Effects of glyphosate-based herbicides and their active ingredients on earthworms, water infiltration and glyphosate leaching are influenced by soil properties

Johann G. Zaller¹*, Maureen Weber¹, Michael Maderthaner¹, Edith Gruber¹, Eszter Takács², Mária Mörtl², Szandra Klátyik², János Győri³, Jörg Römbke⁴, Friedrich Leisch⁵, Bernhard Spangl⁶ and András Székács²

Abstract

Background: Glyphosate-based herbicides (GBHs) are among the most often used pesticides. The hundreds of GBHs used worldwide consist of the active ingredient (AI) glyphosate in form of different salts, possibly other AIs, and various mostly undisclosed co-formulants. Pesticide risk assessments are commonly performed using single AIs or GBHs at standard soil conditions without vegetation. In a greenhouse experiment, we established a weed population with common amaranth (Amaranthus retroflexus) to examine the effects of three GBHs (Roundup LB Plus, Roundup PowerFlex, Touchdown Quattro) and their corresponding AIs (salts of glyphosate isopropylammonium, potassium, diammonium) on the activity and physiological biomarkers (glutathione S-transferase, GST; acetylcholine esterase, AChE) of an ecologically relevant earthworm species (Lumbricus terrestris). GBHs and AIs were applied at recommended doses; hand weeding served as control. Experiments were established with two soil types differing in organic matter content (SOM; 3.0% vs. 4.1%) and other properties.

Results: Earthworm activity (casting and movement activity) decreased after application of glyphosate formulations or active ingredients compared to hand weeding. We found no consistent pattern that formulations had either higher or lower effects on earthworm activity than their active ingredients; rather, differences were substance-specific. Earthworm activity was little affected by soil organic matter levels. Biomarkers remained unaffected by weed control types; GST but not AChE was decreased under high SOM. Water infiltration after a simulated heavy rainfall was interactively affected by weed control types and SOM. Leachate amount was higher after application of formulations than active ingredients and was higher under low SOM. Glyphosate concentrations in soil and leachate were strongly affected by application of formulations or active ingredients and varied with SOM (significant weed control type x SOM interaction).

Conclusions: We found that both commercial formulations and pure active ingredients can influence earthworms with consequences on important soil functions. Glyphosate products showed increased, reduced or similar effects than pure glyphosate on particular soil functions; soil properties can substantially alter this. Especially at lower SOM, heavy rainfalls could lead to more glyphosate leaching into water bodies. A full disclosure of co-formulants would be necessary to further decipher their specific contributions to these inconsistent effects.
Keywords: Amaranthus retroflexus, Co-formulants, Ecosystem services, Herbicides, Humus content, Lumbricus terrestris, Non-target effects, Soil fauna, Soil functions, Weed control

Background
Glyphosate-based herbicides (GBHs) are the globally most widely used pesticides applied in many sectors of agriculture, forestry, landscape planning, municipalities, and in private gardens [1–3]. Several hundred GBHs are in use and it is estimated that about 825 million kilograms of the active ingredient (AI) glyphosate (N-phosphonomethyl-glycine) is globally used per year [2, 4]. It is rarely acknowledged that the AI glyphosate is not a single chemical but rather used in various salt forms with different chemical, physical and toxicological properties: e.g., as ammonium, dimethylammonium, isopropylammonium, potassium or trimesium salt of glyphosate [1, 5]. In order to formulate the commercial GBHs a multitude of co-formulants is added to these AIs for instance to improve the adhesion at the plant surface or to facilitate the intrusion into the target plant [1]. These co-formulants are considered inert although many AIs are only fully effective with these substances in the formulation.

Studies have shown that herbicide formulations can be differently effective against weeds [6] and non-target organisms than the mere AIs [7–12]. The proportion of co-formulants in GBH can reach up to 20% and GBHs toxic effects and endocrine disrupting properties were mostly due to the co-formulants even at concentrations much lower than used in practice [13, 14]. Therefore, in the European Union, environmental risk assessments for pesticide approval include the testing of AIs of potential side-effects on non-target species and of at least one lead formulation depending on its characteristic and usage according to standard guidelines [15, 16]. However, it is difficult to evaluate whether these risk assessments adequately assess non-target effects of the various products in use since the ingredients of commercial pesticides are considered as confidential business information and not declared.

After GBH application glyphosate residues remain in the soil with a half-life ranging from 2 to 215 days [17], depending on soil biological properties, the content of soil organic matter (SOM) and nutrients, or climatic factors [18–20]. Glyphosate residues (and its metabolites) can detrimentally influence crops, non-target plants and other organisms and the long half-life period is a concern [21]. In boreal soils 19% of the applied glyphosate was found even 20 months after the application; most glyphosate came into the soil via plant roots [22, 23]. Glyphosate and its metabolite aminomethylphosphonic acid (AMPA) are among the most commonly found pesticide residues in soils [24–26] and water bodies around the world [2, 17, 27–30]. In aquatic systems glyphosate residues can affect the growth of algae and development of amphibians [31–33].

Earthworms (Lumbricidae) constitute the majority of soil faunal biomass in many temperate agroecosystems with up to 1000 individuals and 300 g of biomass in each square metre of land [34, 35]. They modulate agroecosystem function by affecting nutrient cycling and decomposing organic material [36, 37], recovering soil carbon pools after disturbance [38], maintaining soil microbial diversity [39, 40], controlling plant pathogens [41–43], influencing water infiltration, and interacting with above ground organisms [44–47]. Thus, any herbicide-induced effect on earthworm activity will impact these ecosystem functions and influence water infiltration, and the binding and leaching of glyphosate [48].

Glyphosate has been shown to cause acute and chronic toxicological effects on a variety of animals [49]. Most studies on earthworms have been conducted using single glyphosate AIs or GBHs and only a few compared commercial GBHs with their respective AIs [50]. Earthworms of the species Eisenia fetida in soil contaminated with GBH residues (Roundup Ready-to-Use III, Roundup Super Concentrate) show less body mass decline and stress than those living in soil contaminated with the AI (glyphosate isopropylammonium salt) or in uncontaminated soil [51]. Others found that neither herbicide products (Spasor and Stam Novel Flo 480) nor their corresponding AIs (glyphosate and propanil) affected earthworm avoidance behaviour of E. andrei [52]. Other non glyphosate herbicides showed similar impacts on earthworms (E. andrei) for one product-AI pair (Mikado vs. AI sulcotrione), but higher toxicity of the other product-AI pair (Viper vs. AI penoxsulam) [53]. An excellent overview of earthworm studies dealing with GBHs and/or AIs provides Pochron et al. 2019 [54]; authors conclude that inconsistent results across studies most likely arise from variations in methodological approaches and the use of different earthworm species.

Besides behavioural measures, also biomarkers have been used to assess the effect of pesticides on earthworms. Changes in the activity of antioxidant enzymes including superoxide dismutase and catalase were used [55–57]. Membrane bound glutathione S-transferase (GST) activity, a major metabolic enzyme that contributes to the detoxification and neutralization of pollutants, was significantly elevated in earthworms (Lumbricus
variegatus) exposed to Roundup Ultra [55]. Elevated activities of GST were also observed in three earthworm species (Alma millsoni, Eudrilus eugeniae and Libyodrilus violaceus) exposed to Roundup Alphée while acetylcholine esterase (AChE), a neurotransmitter inactivating enzyme affecting neurotransmission and muscular activities, was found to be insensitive to this herbicide [58].

To the best of our knowledge, so far, no study compared impacts of several GBHs and their respective AIs on ecologically relevant earthworm species and associated ecosystem functions under different soil conditions. Therefore, we conducted a greenhouse experiment with a model weed population to assess (i) the effects of three widely used commercial GBHs and their pure AIs on the activity and physiology of the anecic earthworm species Lumbricus terrestris, (ii) whether different soil properties alter these responses, and (iii) the fate of glyphosate in soil and water samples after simulating a heavy rainfall event. We hypothesized that both GBHs and AIs affect earthworms, but that GBH-effects will be stronger because they contain co-formulants that can be toxic to earthworms [13]. We expected to be able to explain potential changes in earthworm activity and physiology by altered biomarker activity. We further hypothesized that GBH/AI-induced changes in earthworm activity will influence water infiltration and the absorption of glyphosate in soil and leachate. Due to a higher absorption of glyphosate onto soil organic matter we expected to find higher glyphosate concentrations in soils and consequently less glyphosate in leachate under higher SOM. Earthworm activity was examined over four weeks and effects were expected to decrease over time.

Materials and methods
Experimental design
The study was conducted as a pot experiment in a greenhouse of the University of Natural Resources and Life Sciences Vienna. Soil type was in both cases a calcic Chernozem [59] cultivated using common crop rotations following good agricultural practice. As a consequence of their historically different cultivation the two soil types did not only differ in their SOM, but also in their P and K contents, however for the sake of clarity we refer mainly as low and high SOM soils. Overall, these SOM levels also reflect the average situation in conventional and organic arable farms in the region. Low SOM soils had a SOM content of 3.0%, P = 73 mg kg$^{-1}$, K = 140 mg kg$^{-1}$, pH (CaCl$_2$) = 7.7; high SOM soils had a SOM content of 4.1%, P = 113 mg kg$^{-1}$, K = 234 mg kg$^{-1}$, pH (CaCl$_2$) = 7.7. All soil properties were determined according to standard methods: SOM following [60], P and K following [61], pH following [62].

