LONG TERM GLYPHOSATE EFFECTS ON ROUNDUP® READY SOYBEAN RHIZOSPHERE MICROORGANISMS

THESIS

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ABSTRACT

The herbicide glyphosate (N-(phosphonomethyl) glycine) was first introduced in 1974 as a non-selective, broad spectrum, post-emergent agrochemical, branded under the trade name Roundup® and intended to control weed competition in agricultural farming. It gained large popularity and increased usage in 1996 with the introduction of glyphosate resistant soybean (*Glycine max*) cultivars and again in 1998 with resistant corn (*Zea mays*) cultivars. Its widespread usage has increased the concern of unknown long-term effects on the soil rhizosphere microbial community. In the same long-term context there is also increased concern over glyphosate's toxicity and accumulation of degradation products, notably aminomethylphosphponic acid (AMPA), which accounts for the majority of detected metabolites in the soil. Chapter one of this thesis will review the current literature on the toxicity and degradability of glyphosate and AMPA in the soil. In chapter two of this thesis a long-term glyphosate greenhouse experiment was designed with two main objectives, (1) determine the effects of long-term glyphosate application for three different glyphosate formulations on glyphosate resistant (GR) soybean rhizosphere microbial communities of two different soil managements, one with and one without a history of glyphosate exposure, and (2) use stable isotope probing (SIP) to identify possible glyphosate degrading microbial functional groups in these two soil managements. The objective of chapter three was to expand on chapter two by

investigating the accumulation of glyphosate and AMPA in both the rhizosphere and bulk soil of the same long-term glyphosate greenhouse experiment. Research from a greenhouse study showed that repeated application of glyphosate increased the abundance of gram-negative microorganisms relative to a single application as detected by FAMEs. Likewise a field study also showed that repeated application of glyphosate increased *Fusarium* fungal colonization on both corn and soybean root systems over multiple growing seasons. Another study demonstrated that a glyphosate infused medium drastically reduced the abundance of arbuscular michorizae colonies. Additionally, the literature reveals that both glyphosate and AMPA have a higher absorption potential within the soil matrix than most other herbicides, and that microbial communities are mostly responsible for their degradation. AMPA is also found to persist longer in the soil matrix than glyphosate and can be used by some organisms as a source of phosphate. These studies suggest: (1) that long-term glyphosate application could upset the delicate balance between beneficial and non-beneficial microorganisms in the glyphosate resistant soybean rhizosphere by increasing the abundance of gram-negative and fungal microbial communities; (2) glyphosate resistant plants may have an increased risk of fungal disease and mycotoxicity stress from *Fusarium* fungal species; (3) fungal and gram-negative microorganisms may be major players in the degradation of glyphosate and; (4) accumulation of glyphosate and its metabolites is likely more pronounced in areas of the soil with less microbial activity. The major findings of this thesis indicate that glyphosate induces changes in the rhizosphere microbial communities of a soil that has never received glyphosate application and likely adapts to use glyphosate as a substrate, most

notably by fungus. Additionally, glyphosate and its degradation product AMPA

accumulate heavily in the bulk soil, and greater microbial biomass in the rhizosphere soil

is associated with low levels of GLY and AMPA.

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CHAPTER 1. TOXICITY AND DEGRADATION OF GLYPHOSATE AND AMPA: A REVIEW

ABSTRACT

Glyphosate is a widely used herbicide that has gained popularity due to its ease of use, economic return and weed control efficacy when used with glyphosate resistant (GR) cultivars. Aminomethylphosphonic acid (AMPA) is the most commonly detected glyphosate metabolite found in soil and water samples. AMPA persists longer in the soil matrix, has a longer half-life, and has higher concentrations in environmental samples than glyphosate. However, research on the environmental toxicity of glyphosate or AMPA has given conflicting results. Some research has shown that glyphosate can increase fungal abundance in soils under GR cropping, while others showed no change. In aquatic ecosystems, AMPA has a negative effect on aquatic invertebrates when in high concentrations. Furthermore, with the introduction of GR soybean cultivars in the last 20 years glyphosate has become the most extensively used agriculture herbicide in history, and thus further increases the need to understand its toxicological effects and environmental persistence. Therefore, the objective of this review is to examine the literature specifically related to the environmental toxicity and degradation of glyphosate and AMPA so as to better understand their potential non-targeted effects and environmental persistence. Findings from this review show that both GLY and AMPA have strong soil sorption properties, but still are mobile and found extensively in many aquatic and soil environments. Their toxicity is debated, but have been shown to have toxic effects on aquatic organisms, rodents, and humans, under very high doses. Additionally, microorganisms have developed efficient metabolic pathways for AMPA and GLY degradation.

