activated to pump out the toxic pesticide. While no other studies have assessed the impact of the here-investigated pesticides on MXR activity and it is still not regularly used as an endpoint for earthworm toxicity studies, the impact of various pesticides and their commercial formulations on MXR activity has been reported before [31,32,59]. This shows that MXR activity can give valuable insight into the toxicity of pesticides and the following activation of complex defense mechanisms on a cellular level.

5. Conclusions

In this study, *E. andrei* earthworms were exposed to two insecticide and two herbicide commercial preparations for 48 h to determine the short-term toxicity in standardized soil using various endpoints, namely, acute toxicity, avoidance behavior, biomarkers, and multixenobiotic resistance. Overall, it was shown that the commercial pesticide preparations caused adverse effects on a key organism of the soil ecosystem not only on a cellular level, but also indicated the potential for a change in habitat function after short-term exposures. The results indicate that the usage and effects of commercial pesticide preparations on the soil ecosystem should be a bigger focus compared to the testing of active ingredients in a regulatory context. Furthermore, the test design for the assessment of pesticide effects should include exposures in soil to gain clearer understanding of the implications the soil matrix might have on the uptake and thus toxicity of pesticides. As the exposures in this study were fairly short, future studies should assess the effects of pesticides after long-term exposures and the potential for recovery. Furthermore, other sensitive endpoints on higher

levels of biological organization, effects on reproduction, and the implications of natural soil matrices should also be included in test strategies.

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