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Glyphosate Effects on Earthworms: Active Ingredients vs. Commercial Herbicides at Different Temperature and Soil Organic Matter Levels

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Abstract: Little is known about the non-target effects of glyphosate active ingredients (GLY) versus glyphosate-based herbicide (GBH) formulations on soil organisms, and whether effects are influenced by environmental conditions. We investigated the avoidance behavior, biomass growth, and reproduction of earthworms (*Eisenia fetida*, *E. andrei*) in response to two GLYs (glyphosate ammonium and potassium salt), the corresponding GBHs (Touchdown Quattro, Roundup PowerFlex) containing these GLYs, and the "inert" co-formulant alkylpolyglycoside (APG) at two temperature (15 °C vs. 20 °C) and soil organic matter levels (3.2% vs. 4.3%). Earthworm avoidance was lower at high soil organic matter content, but remained unaffected by substances and temperature. Earthworm biomass growth and reproduction (cocoons and juveniles) were significantly affected by substances and temperature; reproduction was also affected by a substance and temperature interaction. Biomass growth was almost zero at higher temperature; reproduction was generally higher at higher temperature. More cocoons were produced under Roundup PowerFlex than under the corresponding AI, due to the impact of the co-formulant APG. No other differences were observed between GBH and the corresponding AIs. We conclude that the non-target effects of pesticides can only be fully assessed if all ingredients in a formulation are known and environmental parameters are included in environmental risk assessments.

Keywords: compost worms; ecotoxicology; earthworm behavior; earthworm reproduction; environmental risk assessment; weed control

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1. Introduction

Glyphosate-based herbicides (GBH) are broad-spectrum, non-selective herbicides used to control weeds or desiccate crop plants to facilitate harvesting [1]. There are hundreds of GBHs on the market worldwide, and the number of applications increased nearly 15-fold between 1996 and 2016 [2]. GBHs usually consist of 35–50% of the active substance glyphosate GLY [3], mainly in the form of various salts (e.g., isopropylamine, ammonium, sodium, and potassium salts) [4,5]. To further increase the efficiency of the active ingredient (AI), coformulants such as surfactants, antifoam agents, and dyes are added to the GLYs [6]. Pesticide approval in the European Union (EU) [7,8], the USA [9], and other OECD countries make a distinction between AI and inert coformulants [7]. Active ingredients or substances are toxic for the target species, while all other coformulants are classified as inert [10]. However, there is increasing evidence that coformulants can also have toxic effects on humans and the environment, yet current environmental risk assessment focuses almost exclusively on the active ingredient [11,12]. Even the European Union lists 144 "unacceptable coformulants" to be banned due to their inherent hazardous properties [13]. Independent research on effects is hampered by the fact that the identity and concentration of coformulants in pesticide products are treated as confidential business information and are usually not listed on product labels [10,14].

In soil, GLY undergoes various physical and chemical changes that affect its retention, transport, degradation, uptake by plants, and leaching [5]. The binding of GLY to soil particles is high compared to other pesticides, and the degree of sorption depends on the mineral content and type of soil, pH, soil phosphate content, and soil organic matter [15,16]. Soils with higher organic matter content and minerals such as aluminum and iron oxides can bind glyphosate for longer [15]. The remaining GLY remains available in soil for plant uptake, interaction with metal cations, and degradation [17,18]. In soil, GLY has a half-life of 47 days [5] to 197 days [19]. Decomposition in soil depends on the type and characteristics of the soil microorganisms, as well as climatic conditions such as moisture and temperature [20–22]. Studies also confirm that GLY is used as a source of carbon, nitrogen, or phosphorus by microorganisms [23,24].

Earthworms are among the most important animal group in soils and serve as surrogate species in environmental risk assessments [25]. Of the approximately 3600 earthworm species worldwide [26], the compost worm *Eisenia fetida* is most commonly used in ecotoxicological studies. Earthworms are sensitive to chemicals in the soil distributed on their body surface due to chemo-sensitive receptors [27]. Earthworm avoidance behavior, growth, reproduction, and mortality have been identified as ecologically relevant endpoints in these ecotoxicological studies [28]. A meta-analysis of 25 ecotoxicological studies comparing the effects of GBH and GLY on earthworms reports widely varying results for the same endpoints and attributed this to the use of different earthworm species, different glyphosate formulations, different soil temperatures, and different soil properties [29]. Temperature has a significant effect on the degradation of GBHs and thus indirectly on the toxic effects, while soil properties affect herbicide absorption and half-life [21,22,30].

The objectives of this study were to (i) evaluate the effects of two commercial GBH formulations (Roundup PowerFlex and Touchdown Quattro) and their respective active ingredients GLY (glyphosate potassium and ammonium salt) and the co-formulant alkylpolyglucoside (APG) contained in both formulations on earthworm avoidance behavior, biomass growth, and reproduction and (ii) determine the extent to which this response is affected by temperature and different soil organic matter levels. We expected that earthworm avoidance behavior, reproduction rate, and biomass would be negatively affected by GBHs, GLYs, or APGs, and that GBHs would show a stronger effect than GLY alone because of coformulants. We also expected that earthworm response would be lower at high soil organic matter levels due to buffering effects, but stronger at higher temperatures because of the additional stress this places on earthworms. To our knowledge, this is among the first studies to adapt standardized protocols for environmental risk assessments with temperature and soil organic matter levels.