Soil was thoroughly mixed, sieved (mesh size 1 cm) and equal amounts were filled in the respective pots. The experimental soil was not sterilized and contained original soil micro- and mesofauna. Arable soil with low SOM was treated with synthetic insecticides (AIs deltamethrin, pymetrozine) three years prior to soil sampling; no herbicides were applied on these fields for at least five years. Soils with high SOM were organically farmed for 25 years and not treated with synthetic insecticides or herbicides ever since.

Amaranthus retroflexus was used as a model weed population, because it is one of the most commonly occurring weeds in arable fields in the study area. To generate a plant cover, A. retroflexus was sown on 20 April 2018 in four rows about 6–7 cm apart from each other. Each pot received 0.3 g of seeds according to the sowing recommendations for A. retroflexus. Seeding material was provided by ATC—Austrian Technology Corporation GmbH. Pots were irrigated with 0.2 l pot$^{-1}$ of tap water on six days a week with a watering can.

As a bioindicator for possible non-target effects of GBH/AI applications we introduced three adult and ciliellated Lumbricus terrestris earthworm specimens to each pot (mean body mass 5.00 ± 1.22 g). Earthworms were purchased in a shop for fishing supplies in Vienna and added to the mesocosms on 12th May (22 days after seeding). To provide extra food for earthworms, chopped hay (0.5 g pot$^{-1}$) was added to all pots once a week. Most earthworm studies on herbicide effects have been conducted using epigeic compost worms (Eisenia species) [54, 63, 64] that is a surrogate species in environmental
### Table 1
Relevant ecological information of glyphosate-based herbicides (GBHs) or active ingredients (AIs) used in the current experiment according to safety data sheets (SDSs) of the manufacturers, and for AIs only according to the Pesticide Properties Database (PPDB; site.mhers.ac.uk/aeru/ppdb/en/index.htm)

| GBH       | AI                        | Conc. Al (g L\(^{-1}\)) | GBH tested on earthworms (spp.) | AI tested on earthworms (spp.) | Persistence and degradability | Mobility in soil | Known co-formulants                                                                 |
|-----------|---------------------------|--------------------------|---------------------------------|---------------------------------|------------------------------|------------------|-----------------------------------------------------------------------------------|
| Roundup LB Plus | Isopropyl-ammonium salt (Ipa) | 486                      | No info in SDS and PPDB         | No info in SDS and PPDB         | No info in SDS and PPDB | No info in SDS and PPDB | No info in SDS and PPDB                                                                 |
| Roundup Power Flex | Potassium salt (Po)       | 588                      | Yes (Eisenia fetida)            | No info in SDS and PPDB         | Half-life: 2–174 days       | Strong adsorption in SDS; no info in PPDB | <20% alkyl polyglycosides; <3% nitro-yl; >33% water & formulation additives in SDS; no info in PPDB |
| Touchdown Quattro | Diammonium salt (Am)      | 435                      | No info in SDS and PPDB         | No info in SDS and PPDB         | No info in SDS and PPDB     | Immobile in SDS; no info in PPDB | 10–20% D-glucopyranose, oligomeric, decyl octyl glycosides in SDS; no info in PPDB |

Ecotoxicological assessment for all three GBHs and AIs attest them acute aquatic toxicity.
risk assessments [65, 66] but commonly do not inhabit agroecosystems. Here, we wanted to test GBH/AI effects on L. terrestris, an anecic species, that indeed inhabits arable fields [41, 67].

**Weed control types**

The GBH/AI treatments were applied on 13th June 2018 (54 days after seeding) onto A. retroflexus that had an average plant height of 22 cm. The used GBHs (herbicide formulations) were: Roundup LB Plus (LB), Roundup PowerFlex (PF) (both products of Monsanto Agrar Deutschland GmbH) and Touchdown Quattro (TQ) (a product of Syngenta Agro GmbH), purchased at a local garden shop. Product information relevant for the current study according to the safety data sheets is provided in Table 1.

The corresponding AIs were salts of glyphosate: isopropylammonium salt (Ipa, AI of LB), potassium salt (Po, AI of PF) and diammonium salt (Am, AI of TQ). Glyphosate isopropylammonium salt was purchased from Toronto Research Chemicals (North York, Canada), while the potassium and diammonium salts were prepared and synthesized at the Agro-Environmental Research Centre, Institute of Environmental Sciences, Hungarian University of Agriculture and Life Sciences, Budapest, according to the procedures described previously [68]. The paste-like AIs were stirred in deionized water to make them applicable via a sprayer.

GBHs were applied once at recommended dosages just as they would be applied to kill weeds before sowing (psmregister.baes.gv.at): 5 l ha\(^{-1}\) for LB, 3.75 l ha\(^{-1}\) for PF and 5 l ha\(^{-1}\) for TQ. The dosages for the AI glyphosate salts were determined according to the concentration levels as given in the list of ingredients of the corresponding herbicide formulations. Different dosages recommended for the formulations resulted in a slightly different amount of glyphosate applied of 243, 221, 218 mg a.i. m\(^{-2}\) for LB, PF and TQ, respectively. These different dosages would also be applied in the field when recommendations are followed. Overall, we assumed that manufacturers gave their dosage recommendations in order to achieve the highest efficacy for their product while posing the least harm for the environment and it is therefore appropriate to compare the GBHs and AIs among each other despite differences in application rates.

All substances were sprayed on plant leaves using spray bottles with brass pump mist nozzles; we used separate spray bottles for each treatment. At the time of application plants in the pots covered the soil surface and direct spray on the soil was avoided. Most of the plants died within 5 days after spraying. In control treatments plants were hand weeded (pulled out) and left on the soil surface; afterwards tap water was applied in equal amounts as for the GBH/AI treatments.

**Measurements**

**Determination of earthworm activity and biomarkers (GST and AChE activity)**

Earthworm activity was assessed once a week by taking two measures of surface activity. First, casts deposited on the soil surface were collected during 4 weeks before and 4 weeks after GBH/AI application, counted, dried for 48 h at 50 °C and weighed. Earthworm activity was based on number and mass of casts produced per sampling event for the period before and after application of weed control type. Second, earthworm surface movement activity was assessed using the toothpick method [69]. Therefore, 10 wooden toothpicks mesocosm\(^{-1}\) were slightly inserted in a uniform pattern into the soil surface in a vertical position in the evening. Then, in the following morning the number of inclined or knocked-over toothpicks was counted. Activity was categorized according to the position of toothpicks: not disturbed (0 score), inclined (0.5 score), knocked-over (1 score). The category values were multiplied with the number of toothpicks in the respective category, summed up and used as a measure for earthworm surface activity.

Physiological reaction of earthworms to weed control treatments were assessed by measuring activities of the glutathione S-transferase (GST) and acetylcholine esterase (AChE) enzymes, as commonly used biomarkers. Chemicals used for the assays were obtained from Sigma-Aldrich Kft. (Budapest, Hungary): 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide, L-glutathione (reduced form), Bradford Reagent, bovine serum albumin (BSA), sodium hydrogen carbonate. All chemicals were of the highest commercially available grade.

At the end of the experiment (26 days after GBH/AI treatment) the survived adult and juvenile earthworms were collected by flipping over the pots; the collected earthworms were immediately frozen at −20 °C and delivered to Hungary, where the samples were kept at −80 °C until sample preparation. One adult earthworm from three randomly selected pots per treatment was weighed individually after the rinse and wiping process; thus three earthworms were used in enzymatic assays for GBHs and AIs. There were too few juvenile specimens to analyse. Weighed samples were added (1:2 w/v ratio) to ice cold sodium phosphate buffer (0.1 M, pH 7.2) and homogenized in ice using an T10 basic Ultra-Turrax (IKA – Werke GmbH & Co. KG, Staufen, Germany) at 15,000 rpm for 30 s, 20,000 rpm for 30 s and at 25,000 rpm for 30 s. 200 mg of homogenized sample was added to pre-cooled Eppendorf tubes with 800 µl ice cold
sodium phosphate buffer (0.1 M, pH 7.2) resulted in the recommended 1:10 w/v ratio [70, 71]. The homogenates were kept at −80 °C until usage, then were centrifuged for 30 min at 12,000 × g and 4 °C. 450 µl of the supernatants were transferred into pre-cooled LoBind microcentrifuge tubes and stored on ice until the measurement of enzyme activities. AChE and GST activities were measured in 3 replicates sample⁻¹. The activity of enzymes was calculated from the slope of the absorbance curve for 30 min at 12,000 × g until usage, then were centrifuged

At the end of the experiment (9th July 2018), we simulated a heavy rainfall event by pouring three litres of tap water (39.7 l m⁻²) on the surface of the experimental pots using a watering can with a sprinkler and measured the time needed for complete infiltration of the water. Water leaching out of the experimental pots was collected in pot saucers, volume measured and frozen at −20 °C until further analysis.

