MICROBIAL COMMUNITY STRUCTURE IN SOILS AMENDED WITH GLYPHOSATE TOLERANT SOYBEAN RESIDUE

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By
Mark Edward Nye, B.S.
Environmental Science Graduate Program

The Ohio State University
2014

Master’s Examination Committee:
Dr. Richard P. Dick, Advisor
Dr. Warren Dick
Dr. Olli Tuovinen
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ABSTRACT

Glyphosate is a broad-spectrum herbicide used extensively worldwide to control broadleaf weeds in agriculture. Research suggests that repeated application causes a change in soil microbial properties which could be affecting soil quality and productivity. Glyphosate tolerant (GT) soybean technology is valuable to farmers because it reduces the use of other herbicides, is as a critical for weed control in reduced tillage systems, and is generally regarded as having low environmental impacts. However, after using this technology for 10 or more years, field observations by farmers and emerging research suggest that long-term glyphosate usage is having cumulative non-target effects on soils and crops.

The research of GT relative to soils and crop productivity has been mixed and often inconclusive. However, most of this research was short-term lab and field studies that did not investigate soils that had been under long-term GT cropping (10+ years). Indeed, there is now anecdotal evidence that long-term repeated application of glyphosate has a detrimental effect on GT crop yields, including non-GT crops grown in rotation with GT crops. Another aspect of GT cropping is the potential effects on soils by the GT crop residue. Nearly all of the research to date has been on glyphosate added directly to soil with little information on the effects of GT plant residue that was exposed to glyphosate.
Therefore, the objective of this study was to analyze soil microbial communities during decomposition of biomass from plants grown in soil that have been exposed to glyphosate in soils with and without a history of glyphosate exposure. An experiment was designed to mimic eight years of field applications in a 24 month greenhouse study. Soybean residues from this experiment were used in an laboratory incubation study and incubated in soils with and without a history of glyphosate exposure. These soils were profiled using phospholipid fatty acid analysis to determine shifts in soil microbial community structure due to the addition of GT residues to soil. The results showed that microbial shifts during decomposition of GT soybean residue varied between soils with or without long-term exposure to glyphosate. There was also a trend that GT material that had been exposed to glyphosate cause a differential shift in the communities over GT residue that had not been exposed to glyphosate.

Commercially available glyphosate formulations have two major types of salt carriers; potassium salt and isopropylamine salt, which could be a factor besides glyphosate in affecting the chemistry of GT residues and subsequently microbial response during decomposition. However, the results showed that carrier did not significantly affect PLFA profiling in soils with or without history of glyphosate exposure.

Ratios of saturated to monounsaturated PLFAs in combination with ratios of specific monounsaturated PLFAs to their cyclopropane precursors are used as indicators of microbial stress. Our results showed that there were significant differences in nutritional and water stress between soils with and without a history of glyphosate exposure. There were also significant differences in stress between glyphosate residue treatments in soil with a history of glyphosate exposure.
Dedicated to all those who have supported me in my educational endeavors: my dearest Erica, my family, my friends, my advisors, and my colleagues.

“I am enthusiastic over humanity’s extraordinary and sometimes very timely ingenuity. If you are in a shipwreck and all the boats are gone, a piano top buoyant enough to keep you afloat that comes along makes a fortuitous life preserver. But this is not to say that the best way to design a life preserver is in the form of a piano top. I think that we are clinging to a great many piano tops in accepting yesterday’s fortuitous contrivings as constituting the only means for solving a given problem.”

― R. Buckminster Fuller
ACKNOWLEDGEMENTS

This research was made possible by the knowledge and experience lent to me by my advisor, Dr. Richard P. Dick, as well as the members of my thesis committee, Dr. Warren Dick and Dr. Olli Tuovinen. Additionally, I appreciate the support given by the Environmental Science Graduate Program and The Ohio State University as a whole.

I am indebted to the staff and students of the Soil Microbial Ecology Lab. This research would not have been possible without the guidance of Dr. Nigel Hoilett, nor without the assistance of Nathan Lee, Amanda Davey, and Dr. Linda Dick. I am also grateful for the support of my fellow students, Emma Snyder, Lumarie Pérez-Guzmán, Kylienne Clark, and Peter Renz.

I would also like to extend my gratitude to the farmers who allowed me to sample their soil: Doug Molpus of Yoakam Farms, Centerburg, Ohio and Jeff Dickinson of Stratford Ecological Center, Delaware, Ohio.

Funding for both this research and my education was generously provided by the Ohio Soybean Council and Ag Spectrum.
VITA

June 1998................................................. Poland Seminary High School
March 2006............................................ B.S., Microbiology, The Ohio State University
September 2011-present.......................... Graduate Research Associate, School of Environment and Natural Resources, The Ohio State University

FIELDS OF STUDY

Major Field: Environmental Science
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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW
INTRODUCTION

Glyphosate is a general use, broad-spectrum, non-selective systemic herbicide that is very effective against broadleaf weeds and grasses. It was developed by the Monsanto Corporation in the early 1970s under the trade name Roundup®, and held under patent until 2000. In 1996, genetically modified glyphosate-tolerant (GT) crops (Roundup Ready®) were made commercially available, and in that year alone were grown on 1.7 million hectares worldwide. Since that time, the use of glyphosate to control weeds in agriculture has dramatically increased. The US agricultural market used 180 to 185 million pounds of glyphosate in 2007 alone. By 2008, GT crops were planted on 79 million hectares in 25 countries, with GT soybeans representing 70% percent of the 95 million acres of soybeans planted globally (James, 2008).

The use of glyphosate and GT soybeans is widespread in Ohio and throughout the Midwest. In 2011, GT soybeans accounted for 94 percent of US soybean acreage, or 71.7 million acres (source: USDA-NASS) (Grube et al., 2011). Glyphosate has greatly reduced the use of other herbicides and is a critical component of reduced tillage systems (Young, 2006), which improve surface water quality and soil water retention while reducing soil erosion and herbicide leaching in comparison to conventional tillage (Fawcett et al., 1994; Sprague and Triplett, 1986). It is generally regarded as having low environmental impact. It has a low mammalian toxicity, and because it is water soluble
has a low risk of accumulation in food webs. Glyphosate is relatively non-volatile, adsorbs to clay particles as well as iron and aluminum oxides in soil, thus has little potential to contaminate ground and surface water. Once it adsorbs, it rapidly degrades by microbial activity to innocuous molecules of CO$_2$, inorganic phosphate, and ammonium (Rueppel et al. 1977, Franz et al. 1997).

