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Halsey, K. and Stroud, J. L. 2023. The post-registration monitoring of glyphosate-treated plants using anecic earthworms. *Annals of Applied Biology - AAB.* pp. 1-7. https://doi.org/10.1111/aab.12838

Received: 19 August 2022 | Revised: 17 February 2023 | Accepted: 18 April 2023
DOI: 10.1111/aab.12838

ORIGINAL ARTICLE

Annals of Applied Biology
An international journal of the aab
WILEY

The post-registration monitoring of glyphosate-treated plants using anecic earthworms

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Funding information
Natural Environment Research Council, Grant/Award Number: NE/N019253/1

Abstract

Glyphosate *N*-(phosphonomethyl) glycine is a widely-used herbicide in agriculture. The anecic earthworm, *Lumbricus terrestris* feeds and forages for surface plant materials meaning that this species has a unique and direct exposure to agrichemicals. At the recommended product rates, significantly ($F_{1,44} = 8.67$, $p = .005$) higher numbers of *L. terrestris* middens were found in the glyphosate treated areas of an arable crop field. Laboratory feeding assays using field aged plant materials indicated that previous glyphosate treatment was a statistically significant factor affecting earthworm *L. terrestris* biomass ($F_{1,12} = 5.75$, $p = .03$). Negligible glyphosate residues were detectable, and the field aged plant materials were encrusted with fungal hyphae. This suggests that glyphosate influences the colonisation of plant material by a litter-fungus complex which improves the food quality to earthworms. Concentrations of epoxiconazole, a fungicide, were detected in some plant materials and may influence overall food quality to earthworms. Glyphosate treatment on fresh volunteer plant leaves (unwanted crop seedlings) was not a statistically significant factor affecting earthworm *L. terrestris* biomass ($F_{1,6} = 0.16$, $p = .92$). These results indicate fungal communities influence feeding behaviours, and plant materials are a direct source of agrichemicals to anecic earthworms.

KEYWORDS
earthworm, glyphosate, midden, pesticide, soil health

1 | INTRODUCTION

The first author had observed a curious phenomenon in arable field experiments. There was an increase in midden-building activities by *Lumbricus terrestris* approximately 6-weeks after applications of the glyphosate (*N*-(phosphonomethyl) glycine) based herbicides compared to untreated areas in some field trials. This phenomenon was observed for a number of years, prompting an investigation via midden counting to quantify the observation, laboratory feeding assays and characterisation of the physical and chemical properties of these 'treated' (with glyphosate) and untreated plant materials.

To date, there is little post-registration monitoring of pesticides, in terms of studies conducted within the context of conventional field management (fertilisers, seed treatments, pesticides etc.). A meta-analysis indicated that glyphosate-based herbicides have no impact on general earthworm populations, and a trend of increased abundance and biomass were suggested when glyphosate was included in the rotation (Briones & Schmidt, 2017). *Lumbricus terrestris* is an anecic earthworm that feeds and forages for surface plant residues to form a distinctive midden (collected surface debris) which overlies a deep vertical burrow. Their innate feeding and foraging behaviour means that this species has a unique and direct exposure to crops treated with agrichemicals, and they are also common in reduced tillage agriculture where plant residues are retained. Farmers who depend on herbicides for weed control have requested research into: 'the impact, if any, of glyphosate (*N*-(phosphonomethyl) glycine) on soil life' (Stroud, 2020).

Glyphosate-based herbicides are typically mixed with an adjuvant and sprayed on the emerged plants (weeds, cover crops or crops to desiccate prior to harvest) where it is intercepted and adsorbed by the leaves, acting by inhibiting the shikimic acid metabolic pathway. This pathway is found in plants and some microorganisms, thus no direct impact on animals is expected. However, glyphosate influences saprotrophic fungal community structure (Wardle & Parkinson, 1992), can have fungicidal effects, or can be used as a nutritional source of Phosphorus by some fungal species (Spinelli, Ceci, Dal Bosco, Gentili, & Persiani, 2021). *Lumbricus terrestris* is a selective feeder, with a preference towards plant pathogens and early successional fungal species (Bonkowski, Griffiths, & Ritz, 2000; Doube, Schmidt, Killham, & Correll, 1997; Oldenburg, Kramer, Schrader, & Weinert, 2008).

