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Glyphosate but not Roundup® harms earthworms (*Eisenia fetida*)

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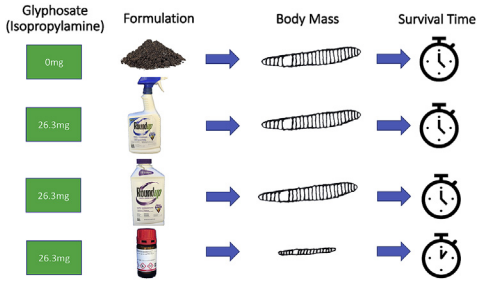
HIGHLIGHTS

- Exposure to pure glyphosate caused a 14.8–25.9% loss in worm body mass.
- Exposure to glyphosate caused worms to die 22.2–33.3% faster in a stress test.
- Exposure to two Roundup® formulations did not impact worm health.
- Soil microbial and fungal biomass was unaffected by pesticide contamination.

GRAPHICAL ABSTRACT



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ABSTRACT

Glyphosate is the active ingredient in Roundup® formulations. While multiple studies have documented the toxicity, environmental persistence, and tendency to spread for glyphosate and Roundup®, few studies have compared the toxicity of glyphosate-based formulations to the toxicity of pure glyphosate for soil invertebrates, which contact both the herbicide and the formulations. Hundreds of formulations exist; their inert ingredients are confidential; and glyphosate persists in our food, water, and soil. In this experiment, we held glyphosate type and concentration constant, varying only formulation. Using Roundup Ready-to-Use III®, Roundup Super Concentrate®, and pure glyphosate, we delivered 26.3 mg glyphosate in the form of isopropylamine salt per kg of soil to compost worms (*Eisenia fetida*). We found that worms living in soil spiked with pure glyphosate lost 14.8–25.9% of their biomass and survived a stress test for 22.2–33.3% less time than worms living in uncontaminated soil. Worms living in soil spiked with Roundup Ready-to-Use III® and Roundup Super Concentrate® did not lose body mass and survived the stress test as well as worms living in uncontaminated soil. No contaminant affected soil microbial or fungal biomass over the 40-day period of this experiment. We suggest that the nitrates and phosphates in the formulations offset the toxic effects of glyphosate by spurring microbial growth and speeding glyphosate degradation. We also found a 26.5–41.3% reduction in fungal biomass across all treatments over the course of this experiment, suggesting that the worms consumed fungi and spores.

1. Introduction

Humans extensively use glyphosate-based herbicides, including Roundup® and its many formulations in agriculture, horticulture, forestry and urban settings (Myers et al., 2016). Roundup Ready® crops, plants modified to withstand Roundup® applications,

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entered the US market in 1990's, and this tool allowed agriculture to undergo a paradigm shift, implementing zero tillage, genetically modified seeds, and direct seeding (Myers et al., 2016; Bach et al., 2018). Roundup® herbicides are currently the most frequently used pesticide in the agricultural sector, and the second most frequently used pesticide in urban settings (Battaglin et al., 2014). They are also common in forestry and horticulture (Sihtmäe et al., 2013). Given the abundance of glyphosate-resistant weeds and the pre-harvest, desiccant-use patterns (e.g. "green burndown") that rely on Roundup® products, researchers expect the use of this herbicide to increase in upcoming years (Myers et al., 2016).

Researchers estimate that less than 0.1% of pesticides applied to crops worldwide reach their specific targets, freeing large amounts to move throughout connected ecologies (Nguyen et al., 2016; de Brito Rodrigues et al., 2017). Consequently, researchers find glyphosate in our water systems, including precipitates (Battaglin et al., 2014; Alonso et al., 2018), our soil systems (Lane et al., 2012; Battaglin et al., 2014; Alonso et al., 2018; Niemeyer et al., 2018) and, because current agricultural practices prescribe the spread of glyphosate-based herbicides on edible genetically modified plants, we also find glyphosate in our food systems (Conrad et al., 2017; Mertens et al., 2018). Glyphosate can persist in soil for months or years; AMPA, its major metabolite, degrades even more slowly (Laitinen et al., 2009; Mertens et al., 2018). The environmental fate of the Roundup® ingredients associated with glyphosate remains largely unknown.

US regulators divide pesticide ingredients into two categories: active and inert. Despite their name, inert ingredients may be biologically or chemically active; manufacturers label them inert only because of their function in the formulated product. Most regulatory tests required to register a pesticide are performed using only the active ingredient, not the full pesticide formulation, and product labels generally do not identify inert ingredients. Additionally, inert ingredients are often claimed to be confidential business information (Cox and Surgan, 2006; Mesnage et al., 2019), making toxicity assessments of the actual products used on our foods and in our gardens even more difficult.

Manufacturers produce between 130 and 750 unique Roundup formulations (de Brito Rodrigues et al., 2017; Reno et al., 2018). These formulations use glyphosate in the forms of isopropylamine, diammonium, monoammonium, potassium, trimethylsulfonium and sesquisodium salts (Travlos et al., 2017). Aside from the various salts, the inert ingredients are numerous, varied, and mostly unidentified. For example, some European glyphosate-based formulations consist of 1–15.5% polyoxyethylenamines (POEAs) with an average ethoxylation of 15 carbons (POE-15); others use a combination of C8-10 ethoxylated alcohol, triethylated glycol and monobutyl ether. One formulation contains 10–20% alkyl polyglucoside; another contains petroleum distillate; still another uses quaternary ammonium compounds (Defarge et al., 2018). The majority of the formulants remain unidentified (Defarge et al., 2018).