Despite the usefulness of glyphosate as a post-emergence herbicide in glyphosate-tolerant crop fields, growers are now reporting the emergence of “superweeds” which do not respond to glyphosate application (Foresman and Glasgow, 2008). In addition to increasing resistance in target plants, glyphosate-tolerant crops systems have also been anecdotally linked in recent years to lower yields. Currently, there is disagreement and speculation as to why glyphosate-tolerant crops are producing lower yields. One theory suggests that the original genetic source material used in breeding glyphosate-tolerant varieties comes from an older and lower-yielding Group V soybean genetic line. Other studies suggest that the diminished yield associated with glyphosate-tolerant varieties cannot be entirely explained by the differences in cultivar genetics. Still other studies suggest that glyphosate can have adverse interactions with plant nutrition, negative effects on soil microbial communities including increase in plant pathogens (Yamada et al., 2009).
Glyphosate is usually formulated as an isopropylamine salt. It kills vegetation by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form 5-enolpyruvyl-shikimate-3-phosphate (ESP). This enzyme inhibition preempts dephosphorylation of ESP to chorismate, an essential precursor to the amino acids phenylalanine, tyrosine and tryptophan, which are used in protein synthesis. It has long been claimed by Monsanto that glyphosate is harmless to animals, mostly due to their assertion that the shikimate pathway is only present in plants. It is also water soluble, and thus poses a low risk of accumulation in the food chain. Glyphosate is moderately persistent in soil, with an estimated average half-life of 47 days and a wide range of reported field half-lives from 1 to 174 days (Wauchope et al.; 1992, WSSA, 1994).

Mineralization to carbon (C) dioxide is the major endpoint for glyphosate degradation (Rueppel et al., 1977). Glyphosate has a low C:nitrogen (N) ratio of 3:1, and as such is readily mineralized by microorganisms (Haney et al., 2000). Studies indicate that soil microorganisms can use glyphosate as a source of C (Neumann et al., 2006) and phosphorus (Liu et al., 1991). Based on degradation kinetics, glyphosate is co-metabolized by microorganisms rather than directly supporting microbial growth (Forlani et al., 1999; Sprankle et al., 1975a).

Several studies have examined the effect of glyphosate on C mineralization. Some of these have shown that glyphosate can significantly increase microbial respiration in the short term (Sprankle et al., 1975a). These effects can be seen 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). A study by Lane et al. (2012) found that application of glyphosate application increased microbial respiration and that long-term repeated application of glyphosate may increase the populations of microorganisms that are able to degrade glyphosate.

It is not common practice in GT crop systems to apply glyphosate directly to soil. However, the herbicide often comes in contact with bare soil during spraying. Treated plants translocate glyphosate rapidly through the leaves and stems to the roots, where it is exuded into the soil (Coupland and Caseley, 1979). The rate at which soybeans can exude glyphosate from their roots can exceed 1000 ng per plant over the sixteen days after application (Kremer et al., 2005). Glyphosate is strongly adsorbed on most soils, especially those with high organic content, and thus has limited potential to leach through soil profiles to groundwater (Mamy and Burriuso, 2005). Due to the structure of the glyphosate molecule, it is primarily adsorbed onto soil particles with variable charge surfaces such as Fe and Al oxides, and is not easily desorbed once bound (Duke et al., 2012). When glyphosate binds to soil, it becomes inactive (Sprankle et al., 1975b), and once immobilized, can serve as a source of nutrients for soil microorganisms. Because degradation of glyphosate in the soil is a result of microbial activity (Rueppel et al., 1977), degradation rates of glyphosate can vary with the conditions that affect microbial activity, such as temperature and moisture content.
SOIL MICROBIAL ECOLOGY

Although research on the effects of glyphosate has yielded varying results, the majority of this research was done on short term glyphosate exposure. Since the commercial release of GT cropping systems in 1996, glyphosate has been in heavy use in some fields, often with several applications per year. It may be necessary to investigate communities after several years of glyphosate applications in order to detect a measurable shift in the microbial community structure. It has been shown that 2-4 applications of glyphosate can reduce C mineralization and increase glyphosate half-life when compared to a single application (Andrea et al., 2003). The degradation patterns of glyphosate have also been shown to change after repeated glyphosate applications (Lancaster et al., 2010). This may suggest a shift in the microbial population toward microorganisms capable of metabolizing glyphosate. Quinn et al. (1988) showed that repeated application of glyphosate can change the response of soil microorganisms over time. Field studies and unpublished data suggest that the effects on soil microbial communities can only be detected after long-term repeated application of glyphosate (Fernandez et al., 2005; Huber, 2007; Locke et al., 2008; Fernandez et al., 2009; Johal and Huber, 2009; Kremer and Means, 2009; Yamada et al., 2009). However, many studies have yielded inconsistent results, showing that the use of GT cropping systems has little to no effect on soil microbial communities, and that negative effects can be mitigated by growing GT crops in rotation with non-GT crops. (Lupwayi and Blackshaw, 2012).

The primary mode of glyphosate degradation in soils is microbial decomposition (Shushkova et al., 2009). Following application, glyphosate translocates from the leaves
of treated plants through the stems and into the roots. It undergoes little to no metabolism in plants, and thus is eventually secreted into the soil. Root exudates from treated glyphosate-tolerant plants contain not only unbound glyphosate, but also elevated levels of carbohydrates and amino acids as compared to untreated plants (Kremer, 2005). As the herbicide residue in root exudates is degraded in the rhizosphere, the C present in glyphosate molecules is utilized by a subset of fungal species during their metabolic processes. By this metabolic mechanism, glyphosate-derived C is incorporated into cytoplasmic carbohydrates by certain species of fungi (Charnay, 2004). The subsequent stimulation of these fungi are possibly attributable not only to a novel source of C and N in the form of glyphosate, but also to the aforementioned increase in production of carbohydrates and amino acids by plant roots of GT crops.

Kremer (2005) found that GT soybeans exposed to glyphosate stimulated fungal populations, specifically *Fusarium*, in the plant rhizosphere to such a degree that he considers the elevation of *Fusarium* levels to be glyphosate's "secondary mode of action." While he found enhanced *Fusarium* colonies in the roots of his plants, which could potentially reduce the crop yield, he did not find it in the harvested soybeans themselves. Nonetheless, he expressed concern that the widespread use of glyphosate could be devastating by causing extensive stimulation of *Fusarium* across cropped landscapes.