To date, laboratory studies have reported *L. terrestris* surface casting activities were reduced by the use of glyphosate-based herbicides (Zaller et al., 2021). This includes a suggestion of an avoidance of glyphosate treated residues given their food supply would have increased (Gaupp-Berghausen, Hofer, Rewald, & Zaller, 2015). In comparison, a laboratory study stimulating glyphosate spraying for cereals did not negatively affect *L. terrestris* earthworms (Nuutinen et al., 2020). However, glyphosate seems to bioaccumulate in earthworms with implications for the animals which consume them for food (Pelosi et al., 2022).

Here, firstly the observation of midden activity differences between treated/untreated areas was quantified. Subsequently, fields were informally observed for the onset of this specific activity to better understand why this was happening. Plant materials were collected and bulked from 'treated/field-aged' and 'untreated' areas from two cereal field experiments. These were trials both being studied in terms of organic matter applications (Whitmore et al., 2017), one field had a small abundance of *L. terrestris* and middens (Stroud, Irons, Watts, & Whitmore, 2016) and the other did not, which was assumed (by the author) to be caused by tillage-related abundance differences (Stroud, Irons, Carter, et al., 2016), but was perhaps associated with unmeasured plant/soil properties. These results led to further questions about the timeline of anecic earthworm interactions with plant materials treated with glyphosate in the field (e.g., initial avoidance behaviour?). A field experiment with an abundance of plant volunteers (unwanted seedlings of the previous crop which was due to be sprayed off) was used. The experiment was extended to *Aporrectodea longa* because this species was abundant in this field, and before/after/rate of consumption of glyphosate-treated plant residues in terms of earthworm biomass was studied.

2 | MATERIALS AND METHODS

Plants and soils were collected from field trials at the Rothamsted Experimental Farm, Harpenden (51.80° N, −0.36° W, 128 m altitude), which has a temperate climate in the South of England. The soil is characterised as a flinty clay loam of the Batcombe soil series (on the NZ field trial, Fosters Field trial and Great Field trial).

2.1 | Midden counting

The NZ field trial is a non-inversion tillage experiment and has an active *L. terrestris* earthworm population as previously described (Stroud, Irons, Carter, et al., 2016). The experiment was under Winter Wheat (*Triticum aestivum* cv. Crusoe) and had received 15 active ingredients during cropping (Table S1). Two 0.5 × 4 m^2 strips on each experimental plot (8 × 4 m^2) were sprayed at 4 L ha^{-1} with a glyphosate-based herbicide (Samurai® containing 360 g L^{-1} glyphosate, as 441 g L^{-1} of the potassium salt of glyphosate) with 1 L ha^{-1} adjuvant (Firebrand™ 500 g L^{-1} ammonium sulphate). The Fosters field trial is 300 m from the NZ field trial, and is fully described elsewhere (Whitmore et al., 2017). Briefly, it is a plough-based experiment to develop models to estimate the effect of organic amendments on crop yields. In the summer of 2015, 12-weeks after treatment with glyphosate midden counting was performed on three replicate blocks (45 plots in total) using a 0.5 m^2 quadrat per plot, to count the number of middens in the herbicide-treated compared to the adjacent non-herbicide treated plot areas. Middens were identified as surface piles of plant debris, at least 5 cm in diameter, which when gently lifted by hand were underlain by a *c.* 5–10 mm diameter burrow, often lined with plant debris. On Fosters, as had been detected for several years (Stroud, Irons, Watts, White, et al., 2016) there was just surface straw.