Comparative toxicity assessments between Roundup® formulations and pure glyphosate are most common amongst aquatic ecosystems and, more recently, mammalian cell lines. These studies consistently show that Roundup® formulations are more toxic than glyphosate alone. (See Gill et al. (2018) for a review.) In fact, the toxicity of Roundup® formulations to aquatic systems prompted the European Union to replace the first-generation POEAs with propoxylated quaternary ammonium surfactants. This updated class of POEA surfactants is about 100 times less toxic to aquatic ecosystems and human cells than previous versions (Mesnage et al., 2019).

Since Roundup® products are so popular, soil microbes frequently encounter glyphosate-based contamination before aquatic ecosystems and cell lines do. Not only do the formulations reach the soil directly when used on commercial crops and urban weeds, but after foliar application, plants translocate both Roundup® and glyphosate through the leaves and out through the roots (Laitinen et al., 2007). This delivers the contamination directly into the soil microbial community (Laitinen et al., 2007). If, as in aquatic systems, soil microbes find Roundup® formulations more toxic than pure glyphosate, soil ecologies across the globe will be impacted.

As soil dwellers, earthworms are also easy and probable non-target organisms for glyphosate and its formulations. The impact of this contamination on worms concerns soil ecologists because worms play a critical role in soil ecology (reviewed in Edwards, 2004). Earthworms' contributions to soil health are so critical that the European Union (EU), the Organization for Economic Co-operation and Development (OECD), the International Organization for Standards (ISO), and the Food and Agriculture Organization of the United Nations (FAO) all use earthworms (*Eisenia fetida*) as an indicator organism for ecotoxicological testing (Piola et al., 2013; Santadino et al., 2014).

In sum, at the regulatory level glyphosate is tested alone, formulations differ in glyphosate concentrations and salt type, and formulations vary in their types and proportions of ostensibly inert (but still potentially toxic) ingredients. In this experiment, we evaluate and compare the acute toxicity of two glyphosate-based herbicides commonly used in an urban setting—Roundup Ready-to-Use III® and Roundup Super Concentrate ®—as well as pure glyphosate to soil dwellers. We hold glyphosate type (isopropylamine) and concentration (26.3 mg a.i./kg) constant and evaluate the effects of the three solutions on compost worms (*Eisenia fetida*), soil microbe and soil fungal biomass.

2. Materials and methods

2.1. Data analysis

Throughout this research, after determining that all variables were distributed normally using a Kolmogorov-Smirnov/Lilliefor test, we tested for differences between means using one-way, parametric ANOVAs significant at the 0.05 level. When we detected a difference in means between the four treatment groups, we followed up with post-hoc Scheffe tests, which is designed for unplanned comparisons, to determine where the differences lay. We ran all analyses in StatPlus version v6.

2.2. Soil preparation

For this experiment, as in Pochron et al. (2019), we used OMRI-listed compost. OMRI-listed materials are certified by the Organic Materials Review Institute. OMRI is accredited to ISO 17,065 standards by the USDA Quality Assessment Division and ensures that materials used in organic food production, such as our compost, meet organic standards. This means that the compost used in this experiment was free of fertilizer, pesticides and animal-care products.

We placed 64 kg of OMRI-listed Black Gold Garden Compost Blend® soil into a clean cement mixer and rotated the soil for 10 min in order to homogenize soil moisture and distribution of soil microbes and fungus. We prepared 13 glass mesocosms (21 cm × 40 cm x 25 cm) by washing with Sparkleen™ and rinsing with Reverse Osmosis (RO) water. We placed 4.0 kg (weighed using a WASING Touch Digital Tempered Glass Scale 10 kg WS-YHC1518B, accurate to 1.0 g) of soil and 0.5 L RO water into each mesocosm; we then mixed with gloved hands until homogenized. Twelve of these mesocosms housed worms for the experiment. From the thirteenth

mesocosm, we extracted a soil sample and sent it to the Cornell Nutritional Analysis Laboratory (CNAL) for analysis which reported a pH of 7.51, a LOI of 18.16%, and organic matter of 12.49%.

At the experiment's completion, we relied on CNAL to quantify the soil respiration rates of the four treatments. CNAL uses an alkali absorption method to measure soil respiration (Cornell University, 2016). At the experiment's completion, while wearing nitrile gloves, we extracted 125 mL soil from each of the three replicates within a treatment and placed the soil in a stainless-steel bowl. We mixed the contents thoroughly by hand. Following CNAL's instructions, we placed 250 mL soil from each mixture into a ziplock bag, placed that bag into a second ziplock bag, and labeled it. We shipped the sample via USPS Express Mail.

2.3. Earthworms

To ensure that our test subjects responded only to purposefully introduced contaminants rather than accidental ones (such as glyphosate-tainted food [sensu Séralini et al., 2014], contaminated soil, or leaching from BPA-based plastic bins), we used a stock of organically raised *Eisenia fetida* earthworms as described in Pochron et al. (2019). Although some categorize this species as epigeic (e.g. Gomez-Brandon et al., 2011), it generally lives in manure piles and in compost, rather than in fields. We used this compost worm species rather than a crop species because its status as an indicator species makes it popular in ecotoxicological studies (e.g. Correia and Moreira, 2010; García-Torres et al., 2014; Santadino et al., 2014), and therefore we have a lot of information about how it responds to contamination. Additionally, it is easily grown and maintained in a laboratory setting.