There is evidence of increased fungal growth and populations in soil treated with glyphosate, perhaps 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 application did not significantly affect bacterial populations, but that it did increase populations of fungi and actinomycetes, and that this effect was greater in soils that had a
history of glyphosate exposure. Glyphosate application has also been shown to coincide with an increase in the fungal plant pathogens of the genera *Fusarium* and *Pythium* (Kremer et al., 2005; Levesque et al., 1993; Meriles et al., 2006) and can also stimulate the growth of mycorrhizal fungi in vitro (Laatikainen and Heinonen-Tanski, 2002). Zobiole et al. (2011) showed that the root colonization by *Fusarium* increased in response to glyphosate applications and was further enhanced as soybean growth progressed.

Other responses to glyphosate reported are decreased populations of pseudomonads and Mn-reducing bacteria, and that soybean biomass also decreased with increasing glyphosate (Zobiole et al., 2011). This may indicate that glyphosate influences plant growth through its effect on rhizosphere microbial communities. In contrast to these studies, Druille et al. (2013) showed that glyphosate application had a profound negative effect on arbuscular mycorrhizal fungi (AMF) spore viability which significantly lowered the root colonization rate. This suggests that this plant-fungus symbiosis was also negatively affected, as the arbuscules are the primary site for nutrient exchange.

One method of determining fungal responses to herbicides has been to observe fungal growth rates in sterile media in the presence of the herbicide in question. In reality, field conditions vary widely based on the structure of the soil microbial community, the plants and soil fauna present, and the physical and chemical properties of the soil. To gain a better understanding of the mechanisms by which glyphosate influences fungal populations, the fungi must be allowed to interact with the other living and nonliving components of the soil. Studies comparing the growth of the pathogenic fungus *Gaeumannomyces graminis* in the presence of glyphosate on both sterilized and unsterilized soil samples showed that glyphosate increased the fungal growth in the
unsterilized soil, constituting a reasonable representation of how the interaction may occur under field conditions (Mekwatanakarn and Sivasithamparan, 1987). Glyphosate also caused a decrease in bacterial populations and in the populations of other non-pathogenic fungi that are considered to be antagonistic to G. graminis, which poses the question of whether growth of pathogens in general is directly stimulated by glyphosate, or whether it is through suppression of competitors and antagonists that certain pathogens are allowed to thrive. As it has been shown that glyphosate can dramatically affect interspecific interactions between fungal species, it can be reasonably assumed that both direct growth stimulation and changes in competition among soil microorganisms are important factors to consider in fungal community structure as a result of exposure to glyphosate (Wardle and Parkinson, 1992).

In general, overall fungal biomass in a soil sample can be measured using phospholipid fatty acid (PLFA) analysis (Frostegård and Bååth, 1996). While PLFA can generate data indicative of general fungal populations (Zelles, 1999), the most useful method of determining the fate of pesticides degraded by fungi is through the use of isotopic labeling experiments. $^{13}$C is a commonly used stable isotope in tracer experiments, and when incorporated into a given pesticide, allows for accurate tracking of the pesticide-derived C to its microbial and soil samples. Fungal cultures incubated in the presence of $^{13}$C-labeled glyphosate incorporate measurable levels of $^{13}$C into their biomass (Charnay, 2004). Fungal biomass can be fractionated into lipids, proteins, and polysaccharides in order to further assess the exact ultimate endpoint of the $^{13}$C derived from the metabolism of glyphosate. Using this method, researchers are able to determine into which portions of the cell structure of a given species glyphosate is incorporated.
GLYPHOSATE/PLANT INTERACTIONS

There are many species of fungi that are pathogenic to plants, as well as a number of species that are potentially pathogenic, infecting plants under favorable conditions. Researchers have suggested that following application of glyphosate, a short-term depression in aromatic amino acid levels occurs in glyphosate-tolerant plants, leading to a delayed plant immune response. This renders the plant more susceptible to fungal diseases caused by *Fusarium* and *Pythium* (Johal and Rahe, 1990). For example, glyphosate treated wheat appears to have higher levels of *Fusarium* head blight than wheat fields where no glyphosate had been applied (Fernandez et al., 2007). However, the consequences of an enhanced *Fusarium* population in soil are still unknown. It has also been suggested that the glyphosate-tolerant varieties of soybeans are less efficient at producing compounds called phytoalexins, which are thought to play a significant role in prevention of fungal infections in the plants themselves. These glyphosate-related increases in fungal populations combined with the physiological changes in glyphosate-tolerant cultivars can lead to widespread fungal infection of cereal crops and subsequently diminish or destroy crop yields (Kremer, 2005).

Glyphosate-tolerant plants are themselves largely unaffected by the application of glyphosate-based herbicides, whose intended use is to kill only competing plants in a given area. However, studies have shown that glyphosate application has adverse effects on plants’ ability to uptake iron, manganese, zinc, and boron (Eker et al., 2006; Neumann et al., 2006), and that that frequent applications of glyphosate may cause micronutrient deficiencies in GT and non-GT plants (Huber and McCaybius, 1993; Huber, 2007). This
has been attributed to the effect of glyphosate on the soil microbial community composition, which can change soil nutrient dynamics (Johal and Huber, 2009; Kremer and Means, 2009). Additionally, Santos et al. (2006) showed that various application rates of glyphosate can reduce plant height, vigor, and marketable and non-marketable fruit weight among tomato (*Lycopersicon esculentum*) plants. A concentration of 25 mg L\(^{-1}\) glyphosate, (approximately 2% of recommended field application rate), reduced fruit yield by 52%, plant height by 31%, and plant vigor by 44%.

A limited amount of research has been conducted on the decomposition of GT plant residues. Glyphosate use has been shown to significantly reduce crop residue decomposition (Powell et al., 2010). However, the results were inconsistent, and varied widely with weather and geography. Furthermore, this effect was shown to be dependent on the location of residue placement, reducing decomposition of surface residues but not residues that had been incorporated into the soil. It was also shown that decomposition was not significantly different between GT and non-GT varieties.

**SUMMARY AND OBJECTIVES**

It is evident after more than 25 years of related research that glyphosate has a profound effect on soil microbial ecology, especially on fungal populations. With the advent and now widespread use of glyphosate-tolerant cultivars, it is becoming increasingly important that the long-term effects of repeated glyphosate exposure be researched and well understood. As conservation tillage operations use glyphosate rather
than tilling to control weeds, it is also important that research be conducted on the microorganisms that participate in the decomposition of glyphosate-treated crop residues left on farm fields after harvest. Fungal diseases of economically important crops will always be of concern, so long as any agricultural chemicals are in use that may stimulate fungal growth. It is important to understand the mechanisms by which glyphosate affects the microbial communities in crop soils in order that soils can remain suitable for crops and fungal diseases can be mitigated.