2.2 | Plant collection for the earthworm feeding experiment

Plant sampling was performed in 2017 to investigate the effect of feeding these plant residues on *L. terrestris* biomass. All samples were bulked into 'treated/untreated' per field trial: spring barley (*Hordeum vulgare* cv. Irina) from the NZ field trial, winter wheat (*Triticum aestivum* cv. Crusoe) from the Fosters field trial and Oil Seed Rape (OSR, *Brassica napus* cv. Imperial) volunteers (small plants after harvest) plants from the Great Field trial. The spring barley had received four active ingredients during cropping (Table S1) and two 0.5 × 4 m^2 strips on each plot were sprayed at 3 L ha^{-1} with a glyphosate-based herbicide (Samurai® containing 360 g L^{-1} glyphosate, as 441 g L^{-1} of the potassium salt of glyphosate) along with 1 L ha^{-1} adjuvant (Buffalo Elite, ammonium sulphate). The Fosters winter wheat had received nine active ingredients during cropping and two 0.5 × 6 m^2 strips on each plot were sprayed at 3 L ha^{-1} with a glyphosate-based herbicide (Samurai® containing 360 g L^{-1} glyphosate, as 441 g L^{-1} of the potassium salt of glyphosate) along with 1 L ha^{-1} adjuvant (Buffalo Elite, ammonium sulphate). Cereal plants were cut at 3 cm above the soil surface on the glyphosate-based herbicide treated and non-treated areas on each plot when it was observed this plant material was being actively incorporated into middens on the NZ field trial, approximately 6-weeks after herbicide treatment. Plant material was collected at the same time on the Fosters field trial from the control, compost and FYM plots (matching its sister NZ experiment), although no midden formation was observed. OSR seedlings (volunteers after harvest) were treated with 4 L ha^{-1} with a glyphosate-based herbicide (Samurai® containing 360 g L^{-1} glyphosate, of 441 g L^{-1} as the potassium salt of glyphosate) along with 1 L ha^{-1} adjuvant (Buffalo Elite, ammonium sulphate). OSR seedlings were collected immediately prior and within 12 h of spraying with glyphosate. This was to compare a 'glyphosate' only treatment (the cereals were treated with a range of active ingredients, Table S1) and check the N-content because the adjuvant is ammonium sulphate. The reason why 'field aged' samples could not be collected 6-weeks later is because they were ploughed-in (conventional tillage arable rotation).

2.3 | Cereal straw preparation and analysis

The straw from each experiment was bulked into 'treated' or 'untreated' with glyphosate. Cereal straw was oven-dried at 80°C to enable fine milling using a hammer mill given the sensitivity of *L. terrestris* to cereal particle size (Sizmur et al., 2017). Subsamples were analysed for particle size using a 1 mm sieve and a balance; total N and C using a LECO TruMac Combusion Analyser and total elements using an acid digest followed by Inductively Coupled Plasma optical (ICP-OES) Emission Spectrometry by the Rothamsted Analytical Chemistry Unit; gross energy content using a PAR 6100 Bomb Calorimeter by Scientec Analytical Services Limited. Pesticide analysis was performed on the Fosters wheat straw and NZ barley straw (however there was insufficient glyphosate-treated barley straw for glyphosate analysis) using a standard acidified methanol/water extraction followed by analysis by liquid chromatography with mass spectrometric detection (HPLC-MS/MS) by FERA. Light and fluorescent microscopy was used to examine the cell wall size and structure. This was used to determine cell damage/decomposition processes had been initiated. Samples were mounted on glass slides in a drop of distilled water with a cover slip and imaged with a Zeiss Axiophot epifluorescence microscope using a Retiga EXT CCD digital camera (QImaging) and Metamorph software (Molecular Devices, USA). Images were taken using brightfield illumination and UV reflected light with fluorescent filter ex. 450–490 nm em. 520 nm LP. Fosters wheat straw phosphorus distributions were mapped in relation to the observed fungal hyphae by the Rothamsted Bioimaging department using energy dispersive x-ray spectroscopy. There was insufficient barley straw for this analysis. The properties of the straw are shown on Table S2 and microscope images in Figures S1 and S2. Please note, these tests were performed on a single bulked sample (rather than the analysis of pseudo-replicates) to inform interesting trends, so the data cannot be statistically confirmed.