Degradation and accumulation of glyphosate were measured in soil samples, collected over the course of the experiment (22nd June, 4 July and 9 July 2018) using a soil core sampler (1 cm diameter, 5 cm depth), by HPLC analysis at the Agro-Environmental Research Centre in Budapest, Hungary. Prior to HPLC analysis the soil samples were prepared in five steps. First, 5 g air-dried soil was extracted (25 ml of 0.03 mol l⁻¹ phosphate, 0.01 mol l⁻¹ citrate buffer), by using 30 min ultrasound agitation and the phases were separated by 10 min centrifugation at 3000 rpm. Ten ml of the supernatant was derivatized in the second step by adding 0.9 ml of 130 mM borate buffer (pH=9) and 0.3 ml of 10 mM FMOC-Cl reagent. Solutions were homogenized by shaking and vortex, then kept for 2 h at room temperature in the dark. Third, removal of derivatizing agent was carried out by extraction of FMOC-Cl with 2 × 3 ml diethyl ether, 5 min centrifugation at 3000 rpm between extractions and removal of organic phase. Fourth, set pH=3 with HCl solution. Fifth, the aqueous phase was subjected to solid phase extraction (SPE) to concentrate the samples. Cartridges (Strata-X sorbent, 33 µm, 200 mg, 3 ml; Phenomenex) were conditioned (5 ml of MeOH, 5 ml of 2xd. H₂O, 5 ml of 50 mM phosphate buffer at pH 3), then samples were loaded followed by washing (3 ml of 2xd. H₂O, 2–3 min drying, and elution with 5 ml of MeOH. The eluates were evaporated to dryness and re-solved in 0.5 ml of the initial HPLC eluent.

Concentrations of glyphosate in leachate were determined at the Agro-Environmental Research Centre in Budapest, Hungary, by applying the HPLC method reported earlier [76]. Briefly, glyphosate was separated on a C18 column (Kinetex Core Shell, Phenomenex, 150 mm × 4.6 mm, i.d., 5 µm) at 40 °C and UV detector signals were recorded at λ = 260 nm. The eluent flow rate was 0.7 ml min⁻¹ with gradient elution. Initially, the eluent consisted of a 1:9 mixture of A:B eluents (A = 100% ACN, B = 10 mM aqueous sodium acetate buffer, pH=6) that was gradually increased to 90% A at 6 min and maintained for 3 min. Prior to HPLC analysis the water samples were prepared following the steps 2–5 as described above for the preparation of the soil samples, starting with 10 ml water samples.
At the end of the experiment (26 days after applying the treatments), *A. retroflexus* biomass in the pots was separated in dead and green biomass; biomass was dried at 55 °C for 5 days and weighed afterwards.

**Statistical analyses**

Statistical analyses were conducted with the software R version 4.0.3 (The R Foundation for Statistical Computing; http://www.R-project.org) using the packages car [77] and multcomp [78]. Our statistical approach was to first perform analyses testing effects of weed control types (GBHs, AIs, hand weeding) and secondly to perform more detailed analyses among individual substances in order to assess differences between individual GBHs and their AIs. When the main treatment effects were significant, post-hoc comparisons were performed. We used type II tests for the analysis of variance or deviance to cope for possible imbalances.

Pre-treatment period: to examine possible differences in experimental units prior to application of weed control types effects on cast numbers and surface activity over a period of 4 weeks were tested using a generalized linear model (GLM) and analysis of deviance with quasi-Poisson distribution with the factors weed control type (3 levels: GBHs, AIs and control) and SOM (2 levels: 3.0% vs. 4.1% SOM) and their interactions; effects on cast mass production were analysed using a linear model (LM) and ANOVAs with Gaussian distribution using the same factors. Time, soil moisture, temperature and electrical conductivity were included as covariates in both models. Multiple comparisons (Tukey) were performed when main effects were significant (p < 0.05).

Treatment period (4 weeks): analyses were performed in a similar manner as described above. First, analyses (GLMs or LMs) considered weed control type (GBHs, AIs, control), SOM and their interactions; secondly more detailed analyses considering individual substances were performed. Time was included as covariate for earthworm activity parameters (4 sampling dates) and glyphosate in soil (3 sampling dates). Additional covariates included in the statistical models were soil moisture, temperature and conductivity. Treatment effects of one-time measured parameters AChE, GST, infiltration time, leachate amount and glyphosate in water were analysed using LMs and ANOVAs; for glyphosate in soil, time was additionally used as covariate to account for the three measurement dates. For both analyses post-hoc comparisons (Tukey) were made as simultaneous tests for general linear hypotheses but only performed when main effects were significant (p < 0.05).

**Results**

**Pre-treatment conditions and efficacy of weed control**

Earthworm activity (number and mass of casts, surface movement) in the period before application of weed control types was similar in experimental units that were later assigned to particular treatments and not influenced by SOM levels (Table 2). Cast mass produced and surface activity but not cast numbers during the pre-treatment period varied with time; cast numbers and cast mass were positively influenced by the covariate soil temperature.

At the end of the experiment, aboveground plant material was collected. Proportion of green biomass was significantly influenced by weed control types but not by SOM levels. Across SOM, hand weeding showed the highest efficacy for weed control at a dry mass basis with 2±6% green biomass remaining at the end of the

| Factors                          | Mean cast numbers (no. pot⁻¹) | Mean cast mass (g pot⁻¹) | Mean surface activity (toothpick index) |
|----------------------------------|-------------------------------|--------------------------|----------------------------------------|
|                                  | Df   | LR X² | Pr (> X²) | Df   | F value | Pr (> F) | Df   | LR X² | Pr (> X²) |
| Weed control type               | 2    | 1.021 | 0.600    | 2    | 1.429   | 0.241    | 2    | 0.264 | 0.876     |
| SOM                             | 1    | 0.138 | 0.710    | 1    | 0.089   | 0.766    | 1    | 0.357 | 0.550     |
| Covar. time                     | 1    | 0.215 | 0.643    | 1    | 24.727  | <0.001   | 1    | 7.690 | 0.006     |
| Covar. soil moist. (%)          | 1    | 0.092 | 0.761    | 1    | 0.953   | 0.330    | 1    | 0.213 | 0.644     |
| Covar. soil temp. (°C)          | 1    | 17.163 | <0.001  | 1    | 25.075  | <0.001   | 1    | 0.688 | 0.407     |
| Covar. soil cond. (mS m⁻¹)      | 1    | 0.198 | 0.657    | 1    | 0.649   | 0.421    | 1    | 0.592 | 0.442     |
| WCT x SOM                       | 2    | 0.092 | 0.955    | 2    | 0.311   | 0.733    | 2    | 0.133 | 0.936     |
| Residuals                       | NA   | 261   | NA       | NA   | NA      | NA       |

GLMs/analysis of deviance was used for cast numbers and surface activity, LM/ANOVA for cast mass; time period (4 weeks), soil moisture, temperature and electrical conductivity was used as covariates. Significant treatment effects are indicated in bold. NA not applicable. No multiple comparisons were made because of lacking main effects.
experiment, while $32\pm 13\%$ green mass was found after GBH treatment and $51\pm 25\%$ after AIs. More details on the efficacy of weed control types on plants are reported elsewhere [68].

**Treatment effects on earthworm activity**

Cast numbers and surface movement activity was affected by weed control types (GBHs, AIs, control), but not by SOM levels (Table 3; Fig. 1). Cast numbers were significantly lower after GBH or AI application than after hand weeding; GBHs and AIs showed similar effects on cast numbers (Table 3). Surface movement activity was significantly lower after GBH or AI application than after hand weeding; effects of GBH and AI were similar (Table 3). Cast mass was unaffected by weed control types or SOM.

Comparing effects of individual treatments it was seen that cast numbers (Fig. 1a, b) and surface movement (Fig. 1e, f) were significantly affected by weed control types while cast mass remained unaffected (Fig. 1c, d). SOM level had no effect on individual measures, however, cast numbers were significantly affected by a weed control type x SOM interaction. Post-hoc comparisons between GBH-AI pairs revealed no significant results.

**Biomarkers GST and AChE in earthworms**

Activity of biomarkers in earthworm tissue was unaffected by weed control types (Table 4). However, GST across weed control types was significantly lower under higher SOM levels; AChE was unaffected by SOM (Table 4).

**Water infiltration and leachate**

Water infiltration was significantly affected by weed control types, SOM and their interactions (Table 5; Fig. 2). Comparisons between weed control types showed that water infiltration was significantly lower after GBH or AI application than after hand weeding, but there was no difference between GBHs and respective AIs (Table 5; Fig. 2a, b). Multiple comparisons of GBH-AI pairs showed no significant difference for water infiltration but a significant weed control type x SOM interaction with less infiltration under high SOM (Fig. 2a, b).