2. Materials and Methods

The experiments were performed between March and May 2019 at the Institute of Zoology of the University of Natural Resources and Applied Life Sciences, Vienna, Austria.

2.1. Test Substances

Roundup PowerFlex (RP; Bayer AG, Leverkusen, Germany) was purchased in a garden center in Vienna, Austria, Touchdown Quattro (TQ; Syngenta Czech, Prague, Czech Republic) in an online store (VMD Drogerie, Veselí nad Moravou, Czech Republic). RP contains the AI potassium salt (potassium N-[(hydroxyphosphinato)methyl]glycine) at 43.8%, and TQ the AI ammonium salt (carboxymethylamino)methyl-hydroxyphosphinate) at 34%. Both formulations contain the co-formulant alkylpolyglucoside (APG) at 20% in RP and 10% in TQ, according to the safety data sheets [31,32].

The glyphosate salts and APG were provided by our project partner the Agro-Environmental Research Institute in Budapest, Hungary. The potassium and ammonium salt, produced from glyphosate, was purchased from Sigma-Aldrich, Hungary (Budapest, Hungary). Glyphosate potassium salt was synthesized by adding 1.66 g (9.82 mmol) of glyphosate under continuous stirring to a cooled 0.84 mL aliquot of a 45% (w/w) aqueous potassium hydroxide solution. The mixture was stirred overnight at 4 °C and the resulting precipitate was filtered and lyophilized to obtain 1.04 g (5.02 mmol, 51.1%) of glyphosate potassium salt. In the same procedure, 1.66 g (9.82 mmol) of glyphosate was added to 1.33 mL aliquot of a 28% (w/w) aqueous ammonium hydroxide solution to obtain 1.01 g (4.97 mmol, 50.6%) of glyphosate ammonium salt.

To calculate the desired concentration in mg kg^{-1} or µL kg^{-1} dry soil, we first calculated the mass of soil of one hectare at a depth of 5 cm and a bulk density for Chernozem soils of 1.5 g cm^{-3} [33]: 1.5 g cm^{-3} × 5 cm × 1 ha = 750,000 kg. Then, the dosage amount ha^{-1} was divided by soil mass to obtain the concentration of the substance kg^{-1} dry soil (Table 1). The substances were diluted with the appropriate amount of tap water to achieve a water content of 40% when mixed with the soil.

Table 1. Treatments and appropriate dosages for avoidance and reproduction testing.

Substance	Conc. AI (g l^{-1})	Recomm. Applic. Rate (l ha^{-1})	Field Rate (l ha^{-1})	Dosage in Lab. Experiments (µL kg^{-1})
Roundup PowerFlex (RP)	480	3.75	3.75	5.00
Potassium salt in RP	588	3.75	2.21 §	2.94 §§
Alkyl polyglucoside in RP	20%	3.75	0.75	1.00
Touchdown Quattro (TQ)	360	5.00	5.00	6.67
Ammonium salt in TQ	435	5.00	2.18 §	2.90 §§
Alkyl polyglucoside in TQ	10%	5.00	0.50	0.70

§ field rate in kg ha^{-1}; §§ dosage in mg kg^{-1}.

2.2. Test Substrate

Topsoil (0–15 cm) from two different arable fields of the BOKU experimental farm in Großenzersdorf near Vienna was used for the experiments. The soil was a Chernozem on loess [34], which was cultivated in crop rotations according to good agricultural practice. The soil with the lower SOM content of 3.2% was from a conventionally farmed field where synthetic insecticides (AI deltamethrin, pymetrozine) had been applied in the previous 3 years but no herbicides in the previous 5 years. The soil with the higher SOM content of 4.3% was from a field that had been managed organically for 25 years. These different SOM values reflect the average situation in conventional and organic arable farms in the region but are relatively high compared to arable cropland worldwide. Nevertheless, the SOM levels we differentiated were substantially (34%) different between the two levels. More detailed information on nutrients is provided in Table 2. Soil moisture content during the experiment was kept constant at 20 ± 2 vol.% during the experiment.

Table 2. Characteristics of the two soil types used in the experiment. Soil analysis was performed by the Austrian Agency for Health and Food Safety (AGES). SOM . . . soil organic matter.

Parameter	Low SOM	High SOM	Intermediate SOM §
pH	7.5	7.5	7.6
Phosphorus (mg kg^{-1})	81	125	113
Potassium (mg kg^{-1})	173	245	227
Magnesium (mg kg^{-1})	140	97	106
Soil organic matter (%)	3.2	4.3	3.9

§ this mixture of 50:50 low:high SOM was used for the reproduction experiment.

2.3. Earthworms

Earthworms were kindly provided by a vermicomposting company (Vermigrand Natur GmbH, Absdorf, Austria). Two thousand adult earthworms were selected and maintained in their rearing compost substrate in plastic bags during a dark:light period of 8:16 h at 10 °C in a climate chamber. The earthworms consisted of the species *Eisenia fetida* and *E. andrei*, which are morphologically identical except for their different pigmentation: *E. andrei* is uniformly red, while *E. fetida* is striped [35]. Both species are standard test organisms in terrestrial ecotoxicology [36]. For simplicity, only *E. fetida* is mentioned throughout the text.