On October 23, 2018, we extracted 200 adult earthworms (maturity indicated by a developed clitellum) from the organic stock population. Because earthworms lose weight due to handling (Pochron et al., 2017), we allowed them to remain overnight in a container before we weighed them using American Weigh Scales ACP-200, accurate to 0.01 g. We excluded the eight smallest worms, which weighed less than 0.10 g. We then sortied worms by body mass, dividing the population into 12 groups of 16 earthworms so that each group had similar mean body mass. After assessing initial soil microbial and fungal biomass (see below) and adding the contaminants (see below), we released 16 earthworms into each of the 12 mesocosms and stored the mesocosms in a Convrion® growth chamber set to a 12/12 light/dark cycle and a temperature of 22 °C. We placed screened lids over the mesocosms to deter earthworm migration. We watered the mesocosms regularly throughout the experiment: mesocosms received equal amounts of water on days when the soil appeared dry (García-Torres et al., 2014; Pochron et al., 2017, 2018; 2019).

2.4. Roundup® and glyphosate contamination

We created three replicates per treatment and three replicates of the control. Since the goal of this project was to determine if Roundup formulations caused more health problems for earthworms than glyphosate alone, we created three treatment groups using equal amounts and type of glyphosate but different Roundup formulations. We contaminated the soil of all three treatments (each consisting of three mesocosms housing 16 worms) with 26.3 mg of glyphosate per kg soil. We selected this concentration because it is consistent with many ecotoxicology experiments using earthworms (e.g. Correia and Moreira, 2010; Buch et al., 2013; García-Torres et al., 2014; Pochron et al., 2019) and because it is consistent with contamination amounts when these particular products are used as directed (Pochron et al., 2019). A full review of concentrations used in earthworm ecotoxicology studies involving Roundup® is available in Pochron et al. (2019), Table S1.

To keep liquid content constant across mesocosms, we mixed 5.2 mL of Roundup Ready-to-Use-III® into 4.0 kg soil (Treatment 1); 0.174 mL Roundup Super Concentrate® plus 5.026 mL Reverse Osmosis water into 4.0 kg soil (Treatment 2); and 0.105 g glyphosate plus 5.09 mL of Reverse Osmosis water into 4.0 kg of soil (Treatment 3). For the control, we added 5.2 mL of Reverse Osmosis water into 4.0 kg of soil. We then added 500 additional mL of Reverse Osmosis water to all 12 mesocosms and mixed by hand wearing nitrile gloves until the soil was homogenized.

We purchased the glyphosate (*N*-(Phosphonaomethyl) glycine), which is an isopropylamine salt, from Aldrich in a form with 96% purity. The two Roundup formulations—Roundup Super Concentrate® and Roundup Ready-to-Use-III®—were purchased from local lawn and garden stores. Both formulations list isopropylamine salt as the active ingredient.

2.5. Soil microbial and fungal biomass

Taking three samples of 0.5 mL soil from each mesocosm and using microBIOMETER® (Prolific Earth Sciences, Montgomery, NY) soil test kits, we determined the microbial and fungal biomass in each of the mesocosms prior to exposure to contaminants. Kit users separate microbes from the soil particles to which they are attached by combining the soil with a solution of salt and detergent and whisking for 30 s. Within 10 min of whisking, the soil particles have settled, and users apply the extracted microbes to a membrane. Fungi are measured by staining the microbial biomass on the membrane. The intensity of the resulting color correlates with microbial biomass and separately with fungal biomass, and can be estimated using cell phone cameras and the microBIOMETER® app. MicroBIOMETER developers report that kit data has been correlated with PLFA (Phospholipid-derived Fatty Acid) analysis with $r = 0.91$. It delivers biomass in units of μg microbes per g of soil or μg fungus per g of soil. At the experiment's inception, all mesocosms had equal biomass of earthworms, microbes and fungus.

2.6. Earthworm health parameters

Consistent with many earthworm ecotoxicology studies (see Pochron et al., 2019, Table S1, for a review of methodologies), worms in this study had been exposed to contaminants for 40 days (October 31-December 10, 2018) when we extracted the worms to collect data on health parameters. These parameters include survivorship, body mass (g), and survival time (min) under conditions of heat and light stress. Most earthworms would not reproduce during the short period of this experiment; body size and clitellum development enable us to distinguish adult test subjects from any offspring that might occur (Pochron et al., 2017, 2018; 2019). After extraction, we thus counted and weighed the adult worms to determine survivorship and final body mass. We then placed each worm in a labeled 35 mm Petri dish to quantify survival time, as per below.

To quantify stress-test survival time, we followed Pochron et al. (2017, 2018, 2019). Specifically, we used a Conviron CMP6050 growth chamber programmed to a temperature of 35 °C and a light intensity (photon flux) of 250 μmol m^2 s^{-1} to detect sublethal effects caused by exposure to the contaminants. We placed the Petri dishes containing weighed earthworms into the chamber, exposing them to heat and light stress. We had labeled the bottom of each dish so as avoid providing shade to the worms within. All Petri dishes were inspected at 5-min intervals to determine the time of death for each earthworm. Following Pochron et al. (2017, 2018, 2019), earthworms were classified as dead when they failed to respond to gentle mechanical stimulus.

