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Correspondence and requests for materials should be addressed to J.G.Z. (johann.zaller@boku.ac.at)

Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem

Johann G. Zaller[1], Florian Heigl[1], Liliane Ruess[2] & Andrea Grabmaier[1]

[1]Institute of Zoology, University of Natural Resources and Life Sciences Vienna, Gregor Mendel Straße 33, 1180 Vienna, Austria, [2]Institute of Biology, Ecology Group, Humboldt-Universität zu Berlin, Philippstrasse 13, 10115 Berlin, Germany.

Herbicides containing glyphosate are widely used in agriculture and private gardens, however, surprisingly little is known on potential side effects on non-target soil organisms. In a greenhouse experiment with white clover we investigated, to what extent a globally-used glyphosate herbicide affects interactions between essential soil organisms such as earthworms and arbuscular mycorrhizal fungi (AMF). We found that herbicides significantly decreased root mycorrhization, soil AMF spore biomass, vesicles and propagules. Herbicide application and earthworms increased soil hyphal biomass and tended to reduce soil water infiltration after a simulated heavy rainfall. Herbicide application in interaction with AMF led to slightly heavier but less active earthworms. Leaching of glyphosate after a simulated rainfall was substantial and altered by earthworms and AMF. These sizeable changes provide impetus for more general attention to side-effects of glyphosate-based herbicides on key soil organisms and their associated ecosystem services.

Earthworms and arbuscular mycorrhizal fungi (AMF) are important components in temperate ecosystems, influencing nutrient cycling and overall ecosystem functioning[1,2]. Earthworms are considered to be ecosystem engineers because they shred and redistribute organic material in soil, increase soil penetrability for roots, thus improving overall soil fertility[3,4]. Because of their importance, earthworms have also been used as bioindicators of soil health and quality[1,5,6]. Mycorrhizal fungi form a symbiosis with over 80% of vascular plant species and are also considered keystone species in temperate ecosystems because of their influence on plant nutrient supply[7] and soil aggregation[8]. In arable soils AMF are the dominant root symbionts that sustain plant growth[9]. Mycorrhized plants commonly show a higher uptake of phosphorus and nitrogen, as the fungal mycelium has more efficient mechanisms for absorbing mineral nutrients than roots and by extending the root system enabling further exploration of the soil resources[5,10,11]. In return, host plants provide photoassimilates (predominantly glucose and fructose) that are converted to lipids by the fungus and used for carbon transport and storage[9,12]. Recently, the analysis of fatty acids as biochemical markers considerably improved our knowledge in AMF distribution and foraging activity in soil[13]. Thereby, the soil phospholipid fatty acid (PLFA) 16:1ω5 represents viable hyphal biomass, while the neutral lipid fatty acid (NLFA) reflects fungal storage reserves such as spores, vesicles and propagules[14,15]. Moreover, the ratio of 16:1ω5 NLFA to PLFA indicates fungal phenology such as senescence or active colonization phases[12,16].

Despite their important roles in ecosystems, our understanding on ecological interactions between earthworms and AMF is rather limited. The few studies investigating earthworm-AMF interactions suggest that the response patterns are dependent on the species involved; as a result effects range from additive, synergistic, antagonistic or no interactive effects[17–20]. Here we examined, whether the interactions between earthworms and AMF are affected by herbicide application. We experimented with two essential players in temperate soil ecosystems: the anecic, vertically burrowing earthworm *Lumbricus terrestris* (Linnaeus 1758) and the arbuscular mycorrhizal fungi *Glomus mosseae* (T.H. Nicolson & Gerd.). As a herbicide we used Roundup (RU), the most widely used pesticide worldwide[21] containing the active ingredient glyphosate. Glyphosate is a broad-spectrum, post-emergence, non-selective chemical that kills plants by affecting the shikimate-pathway during photosynthesis[22]. Generally, glyphosate is regarded as environmentally friendly due to its fast biodegradation and strong adsorption to soil

particles[23]. However, there is mounting evidence that many amphibian species[24–26] and other wildlife[27] can be detrimentally affected by glyphosate-based herbicides.

Contrary to the wide use of glyphosate surprisingly little is known on potential side effects on interactions between key soil organisms such as earthworms or AMF. Glyphosate effects on earthworms vary from detrimental[28–30] to no effects[31–33], however, to what extent their interaction with other soil organisms is affected by glyphosate has never been investigated. Studies testing glyphosate effects on AMF show an inhibition of AM fungal spore germination and germ tube growth[34] or reduced mycorrhiza in soil[35], however only at concentrations greater than those recommended for field use. Several other reports show no effect of glyphosate on mycorrhiza when applied at recommended doses[36–40]. The fate of glyphosate in ecosystems is another aspect which has rarely been investigated[41,42]. While glyphosate sorbs strongly to soil minerals[43], leaching and soil erosion by water or wind can transport glyphosate from land to water environments[44]. This glyphosate leaching is assumed to be affected by earthworms and/or AMF. Earthworms maintain soil structure and foster macropores, which may influence water infiltration[45] and thereby increase glyphosate leaching. On the other hand, mycorrhiza could lead to stronger absorption of glyphosate by binding and enmeshing soil particles into larger aggregates[46].