2.4. Earthworm Avoidance Test

The avoidance test was conducted using a 3-factorial design consisting of

- Factor substances (6 levels):
    - ○ 2 GBHs (Roundup PowerFlex vs. Touchdown Quattro)
    - ○ 2 GLYs (potassium salt vs. ammonium salt)
    - ○ Adjuvant (alkylpolyglucoside APG vs. water);
- Factor temperature (2 levels): 15 ± 2 °C vs. 20 ± 2 °C air temperature;
- Factor soil organic matter (2 levels): 3.2% vs. 4.3% SOM.

Each level was replicated 5 times: (5 substances + control) * 2 temperature levels * 2 SOM * 5 replicates = 120 experimental units.

White plastic boxes (length: 18 cm; width: 13.5 cm; height: 4.5 cm) with a removeable vertical partition were used as experimental units to create two equally sized treatment areas (Figure 1A). The test substances (Roundup, Touchdown Quattro, ammonium, potassium salt, and APG) were prepared and mixed into the substrate (40% water content) immediately before the start of the experiment. The soils were mixed and 200 g (dry weight) was filled into the plastic containers, with the control soil in one half and the test soil containing the substances in the other half of the container. The control treatments were set up in parallel and contained only water [36,37].

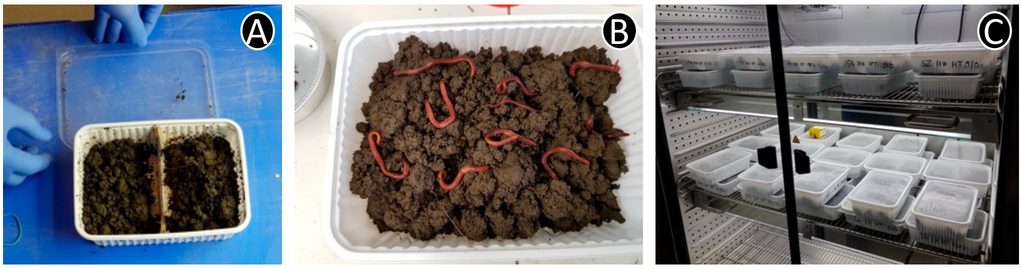

Figure 1. Experimental units separated in two halves for avoidance test (**A**), experimental units used for reproduction test (**B**), and arrangement of units in climate chambers to test for temperature effects (**C**).

Adult *E. fetida* with similar biomass (200–300 mg) were selected from the rearing containers, carefully cleaned from adhering soil, and dried on a paper towel. The partition of the plastic boxes was removed, and 10 adult earthworms were introduced to the boxes. To prevent escape of the worms, the test containers were covered with a perforated transparent plastic lid. Containers were randomly placed in a climate chamber and incubated at 15 ± 2 °C and 20 ± 2 °C with a 16:8 h light:dark period for 48 h (Binder Climate Chamber KBLF 720, Tuttlingen, Germany) (Figure 1C). According to DIN ISO 17512 [36], no feeding of the animals is required during the test. After 48 h the divider was inserted to keep the worms on the respective half and worms counted separately in each half of the soil. Worms found in the center line of the test vessel were scored with 0.5 [36,37]. Worms that did not show activity were classified as dead [36].

Soil pH and moisture content were measured at the beginning and end of the test phase in five test containers with untreated soil [36,37].

Avoidance behavior of the test substance was calculated according to DIN ISO 17512 [36], using the mean earthworm number in the test soil and the mean earthworm number in the control (Equation (1)). This means that with negative values, the earthworms prefer the test soil, 0% means no reaction, a positive value means avoidance of the test substance.

$$\boldsymbol{x} = \left(\frac{\boldsymbol{nc} - \boldsymbol{nt}}{\boldsymbol{N}}\right) * \mathbf{100} \tag{1}$$

where $\boldsymbol{x}$ is the avoidance in percent (%), $\boldsymbol{nc}$ is the number of earthworms in the control soil, $\boldsymbol{nt}$ is the number of earthworms in the test soil, and $\boldsymbol{N}$ is the total number of earthworms. ISO 17512 sets a threshold value according to which a test soil has a limited habitat function if >80% of earthworms are present in the control soil or <20% in the test soil. The 20% threshold used in the above formula at $\boldsymbol{N}$ = 10 corresponds to 60% avoidance behavior [27].

2.5. Earthworm Biomass Growth and Reproduction

This experiment was conducted using a 2-factorial test design with the following factors:

- Factor substances (6 levels):
    - ○ 2 GBHs (Roundup PowerFlex vs. Touchdown Quattro)
    - ○ 2 GLYs (potassium salt vs. ammonium salt)
    - ○ Adjuvants (alkylpolyglucoside vs. water);
- Factor temperature: 15 ± 2 °C vs. 20 ± 2 °C air temperature.

Each level was replicated 5 times: (5 substances + control) * 2 temperatures * 5 replicates = 60 experimental units.

We used similar white plastic boxes with perforated transparent lids as experimental units for this experiment (Figure 1B). Each box was filled with control soil or test substrate with 500 g (dry soil) approx. 4 cm high.