3. Results

3.1. Earthworm body mass

At the experiment's initiation, we verified that our sorting method generated 12 groups of worms with equal mass with a one-way ANOVA (DF = 11, 180, F = 0.12,493, P = 0.99). After completing the experiment but before examining the effect of contamination type on worm body mass, we used a one-way ANOVA to determine if we could treat all earthworms from each of the three replicates in their treatment as if they came from one population. Three of the four treatments produced consistent results; the glyphosate treatment produced one mesocosm with a higher mean body mass than the other two. Table 1 provides the means, standard deviations, and test statistics for this test. Because viable arguments exist in support of both including and excluding the data (see Discussion), we report the findings below with and without the outlying mesocosm. Additionally, since that one glyphosate-contaminated mesocosm consistently produced outlying worm-health data, we report analyses with and without it throughout this report.

Contamination significantly impacted body mass in adult earthworms as per a one-way ANOVA (outlier excluded: F = 8.91, DF = 3, 133, P = 0.00002; all data: F = 8.91, DF = 3, 146, P = 0.007). See Table 1. A post-hoc Scheffe test on the outlier-free data indicates that the mean mass of the worms living in glyphosate-contaminated was significantly lower than the mean for those living in uncontaminated soil, soil contaminated with Roundup Ready-to-Use III®, and soil contaminated with Roundup Super Concentrate®. See Fig. 1 and Table 2. Using all data, a post-hoc Scheffe test indicates that the mean mass of the worms living in glyphosate-contaminated was significantly lower than the mean for those living in Roundup Ready-to-Use III® and perhaps Roundup Super Concentrate®, which had a p-value of 0.08. See Table 2.

At the project's initiation on October 24, 2018, the mean body mass of the worms was 0.27 g ± 0.09, with a minimum of 0.11 g and a maximum of 0.52 g. The final body mass was 0.20 g ± 0.06 (outlier excluded) or 0.23 g ± 0.07 (all data); worms living in the glyphosate-contaminated mesocosms showed a 14.8–25.9% decrease in body mass over the 40-day duration of the experiment.

3.2. Stress-test survival time

We used a one-way ANOVA to determine if we could treat all earthworms from each of the three mesocosms in their treatment as if they came from one population. As above, three of the four treatments produced consistent results; the same glyphosate-contaminated mesocosm that produced outlying body mass data also produced worms with a higher mean stress-test survival time than the other two. When we excluded this mesocosm, we found that the mean survival time of earthworms living in the two mesocosms contaminated with glyphosate did not significantly differ from each other. Table 1 provides the means, standard deviations, and test statistics for this test. We report the findings below with and without data from the outlying mesocosm.

Contamination significantly impacted stress-test survival time as per a one-way ANOVA (outlier excluded: F = 7.66, DF = 3, 133, P = 0.00009; all data: F = 4.73, DF = 3, 146, P = 0.003). See Table 1. Post-hoc Scheffe tests indicate that the mean stress-test survival time of worms living in glyphosate-contaminated was significantly lower than the mean for those living in uncontaminated soil, soil contaminated with Roundup Ready-to-Use III®, and soil contaminated with Roundup Super Concentrate®, regardless of dataset. See Fig. 2 and Table 2.

The average stress-test survival time of worms exposed to uncontaminated soil, soil contaminated with Roundup Ready-to-Use III, and Roundup Super Concentrate produces was 176.98 ± 59.20 min; worms exposed to glyphosate alone had a mean survival time of 117.96 ± 46.59 min (outlier excluded) or 137.9 ± 54.02 min (all data). Worms from glyphosate-contaminated soil had a 22.2–33.3% drop in stress-test survival time relative to the other worms.

3.3. Earthworm mortality/migration

We originally placed 16 earthworms into each of 12 mesocosms; 40 days later we retrieved between nine and 15 adult worms from each mesocosm. A one-way ANOVA found no difference across treatments (outlier excluded: F = 0.81, DF = 3, 172, P = 0.49; all data: F = 0.52, DF = 3, 188, P = 0.67). Despite the fact that the mesocosms were covered, earthworms sometimes escape (pers. obs.), and we cannot determine whether the earthworms died or migrated. Percentages of deaths or migrations are provided in Table 1.

3.4. Soil microbial biomass

After taking three readings from each mesocosm before adding contaminants, we calculated 687.3 μg microbes g^{-1} soil ±115.0 (N = 36). A one-way ANOVA detected no significant difference between the mean microbial biomass of the four groups (F = 0.99, DF = 3, 32, P = 0.41); at the experiment's inception, all mesocosms had equal microbial biomass. Because one of the glyphosate-contaminated mesocosms produced outlying worm-health data, we include results with and without that mesocosm below. However, that mesocosm did not differ from the others in its microbial biomass.

The final mean microbe biomass, based on three readings from each of three replicates for each treatment type, are provided in

Table 1
Earthworm health parameters. The first columns provide mean and standard deviations of earthworm body weights (g) and stress-test survival time (min) after 40 days exposure to contamination. We show results from analysis with and without data from the one mesocosm that generated the outlying data. The F test statistic, degrees of freedom (DF) and P-values were generated from within treatment comparisons executed to ensure that worms from each of the mesocosms could be treated as if they came from the same treatment population. Survivorship shows the numbers of worms recovered at the end of the experiment divided by the number of worms subjected to each treatment.

