THE EFFECT OF GLYPHOSATE ON SOIL MICROBIAL COMMUNITIES

THESIS

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By

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ABSTRACT

Glyphosate [N-(phosphonomethyl)glycine] is the most widely used herbicide in the world. First sold in 1974 under the trade name Roundup, its use has increased dramatically in recent years with the introduction of genetically modified, glyphosate resistant (GR) crops. There is growing anecdotal evidence in the Midwestern United States of potassium (K) deficiency in corn, which appears to be related to the adoption of GR soybeans grown in rotation with corn. It is possible that the use of glyphosate in GR cropping systems is creating a selection pressure in soil microbial communities which could affect soil K dynamics. The first objective of Chapter 1 was to determine the effect of rates of glyphosate on microbial respiration. The second thesis objective of Chapter 1 was to determine the effect of glyphosate on microbial community structure, exchangeable, non-exchangeable, and microbial K in soils that had no, limited, or high amounts of glyphosate exposure under field conditions. The objectives of Chapter 2 were to determine the effect of foliar glyphosate applied to GR soybean on: 1) the rhizosphere soil microbial community composition, 2) exchangeable, non-exchangeable, and microbial K; and 3) leaf concentration K. In an incubation experiment, the addition of glyphosate was shown to significantly increase microbial respiration rates. The magnitude and duration of rates of respiration were greater in soils with a previous history of glyphosate applications, indicating that previous exposure to glyphosate may

be associated with an increase in organisms able to metabolize glyphosate. In a second experiment, glyphosate was applied repeatedly to soils over a 180 day period. There were no significant shifts in soil microbial community structure based on ester linked fatty acid methyl ester (EL-FAME) analysis. In addition, glyphosate application did not significantly affect microbial biomass K. In a third experiment, glyphosate resistant soybeans grown in the greenhouse were exposed to repeated glyphosate applications. Glyphosate application did cause a significant decrease in the total microbial biomass in soybean rhizosphere soil, as measured by total extracted EL-FAMEs, in the soil that had no previous exposure to glyphosate at seven days after the glyphosate application. However, no significant changes were observed in the overall microbial community structure. These studies indicated that: 1) glyphosate stimulates microbial respiration; 2) repeated glyphosate application to soil may increase populations of microorganisms able to degrade glyphosate; 3) the application of glyphosate can lower total microbial biomass in the glyphosate resistant soybean rhizosphere; 4) changes in the structural diversity of the soil microbial community due to glyphosate application were not detectible using EL-FAME profiling, and; 5) glyphosate does not appear to reduce the plant availability of K, as no significant decreases in exchangeable or plant tissue K, nor increases in microbial biomass K, were observed after glyphosate application.

Perhaps the most valuable result of all education is the ability to make yourself do the thing you have to do, when it ought to be done, whether you like it or not; it is the first lesson that ought to be learned; and however early a man's training begins, it is probably the last lesson that he learns thoroughly.

-Thomas H. Huxley

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: THE EFFECT OF GLYPHOSATE ON SOIL MICROBIAL ACTIVITY, COMMUNITY STRUCTURE, AND SOIL POTASSIUM: AN INCUBATION STUDY

ABSTRACT

Glyphosate [N-(phosphonomethyl)glycine] is the most widely used herbicide in the world. There is a growing anecdotal evidence of K deficiency in corn, which appears to be related to the adoption of glyphosate resistant soybeans grown in rotation with corn. It is possible that the use of glyphosate is creating a selection pressure in soil microbial communities that could affect soil K dynamics. The primary objectives of this research were to: 1) define the influence of glyphosate on soil microbial activity; 2) describe shifts in the soil microbial community that occur as a result of repeated glyphosate applications using the extraction of fatty acid methyl esters; and 3) measure changes in exchangeable, and microbial biomass K after glyphosate application. In an incubation experiment, the addition of glyphosate was shown to significantly increase microbial respiration rates. The magnitude and duration of the respiration rate increase were greater in soils with a history of previous glyphosate application, indicating that previous exposure to glyphosate may be associated with an increase in the population of organisms able to metabolize glyphosate. In a second experiment, where glyphosate was applied repeatedly to incubated samples, no significant changes were observed soil microbial community structure when examined by ester linked fatty acid methyl ester extraction (EL-FAME). In addition, glyphosate application did not significantly affect microbial biomass K. These studies indicate that: 1) glyphosate stimulates microbial respiration; 2) repeated glyphosate application to soil may increase populations of microorganisms able to degrade glyphosate; 3) changes in the structural diversity of the soil microbial community

due to glyphosate application were not detectible using EL-FAME analysis, and; 4) glyphosate does not appear to reduce the plant availability of K, as no significant decreases in exchangeable K, nor increases in microbial biomass K, were observed after glyphosate application.

INTRODUCTION

Glyphosate [N-(phosphonomethyl)glycine] is the most widely used herbicide in the world. First sold in 1974 under the trade name Roundup by Monsanto, glyphosate works by inhibiting the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase. This enzyme is a critical intermediate, used in the production of three aromatic amino acids vital to plants (Franz et al., 1997).

The rapid adoption of glyphosate resistant (GR), or "Roundup Ready", cropping systems has had a dramatic effect on agriculture. Plants with glyphosate resistance were first released for general use in 1996. That year, GR crops were grown on 1.7 million hectares worldwide. In 2008, GR crops were planted on 79 million hectares in 25 countries. Seventy percent of the 95 million acres of soybeans planted globally in 2008 were GR (James, 2008).

Glyphosate is generally regarded to be an herbicide with low environmental impact, with low mammalian toxicity. Being water soluble, glyphosate has a low risk of bioaccumulation in food webs. In addition, the phosphate group in glyphosate readily adsorbs to clay and aluminum and iron oxides in soil, which limits losses from the field and entry into aquatic ecosystems or groundwater. Once adsorbed, the compound is rapidly degraded by soil microorganisms (Franz et al., 1997). Also, GR crops are typically used in conjunction with conservation and no-till agriculture (Young, 2006). These tillage practices improve surface water quality, soil water retention, and mineral

nutrition while reducing soil erosion and herbicide leaching in comparison to conventional tillage (Fawcett et al., 1994; Service, 2007; Sprague and Triplett, 1986).

However, there is concern that the widespread use of glyphosate may be having unintended, undesirable consequences. These concerns include glyphosate interactions with plant nutrition, the effects on soil microbial communities including an increase in plant pathogens, and the emergence of glyphosate resistant weeds (Yamada et al., 2009).

It has been shown that glyphosate application reduces plant uptake of iron, manganese, zinc, and boron (Eker et al., 2006; Neumann et al., 2006). Observers in Brazil and the United States have reported that frequent applications of glyphosate may cause micronutrient deficiencies in GR and non-GR plants (Huber and McCaybius, 1993; Huber, 2007). It has been suggested that glyphosate is affecting soil microbial community composition, which alters nutrient dynamics in the soil (Johal and Huber, 2009; Kremer and Means, 2009).

There is a growing anecdotal evidence for potassium (K) deficiency in corn grown in rotation with GR soybeans (Cliff Ramsier, personal communication, 2006). Fast growing, high yielding corn hybrids are more susceptible to K deficiency, but K deficiency has occurred with other cultivars as well. The dominant conditions for this observed K deficiency are poorly drained soils, particularly after high rainfall (Cliff Ramsier, personal communication, 2006).

It is possible that glyphosate affects soil microbial community composition. Although glyphosate is not intentionally applied directly to soil, the herbicide may reach bare soil during broadcast spraying. In addition, treated plants translocate and

concentrate glyphosate in roots, where it is exuded into the soil (Coupland and Caseley, 1979). Fourteen day old, GR soybeans plants treated at 0.84 kg ae ha^{-1} exude >1000 ng glyphosate per plant through the roots over the sixteen days after application (Kremer et al., 2005).

Glyphosate can adversely affect microorganisms when applied *in vitro*. This result could be expected, since the shikimic acid pathway disrupted by glyphosate is present in bacteria and fungi. However, the experiments that have shown the greatest inhibition of microorganisms may be of little ecological relevance, since they were performed *in vitro* or in pure culture (Grossbard, 1985).

When glyphosate binds to soil, it becomes inactive, losing its antimicrobial properties (Sprankle et al., 1975b). Soluble and immobilized glyphosate can be a source of nutrients for soil microorganisms. Degradation of glyphosate in the soil is a primarily a biological process, performed by microorganisms (Rueppel et al., 1977). Therefore, degradation rates of glyphosate can vary, depending on conditions that affect microbial activity, such as temperature and moisture content. A thirteen site study performed by the European Commission found the average half-life of glyphosate to be 33 days, with a range of 1-130 days (European Commission, 2002).

Mineralization to carbon dioxide is the major endpoint for glyphosate degradation (Rueppel et al., 1977). Glyphosate has a low C:N ratio of 3:1, making it a readily mineralizable (Haney et al., 2000). Studies indicate that soil microorganisms can use glyphosate as a source of C (Neumann et al., 2006). In addition, some bacteria can use glyphosate as a source of phosphorus (Liu et al., 1991). It has been suggested that the

degradation kinetics of glyphosate indicate that glyphosate does not support microbial growth. Instead, the herbicide is co-metabolized by the indigenous microorganisms (Forlani et al., 1999; Sprankle et al., 1975a).

The effect of glyphosate on soil microorganisms has been widely studied, with conflicting results. In part this may be because soil microbial communities are diverse and live in diverse soil ecosystems. A number of studies have found glyphosate has no significant effect on microbial community activity and composition (Busse et al., 2001; Liphadzi et al., 2005; Ratcliff et al., 2006; Weaver et al., 2007).

Several studies have examined the effect of glyphosate on C mineralization. Some studies have shown that glyphosate can significantly increase microbial respiration in the short term (Sprankle et al., 1975a), which typically happens from 7-14 days (Haney et al., 2002), and up to 38 days (Haney et al., 2000). Other studies have reported that glyphosate has no effect on microbial respiration (Hart and Brookes, 1996; Wardle and Parkinson, 1990; Wardle and Parkinson, 1992).

Although the effect of glyphosate has been studied before and shown mixed results, most research was done with only short term applications of glyphosate. Since the introduction of GR crops, glyphosate has been in heavy use in some fields for fifteen years, with applications several times a year. There is the possibility that it takes a number of years of glyphosate applications for a shift of the microbial community to occur. It has been found that two, three, or four applications of glyphosate can reduce carbon mineralization and increase glyphosate half-life relative to a single application (de Andrea et al., 2003). Also, the degradation patterns of glyphosate change following

repeated glyphosate applications, possibly indicating an increase in soil microorganisms capable of metabolizing glyphosate (Lancaster et al., 2010). It has been shown that repeated application of glyphosate can change the response of soil microorganisms to subsequent glyphosate treatments (Quinn et al., 1988). Some studies from the field (Fernandez et al., 2005; Fernandez et al., 2009; Johal and Huber, 2009; Kremer and Means, 2009; Locke et al., 2008), anecdotal evidence, and unpublished data (Huber, 2007; Yamada et al., 2009) suggest that only with long-term and repeated applications there is an effect on the soil microbial community.

There is evidence of increases in fungal activity and populations in soil treated with glyphosate. This stimulation may be due to the fact that fungi are the main microbial degraders of glyphosate (Krzysko-Lupicka et al., 1997). Araujo, et al., (2003), found that glyphosate amendment did not affect culturable bacterial population, while fungi and actinomycetes populations increased. This effect was larger in soils that had greater previous exposure to glyphosate. Other studies have shown that glyphosate use is associated with an increase in the plant pathogens *Fusarium* and *Pythium* (Kremer et al., 2005; Levesque et al., 1993; Meriles et al., 2006). Glyphosate can stimulate the growth of mycorrhizal fungi *in vitro* (Laatikainen and Heinonen-Tanski, 2002).

It has been shown that fungi have the ability to rapidly take up K (Weed et al., 1969). Therefore, it is possible that if glyphosate stimulates fungal biomass, in turn it could immobilize biologically available K and cause K deficiency in plants. In addition to storing K in microbial biomass K, fungal hyphae could also be transferring exchangeable K to nonexchangeable K sites. Alternatively, there could be other shifts in

subpopulations that potentially impact crops. If this does occur, the US Midwest would be likely region for this to happen because glyphosate has been extensively and regularly used for > 10 years.

Consequently, in order to study the long-term effects we collected a soil that had high and repeated applications of glyphosate over the last 10 years to be used in comparison to one soil that had moderate levels of glyphosate and another that, as far as we know, never received glyphosate. Our goal was to simulate long-term applications with repeated applications in a lab incubation study that would mimic six years of intensive glyphosate applications, and then compare microbial community structure and K dynamics among these three soils. Except for the glyphosate application history, these three soils were of similar classification and very similar in terms of chemistry and texture.

The first objective of these experiments was to determine the effect of rates and repeated applications of glyphosate on microbial respiration. The second objective was to determine the effect of glyphosate on microbial community structure, exchangeable, non-exchangeable, and microbial K in soils that had no, limited, or high amounts of glyphosate exposure under field conditions.

MATERIALS AND METHODS

Soils

Three soils were chosen that had similar physical and chemical characteristics, yet different levels of previous exposure to glyphosate. All three sites were within an eleven kilometer radius in eastern Delaware County, Ohio.

The first soil was a Blount silt loam (fine, illitic, mesic Aeric Epiaqualf). This soil was from an organically managed farm utilizing a continuous rotation, the previous five years being alfalfa-orchard grass, corn, oats-alfalfa-orchard grass, spelt-timothy-clover, and timothy-clover. The soil has no recorded exposure to glyphosate (Blount0).

The second soil also was a Blount silt loam. It was from a farm practicing no till, growing corn and soybeans in rotation. The soybeans grown were not GR, therefore glyphosate was applied to the field once a year for a pre-planting burn down (Blount1).

The third soil was a Bennington silt loam (fine, illitic, mesic Aeric Epiaqualf). It was from a farm practicing no till, growing corn and GR soybeans in rotation. Glyphosate was applied up to three times a year while growing soybeans, and once a year while cultivating corn, for an average of two yearly glyphosate applications (Bennington2). Corn grown in this field had experienced symptoms of K deficiency. The primary distinguishing factor between a Bennington and Blount silt loam, based on soil classification, is the calcium carbonate concentration within the C horizon, a depth not sampled in this experiment.

Soils were sampled in the spring of 2009. Approximately 20 cores (2.5-cm diameter x 10-cm depth) were taken at each site and composited. The samples were passed through a 2-mm sieve and stored at 4ºC.