To investigate interrelationships between herbicide application, earthworms and AMF we set up a full-factorial mesocosm greenhouse experiment. We planted the mesocosms with the leguminous forb white clover (*Trifolium repens* L.), which is frequently used as green manure in agriculture. Three hypotheses were tested. First, herbicide application will increase earthworm activity as an increased amount of dead plant material will be available as food for earthworms. Second, herbicide application will not affect AMF in soil because of the very plant-specific mode of symbiotic interaction. Third, herbicide-stimulated earthworm activity increases the preferential flow of rainwater through burrows and therefore increase leaching of glyphosate; whereas AMF counteract glyphosate leaching as they enhance soil aggregation. Such terrestrial model ecosystems have been proposed as an ideal tool to evaluate the effects of chemicals in soil ecosystems in order to achieve a greater realism in the ecotoxicological evaluation of chemicals to non-target organisms[47].

Results

Plants. *Trifolium* leaves were killed by the herbicide within several hours, whereas stolons remained partly green. Shoot biomass of *T. repens* at harvest was significantly reduced by earthworms ($F_{1;16}$ = 5.485, P = 0.032) but not significantly affected by RU application ($F_{1;16}$ = 2.529, P = 0.131) or AMF ($F_{1;16}$ = 0.220, P = 0.645; shoot biomass across AMF and RU treatments: −EW 23.724 ± 2.283 g, +EW 18.812 ± 3.169 g). Root biomass of *T. repens* was unaffected by RU ($F_{1;16}$ = 0.190, P = 0.668), AMF ($F_{1;16}$ = 0.682, P = 0.421) or earthworms ($F_{1;16}$ = 0.082, P = 0.778; root biomass across treatments: 1.775 ± 0.361 g).

Earthworms. Earthworm activity was similar across treatments prior to herbicide application with mean surface cast production of 1.5 ± 0.1 casts day^{-1} mesocosm^{-1} and 3.6 ± 0.4 moved toothpicks day^{-1} mesocosm^{-1}. Earthworm activity measured by toothpicks was marginally significantly lower after herbicide application ($F_{1;10}$ = 4.490, P = 0.060), however was not influenced by AMF ($F_{1;10}$ = 0.001, P = 0.977; Figure 1a+1b). Herbicide application reduced earthworm activity (toothpicks) in +AMF mesocosms ($F_{1;4}$ = 9.042, P = 0.040; Figure 1b) but had no influence on earthworm activity in −AMF mesocosms (Figure 1a). Earthworm activity measured by surface cast production was neither influenced by RU nor AMF (Figure 1).

Earthworm fresh mass at harvest was on average 72% of the initially added fresh mass; neither AMF inoculation ($F_{1;10}$ = 0.138, P = 0.720) nor RU application ($F_{1;10}$ = 2.903, P = 0.127) affected recaptured earthworm fresh mass, but a significant AMF × RU interaction occurred ($F_{1;10}$ = 6.388, P = 0.035). Earthworm mass in the different treatments was: −RU/−AMF 11.0 ± 7.0 g, +RU/−AMF 9.5 ± 5.7 g; −RU/+AMF 5.3 ± 9.1 g, +RU/+AMF 16.4 ± 3.4 g. Earthworm activity (both moved toothpicks and surface castings) was not correlated to earthworm biomass or greenhouse mean air temperature or relative humidity (data not shown).

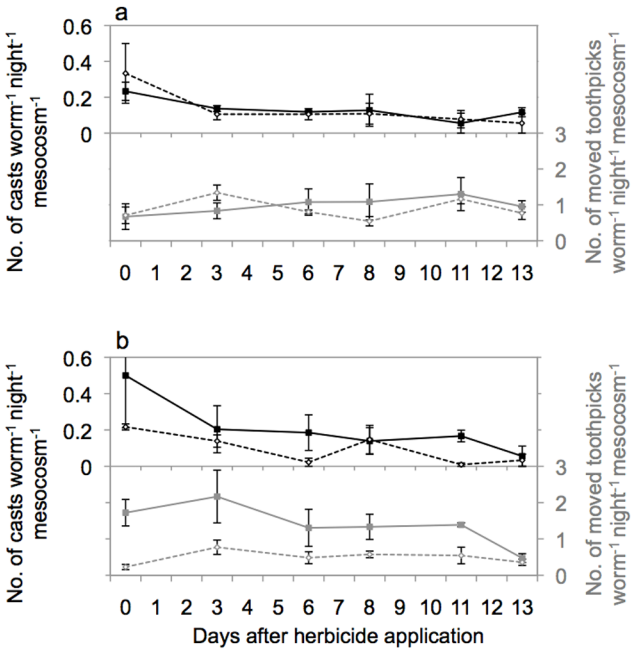

Figure 1 | Earthworm activity measured by surface cast production and moved toothpicks in mesocosms without (a) and with (b) AMF inoculation and without (continuous line) and with herbicide application (dotted line). Means ± SE, n = 3.