Ten adult worms (total biomass of 2.8–3.5 g) were selected from the rearing substrate, washed free of soil, carefully dried on a paper towel, and introduced to the experimental units. All test boxed were placed in the corresponding climate chamber programmed to 15 ± 2 °C and 20 ± 2 °C for a 16:8 h light:dark period. Boxes were randomly arranged in the climate chamber to avoid systemic errors.

Earthworms were fed 5 g of dried horse manure once a week and sprayed with 5 mL of tap water per test box. The horse manure came from the provider of the worms and was not medicated or treated with substances such as growth-promoting nematicides or similar veterinary products that could adversely affect the worms. These feeds therefore fulfilled the requirements of the Austrian standard ISO 11268.

After completion of the first experimental phase after 31 days, the total number of earthworms and the mass of living adult worms were recorded. Then, 5 g of horse manure was mixed into the test and control soil and placed back into the respective test boxes. The boxes were incubated for another 32 days and then the juveniles and cocoons counted during 15 min per box.

Earthworm biomass change was expressed as a percentage of the mass change in relation to the initial weight, taking into account the number of missing worms (Equation (2)):

$$\boldsymbol{mInd} = (\frac{\boldsymbol{me}}{\boldsymbol{mb}} * \frac{\boldsymbol{nb}}{\boldsymbol{ne}} - \mathbf{1}) * \mathbf{100} \tag{2}$$

with $\boldsymbol{mInd}$ meaning the relative biomass change per individual in percent (%) per test vessel, $\boldsymbol{me}$ meaning total earthworm biomass of each test vessel at the end of the test, $\boldsymbol{ne}$ meaning the number of worms at the end of the test per test vessel, $\boldsymbol{mb}$ meaning total earthworm biomass of each test vessel at the start of the test, and $\boldsymbol{mb}$ meaning the number of worms at the end of the test per test vessel.

Two samples (21 HT-TQ with 7 worms and 30 LT-TQ with 2 worms) were excluded from the calculation because of more than 10% of worms being missing.

Each test block (5 replicates) was removed from the climatic chamber immediately before counting. On the penultimate day of the experiment (18 May), a technical error occurred and the remaining 13 samples incubated in the climatic chamber at 20 °C were exposed to 60 °C for 3 h. Therefore, only the number of cocoons could be determined, but not the number of juvenile worms due to the high temperature. Soil pH and moisture content were measured at the beginning (day 0) and end (day 34) of the experiment.

The pH value of the test and control soils was measured before and after the experiments with distilled water in a ratio of 1:5 soil. Soil moisture was determined gravimetrically on 30 g soil taken from the 5 repetitions before and after the experiment after drying at 105 °C for 24 h. Air temperature in the climatic chambers was monitored using data loggers (Tinytag TPG 4901, West Sussex, UK).

2.6. Statistical Analyses

All analyses were performed with R-Studio, R version 3.6.1 for Windows [38] using the "car" and "multcomp" packages. The factor substances had six levels: Roundup PowerFlex (RP), Touchdown Quattro (TQ), potassium salt (po), ammonium salt (am), alkypolyglucoside (APG), and control. The factor temperature had two levels (20 °C vs. 15 °C), and the factor SOM also two levels (4.3% vs. 3.2%). All raw data are provided in the Supplementary Materials.

Avoidance behavior was tested using a three-factorial analysis of variance (ANOVA) with the factors: substance, temperature, and SOM. Prerequisites such as normal distribution (QQ plot) and variance homogeneity (Levene Test) of residuals were tested and were met. Such statistical analysis and the threshold method are often used in avoidance testing [27,39].

Biomass changes were tested using two-factorial ANOVAs with the factors: substances and temperature, followed by glht-Post-Hoc-Tests (General Linear Hypothesis Test with Tukey Contrast) when main effects were significant. The residuals of the dependent variable were tested for normal distribution (Q/Q plot) and homoscedasticity (Levene test).

The numbers of juveniles and cocoons were tested using a general linear model with a Poisson distribution with factors: substances and temperature together with their two-way interactions and subsequent analysis of deviation, followed by glht-Post-Hoc-Test with significant results. Control and coformulant (ADJ) were excluded from the GLM model for the variable juveniles because of incomplete datasets. The assumption of normal distribution of the residuals was verified by a Q/Q plot.

Statistical significance was generally defined at $p < 0.05$.

3. Results

3.1. Avoidance Behavior

Statistical analysis showed that avoidance behavior was unaffected by substances and temperature but significantly influenced by SOM (Figure 2, Table 3). At both temperature levels, the substances are usually more avoided at low SOM; no significant interaction effects between substances, SOM, and temperature were found (Table 3).