Leachate amount was also significantly affected by weed control types and SOM, but without an interactive effect (Table 5; Fig. 2). Comparisons between weed control types showed a significant difference between GBHs and AIs but not between GBH or AI and control (Table 5). Multiple comparisons of individual GBH-AI pairs showed no effects (Fig. 2c, d).

**Glyphosate concentration in soil and leachate**

Both glyphosate concentrations in soil (Fig. 3a, b) and leachate (Fig. 3c, d) were highly significantly affected by weed control types and their interactions (Table 5). Only glyphosate in water was additionally affected by SOM.

### Table 3  
**After-treatment period: earthworm activity in response to weed control types (WCT: glyphosate-based herbicides GBH, active ingredients AI, or hand weeding as control) and soil organic matter levels (SOM). GLM/analysis of deviance was used for cast numbers and surface activity, LM/ANOVA for cast mass; time period (4 weeks), soil moisture, temperature and electrical conductivity was used as covariates**

| Factors          | Mean cast numbers (no. pot$^{-1}$) | Mean cast mass (g pot$^{-1}$) | Mean surface activity (toothpick index) |
|------------------|-----------------------------------|-------------------------------|----------------------------------------|
|                  | DF  | LR $\chi^2$  | Pr ($\chi^2$) | DF  | F value  | Pr (>$F$) | DF  | LR $\chi^2$  | Pr ($\chi^2$) |
| Weed control type| 2   | 7.558  | 0.023  | 2   | 2.213  | 0.120  | 2   | 11.533  | 0.003  |
| SOM              | 1   | 0.421  | 0.517  | 1   | 0.000  | 0.996  | 1   | 0.667  | 0.414  |
| Covar time       | 1   | 1.075  | 0.300  | 1   | 0.004  | 0.952  | 1   | 0.021  | 0.638  |
| Covar soil moist. (%) | 1   | 0.391  | 0.501  | 1   | 0.000  | 0.986  | 1   | 0.023  |
| Covar soil temp. (°C) | 1   | 0.052  | 0.819  | 1   | 0.000  | 0.998  | 1   | 0.417  | 0.627  |
| Covar soil cond. (mS m$^{-1}$) | 1   | 0.054  | 0.816  | 1   | 0.156  | 0.694  | 1   | 0.079  | 0.387  |
| WCT x SOM        | 2   | 0.905  | 0.636  | 2   | 1.042  | 0.360  | 2   | 1.281  | 0.527  |
| Residuals        | NA  | 261    | NA    |

Multiple comparisons of means (Tukey)

| AIs – control   | $-2.188$  | 0.073  | NA   | NA   | $-2.822$  | 0.013  |
| GBH – control   | $-2.838$  | 0.018  | NA   | NA   | $-3.498$  | 0.001  |
| GBH–AI          | $-0.688$  | 0.771  | NA   | NA   | $-0.693$  | 0.768  |

Significant treatment effects are indicated in bold. No multiple comparisons were performed for cast mass because of lacking main effects

NA not applicable
...is important for more realistic ecotoxicological assessments [48, 69, 79]. We found a decreased surface activity of earthworms after GBHs or AIs compared to hand weeding but no influence of SOM. Earthworm activity was similarly affected by GBHs and AIs and weed control types had no influence on biomarkers. Both leachate and glyphosate concentrations in water were significantly higher under GBHs than under AIs. SOM levels significantly affected GST biomarker activity in earthworms, water infiltration and leaching as well as glyphosate concentrations in water. Of particular importance were interactions between weed control types and SOM because they indicate that effects are soil-type specificity, an aspect that is commonly not considered in environmental risk assessments. Below, these effects will be discussed in more details.

**Effects on earthworms**

We expected to see different effects between GBHs and AIs because additional co-formulants in GBHs might have detrimental effects on earthworms [13]. However, our findings showed no consistent difference between effects on earthworm activity of the group of GBHs compared to the group of AIs. Also, we could not confirm findings that glyphosate (isopropylamine salt) is more harmful than the respective GBHs (Roundup Ready-to-Use III, Roundup Super Concentrate) [51]. In our experiment all treatments left dead plant material as food on the soil surface. Thus, we explain a reduced earthworm activity after GBH or AI application compared to hand weeding as an avoidance of glyphosate contaminated leaf material on the soil surface. Further, this difference might be due to (i) known detrimental effects of GBHs and AIs on earthworm activity [48, 51, 54, 69, 80, 81], (ii) a slightly higher soil moisture in control pots as a result of a higher weed control efficacy of hand weeding compared to GBH/AI applications and thus lower plant transpiration [48, 82]. Another explanation might be that dying roots after GBH/AI application provided a food source for earthworms belowground which shifted their activity from aboveground to belowground [83]. Earthworm activity was assessed over four weeks but we found no indication that effects would decrease with time. Others have shown that earthworms (*E. fetida*) can recover from effects of GBHs (Roundup Ready-to-Use III) after three weeks [84].

Although earthworm activity (cast numbers, surface activity) was significantly affected by GBHs and AIs, multiple comparisons of individual GBHs and AIs showed no significant differences. This is mainly due to complex interactions with SOM levels indicating that impacts of GBHs and AIs depend on soil properties. Effects of GBHs were in some cases higher, in others lower than effects of the respective AIs. The only

with higher concentrations under low SOM. Comparisons between weed control types showed significantly more glyphosate in soil and water under GBH than control, significantly higher glyphosate in soil under AI than control and significantly more glyphosate in water under GBHs than AIs (Table 5). Multiple comparisons of individual treatments showed significantly higher glyphosate concentrations in water under LB compared to its AI Ipa and between TQ and its AI Am at low SOM (Fig. 3c), while all other GBH-AI pairs were similar (Fig. 3a–c).

**Discussion**

We studied the impacts of three commercial glyphosate formulations and their respective pure glyphosate active ingredients on earthworms and soil parameters under two soil organic matter levels. Understanding such interactions is important for more realistic ecotoxicological

**Fig. 1** Mean surface cast numbers (a, b), cast mass (c, d) and surface movement activity (e, f) in response to hand weeding (CO), or chemical weed control with glyphosate-based herbicides (LB—Roundup LB, PF—Roundup PowerFlex, TQ—Touchdown Quattro) or their respective active ingredients (Ipa—isopropylammonium salt, Po—potassium salt, Am—diammonium salt) under low (a, c, e) and high (b, d, f) soil organic matter (SOM) levels. Statistical results from analyses of deviance and ANOVAs on individual treatments are given below panels; subsequent Tukey tests compared GBH-AI pairs. Significant results in bold; ns not significantly different (p > 0.05). Means ± SE across a 4-week sampling period, n = 5

assessments [48, 69, 79]. We found a decreased surface activity of earthworms after GBHs or AIs compared to hand weeding but no influence of SOM. Earthworm activity was similarly affected by GBHs and AIs and weed control types had no influence on biomarkers. Both leachate and glyphosate concentrations in water were significantly higher under GBHs than under AIs. SOM levels significantly affected GST biomarker activity in earthworms, water infiltration and leaching as well as glyphosate concentrations in water. Of particular importance were interactions between weed control types and SOM because they indicate that effects are soil-type specificity, an aspect that is commonly not considered in environmental risk assessments. Below, these effects will be discussed in more details.

**Effects on earthworms**

We expected to see different effects between GBHs and AIs because additional co-formulants in GBHs might have detrimental effects on earthworms [13]. However, our findings showed no consistent difference between effects on earthworm activity of the group of GBHs compared to the group of AIs. Also, we could not confirm findings that glyphosate (isopropylamine salt) is more harmful than the respective GBHs (Roundup Ready-to-Use III, Roundup Super Concentrate) [51]. In our experiment all treatments left dead plant material as food on the soil surface. Thus, we explain a reduced earthworm activity after GBH or AI application compared to hand weeding as an avoidance of glyphosate contaminated leaf material on the soil surface. Further, this difference might be due to (i) known detrimental effects of GBHs and AIs on earthworm activity [48, 51, 54, 69, 80, 81], (ii) a slightly higher soil moisture in control pots as a result of a higher weed control efficacy of hand weeding compared to GBH/AI applications and thus lower plant transpiration [48, 82]. Another explanation might be that dying roots after GBH/AI application provided a food source for earthworms belowground which shifted their activity from aboveground to belowground [83]. Earthworm activity was assessed over four weeks but we found no indication that effects would decrease with time. Others have shown that earthworms (*E. fetida*) can recover from effects of GBHs (Roundup Ready-to-Use III) after three weeks [84].