2.4 | OSR preparation and analysis

Oil seed rape seedlings were used both as collected (fresh) and air-dried and finely ground (rate feeding assay and chemical analysis). Glyphosate analysis was performed using a standard acidified methanol/water extraction followed by analysis by liquid chromatography with mass spectrometric detection (HPLC-MS/MS) by FERA. Subsamples were finely milled using a hammer mill and analysed for total N and C and total elements by the Rothamsted Analytical Chemistry Unit as above. Light and fluorescent microscopy was used to examine the cell wall structure (as above). The properties of the OSR are shown on Table S2 and microscope images in Figure S3.

2.5 | Earthworm feeding bioassay

Soil was collected from the NZ and the Fosters field trials (cropping from 2012 to 2017 reported elsewhere; Whitmore et al., 2017) for the earthworm bioassay and was sent to Eurofins Limited to be screened for over 400 compounds (organo-chlorine pesticides, pyrethroids, organophosphorus pesticides, organonitrogen pesticides) using the PSP0C standard method. The pint glass method (Sizmur et al., 2017), was adapted for this quick screening bioassay. A 0.6 L Tupperware box was filled with soil for the 2-week screening assays and five air holes were placed in the lid. The box microcosm test was performed using four replicates per treatment, arranged in a randomised block design in the incubator (15°C in the dark) for 2 weeks. The difference between the initial mass and final mass of each earthworm was recorded and calculated as a percentage change.

2.5.1 | Glyphosate treated cereal straw using *L. terrestris* earthworms

Adult *L. terrestris* (5.19 ± 0.18 g) were used within 24 h on receipt from wormsdirect.co.uk, and the experiment was conducted using Fosters soil (560 ± 12 g per assay). A control (no straw), or 2 g of ground straw (barley, glyphosate treated barley, wheat, glyphosate treated wheat) were sprinkled over the soil surface. A total of 50 mL of water was dispensed onto the soil surface (soil was at a gravimetric moisture content of 29.5% ± 0.6%) and one weighed earthworm was added to each bioassay box. The experiment was repeated using the wheat and glyphosate treated wheat, with *L. terrestris* (5.38 ± 0.27 g) that had been incubated in Fosters soil for 1 week prior to use. The Fosters soil (500 ± 11 g per assay) had a gravimetric moisture content of 26.4% ± 0.5% after water application. The wheat experiment was repeated again using *L. terrestris* (5.71 ± 0.14 g) that had been incubated in NZ soil for 1 week prior to use, using NZ soil (473 ± 11 g per assay) with a gravimetric moisture content of 31.0% ± 0.8%. The reason for using the different field soils was that at that time, the pesticide analysis results were unknown, but NZ field had an abundance of middens compared to none on Fosters field. The differences between these fields had been assumed to be caused by tillage intensity (Stroud, Irons, Watts, White, et al., 2016).

2.5.2 | Glyphosate treated fresh OSR seedling leaves

Adult *L. terrestris* (5.54 ± 0.16 g) were used within 24 h on receipt from wormsdirect.co.uk, and the experiment was conducted using NZ soil (460 ± 12 g per assay). A control (no amendment), or 20 g fresh (equivalent of 2 g dried) seedlings (OSR prior to glyphosate spraying or 24 h after spraying) were added to the soil surface. A total of 50 mL of water was dispensed onto the soil surface (to a gravimetric moisture content of 32.3% ± 1.7%) and one weighed earthworm was added to each bioassay box.

