Investigating the Toxicity and Accumulation of 2,4-Dichlorophenoxyacetic Acid (2,4-D) and Glyphosate in *Eisenia fetida*

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Glyphosate, the main ingredient in Roundup®, is the most widely used herbicide in the world. It is also used in combination with another effective herbicide, 2,4-D, in the formulation called Enlist Duo®. The EPA approved the use of Enlist Duo® on certain crops including those genetically modified to be resistant to both herbicides. The predicted significant increase in the use of these herbicides raised concerns from the general public because both compounds have been classified as possibly or probably carcinogenic. Since herbicides are applied directly to vegetation, the safety of organisms that come in contact with the herbicides is at risk. In this toxicity study the responses of earthworms exposed to various concentrations of 2,4-D, glyphosate, or both were investigated. Results of direct contact studies show severe effects of 2,4-D on worms, including death. Despite seeing ill effects in direct contact trials, we saw no significant difference in the mortality rates, reproductive health, and weight of the earthworms treated via ingestion of contaminated soil or vegetation. As earthworms constitute a food source for other organisms, bioaccumulation is possible. A HPLC method to detect herbicide uptake in earthworm tissues was also developed. These methods were suitable for analyzing worms obtained from soil and plants treated with 2,4-D, glyphosate, or both. HPLC analysis showed uptake of both 2,4-D and glyphosate but no evidence indicating that the presence of 2,4-D changed the uptake of glyphosate.

Introduction

Glyphosate and 2,4-dichlorophenoxyacetic acid (2,4-D) are two widely used herbicides in both agricultural and residential areas to control broadleaf weeds and grasses. A combined formulation known as Enlist Duo® (Dow Chemical Company) is very effective in killing tough weeds that are otherwise resistant to either herbicide used alone. The use of Enlist Duo® was approved by the Environmental Protection Agency to control weeds in corn and soybeans that are genetically modified to tolerate these herbicides (EPA 2016). Both Dow Chemical and the US Department of Agriculture predicted a significant increase in the use of these herbicides worldwide (CFS 2015).

In 2010, Correia and Moreira reported 30-40% mortality rate in worms exposed to 2, 4-D at concentrations ranging from 1 - 500 mg/Kg dry soil within 14 days and a 100% mortality rate at concentrations of 500-1000 mg/Kg within a few hours of exposure. They also claimed that glyphosate significantly affected worm reproductive health (Correia and Moreira 2010). More recently, Mérey and colleagues reported no significant effects when they exposed worms up to 473 mg/Kg concentrations of glyphosate (Mérey et al. 2016).

There are conflicting reports regarding the safety of 2,4-D and its possible carcinogenic effect in humans. An epidemiological study has linked 2,4-D with Non-Hodgkin's Lymphoma (Zahm et al. 1990). A subsequent review of the scientific evidence regarding the safety of this herbicide and the hypothesis linking it with cancer was inconclusive (Munro et al. 1992). In February 2015, the Center for Food Safety and other public interest groups sued the EPA for their approval of the use of Enlist Duo® despite conflicting reports that implicate 2,4-D as a potential carcinogen (CFS 2015). In June 2015, both 2,4-D and glyphosate were classified as possibly or probably carcinogenic by the International Agency for Research on Cancer (IARC 2015a, 2015b). This raises concerns about the health risk of using these herbicides, consuming treated food, and the long-term effects upon soil quality affecting important soil biota such as earthworms.

This study aimed to analyze the effects of glyphosate and 2,4-D applied separately and together on earthworms, and to develop HPLC methods that would detect herbicide uptake in earthworm tissue. The HPLC methods would enable comparison of the amount of uptake with earthworm mortality rates, reproductive health, and weight gain. Earthworms have been used as test organisms in toxicity studies as they are an important part of the soil biomass. They are in direct contact with the soil and ingest the soil along with its contaminants. Since they constitute a food source for other organisms, bioaccumulation of 2,4-D or glyphosate or their derivatives would be particularly concerning.

Methods

A. Worm Culture and Maintenance

Eisenia fetida (Carolina Biological Supply Company) were maintained in 10-gallon glass aquaria containing worm bedding and fed commercial worm food under controlled conditions (12 h light: dark cycle, 21-23°C, 58-60% humidity). Prior to treatments, adult earthworms (age less than 2 months with visible clitellum; weight from 300-490 mg) were randomly selected from the culture and used for the various experiments.