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INTRODUCTION

The introduction of glyphosate resistant (GR) soybeans (*Glycine max*) in 1996 and corn (*Zea mays)* in 1998 has resulted in the widespread use of glyphosate (*N*-(phosphonomethyl)glycine). According to the National Agricultural Statistics Service, in 2017 94 % of soybean, 91 % of cotton (*Gossypium hirsutum*) and 89 % of corn in the United States were GR cropping cultivars. GR cropping is an attractive form of crop management because it simplifies weed management, is highly effective in weed control, lowers cost, and reduces labor.

However, the environmental and ecological effect of glyphosate (GLY) is of major concern because of its widespread use, short history in modern agriculture, and the lack of long-term research on its biological toxicity. It may also have non-targeted effects on soil biology and long-term crop productivity. For example, since 1996 there has been an average of eight reported cases of new GLY resistant weed species identified each year (Heap, 2018). Additionally, Schafer et al. (2014) showed that the microbial community structure in the rhizosphere of GLY resistant ragweed was lower in abundance and diversity than the rhizosphere of non-resistant giant ragweed.

Ecological toxicity of glyphosate

GLY is considered to be less environmentally toxic than most other herbicides because it can be readily degraded and strongly sorbed to the soil matrix (Schnurer et al., 2006). Thus, dispersion and contamination of ground and surface water of GLY is highest when there is higher rainfall shortly after application (Coupe et al. 2012). Van Stampvoort et al. (2016) showed that GLY can also be leached from soil into shallow

groundwater and subsequently be transferred into surface water. However, Al-Rajab and Hakami (2014) suggested that this is a rare event in silty clay loam soils, and showed that less than 1 % of GLY is leached out of surface soil over a 2 month period. Thus, soil texture is a major controller in the amount of GLY leaching. Additionally, soil chemical properties are important in controlling absorption and desorption of GLY, and therefore its environmental mobility.

From an agricultural perspective GLY's toxicity on the rhizosphere microbial community is extremely important. Microorganisms are in a symbiotic relationship with all plant species, therefore GLY's potentially non-targeted disruption of the rhizosphere microbial community could affect plant health and productivity. For example Zobiole et al. (2010) suggested that application of GLY on GR soybeans can negatively impact soil rhizosphere microbial communities by reducing their abundance. Likewise Gomez et al. (2009) observed transient decreases in microbial biomass, respiration rate, and dehydrogenase activity following GLY application directly to soil at varying doses. In contrast, Lane et al. (2012) applied GLY to two soils, one that had a history and one that had no history of GLY exposure and found an increase of microbial respiration in the soil that had a history, compared to the soil with no history. Bohm et al. (2007) also found that GLY application resulted in increased microbial respiration, but also lower rhizosphere microbial biomass carbon in GR soybeans. However, Haney et al. (2000) and Busse et al. (2010) reported limited effects of GLY on soil microbial respiration and carbon and nitrogen mineralization.

GLY is considered to have minor toxicity toward birds and aquatic wildlife, ranging from $4.7x10^3 - 7.9x10^3$ nmol g^{-1} in mammals, and $6.9x10^3 - 1.2x10^4$ nmol g^{-1} in amphibians (McComb, 2008). One study examined GLY levels in aquatic systems and found it to display toxic effects to some aquatic organisms, including fish and aquatic invertebrates (Alberdi et al., 1996). Other research suggested there was a synergistic interaction of GLY with aquatic parasites that reduced fish survival (Kelly et al., 2010).