2.5.3 | Glyphosate treated OSR seeding leaves at different feeding rates

Lumbricus terrestris (5.86 ± 0.2 g) was used within 24 h on receipt from wormsdirect.co.uk. *A. longa* adults (2.32 ± 0.11 g) were used within 24 h after collection from the margins of the Highfield field experiment (which is adjacent to the Great Field Experiment where the seedlings were collected) by using a mustard solution (1 tablespoon mustard powder to 1 L of water) to bring the earthworms to the surface on areas with extensive earthworm casting activities. The experiment was conducted in the NZ field trial soil using finely ground glyphosate treated OSR leaves at 0, 1, 2, or 4 g rate. The reason for comparing the anecic earthworms is that *L. terrestris* were large (*c.* 5–6 g), purchased and not previously exposed to agrichemicals whereas *A. longa* is much smaller (*c.* 2 g), arable field collected and locally abundant on the Great Field experiment (extensive casting activities). That is, NZ field had an abundance of *L. terrestris* middens, Fosters field had neither middens nor casting, and Great Field had an abundance of *A. longa* (as indicated by earthworm castings). The reason for this range of feeding rates (1–4 g, which is *c.* 4–16 g kg^{-1} per month) is that previous authors have found up to 1 g increase in *L. terrestris* earthworm biomass with feeding rates of 6 g kg^{-1} per month (Sizmur et al., 2017).

2.6 | Statistical analyses

All plant chemical characterisation is reported on a dry weight basis. Genstat (18th edition, 18.1.0.17008, VSN International Ltd., UK) was used to perform the statistical analyses. General ANOVA (Analysis of Variance) was used for midden counting assessments with the following parameters: Block = block/plot, Treatment = treatment; where 'treatment' was a two-factor category, comparing glyphosate treated to untreated areas. The residual graphs indicated that no transformation was required to meet the normality assumption. For the feeding assay comparing crop