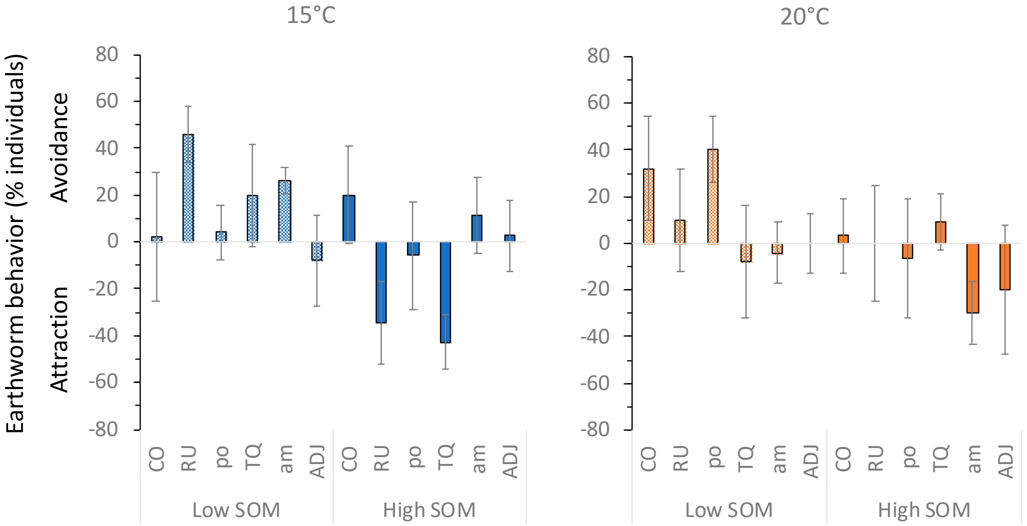

Figure 2. Avoidance behavior of earthworms in response to the test substances: CO—control (water), RU—Roundup Power Flex, po—potassium salt active ingredient (AI) of RU, TQ—Touchdown quattro, am—diammonium salt AI of TQ, and adjuvant (ADJ) contained both in RU and TQ, at low (3.2%) and high (4.3%) soil organic matter level (SOM) under 15 °C or 20 °C air temperature. Means ± SD = 5.

Table 3. ANOVA results of avoidance behavior of earthworms in response to substances (2 glyphosate-based herbicides, 2 glyphosate-salts, coformulant, water), two soil organic matter levels (3.2% vs. 4.3%), and two air temperatures (15 °C vs. 20 °C). Significant effects in bold.

Factors	Df	F Value	Pr (>F)
Substances	5	0.649	0.663
Soil organic matter	1	7.434	**0.008**
Temperature	1	0.026	0.871
Substances × Soil organic matter	5	0.292	0.916
Substances × Temperature	5	1.559	0.179
Substances × Soil organic matter × Temperature	5	2.107	0.071

Avoidance was never higher than 60%, which is the threshold value for the restriction of the habitat function according to DIN ISO 17512.

Nine samples of a total of 120 samples only had nine worms instead of the initial 10 and were discarded according to ISO 17512. The validation of the homogeneity of earthworm distribution was carried out with control soils without substance addition and resulted in average ratios between the two halves of the test system of 40–60% [36].

3.2. Growth, Cocoons and Juveniles

Earthworm biomass change was significantly affected by substances and temperature, with a marginally significant substance × temperature interaction (Table 4, Figure 3a). Post-hoc comparisons showed no significant differences between substances within temperature levels (Figure 3a). Only one earthworm died or was missing in the control group, so the data were considered valid [40]. No earthworm mortality was observed at the substance concentrations used.

Table 4. Analysis of deviance results of earthworm biomass change, cocoon and juvenile production in response to substances (2 glyphosate-based herbicides, 2 glyphosate-salts, adjuvant, water) and temperature (15 °C vs. 20 °C) and their interaction. Df = 5 for substances, df = 1 for temperature, and df = 5 for interactions. Due to missing values for control and APG for juveniles, these levels were deleted from the model (df = 3 for substances). F-values for biomass change, Chi^2 for cocoon and juvenile production. Values in bold indicate significant results.

Parameters	Substances		Temperature		Subst. × Temp.	
	F/Chi^2	*p*	F/Chi^2	*p*	F/Chi^2	*p*
Biomass change (%)	2.644	**0.035**	165.160	**<0.001**	2.090	0.083
Cocoon production (no.)	11.795	**<0.001**	59.170	**<0.001**	14.275	**0.014**
Juvenile production (no.)	71.198	**<0.001**	178.89	**<0.001**	8.410	**0.038**