Experiment I: Glyphosate Use History and Rate of Application

The form of glyphosate used in this treatment was a K salt, branded Roundup PowerMax (Monsanto, St. Louis, MO, 540 g acid equivalent L^{-1}). This herbicide was applied at rates of 0.5, (29.5 µg ae g^{-1} dry soil), 1x (59 µg g^{-1}), 2x (118 µg g^{-1}), and 3x (177 µg g^{-1}) the maximum recommended field application rate. The application rate of glyphosate was calculated based on the maximum recommended field application rate of 1.74 kg ae ha^{-1} and a 2 mm interaction depth in the soil (Haney et al., 2000). For all concentrations, the herbicide was diluted in 1 mL of deionized water to improve evenness of application. In this manuscript the term glyphosate will refer to this commercial formulation, which includes the chemical glyphosate and other ingredients, including surfactants. Many laboratory studies use analytical grade glyphosate, which lacks the extra ingredients found in the commercial formulation. The use of the commercial formulation allows for a more accurate representation of field use. However, it has been shown that surfactants or other ingredients present in the commercial formulation may have a slightly inhibitory effect on microbial respiration when compared to analytical grade glyphosate (Haney et al., 2002).

The experimental design was a 3 x 5 factorial with the three soil types (as described above) and five rates of glyphosate application: 0 (control), 0.5, 1, 2, and 3 times the recommended field application rate.

Thirty g (dry weight) of field moist soil were placed into a 50 mL beaker. The glyphosate herbicide was applied in a 1 mL aliquot, and 1 mL aliquot of deionized water was added to the control. Then, the soil was wetted to two thirds of the field capacity. The beakers were placed in air tight 1L mason jars alongside a beaker of sodium hydroxide to trap carbon dioxide. The jars were incubated at 20ºC. Each treatment was replicated three times.

At 1, 2, 3, 5, 7, 9, 14, 19, 23, 30, 34, 42, and 54 days the sodium hydroxide traps were replaced. The traps were titrated with 0.01M HCl to determine CO_2-C.

Experiment II: Repeated Application Incubation

The experimental design was a 3 x 3 factorial with the three soil types and three rates of glyphosate application: 0 (control), 1 (59 µg ae g^{-1} dry soil), and 2 (118 µg ae g-1) times the maximum recommended field application rate. For both glyphosate concentrations, the herbicide was diluted in 1 mL of deionized water to improve evenness of application.

Forty g (dry weight) of field moist soil were placed into a 50 mL beaker. The glyphosate herbicide was applied in a 1 mL aliquot, and 1 mL aliquot of deionized water was added to the control. Then, the soil was wetted to two thirds of the field capacity. The beakers were placed in air tight 1L mason jars alongside a beaker of sodium

hydroxide to trap evolved carbon dioxide. The jars were incubated at 20ºC. Each treatment was replicated three times.

To prevent saturation of the NaOH trap, the jars were opened and the trap was replaced every fifteen days. At this time the soil moisture was measured gravimetrically and adjusted to two thirds field capacity. Glyphosate was applied monthly and soils were destructively sampled at 0.5, 1, 2, 3, 4, and 6 months and analyzed for ester linked fatty acid methyl esters (EL-FAME) and microbial biomass K.

Soil Analytical Methods

Chemical Analyses Soil pH was determined in 0.01 M $CaCl_2$ at a soil:solution ratio of 1:2.5 after 2 h using a glass membrane electrode. Soil organic C was measured by dry combustion at 550 °C using a C elemental analyzer (LECO). Soil texture was determined by the hydrometer method. The field capacity of the soil was determined by drying a saturated core on a pressure plate at -30 kPa, weighing, then oven drying the core at 110ºC, and re-weighing. Soil characteristics are presented in Table 1.1.

Exchangeable K was measured by shaking soil in a 1M solution of ammonium acetate for one hour. The solution was then filtered through Whatman no. 2 filter paper. The filtered extracts were analyzed with a flame photometer (Model 2655, Cole Parmer Instrument Co.) (Knudsen et al., 1982).

Nonexchangeable K was measured using the boiling nitric acid method (Helmke and Sparks, 1996). Briefly, soil was boiled in 1M nitric acid for 15 minutes, and then filtered through Whatman no. 50 paper. The filtered extracts were analyzed with a flame

photometer. Exchangeable K was subtracted from this result to obtain the nonexchangeable K concentration.

Microbial Analyses Microbial K was measured using a chloroform fumigation-extraction method (Lorenz et al., 2010). Briefly, soil was fumigated with ethanol-free chloroform for twenty-four hours. Unfumigated samples served as controls. Potassium was then extracted by adding a 1M ammonium acetate solution and shaking. The solution was then filtered through Whatman no. 2 filter paper. The filtered extracts were analyzed with a flame photometer. Microbial biomass K was calculated by first subtracting the unfumigated control K (exchangeable K) from the fumigated K, then dividing by an extraction efficiency coefficient (0.18).

Fatty acid methyl esters (FAMEs) were extracted by the method described by Schutter and Dick (2000). Briefly, lipids were extracted and methylated in a K hydroxide solution. The resulting solution was neutralized with acetic acid. FAMEs were partitioned into an organic phase by the addition of hexane and subsequent centrifuging. The organic hexane layer was separated and evaporated. The FAMEs were then dissolved in 0.5 hexane methyl-*tert* butyl ether. FAMEs were separated and quantified on a HP 5890 gas chromatograph with automated peak identification software (MIDI Inc.). Quantification of FAMEs was accomplished using the addition of nonadecanoic methyl ester (19:0) as an analytical standard, allowing GC peak areas to be converted to a molar basis.

The standard nomenclature is used to describe FAMEs. Each is designated by the total number of carbon atoms. The number of double bonds is given after the colon,

followed by the position of the double bond from the methyl end of the molecule. The prefixes “a” and “i” refer to anteiso- and iso-branching, the suffixes “c” and “t” indicate c*is* and *trans* geometry, 10Me indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule, position of hydroxy (OH) groups are noted, and cy indicates cyclopropyl fatty acids.

The total amount of FAME extracted was used as an index of total biomass. The sum of FAMEs characteristic to general bacteria, gram positive bacteria, gram negative bacteria, actinomycetes, saprotrophitic fungi, and protozoans were used to identify these larger taxonomic groups. The total of 16:1ω5c, the marker for abruscular mycorrizal fungi (AMF), cannot be used directly as indicative of the total population, since this FAME also occurs in bacteria. Therefore, the AMF data was based on the ratio of 16:1ω5c to total bacterial FAMEs (Table 1.2).

The ratio of total fungal to total bacterial FAMEs was calculated. Physiological or nutritional stress markers were calculated as follows: total monounsaturated to total saturated FAMEs, and the sum of cyclopropyl FAMEs to the sum of their monounsaturated precursors (Table 1.3).

Statistical Analysis

All statistical analyses were conducted using PSAW Statistics (version 18.0) and PC-ORD (version 5.32) statistical software.

For Experiment I, at each time interval, a Dunnett's test was used to determine if the application of glyphosate caused a significant deviation in respiration rate and total CO_2-C evolved from the untreated control. A 5% level of significance was used.

Linear regression was used to assess relationships between carbon added as glyphosate and CO_2-C evolved.

For Experiment II, FAMEs were calculated using both absolute concentration (nanomoles per gram soil) and relative concentration (mole percent of total moles of FAME). Prior to data analysis, FAMEs that were present in fewer than 6.5% of all samples were discarded (McCune and Grace, 2002). Forty-six FAMEs were used for analysis.

A Kruskal-Wallis nonparametric one-way analysis of variance was used to evaluate the significance of glyphosate application on each FAME and the FAME taxonomic groupings.

Non-metric multidimensional scaling (NMS) was performed using PC-ORD based on Sørensen distance. Two hundred and fifty runs were made with real data and compared to 250 randomized runs. A stability criterion of 0.00001 was used. NMS was performed on both the absolute and relative concentration data sets (nanomoles per gram soil and mole percent of total FAME). Before NMS, the data were transformed using a monotonic square root transformation to improve normality and reduce the coefficient of variation among FAME's.

A general linear model univariate analysis of variance (ANOVA) was used to evaluate the significance of glyphosate application on microbial and exchangeable K.

RESULTS

Experiment I: Glyphosate Use History and Rate of Application

Respiration In the Bennington2, total respiration increased with increasing glyphosate application rate (Figure 1.1). The respiration rate was significantly higher than the control at all application rates on Day 1 (Table 1.4). On Day 2 and 3, the respiration rate was significantly higher at the 2x and 3x application rates. The respiration rate of the 3x application remained at a statistically significantly greater rate through all the sampling dates except Days 9 and 14. While not significant at every sampling, the respiration rates of the 1x, and 2x treatments remained greater than the control through Day 42. Significant differences in the respiration rates reemerged at later sampling periods. By Day 19, the respiration rate at the 1x, 2x, and 3x application rates were significantly greater than the control. On Day 23, the respiration rates of all four treatments were significantly greater than the control.

The respiration rate of the Blount0 was elevated for a short period of time when compared to the Bennington2 soil. At day 2, the 1x, 2x, and 3x application rates showed a significant increase in respiration rate over the control. By Day 3, only the 3x application rate still exhibited a significantly higher rate. At Day 5 there was no significant difference in respiration rate. By Day 30, the respiration rates for all four treatments trended lower than the control. At Day 42, the 1x, 2x, and 3x application levels were all significantly lower than the control.

The Blount1 did not exhibit the same significant immediate increase in respiration rate observed in the other soils. To the contrary, the 0.5x and 3x application rates showed a significant decrease in microbial respiration rate at Day 1. This respiration rate suppression was temporary, and by Day 3 the respiration rates of all treatments were not significantly different. Then, respiration rates increased with increasing glyphosate application rate. By Day 14, the 1x, 2x, and 3x application rates showed a significant increase in respiration rate over the control. The 2x and 3x application rates remained at least 12.3% greater than the control through Day 30.

In the soil Blount0, there was little relationship between carbon added as glyphosate and increase in mineralized carbon (Figure 1.2). The slope of the regression line was negative, indicating that larger doses of glyphosate decreased respiration. However, the low coefficient of determination (R^2=0.06) indicates that the level of glyphosate did not explain much of the variation observed.

The Blount1 soil showed a moderate relationship between glyphosate carbon added and CO2-C evolved (R^2=0.80). The slope of the regression line was less than 1 (0.8848), suggesting that the carbon added as glyphosate was not completely mineralized.

In the Bennington2 soil, there was a strong relationship between glyphosate carbon added and CO2-C evolved (R^2=0.99). The slope of the regression line was greater than 1 (1.39), indicating that the glyphosate stimulated microbial activity beyond glyphosate decomposition.

Experiment II: Repeated Application Incubation

Ester linked fatty acid analysis The Kruskal-Wallis nonparametric one-way analysis of variance discerned no significant effect of glyphosate on the relative abundances of seven functional microbial groups. Of these 126 discrete nonparametric analyses of variance, only 2 found a significant effect at $P< 0.05$. The two significant results showed that in the Blount1 soil on Day 60, gram positive and total bacterial FAME's were less in both glyphosate treatments compared to the control.

Fungal to bacterial ratio is a way to determine if fungi were stimulated over bacteria by glyphosate. Kruskal-Wallis nonparametric one-way analysis of variance found no differences in the ratio of fungal to bacterial FAMEs in all three soils on all sampling days (Figure 1.3).

The analysis of individual FAMEs also yielded no significant effects of glyphosate on the relative abundances individual FAMEs. Of 828 nonparametric analyses of variance only 16 tests, or 1.9%, found a significant effect at $P< 0.05$. The significant results were randomly spread across soil, days, and FAMEs. Setting $P< 0.10$ yielded a higher number of significant results. At this level, 11% of the individual tests showed a significant effect by glyphosate treatment. These significant results also showed no pattern of distribution by day nor FAME nor soil. Analysis of the data using the absolute concentration of FAMEs gave similar results.

Initial NMS analysis of the FAME data showed that soil type was the main structuring variable in this experiment. At day 180, the differences in the soil microbial

communities were readily distinguishable by soil type (Figure 1.4). 85.6% of the variation in the FAME data could be explained by one axis, and the Blount0 soil was distinctly separate from Blount1 and Bennington2. No grouping was apparent by glyphosate treatment (not shown). This result indicates that the initial soil microbial communities of these three soils were significantly different, and that the repeated glyphosate treatments and incubation did little to change the composition of the communities relative to one another.

NMS analysis of each soil type also found no significant effect of glyphosate treatment on soil microbial community makeup as measured by the relative concentration of FAMEs. These 18 analyses yielded either no useful NMS ordination or ordinations that showed no separation by glyphosate treatment (not shown). NMS analysis of the data by absolute concentration of FAMEs gave similar results.

Exchangeable and Microbial Potassium Exchangeable K increased with glyphosate treatment. This result could be expected, since the glyphosate formulation contains K salt. Each field application rate of glyphosate included 11.4 μg K g^{-1} dry soil (2x = 22.8 μg K g^{-1}). Therefore, after six applications of glyphosate, the 1x and 2x glyphosate treatments received a total of 68.4 and 136.8 μg K g^{-1} respectively. Increases in exchangeable K reflect these additions, with each soil showing an increase of approximately 100 μg K g^{-1} after 6months at the 2x glyphosate treatment level (Figure 1.5). These results indicate that K added to the soil with glyphosate is not readily converted to microbial biomass K, nor fixed between clay layers as nonexchangeable K. The majority of K added stayed in the plant available, exchangeable form.

The measurement of microbial biomass K was marked by high variability (Figure 1.6). Two sampling dates (Days 90 and 120) were not used for analysis due to a high number of samples showing a negative microbial biomass K measurements. Variability was less in the Blount0 soil. A general linear model univariate ANOVA found that microbial biomass K was not significantly affected by glyphosate treatment at all sampling date and soils.

DISCUSSION

The Blount0 and Bennington2 soils had an immediate spike in respiration rate that lasted less than nine days. These results agree with previous experiments that show glyphosate causes an immediate, short lived stimulus to microbial activity (Haney et al., 2000; Haney et al., 2002; Lancaster et al., 2010; Sprankle et al., 1975a).

The Blount0 shows two responses to the glyphosate. At first, microbial activity is stimulated. At Day 1, all glyphosate treatments showed significant increases in respiration, with respiration rates increasing with increasing glyphosate application rate. This stimulation is short lived, and by Day 5, there were no significant differences in respiration rates by treatment. Later, the respiration rates of the treated soil were less than the control. By Day 42, the 1x, 2x, and 3x treatments are significantly less than the control. Perhaps the initial elevated stimulation is caused by the decomposition of microorganisms susceptible to glyphosate. Then, later in the incubation, the respiration rates of the treated samples are lower because the total microbial population has been lowered compared to the untreated control.

The Blount1 soil did not show an immediate increase in microbial respiration with glyphosate application. Other studies of glyphosate degradation in various soil types have found a similar response to glyphosate. The slower rate of degradation was attributed to either a lower initial microbial population or stronger binding of glyphosate to the soil (Rueppel et al., 1977; Sprankle et al., 1975a). The initial microbial biomass of these three soils, as measured by total extractable EL-FAME, was not significantly different (data not shown). The strength of glyphosate bonding, and subsequent availability to microorganisms, correlates with the amount of available phosphate sorption sites. Greater increases in microbial respiration after glyphosate exposure correlated with high levels of extractable soil phosphate (Sprankle et al., 1975a). In this experiment, initial soil extractable phosphate was not tested.