Mycorrhizae. Thirty-six weeks after AMF inoculation, average mycorrhization rates of *Trifolium* roots were 26% in +AMF and 15% in −AMF treatments. Across soil layers, herbicide application significantly reduced mycorrhization in +AMF ($F_{1;10}$ = 7.887, P = 0.023) but had no effect on mycorrhization rates in −AMF mesocosms (Figure 2). The reduction in mycorrhization due to herbicide application was even more pronounced when soil layers were considered separately (Table 1, Figure 2). In −RU/−EW mesocosms mycorrhization was significantly different between layer 0–5 cm and layer 5–10 cm ($F_{1;10}$ = 14.756, P = 0.018). In −RU + EW mesocosms mycorrhization differed significantly between layer 0–5 cm and layer 10–30 cm ($F_{1;10}$ = 23.093, P = 0.009). In +RU/−EW mesocosms mycorrhization differed significantly between layer 0–5 cm and layer 10–30 cm ($F_{1;10}$ = 20.050, P = 0.011). Earthworms had no influence on root AMF colonisation

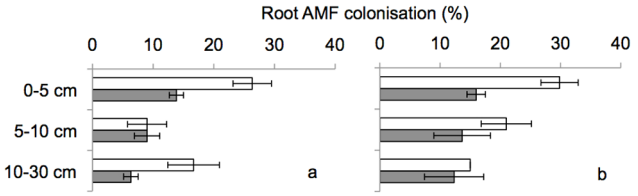

Figure 2 | Mycorrhization of *T. repens* roots in different soil layers without (a) and with (b) earthworms in mesocosms without (white) or with (grey) herbicide application. Means ± SE, n = 3.

Table 1 | ANOVA results on the effects of herbicide application (RU) and earthworms (EW) on *Trifolium repens* root AMF colonisation. ANOVA with * for P < 0.05, ** for P < 0.01, *** for P < 0.001

Soil layer	Roundup (RU)		Earthworms (EW)		RU × EW	
	F-value	P-value	F-value	P-value	F-value	P-value
0–5 cm	29.826	0.001**	1.381	0.274	0.076	0.789
5–10 cm	0.994	0.348	5.133	0.053	0.994	0.348
10–30 cm	3.870	0.085	0.430	0.530	1.346	0.279

across soil depths ($F_{1;10}$ = 2.575, P = 0.147; also no RU × EW interaction).

Hyphal biomass in soil assigned by 16:1ω5 PLFAs was not enhanced by AMF inoculation (Table 2, Figure 3). Highest PLFA concentrations of 16:1ω5 in soil were found in the layer 0–5 cm in mesocosms without any manipulation (−EW, −AMF, −RU). A significant herbicide AMF interaction occurred (no interaction between the three treatment factors). We found higher PLFA concentrations of 16:1ω5 in mesocosms with herbicide application, especially in combination with earthworms. AMF spores, vesicles and propagules assigned by 16:1ω5 in soil NLFAs were significantly enhanced by AMF inoculation (Table 2, Figure 3). Most storage reserves were found in mesocosms without any manipulation in layer 0–5 cm. Earthworms reduced the concentration of 16:1ω5 NLFAs and had a strong negative effect on storage structures of AMF assigned by NLFA/PLFA ratio (Table 2, Figure 3). This effect diminished in presence of herbicide, but was still visible. A herbicide-earthworm interaction occurred in layer 5–10 cm: means in NLFA concentration in −RU/−EW was higher than in +RU/+EW mesocosms (Figure 3).

Water infiltration and herbicide leaching. Water infiltration rate was unaffected by earthworms or AMF (Figure 4). Herbicide application showed a trend towards reduced water infiltration ($F_{1;22}$ = 3.796, P = 0.069; there was no Roundup × AMF interaction).

Concentration of glyphosate or its metabolite AMPA in the leachate was unaffected by earthworms or AMF (Figure 5). However, concentrations of glyphosate were significantly ($F_{1;10}$ = 7.572, P = 0.025) and of AMPA marginally significantly ($F_{1;10}$ = 4.515, P = 0.066) interactively affected by earthworms and AMF with increasing earthworm effects in −AMF and decreasing earthworm effects in +AMF mesocosms (Figure 5). In −AMF mesocosms earthworms significantly increased glyphosate leaching ($F_{1;4}$ = 9.439, P = 0.037).