B. Worm Exposure to Herbicides

Adult earthworms were exposed to varying concentrations of either glyphosate, 2,4-D, or a combination of 2,4-D and glyphosate. Treatment solutions were prepared by dissolving 2,4-D (Sigma D7299**)** or diluting Roundup® Weed & Grass Killer Super Concentrate (Active Ingredient: 50.2% glyphosate isopropylamine salt) in water. Distilled water was used for the control. Exposure was either by direct contact with the herbicide, or by ingestion of herbicide-treated soil or organic matter. For each experiment, 3 replicates of 10 samples each were used. For the direct contact exposure, individual worms were placed in vials lined with filter paper moistened with 1 mL of the appropriate concentration of herbicide and observed after 48 hours. The worms were weighed and examined for physical abnormalities and percent mortality.

For exposure via ingestion of contaminated soil, worms were placed in each test chamber filled with either sterilized or unsterilized artificial soil. The soil was prepared according to the procedure described by Paradise (2001) with few modifications (119 g sand, 35 g peat, 17 g clay, 3 g calcium carbonate, and 121 mL distilled water). The dry ingredients were mixed thoroughly prior to addition of distilled water to reach a consistency of the standard worm bedding. The soil was sprayed with 1.6 mL of various herbicide concentrations. The worms were harvested and observed after 4 weeks and examined for percent mortality, weight, and overall health conditions.

For exposure via ingestion of herbicide-treated plants, clover and rye seeds were planted in pots containing equal amounts of the artificial soil. After 10 days, ten worms were placed in each pot and allowed to burrow. After 3 days, the leaves were sprayed with 20 mL of the appropriate herbicide concentrations. The pots were watered with equal amounts of distilled water as needed to keep the soil moist. After 4 weeks, the worms were recovered, weighed, and examined for any physical abnormalities and percent mortality. All live worms were stored at -80°C for HPLC analysis.

C. Development of HPLC Methods to Detect Herbicide Uptake in Earthworm Tissue

Worm Tissue Extraction

Untreated worms stored at -80°C were combined with 10 mL of solution per gram worm, and homogenized using a glass-glass homogenizer (Kontes Glass). This was microfuged at 11,000 g for 15 min, and the supernatant was collected for analysis. The extraction solution was 40 mM sodium acetate pH 4.0. Solid phase extraction (SPE) was attempted using a Strata-X-C 33 µm device (strong cation; Phenomenex) that would be best for 2,4-D. The supplier's suggested method was used to process worm tissue spiked with a known amount of 2,4-D. The flow through and eluted fractions were analyzed. A Strata-X-AW device (weak anion; Phenomenex) was also used. Both retained some 2,4-D but other retained material from the worm extracts eluted in the HPLC at approximately the same time as 2,4-D. These procedures were repeated using a pH 5.0 extraction buffer with little improvement. Ultrafiltration using Amicon 10,000 KD nominal molecular weight limit filters (Millipore) were used next. SPE showed no clear benefits over ultrafiltration. To improve 2,4-D extraction, a 50:50 mixture of 0.1 M HCl and ethanol was used. Different alcohols and different concentrations were tested, but the best results were obtained using this mixture. This method of extraction proved to be effective for glyphosate as well.

2,4-D HPLC Program Development

This method used a Kinetex C18 100A column (5µ, 150 x 4.6 mm; Phenomenex). A biphenyl column was used for most of the 2,4-D method development but it was no better than C18. Different concentrations of acetonitrile and various sodium acetate buffers were tested. A gradient program was developed that used Solvent A - 40 mM sodium acetate adjusted to pH 4.0 with acetic acid and Solvent B – acetonitrile. The program was 0-1 min 25% B, 1-7 min 25% to 50% B, 7 -8 min 50% B, 8-9 min 50% to 25% B, and 9-12 min 25% B. The 2,4-D retention time was 4.7 min separated from all major background peaks in untreated worm samples (Fig. 1). Two minor peaks co-eluted with 2,4-D which limited sensitivity. The photodiode array settings were optimized for 2,4-D with quantification at 227 nm.