Although earthworm activity (cast numbers, surface activity) was significantly affected by GBHs and AIs, multiple comparisons of individual GBHs and AIs showed no significant differences. This is mainly due to complex interactions with SOM levels indicating that impacts of GBHs and AIs depend on soil properties. Effects of GBHs were in some cases higher, in others lower than effects of the respective AIs. The only

**Fig. 1** Mean surface cast numbers (a, b), cast mass (c, d) and surface movement activity (e, f) in response to hand weeding (CO), or chemical weed control with glyphosate-based herbicides (LB—Roundup LB, PF—Roundup PowerFlex, TQ—Touchdown Quattro) or their respective active ingredients (Ipa—isopropylammonium salt, Po—potassium salt, Am—diammonium salt) under low (a, c, e) and high (b, d, f) soil organic matter (SOM) levels. Statistical results from analyses of deviance and ANOVAs on individual treatments are given below panels; subsequent Tukey tests compared GBH-AI pairs. Significant results in bold; ns not significantly different (p > 0.05). Means ± SE across a 4-week sampling period, n = 5

with higher concentrations under low SOM. Comparisons between weed control types showed significantly more glyphosate in soil and water under GBH than control, significantly higher glyphosate in soil under AI than control and significantly more glyphosate in water under GBHs than AIs (Table 5). Multiple comparisons of individual treatments showed significantly higher glyphosate concentrations in water under LB compared to its AI Ipa and between TQ and its AI Am at low SOM (Fig. 3c), while all other GBH-AI pairs were similar (Fig. 3a–c).

**Discussion**

We studied the impacts of three commercial glyphosate formulations and their respective pure glyphosate active ingredients on earthworms and soil parameters under two soil organic matter levels. Understanding such interactions is important for more realistic ecotoxicological assessments [48, 69, 79]. We found a decreased surface activity of earthworms after GBHs or AIs compared to hand weeding but no influence of SOM. Earthworm activity was similarly affected by GBHs and AIs and weed control types had no influence on biomarkers. Both leachate and glyphosate concentrations in water were significantly higher under GBHs than under AIs. SOM levels significantly affected GST biomarker activity in earthworms, water infiltration and leaching as well as glyphosate concentrations in water. Of particular importance were interactions between weed control types and SOM because they indicate that effects are soil-type specificity, an aspect that is commonly not considered in environmental risk assessments. Below, these effects will be discussed in more details.

**Effects on earthworms**

We expected to see different effects between GBHs and AIs because additional co-formulants in GBHs might have detrimental effects on earthworms [13]. However, our findings showed no consistent difference between effects on earthworm activity of the group of GBHs compared to the group of AIs. Also, we could not confirm findings that glyphosate (isopropylamine salt) is more harmful than the respective GBHs (Roundup Ready-to-Use III, Roundup Super Concentrate) [51]. In our experiment all treatments left dead plant material as food on the soil surface. Thus, we explain a reduced earthworm activity after GBH or AI application compared to hand weeding as an avoidance of glyphosate contaminated leaf material on the soil surface. Further, this difference might be due to (i) known detrimental effects of GBHs and AIs on earthworm activity [48, 51, 54, 69, 80, 81], (ii) a slightly higher soil moisture in control pots as a result of a higher weed control efficacy of hand weeding compared to GBH/AI applications and thus lower plant transpiration [48, 82]. Another explanation might be that dying roots after GBH/AI application provided a food source for earthworms belowground which shifted their activity from aboveground to belowground [83]. Earthworm activity was assessed over four weeks but we found no indication that effects would decrease with time. Others have shown that earthworms (*E. fetida*) can recover from effects of GBHs (Roundup Ready-to-Use III) after three weeks [84].

Although earthworm activity (cast numbers, surface activity) was significantly affected by GBHs and AIs, multiple comparisons of individual GBHs and AIs showed no significant differences. This is mainly due to complex interactions with SOM levels indicating that impacts of GBHs and AIs depend on soil properties. Effects of GBHs were in some cases higher, in others lower than effects of the respective AIs. The only
consistent pattern across GBHs and AIs was that after GBHs/AIs application earthworm activity was in the majority of cases lower and never higher than after hand weeding. Our observations also confirm earlier findings that different agrochemicals affect different parameters in earthworms [85].

In contrast to our current findings are results from other experiments, where no short-term effects of GBHs on the activity (and growth) of *L. terrestris* was observed, e.g., for the product Rodeo XL with 360 g l$^{-1}$ [86] or for Roundup Power Flex with 588 g l$^{-1}$ both with potassium salt as AIs [87]. However, it was also shown that GBHs (Roundup Ready-to-Use III, Roundup Super Concentrate) were actually less harmful for earthworms (*E. fetida*) than pure isopropylamine glyphosate salt [51]. Unfortunately, a further exploration of underlying mechanisms is precluded by the concealment of the complete list of ingredients of the studied GBHs. Studies show that co-formulants in GBHs were cytotoxic and endocrine disrupting well below the doses used in agriculture [14]. It is suggested that glyphosate is only slightly toxic to plants at the recommended doses in agriculture and most of the phytotoxic effect of GBHs come from the co-formulants including the heavy metals arsenic, chromium, cobalt, lead and nickel which are known to be endocrine disruptors and also toxic to animals [13]. A lower phytotoxicity of the AIs compared to the GBHs could also be confirmed in a previous study using the same setting as in the current one [68].

These inconsistent results across studies are difficult to interpret but most likely arise from variations in tested application rates, differences in compared formulations and glyphosate salts, different assessments of earthworm activity and different earthworm species investigated [54]. Our current findings showed that additionally soil properties play an important interactive role. Moreover, earthworm responses to GBH or AI can also vary with intrinsic worm characteristics such as the initial body mass and that their stress reaction against herbicides can be higher at cooler temperatures [54]. In our study surface movement activity was affected by soil temperature (as covariate) with decreased activity with increased temperature. However, when comparing these findings it is important to note that different parameters were assessed and the former study [54] was conducted with a different earthworm species (*E. fetida*) that is most likely differently susceptible to herbicides and temperature than *L. terrestris* in our experiment [88]. In previous studies we also found species-specific responses of earthworms to herbicides. In one study the activity of the anecic *L. terrestris* was reduced after GBH application while the soil-dwelling species *A. caliginosa* remained almost unaffected [48]. In field studies a lack of earthworm response to GBH treatment might also be the result of only those species or individuals remaining that were adapted to year-long pesticide applications [87, 89]. Another issue are legacy effects of previous herbicide treatments that might have interfered with actual applications. However, this is unlikely in our study as low SOM soils did

| Weed control type | SOM level | AChE activity µMol min$^{-1}$ mg$^{-1}$ | GST activity µMol min$^{-1}$ mg$^{-1}$ |
|-------------------|-----------|----------------------------------------|---------------------------------------|
| GBH               | Low       | 523.54 ± 98.48                         | 463.27 ± 115.13                       |
| AI                |           | 565.59 ± 95.99                         | 469.79 ± 69.67                        |
| Control           |           | 603.37 ± 159.65                        | 446.99 ± 74.24                        |
| GBH               | High      | 521.79 ± 127.42                        | 366.87 ± 105.26                       |
| AI                |           | 463.18 ± 86.69                         | 396.90 ± 127.49                       |
| Control           |           | 499.76 ± 9.09                          | 398.99 ± 50.48                        |

ANOVA results

| Factors          | AChE activity | GST activity |
|------------------|---------------|--------------|
|                  | Df  | F value | Pr (>F) | Df | F value | Pr (>F) |
| Weed control type| 2   | 0.385   | 0.683    | 2  | 0.152   | 0.860   |
| SOM              | 1   | 3.071   | 0.088    | 1  | 6.209   | 0.017   |
| WCT × SOM        | 2   | 1.115   | 0.339    | 2  | 0.132   | 0.877   |
| Residuals        | 36  |         |          | 36 |         |         |

Means ± SD; n = 15 for GBH and AI; n = 5 for control. Significant results in bold. No multiple comparisons were made because of lacking main effects.
not receive GBH treatments at least in the previous three years and high SOM soils were under organic cultivation for 25 years without any herbicide application ever since. Glyphosate concentrations in control soils of our current experiment were below detection levels.

Besides earthworms, comparisons between GBHs and their AIs have rarely been studied on other soil animals [49]. A study testing the same GBHs and AIs on springtails (soil mesofauna) shows increased surface activity of springtails both under GBHs and AIs compared to hand weeding with a higher stimulation of springtail activity under higher SOM levels [68]. Another study found only minor effects of GBHs (Roundup Gold, 450 g l$^{-1}$ potassium salt) on enchytraeids and nematodes [90]. The lack of response of the biomarkers GST and AChE to GBH or AI is confirmed by findings of others [54–57]. However, because we analysed GST and AChE only 26 days after GBH/AI application we might have missed the strongest biochemical response. Others indeed found an inhibition of GST in the endogeic earthworm *Octolasion cyaneum* 28 days after a commercial GBH (Atanor SCA; AI potassium salt) was applied [56]. The lack of response in AChE is in accordance with the information in the safety data sheets of Roundup LB Plus and Roundup Power Flex mentioning that the products are not AChE inhibitors. Our findings suggested that Touchdown Quattro also does not appear to be an AChE inhibitor. However, more specific studies would be necessary to confirm this.