The exact nature and magnitude of the effects of long-term, repeated glyphosate application on soil microbial ecology are still largely unknown, as are the ramifications of a microbial community that has participated in the degradation of glyphosate treated GT plant residues. Therefore, the objective of this study was to observe the decomposition of residues from plants grown in soil with a simulated history of repeated glyphosate application to determine the nature of the community shift in each soil during decomposition.
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CHAPTER 2
EFFECTS OF DECOMPOSITION OF GLYPHOSATE-TREATED SOYBEAN RESIDUES ON MICROBIAL COMMUNITY STRUCTURE IN SOILS WITH HISTORY OF LONG-TERM GLYPHOSATE EXPOSURE
ABSTRACT

Glyphosate is a broad-spectrum herbicide used extensively worldwide to control broadleaf weeds in agriculture. Research suggests that repeated application causes a change in soil microbial properties which could be affecting soil quality and productivity. Although glyphosate is generally regarded as having relatively low environmental impact, after 10 or more years of widespread use, field observations by farmers and emerging research suggest that long-term glyphosate tolerant (GT) cropping is having cumulative and non-target effects on soils and crop productivity. There is very little information on the effects of GT residue when added to soils.

Therefore, the objective was to determine the effects of GT residue during decomposition in soils with and without a history of glyphosate exposure. Soybean residues from a simulated long-term GT cropping system were used in a laboratory incubation. The experiment was a 2x3x4 factorial design with 2 soils (with/without glyphosate), three residue types (leaf, stem, root), and 4 soybean residue treatments (GT residue exposed to glyphosate with potassium salt carrier, GT residue exposed to glyphosate with isopropylamine salt carrier, untreated GT genotype, and untreated non-GT genotype). These soils were profiled using phospholipid fatty acid analysis to determine shifts in soil microbial community structure due to the addition of GT residue to soil. The results showed that microbial shifts during decomposition of GT soybean residue varied between soils with or without long-term exposure to glyphosate. There was also a trend that GT material that had been exposed to glyphosate cause a differential shift in the communities over GT residue that had not been exposed to glyphosate.
Commerially available glyphosate formulations have two major types of salt carriers; potassium salt and isopropylamine salt which could be a factor besides glyphosate in affecting the chemistry of GT residues and subsequently microbial response during decomposition. However, the results showed that carrier did not significantly affect PLFA profiling in soils with or without history of glyphosate exposure.

Ratios of saturated to monounsaturated PLFAs in combination with ratios of specific monounsaturated PLFAs to their cyclopropane precursors are used as indicators of microbial stress. Our results showed that there were significant differences in nutritional and water stress between soils with and without a history of glyphosate exposure. There were also significant differences in stress between glyphosate residue treatments in soil with a history of glyphosate exposure.
INTRODUCTION

Glyphosate is a broad-spectrum, non-selective systemic herbicide that is very effective against broadleaf weeds and grasses. It was developed and patented by the Monsanto Corporation in the early 1970s. In 1996, genetically modified glyphosate-tolerant (GT) crops (Roundup Ready®) were made commercially available, and in that year were grown on 1.7 million hectares worldwide. Since that time, the use of glyphosate to control weeds in agricultural scenarios has dramatically increased.

The use of glyphosate and GT soybeans is widespread in Ohio and throughout the Midwest. In 2011, GT soybeans accounted for 94 percent of US soybean acreage, or 71.7 million acres (source: USDA-NASS) (Grube et al., 2011). Glyphosate has greatly reduced the use of other herbicides and is a critical component of reduced tillage systems (Young, 2006), which improve surface water quality and soil water retention while reducing soil erosion and herbicide leaching in comparison to conventional tillage (Fawcett et al., 1994; Sprague and Triplett, 1986). Glyphosate is generally regarded as having a low environmental impact and a low mammalian toxicity because it is water soluble and does not accumulate in food webs. Glyphosate is relatively non-volatile, adsorbs to clay particles as well as iron and aluminum oxides in soil, thus has little potential to contaminate ground and surface water.
Research on the impact of GT cropping on soils has had mixed results. However, the majority of this research was done on short term glyphosate exposure. Since the commercial release of GT cropping systems in 1996, glyphosate has been in heavy use with several applications per year. It is important to investigate long-term effects of GT cropping, because it is likely that only after repeated glyphosate exposure would there be a shift in microbial communities. It has been shown that 2-4 applications of glyphosate can reduce C mineralization and increase glyphosate half-life when compared to a single application (Andrea et al., 2003). The degradation patterns of glyphosate have also been shown to change after repeated glyphosate applications (Lancaster et al., 2010). This may suggest a shift in the microbial population toward microorganisms capable of metabolizing glyphosate. Quinn et al. (1988) showed that repeated application of glyphosate can change the response of soil microorganisms over time. Field studies and unpublished data suggest that the effects on soil microbial communities can only be seen after long-term repeated application of glyphosate (Fernandez et al., 2005; Huber, 2007; Locke et al., 2008; Fernandez et al., 2009; Johal and Huber, 2009; Kremer and Means, 2009; Yamada et al., 2009).

Glyphosate-tolerant plants are largely unaffected by the application of glyphosate-based herbicides. However, studies have shown that glyphosate application can have adverse effects on plants’ nutrient uptake (Eker et al., 2006; Neumann et al., 2006), and that frequent applications of glyphosate may cause micronutrient deficiencies in GT and non-GT plants (Huber and McCaybius, 1993; Huber, 2007). This has been attributed to the effect of glyphosate on the soil microbial community composition, which can change soil nutrient dynamics (Johal and Huber, 2009; Kremer and Means, 2009).
Most of the glyphosate applied is dispersed onto plant surfaces rather than directly onto the soil. However, the herbicide often comes in contact with bare soil during spraying. Treated plants translocate glyphosate rapidly through the leaves and stems to the roots, where it is exuded into the soil (Coupland and Caseley, 1979). The rate at which soybeans can exude glyphosate from their roots can exceed 1000 ng per plant over the sixteen days after application (Kremer et al., 2005). Because degradation of glyphosate in the soil is a result of microbial activity (Rueppel et al., 1977), degradation rates of glyphosate can vary with the conditions that affect microbial activity, such as temperature and moisture content.

