Examining the Individual and Combined Effects of Roundup's Disclosed Ingredients on the Activity Levels of *C. elegans*

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ABSTRACT

Roundup, produced by the company Monsanto, is the most widely used commercial herbicide. It is a selective, broad-spectrum preparation containing the active ingredient glyphosate, polyethoxylated tallow amine (POEA), and other undisclosed ingredients. Monsanto claims Roundup does not pose a risk to humans as glyphosate targets the shikimic acid pathway, which is not found in animals. Despite this line of reasoning, glyphosate was classified as "probably carcinogenic to humans" by the International Agency for Research on Cancer in 2015. POEA is a surfactant that facilitates glyphosate adherence to foliage. Recently published studies suggest that the addition of POEA to glyphosate increases its efficacy as an herbicide. This investigation examines the individual and combined effects of Roundup's known ingredients, glyphosate and POEA, as well as the complete commercial preparation, on the activity of the non-parasitic nematode, *C. elegans*. The activity levels of the worms were monitored during exposure to varying concentrations of: a) Roundup Weed and Grass Killer Super Concentrate at concentrations below and above that recommended for residential use, b) pure glyphosate, c) pure POEA, and d) the combination of glyphosate and POEA. The substances were serially diluted in M9 growth medium. The responses of unsynchronized *C. elegans* were measured over 8 different time periods (from 0 to 150 minutes) using an activity level scale ranging from 0 to 3, with 0 indicating no movement and 3 indicating rapid sinusoidal movement. Exposure to Roundup, glyphosate alone, and the combination of glyphosate with POEA resulted in a statistically significant dose-response pattern with the activity levels declining more rapidly over time with increased concentration. Exposure to POEA alone demonstrated no significant effect on activity. When effects were compared across substances, glyphosate alone caused a greater decrease in worm activity compared to POEA alone. A modest, however statistically significant difference ($p < 0.05$) was observed between exposing the worms to glyphosate alone and the combination of glyphosate and POEA, with the combination having a more detrimental effect. This result suggests an additive effect between POEA and glyphosate. However, further investigation is necessary to verify a potential interaction between glyphosate and POEA. Future research will also investigate the effects of Roundup, POEA, and glyphosate at additional concentrations over greater periods of time. This will include replacing the activity level as a measure of toxicity with the measurement of fecundity with synchronized larval stages as a more accurate scale to understand the compounding effects of Roundup's known ingredients.

INTRODUCTION

Glyphosate is the active ingredient in Roundup, the most heavily used herbicide in history, with over 9.4 billion tons applied since 1974 [1, 2]. Glyphosate use increased 15-fold since the introduction of genetically modified glyphosate tolerant Roundup Ready® crops which account for 56% of the herbicide global application [3]. Roundup is also used in pre harvest crop desiccation. [4]

Glyphosate herbicidal activity relies on the interruption of the shikimate pathway exclusive to plants, bacteria, archaea, and fungi, preventing the synthesis of amino acids essential to plant life [5, 6]. Monsanto denies adverse effects to humans, wildlife and environment [7]. Many of the studies that the company cites in support of this claim are poorly designed and shortened before negative results can emerge. In addition, these investigations are conducted by scientists and companies affiliated with and supported by Monsanto [8].

Polyethoxylated tallow amine (POEA), the only disclosed surfactant in Roundup, enables herbicide adherence and functions to promote the penetration of glyphosate into plant cuticles [10]. Its toxicity in species of amphibian larvae [9] and aquatic life [11] has been previously suggested. Glyphosate effects on embryonic, placental, and umbilical cells was studied alone and in combination with POEA, at concentrations below the recommended lawn and agricultural dose. The results revealed individual toxicity of POEA and glyphosate, and added toxicity when the two chemicals were combined [12, 13]. Despite these results, the U.S. Environmental Protection Agency (EPA) upon review of all reliable toxicity data related to POEA concluded that there is reasonable certainty that "no harm will result ... from dietary exposure to this class of surfactants" [14].

The objective of this study was to examine the individual and combined effects of Roundup's disclosed components on activity levels of a non-parasitic nematode. *Caenorhabditis elegans* a model organism often used in toxicological studies, was selected for this project as it is found in the soil, lacks a shikimic pathway, and is convenient to work with under laboratory conditions.