Table 2 | ANOVA results for effects of Arbuscular mycorrhizal fungi (AMF), Earthworms (EW), Roundup (RU) and their interactions on PLFA amount of 16:1ω5 and NLFA amount of 16:1ω5 in different soil layers. ANOVA with * for P < 0.05, ** for P < 0.01, *** for P < 0.001

	PLFA		NLFA		NLFA : PLFA ratio	
Parameter	F	P	F	P	F	P
Soil depth 0–5 cm						
AMF	4.926	0.041*	0.296	0.594	0.046	0.832
EW	0.029	0.866	4.595	0.048*	5.445	0.033*
RU	1.154	0.299	0.064	0.803	0.520	0.481
AMF × EW	0.751	0.399	0.425	0.524	1.207	0.288
AMF × RU	9.069	0.008**	6.353	0.023*	2.730	0.118
EW × RU	5.957	0.027*	2.543	0.130	0.734	0.404
AMF × EW × RU	0.031	0.862	3.486	0.080	4.267	0.055
Soil depth 5–10 cm						
AMF	0.011	0.918	4.485	0.050*	4.165	0.058
EW	8.047	0.011*	4.399	0.052	13.135	0.002**
RU	0.470	0.503	2.241	0.154	3.448	0.082
AMF × EW	4.146	0.059	3.126	0.096	0.411	0.530
AMF × RU	4.522	0.049*	3.424	0.083	0.454	0.510
EW × RU	2.050	0.171	5.449	0.033*	2.346	0.145
AMF × EW × RU	0.948	0.345	0.038	0.849	0.522	0.480

Discussion

To our knowledge, this is among the first studies investigating the impact of a glyphosate-based herbicide on ecological interactions between a vertically burrowing earthworm species (*Lumbricus terrestris*) and symbiotic mycorrhizal fungi. Contrary to our hypothesis, Roundup did not stimulate but rather decrease earthworm activity, especially in mesocosms with AMF amendment. Also in the +AMF mesocosms, earthworm biomass was 50% higher after Roundup application, than in −AMF mesocosms. This suggests that over the short duration of our experiment, Roundup led to heavier earthworms that were less active at the surface, probably because there was abundant food in form of dead roots or AMF in the soil that precluded earthworms from foraging food from the surface. Other studies showed that earthworm biomass was unaffected by glyphosate-based herbicides for endogeic species[48], whereas in temperate epigeic species[30,49] and tropical earthworms strong mass loss after glyphosate application was found[50]. Studies investigating effects of Roundup on soil dwelling endogeic earthworm species (*Aporrectodea caliginosa*) found no alteration of the energy status after acute exposure[31]. Glyphosate had no effect on growth of *A. caliginosa* in a pot experiment where the herbicide was mixed with soil[51], in contrast, another study showed that glyphosate reduces the growth of *A. caliginosa* even at a rate lower than recommended by the manufacturer[52]. Surface dwelling, epigeic earthworms showed no avoidance of Roundup treated leaves (*Eisenia andrei*[32]) or response in their depth distribution (*E. fetida*[53]) but avoided glyphosate treated soil[28,29]. Previous studies found no influence of glyphosate on the survival rate in temperate earthworm species *Aporrectodea trapezoides*, *A. rosea*, *A. caliginosa* or *A. longa* populations[30,48], whereas a 50% reduction in mortality was found for the tropical earthworm species *Pheretima elongata*[54]. Effects of glyphosate had no influence on reproduction of *E. fetida*[49], whereas others reported a significant reduction of hatched cocoons in glyphosate treated soil for this species[29,30].

Two things are important to note, when evaluating our current results and previous results from the literature. First, we monitored the surface activity of earthworms over a period of only two weeks after Roundup application and therefore no conclusions on long-term effects, consequences for reproduction or changes in below-ground activity can be derived from this study. Second, findings on herbicide effects on epigeic species such as *E. fetida* are important contributions when testing possible mode of actions in ecotoxicological tests, however they are of limited value when aiming to evaluate pesticide effects under field situations as these species preferably live in habitats with an abundant surface litter layer which is not the case in arable agroecosystems where these herbicides are applied.

We found a 40% reduction of mycorrhization after Roundup application in soils amended with the mycorrhizal fungi *G. mosseae*. This is in contrast to what we hypothesized, based on the allegedly fast biodegradation of the herbicide and the very plant-specific mode of action. We explain this mainly by direct and indirect influences. Roundup could have directly affected active metabolite production in the plant with detrimental effects on root AMF colonisation[38]. Indirect effects of Roundup on AMF could have affected the intraradical phase of AMF that has been shown to be sensitive to several host plant metabolites which regulate AMF abundance[55–57]. Mycorrhizal infection of maize, soybean and cotton was influenced by glyphosate in pasteurized soil but not in non-pasteurized soil[38]. Our soil mixture was steam-sterilized, but afterwards amended with a microbial wash from field soil, therefore only differing from field