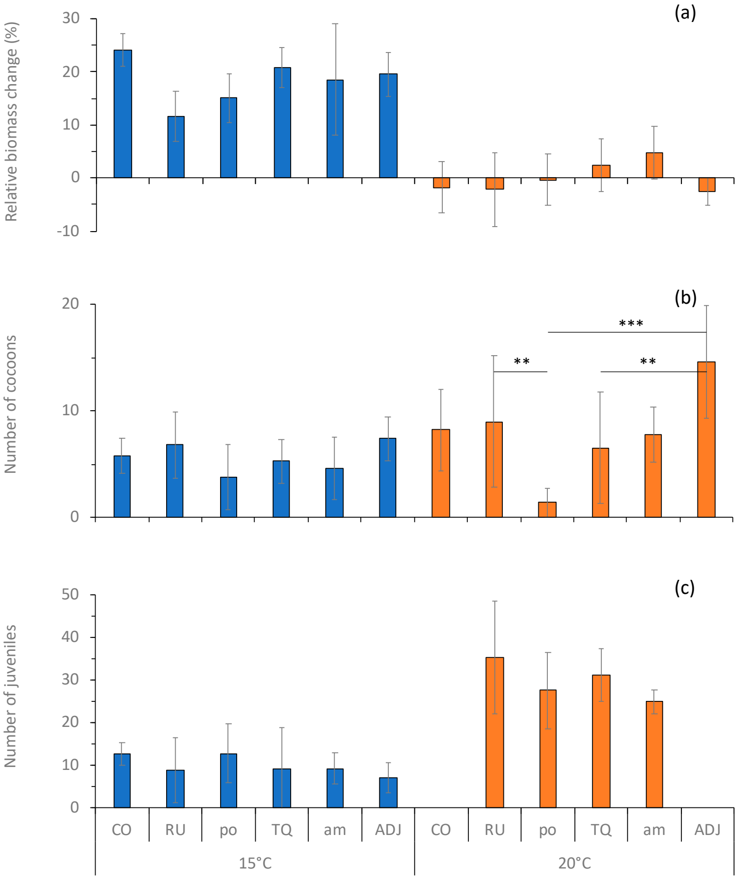

Figure 3. Earthworm biomass change from initial biomass (**a**), number of cocoons (**b**), and number of juveniles produced (**c**) of E. fetida in soil contaminated with substances (CO—water, RU—Roundup Power Flex, po—potassium salt active ingredient of RU, TQ—Touchdown quattro, am—diammonium salt AI of TQ, ADJ-surfactant contained in RU and TQ) under 15 °C or 20 °C air temperature. Significant mean comparisons denoted with asterisks: ** $p < 0.05$; *** $p < 0.001$. Missing values of juveniles for CO and ADJ due to technical problems. Means ± SD, $n = 5$.

Cocoon production was significantly affected by substances and temperature, with a significant substance × temperature interaction (Figure 3b, Table 4). Post-hoc comparisons showed significant differences between substances only at high temperature (Figure 3b). Cocoon production was highest under the coformulant ADJ, with significant differences from potassium salt and TQ. Only the formulation RU was significantly different to its AI potassium salt (Figure 3b).

Juvenile numbers were significantly affected by substances and temperature, with a significant substance x temperature interaction (Figure 3c, Table 4). Post-hoc comparisons at temperature levels revealed no significant differences (Figure 3c).

4. Discussion

In this study, we attempted to provide a more realistic environmental risk assessment of two widely used glyphosate herbicide formulations (Roundup PowerFlex, Touchdown Quattro), their active ingredients (potassium salt, ammonium salt), and a common coformulant (alkylpolyglcoside) on earthworm behavior, growth, and reproduction by extending ecotoxicological standard tests with two temperature and two soil organic matter levels. Earthworms were affected either by GBHs or GLYs, but there was no general pattern that GBHs had a stronger effect than its corresponding GLYs. The most surprising result was a significant effect of the "inert" coformulant APG on earthworms. The interactions between the impact of GBH or AIs and soil organic matter or temperature suggest that these environmental parameters should no longer be ignored in standard environmental risk assessments.

4.1. Avoidance Behavior

Avoidance behavior varied considerably but was not significantly affected by substances or temperature. However, avoidance was significantly lower at high SOM across all substances and temperatures.

Since avoidance tests are common in environmental risk assessments for pesticide registration, it was not surprising that we did not find strong effects. While we did not find differential effects between GBHs and their respective AIs, others [41] found higher avoidance of *E. andrei* to GLY compared to GBH (herbicide Pica Pau 480 SC, 480 g a.i. l^{-1}). However, the dose applied in the previous study was >10 times higher (30 mg AI kg^{-1}) than in our study. Three other studies with GBH at the recommended field dose yielded conflicting results. A meta-analysis of 25 ecotoxicological studies comparing earthworm response to GLY vs. GBH revealed very different results, even for the same endpoints [29]. In the meta-analysis, the contrasting results were attributed to several factors, including the use of different earthworm species (epigenetic and anecic species), different glyphosate formulations (consisting of different coformulants), different soil temperatures, and different soil types [29].

We observed a lower avoidance of the substances by the earthworms at high SOM levels. It seems that the higher sorption of glyphosate in soils with higher SOM content [30] influences the behavior of earthworms towards the test substances. Independent of SOM content, others researchers also found differences in earthworm (*Eisenia andrei)* avoidance between two herbicide AIs (sulcotrione and penoxsulam) and their respective commercial formulations (Mikado and Viper) [27]. Further experiments would be needed to better understand the interactions between SOM, sorption processes, and soil biota response.

Several factors could lead to interactive effects of SOM on the toxicity of substances. First, higher SOM leads to higher soil moisture and better living conditions for earthworms, which could then better cope with harmful substances [42]. Second, higher SOM could lead to improved microbial degradation of glyphosate [23] or higher microbial biomass which provides an additional food source for earthworms [24,43]. Third, soils with a higher SOM also have higher p and K contents as inherent properties that could adsorb GLY and make them less available to earthworms [30,44]. Fourth, GLY has been shown to sorb to SOM and earthworms might not readily get in contact with GLY at high SOM contents [30,45]. Additional soil parameters need to be studied to clarify the influence of each soil property [15]. Further studies are needed to determine whether higher tolerance of GBHs and GLYs at high SOM would also reduce earthworm hazards in the long-term, or whether prolonged contact with these substances would result in greater harm to earthworms.