The highly significant relationship between glyphosate C added and C mineralized indicates the glyphosate was a source of energy for microbial activity (Haney et al., 2000). Interestingly, this significant relationship was only observed in the two soils that had previous exposure to glyphosate. In fact, the soil with the highest long-term exposure exhibited the strongest relationship.

In the soil with the highest long-term exposure the regression line had a slope greater than 1. The steep slope suggests that the addition of glyphosate stimulated the mineralization of existing soil C or the decomposition of microorganisms susceptible to glyphosate (Wardle and Parkinson, 1990).

This phenomenon was not clearly observed in the Blount0 soil. While there is an initial stimulation of microbial activity at some glyphosate levels, the stimulation is not as clear and consistent.

The difference in the respiration response between the previously untreated soil and the treated soil suggests that glyphosate exposure shifts the microbial community to a population that can more readily use glyphosate as a substrate, an observation supported by Araujo et al. (2003). While organisms that can readily utilize glyphosate may exist in the Blount0 soil, their populations may be too low to have as rapid response as the other soils. This conclusion agrees with Lancaster et al. (2010), who found that after repeated application of glyphosate, microorganisms were better able to utilize glyphosate.

However, EL-FAME data showed no significant changes in microbial community composition due to glyphosate addition. These results are consistent with other experiments that were unable to characterize long term shifts in microbial communities due to glyphosate using fatty acid analysis (Lancaster et al., 2010; Ratcliff et al., 2006; Weaver et al., 2007).

Weaver (2007) did find small, short lived changes in microbial communities after glyphosate application. However, these changes lasted less than seven days. Ratcliff (2006) also found a transient suppression of general bacterial and fungal phospholipid fatty acids after glyphosate application to forest soils. However, this suppression lasted 1 to 3 days.

Our study sampled soils 15 days after the first application, and 30 days after the first, second, fourth, fifth, and sixth applications of glyphosate. This longer period

between glyphosate application and sampling for EL-FAME analysis would allow transient changes to the microbial communities to go undetected. However, the goal of the experiment was to determine if more permanent changes in community structures were occurring after repeated applications.

Lancaster et al. (2010) and Weaver at al. (2007) also reported an increase in the fatty acid 20:0 relative to glyphosate application. In our experiment we found no significant changes in 20:0 in any soil at any sampling date.

Exchangeable K did increase with glyphosate application. This could be because the glyphosate formulation was a K salt. Exchangeable K increased proportionally to the amount of added glyphosate and K. The large majority of K added with glyphosate was able to be extracted and detected as exchangeable K. Therefore, it seems unlikely that soil fungi are rapidly incorporate K into microbial biomass K, nor transfer K to nonexchangeable K sites. If soil fungi are transforming exchangeable K to plant unavailable forms of K, the transformation is not proceeding rapidly enough to overcome the rate of K addition by glyphosate or from other soil K pools.

Table 1.1. Properties of Soils.

Soil Name	Soil Type	Soil Management	Average Glyphosate Applications per Year	pH	C	Soil Texture		
						Clay	Silt	Sand
					---------- % ----------			
Blount0	Blount Silt Loam	Organic	0	6.95	1.47	41	48	11
Blount1	Blount Silt Loam	Non-GMO	1	6.85	2.24	42	41	18
Bennington2	Bennington Silt Loam	GMO	2	6.95	2.46	45	43	12

Table 1.2. Fatty Acid Methyl Esters used as Biomarkers for Taxonomic Grouping

Taxonomic Group	Biomarker Characteristic	FAMEs	Reference
Gram Positive Bacteria	Branched FAMEs	Sum of a15:0, i15:0, a16:0, i16:0, a17:0, i17:0	(Frostegard and Baath, 1996; Tunlid et al., 1989; Zelles et al., 1995)
Gram Negative	Cyclopropyl and monounsaturated FAMEs	Sum of 16:1ω7c, cy17:0, 18:1ω7c, cy19:0ω8c, cy19:0ω10c	(Moore-Kucera and Dick, 2008; Zelles et al., 1995)
General Bacteria	Saturated odd length, ω7 FAMEs	Sum of 15:0, 17:0, 20:1ω7c, Gram positive, and Gram negative Bacteria	(Tunlid et al., 1989)
Actinomycetes	10Me FAMEs	Sum of 10Me16:0, 10Me17:0, 10Me18:0	(Zelles, 1999)
Saprotrophitic Fungi	18:2ω6,9c	18:2ω6,9c	(Frostegard and Baath, 1996)
Abruscular Mycorrhizal Fungi	16:1ω5c	16:1ω5c / (Sum of 16:1ω5c and General Bacteria)	(Olsson, 1999)
Protozoa	20:4ω6,9,12,15	20:4ω6,9,12,15c	(Moore-Kucera and Dick, 2008)

Table 1.3. Fatty Acid Methyl Ester Ratios used in Data Analysis

FAME Ratio	FAMEs	Reference
Fungi / Bacteria Ratio		(Frostegard and Baath, 1996)
Fungi	18:2ω6,9	
Bacteria	15:0, a15:0, i15:0, a16:0, i16:0, 16:1ω7c, 17:0, a17:0, cy17:0, i17:0, 18:1ω7c, cy19:0ω8c, cy19:0ω10c, 20:1ω7c	
Saturated / Monounsaturated Ratio		(Bossio and Scow, 1998)
Saturated	14:0, 15:0, 16:0, 17:0, 18:0	
Monounsaturated	16:1ω5c, 17:1ω9c, a17:1, 17:1ω8c, 18:1ω9c, 18:1ω7c, 18:1ω5c, 19:1ω11c, 20:1ω9c, 20:1ω7c	
Cyclopropyl / monounstaturated precursor Ratio		(Bossio and Scow, 1998)
Cyclopropyl	cy17:0, cy19:0	
Monounsaturated precursor	16:1ω7c, 18:1ω7c	

Table 1.4. Percentage difference in respiration rate between glyphosate amended and unamended control of three soils having different histories after 55 days. * Indicates a significant difference from the control using Dunnett test at 5% significance.

Soil	Glyphosate Application Rate	Incubation Day												
		1	2	3	5	7	9	14	19	23	30	34	42	55
		Respiration Rate Difference from Control (%)												
Blount0	0.5	11.7 *	2.9	2.5	1.3	-1.9	1.0	-0.5	8.3	3.7	-3.4	-1.4	-5.8	-5.0
Blount0	1	26.0 *	22.2 *	6.8	1.3	7.5	9.3	3.1	0.8	15.1	-4.9	-6.8	-8.1 *	-0.4
Blount0	2	33.8 *	17.5 *	3.7	-3.1	-4.2	-1.5	-7.1	-8.3	-8.6	-9.9	-10.4 *	-13.5 *	-8.8
Blount0	3	43.1 *	40.9 *	21.0 *	5.8	3.8	2.9	-3.1	-1.9	-7.3	-7.6	-8.6	-14.7 *	-10.1
Blount1	0.5	-7.6 *	0.6	-3.3	1.5	-0.6	2.0	4.8	2.0	8.0	2.8	-3.0	1.0	5.3
Blount1	1	-4.0	5.4	8.6	7.4	7.4	9.8	9.5 *	3.6	12.9	30.6	10.2	10.4	11.0 *
Blount1	2	-4.6 *	3.6	7.3	9.4	11.9 *	13.7 *	15.6 *	12.3 *	18.4 *	33.4	8.4	7.2 *	4.0
Blount1	3	3.3	15.0 *	9.9	15.3 *	23.3 *	24.2 *	21.8 *	15.0 *	21.5 *	17.4	18.5 *	10.4 *	6.4
Bennington2	0.5	18.3 *	4.0	3.8	-1.7	4.6	4.1	2.8	3.2	7.0 *	4.1	0.3	0.5	-1.5
Bennington2	1	26.7 *	11.4	11.5	1.3	7.3 *	3.2	5.0	6.2 *	7.7 *	3.5	0.3	0.9	-1.9
Bennington2	2	49.2 *	18.2 *	24.4 *	6.5 *	11.0 *	13.1	10.5	12.7 *	3.3 *	9.7 *	5.5	5.7 *	1.5
Bennington2	3	48.9 *	28.4 *	39.1 *	20.8 *	16.0 *	16.2	14.2	15.9 *	5.4 *	11.7 *	9.1 *	8.4 *	1.5

LIST OF FIGURES

Figure 1.4. Nonmetric-multidimensional scaling plot of FAME profiles (as square root of mole percent) from soils with three different levels of previous glyphosate exposure after a six month incubation and six applications of glyphosate. The proportion of variance explained the axis is based on the correlation (R^2) between distance in the reduced NMS space and distance in the original space.

Figure 1.5. Relationship of glyphosate application rate and exchangeable K in three soils after six month incubation with six glyphosate applications. The soils were: a) a soil with no reported application of glyphosate, (b) a soil with a history of moderate glyphosate application, and (c) a soil with a history of heavy glyphosate application. Vertical bars represent ±SD (n=3), and most are smaller than treatment symbols.

Figure 1.6. Relationship of glyphosate application rate and microbial biomass K in three soils after six month incubation with six glyphosate applications. The soils were: a) a soil with no reported application of glyphosate, (b) a soil with a history of moderate glyphosate application, and (c) a soil with a history of heavy glyphosate application. Vertical bars represent ±SD (n=3).

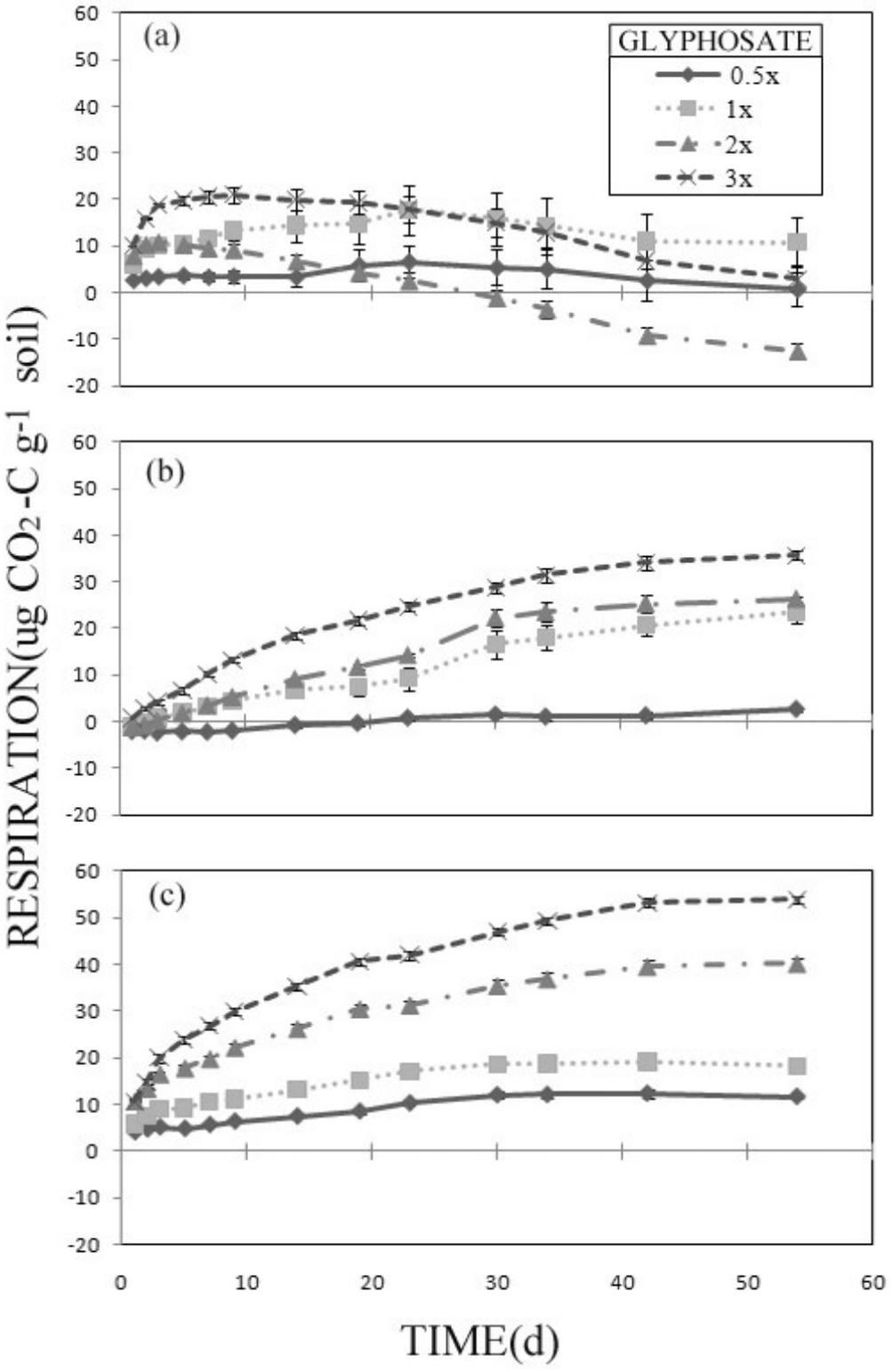
(a)
GLYPHOSATE
0.5x
1x
2x
3x
(b)
(c)
RESPIRATION(ug CO2-C g-1 soil)
TIME(d)