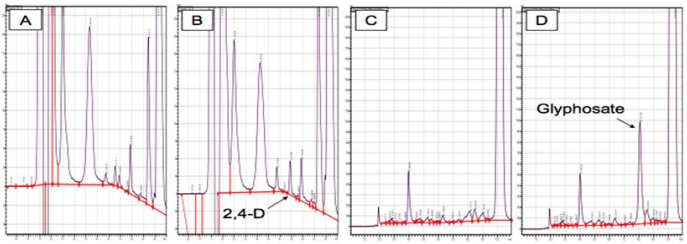

Figure 1. Sample chromatograms from HPLC analysis of 2,4-D or glyphosate. An untreated worm extract was injected without (A) or with (B) added 2,4-D – 108 ng (amount of 2,4-D injected). For glyphosate analysis, 10 µl of untreated worm extract was derivatized with fMOC without (C) or with (D) added glyphosate – 1 ng (amount of glyphosate injected).

Glyphosate HPLC Program Development

Glyphosate analysis used sample derivatization of glyphosate with 9-fluorenylmethyloxycarbonyl chloride (fMOC) similar to a method by Buha et al. (2011). The best HPLC solvents and column were the same as above. The pH and salt concentration of the solvents were important for separation of glyphosate from co-eluting peaks. The gradient program was 0-9 min 20% B, 9-10 min 20% to 70% B, 10-12 min 70% B, 12-13 min 70% to 20% B, and 13-15 min 20% B. The fluorescence detector settings were Ex: 266 nm and Em: 305 nm. The glyphosate derivative eluted at 8.0 min with good but not complete separation from co-eluting worm extract compounds (Fig. 1). Prior treatment of samples with o-phthalaldehyde to remove primary amines did not eliminate these peaks and was not used.

Results

A. Worm Exposure to Herbicides

Direct contact with 2,4-D or a combination of 2,4-D and glyphosate caused severe harm and eventual death of worms. At low concentrations, direct contact with glyphosate had no effect on worms (Fig. 2). At higher concentrations, ill effects on worms were evident. The lethal concentration that killed 50% of the worm population (LC50) was 6.5 mg/mL of glyphosate or 1.0 mg/mL of 2,4-D. However, when tested with both herbicides in a ratio similar to Enlist Duo®, the LC50 was only 0.8 mg/mL of glyphosate and 0.7 mg/mL of 2,4-D (Fig. 2). Ingestion of herbicide-treated soil (0.68 - 6.8 mg 2,4-D and/or 0.72 - 7.2 mg glyphosate applied to 25 cm^2 soil surface) did not affect the worms. Similarly, ingestion of herbicide-treated plant material (8.6 - 86 mg 2,4-D and/or 9.1 - 91 mg glyphosate applied to plant canopy over 353 cm^2 soil surface) did not affect worms. The Enlist Duo®, recommended season use rate is equivalent to 1291 g 2,4-D/acre (32 µg/cm^2) and 1372 g glyphosate/acre (34 µg/cm^2). There was no significant difference in the mortality rates, reproductive health, and weight of the worms.

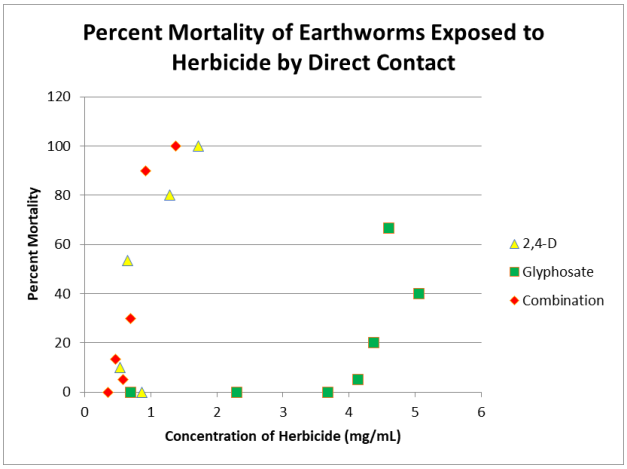

Figure 2. Results of the effect of direct contact with varying concentrations of glyphosate, 2,4-D or a combination of these two herbicides on worms.

B. HPLC Analysis

Selected results of analysis of 2,4-D or glyphosate in treated worms is shown in Table 1. Eight sets of twelve earthworms treated with 2,4-D, glyphosate, or both were analyzed using the methods described above. Both 2,4-D and glyphosate were detected in contact treated worms. Only glyphosate was detected in worms cultured in treated soil or with treated plants.