The U.S. Environmental Protection Agency (EPA) has classified GLY as a group E carcinogen, which has a non-carcinogenic effect on humans with a low oral and acute dermal toxicity (U.S.E.P.A., 1993). However, the International Agency for Research on Cancer (IARC) classifies it as a group 2A carcinogen meaning that it is carcinogenic to humans (Guyton, 2015). The World Health Organization (WHO) sets the acceptable daily intake (ADI) of GLY at 5.9 nmol g^{-1} and estimated in 2011 that no more than 2 % of the ADI was consumed by people (W.H.O., 2011). However, there are reports in the literature of negative effects on humans and animals. In most cases severe detrimental effects only occurred at very high doses. For example, Chan and Mahlar (1992) fed rats daily diets of $1.8x10^7$, $3.6x10^7$, $7.4x10^7$, $1.5x10^8$, and $3.0x10^8$ nmol L^{-1} of pure GLY, and found the two highest rates significantly reduced sperm production in male rats and significantly increased the length of estrus cycles in female rats. Additionally, Arbuckle et al. (2001) studied farm operators and their partners who routinely used GLY, and found an increase in late spontaneous abortions. However, there are no known examples of human deaths linked to GLY toxicity or ingestion, but detectable levels have been measured in blood and urine samples of farmers and their families (Acquavella et al.,

2004). A potential hazard for farmers arises from the presence of surfactants in commercial GLY formulations such as Roundup®. The presence of these surfactants increases GLY's wetting potential, thus increasing its potential dermal absorptivity to humans and wildlife that come into contact with the concentrated commercial formulations (Folmar et al., 1979).

As with any potentially toxic chemical the relative toxicity of that chemical is almost always dose dependent. GLY dosage can be affected by its ability to persist in the environment, which depends on its rate of degradation. If GLY is applied at a higher rate than it can be degraded, then it would be expected to accumulate in the soil. Due to the many carboxyl groups present on the structure of GLY it has a very large capacity to sorb to the soil matrix. This sorption makes it less bioavailable and thus more persistent in the environment especially when there is less microbial activity (Schnurer et al., 2006). A decrease in plant bioavailability may be good for crops but an increase in GLY persistence means that long-term residual accumulation is possible, which overtime may have detrimental effects on soil microorganisms and the surrounding ecosystem.

Degradation and plant resistance of glyphosate in soils

GLY acts as an enzyme inhibitor in the shikimic acid pathway in plants and microorganisms where the synthesis of compounds such as aromatic amino acids occur that are important for plant and microbial functions, and tissue composition (Funke, 2006). Many of the downstream products are also important for the synthesis of compounds used for immunological functions and plant defenses (Duke, 2012). GLY is able to inhibit the shikimic acid pathway in microorganisms in the same way as plants,

but because of their high rate of generation turnover they can rapidly adapt to the presence of GLY. In fact the gene that confers GLY resistance in the commercially distributed GR plants was isolated from microorganisms, *Agrobacterium sp*. strain CP4, *Achromobacter* sp. strain LBAA, and *Pseudomonas sp*. strain PG2982 (Funke et al., 2006). The enzymes that these genes encode for are known as II EPSP synthases and have high catalytic efficiency in the presence of GLY. Some plant species have the GLY-insensitive EPSP synthase, but the resistance of these enzymes to GLY was not fully effective even with selective evolution and site-directed mutagenesis (Funke et al., 2006). Therefore, because the II EPSP gene has high efficacy it has been widely incorporated into genetically modified crop species such as *Zea mays*, *Glycine max* and *Gossypium hirsutum* which make up the majority of the GR cultivars that are used today.

The microbial derived EPSP synthase gene confers plant resistance via continued catalytic activity in the presence of GLY; however microorganisms have also developed ways to counteract the toxicity of GLY by degrading it directly. In fact most of the ecological degradation is mediated by microorganisms (Franz et al., 1997) which have evolved two main pathways. The less common degradation pathway involves GLY being reduced to sarcosine and inorganic phosphate; sarcosine then is reduced to formaldehyde and glycine. This pathway and these products have been shown to be utilized by various microorganisms including those belonging to the family Rhizobiaceae (Duke et al., 2012). The second and more common pathway involves the reduction of glyphosate to glyoxylate and AMPA. This pathway is dependent on inorganic phosphate deficiency within the cells of some organisms. Glyoxylate can then be further used to support

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microbial growth while AMPA has not been definitively shown to have any metabolic

benefit (Duke et al., 2012; Sviridov et al., 2015).