Figure 1.1

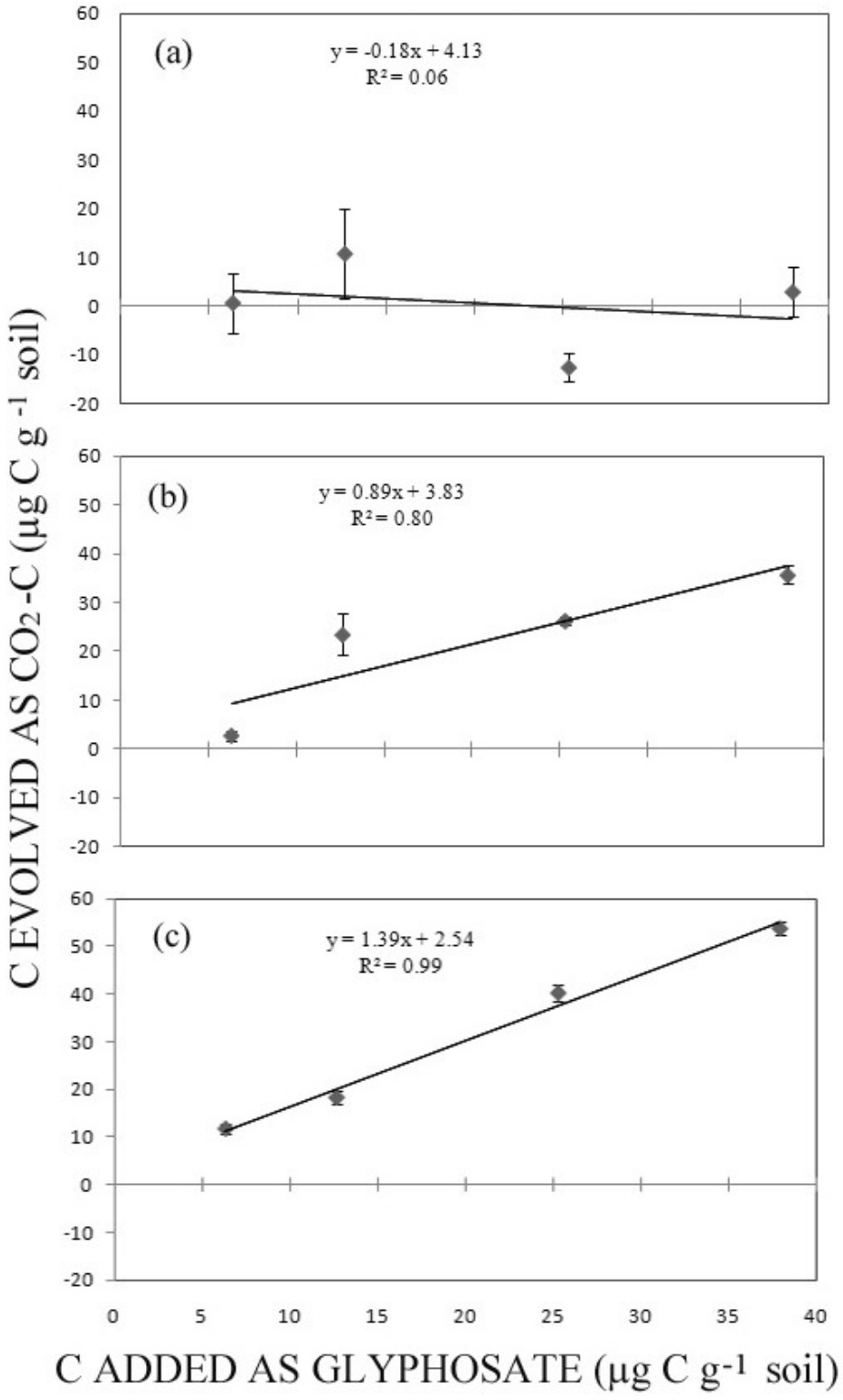
(a)
y = -0.18x + 4.13
R² = 0.06
(b)
y = 0.89x + 3.83
R² = 0.80
(c)
y = 1.39x + 2.54
R² = 0.99
C EVOLVED AS CO2-C (µg C g-1 soil)
C ADDED AS GLYPHOSATE (µg C g-1 soil)

Figure 1.2

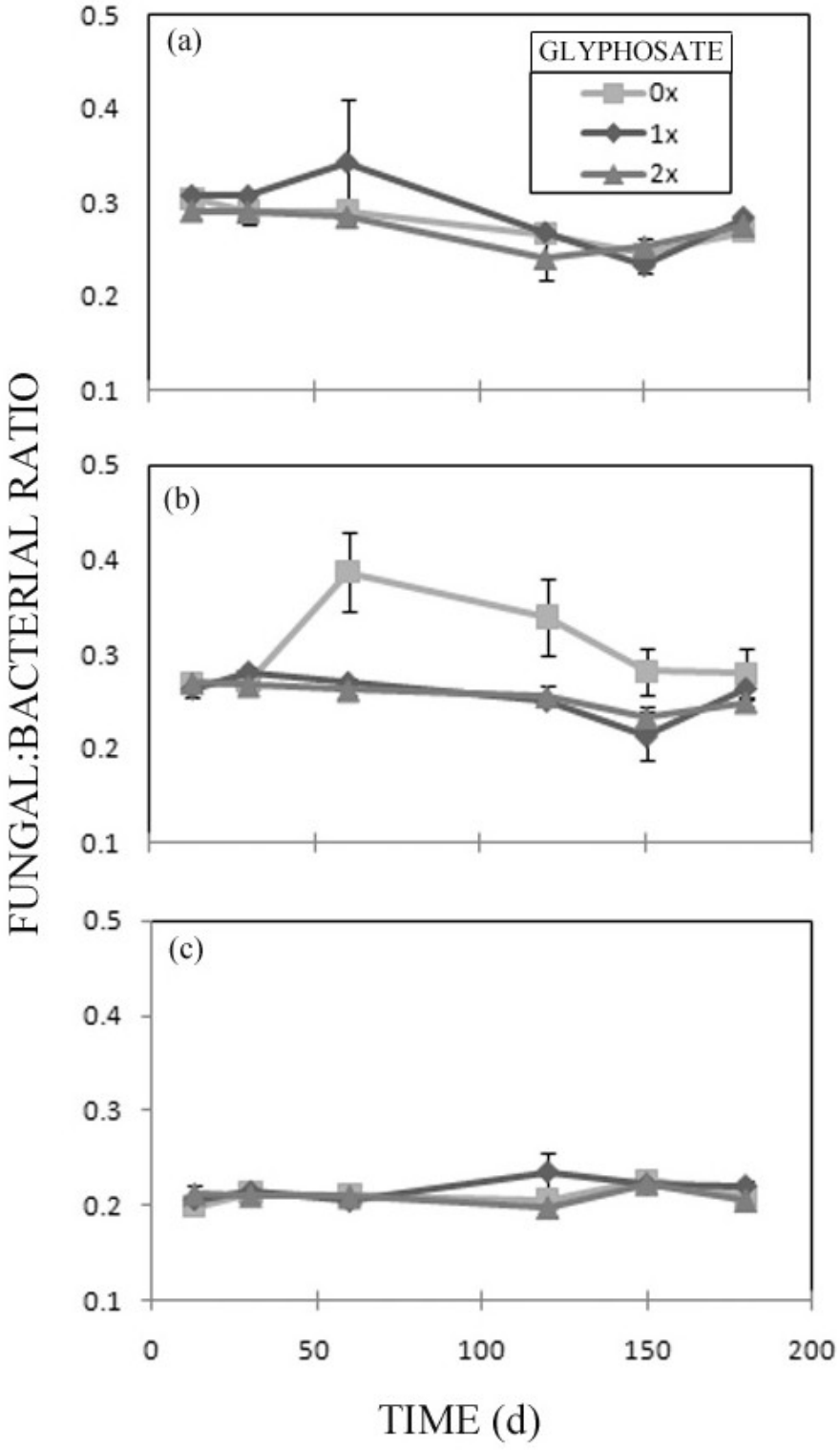
(a)
GLYPHOSATE
0x
1x
2x
(b)
(c)
FUNGAL:BACTERIAL RATIO
0.5
0.4
0.3
0.2
0.1
0
50
100
150
200
TIME (d)

Figure 1.3

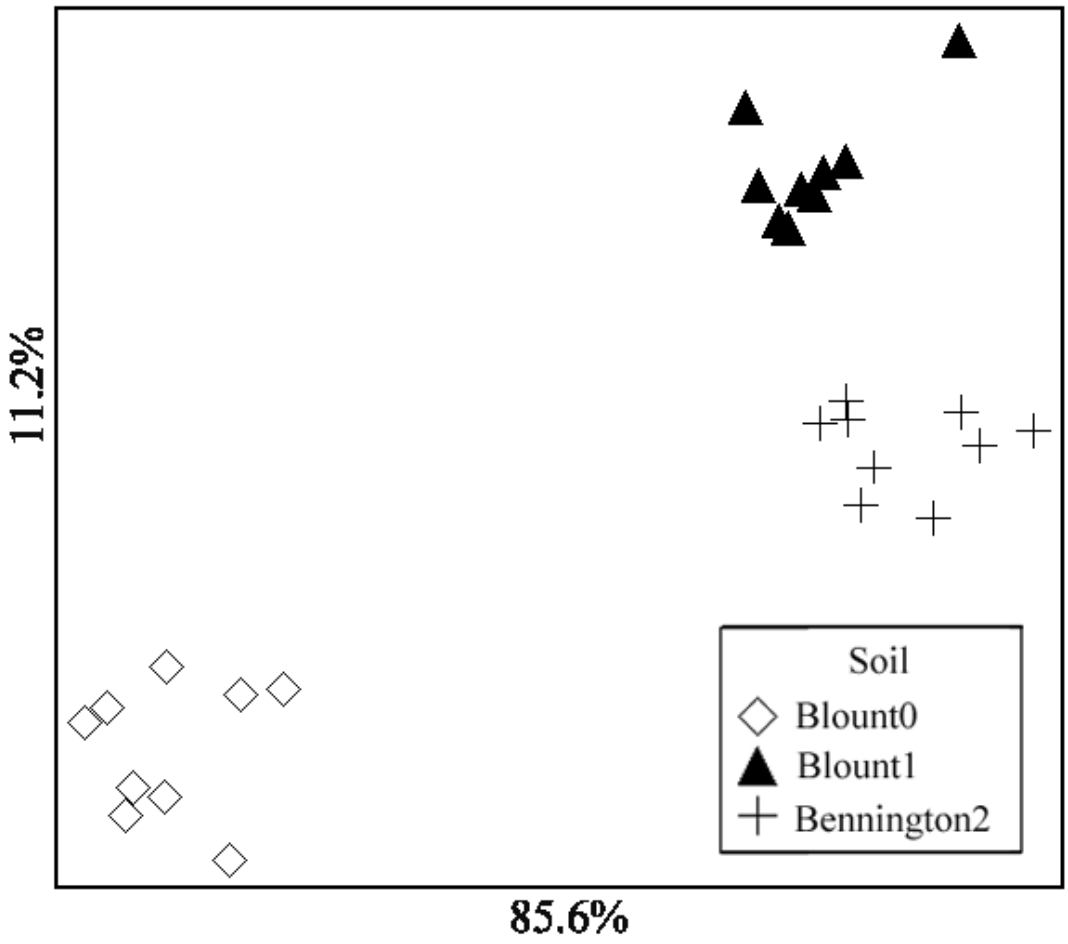

Figure 1.4

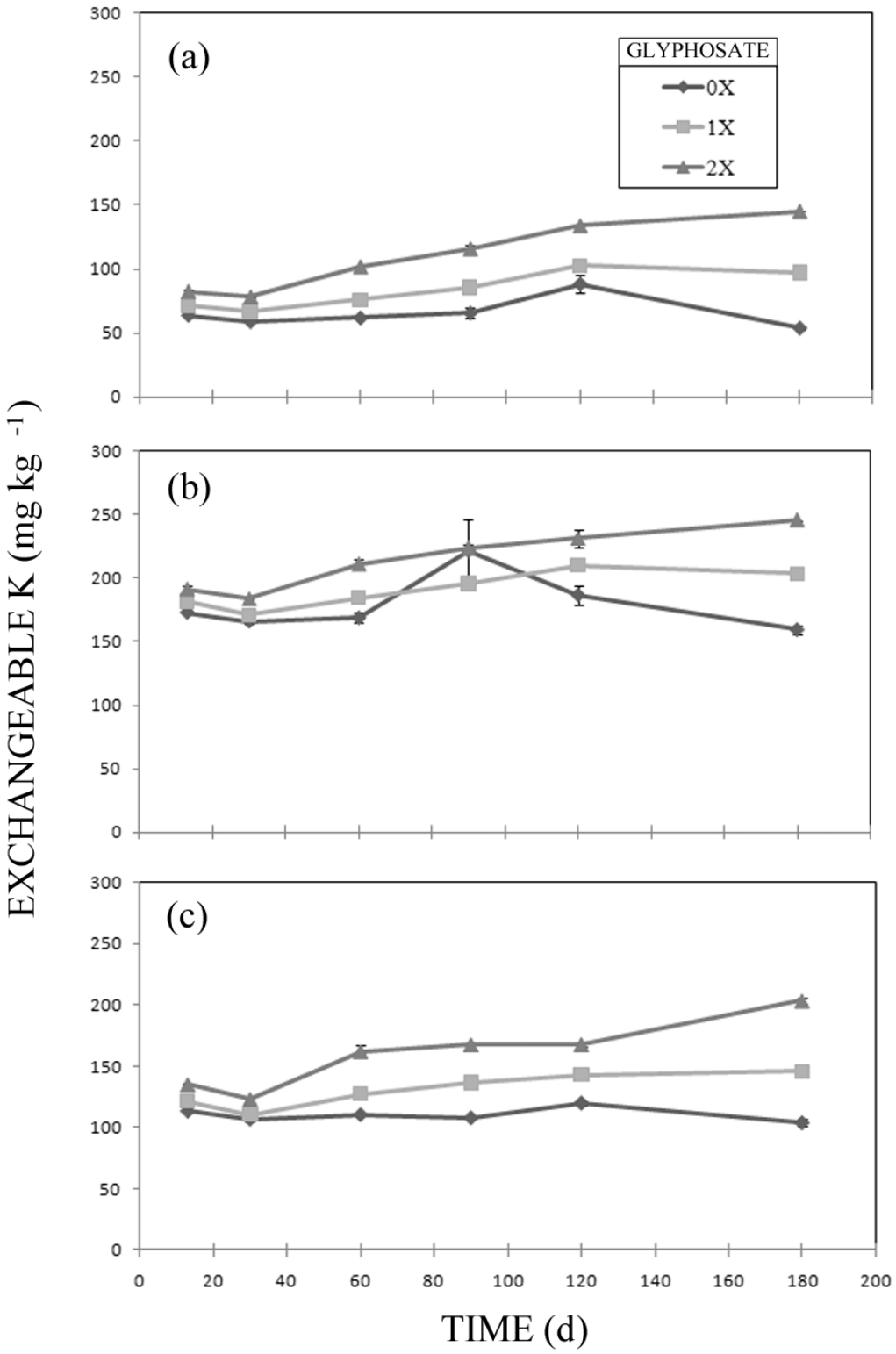
(a)
(b)
(c)
GLYPHOSATE
0X
1X
2X
EXCHANGEABLE K (mg kg -1)
TIME (d)
0
50
100
150
200
250
300
0
20
40
60
80
100
120
140
160
180
200