Discussion and Conclusions

Our results indicate a possible, small synergistic effect of the herbicides on earthworms treated by direct contact. Despite ill effects of direct contact of herbicides on worms, ingestion of herbicide-treated soil or vegetation did not affect the worms. Sterilization of the soil did not make a difference in worm response to herbicide treatments either. This suggests that 2,4-D was not degraded by bacteria prior to reaching the worms. Despite the high sensitivity of HPLC to very minute concentrations of herbicides, no 2,4-D residues were detected in tissues of these worms. 2,4-D may bind to soil particles and be broken down by other means before it reaches the worms, or the 2,4-D amount in these worms was below the limit of detection. The 2,4-D limit of detection was 2.2 µg/g worm which could be expected if worms took up about 2% of the total applied herbicide. The glyphosate limit of detection was 0.9 µg/g worm, which would be about 1% of the total applied herbicide. Although glyphosate residues were detected in worm tissues, there was no evidence that the presence of 2,4-D changed the uptake of glyphosate.

Robust HPLC methods for the analysis of 2,4-D and glyphosate in earthworm extracts were developed. Over a two-month period, these methods were used to analyze eight sets of herbicide-treated worms.

Set 1: Contact	Worm Mass	Treatment		2,4-D		Glyphosate		Uptake			
		2,4-D	Glyphosate	RT	PA	RT	PA	2,4-D	Glyphosate	2,4-D	Glyphosate
	mg	µg		min		min		µg/worm		%	
2,4-D				4.678	2.38E+06						
Control Worm 2 + 2,4-D				4.734	3.90E+05						
Glyphosate						8.018	2.05E+07				
Control Worm 2 + Glyphosate						7.995	1.82E+07				
Control Worm 1	605										
Control Worm 2	621										
Control Worm 3	478										
2,4-D Worm 1 (1.5x)	376	645		4.727	3.54E+06			176.8		27.4	
2,4-D Worm 2 (1.5x)	497	645		4.714	4.12E+06			272.1		42.2	
2,4-D Worm 3 (1.5x)	465	645		4.754	6.06E+05			36.6		5.7	
Control Worm 2 + 2,4-D				4.714	3.97E+05						
Control Worm 2 + Glyphosate						7.990	1.72E+07				
Glyphosate Worm 1 (8x)	473		3655			7.983	1.47E+08		55.1		1.5
Glyphosate Worm 2 (8x)	434		3655			7.980	1.42E+08		48.9		1.3
Glyphosate Worm 3 (8x)	451		3655			7.990	1.49E+08		53.3		1.5
Combination Worm 1 (1.5x)	550	645	685	4.732	5.30E+05	7.999	8.58E+06	37.7	3.3	5.8	0.5
Combination Worm 2 (1.5x)	602	645	685	4.723	4.13E+05	7.993	2.17E+07	31.9	9.9	4.9	1.4
Combination Worm 3 (1.5x)	533	645	685	4.726	4.61E+05	7.980	1.52E+07	31.6	6.0	4.9	0.9

Table 1. From top are shown results of analysis of herbicides without worm extract, untreated control worm extract spiked with 2,4-D or glyphosate, extracts of three untreated worms, extracts of three worms contact treated with 2,4-D at 1.5 x the recommended 2,4-D field rate, extracts of three worms contact treated at 8x the recommended glyphosate field rate and extracts of three worms contact treated with both herbicides.

These analyses demonstrated the effectiveness and reproducibility of the methods. Herbicide standards run with each set of treated worms were used to generate a final set of standard curves (Fig. 3). Manual inspection of the chromatograms for untreated worms or worm treated with only one herbicide resulted in an initial set of results to use to further evaluate the size range of the small co-eluting peaks. The limit of detection for each herbicide was near the anticipated level of herbicide uptake. Further improvements in sensitivity will likely require changing the worm extraction procedure to get more extracted herbicide injected into the HPLC.

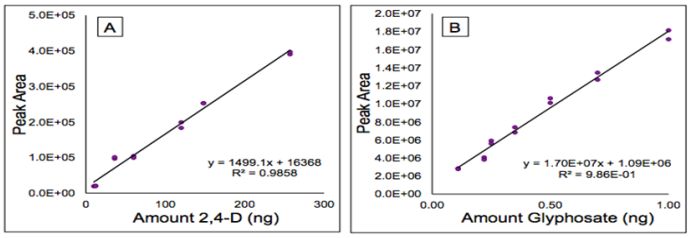

Figure 3. Standard curves for HPLC analysis of 2,4-D or glyphosate. Untreated worm extracts were spiked with 2,4-D (A) or glyphosate (B). The data shown was collected with eight sets of analyses done over a two-month period. The amounts shown

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