The primary mode of glyphosate degradation in soils is microbial decomposition (Shushkova et al., 2009). It undergoes little to no metabolism in plants, and thus is eventually secreted into the soil. Root exudates from treated glyphosate-tolerant plants contain not only unbound glyphosate, but also elevated levels of carbohydrates and amino acids as compared to untreated plants (Kremer, 2005). As the herbicide residue in root exudates is degraded in the rhizosphere, the C present in glyphosate molecules is utilized by select fungi during their metabolic processes (Charnay, 2004). By this metabolic mechanism, glyphosate-derived C is incorporated into cytoplasmic carbohydrates by certain species of fungi. The subsequent stimulation in growth of these fungi are possibly attributable not only to a novel source of C and nitrogen in the form of glyphosate, but also to the increase in carbohydrates and amino acids, as mentioned above.

A limited amount of research has been conducted on the decomposition of GT plant residues. Glyphosate use can significantly reduce crop residue decomposition.
However, Powell et. al (2010) found inconsistent results that varied widely with weather and geography. Furthermore, this effect was shown to be dependent on the location of residue, where decomposition rates were reduced for surface residues but not incorporated residues. It was also shown that decomposition rates were not significantly different between GT and non-GT crop varieties.

To our knowledge, there have been no studies on the effects of GT residue, exposed or unexposed to glyphosate, amended to soil microbial communities. Furthermore, few studies have investigated the effect of long-term GT cropping systems on microbial communities. Therefore, the objective of this study was to profile soil microbial communities using PLFA analysis during decomposition of GT residues added to soils with and without history of GT cropping.
MATERIALS AND METHODS

Soils

Two soils were chosen, with one having had a history of GT cropping of >10 years (GLY$^+$), and the other having had no known history of exposure to glyphosate (GLY$^-$) (Table 2.1). The GLY$^-$ soil was a Blount silt loam (fine, illitic, mesic Aeric Epiaqualf). This soil was from an organically managed farm located in Delaware County, Ohio utilizing a continuous rotation; the previous five years were alfalfa-orchard grass-corn, oats-alfalfa-orchard grass, spelt-timothy-clover, and timothy-clover. The GLY$^+$ soil was a Bennington silt loam (fine, illitic, mesic Aeric Epiaqualf) from a farm in Knox County, Ohio practicing a no-till corn-soybean rotation (soybeans were GT). Glyphosate was applied up to three times per year while growing soybeans, and once per year while cultivating corn. Bennington and Blount silt loams are taxonomically identical except that the Blount has greater calcium carbonate concentration in the C horizon, a depth not sampled in this experiment.

Soil samples were collected with probes (2.5 cm x 20 cm) randomly at each of the field sites in January 2012. The samples were inspected in the lab, where stones, large pieces of organic matter and roots were removed. The soils from each site were homogenized, sieved to pass 2 mm mesh size, and stored in sealed plastic bags at 4°C.
Plant residue

The soybean residues used in this experiment came from a greenhouse study where corn (*Zea mays*) and soybeans (*Glycine max*) were grown in rotation for 8 growth periods that ran 58 days each. The two glyphosate treatments used had different carrier molecules: Powermax® (Monsanto, Inc., St. Louis, MO) potassium salt of N-(phosphonomethyl)glycine and Cornerstone® (AgriSolutions, Brighton, IL) isopropylamine salt of N-(phosphonomethyl)glycine. Additionally, there were GT and non-GT plant controls to which no glyphosate was applied. At the end of each period, plant biomass was harvested, dried, and stored in paper bags at ambient temperature.

Residues from each box were separated into leaf, stem, and roots. All tissues were dried and weighed. Roots were rinsed with distilled water to remove any excess soil. The distribution of leaf, stem and root residues calculated from the dry weights of the harvested plants in the greenhouse study was 32.7 : 51.8 : 15.5 leaf, stem, and root, respectively. This was similar to the distribution reported by Bergersen, et. al (1992). This distribution of leaf, stem, or root residue was used in the incubation study described below.

Incubation

The incubation study used a 2 (soils) x 3 (residue types) x 4 (glyphosate treatments) factorial design: two soils (with and without a history of long-term GT cropping), three residue types (leaf, stem, root), and four glyphosate residue treatments the greenhouse experiment:

1: Powermax®-treated GT plants
2: Cornerstone®-treated GT plants
3: untreated GT plants
4: untreated non-GT plants

Prior to incubation, 28 g of soil was weighed in each glass sample jar (small Whatman jar, 15mm radius). Water was added by weight to attain 66 % field capacity, and the total weight of each sample jar containing soil was recorded for future soil moisture adjustments. Moisture content of the samples was maintained gravimetrically at 66 % field capacity by adding distilled water as needed (Linn and Doran, 1984) after each destructive sampling day.

Into each 28 g soil sample was added either 132 mg stem residue, 83 mg stem residue, or 39 mg root residue. The jars were closed with a plastic cap and shaken briefly to ensure thorough distribution of residue. Sample jars were placed in plastic storage bins, each with an open beaker of distilled water, and were then covered with plastic wrap. The plastic wrap was punctured several times with a hypodermic needle, and the bins were then placed in an incubator at 22°C. Microcosms were destructively sampled in triplicate at 3, 7, and 30 days. After sampling, each jar was sealed with a plastic cap and stored at -20°C until phospholipid fatty acid analysis.

**PLFA Analysis**

A modified version of the phospholipid fatty acid (PLFA) extraction method as described by Frostegård et al. (1991) was used in this experiment. Total lipids were extracted from 2 g of soil from each sample using a chloroform: methanol: aqueous citrate buffer (1:2:0.8) extractant (Bligh and Dyer, 1959). The total lipid extract was then separated into neutral lipids, glycolipids, and phospholipids using silicic acid columns.
Phospholipids were then subjected to alkaline methanolysis and dried under N$_2$ in a 35°C heating block. The dry sample was reconstituted in 170 µl of 1:1 (v/v) hexane: methyl tert-butyl ether (MTBE), transferred to GC vials, and combined with an internal standard (30 µl, 0.01 M C19:0-ME in 1:1 Hexane: MTBE).

PLFA detection and quantification were performed on Agilent N6890 gas chromatograph (Agilent Technologies, USA) equipped with an Agilent 7683 Series Injector and a flame ionization detector (FID). The MIDI System, PLFAD1 protocol (MIDI Inc., Newark, DE, USA), in combination with the Agilent ChemStation Software was then used to measure peak area (response) and to identify PLFAs. These PLFAs were associated with a specific lipid biomarker.