Figure 1.5

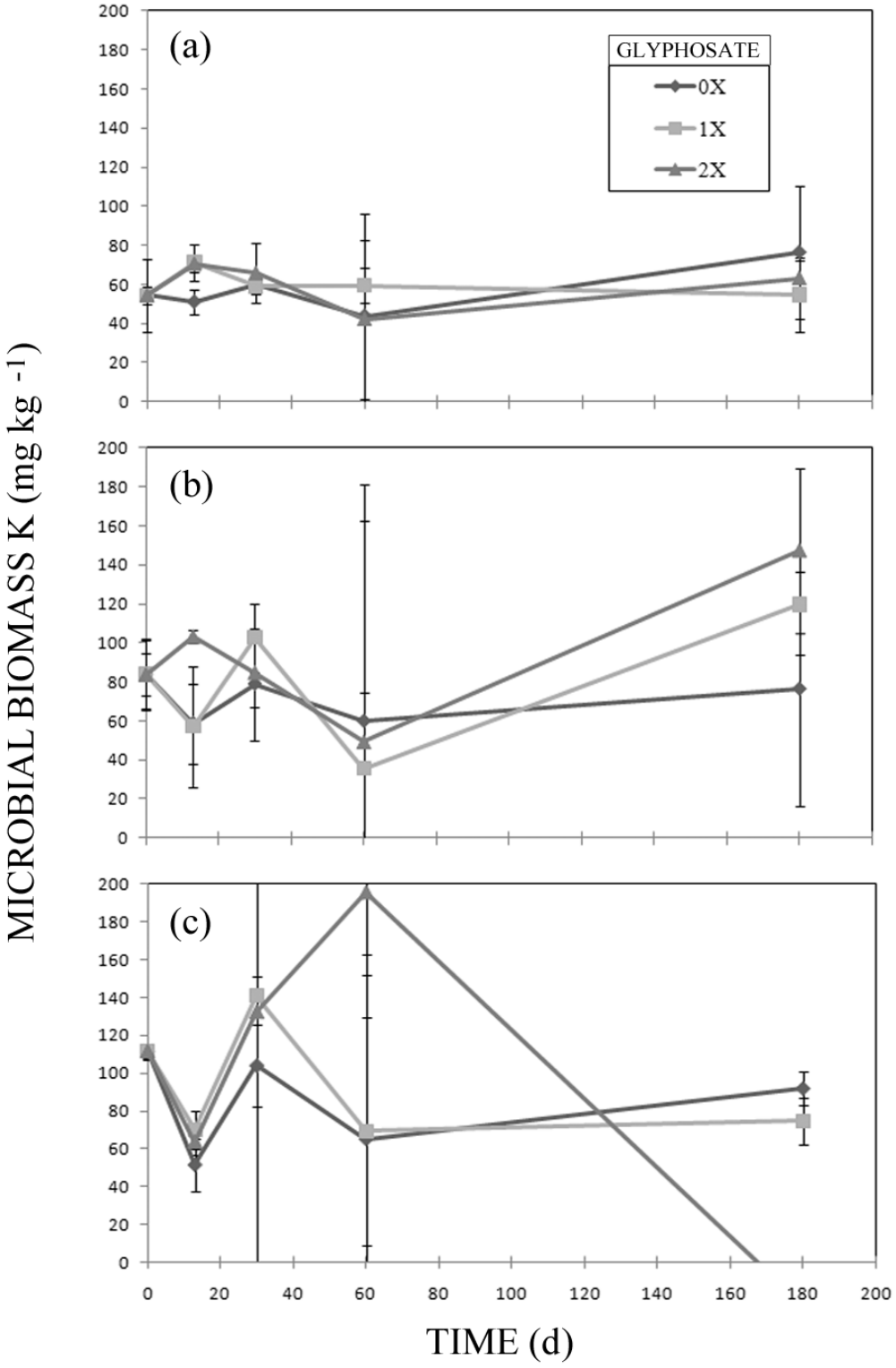
(a)
(b)
(c)
GLYPHOSATE
0X
1X
2X
MICROBIAL BIOMASS K (mg kg -1)
TIME (d)
0
20
40
60
80
100
120
140
160
180
200

Figure 1.6

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CHAPTER 2: THE EFFECT OF GLYPHOSATE ON MICROBIAL ACTIVITY, COMMUNITY STRUCTURE, AND POTASSIUM DYNAMICS: A GREENHOUSE STUDY

ABSTRACT

With advent of glyphosate [N-(phosphonomethyl)glycine] tolerant crops, soils have now been receiving repeated applications for over ten years in the Midwestern US. Relatively little is known about long term or repeated glyphosate affects the soil microbial affects on the soil community which ultimately could impact nutrient availability for crops. One possibility is that repeated use of glyphosate is creating a selection pressure in soil microbial communities that could affect soil K dynamics for which there is anecdotal evidence. The objective of this study was to determine the effect glyphosate applications on microbial communities and K dynamics in soils and uptake by plants. A greenhouse study was done that had a 2 X 2 X 3 factorial design with two soil treatments (with or without long-term field applications of glyphosate), two plant treatments (presence and absence of soybean plants), and three rates of glyphosate treatment (0, 1, and 2 applications of 0 or 0.87 kg ae ha^{-1}). After each glyphosate application soils were sampled and analyzed for microbial community structure using ester linked fatty acid methyl ester biomarkers (EL-FAME) and exchangeable, plant tissue, and microbial biomass K. Glyphosate application caused a significant decrease in the total microbial biomass in soybean rhizosphere in soil that had no previous exposure to glyphosate at seven days after glyphosate application, as measured by total extracted EL-FAMEs. However, no significant changes were observed in the overall microbial community structure. The conclusions were that glyphosate: 1) caused lower total microbial biomass in the glyphosate resistant soybean rhizosphere; 2) caused no changes

in the microbial community structure (EL-FAME), and; 3) did not reduce the plant availability K (soil exchangeable or plant tissue K).

INTRODUCTION

One of the most dramatic changes to agriculture in recent history has been the rapid adoption of glyphosate resistant (GR), or "Roundup Ready," crops. When GR crops first became available in 1996, GR crops were grown on 1.7 million hectares worldwide. In 2008, GR crops were planted on 79 million hectares in 25 countries. Seventy percent of the 95 million acres of soybeans planted globally in 2008 were GR (James, 2008).

Glyphosate [N-(phosphonomethyl)glycine] is now the most commonly used herbicide in the world. This broad-spectrum, postemergence herbicide was first sold in 1974 by Monsanto under the trade name Roundup. The mode of action is the inhibition of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase, a critical intermediate, used in the production of three aromatic amino acids vital to plants (Franz et al., 1997).

There is growing concern that the widespread use of glyphosate may be producing unintended, undesirable consequences. These consequences include adverse effects on plant nutrition and shifts in soil microbial communities, including an increase in plant pathogens (Yamada et al., 2009).

Anecdotal evidence suggest there is an increasing amount of potassium (K) deficiency in corn grown in rotation with GR soybeans (Cliff Ramsier, personal communication, 2006). This K deficiency has been observed in several corn cultivars, but is most prevalent in fast growing, high yielding corn hybrids. Also, this K deficiency

has been predominantly observed in poorly drained soils, particularly after high rainfall (Cliff Ramsier, personal communication, 2006).

Some plant nutrient deficiencies associated with glyphosate are attributed to glyphosate affecting the ability of the plant to take up nutrients. Ozturk et al. (2008) found that glyphosate lowered the ability of sunflower plants to take up iron, due to a reduction in root ferric reductase activity. Additional studies found that glyphosate application reduces plant tissue concentrations of iron, manganese, zinc, and boron (Eker et al., 2006; Neumann et al., 2006).

Plant nutrient deficiencies associated with glyphosate use have also been attributed to reduced availability of soil nutrients due to a change in soil microbial community (Johal and Huber, 2009; Kremer and Means, 2009). For example, glyphosate has been shown to reduce populations of Mn-reducing microorganisms active in soil nutrient cycling (Huber, 2007). It is possible that the widespread use of glyphosate is causing a shift in soil microbial community composition which affects soil K dynamics.

The effect of glyphosate on soil microorganisms has been widely studied, with conflicting results. As soil microbial communities are diverse, responses to glyphosate use are varied. A number of studies have found that glyphosate amendment has no significant effect on microbial community activity and composition (Busse et al., 2001; Liphadzi et al., 2005; Ratcliff et al., 2006; Weaver et al., 2007). A review of the effect of GR crops on soil microbial communities concluded that microbial diversity can be altered

by the use of GR plants in conjunction with glyphosate, although the observed changes were variable and transient (Dunfield and Germida, 2004).

However, most previous research was done with only short term applications of glyphosate. Since 1996, glyphosate has been in heavy use in some fields, with applications several times a year. There is the possibility that a shift in microbial communities occurs after extended glyphosate use. A number of field studies, (Fernandez et al., 2005; Fernandez et al., 2009; Johal and Huber, 2009; Kremer and Means, 2009; Locke et al., 2008), anecdotal evidence, and unpublished data (Huber, 2007; Yamada et al., 2009) suggest that an glyphosate has an effect on microbial communities after long term, repeated applications.

Glyphosate can adversely affect microorganisms when applied *in vitro*. This result could be expected, since the shikimic acid pathway disrupted by glyphosate is present in bacteria and fungi. However, the experiments that have shown the greatest inhibition of microorganisms may be of little ecological relevance, since they were performed *in vitro* or in pure culture (Grossbard, 1985).

The chemical structure of glyphosate includes a phosphate group that readily adsorbs to soil clays and aluminum and iron oxides. When glyphosate binds to soil, it loses its antimicrobial properties (Sprankle et al., 1975b), and is rapidly degraded by soil microorganisms, which use glyphosate as a source of nutrients (Franz et al., 1997). Degradation of glyphosate in the soil is a primarily a biological process, performed by microorganisms (Rueppel et al., 1977). Therefore, degradation rates of glyphosate are determined by conditions that affect microbial activity, such as temperature and moisture

content. A thirteen site study performed by the European Commission found the average half-life of glyphosate to be 33 days, with a range of 1-130 days (European Commission, 2002).

There is evidence of increases in fungal activity and populations in soil treated with glyphosate. This stimulation may be due to the fact that fungi are the main microbial degraders of glyphosate (Krzysko-Lupicka et al., 1997). Araujo, et al., (2003), found that glyphosate amendment did not affect culturable bacterial populations, while fungi and actinomycetes populations increased. This effect was larger in soils that had greater previous exposure to glyphosate. Other studies have shown the glyphosate use is associated with an increase in the plant pathogens *Fusarium* and *Pythium* (Kremer et al., 2005; Levesque et al., 1993; Meriles et al., 2006). Glyphosate can stimulate the growth of mycorrhizal fungi *in vitro* (Laatikainen and Heinonen-Tanski, 2002).

It has been shown that fungi have the ability to rapidly take up K (Weed et al., 1969). Therefore, it is possible that repeated glyphosate addition is causing a selection pressure in soil microbial communities which favors fungal growth. In turn, the fungi are able to rapidly uptake K, making it unavailable to plants. In addition to storing K in microbial biomass K, fungi hyphae could also be transferring exchangeable K to nonexchangeable K sites. Alternatively, there could be other shifts in subpopulations that potentially impact crops. If this does occur, the US Midwest would be likely region for this to happen because there has been over 10 years of extensive use of glyphosate.

Consequently, in order to study the long-term effects we collected a soil that had high and repeated applications of glyphosate over the last 10 years to be used in comparison to a soil that, as far as we know, never received glyphosate.

This experiment incorporated the GR soybean plant as a conduit to apply glyphosate to rhizosphere soil, since treated plants translocate and concentrate glyphosate in roots, where it is exuded into the soil (Coupland and Caseley, 1979; Laitinen et al., 2007). Hydroponically grown GR soybean roots were shown to release greater amounts of glyphosate, carbohydrates, and amino acids than a non-GR cultivar after foliar application of glyphosate. These exudates were shown to stimulate fungal growth *in vitro* (Kremer et al., 2005).

The primary objectives of this experiment were to characterize the effect of foliar applied glyphosate on the GR soybean and the surrounding soil through: 1) ester linked fatty acid methyl ester (EL-FAME) analysis of rhizosphere soil microbial community composition, 2) measurement of exchangeable and microbial K in the rhizosphere, and 3) analysis of leaf K. Two soils, one with a history of high amounts of glyphosate exposure, and one with no previous application history, were used to examine for different responses.

MATERIALS AND METHODS

Soils

Two soils were used in this study. The goal was to use two soils with similar physical and chemical characteristics, yet different levels of previous exposure to

glyphosate. Both sites were within an eleven kilometer radius in eastern Delaware County, Ohio.

The first soil was a Blount silt loam (fine, illitic, mesic Aeric Epiaqualf). This soil was from an organically managed farm utilizing a continuous rotation, the previous five years being alfalfa-orchard grass, corn, oats-alfalfa-orchard grass, spelt-timothy-clover, and timothy-clover. The soil had no previous recorded exposure to glyphosate (Blount0).

The second soil was a Bennington silt loam (fine, illitic, mesic Aeric Epiaqualf). It was from a farm practicing no till, growing corn and GR soybeans in rotation. Glyphosate was applied up to three times a year while growing soybeans, and once a year while cultivating corn, for an average of two yearly glyphosate applications (Bennington2). Corn grown in this field had experienced symptoms of K deficiency. The primary distinguishing factor between a Bennington and Blount silt loam, based on soil classification, is calcium carbonate concentration C horizon, a depth not sampled in this experiment.

Soils were sampled in the August of 2009. Approximately 40 kg of soil were taken from the top 10 cm at each site. The soil was randomly selected within a 15 meter radius, then composited. The samples were passed through a 6.4 mm sieve and stored at 4ºC.

Greenhouse Experiment

A greenhouse study was done that had a 2 X 2 X 3 factorial design with two soil treatments (with or without long-term field applications of glyphosate), two plant

treatments (presence and absence of soybean plants), and three rates of glyphosate treatment (0, 1, and 2 applications). Each treatment was replicated four times.

2.5 kg of soil was placed in a plastic pot. Soil moisture in the pot was taken to two thirds water holding capacity (16% w/w for Blount0, 22% w/w for Bennington2). Roundup ready soybean seeds (Asgrow AG 3205) were placed between wet paper towels until germination. Six germinated seeds were planted in each pot. Eight days after emergence the plants reached stage V1. At this point, the plants were manually thinned, leaving the two largest plants in each pot. To assess the affect of glyphosate on soil without a plant, pots were filled with soil, but soybeans were not planted. These pots received the same fertilizer, pesticide, and watering treatments as the planted pots.

To ensure that nutrients, besides K, were not a limiting factor, each pot was fertilized. At 33 days post emergence the pot was fertilized with N as $Ca(NO_3)_2$ at 100 mg N kg^{-1} soil. At day 40, the pot was fertilized with N and P as monobasic ammonium phosphate ($NH_4H_2PO_4$) at 22.5 mg N kg^{-1} soil and 50 mg P kg^{-1} soil. To prevent insect damage, the plants were sprayed with spinosad (Conserve SC, Dow AgroSciences) and pymetrozine (Endeavor, J.R. Johnson Supply) on day 36.

For the first ten days after planting, water loss from each pot was determined gravimetrically daily. Thereafter, water loss was determined every two days. Water was replaced with deionized water to maintain soil moisture at two thirds of water holding capacity.