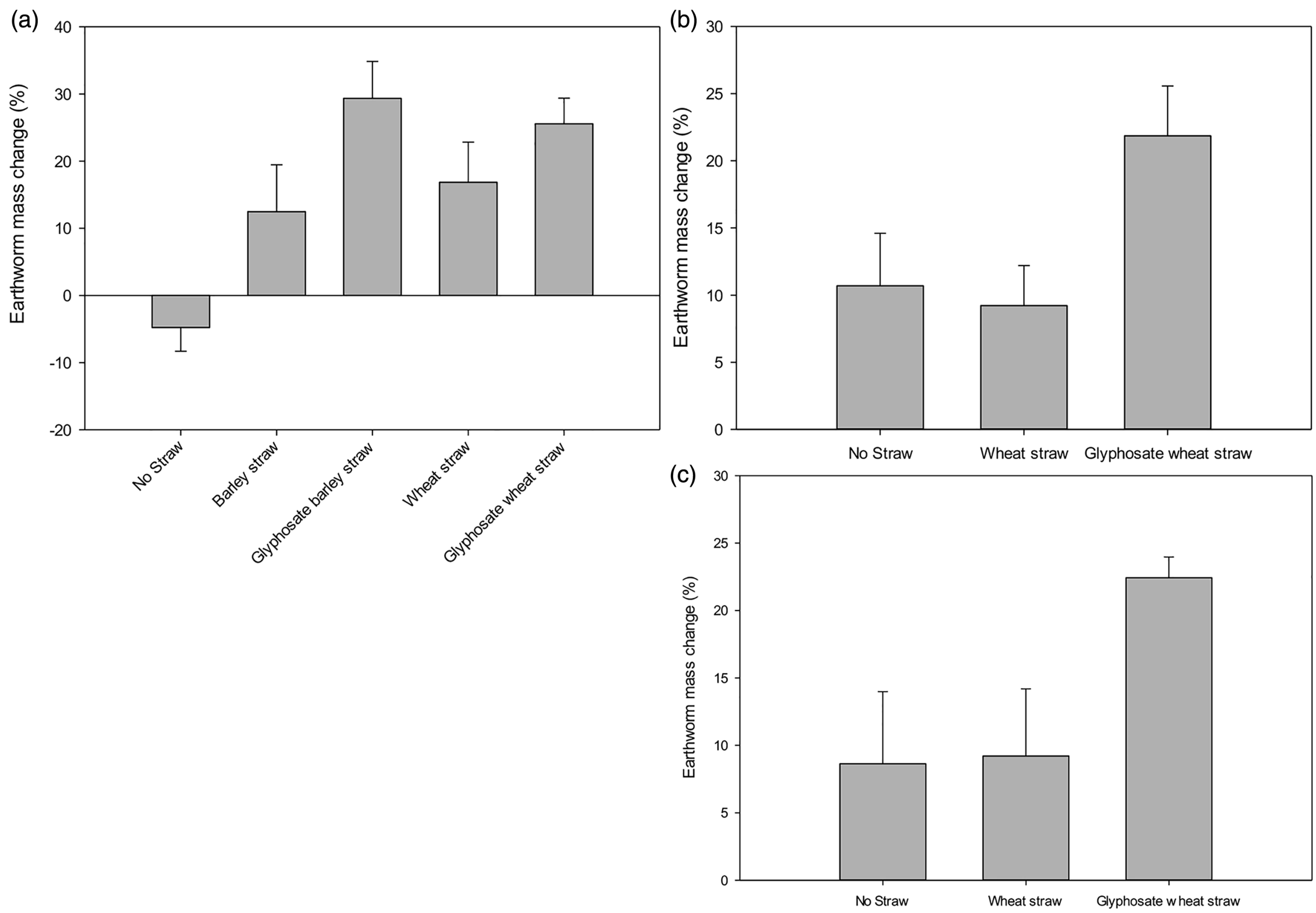

FIGURE 1 Percentage earthworm biomass change from feeding with cereal straw treated with or without glyphosate (a) wheat from the Fosters experiment and barley straw from the NZ experiment, (b) repeat of the wheat experiment in Fosters soil (c) repeat of the wheat experiment in NZ soil.

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types the parameters were: Block = block, Treatment = rate/(crop × treatment), where 'rate' was the amount of straw (0–4 g), 'crop' was barley or wheat, and 'treatment' was glyphosate treated or untreated straw. For the repeated feeding assay and OSR feeding assays the parameters were: Block = Block, Treatment = rate/treatment, as above. The residual graphs indicated that no transformation was required to meet the normality assumption. There was one *A. longa* death in experiment 2.5.3 and managed as a 'missing' result as this was the only mortality recorded during these bioassays ($n = 100$ earthworms).

3 | RESULTS

3.1 | Midden counting

There were significantly ($F_{1,44} = 8.67$, $p = .005$) more middens found on the glyphosate-treated areas than the non-glyphosate treated areas on the NZ winter wheat field trial.

3.2 | Characterisation of the soil and plant materials

The agricultural field soils and straws used for the earthworm bioassay had received a range of pesticides (Table S1) and were screened for general pesticide residues to provide the agricultural context for this post-monitoring of pesticides research activity. Concentrations of epoxiconazole were detected in Fosters soil at 0.11 mg kg^{-1} and straw at 0.070 mg kg^{-1} and NZ soil at 0.10 mg kg^{-1}. Epoxiconazole was not used on the NZ spring barley experiment and was not detected in the spring barley straw. No glyphosate was detected in the non-glyphosate treated plants and the wheat straw had a glyphosate residue level of 2.7 mg kg^{-1}. There was an indication that there were higher concentrations of N and P (and other macro-nutrients) in the glyphosate treated straws, and they had the same energy content (Table S2). There was no evidence for plant cell wall breakdown (i.e., decomposition) in the glyphosate treated or untreated plants (Figure S1), and there was little evidence of a spatial relationship between fungal hyphae and elemental P distributions (however, fungal hyphae encrusted glyphosate treated straw) (Figure S2). OSR seedlings measured before and after glyphosate spraying had the same N content (Table S2) and there was no change in cell wall structure (Figure S3). The glyphosate treated leaves had a glyphosate residue level of 62 mg kg^{-1}.