Comparing biomarker studies further confirms that different earthworm species respond differently to different GBHs: in the three earthworm species (*Alma millsoni*, *Eudrilus eugeniae* and *Libyodrilus violaceus*) higher activities of GST were observed after GBH (Roundup Alphée; isopropylammonium salt of glyphosate as AI) application while no response on AChE activity was seen [58]. Overall, the application of various pesticides have

### Table 5 ANOVA results testing effects of weed control types (WCT: glyphosate-based herbicides GBHs, their active ingredients AIs, or hand weeding as control) and soil organic matter level (SOM) on water infiltration rate and leachate, and glyphosate concentrations in soil and water

| Factors       | Measurement parameters | Water infiltration rate (mm min$^{-1}$) | Water leachate (l m$^{-2}$) |
|---------------|------------------------|----------------------------------------|----------------------------|
|               | Df | F value | Pr (> F) | Df | F value | Pr (> F) |
| Weed control type | 2  | 4.725   | 0.012    | 2  | 4.996   | 0.010    |
| SOM           | 1  | 45.991  | <0.001   | 1  | 28.072  | <0.001   |
| WCT x SOM     | 2  | 5.935   | 0.004    | 2  | 1.279   | 0.285    |
| Residuals     | 64 |         |          | 64 |         |          |

Multiple comparisons of means (Tukey)

| AI—Control  | 3.006 | 0.010 |
| GBH—Control | -2.680 | 0.024 |
| GBH—AI      | 0.461 | 0.888 |

#### Glyphosate conc. in soil (ng g$^{-1}$)

| Factors       | Measurement parameters | Glyphosate conc. in soil (ng g$^{-1}$) | Glyphosate conc. in water (ng g$^{-1}$) |
|---------------|------------------------|----------------------------------------|----------------------------------------|
|               | Df | F value | Pr (> F) | Df | F value | Pr (> F) |
| Weed control type | 2  | 5.910   | 0.003    | 2  | 11.317  | <0.001   |
| SOM           | 1  | 2.745   | 0.099    | 1  | 22.144  | <0.001   |
| Time          | 1  | 52.727  | <0.001   | NA | NA      | NA       |
| WCT x SOM     | 2  | 7.242   | 0.001    | 2  | 4.163   | 0.020    |
| Residuals     | 200 |         |          | 62 |         |          |

Multiple comparisons of means (Tukey)

| AI—Control  | -3.135 | 0.006 |
| GBH—Control | 3.317  | 0.003 |
| GBH – AI    | 0.269  | 0.960 |

#### Glyphosate conc. in water (ng g$^{-1}$)

See Figs. 2, 3 for illustrations of these effects. Significant treatment effects are in bold.

NA not applicable
It was interesting to see that water infiltration at lower SOM can be attributed to permanent and stable burrows that are important for worm species, such as Allolobophora chlorotica (anecic earthworm). Anecic earthworms are not plausible we believe that herbicide interactions in infiltration. As direct effects of GBH or AI on SOM are mediated by earthworms. Anecic earthworms have been shown to reduce AChE activities in earthworms (Allolobophora chlorotica) in apple orchards [91].

**Water infiltration, leaching and glyphosate in soil and leachate**

After simulating a heavy rainfall event with 40 mm, we found that the water infiltration rate and leachate amount were affected by both GBH/AI application and SOM. We interpret this as a consequence of GBH/AI effects on earthworm burrowing activity and consequences for soil hydrology [44, 48, 92, 93]. A higher water infiltration at lower SOM can be attributed to lower water-holding capacities in soils with less organic matter [94]. It was interesting to see that water infiltration under low SOM was twice as high than under high SOM (significant herbicide x SOM interaction) although SOM levels of the two soil types only differed by 1.1% (relative difference in SOM between soil types 36%). More detailed studies would be needed to examine whether there is a tipping point in SOM effects on infiltration. As direct effects of GBH or AI on SOM are not plausible we believe that herbicide interactions with SOM are mediated by earthworms. Anecic earthworm species, such as L. terrestris studied here, form permanent and stable burrows that are important for water infiltration and soil aeration [95] and earthworm activity has been shown to affect glyphosate leaching [69].

When heavy rainfalls become more prevalent under climate change [96] a higher infiltration would be beneficial after heavy rainfalls. However, a higher infiltration might also increase glyphosate leaching. Indeed, we found that both GBH and AI application strongly increased glyphosate concentrations in soil and leachate and both parameters were highly influenced by a herbicide x SOM interaction. Generally, after GBH application twice as high glyphosate concentrations were found in soils at high SOM than at low SOM. Contrastingly, after AI application lower glyphosate concentrations were found at high SOM than at low SOM. Sorption and degradation of glyphosate depends on various environmental conditions like microbial activity, temperature, soil moisture, pH and soil minerals [97], and in our experiment regular watering most likely increased the degradation of glyphosate in the soil [98, 99]. More detailed studies would be necessary to reveal the underlying mechanisms also regarding the interaction with soil biological activity and chemistry.
The current study focused only on responses to a one-time GBH/AI application and we cannot predict long-term consequences. Glyphosate is commonly considered to easily degrade with an estimated half-life of 2–215 days depending on soil types and environmental factors [17]. Others found that 19% of the applied amount of glyphosate was present in the topsoil even 20 months after its application [22,23]. These results indicate rather long persistence for glyphosate (at least in boreal soils) and that residues might affect earthworms over a longer time. To what extent such residues affect earthworm population dynamics or whether glyphosate is bioaccumulated or biomagnified by earthworms remains to be studied [58]. Our analyses were conducted 26 days after GBH/AI applications and indicate that substantial amounts of glyphosate are still available in soils especially under high SOM (82 ± 23 µg kg⁻¹ across GBHs, 41 ± 18 µg kg⁻¹ across AIs) and would thus be prone to leaching. Glyphosate residues have been found in topsoils across Europe and 45% of the soils contained > 50 µg kg⁻¹ [100]. In Argentina, due to intensive cultivation of glyphosate-tolerant genetically modified crops glyphosate was almost ubiquitous in soils with very high concentrations up to 8105 µg kg⁻¹ [101]. Experimental results show that 88% of the applied glyphosate was retained in the upper 0–9 cm surface soil layer, but that glyphosate residues were also found down to 1 m soil depth [102].

We also found considerable amounts of glyphosate in leachate (up to 66 µl l⁻¹ under low SOM) with interactive effects between weed control types and SOM. At low SOM glyphosate concentrations in leachate was 41% higher under GBHs but 76% lower under AIs compared to glyphosate concentrations in the soil. In contrast, at high SOM glyphosate concentrations in leachate was 87% lower under GBHs and 88% lower under AIs than glyphosate in the soil. This finding indicated that co-formulants in GBH might have influenced the mobility of AI in the soil–water interface. To elucidate the underlying mechanisms including biological and chemical interactions it would be necessary to know the exact ingredients of the GBHs. Studies confirm that glyphosate leaching is a problem. When rain falls shortly after GBH application, up to 47% of the applied glyphosate has been shown to be dissipated with surface run-off [103]. In a greenhouse pot study with field soil glyphosate was also easily leached with concentrations > 250 µg l⁻¹ in leachate [69]. In Argentina areas of intensive glyphosate use, maximum glyphosate concentrations in surface water were 1.80 µg l⁻¹ [101].

Conclusions

We found that both commercial glyphosate formulations and pure active ingredients can reduce earthworm activity compared to hand weeding, with cascading consequences for the fate of glyphosate in soil and leachate. Because water infiltration and glyphosate in soil and water were also interactively influenced by soil properties we suggest that relationships between formulations and active ingredients, earthworms and ecosystem functions are likely to be soil type specific. In order to fully understand the differential effects of formulations vs. their active ingredients a full disclosure of all ingredients in the formulations would be mandatory. Clearly, more investigations considering long-term effects at different trophic levels seem necessary for a more realistic evaluation of ecological side-effects of glyphosate herbicides.

Acknowledgements

We are grateful to the University of Natural Resources and Life Sciences Research Farm in Groß Enzersdorf for providing the soil, and to ATC Agro Trial Center-Gerhaus, Parndorf, Austria, for providing the A. retroflexus seed material. Suggestions and comments by anonymous reviewers helped to improve the manuscript.

Authors’ contributions

ASz, ET, EG, JGZ planned and conceptualized the study; MW, MM conducted the experiments; JR provided ecotoxicological expert knowledge; BS, FL, JGZ performed statistical analyses; ET, MM, SK, ASz, and JGy synthesized AIs and performed chemical and biochemical analyses; MW, JGZ wrote the first draft; JGZ made graphs; all authors reviewed the final manuscript.