At 47 and 61 days post emergence, the pots were sprayed with glyphosate in the form of Roundup PowerMax (Monsanto, St. Louis, MO) (540 g acid equivalent L^{-1}). The

pots were sprayed with a hand sprayer at the rate of 1.61 liters PowerMax per hectare (0.87 kg ae ha^{-1}), or one half the maximum annual recommended application rate. Before application the PowerMax was diluted to 3.6 g ae L^{-1}. Spraying was performed in the morning, outside the greenhouse, in sunny, calm conditions.

At 54 days post emergence (7 days after glyphosate exposure), half the pots with plants were destructively sampled. The soil was carefully removed from each pot and the bulk soil was discarded. Soil that remained attached to the roots was manually separated from the roots and collected as rhizosphere soil. The plants were then frozen. The surface soil of the fallow pots was also sampled. Four cores that were 2 cm in diameter and 1 cm deep were collected from each fallow pot.

At 68 days post emergence (21 days after the first, and 7 days after the second glyphosate exposure), the remaining pots with plants were destructively sampled and rhizosphere soil was collected in the same manner. Surface soil was again sampled from the fallow pots.

Soil and Plant Analytical Methods

Chemical Analyses Soil pH was determined in 0.01 M $CaCl_2$ at a soil:solution ratio of 1:2.5 after 2 h using a glass membrane electrode. Soil organic C was measured by dry combustion at 550 °C using a C elemental analyzer (LECO). Soil texture was determined by the hydrometer method. The field capacity of the soil was determined by drying a saturated core on a pressure plate at -30 kPa, weighing, then oven drying the core at 110ºC, and re-weighing. Soil characteristics are presented in Table 2.1.

Exchangeable K was measured by shaking soil in a 1M solution of ammonium acetate. The solution was then filtered through Whatman no. 2 filter paper. The filtered extracts were analyzed with a flame photometer (Model 2655, Cole Parmer Instrument Co.) (Knudsen et al., 1982).

Nonexchangeable K was measured using the boiling nitric acid method (Helmke and Sparks, 1996). Briefly, soil was boiled in 1M nitric acid for 15 minutes, and then filtered through Whatman no. 50 paper. The filtered extracts were analyzed with a flame photometer. Nonexchangeable K concentration was calculated as nitric acid extractable K minus exchangeable K.

Microbial K was measured using a chloroform fumigation-extraction method (Lorenz et al., 2010). Briefly, soil was fumigated with ethanol-free chloroform for twenty-four hours. Unfumigated samples served as controls. Potassium was then extracted by adding a 1M ammonium acetate solution and shaking. The solution was then filtered through Whatman no. 2 filter paper. The filtered extracts were analyzed with a flame photometer. Microbial biomass K was calculated by first subtracting the unfumigated control K (exchangeable K) from the fumigated K, then dividing by an extraction efficiency coefficient (0.18).

Above ground plant biomass was measured by separating the roots from the rest of the plant. The resulting stems and leaves were dried at 40ºC to a stable weight.

Plant tissue K was measured using a dry ashing procedure. 0.5 g of oven dried plant leaves were combusted in a 500ºC muffle furnace. The resulting ash was dissolved

in dilute acid (3.6 M HCl and 2.6 M HNO_3). This solution was diluted and analyzed in a flame photometer.

Microbial Analyses Ester linked fatty acid methyl esters (FAMEs) were extracted by the method described by Schutter and Dick (2000). Briefly, lipids were extracted and methylated in a K hydroxide solution. Then resulting solution was neutralized with acetic acid. FAMEs were partitioned into an organic phase by the addition of hexane and subsequent centrifuging. The organic hexane layer was separated and evaporated. The FAMEs were then dissolved in 0.5 hexane methyl-*tert* butyl ether. FAMEs were separated and quantified on a HP 5890 gas chromatograph with automated peak identification software (MIDI Inc.). Quantification of FAMEs was accomplished using the addition of nonadecanoic methyl ester (19:0) as an analytical standard, allowing GC peak areas to be converted to a molar basis.

The standard nomenclature is used to describe FAMEs. Each is designated by the total number of carbon atoms. The number of double bonds is given after the colon, followed by the position of the double bond from the methyl end of the molecule. The prefixes “a” and “i” refer to anteiso- and iso-branching, the suffixes “c” and “t” indicate c*is* and *trans* geometry, 10Me indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule, position of hydroxy (OH) groups are noted, and cy indicates cyclopropyl fatty acids.

The total amount of FAME extracted was used as an index of total biomass. The sum of FAMEs characteristic to general bacteria, gram positive bacteria, gram negative bacteria, actinomycetes, saprotrophitic fungi, and protozoans were used to identify these

larger taxonomic groups. The total of 16:1ω5c, the marker for abruscular mycorrizal fungi (AMF), cannot be used directly as indicative of the total population, since this FAME also occurs in bacteria. Therefore, the AMF data was normalized to the total of all bacterial FAMEs (Table 2.2).

The ratio of total fungal markers to total bacterial markers was used as a broad indicator to characterize the state of the microbial community. The following ratios were used to identify possible physiological or nutritional stress: total monounsaturated to total saturated FAMEs, and the sum of cyclopropyl FAMEs to the sum of their monounsaturated precursors (Table 2.3).

Statistical Analysis

All statistical analyses were conducted using PSAW Statistics (version 18.0) and PC-ORD (version 5.32) statistical software. FAMEs were calculated using both absolute (nanomole per gram soil) and relative concentrations (mole percent of total FAME). Prior to data analysis, FAMEs that were present in fewer than 5% of all samples were discarded (McCune and Grace, 2002). Forty-two FAMEs were used for analysis.

Non-metric multidimensional scaling (NMS) was performed using PC-ORD based on Sørensen distance. Two hundred and fifty runs were made with real data and compared to 250 randomized runs. A stability criterion of 0.00001 was used. NMS was performed on both the absolute and relative abundance data sets (nanomoles per gram soil and mole percent of total FAME). Before NMS, the data were transformed using a monotonic square root transformation to improve normality and reduce the coefficient of variation among FAME's.

Kruskal-Wallis nonparametric one-way analysis of variance was used to evaluate the significance of glyphosate application on each FAME and the FAME taxonomic groupings.

A general linear model univariate analysis of variance (ANOVA) was used to evaluate the significance of glyphosate application on microbial, exchangeable, and plant K.

RESULTS

Ester Linked Fatty Acid Analysis

No significant effects of glyphosate on the relative concentrations of seven broad taxonomic microbial groups in the rhizosphere soil were detected in one-way analysis of variance. Twenty eight discrete nonparametric analyses of variance within 7 seven microbial functional groups at all sampling dates showed no significant effect of glyphosate at $P< 0.05$.

The analysis of variance for the fungal:bacterial ratio shower no significant differences in the rhizosphere of both soils on both sampling days (Figure 2.1).

The analysis of individual FAMEs also yielded few significant effects of glyphosate on the relative abundances individual FAMEs. Of 168 nonparametric analyses of variance only 9 tests, or 5.4%, found a significant effect at $P< 0.05$. The significant results were randomly spread across soil, days, and FAMEs. In the Blount0 soil, the only significant effect observed was higher levels of i16:1H in the treated rhizosphere on Day 68. The Bennington2 rhizosphere soil treated with glyphosate had

significantly lower levels of cy17:0 on Day 54. On Day 68, levels of 17:0 , i17:0 , 10Me17:0 ,and 18:1ω9c were significantly lower, and levels of a16:0 were significantly higher.

Setting P< 0.10 yielded a higher number of significant results. At this level, 8.9% of the individual tests showed a significant effect by glyphosate treatment. These significant results also showed no pattern of distribution by day nor FAME nor soil. Analysis of the data using the absolute concentration of FAMEs gave similar results.

Glyphosate application did significantly reduce the total FAMEs extracted in the Blount0 soil at the first sampling date (after one glyphosate exposure) (Figure 2.2). The total FAME extracted was also lower in the glyphosate treated soil at the second sampling date, but the difference was not significant. The same trend was found in the Bennington2 soil on both dates: total extracted FAME was lower in the glyphosate treated rhizosphere, but not at a level of statistical significance.

Initial NMS analysis of the FAME data showed that soil was the main determinant of microbial community composition (Figure 2.3). The NMS ordination also showed that the presence of the soybean plant also caused obvious grouping in both the Blount0 (Figure 2.4) and Bennington2 (Figure 2.5) soils. No groupings were apparent by glyphosate treatment. This result indicates that the foliar glyphosate treatments caused no measurable change to the composition of the communities.

A NMS analysis of each sampling date, soil, and plant factorial combination also showed no significant effect of glyphosate treatment on soil microbial community makeup as measured by the relative concentration of individual FAMEs. These 8

analyses yielded either ordinations that showed no separation by glyphosate treatment (not shown) or ordinations that did not produce stronger axes than expected by chance as evaluated by a stress test. NMS analysis of the data by absolute concentration of FAMEs yielded similar results.

Exchangeable, Nonexchangeable, and Microbial Potassium

In the Blount0 soil, the glyphosate treatment showed no significant effect on exchangeable K. In the Bennington2 soil, the single application of glyphosate showed no effect on exchangeable K. After the second application, exchangeable K was significantly higher (Figure 2.6).

The nonexchangeable K did not vary significantly at both sampling dates in both soils (not shown).

Microbial biomass K had high variability. ANOVA showed that microbial biomass K was not significantly affected by glyphosate treatment at both sampling dates in both soils.

Plant Biomass and Tissue Potassium

Glyphosate treatment had no significant effect on plant biomass in both soils on both sampling dates (not shown).

ANOVA showed that soybean leaf K was not significantly affected by glyphosate treatment at both sampling dates in both soils (Figure 2.7).

DISCUSSION

No significant differences in soil microbial community composition were detected using NMS ordination and analysis of taxonomic groupings of FAMEs. Previous experiments using fatty acid analysis have shown that glyphosate causes small and short lived changes to microbial communities. These detected changes were transient, and lasted less than seven days (Lancaster et al., 2010; Ratcliff et al., 2006; Weaver et al., 2007). In contrast to these studies, our experiment was designed to follow what happens in farmers' fields, where most of the glyphosate applied moves from the leaves to the roots and is then released into rhizosphere soil. Furthermore, there is co-release of other organic compounds from roots releasing glyphosate (Kremer et al., 2005) that would be expected to affect transformations of glyphosate in rhizosphere soil.

Our experiment was sampled later after the glyphosate application (21 days after the first application and 7 days after the second application) than the previous research in the literature. In part this was done to allow enough time for glyphosate translocation to the roots and to release it into the rhizosphere soil. It may be possible that we missed a transient change in community structure after the glyphosate application.

One result of our experiment not found in previous investigations is that glyphosate caused a decrease in microbial biomass in the previously unexposed soil seven days after the first application. This result indicates that soil microbial community may be negatively impacted by glyphosate. A significant decrease in biomass caused by glyphosate could be classified as a selection pressure able to cause a shift in microbial

populations. However, the FAME data, including the fungal to bacterial ratio, showed no significant shifts in microbial community structure.

Our results stand in contrast to Kremer et al., (2005), who found glyphosate increased root exudates and stimulated microbial biomass, most notably *Fusarium* spp. However, this study was done in hydroponic mesocoms that allowed detection of these compounds and isolation of *Fusarium* at very low levels. It may be that the FAME analysis we used was unable to detect these small and perhaps subtle changes in the subpopulations of soils receiving glyphosate.

Foliar glyphosate application had no significant effect on the microbial biomass K and nonexchangeable K in the rhizosphere soil. This would suggest K deficiency is not due to K immobilization in microbial biomass after glyphosate application. In fact, exchangeable K was significantly higher in the Bennington2 rhizosphere soil after two applications of glyphosate. This result indicates that the distribution of K in the soybean rhizosphere, and the plant availability of K, is not significantly affected by glyphosate application.

Leaf tissue analysis shows that glyphosate application had no significant effect on K uptake in the GR soybean plant. Under field conditions, anecdotal information indicates that it is corn grown in rotation with GR soybeans that are mainly having K deficiency. It is possible that there is a phenomenon occurring in the corn plant rhizosphere which limits K uptake that is caused by application of glyphosate during the soybean phase of the crop rotation. However, there is no evidence that glyphosate lowered exchangeable K, increased microbial biomass K, increased nonexchangeable K,

or inhibited K uptake by soybean. Perhaps there is immobilization or a microbial response occurring in corn that does not happen in soybean.

This research found that glyphosate did not cause a K deficiency in GR soybeans. Perhaps the observed K deficiency should not be attributed to solely to glyphosate, but to other practices associated with GR crops, such as no tillage, which can cause K deficiency (Moncrief and Schulte, 1982). Long term use of NT can cause a stratified distribution of K within the soil profile, with concentrations highest at the soil surface that decrease with depth, compared to conventional tillage systems that are relatively uniform in K content in the top 15 cm (Vyn et al., 2002). However, K deficiency has been observed in both NT and conventional tillage systems across the Midwest (Cliff Ramsier, personal communication, 2006). Alternatively, there may be direct or indirect (mediated by microorganism stimulated by glyphosate) physiological effects that affects the ability of crops to take up K without measurable changes in soil microbial properties.

Table 2.1. Properties of Soils.

Soil Name	Soil Type	Soil Management	Average Glyphosate Applications per Year	pH	C	Soil Texture		
						Clay	Silt	Sand
						%		
Blount0	Blount Silt Loam	Organic	0	6.95	1.47	41	48	11
Bennington2	Bennington Silt Loam	GMO	2	6.95	2.46	45	43	12

Table 2.2. Fatty Acid Methyl Esters used as Biomarkers for Taxonomic Grouping

Taxonomic Group	Biomarker Characteristic	FAMEs	Reference
Gram Positive Bacteria	Branched FAMEs	Sum of a15:0, i15:0, a16:0, i16:0, a17:0, i17:0	(Frostegard and Baath, 1996; Tunlid et al., 1989; Zelles et al., 1995)
Gram Negative	Cyclopropyl and monounsaturated FAMEs	Sum of 16:1ω7c, cy17:0, 18:1ω7c, cy19:0ω8c, cy19:0ω10c	(Moore-Kucera and Dick, 2008; Zelles et al., 1995)
General Bacteria	Saturated odd length, ω7 FAMEs	Sum of 15:0, 17:0, 20:1ω7c, Gram positive, and Gram negative Bacteria	(Tunlid et al., 1989)
Actinomycetes	10Me FAMEs	Sum of 10Me16:0, 10Me17:0, 10Me18:0	(Zelles, 1999)
Saprotrophitic Fungi	18:2ω6,9c	18:2ω6,9c	(Frostegard and Baath, 1996)
Abruscular Mycorrhizal Fungi	16:1ω5c	16:1ω5c / (Sum of 16:1ω5c and General Bacteria)	(Olsson, 1999)
Protozoa	20:4ω6,9,12,15	20:4ω6,9,12,15c	(Moore-Kucera and Dick, 2008)

Table 2.3. Fatty Acid Methyl Esters Ratios used in Data Analysis

FAME Ratio	FAMEs	Reference
Fungi / Bacteria Ratio		(Frostegard and Baath, 1996)
Fungi	18:2ω6,9	
Bacteria	15:0, a15:0, i15:0, a16:0, i16:0, 16:1ω7c, 17:0, a17:0, cy17:0, i17:0, 18:1ω7c, cy19:0ω8c, cy19:0ω10c, 20:1ω7c	
Saturated / Monounsaturated Ratio		(Bossio and Scow, 1998)
Saturated	14:0, 15:0, 16:0, 17:0, 18:0	
Monounsaturated	16:1ω5c, 17:1ω9c, a17:1, 17:1ω8c, 18:1ω9c, 18:1ω7c, 18:1ω5c, 19:1ω11c, 20:1ω9c, 20:1ω7c	
Cyclopropyl / monounstaturated precursor Ratio		(Bossio and Scow, 1998)
Cyclopropyl	cy17:0, cy19:0	
Monounsaturated precursor	16:1ω7c, 18:1ω7c	

LIST OF FIGURES

differences in the treatments using a general linear model univariate analysis of variance (alpha=0.05).