Treatment	Body Mass					Stress-test Survival Time					Survivorship
	Mean(g)	Standard Deviation	F	DF	P	Mean (mins)	Standard Deviation	F	DF	P	Percent
Control	0.25	0.003	4.31	2, 37	0.35	180.25	62.69	3.18	2, 37	0.06	(40/48) 83.3
Roundup® Ready-to-Use III	0.28	0.06	2.53	2, 34	0.10	176.08	59.20	1.91	2, 34	0.16	(37/48) 77.1
Roundup® Super Concentrate	0.27	0.07	0.15	2, 35	0.86	174.61	37.44	1.35	2, 35	0.28	(38/48) 79.2
Glyphosate (no outlier)	0.20	0.06	0.02	1,20	0.90	117.96	46.59	0.98	1, 20	0.33	(22/32) 68.8
Glyphosate (all data)	0.23	0.07	8.31	2, 32	0.001	137.86	54.03	5.56	2, 32	0.01	(35/48) 72.9

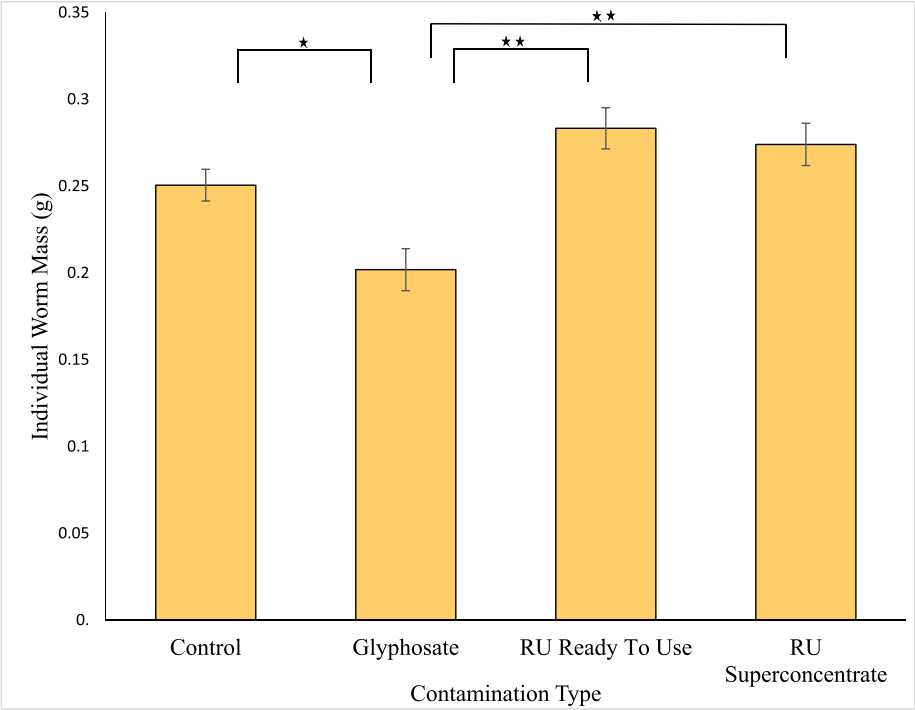

Fig. 1. The impact of contamination type on earthworm biomass (g). A one-way ANOVA detected a significant difference between the four groups (outlier excluded: F = 8.91, DF = 3, 133, P = 0.00002; all data: F = 8.91, DF = 3, 146, P = 0.007). Excluding the outlier, a post-hoc Scheffe test finds the significant differences shown above, with one star showing a p-value below 0.05 and two stars showing a p-values below 0.001. When we include the outlier, the mean worm mass in the glyphosate does not differ from that of the control worms, and the difference between Roundup® Superconcentrate and glyphosate approaches significance.

Table 2
Post-hoc Comparisons. After one-way ANOVAs indicated that contamination significantly impact earthworm body mass and stress-test survival time, we use post-hoc Scheffe tests to determine where those differences occurred. When we excluded data from the one glyphosate-contaminated mesocosm (culled data), for worms living in glyphosate-contaminated soil, the body mass was significantly lower and the stress-test survival time was significantly shorter than that of worms from all other treatments. When we used all data, worms living in glyphosate-contaminated soil survived the stress test for significantly fewer minutes, but the results concerning body mass became less consistent.

Comparisons	Body Mass				Stress-test Survival Time			
	Culled Data		All Data		Culled Data		All Data	
	F	P	F	P	F	P	F	P
Glyphosate vs. Control	2.93	<0.05	1.10	0.75	4.41	<0.001	3.37	≤0.01
Glyphosate vs. Roundup® Ready-to-Use III	4.83	<0.0001	3.20	≤0.02	4.06	<0.001	2.98	≤0.05
Glyphosate vs. Roundup® Super Concentrate	4.30	<0.001	2.61	0.08	3.97	<0.002	2.88	≤0.05

Table 3
Soil respiration, mean and standard deviations of the final soil microbial and fungal biomasses and the difference between the initial and final biomasses. An ANOVA detected no significant differences in any of the biomass comparisons.