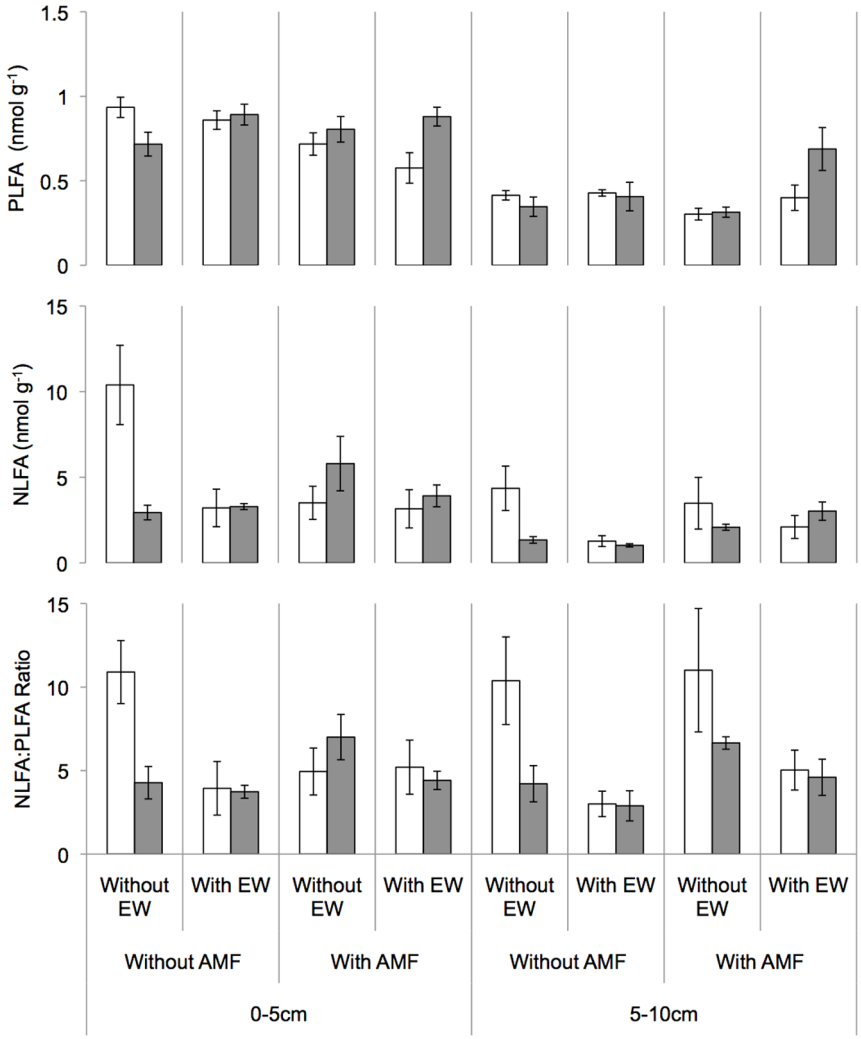

Figure 3 | PLFA amount of 16:1ω5, NLFA amount of 16:1ω5 in nmol g^{-1} DW soil and the ratio of 16:1ω5 NLFA to PLFA in different soil layers without (white) and with (grey) Roundup application, without/with earthworms in mesocosms without/with AMF inoculation. Mean ± SE, n = 3.

soil by the presence or absence of the inoculated AMF taxa. However, the latter did not enhance AMF hyphal biomass measured by 16:1ω5 PLFA, whereas spores assigned by fungal storage lipids, i.e. 16:1ω5 NLFA, were highest in soils without any manipulations (−AMF, −RU, −EW). These fungal propagules obviously have survived the steam-sterilization procedure. The generally low impact of soil amendment by the mycorrhiza inoculum points to competition with the indigenous soil community hampering the establishment of introduced *G. mosseae*. However, this cannot be assigned by the used biomarker fatty acid, 16:1ω5, as it is a measure for viable fungal hyphae biomass and storage fat in spores across the genus *Glomus*[15,58,59].

Direct influence of Roundup on AM fungi are generally regarded to be minor as soil fungi are well protected from direct contact with the herbicide. Indeed reports show rather insignificant influence of glyphosate on hyphal growth and germination of spores as well as root AMF colonisation[34,37,38,40,57]. However, in the present experiment Roundup application affected hyphal (i.e. amount of 16:1ω5 PLFA) and spore (i.e. amount of 16:1ω5 NLFA) biomass in the soil. Spore biomass generally declined with herbicide application, which is in accordance with others who showed reduced spore viability even under the lowest glyphosate rate[60]. Interestingly, the presence of earthworms resulted in a comparable negative effect on fungal storage structures. Earthworms are reported to influence AMF positively

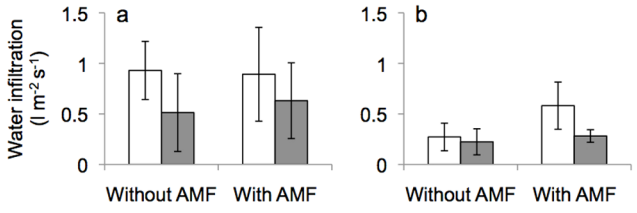

Figure 4 | Water infiltration rate measured in mesocosms without earthworms (a) and with earthworms (b) in response to AMF, without (white) and with (grey) Roundup application. Means ± SE, n = 3.