**Statistical Analysis**

PLFA concentrations were analyzed using both absolute concentration (nmol g$^{-1}$ soil) and relative concentration (percent of total moles of PLFA). Seventeen PLFAs were used for analysis. PLFAs used as biomarkers for functional groups were summed, and statistical analysis was performed on totals PLFA amounts representing six functional taxonomic groups: gram positive bacteria, gram negative bacteria, fungi, actinomycetes, arbuscular mycorrhizal fungi (AMF), and eukaryotes (Table 2.2). The ratio of cy19:0 to 18:1$\omega$7c was calculated as an indicator of water stress and the ratio of saturated to monounsaturated fatty acids (SAT/MONO) was calculated as an indicator of nutrient deprivation (Bossio and Scow, 1998; Guckert et al., 1986; Larkin, 2003; Lundquist et al., 1999; Moore-Kucera and Dick, 2008).

Two-way analysis of variance (ANOVA) and Student-Newman-Keuls stepwise multiple comparison test were used to evaluate the significance of soil glyphosate history,
glyphosate treatment, residue type, and sampling day for each functional taxonomic group. This analysis was also used to evaluate differences in microbial stress between soils and glyphosate treatments. ANOVA analyses were conducted using SAS 9.0 software.

Non-metric multidimensional scaling (NMS) was performed using PC-ORD based on Sørensen distance. Two hundred and fifty runs were conducted 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 (nmol g⁻¹ soil and percent of total moles of PLFA). Prior to NMS, the data were transformed using a monotonic square root transformation to improve normality and reduce the coefficient of variation among PLFAs.
RESULTS

Soil Type

The most significant effects on PLFA diversity were due to the soil source, where the main difference was glyphosate-tolerant (GT) and non-GT cropping. All six functional taxonomic groups, were significantly different at various sampling dates over the course of the experiment due to soil type. PLFA concentrations for gram positive bacteria showed a significantly higher concentration in GLY+ soil when compared to GLY− soil at Day 3 than at Day 30 (Figure 2.1). Both concentrations decreased, however, the rate of decrease was faster for gram positive bacteria in the GLY+ soil than in the GLY− soil. PLFA concentrations for gram negative bacteria were significantly different between the two soils at all three sampling days and decreased throughout the course of the incubation (Figure 2.2). Concentrations of gram negative PLFA in the two soils decreased at approximately the same rate. PLFA concentrations for fungi were not significantly different at Day 3 or Day 7, but were significantly different at Day 30 (Figure 2.3). The rate of decrease was faster for the fungal PLFAs in GLY− soil than in GLY+ soil.

The only significant differences due to residue glyphosate treatments (averaged across all residue types) in the GLY+ soil were found on Day 7 in the AMF (Figure 2.4)
and eukaryote groups (Figure 2.5). In both cases, there was a significant difference in PLFA concentrations between soils amended with Powermax treated residue and soils amended with untreated residues (GT and non-GT). In each case, there was no significant difference between the Powermax and Cornerstone treatments, nor was there a significant difference between the Cornerstone treatments and the untreated samples. There were no significant differences between the individual residues (averaged across all treatments) in the GLY⁺ soil in any functional taxonomic group on any sampling day (data not shown).

In contrast GLY⁺ soil, there were more treatment effects on the GLY⁻ soil. For this soil, significant differences were found on Day 3 for the fungal PLFA values averaged across residues and across treatments (Figure 2.6). There was a significant difference between values averaged across all treatments of the fungal PLFA concentration for leaf residue samples when compared to the stem and root residue samples. There was also a significant difference in values averaged across all residues of the fungal PLFA concentration for the samples amended with Powermax treated residues when compared to the other treatments. Within residue types, there were significant differences in fungal PLFA concentrations 1) between Powermax and the other treatments for leaf residue samples and 2) between the glyphosate treated and non-treated root residue samples. These significant differences were not found in the Day 7 and Day 30 samples. Similarly, significant differences were found on Day 3 for the actinomycete PLFA biomarkers (Figure 2.7), and like the fungal PLFAs, the significant differences were not found on Days 7 or 30.
Ordination Analysis

Clustering of community PLFA profiles (all PLFAs) was distinct at Day 3 and Day 30 (Figure 2.8), but the soils were closer by Day 30 than at Day 3. Within soil types, the clustering patterns varied due to sampling day and due to glyphosate residue treatment. For example, root residue samples in the GLY− soil showed clustering of profiles by sampling day (Figure 2.9). Days 3 and 7 are clustered more closely together than Day 30. In contrast, this effect is not found in samples taken from soil with stem residue in GLY+ soil. However, in the GLY− soil, the controls (no residue) are clearly separated; whereas in the GLY+ soil, the controls are clustered closely together. Furthermore, on Day 30 (the end of the incubation), the GLY− soil showed clustering of profiles from soils amended with untreated plant residues (GT and non-GT), an effect not found in the GLY+ (Figure 2.10). Clustering was not distinct in either soil due to residue type (leaf, stem, root) (data not shown).

Microbial stress

Significant differences were found between the two soil types for microbial nutrient and water stress indicators when averaged across all treatments. The GLY+ soil had greater stress than the GLY− soil at Day 3 and Day 30 (Figure 2.11). Significant differences were found in the SAT/MONO ratio due to residue glyphosate treatments in the GLY+ soil, but not in the GLY− soil (Figure 2.12). Neither soil showed significant differences in water stress (cy19:0/18:1w7c ratio) between the residue glyphosate treatments. However, there was a significant difference in the GLY+ soil between the control (no residue) and all other treatments that was not found in the GLY− soil. There were significant differences in both stress indicators due to sampling day in both soils.
(data not shown). The SAT/MONO ratio was significantly higher at Day 3, then decreased by Day 7 and then increased again by Day 30. The cy19:0/18:1w7c ratio increased over the incubation, but was not significantly different (P>0.05) between Day 7 and Day 30 in either soil.
DISCUSSION

The most apparent effect on PLFA profiles was soil history of glyphosate exposure. This suggests that long-term repeated glyphosate application has an unintended effect on the soil microbial community, specifically with regard to the manners in which the communities change during decomposition of plant residues. This is supported by ordination plots of community PLFA profiles where PLFA profiles clustered by soil type. This grouping is more evident early in the incubation (Day 3), and while the groups move closer to one another later in the incubation (Day 30), the grouping and separation of groups is still distinct between the GLY$^+$ and GLY$^-$ soils (Figure 2.8). Furthermore, it is clear on Day 30 that the profiles of amended GLY$^-$ soils move further from the non-amended control. This suggests that the initial response of the microbial community during plant residue decomposition differs initially between the two soils, but that this effect diminishes over time. The effect was shown regardless of residue type or the glyphosate treatment applied to the growing plants.