Treatment	Soil Respiration (mg CO2/g soil)	N	Microbe Biomass (μg microbe/g soil)				Fungal Biomass (μg fungus/g soil)			
			Mean Final	Standard Deviation	Mean Difference	Standard Deviation	Mean Final	Standard Deviation	Mean Difference	Standard Deviation
Control	3.25	9	625.5	93.9	−53.6	225.8	833.1	62.0	−344.9	232.4
Ready-to-Use III	3.72	9	657.1	164.2	39.0	189.9	830.2	196.1	−294.0	213.7
Super Concentrate	3.56	9	757.4	99.3	31.1	136.6	849.5	147.2	−227.2	134.0
Glyphosate (no outlier)		6	596.7	91.0	−197.8	227.2	799.2	57.0	−413.7	232.0
Glyphosate (all data)	3.36	9	618.0	104.0	−198.5	227.2	815.1	57.7	−413.7	232.0

Table 3. Including or excluding the outlier did not impact the conclusion that treatment type did not impact the mean microbe biomass (no outlier: F = 2.88, DF = 3, 29, P = 0.06; all data: F = 2.67, DF = 3, 32, P = 0.06). Although both p-values approach significance, a post-hoc Scheffe test found no significant group versus group difference.

Tracking the same mesocosm over time could potentially provide more reliable information so we also calculated the difference between the initial microbial biomass reading and the final biomass reading for each mesocosm. As above, a one-way ANOVA found no

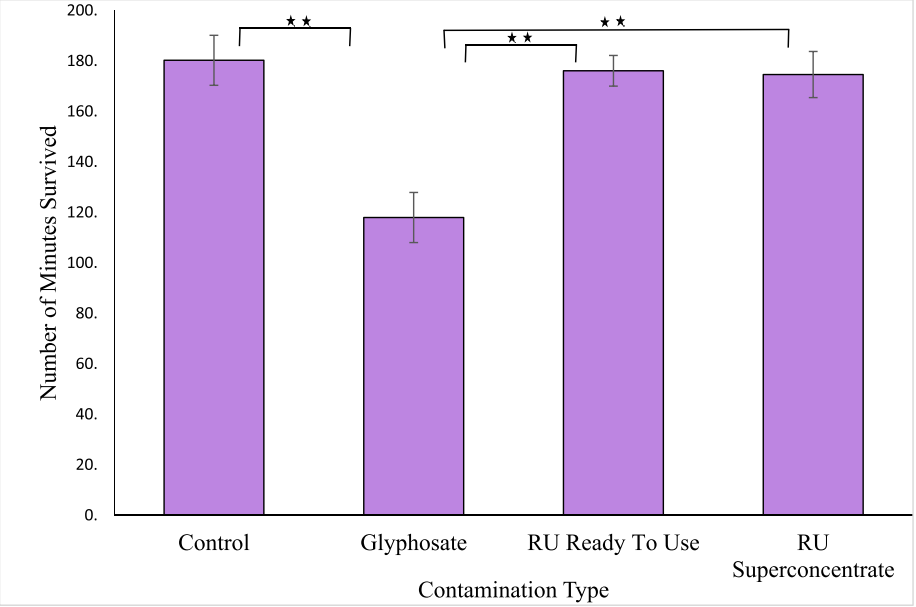

Fig. 2. The impact of contamination type on earthworm survival during a stress test. One-way ANOVAs detected significant difference between the four groups (outlier excluded: F = 7.66, DF = 3, 133, P = 0.00009; all data: F = 4.73, DF = 3, 146, P = 0.003). Post-hoc Scheffe tests find the significant differences shown above, with all p-values falling at or below 0.001 (no outlier) or below 0.05 (all data). One star designates a p-value below 0.05 and two stars designates a p-values below 0.001.

significant differences between treatments, regardless of whether we included the outlier (no outlier: F = 2.21, DF = 3, 29, P = 0.11; all data: F = 1.11, DF = 3, 32, P = 0.36).

Lastly, we asked if microbial biomass changed over the course of this study, combining all data since microbial biomass did not vary with treatment type. A one-way ANOVA (no outlier: F = 0.91, DF = 1, 62, P = 0.34; all data: F = 0.47, DF = 1, 70, P = 0.50) reports that the mean initial microbial biomass did not differ from the mean final microbial biomass. See Table 3.

3.5. Soil fungal biomass

At the experiment's inception, we took three readings from each mesocosm before adding contaminants and calculated 1132.5 μg fungus g^{-1} soil ±171.9, (N = 36). A one-way ANOVA detected no significant difference between the mean fungal biomass of the four groups (F = 0.55, DF = 3, 32, P = 0.65). All mesocosms had equal fungal biomass at the experiment's beginning. Because one of the mesocosms produced outlying worm-health data, we report analysis below with and without that mesocosm.

A one-way ANOVA indicated that treatment type did not impact the mean fungal biomass (no outlier: F = 0.16, DF = 3, 29, P = 0.92; all data: F = 0.11, DF = 3, 32, P = 0.96). See Table 3. We also calculated the difference between the initial fungal biomass reading and the final biomass reading for each mesocosm. A one-way ANOVA found no significant differences between treatments (no outlier: F = 1.11, DF = 3, 29, P = 0.36; all data: F = 0.62, DF = 3, 32, P = 0.61).