METHODS AND MATERIALS

Dilutions

Dilutions were prepared of Roundup Super Concentrate (a 50.2% glyphosate solution) in 1X M9 at concentrations at, below, and above the suggested usage. The recommended use for Roundup Weed & Grass Killer Super Concentrate once diluted in water is 2% Roundup or 1% glyphosate. Concentrations of Roundup containing 3% glyphosate, 1%, 0.33%, and 0.11% were tested. The Roundup was diluted in M9 buffer solution, as it supplies the necessary nutrients, salts, and pH for the growth and sustenance of C. elegans. In order to assess more clearly what targets C. elegans, the only 2 disclosed ingredients in Roundup: glyphosate, the active ingredient, and POEA (polyethoxylated tallow amine), a surfactant, were studied. Dilutions of glyphosate in M9 were prepared at concentrations identical to the Roundup. (3% glyphosate, 1%, 0.33%, 0.11%). Concentrations equivalent to those of glyphosate were prepared of POEA: 3% POEA, 1%, 0.33%, 0.11%. The same concentrations of dilutions were made containing equal parts of glyphosate and POEA included: 3% glyphosate and 3% POEA, 1%, 0.33%, 0.11%) In all experiments, 1 x M9 was used as a positive control.

C. elegans Treatment and Experimental Design

C. elegans were exposed to the dilutions in a 96-well microtiter plate. 200µl of the corresponding dilution were pipetted and ~5-25 worms were transferred by platinum pick into each well. Observation was performed using a Zeiss inverted microscope right as worms were placed in the well and every 15 minutes for the first hour and subsequently every 30 minutes until the *C. elegans* reached 2.5 hours of exposure.

Individual experiments used one set of dilutions (either Roundup, glyphosate, POEA, or POEA and glyphosate) with 15 total wells used; each concentration was tested in 3 wells. 8 total experiments were performed, 2 with each toxin. In order to preserve the integrity of the data collection process, all experiments were performed double blind where: 1) concentrations were randomly assigned to wells and were prepared by a researcher not involved in the recording or observation process, and 2) the researchers recording and observing activity levels were not aware of the placement of the concentrations in the wells.

At each observation, the worms' activity was assessed on a scale from 0-3 using an activity scale, slightly modified from M. Viazmenski, G. Griggs, and S. Miller (2017). The criteria for each activity level are described in *Table 1*.

Activity Level	Description
3	Rapid sinusoidal movement or flailing
2.5	Moderate sinusoidal movement
2	Slow, deliberate sinusoidal movement
1.5	Irregular, slow movement
1	Either twitching, seizing, or only minor movement at distal end
0.5	Only slight movement for the duration of observation (10 seconds)
0	No movement for the duration of observation (at least 10 seconds)

Table 1. Activity level scale for *C. elegans*.

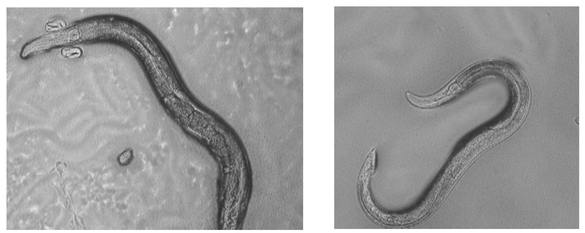
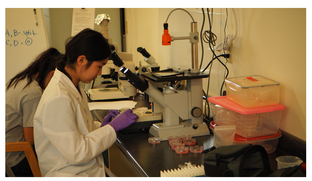


Figure 1. C. elegans nematodes taken at100x. Left: adult hermaphrodite surrounded by 3 expelled eggs, Right: adult male



RESULTS

Statistical Analysis

To conduct the analysis, each well was treated as a separate observation, providing 6 observations per concentration of each substance (3 wells per concentration for each experiment, 2 replicates performed). All statistical analyses were conducted in SPSS using a repeated measures general linear model. Results were considered significant for p-values less than .05.

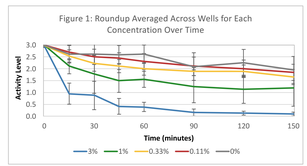
A series of two sets of mixed ANOVAs were conducted. First, two-way mixed ANOVAS were conducted to determine the effects of concentration and time within each substance (Glyphosate, POEA, Glyphosate + POEA, and Roundup). For each analysis, concentration was the between subjects independent variable (5 levels: 0%, .11%, .33%, 1%, 3%) and time was the within subjects independent variable (8 points in time in minutes: 0, 15, 30, 45, 60, 90, 120, 150). The second set of mixed ANOVAs compared the effects between pairs of substances. For each pair of substances compared, three-way mixed ANOVAs were conducted with substance and concentration as between subjects independent variables and time as the within subject independent variable.