Our findings are in contrast with studies that have shown that GT cropping under field conditions has little to no effect on soil microorganisms (Lupwayi and Blackshaw, 2013, Hart and Brookes, 1996; Wardle and Parkinson, 1990; Wardle and Parkinson, 1992). However, none of these studies were done on soils that had been under
long-term GT management, and all were short-term studies. Additionally, our residues were derived from an experiment conducted under greenhouse conditions rather than field conditions. Other studies of soils that received glyphosate under long-term GT management have not shown shifts in fatty acid profiles (Lancaster et al., 2010; Ratcliff et al., 2006; Weaver et al., 2007). However, these studies had direct application of glyphosate to soil over short time periods rather than decomposition of glyphosate-exposed plant residues in soils with and without long-term GT cropping. Our study showed an initial difference between the soils with and without exposure to glyphosate and the differential response of the two soils to the amendment during the decomposition. The shift and distinct response were most prevalent in the GLY− soil. This would suggest that the GLY+ soil already had a community primed for glyphosate residues, whereas the GLY− soil was starting to change in response to those treatments.

Ordination analysis showed that, within each soil type, there are differences in the microbial community PLFA profiles for experimental factors (time, glyphosate treatment of residue, residue type). The GLY− soil showed distinct clustering of communities by sampling day within the incubation, regardless of the glyphosate treatment applied to the plant residue. This was consistent with Ratcliff (2006), who studies forest soils and found clustering of PLFA profiles regardless of glyphosate application under field conditions. PLFA profiles of samples from Days 3 and 7 clustered more closely to one another than Day 30 (Figure 2.9). This was most evident in communities in soil samples to which root residue was applied. This effect was not found in the GLY+ soil for any residue. This suggests that the microbial communities in the soil with a history of long-term glyphosate exposure have adapted to GT residues and thus show fewer microbial responses during decomposition than the GLY− soil. This was
shown in GLY− soil, where there was no clustering of Day 3 or Day 7 communities, but there was clustering at Day 30. This was the opposite for GLY+ soil which presumably was primed for these residues. Furthermore, despite the clear separation of profiles over sampling day in the GLY− soil, the controls (no residue) were very clearly separated from one another. This is in contrast to the GLY+ soil, where only Day 30 clustered, but the controls from all three days were clustered closely together (Figure 2.9).

The effect of glyphosate treatment previously applied to plants was only found at the end of the incubation (Day 30) in the GLY− soil (Figure 2.10). The PLFA profiles in samples to which untreated plant residue (GT and non-GT) was applied and the control (no residue) were more closely clustered and separated from the glyphosate treated residues. This effect was found across the means of all residues, regardless of whether the residue was GT or non-GT. No such grouping was see in the GLY+ soil. This result is consistent with Powell et al. (2010), who found no significant difference between the decomposition of GT and non-GT corn and soybeans. No such grouping was found in the GLY+ soil. This suggests that the microbial communities in the GLY+ soil respond indiscriminately to the addition of treated/untreated plant residues, whereas the communities in the GLY− soil have more distinct microbial profiles between glyphosate treated and untreated residues. Neither soil shows any clear separation between GT and non-GT untreated residues. This would be consistent with the report of Duke et al. (2012) that there are no substantive differences in the nutritional value between GT and non-GT residues. A strong trend in our data suggests that it is the exposure to glyphosate and not the GT plant material itself that causes microbial responses.

The dominant PLFA markers changed over the incubation, with differential shifts in the PLFAs profiles between the GLY+ and the GLY− soils. For each functional
group analysed, the initial PLFA concentration was higher in the GLY$^+$ soil than in the GLY$^-$ soil. The PLFA concentrations representing each functional taxonomic group decreased over the course of the incubation. However, the changes over time varied between the two soils. Statistically significant differences were present between the PLFA concentrations in the two soils at different time points for different functional taxonomic groups. This suggested that soil history of glyphosate exposure was important in controlling microbial response to GT residues added to soils.

The concentration of gram positive bacterial PLFAs were significantly different between the two soils on Day 3, and although the concentration in both soils decreased by Days 7 and 30, there were no significant differences at these days (Figure 2.1). This was the same for fungal PLFAs except that significant differences were only seen at Day 30 (Figure 2.3). In contrast, gram negative bacterial PLFAs were significantly different between the two soils at all time points (Figure 2.2). In all three functional groups, the concentration of PLFAs were higher in the GLY$^+$ soil than in the GLY$^-$ soil.

Our results are consistent with Ratcliff et al. (2006). PLFA markers for bacteria and fungi decreased over the 30 day incubation, and the differences in PLFA community profiles are more strongly tied to sampling date rather than herbicide treatment. However, Ratcliff et al. (2006) did not address the potential effects of long-term GT cropping on PLFA profiles. There may be differences because Ratcliff et al. (2006) studied forest soils rather than agricultural soils.

Significant differences between glyphosate treatments (Powermax, Cornerstone, untreated GT, untreated non-GT residues) were not found in the GLY$^+$ soil, with the following exception: arbuscular mycorrhizal fungal PLFA markers were significantly different (averaged across all residues) between Powermax treated residues and either of
the untreated residues on Day 7 (Figure 2.4). However, for the same group, there was no significant difference between the samples with Cornerstone treated residues and the samples with untreated residues, nor between the Cornerstone and Powermax treated residues. The same result was found on Day 7 for the eukaryote PLFAs in the/GLY + soil (Figure 2.5). Nonetheless, based on PLFA biomarkers across all functional groups, the data suggests limited microbial response in the/GLY + soil due to GT residues during decomposition. In the/GLY - soil, there were two cases of significant differences between functional group PLFA concentrations due the residue glyphosate treatments, both of which were found on Day 3. In this case, the groups were fungi (Figure 2.6) and actinomycetes (Figure 2.7). PLFA markers for each group were significantly different in samples with Powermax treated residues compared to all other treatments.

Neither soil showed a significant difference in PLFA concentrations for any functional group between samples with Powermax treated residue and samples with Cornerstone treated residue (data not shown). This suggests that, regardless of soil history of glyphosate exposure, there is no difference in microbial response due to carrier molecules, which were a K salt with Powermax and an isopropylamine salt with Cornerstone. We found no other research on the effects of glyphosate carriers on microorganisms with which to compare our own research.