Figure 2.5. Relationship of glyphosate application and leaf tissue K from soybeans grown in two different soils. The soils were: a) a soil with no reported application of glyphosate, (b) a soil with a history of heavy glyphosate application. Vertical bars represent ±SD (n=4). A general linear model univariate analysis of variance found no significant differences between glyphosate treatments at both sampling dates in both soils (alpha=0.05).

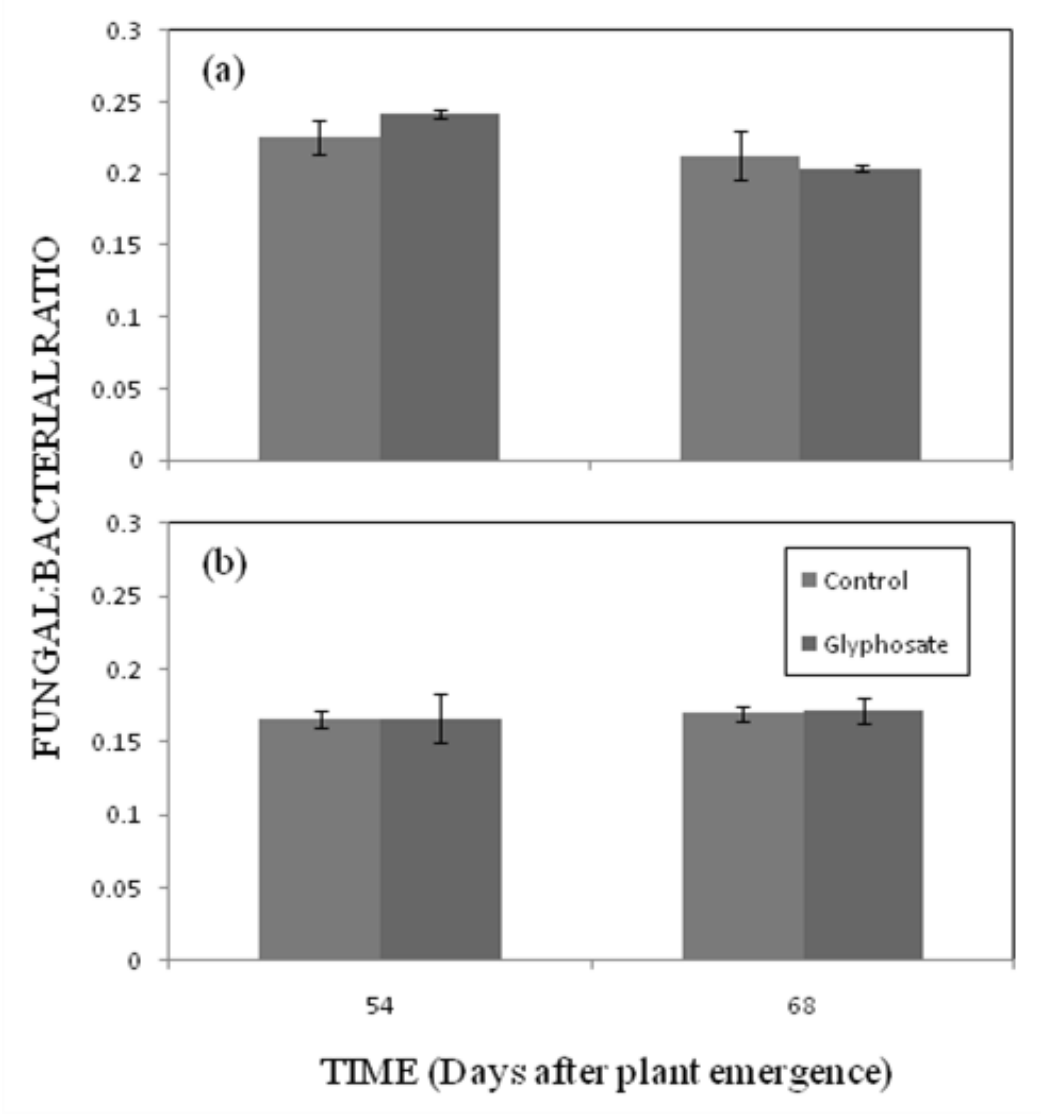
(a)
(b)
0.3
0.25
0.2
0.15
0.1
0.05
0
FUNGAL:BACTERIAL RATIO
Control
Glyphosate
54
68
TIME (Days after plant emergence)

Figure 2.1

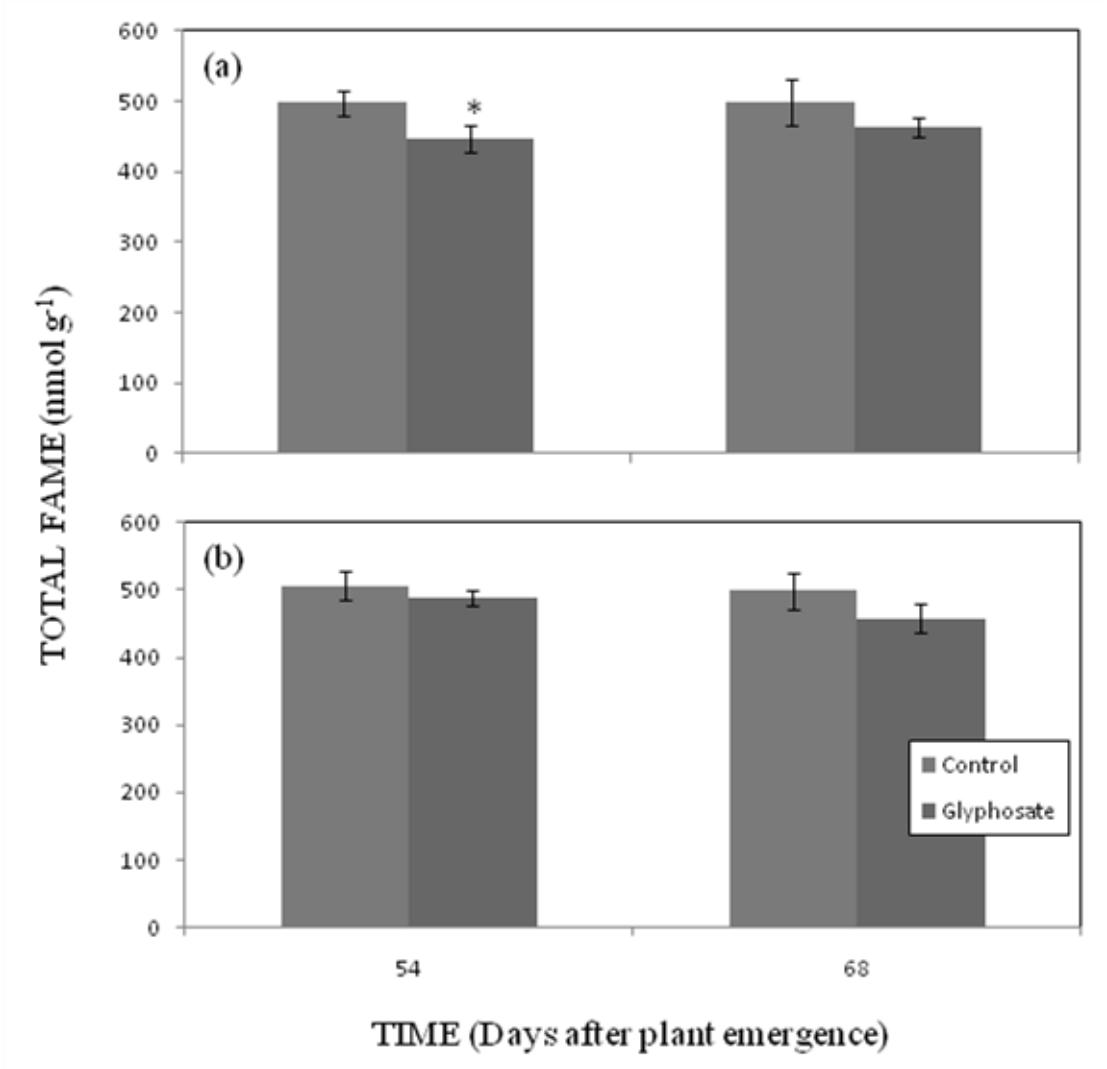
(a)
(b)
600
500
400
300
200
100
0
*
TOTAL FAME (nmol g-1)
Control
Glyphosate
54
68
TIME (Days after plant emergence)

Figure 2.2

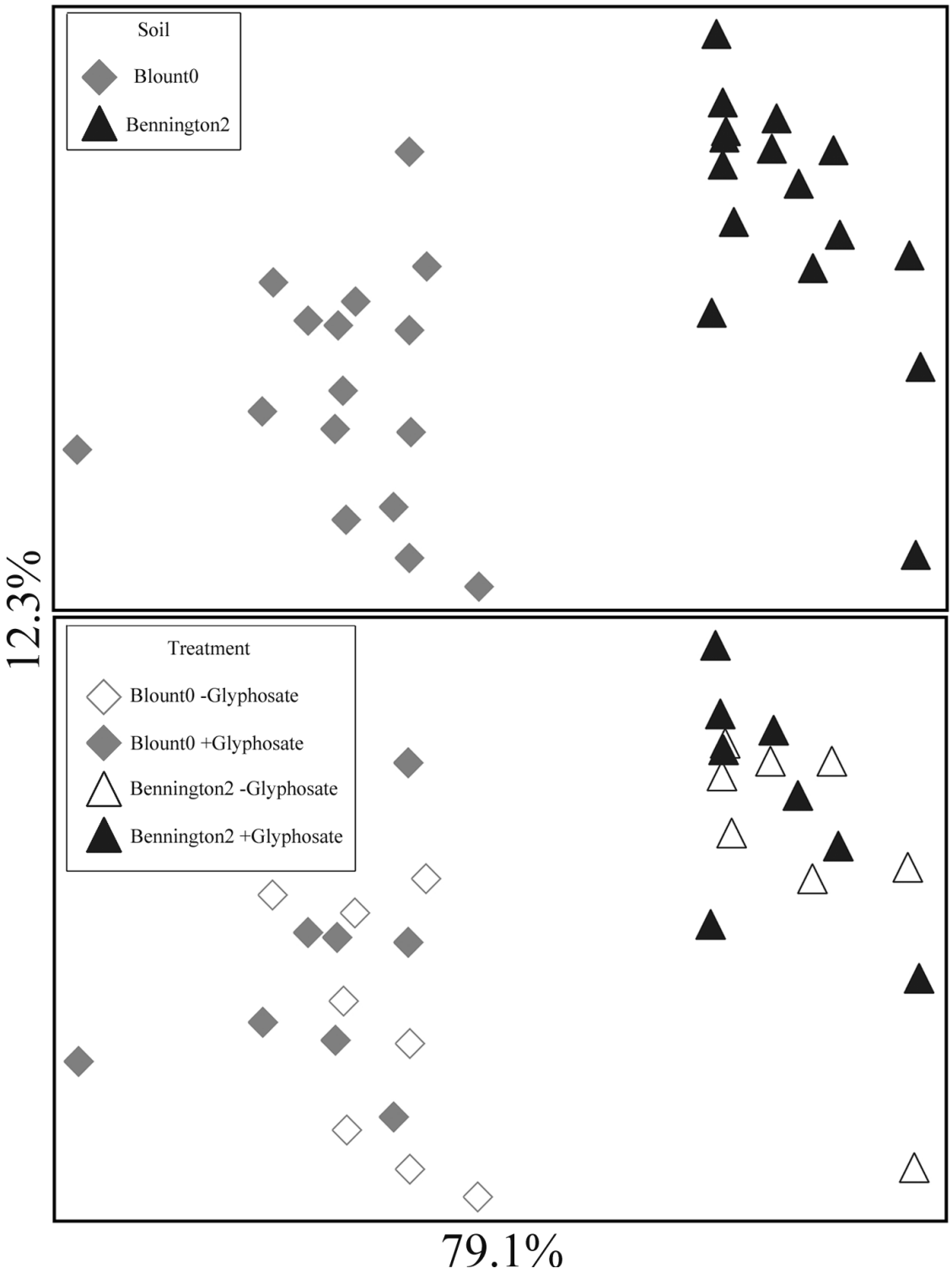
Soil
Blount0
Bennington2
Treatment
Blount0 -Glyphosate
Blount0 +Glyphosate
Bennington2 -Glyphosate
Bennington2 +Glyphosate
12.3%
79.1%