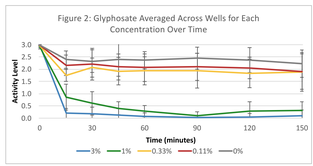
Effects of Concentration and Time Within Substance

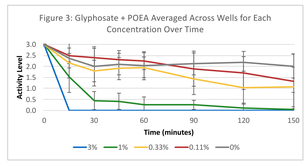
Previous research suggests that increasing concentrations and exposure time of herbicides (glyphosate, Roundup) results in decreased activity levels. For glyphosate, glyphosate + POEA, and Roundup, the mixed ANOVA revealed significant main effects of time (all ps < .001) and concentration (all ps < .001), with activity levels declining more rapidly with increased concentration and over time (See Figures 1-4). However, the same pattern of results for POEA were not necessarily expected, as it has been described as harmless in the preparation since it is not the active ingredient. The same mixed ANOVA for POEA revealed a significant main effect of time ($p < .001$), but the effect of concentration was not significant ($p = .73$, see Figure 4).

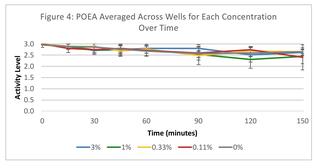
Effects of Concentrations and Time Between Substances

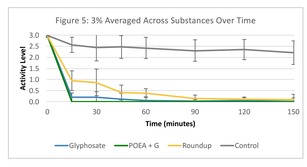
A series of mixed ANOVAs comparing the effects on activity levels across substances for each concentration were also conducted. In order to understand the effects of POEA in the Roundup preparation, the effects of glyphosate v. POEA, glyphosate v. glyphosate + POEA, and Roundup v. glyphosate + POEA were compared. The analyses revealed a significant main effect of substance in all three comparisons (glyphosate v. POEA: $p < .001$; glyphosate v. glyphosate + POEA: $p < .001$; Roundup v. glyphosate + POEA: $p < .001$). Graphs comparing the effects of the substances glyphosate, glyphosate + POEA, and Roundup for each concentration are included (figures 5-8), with all error bars representing standard deviations.

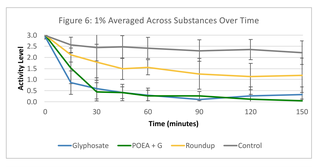


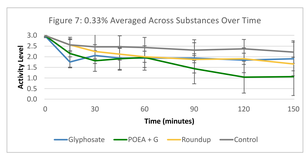


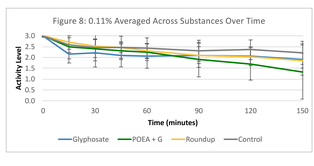












DISCUSSION

Within the three experimental conditions of Roundup, glyphosate, and the combination of glyphosate and POEA, the analysis indicated a significant dose – response pattern with activity level declining more rapidly with increasing concentrations over time. POEA alone only exhibited a main effect of time and not of concentration. This suggests that increasing the concentration of POEA does not have a detrimental effect on activity level.

The mixed ANOVA comparing the effect of glyphosate and POEA suggests that the impact of glyphosate on the activity level the *C. elegans*' activity level results in a significantly more detrimental decline in activity than that of POEA. Increased concentrations of POEA did not have an adverse effect on the activity level; therefore, one might expect that the effect of glyphosate and the effect of glyphosate combined with POEA would be similar Surprisingly, the results of the mixed ANOVA comparing glyphosate and the combination of glyphosate and POEA indicate that the combination has a significantly more detrimental effect on activity level compared to glyphosate. These results suggest that the herbicide preparation may not solely operate upon the effect of glyphosate alone, but rather the interaction between glyphosate and POEA.

The additional ANOVA comparing the effects of Roundup and the POEA and glyphosate combination was performed to examine whether the combination had similar effects to that of Roundup, as each of the substances contain both a glyphosate and POEA formulation. Our results reveal that the POEA and glyphosate combination causes a more inimical decline in activity level compared to the Roundup. In addition, glyphosate alone seems to have more deleterious effects on the activity level compared to Roundup (Figure 1). Further research will focus on optimizing the concentrations of POEA in our dilutions and gaining a more thorough understanding of the interaction between POEA and glyphosate to further comprehend why such differences described above may occur. Since the herbicide preparation also incorporates other unidentified ingredients, it is possible that these unknown ingredients may possibly slightly alleviate the effects of the combination of glyphosate and POEA. These data suggest that studying the effects of glyphosate alone is not a sufficient way to evaluate the potential toxicity of Roundup. Further investigation of components other than the active ingredient, and the interaction between these components should be undertaken.

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ACKNOWLEDGEMENTS

We would like to thank Dr. Chery Whipple for her patience, encouragement, and extensive time dedicated to helping us design and execute our experiments as well as guiding us through technical challenges along the entire course of our work. We also extend our thanks Dr. Peter Faletra, Dr. Jessica Day, and Elaine Faletra for their extensive mentorship and technical assistance. Finally, we would like to thank our families for their continuing support.

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