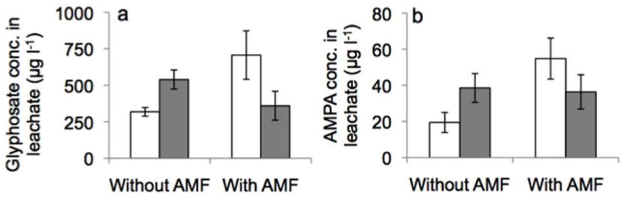

Figure 5 | Glyphosate concentration (a) and its metabolite AMPA (b) in soil leachate. Illustrated are concentrations in mesocosms with herbicide application, without and with AMF inoculation and without (white) and with (grey) earthworms. Means ± SE, n = 3.

by contribution to the dispersal of spores[61,62] yet our results indicate feeding rather than propagating fungal spores[63], which is supported by the corresponding changes in the NLFA to PLFA ratio. Spores, hyphae and infected root pieces form the three types of AMF propagules in soil, however their importance varies due to fungal species. In Glomeraceae, the extraradical mycelium is the most important source of inoculum, whereas spores are the main propagules in Gigasporaceae[64]. Thus, hyphae of *Glomus* are responsible for rapid colonization of new hosts, and hyphal biomass was positively affected by herbicide application especially in combination with earthworms. This indicates that glyphosate application alters fungal phenology, i.e. fosters fungal foraging over resting structures. Such effects likely are mediated through the modification of host plant physiology[57]. In sum the performance of AMF was distinctly altered by both Roundup and earthworms, albeit the impact varied with AMF propagule structures. Given the immense importance of AMF for plant nutrition and soil structure[9,46], these effects can have ramification for the functioning of ecosystems.

Will effects of herbicide on earthworms and mycorrhiza influence herbicide leaching? We found a tendency that water trickled away more slowly after a simulated heavy rainfall in mesocosms treated with Roundup as compared to those without Roundup application, however water infiltration rate was not influenced by earthworms or AMF. As Roundup had no effect on shoot and root mass of *T. repens*, we assume that after Roundup application dead plant material soaked up the excessive water and blocked the downflow of water. We hypothesized that earthworms increase glyphosate leaching by a preferential flow of contaminated rainwater through burrows, but also expected AMF to decrease glyphosate leaching by binding and enmeshing soil particles into larger aggregates[46,65]. Interestingly, earthworms significantly increased glyphosate leaching only in absence of AMF, while in presence of AMF earthworms tended to decrease glyphosate leaching. It remains to be tested whether this is due to glyphosate uptake and accumulation by AMF or whether these AMF hyphae might have been consumed by earthworms thus protecting glyphosate from leaching. Other studies have shown that glyphosate is mostly located in the earthworm mucus[31] which is smeared along the walls of earthworm burrows[66] and could therefore increase herbicide leaching. Furthermore, a rapid preferential transport for even strongly sorbing pesticides such as glyphosate and pendimethalin was demonstrated[44]. In contrast, AMF hyphae and other microorganisms could play a role in bonding glyphosate in the burrow walls. Biopore walls represent hot spots for microbial activity and pesticide mineralization[67]. The drilosphere of the burrows of *L. terrestris* therefore created an ideal habitat for a diverse microbial community. Many soil bacteria are known to degrade the organophosphonate glyphosate, e.g. dominant rhizosphere colonizer such as *Pseudomonas*[68], bulk soil inhabitants such as *Arthrobacter*[69], or symbiotic groups such as the family *Rhizobiaceae*[70]. A higher phosphorus transport via fungal hyphae was reported after glyphosate application for *G. mosseae*[71]. These processes in turn decreased glyphosate leaching as burrows transmitted clean water past the herbicide-containing soil matrix[72]. For the current results this could mean that without earthworms, water from the simulated heavy rainfall seeped through the whole soil matrix and thus absorbing glyphosate from the soil. The hyphae and other soil microorganisms absorbed glyphosate and so decreased glyphosate leaching; because the burrows transmitted clean water past the herbicide-containing soil matrix[72].

Taken together, our results show for the first time that Roundup can affect important interactions between earthworms and AMF, two of the most important soil organisms. While the short-term influence of Roundup on earthworms seem rather minor, the detrimental effects on AMF in roots and soil can have wide consequences for crop cultivation. Given AMFs and earthworms eminent role in plant nutrition, a glyphosate-induced decline in AM fungi would require more fertilization with economical and ecological consequences for farmland management. The finding that Roundup affects, together with earthworms and AMF, water infiltration requires more attention especially as climate change models prognosticate heavier rainfalls. Results of this study also highlight the importance of more complex experimental settings that investigate interactions of several species in order to better assess potential effects of pesticides on the environment.

Methods

Experimental setup. We conducted a full-factorial mesocosm experiment manipulating the three factors Earthworms (two levels: earthworm addition, +EW vs. no earthworms, −EW), AMF (two levels: AMF inoculation, +AMF vs. no AMF inoculation, −AMF) and Herbicide application (two levels: Roundup application, +RU vs. no Roundup application, −RU; more details on the individual treatments below). The experiment was conducted in December 2011 in a greenhouse of the University of Natural Resources and Life Sciences Vienna (BOKU), Austria. During the course of the experiment mean daytime air temperature inside the greenhouse was 20.1 ± 3.2°C at a mean relative humidity of 55.2 ± 5.4%; mean nighttime air temperature was 15.3 ± 2.6°C at a mean relative humidity of 68.3 ± 6.7%; to ensure optimal light conditions, three 1000-W Radium lamps (type HRI-T100W/D, WE-EF Leuchten, Bispingen, Germany) were installed in 1.5 m distance above the experimental units (14 hours light, 10 hours night).