As was the case with the differences between glyphosate treatments, there were only a limited number of significant differences between residues (root, stem, leaf) when averaged across all glyphosate residue treatments (treated and untreated, GT and non-GT) within a given soil. These significant differences were found in the/GLY - soil on Day 3 only. For concentrations of fungal PLFAs, there was a significant difference on Day 3 between leaf residue amended soil compared to stem and root amended soils (which were
not significantly different from each other) (Figure 2.6). An identical result was observed on Day 3 for concentrations of PLFAs associated with actinomycetes (Figure 2.7). These results suggest that although we found these limited instances of specific differences between residues, it is difficult to assess the relevance of these differences, because under field conditions, residues would not be segregated from one another.

The microbial stress indicators we used showed significant differences in both nutritional and water stress between the two soils at Day 3 and at Day 30. Differences in stress due to sampling day, glyphosate residue treatment, and residue type all varied between GLY$^+$ and GLY$^-$ soils. Stress was greater across all residue treatments at all sampling days for the GLY$^+$ soil than the GLY$^-$ soil. We did not expect to see significant differences in the cy19:0 : 18:1w7c ratio, as water stress was not imposed in our experiment (soil moisture was gravimetrically maintained at 66 % field capacity).

**PERSPECTIVES**

There were several treatment comparisons that did not affect PLFA profiling or stress levels within either soil type. Plant residue type (leaf, stem, root) showed no differential response across the other treatments. This was also the case for GT vs. non-GT soybean residue that had not been exposed to glyphosate. This is likely because major constituents of plant residues such as lignin, cellulose and other compounds which are dominant in controlling decomposition and microbial responses are quite similar between these varietal genotypes. The other comparison of glyphosate carrier also had no significant differences in PLFA profiling or stress levels. Although this should be investigated more thoroughly, it seems that the glyphosate molecule in plant residue is more important in affecting microbial response.
The latter point above was detected in the soil that had not been exposed to GT cropping. Overall, the GLY\(^-\) soil had a number of responses that were temporally meaningful, whereas the GLY\(^+\) soil showed very little response to any of the soil treatments.

The major effect shown in this study was related to history of GT cropping relative to responses. When taken as a whole, the data suggests that there is a difference in the PLFA profiles in soils of the same type and class after a long-term repeated application of glyphosate (> 10 years). Although the soils used in this study were of the same type, there were other factors besides exposure to glyphosate which may have contributed to differences in microbial response (crops, manure application, commercial fertilizers). Because glyphosate use is so widespread, it is increasingly difficult at present to find agricultural soils that have never been exposed to glyphosate or used under GT management.

The data also suggest that there is a shift in PLFA profile from long-term repeated glyphosate exposure, indicating changes in the overall microbial response in soils to the addition of plant residue and the dynamics under which it is decomposed. However, we did not see any indication in the data that there is an overall difference in the PLFA profiles based on the type of residue applied to the soils or the exposure of residues to glyphosate during plant growth. This was shown by community PLFA profiles with separate clustering on ordination plots of glyphosate treated and untreated residues.

Our findings are consistent with some previous studies and in contrast with others, although direct comparison is difficult, as few if any studies investigated
decomposition or used soils with and without long-term history of GT cropping, and many studies involved direct application of glyphosate to soil samples.
Table 2.1. Soil pH, C content, and texture.

| Soil Symbol | Soil Type            | pH  | Total C | Soil Texture |
|-------------|----------------------|-----|---------|--------------|
|             |                      |     |         | Clay | Silt | Sand |
| GLY-        | Blount Silt Loam     | 6.95| 1.47    | 41   | 48   | 11   |
| GLY+        | Bennington Silt Loam | 6.95| 2.46    | 45   | 43   | 12   |
Table 2.2. PLFAs associated with functional taxonomic group.

| Group                        | Phospholipid fatty acid                            |
|------------------------------|----------------------------------------------------|
| Gram positive bacteria       | i14:0, i15:0, a15:0, i16:0, a16:0, 17:0, i17:0, i17:0 |
| Gram negative bacteria       | 16:1 ω7c, 17:1 ω8c, 18:1 ω7c                       |
| Fungi                        | 18:2 ω6,9c/a18:0, 18:1 ω9c                         |
| Actinomycetes                | 10Me16:0, 10Me17:0                                 |
| Arbuscular mycorrhizal fungi | 16:1 ω5c                                           |
| Eukaryotes                   | 20:0                                               |
Figure 2.1. Time course PLFA concentrations for gram positive bacteria averaged over soil type.

*P<0.05.

NS no significant difference P<0.05.
Figure 2.2. Time course PLFA concentrations for gram negative bacteria averaged over soil type.

*P<0.05.

NS no significant difference P<0.05.
Figure 2.3. Time course PLFA concentrations for fungi averaged across soil type.

*P<0.05.

NS no significant difference P<0.05.
Figure 2.4. PLFA biomarker concentrations for arbuscular mycorrhizal fungi, Day 7.

Bars having the same letter are not significantly different at P<0.05. Figures on left are averaged over plant parts. Figures on right are averaged over residue glyphosate treatments.
Figure 2.5. PLFA biomarker concentrations for eukaryotes, Day 7. Bars within soil having the same letter are not significantly different at P<0.05. Figures on left are averaged over plant parts. Figures on right are averaged over residue glyphosate treatments.
Figure 2.6. PLFA biomarker concentrations for Fungi, Day 3. Bars within soil having the same letter are not significantly different at P<0.05. Figures on left are averaged over plant parts. Figures on right are averaged over residue glyphosate treatments.
Figure 2.7. PLFA biomarker concentrations for actinomycetes, Day 3. Bars within soil having the same letter are not significantly different at P<0.05. Figures on left are averaged over plant parts. Figures on right are averaged over residue glyphosate treatments.
Figure 2.8. Ordination plots of all PLFA profiles from all treatments for both soils on Day 3 and Day 30. Each point is the mean (three replications) of PLFA community profile for a given soil-residue-treatment combination.
Figure 2.9. Ordination plot of PLFA profiles from root in soil with and without known history of glyphosate exposure amended with root or stem residue.
Figure 2.10. Ordination plot of PLFA profiles from Day 30 of all residue treatments in soil with and without known history of glyphosate exposure.
Figure 2.11. PLFA ratios indicating microbial stress averaged over all treatments at Day 3 and Day 30. Bars within soil having the same lowercase letter are not significantly different at $P<0.05$. 
Figure 2.12. PLFA ratios indicating microbial stress averaged over the course of 30 day incubation. Bars within soil having the same lowercase letter are not significantly different at P<0.05.
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