4.2. Growth and Reproduction

Studies comparing GBHs vs. AIs are scarce and reach different conclusions. Only one study compared GBH with GLY on *E. fetida* in a similar setting and comes to similar results [29]. Worms living in soil contaminated with AI isopropylamine salt lost biomass and survived a stress test for a shorter time than worms in control groups. In contrast, worms living in soil contaminated with GBH (Roundup Ready-to-Use III, Roundup Super Concentrate) did not lose biomass and survived the stress test just like the worms in the control group. However, it should be noted that dosages in this experiment (26.3 mg kg^{-1}) were almost nine times higher than in the current study. The authors suggest that the nitrates and phosphates in the formulations stimulate microbial activity, thereby accelerating glyphosate degradation [43].

In our experiment, earthworm growth and reproduction in response to different herbicides were tested at two temperature levels without distinguishing between SOM. Biomass was significantly affected by substance and temperature (no interaction). Temperature had a stronger effect on biomass than substances with significantly higher growth at higher temperature, but with only a marginally significant interaction between substance and temperature. Earthworm biomass was similar for GBHs and GLYs, suggesting that coformulants added to GLYs in GBHs had no adverse effects on biomass. To our knowledge, only one study [29] reported effects of GBHs vs. GLYs and temperature on earthworms. In another study, *E. fetida* exposed to a GBH (Roundup Ready-To-Use III; isopropylamine salt as active ingredient) did not impact body mass at about twice the recommended dose (60.7 mg AI kg^{-1}) [46]. The earlier study found that both earthworm responses varied with the initial body mass and soil temperature [29]; only initially heavy worms growing up in warm soil responded to GBH, and they responded by becoming significantly heavier than their uncontaminated counterparts; in contrast, worms with lighter mass and worms growing up in a cooler temperature did not respond to contamination with a change in final body mass.

In general, for all samples, the number of juveniles hatched per cocoon ranged from 2–6, which is common for this species [47]. We found significantly fewer cocoons under potassium salt compared to its GBH Roundup PowerFlex. This is in line with others who also report fewer cocoons at a GLY compared to GBH (95% AI; GLY not further specified) although much higher dosages of $\geq$5000 mg kg^{-1} were used [48]. No difference in cocoon production was found between GBHs and AIs (95% GLY not further specified) at concentrations of 200 mg GLY kg^{-1} [49].

We noted a trend for glyphosate salts to develop fewer juveniles than GBHs. Significantly fewer juveniles were found in *E. fetida* after GBH treatment (Roundup FG with 1440 g AI/ha) [50]. No effect on the reproduction rate was found when testing GBH Glycel S.L (at 2 and 8 mg AI kg^{-1} soil) [51]. In contrast, increased cocoon production but decreased cocoon fertility was observed after GBH application (Roundup, 48%, 6 l GBH/ha and 12 l GBH/ha) [52].

It is also observed that *E. fetida* is less sensitive to pesticides than other earthworm species [53–55]. The discrepancy in results could be due to the unknown coformulants in the formulations [14]. This type of inconsistency is often reported in ecotoxicological experiments [29]. Several studies looking at terrestrial non-target animals show that GBH has an equal or higher toxicity than GLY [56,57]. In contrast, in several studies considering terrestrial non-target organisms, GBH has been observed to be more toxic than GLY, including soil bacteria [58], amphibians [59], collembolans [44].

4.3. Effects of Coformulants

The effects of coformulants have rarely been tested in invertebrates. A recent systematic literature review found that only 19 studies examined the effects of coformulants or ”inert” ingredients on bee health [12]. In these studies, ”inert” ingredients were found to cause mortality in bees through multiple exposure routes, act synergistically with other stressors, and cause colony-level effects.

When used on freshwater worms, GBH with the coformulant POEA (polyethoxylated tallow amine) shows higher cytotoxicity than GLY alone [60]. Even for plants, GLY was only slightly toxic at the recommended dilutions in agriculture, but the strong herbicidal and toxic properties of its formulations were exerted by the POEA formulant family alone [56]. In addition, the authors identified the heavy metals arsenic, chromium, cobalt, lead, and nickel, which are known to be toxic and endocrine disruptors, as contaminants in GBHs. This could also explain some of the adverse effects of GBHs. The effects of POEA and a GBH formulation (Roundup) on different test organisms proved POEA to be more acutely toxic to aquatic organisms [61]. POEA was also proven to be the most toxic component on aquatic invertebrates, compared to the effects of technical-grade glyphosate and the investigated GBH [62]. In another study, ethoxylated adjuvants used in GBH formulations proved to be nearly ten thousand times more toxic to human cells than the toxicity of the AI [63]. This finding has been reconfirmed in numerous additional studies [14,64,65]. In the meantime, POEA-based GBHs were banned in the EU and gradually phased out during the 2015–2017 period [66], but in most other regions POEA-based GBHs are still in use. The EFSA reference GBH formulation is Roundup BioFlow with a quarternary ammonium coformulant/surfactant, however, unfortunately there are no data on the proportion of GBHs containing APG. There is ample evidence that failure to consider the toxicity of coformulants distorts the safety profile of commercial herbicides and other pesticides [6,11,12,67]. Another important issue with indications of genotoxic and carcinogenic effects is the increasing use of GBHs containing additional herbicidal AIs such as 2,4-D, or dicamba [68], but a discussion of this is beyond the scope of this study.