Lastly, we asked if fungal biomass changed over the course of this study, combining all data since microbial biomass did not vary with treatment type. One-way ANOVAs (no outlier: F = 64.11, DF = 1, 62, P < 0.00001; all data: F = 72.35, DF = 1, 70, P < 0.00001) report that the mean initial fungal biomass (1132.5 ± , 171.9 μg g^{-1}) significantly exceeds the final biomass (no outlier: 664.8 ± 128.3 μg g^{-1}; all data: 831.8 ± 124.3 μg g^{-1}). This constitutes a 26.6–41.3% decline in fungal biomass over the course of this study. See Fig. 3.

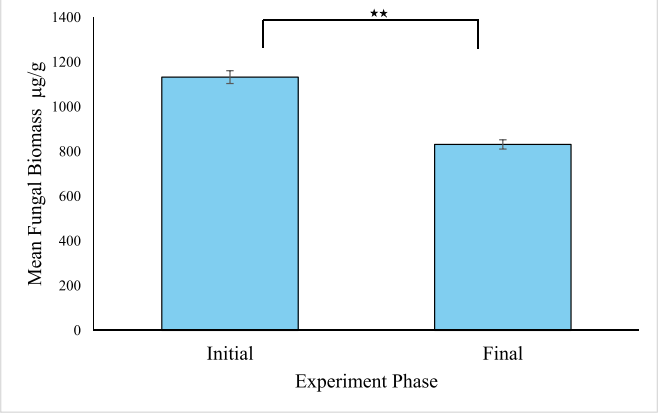

Fig. 3. Change in fungal biomass over time. A one-way ANOVA (no outlier: F = 64.11, DF = 1, 62, P < 0.00001; all data: F = 72.35, DF = 1, 70, P < 0.00001) finds that the mean initial fungal biomass significantly exceeded the final biomass. One star designates a p-value below 0.05 and two stars designates a p-values below 0.001.

3.6. Soil respiration

Soil respiration measures both microbial abundance and microbial activity (Cornell University, 2016). The Cornell Soil Health Laboratory assessed post-treatment soil respiration for each of the treatment types. Values are provided in Table 3. All numbers were consistent with Cornell's soil health score of 100, indicating the highest possible soil respiration rates and therefore high soil quality. Contaminants did not reduce soil health, as measured by soil respiration.

4. Discussion

This experiment found that glyphosate alone was more toxic to earthworms than were either of two Roundup® formulations commonly used in urban settings. Holding both glyphosate concentration and glyphosate type constant, the inert ingredients

found in two formulations did not cause the same harm that glyphosate alone did.

Both datasets used here—one culling outlying data and the other complete—indicate that contamination impacted body mass and stress-test survival time. Both also indicate that worms living with pure glyphosate survive the stress-test for significantly fewer minutes than worms living with either Roundup formula or with clean soil. The culled dataset indicated that living with pure glyphosate causes a significant decrease in body mass compared to all other contaminants, while the full dataset indicated that living with pure-glyphosate causes a significant decrease in body mass compared only to Roundup® Ready-to-Use III. This kind of inconsistency is frequently reported ecotoxicological experiments using body mass as an endpoint, as reviewed in Pochron et al. (2019) and discussed in Pochron et al. (2017, 2018).

Glyphosate-based formulations are notoriously toxic to aquatic animals (Sihtmäe et al., 2013; Babalola and Van Wyk, 2018; Reno et al., 2018; Sánchez et al., 2019, and many others), with formulations demonstrating higher toxicity than glyphosate alone (Sihtmäe et al., 2013; Wagner et al., 2013; Bridi et al., 2017; Janssens and Stoks, 2017; Bach et al., 2018; Lopes et al., 2018). One of the few studies testing the toxicity of both glyphosate and a formulation on earthworms used a freshwater worm *Lumbriculus variegatus* that lives in sediment of marshes, ponds and swamps. Researchers found that, as in other aquatic organisms, the formulation demonstrated stronger toxicity than glyphosate itself (Contardo-Jara et al., 2009). Researchers who studied aquatic organism suggested that the surfactant POEA facilitates glyphosate penetration through plasmatic membranes and consequently makes formulations more toxic than glyphosate alone (Cattani et al., 2014; de Brito Rodrigues et al., 2017).

Comparisons of pure glyphosate to glyphosate-based formulations are rare in non-aquatic organisms, but formulations are generally found to be equally toxic or more toxic than glyphosate alone (Gill et al., 2018). In one of the few such comparisons, glyphosate-based formulations had detrimental effects on the cardiovascular system of swine (*Sus scrofa*) while glyphosate alone had no impact (Lee et al., 2009), and in vertebrate cell lines, usually human cell lines, formulations tended to be more toxic than glyphosate alone (Benachour and Séralini, 2008; Defarge et al., 2016, 2018; de Almeida et al., 2018; Vanlaeys et al., 2018; Woźniak et al., 2018). For a complete review of the effect of glyphosate, AMPA, and Roundup® formulations on animals, see Gill et al. (2018).

The general consensus in the literature is that Roundup® formulations are more toxic than glyphosate alone, although that was not what we found in this study. We could be alone in this finding because comparisons between formulations and glyphosate alone are rare in non-aquatic organisms, but we suggest that the formulations might differ from glyphosate in two ecologically important ways.