We used 24 plastic pots (volume: 20 l, diameter: 31 cm, height: 30 cm; further called mesocosms) which were lined out with two layers of garden fleece at the bottom and extended at the upper rim with a 10 cm high barrier of transparent plastic to prevent earthworms from escaping; the fleece and barriers were also installed in mesocosms containing no earthworms to create similar microclimatic conditions among treatments.

Treatments. AMF treatments were prepared in March 2011 by first filling the mesocosms with 12 l steam-sterilized (3 hours at 100°C) field soil (Haplic Chernozem, silt loam) mixed with quartz sand (grain size 1.4–2.2 mm) in a ratio of 40 : 60 vol/vol. Characteristics of this soil mixture: C_{org} = 24.1 g kg^{-1}, N_{tot} = 0.98 g kg^{-1}, K = 111.2 mg kg^{-1}, P = 58.42 mg kg^{-1}, pH = 7.63. The upper 6 l of the +AMF treatments were filled with the same substrate mixture and amended with 25 g l^{-1} inoculum of *Glomus mosseae* (T.H. Nicolson & Gerd.; synonymously *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler) obtained from a commercial supplier (Symbio-m Ltd., Lanskroun, Czech Republic). The −AMF controls were filled with the same amount of steam-sterilized and thus inactive AMF inoculum. We successfully used this substrate mixture in other experiments involving the same earthworm and AMF taxa[45,73,74]. Then 400 ml of microbial wash was added to each mesocosm to inoculate the steam-sterilized soil with microorganisms present in field soil[75]. This microbial wash contained 300 ml soil suspension (3500 g fresh soil dispensed in 7200 ml distilled H_2O filtered through a sieve-cascade from 2000 µm to 25 µm mesh size) and 100 ml AMF suspension (466 g AMF-inoculum dispensed in 2400 ml distilled H_2O filtered through the same sieve-cascade).

In April 2011 mesocosms were planted with white clover (*Trifolium repens* L.). Therefore, *T. repens* was first propagated from seeds in steam-sterilized potting soil, then 18 seedlings (average height about 10 mm, seedlings consisted of two cotyledons and two real leaves) were transplanted into each mesocosm in a regular hexagonal pattern with an equidistance to each other of 5 cm (240 seedlings m^{-2}). This seed material is commonly used by farmers in mixtures for green manuring and was obtained from the BOKU Department of Crop Sciences. No fertilizers were applied during the course of the experiment.

In December 2011 we added 4 adult individuals of vertically burrowing *Lumbricus terrestris* L. to the +EW mesocosms (16.6 ± 2.1 g $mesocosm^{-1}$, equivalent to 220.6 g m^{-2}). Earthworm densities were roughly oriented on the average earthworm biomass in temperate grasslands ranging between 52–305 g m^{-2} where 50–75% of the biomass consists of anecic species[1]. Earthworms were purchased from a local fishing bait shop. To acquaint earthworms with experimental conditions, we cultivated them in plastic boxes (climate chamber at 15°C) filled with steam-sterilized field soil and ground oat flakes as food before they were introduced to the mesocosms. Before earthworms were randomly added to the +EW mesocosms, they were washed free of attached soil, dried off on filter paper and weighed. All earthworms buried themselves in the soil within a few minutes. The mesocosms were randomly placed on greenhouse tables and randomly repositioned every second week to avoid treatment interactions with potential microclimatic gradients inside the greenhouse. No additional food was provided for earthworms in the mesocosms as there was abundant dead organic material on the soil surface. An automatic irrigation system added on average 0.5 l tap water day^{-1} to each mesocosm.

Herbicide was applied five days after earthworm insertion on half of the mesocosms comprising all treatment combinations. We used Roundup Speed (Monsanto Inc., St. Louis, Missouri, USA), a systemic, broad-spectrum herbicide containing 7.2 g l^{-1} of the active ingredient glyphosate. This herbicide is recommended for use in home and garden areas and was obtained from a garden center in Vienna. Following the instructions for use, we applied the herbicide directly onto the plants from the original bottle with the attached fine mist spray nozzle. We applied the herbicide once on day 5 after earthworm inserting at 4 p.m. without direct sunlight at an air tem-

perature of 25°C. As recommended in the instruction text we sprayed the herbicide so that the plant surface was homogeneously covered and shiny from the herbicide film. This application needed 14 squirts of Roundup Speed with the spray nozzle mesocosm^{-1} amounting to 177.48 ml m^{-2}.

These treatments were replicated three times in a full-factorial design: two earthworm treatments × two AMF treatments × two RU treatments × three replicates equals totally 24 mesocosms.

Measurements and analyses. Earthworm activity was indirectly assessed during nighttime by 30 toothpicks mesocosm^{-1} that were vertically inserted (0.5 cm deep) in a consistent pattern. In the following morning the number of toothpicks differing from the original vertical position was considered as a measure of earthworm activity because earthworms crawl over the soil surface when searching for food. Knocked over toothpicks were counted as 1 and inclined toothpicks were counted as 0.5. As another measure of earthworm activity we additionally measured the number of freshly produced casts on the soil surface[76]. Both activity measurements were done parallel three times before and six times after herbicide application.