Figure 2.3

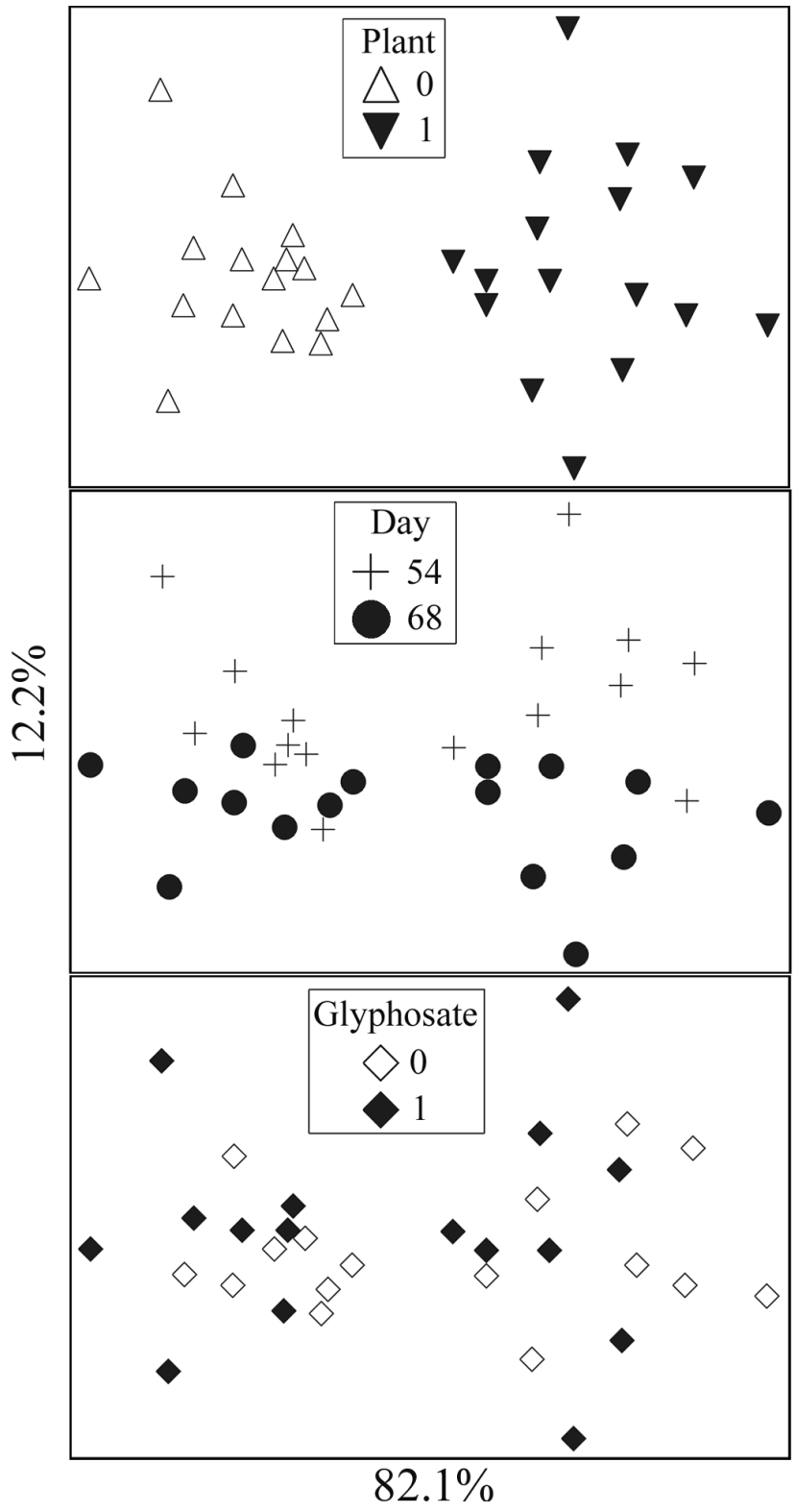

Figure 2.4

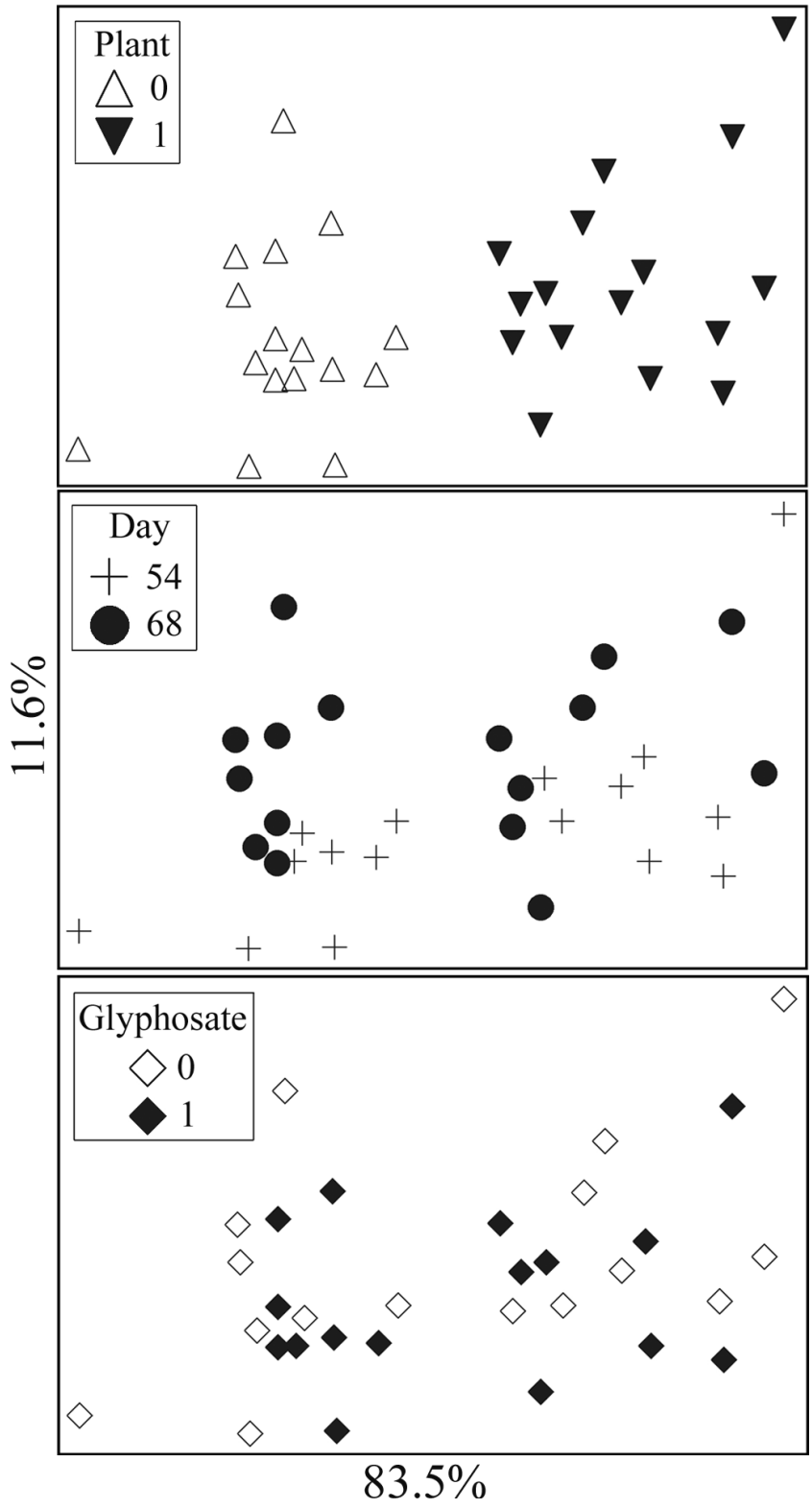

Figure 2.5

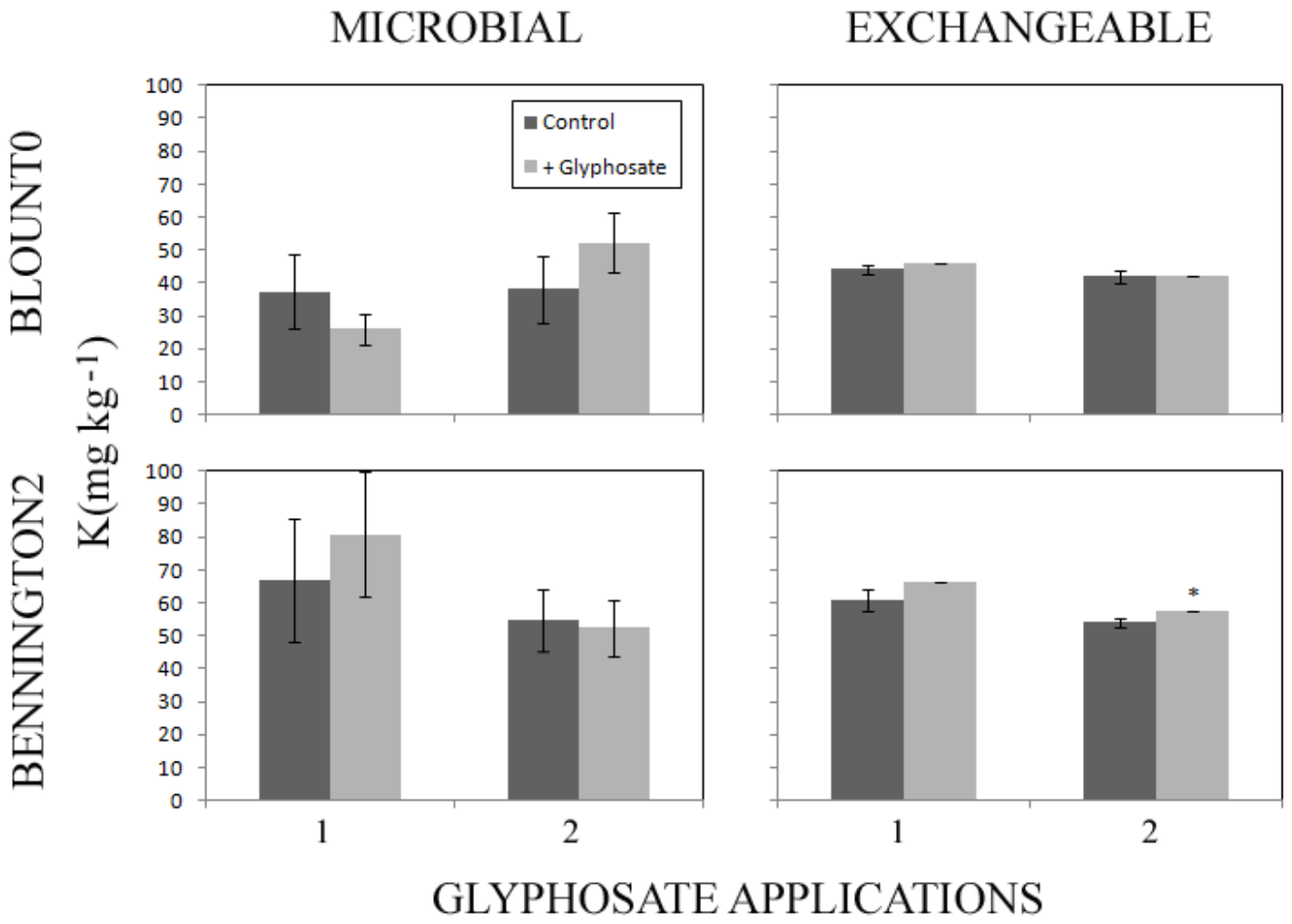
MICROBIAL
EXCHANGEABLE
BLOUNT0
BENNINGTON2
K(mg kg-1)
Control
+ Glyphosate
100
90
80
70
60
50
40
30
20
10
0
*
1
2
GLYPHOSATE APPLICATIONS

Figure 2.6

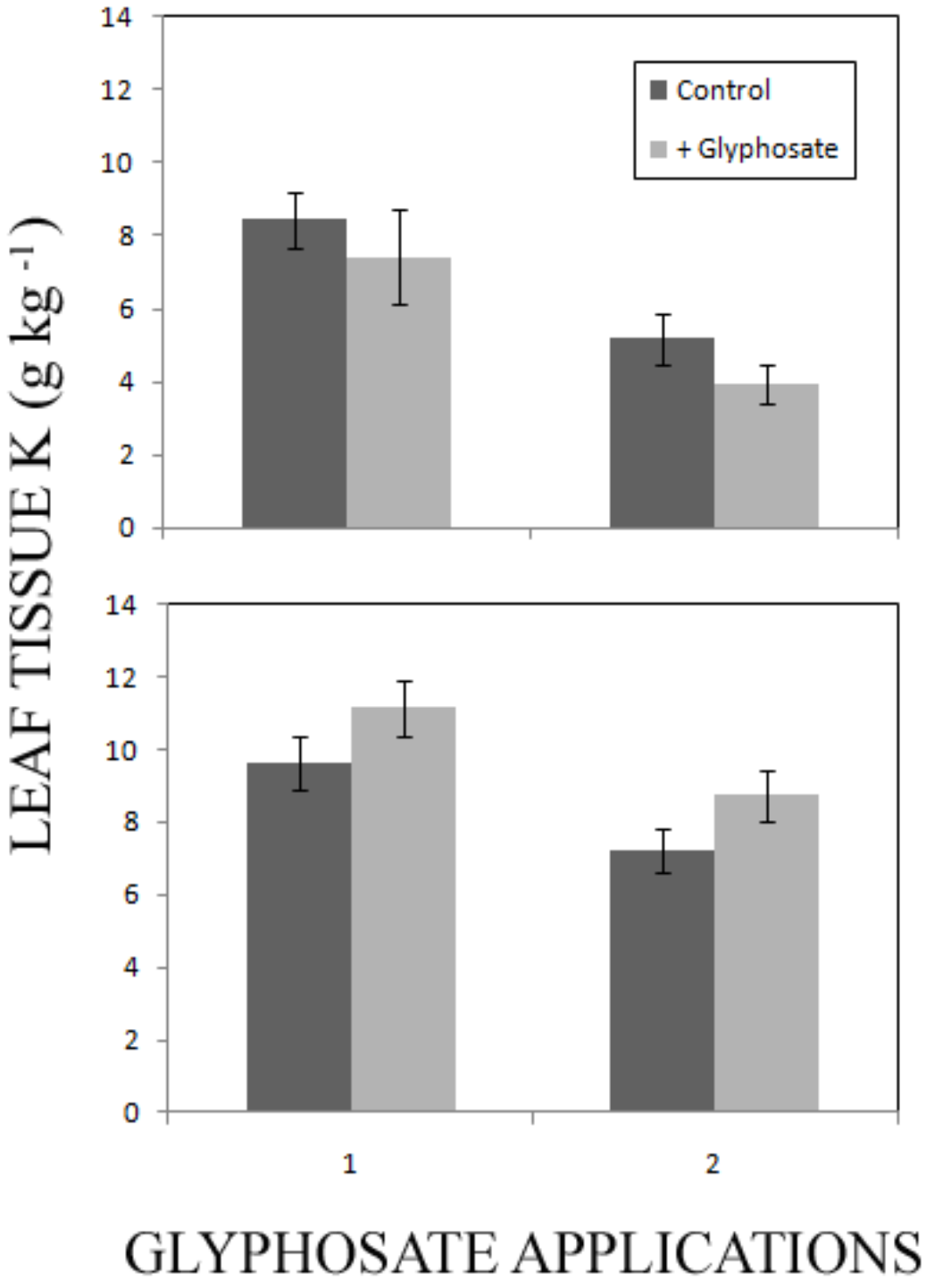
LEAF TISSUE K (g kg -1)
14
12
10
8
6
4
2
0
Control
+ Glyphosate
14
12
10
8
6
4
2
0
1
2
GLYPHOSATE APPLICATIONS

Figure 2.7

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