Funding

This work was supported by a project (No. 970ui3) funded by the foundation action Austria–Hungary of the Osztrák-Magyar Akció Alapítvány (OMAA) and the Austrian Agency for International Cooperation in Education and Research (OED) granted to ASz and JGZ. The funding body had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials

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

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of the University of Natural Resources and Life Sciences, Vienna.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1 Institute of Zoology, University of Natural Resources and Life Sciences Vienna (BOKU), Gregor Mendel Straße 33, 1180 Vienna, Austria. 2 Agro-Environmental Research Centre, Institute of Environmental Sciences, Hungarian University of Agriculture and Life Sciences, Herman Öttö út 15, 1022 Budapest, Hungary. 3 Balaton Limnological Research Institute, Eötvös Loránd Research Network (ELKH), Klebelsberg Külnö utca 3, 8237 Tihany, Hungary. 4 ECT Ökotoksikologie GmbH, Böttgerstraße 2, 65439 Flörsheim, Germany. 5 Institute of Statistics, University of Natural Resources and Life Sciences Vienna (BOKU), Peter-Jordan-Straße 82, 1190 Vienna, Austria.
References

1. Szkács A, Darvas B (2012) Forty years with glyphosate. In: Hasanen N (ed) Herbicides—properties, synthesis and control of weeds. InTech, Rijeka, pp 247–284. https://doi.org/10.5772/32491

2. Szkács A, Darvas B (2018) Re-registration challenges of glyphosate in the European union. Front Environ Sci. https://doi.org/10.3389/fenvs.2018.00078

3. Zaller JG (2020) Daily poison. Pesticides—an underestimated danger. Springer Nature, Cham. https://doi.org/10.1007/978-3-030-50530-1

4. Benbrook CM (2016) Trends in glyphosate herbicide use in the United States and globally. Environ Sci Eur 28:3. https://doi.org/10.1186/s12302-016-0070-0

5. Cuhra M, Bahn T, Cuhra P (2016) Glyphosate: too much of a good thing? Front Environ Sci 4:28

6. Travlos I, Cheimona N, Bilalis D (2017) Glyphosate efficacy of different formulations and adjuvant additives on various weeds. Agronomy 7:60. https://doi.org/10.3390/agronomy7030060

7. Moore LJ, Fuentes L, Rodgers JH, Bowerman WW, Yarrow GK, Chao WY, Bridges WC (2013) Relative toxicity of the components of the original formulation of Roundup® to five North American anurans. Ecotoxicol Environ Saf 78:128–133. https://doi.org/10.1016/j.ecosaf.2011.11.025

8. Cuhra M, Bahn T (2016) Glyphosate: too much of a good thing? Front Environ Sci 4:28

9. Mullin CA, Fine JD, Reynolds RD, Frazier MT (2016) Toxicological risks of agrochemical spray adjuvants: organosilicone surfactants may not be safe. Front Public Health 4:92. https://doi.org/10.3389/fpubh.2016.00092

10. Mesnage R, Defarge N, Vendómosi Jsd, Séralini G-E (2014) Major pesticides are more toxic to human cells than their declared active substances. J Agric Food Chem 44:2442–2446. https://doi.org/10.1021/jf505062x

11. Singh S, Kumar V, Datta S, Wani AB, Dhanjal DS, Romero R, Singh J (2020) Glyphosate uptake, translocation, resistance emergence in crops, analytical monitoring, toxicity and degradation: a review. Env Chem Lett 18:663–702. https://doi.org/10.1007/s10311-020-00969-z

12. Laitinen P, Ramo S, Nikunen U, Jauhianen L, Siimes K, Turtola E (2009) Glyphosate and phosphorus leaching and residues in boreal sandy soil. Plant Soil 323:267–283

13. Laitinen P, Ramo S, Siimes K (2007) Glyphosate translocation from plants to soil—does this constitute a significant proportion of residues in soil? Plant Soil 300:51–60

14. Defarge N, Takács E, Lozano VL, Mesnage R, Vendômois JS, Seralini GE, Ci, Geissen V (2017) Distribution of glyphosate and aminomethylphosphonic acid (AMPA) in agricultural soils of the European Union. Sci Total Environ 621:1352–1359. https://doi.org/10.1016/j.scitotenv.2017.10.093

15. Silva V, Mol HGJ, Somer T, Pientstra M, Ritsema CJ, Geissen V (2017) Pesticide residues in European agricultural soils—a hidden reality unfolded. Sci Tot Environ 653:1352–1545. https://doi.org/10.1016/j.scitotenv.2018.10.441

16. Maggi F, la Cecilia D, Tang FH, McBratney A (2020) The global environmental and health hazard of glyphosate use. Sci Total Environ 717:173767. https://doi.org/10.1016/j.scitotenv.2020.137167

17. Borggaard OK, Gimsing AL (2008) Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. Pest Manag Sci 64:441–456

18. Coupe RH, Kalkhoff SJ, Capel PG, Greigore C (2012) Fate and transport of glyphosate and aminomethylphosphonic acid in surface waters of agricultural basins. Pest Manag Sci. https://doi.org/10.1002/ps.2212

19. Malaj E et al (2014) Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. PNAS 111:9549–9554. https://doi.org/10.1073/pnas.1321082111

20. Sabatier P et al (2014) Long-term relationships among pesticide applications, mobility, and soil erosion in a vineyard watershed. PNAS 111:15647–15652. https://doi.org/10.1073/pnas.141512111

21. Baier F, Jedinger M, Gruber E, Zaller JG (2016) Temperature-dependence of glyphosate-based herbicide’s effects on egg and tadpole growth of common toads. Front Environ Sci. https://doi.org/10.3389/fenvs.2016.00051

22. Relyes RA (2005) The lethal impact of roundup on aquatic and terrestrial amphibians. Ecol Appl 15:1119–1124

23. Relyesa RA, Jones DK (2009) The toxicity of Roundup Original Max® to 13 species of larval amphibians. Environ Toxicol Chem 28:2004–2008. https://doi.org/10.1897/09-021.1

24. Edwards CA, Bohlen PJ (1996) Biology and ecology of earthworms, 3rd edn. Chapman & Hall, London

25. Curby JP (1994) Grassland invertebrates. Ecology, influence on soil fertility and effects on plant growth. Springer Netherlands, Dordrecht, NL

26. Creamer CA, de Menezes AB, Krull ES, Sanderman J, Newton-Walters R, Farrell M (2015) Microbial community structure mediates response of soil labile plant compounds into stabilized soil microbial necromass. Com-Bio 1016/j. cub. 2019. 08. 045

27. Angst G et al (2019) Earthworms act as biochemical reactors to convert labile plant compounds into stabilized soil microbial necromass. Commun Biol 2:441. https://doi.org/10.1038/s42003-019-0684-z

28. Liu T et al (2019) Earthworms coordinate soil biota to improve multiple ecosystem functions. Curr Biol 29:3420–3425. https://doi.org/10.1016/j.cub.2019.08.045

29. Liu M, Cao J, Wang C (2020) Bioremediation by earthworms on soil microbial diversity and partial nitrification processes in oxycracycline-contaminated soil. Ecotoxic Environ Saf 189:109996. https://doi.org/10.1016/j.ecosaf.2019.109996

30. Euteneuer P, Wagentrith H, Steinkellner S, Scheib breithner C, Zaller JG (2019) Earthworms affect decomposition of soil-borne plant pathogen Sclerotinia sclerotiorum in a cover crop field experiment. Appl Soil Ecol 138:88–93. https://doi.org/10.1016/j.apsoil.2019.02.020
42. Li N, Wang C, Li X, Liu M (2019) Effects of earthworms and arbuscular mycorrhizal fungi on preventing Fusarium oxyssporum infection in the strawberry plant. Plant Soil 443:139–153. https://doi.org/10.1007/s11104-019-03228-6

43. Plass E et al (2019) Towards valuation of biodiversity in agricultural soils: a case for earthworms. Ecol Econ 159:291–300. https://doi.org/10.1016/j.ecolecon.2019.02.003

44. Zaller JG et al (2011) Earthworm-mycorrhiza interactions can affect the diversity, structure and functioning of establishing model grassland communities. PLoS ONE 6:e29293

45. Wurst S, Sonnemann I, Zaller JG (2018) Soil macro-invertebrates: their impact on plants and associated aboveground communities in temperate regions. In: Ohgushi T, Wurst S, Johnson SN (eds) Aboveground-belowground community ecology, vol Ecological Studies 234. Springer, Tokyo, pp 175–200. https://doi.org/10.1007/978-3-319-91614-9

46. Hoefnagl K, Monard C, Santonja M, Cluzeau D (2018) Feeding behaviour of epi-anecic earthworm species and their impacts on soil microbial communities. Soil Biol Biochem 125:1–9. https://doi.org/10.1016/j.soilb.2018.06.017

47. Zhang B-G, Li G-T, Shen T-S, Wang J-K, Sun Z (2000) Changes in microbial biomass C, N, and P and enzyme activities in soil incubated with the earthworms Metaphor guillelmi or Eisenia fetida. Soil Biol Biochem 32:2055–2062. https://doi.org/10.1016/S0038-0717(00)00111-5

48. Gaupto-Bergsten M, Hoffer M, Rewald B, Zaller JG (2015) Glyphosate-based herbicides reduce the activity and reproduction of earthworms and lead to increased soil nutrient concentrations. Sci Rep 5:12886. https://doi.org/10.1038/srep12886

49. Gill JPK, Sethi N, Mohan A, Datta S, Girdhar M (2018) Glyphosate toxicity for animals. Env Chem Lett 16:401–426. https://doi.org/10.1007/s10311-017-0689-9