Water infiltration and Roundup leaching was measured seven days after the Roundup application by pouring 3 l of distilled water on top of the mesocosms simulating a rain shower of about 40 l m^{-2} (see also[45]). The time from pouring the water onto the mesocosms until the last water pool disappeared from the soil surface was recorded and used to calculate the water infiltration rate in l m^{-2} s^{-1}. We collected 250 ml of the leachate from the saucers at bottom of the mesocosms immediately stored it in a freezer at −20°C before it was analysed for glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) in the laboratories of the BOKU Department of Forest and Soil Sciences using a HPLC-MS/MS method[77,78].

Harvest of the mesocosms started 14 days after herbicide application by cutting the remaining or untreated plants at the soil surface to obtain aboveground plant biomass production. Afterwards, soil was removed from the mesocosms in three separate layers 0–5 cm, 5–10 cm and 10–30 cm. Earthworms present in these soil layers were carefully washed free of soil, placed on moist filter paper, counted, weighed and released to the BOKU garden. Roots present in these soil layers were washed free of attached soil particles under a jet of tap water over a 1-mm sieve and sorted out. Dry mass of shoots and roots was determined after 48 hours oven-drying at 55°C. A portion of roots per layer was collected, cleared with boiling KOH for four minutes and stained for one minute with black Sheaffer ink[79]. Percentage of root length colonized by AMF considering arbuscules and vesicles (i.e. mycorrhization rate) was determined using the grid-line method by counting at least 100 intersections per sample[80].

Lipid extraction from pot soil was carried out by extracting 3–4 g of soil (wet weight) with Bligh & Dyer solvent (chloroform: methanol: citrate buffer as 1 : 2 : 0.8, pH 4)[81]. The obtained lipids were fractionated into neutral lipid (NLFAs), glycolipid and phospholipid fatty acids (PLFAs) on a silica column (HF Bond Elut - SI, Varian Inc.) by elution with chloroform, acetone and methanol, respectively. NLFAs and PLFAs were subjected to an alkaline methanolysis in 0.2 M methanolic KOH, and the fatty acid methyl esters (FAMEs) were extracted with hexane-chloroform. Samples were dissolved in isooctane and stored at −20°C until analysis. FAMEs were analyzed using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC-FID) using a HP Ultra 2 capillary column (25 m × 0.2 mm i.d., film thickness 0.33 μm). The oven temperature program started with 170°C and increased by 28°C min^{-1} to 288°C, followed by 60°C min^{-1} to 310°C. FAMEs were identified with the Sherlock Pattern Recognition Software (MIDI®) by comparing retention times to a standard mixture, and quantifying based on the internal standard methylnondecanoate (19 : 0). To verify correct identification (chain length and saturation) a range of samples was additionally analyzed with the Agilent 7890A coupled to a Mass Selective Detector (Agilent 7000 Triplequadrupole) equipped with a HP5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm), operated in splitless mode with helium as carrier gas. Oven temperature program started with 40°C and increased by 46°C min^{-1} to 200°C, followed by 5°C min^{-1} to 238°C, 120°C min^{-1} to 300°C. A mass range of 40–400 m/z was monitored in Scan mode. The fatty acid 16:1ω5 was applied as general marker for AMF, predominantly Glomales, with the PLFA fraction representing hyphal membranes and the NLFA fraction storage lipids[58,59].

Statistical analyses. All variables were tested for homogeneity of variances and normality using the tests after Levene and Kolmogorow-Smirnow, respectively. Data on PLFAs and NLFAs were log-transformed to meet the assumptions for parametric tests. We conducted a three way analysis of variance (ANOVA) to test the effects of Earthworms, AMF and Roundup on PLFAs, NLFAs, water infiltration and Roundup leaching. Here analyses for treatment effects on PLFAs and NLFAs were conducted for each soil layer separately. Earthworm activity (moved toothpicks and surface castings) during the course of the experiment was analyzed conducting a repeated measures ANOVA with Roundup and AMF as factors by only including data from mesocosms containing earthworms. Root AMF colonisation was analyzed for each soil layer using a two-way ANOVA considering the factors Earthworms and Roundup; mesocosms without AMF inoculation were not included. We also performed Pearson correlations between earthworm biomass and earthworm activity (moved toothpicks and surface castings) and between earthworm activity and mean air temperature or mean relative humidity. All statistical tests were performed in PASW Statistics 18 (vers. 18.0.0, IBM Corp., Armonk, New York, USA). Values given throughout the text are means ± SE.

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Author contributions

Conceived and designed the experiment: F.H., A.G., J.G.Z. Performed the experiment: F.H., A.G., J.G.Z. Analyzed the data: F.H., J.G.Z., L.R. Contributed reagents/materials/analysis tools: L.R. Wrote the paper: J.G.Z., F.H., L.R., A.G.

Additional information

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