First, fungus killed by the formulations might provide food for earthworms. Glyphosate-based formulations are known fungicides (Tanney and Hutchison, 2010; Druille et al., 2016;Poirier et al., 2017; Przemieniecki et al., 2017), and out of seven tested formulations, Roundup Ready-to-Use® had the strongest fungicidal properties while glyphosate itself had no fungicidal activity (Morjan et al., 2002). Additionally, in a greenhouse experiment where Roundup® exposure led to heavier worms, the researchers suggested that the Roundup® formulation killed soil fungus, providing abundant food for the worms (Zaller et al., 2014). In our study, fungus biomass significantly decreased over the duration of this experiment in all treatments (see Fig. 3.), suggesting that earthworms were eating it but not necessarily that mesocosms contaminated with fungus provided more food to worms. The soil was very rich initially, as discussed below. Worms of various species are known to consume fungus (Tiwari and Mishra, 1993; Bonkowski et al., 2000), even if not all species completely digest it (Gomez-Brandon et al., 2011).

In a gut microbial ecosystem, xenobiotics and infections can cause compositional shifts in the microbial community, a process known as dysbiosis when it exerts a pathophysiological effect on the host (Shaler et al., 2019). Potentially, glyphosate or Roundup® could cause a compositional shift in the soil microbial community, killing the susceptible bacterial species (e.g. those that use the shikimate process as per Zucko et al., 2010) and allowing surviving species to increase population size and fill the emptied ecological niche (Pedersen and Hendriksen, 1993; Xiong et al., 2017).

Roundup provides nutrients to the ecosystem that pure glyphosate does not. Gaupp-Berghausen et al. (2015) found that Roundup application led to increased soil concentrations of nitrate by 1592% and phosphate by 127%; a similar increase would not be possible with glyphosate alone. The glyphosate-resistant bacteria, nourished by the nitrates and phosphates contained in the formulation, might exhibit a period of growth. This period of growth and the associated increase in microbial biological activity could potentially speed the degradation of glyphosate (Okada et al., 2017; Niemeyer et al., 2018) because the degradation process is driven primarily by microbial activity (Battaglin et al., 2014). We did not measure nitrates or phosphates in our experiment, but clearly tying those measurements to an understanding of shifting microbial communities, especially changes in the population sizes of glyphosate-resistant bacteria, would be an important contribution to the field.

While we hypothesized above that Roundup® and glyphosate contamination may alter microbial function by causing a compositional shift in the microbial community, in this experiment we addressed only microbe and fungus abundance. Over the 40-day length of this experiment, we did not find that exposure to any of the contaminants caused changes in microbial biomass. This is not unusual. Although some studies report that Roundup or glyphosate application caused an increase in microbial biomass and respiration (Haney et al., 2002; Lane et al., 2012; Dabney and Patiño, 2018), glyphosate is frequently found to have little effect or a fleeting effect on soil microbial communities (Haney et al., 2000; Busse et al., 2001; Battaglin et al., 2014; Nakatani et al., 2014; Schlatter et al., 2017; Bruckner et al., 2019). A meta-analysis of the impact of glyphosate on soil microbial biomass reports that aside from the addition of glyphosate itself, soil pH, glyphosate concentration, organic carbon, and time after application all significantly affected microbial biomass and its activity (Nguyen et al., 2016). Making a blanket prediction about the effect of glyphosate on soil microbes is difficult, and we would not have caught a fleeting effect, if one existed, with our methodology.

The soil we used for this experiment, OMRI certified Black Gold Garden Compost Blend, had high microbial and fungal biomass (between 618 and 726 μg microbes and between 1076 and 1177 μg fungus per g soil) at the experiment's initiation: well over the 600 μg per g soil that is the microBIOMETER® cutoff for "excellent." Forty days after exposing the soil to contamination, the numbers stayed in the "excellent" range, running between 617 and 757 μg microbes per g soil and 814–848 μg fungus per g soil. Analysis of soil respiration from the Cornell Soil Health Lab concurred, giving samples collected at the end of the experiment the highest grade for soil health. Possibly the soil was initially so healthy at the onset of the experiment that the addition of contaminants at the concentrations we used did not bump the community out of equilibrium. Nguyen et al. (2018) suggest that soils with high organic matter have higher microbial activity and higher inherent nutrient availability, making the soil more resilient to changes induced by either glyphosate or the two formulations.

Many researchers have studied the impact of glyphosate and Roundup on earthworms (reviewed in Pochron et al., 2019, Table S1); tested glyphosate concentrations range from 0.116 mg per kg soil (Salvio et al., 2016) to 46,500 mg per kg (García-Torres et al., 2014). Our selected concentration of 26.3 mg glyphosate per kg soil was consistent with several other studies (Santos et al., 2011; Buch et al., 2013; Zhou et al., 2013; García-Torres et al., 2014; Pochron et al., 2019), as well as with levels of soil contamination after recommended use per the label (Pochron et al., 2019).

5. Conclusion

This experiment shows that for a non-aquatic organism, the compost worm, glyphosate was toxic but two glyphosate-based formulations were not. We suggest two ways in which formulations' inert ingredients may offset glyphosate's toxic effects. First, the inert ingredients in both formulations act as fungicides; the dead fungi may act as food for the worms, providing them with metabolic resources to withstand the toxic glyphosate. We detected no change in microbial or fungal biomass mediated by contamination, but fungal biomass decreased by 26.5–41.3% over the 40-day period of this experiment, suggesting that worms were eating it. Second, the nitrites and phosphates in the inert ingredients potentially nourish soil bacteria, perhaps enabling intense bacterial population growth. This increased microbial activity may speed the degradation of glyphosate.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.125017.

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