3.3 | Earthworm feeding assays

Glyphosate treatment (field aged) was a statistically significant factor affecting earthworm *L. terrestris* biomass ($F_{1,12} = 5.75$, $p = .03$, Figure 1a). In the repeated experiments, glyphosate treatment (field aged) was a statistically significant factor affecting earthworm *L. terrestris* biomass in both soil types ($F_{1,6} = 4.8$, $p = .042$; $F_{1,6} = 6.11$, $p = .048$, Figure 1b,c). Glyphosate treatment (comparison of fresh leaves) was not a statistically significant factor affecting earthworm *L. terrestris* biomass ($F_{1,6} = 0.16$, $p = .92$, Figure 2). There was a statistically significant rate of food (dried, ground, glyphosate treated OSR) leaves affecting earthworm biomass [$r = .72$ (*L. terrestris*), $n = 16$, $p < .05$, F-test of the correlation; $r = .73$ (*A. longa*), $n = 15$, $p < .05$, F-test of the correlation].

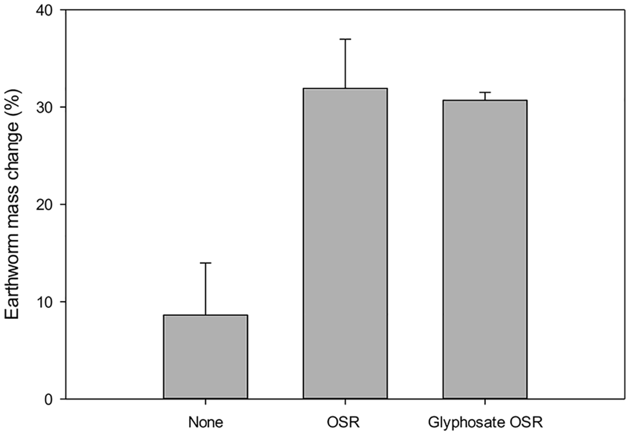

FIGURE 2 Percentage earthworm biomass change from feeding with oil seed rape treated with or without glyphosate in the NZ soil.

4 | DISCUSSION

There were 23% more *L. terrestris* middens on glyphosate-treated areas than on the non-glyphosate treated areas on the NZ winter wheat field trial, and no middens on the control/compost/FYM Fosters field trial plots (in agreement with a larger, previous study; Stroud, Irons, Watts, White, et al., 2016). The differences between the field trials are likely to be caused by differing *L. terrestris* populations, there is an active population on the NZ minimum tillage field trial (Stroud, Irons, Carter, et al., 2016) and negligible populations on the Fosters conventional tillage field trial (Whitmore et al., 2017). Tillage intensity is detrimental to populations of midden-building earthworm species (Briones & Schmidt, 2017). There was no evidence for avoidance behaviours associated with glyphosate-treated, field-aged plant materials, earthworms gained biomass feeding on straw from both field trials (Figure 1). This result differs from the laboratory studies which detected reduced activities (Gaupp-Berghausen et al., 2015) which could be explained by timings, the laboratory studies followed earthworm responses to spraying, whereas here field-aged glyphosate plant materials were used to understand an increase in activity observed in the field.

In terms of the laboratory feeding study, the glyphosate-treated (field aged) plant materials significantly ($p < .05$, F-test) increased earthworm biomass over the untreated plant materials (Figure 1). There was no evidence for decomposition (breakdown of cell walls, Figure S1) or energy content between the plant materials (Table S2). There was an indication this may be linked to an increased nutrient value (N, P and macronutrients, Table S2) and fungal hyphae which