50. Zaller JG, Brühl CA (2021) Direct herbicide effects on terrestrial nontarget organisms belowground and aboveground. In: Messing N, Zaller JG (eds) Herbicides. Chemistry, Efficacy, Toxicology, and Environmental Impacts. Elsevier, Amsterdam, ISBN 978-0-12-823674-1, pp 181–230

51. Pochon S, Simon L, Mirza A, Littleton A, Sahebzada F, Yudell M (2020) Impacts of glyphosate and its formulation Roundup Ultra in Lumbricus terrestris. Bull Environ Contamin Toxicol 105:678–85. https://doi.org/10.1007/s00128-020-0213-5

52. Zaller JG, Heigl F, Russel L, Grabmaier A (2014) Glyphosate herbicide effects on springtails (Collembola) differ from those of their respective active ingredients and vary with soil organic matter content. Envi Sci Poll Res 27:17280–17289. https://doi.org/10.1007/s00128-014-2499-1

53. Martínez Morcillo S, Yela JL, Capowiez Y, Mazzia C, Rault M, Sanchez-Hernandez JC (2013) Avoidance behaviour response and esterase inhibition in the earthworm, Lumbricus terrestris, after exposure to chlorypyrifos. Comp Biochem Physiol C 151:351–359. https://doi.org/10.1016/j.cbpc.2009.12.008

54. O’Hara RB, Anderson DJ (2010) A new protocol for multiple comparisons. Aust Ecol 35:58–67. https://doi.org/10.1111/j.1442-9993.2009.01963.x

55. Owagboriaye F, Dedeke G, Bamidele J, Adeleke M (2020) Biochemical response and vermiremediation assessment of microorganisms and earthworms to residual agricultural pesticide contamination: Field and experimental Impacts. Elsevier, Amsterdam, ISBN 978-0-12-823674-1, pp 181–230

56. Ahmed A (2013) Economic Cooperation and Development, Paris

57. van Hoesel W et al (2017) Single and combined effects of pesticide seed dressings and herbicides on earthworms, soil microorganisms, and earthworm diversity, structure and functioning of establishing model grassland communities. PLoS ONE 6:e29293

58. Pochron S et al (2019) Temperature and body mass drive earthworm activity and optimization. Toxicol Sci 58:60–67. https://doi.org/10.1093/toxsci/kfv035

59. Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new rapid colorimetric method for determination of acetylcholinesterase and butyrylcholinesterase. Biochem Pharmacol 7:88–95. https://doi.org/10.1016/0006-2952(61)90145-9

60. ÖNORM L1080 (2013) Chemische Bodenuntersuchungen - Bestimmung des organischen Kohlenstoffs durch trockene Verbrennung mit und ohne Berücksichtigung von Carbonaten.ÖP
and litter decomposition. Front Plant Sci 8:215. https://doi.org/10.3389/fpls.2017.00215

82. Hurtalová T, Usowicz B, Kossowski J, Matejka F (2001) Effect of plant cover on soil water content and seasonal changes in evapotranspiration. Contr Geophys Geodesy 31:467–476

83. Arnone JA, Zaller JG (2014) Earthworm effects on native grassland root system dynamics under natural and increased rainfall. Front Plant Sci 5:152. https://doi.org/10.3389/fpls.2014.00152

84. Pochron ST et al (2021) Earthworms Eisenia fetida recover from Roundup exposure. Appl Soil Ecol 158:103793. https://doi.org/10.1016/j.apsoil.2020.103793

85. Van Gestel CAM, Dirven-Van Breemen EM, Baerselman R, Emans HJB, Janssen JAM, Postuma R, Van Vliet PJM (1992) Comparison of sublethal and lethal criteria for nine different chemicals in standardized toxicity tests using the earthworm Eisenia fetida. Ecotox Env Safety 23:206–220. https://doi.org/10.1016/0147-6513(92)90059-C

86. Nuutinen V, Hagner M, Jalli H, Jauhiainen L, Rämö S, Sarikka I, Uusimäki J (2020) Glyphosate spraying and earthworm Lumbricus terrestris L. activity: Evaluating short-term impact in a glasshouse experiment simulating cereal post-harvest. Eur J Soil Biol 96:103148. https://doi.org/10.1016/j.ejsobi.2019.103148

87. Zaller JG et al (2018) Herbicides in vineyards reduce grapevine root mycorrhization and alter soil microorganisms and the nutrient composition in grapevine roots, leaves, xylem sap and grape juice. Env Sci Poll Res 25:23215–23226. https://doi.org/10.1002/esp.11356-018-2422-3

88. Pelosi C, Joelm S, Makowski D (2013) Searching for a more sensitive earthworm species to be used in pesticide homologation tests—a meta-analysis. Chemosphere 90:895–900. https://doi.org/10.1016/j.chemosphere.2012.09.034

89. Stellin F, Gavinielli F, Stevanato P, Conchieri G, Squartini A, Paolelli MG (2017) Effects of different concentrations of glyphosate (Roundup360®) on earthworms (Octodrilus complanatus, Lumbricus terrestris and Aporrectodea caliginosa) in vineyards in the North-East of Italy. Appl Soil Ecol doi: https://doi.org/10.1016/j.apsoil.2017.07.028

90. Hagner M, Mikola J, Saloniemi I, Saikkonen K, Helander M (2019) Effects of a glyphosate-based herbicide on soil animal trophic groups and associated ecosystem functioning in a northern agricultural field. Sci Rep 9:13. https://doi.org/10.1038/s41598-019-44988-5

91. Denoyelle R, Raut M, Mazzia C, Mascle O, Capowiez Y (2007) Cholinesterase activity as a biomarker of pesticide exposure in Allobophora chlorotica earthworms living in apple orchards under different management strategies. Env Toxocol Chem 26:2644–2649. https://doi.org/10.1098/06-355.1

92. Ernst G, Felten D, Vohland M, Emmerling C (2009) Impact of ecologically different earthworm species on soil water characteristics. Eur J Soil Biol 45:207–213

93. Shipitalo MJ, Nuutinen V, Butt KR (2004) Interaction of earthworm burrows and cracks in a clayey, subsurface-drained, soil. Appl Soil Ecol 26:209–217. https://doi.org/10.1016/j.apsoil.2004.01.004

94. Bordoli R, Das B, Yarn G, Pandey P, Tripathi O (2019) Modeling of water holding capacity using readily available soil characteristics. Agric Res 8:347–355. https://doi.org/10.1016/j.sis003-018-033-76-9

95. Altmeier J, Schobel S, Emmerling C (2002) Beitrag von Regenwürmern zum Infiltrations- und Abflussgeschehen in Boden in Abhängigkeit von Bodensubstrat und Bodennutzung. Mitt Deutsch Bodenkundl Ges 99:177–178

96. IPCC, et al (eds) (2012) Managing the risks of extreme events and disasters to advance climate change adaptation. Cambridge University Press, Cambridge

97. Henderson AM et al. (2010) Glyphosate technical fact sheet. http://npic.ornst.edu/factsheets/archive/glyphotech.html. Accessed 27 Aug 2019

98. Capri E, Vicari A (2010) Environmental fate and behaviour of glyphosate and its main metabolite. European Glyphosate Environmental Information Source http://www.egeis-toolbox.org/documents/10%20Fate%20and%20behaviour%20v3.2.pdf:3pp

99. Schoil R, Becher HH, Dörfler U, Gayler S, Grundmann S, Hartmann HP, Russi J (2006) Quantifying the effect of soil moisture on the aerobic microbial mineralization of selected pesticides in different soils. Env Sci Technol 40:3305–3312. https://doi.org/10.1021/et052205j

100. Silva V, Montanarella L, Jones A, Fernandez-Ugalde O, Mol HGJ, Ritserma CJ, Geissen V (2018) Distribution of glyphosate and aminomethylphosphonic acid (AMPA) in agricultural topsoils of the European Union. Sci Tot Environ 621:1352–1359. https://doi.org/10.1016/j.scitotenv.2017.10.093

101. Primost JE, Marino DJG, Aparicio VC, Costa JL, Carriquinibe P (2017) Glyphosate and AMPA, “pseudo-persistent” pollutants under real-world agricultural management practices in the Mesopotamic Pampas agroecosystem, Argentina. Environ Poll 229:771–779. https://doi.org/10.1016/j.envpol.2017.06.006

102. Lupi L, Bedmar F, Puricelli M, Marino D, Aparicio VC, Wunderlin D, Miglioranza KS (2019) Glyphosate runoff and its occurrence in rainwater and subsurface soil in the nearby area of agricultural fields in Argentina. Chemosphere 225:906–914. https://doi.org/10.1016/j.chemosphere.2019.03.090

103. Todorovic GR, Rampazzo N, Mentler A, Blum WEH, Eder A, Strauss P (2014) Influence of soil tillage and erosion on the dispersion of glyphosate and aminomethylphosphonic acid in agricultural soils. Int Agrophysics 28:93–100

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Submit your manuscript to a SpringerOpen journal and benefit from:

► Convenient online submission
► Rigorous peer review
► Open access: articles freely available online
► High visibility within the field
► Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com