We are aware of only a few studies addressing the effects of the coformulant APG. Testing the cytotoxic and endocrine effects of GLY or APG on human placental cells showed that APG is 18 times and GBH (Medallon Premium, 350 g GLY l^{-1}) 2000 times more cytotoxic than GLY only [14]. The coformulant APG was found to be more cytotoxic than the formulation GBH which contained APG; however, the formulation was more endocrine-disrupting than APG, and GLY alone had no significant effect [14]. We found increased cocoon production stimulated by APG, which might be explained by the hormesis effect, which exerts a positive effect on organisms at small doses of harmful substances [52,69]. A no-observed-effect concentration (NOEC) for APG of 654 mg kg^{-1} was determined for *E. fetida* [70], therefore, APG is considered only slightly toxic to earthworms and is readily biodegradable under all aerobic and anaerobic environmental conditions [70]. APG is also listed on the Safer Chemical Ingredients List (SCIL) of the US EPA based on the aquatic toxicity and rate of biodegradation [71]. Our results indicate that glyphosate AI may have a more negative effect on reproduction than the GBH formulation, because the adjuvant APG stimulates cocoon production.

The resulting effects of the adjuvant in combination with GLY may explain the inconsistencies of the results in the 25 studies compared by Pochron et al. 2019 [29], as they used different GBHs from different manufacturers. In addition, many studies do not report exact product names, making it impossible to determine the effects of coformulants. None of these studies consider the influence of soil type on the effect of GLYs/GBHs. There is no consensus on the type of soil used or what soil values have been reported. Moreover, some follow the OECD standards, while others use variants of the OECD standards [29]. These differences make it difficult to compare the studies.

However, pioneering studies from Pochron's laboratory suggest that even with a constant soil type (e.g., pH, soil moisture, and SOM), the microbial biomass may vary. They suggest that the microbial biomass may influence the health of earthworms [29]. An increase in microorganisms could serve as a food source for earthworms and/or promote the increase of available nutrients in the soil [24,29,72], which could have a positive effect on the offspring in the current study.

4.4. Effects of Temperature

Interactions between herbicide effects and climate variables get increasingly important with ongoing climate change [73]. In this study, temperature was shown to affect biomass, cocoon production, and the number of juveniles, but not avoidance behavior. Earthworms showed an 18% increase in biomass at low temperature as opposed to an increased temperature. However, a high temperature increased the number of cocoons and juveniles. Reproduction rate was found to interact with treatments and temperature, indicating a dependence.

Very little is known about the interactions between temperature and herbicide effects. Amphibians (*Bufo bufo*) have been shown to be more sensitive to GBH [74,75] at lower temperatures, this pattern was also confirmed for other amphibian species (*Rana temporaria* and *Bufotes viridis*) and the response to a fungicide formulation (Folpan 500 SC) [76]. Interactions between GBHs, their Ais, and temperature have rarely been studied for soil biota. Stress tests of the worms revealed the interaction of the herbicide Roundup Ready to Use III (26.3 mg kg^{-1} dry soil) and temperature [29]. Especially when the earthworms lived in heated soil, exposure to Roundup did not affect their survival time under stress. However, when the earthworms had lived in non-heated soil, Roundup exposure shortened their survival time.

Studies show that tolerance to high and low temperature can be reduced by chemical stressors [77]. The effects of two insecticides (chlorpyrifos and carbofuran) on survival, growth, and reproduction of *E. andrei* are greater at a standard temperature of 20 °C than at 26 °C [78]. In the risk assessment of environmental chemicals, tests are performed under standard conditions 20 ± 2 °C. Our results suggest that the substances interact with temperature, and it would therefore be important in further studies for the ERAs to include tests of the substances as a function of temperature.

5. Conclusions

In general, we found that either GBHs or GLYs affect earthworms, but could not identify a general pattern, and there was only one clear indication for cocoon numbers that a GLY had a stronger effect than its corresponding GBH. The most surprising result was that the coformulant APG showed stimulatory effects on earthworms, resulting in lower effects of GBH on cocoon numbers than pure AI. It was important to observe interactions between the impact of GBH or AIs with either soil organic matter or temperature because in the European Union, environmental risk assessments of pesticide approval typically test AIs and at least one lead formulation using one soil type at one standard temperature [79]. Looking at the literature on this topic, there is a wide variation in the effects of GBHs, GLYs, and coformulants on earthworms. These differences are due to the fact that ingredients in a given branded product may vary geographically and temporally without being indicated on product labels, in safety data sheets, or other publicly available information sources [10]. Therefore, GBHs are black boxes in terms of their complete ingredients [6]. Furthermore, in addition to the coformulants discussed in this study, petroleum and heavy metals were frequently found in formulations with even more toxic effects than the active ingredient itself [56,80]. Thus, our results suggest that current environmental risk assessments, which focus mainly on active ingredients or specific formulations and use standard temperatures and standard soils, most likely underestimate impacts on non-target organisms. To better understand the effects of herbicides on non-target organisms, a full declaration of all formulation ingredients, tank mixtures, and interactions with other agrochemicals applied [81] would be essential.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agrochemicals2010001/s1, Table S1 data avoidance.xlsx; Table S2 data biomass cocoons juveniles.xlsx.

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