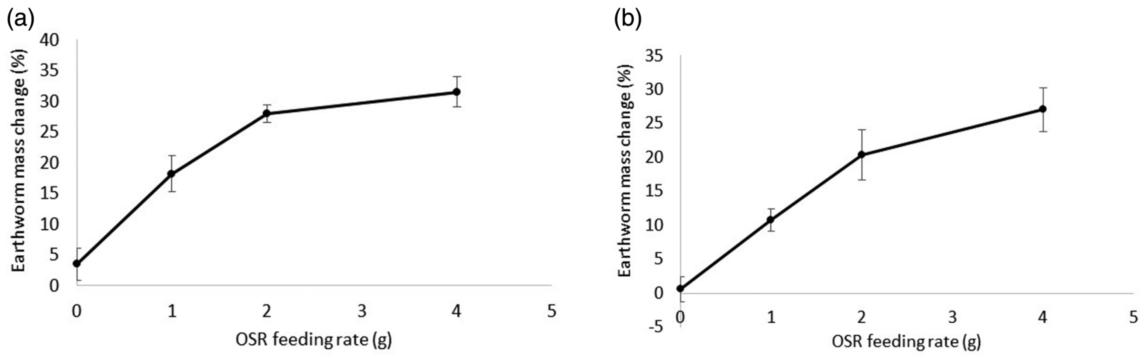

FIGURE 3 Biomass change from feeding with increasing rates of oil seed rape treated with glyphosate (a) *L. terrestris* earthworms and (b) *A. longa* earthworms in the NZ soil.

encrusted the glyphosate treated cereal straw (Figure S2). This suggests the effects were caused by fungal conditioning/priming. That is, the colonisation of the straw by fungi forming a litter-fungus complex that improved the nutrient(s) to C ratio, thus improves macronutrient food quality for the *L. terrestris*. This is a novel finding, and the improved macronutrient food quality would likely explain the stimulation in earthworm activity (midden building) after glyphosate treatment detected on the NZ field experiment. These results suggest that agricultural systems where glyphosate treated plants are retained (e.g., conservation tillage management practices), leads to saprotrophic fungal succession patterns which improve the macronutrient food quality and subsequent biomass of anecic earthworms. This may help to explain the trend of increased abundance and biomass when glyphosate is included in the rotation (Briones & Schmidt, 2017). Other authors have detected an increase in feeding activity by earthworms after the application of glyphosate which could not be explained by the variables (soil moisture, food supply) measured (Reinecke, Helling, Louw, Fourie, & Reinecke, 2002; Santos, Morgado, Ferreira, Soares, & Loureiro, 2011).

Our results are within the context of post-registration monitoring, that is, concentrations of epoxiconazole were detectable in both soil and straw used for these bioassays. The bioavailability of epoxiconazole is beyond the scope of this study, but as a fungicide, it may influence the colonisation of the straw by the hypothesised litter-fungus complex. To date, the effects of epoxiconazole include a tolerance by earthworms to this chemical via an accelerated activation of a detoxification enzyme (Givaudan, Binet, Le Bot, & Wiegand, 2014), increased burrowing behaviour, which stimulates pesticide degradation (Givaudan et al., 2014) and potential bioaccumulation by earthworms (Pelosi et al., 2021).

To determine the effect of glyphosate-only field treated plants on earthworm biomass, OSR volunteers (unwanted seedlings of the previous crop) were collected immediately before and after glyphosate spraying. There was no change in the N content of OSR nor change in cell wall structure, indicating that the adjuvant does not cause increased N contents and no biodegradation had occurred. There was a significant ($p < .05$) difference in *L. terrestris* biomass in comparison to the control where no food was provided (Figure 2). This effect is not limited to *L. terrestris*, as field collected endo-anecic *A. longa* were fed glyphosate treated leaves and gained biomass over the control (no food) (Figure 3b). This could be problematic because anecic earthworms have a unique exposure to pesticides from their foraging and feeding behaviours towards plant materials, and glyphosate may bioaccumulate in earthworms which may have implications for their fitness and has implications for the animals which consume them for food (Pelosi et al., 2022).

In terms of farmers' information needs, specifically the impact of glyphosate on soil life (Stroud, 2020), and scientific calls for improving environmental realism (Topping et al., 2020), it seems that the use of glyphosate stimulates the colonisation of plant residues by a litter-fungus complex that increases foraging and feeding activities by anecic earthworms. Whilst this can be linked to increasing the macronutrient food quality for anecic earthworms, the plant materials may also contain other pesticide residues (e.g., epoxiconazole) and are a direct source of pesticide exposure to soil organisms.

ACKNOWLEDGEMENTS

This work was supported by a NERC (NE/N019253/1) fellowship.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Stroud, J. L., & Halsey, K. (2023). The post-registration monitoring of glyphosate-treated plants using anecic earthworms. *Annals of Applied Biology*, 1–7. https://doi.org/10.1111/aab.12838