GLY is considered to be a very stable compound in air with very minimal

solubility in organic solvents and complete solubility in water; additionally

photodegredation of GLY is widely considered not to occur, indicating that its

degradation is mainly associated with microbial activity (Singh and Singh, 2016). With

the increasing popularity of GR cultivars, GLY application rates will likely also continue

to increase and microorganisms will develop even more pathways of its degradation. It

would therefore be expected that there will be shifts in the rhizosphere microbial

community of GR cultivars toward those which are highly efficient at degrading GLY.

Ecological toxicity of AMPA

AMPA is the primary metabolite of GLY that is detected in the environment, but

research on its toxicity relative to GLY has shown conflicting results. Some findings

show AMPA to be as toxic as GLY to green algae, birds, and terrestrial mammals.

However, Giesy et al. (2000) found it to be more toxic than GLY to aquatic invertebrates,

but less toxic to fish.

The solubility of pure GLY and AMPA in water is around 6.2×10^7 and 5.0×10^8

nmol L^{-1} at 20° C, respectively. However, GLY is most commonly combined with a salt

carrier in commercial formulations that substantially increases solubility and therefore

potential toxicity (Mérey et al., 2000). Additionally, because AMPA has a high water

solubility compared to other herbicides, and its degradation products are composed of

phosphate and ammonium could make it a major contributor to the total phosphate

content in aquatic ecosystems. Excess phosphorous is of concern because it would increase the likelihood and frequency of algal blooms upon AMPA degradation. This would drive the release of various neurotoxins in aquatic bodies.

The potential toxicity of AMPA toward humans is also of major concern, and the research in this area is also conflicting. AMPA has shown slightly toxic effects on human erythrocytes, and mutagenic effects on human lymphocytes at $1.8x10^6$ nmol L^{-1} concentrations in vitro. At $2.5x10^6$ nmol L^{-1}, AMPA was shown to cause significant DNA damage to human lymphocyte cells (Battaglin et al., 2014). However, Li et al. (2013) showed GLY to have positive human health effects by inhibiting cancer cell growth with no observed damage to normal non-cancerous cells. The W.H.O. does not give any recommended ADI for AMPA specifically, but instead puts it in the same group as GLY, and defines GLY as the parent compound and its metabolites.

Table 1.1 adapted from Battaglin et al. (2014) shows the average and maximum detection levels (nmol L^{-1}) of AMPA and GLY as detected from various aquatic and soil sediment environments (nmol g^{-1}). For soils and sediments, 91 % and 93 % of all sites had detectable levels of GLY and AMPA, respectively. This was much higher than all the aquatic environments, and higher than the next highest category (ditches and drains) by 20 % and 12 % for GLY and AMPA, respectively. Additionally, for nearly all aquatic environments AMPA was detected without measurable levels of GLY in 17.9 % of the cases, and in higher concentrations. Whereas GLY was detected without AMPA in only 2.3 % of the cases. This may be due to slower degradation of AMPA than GLY in most aquatic systems, or that AMPA may be more easily desorbed from the soil matrix and

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thus have a much higher mobility than GLY in the overall ecosystem. This could be of concern in soils that are highly susceptible to erosion.

Degradation of AMPA in soils

AMPA tends to have greater persistence in soil, with a half-life of 60 to 240 days compared to an average of 2 to 215 days for GLY (Battaglin et al., 2014); although, one review reported it to be 150 days for AMPA and 80 to 94 days for GLY (Duke, 2011). However, soil chemistry and microbial activity can greatly affect these values. Table 1.2 adapted from Grandcoin et al. (2017) gives an overview of the average half-life of GLY to form AMPA under oxic and anoxic soil conditions in the most common soil classes. Under oxic conditions, loam, loess, and clay loam had the shortest half-life of 1.5 to 53.5 days, whereas clay soil had the longest half-life of 110 days. Oxic conditions generally promoted more rapid degradation of GLY over anoxic conditions.

In most GLY degrading microorganisms AMPA is not mineralized, but is instead exported into the environment (Sviridov et al., 2015). This is an indication that microbial metabolism is less adapted to degrade or utilize AMPA. In fact AMPA is only known to be utilized as a phosphate source in a small number of organisms such as *Pseudomonas sp*. Strain PG2982 (Sviridov et al., 2015). AMPA is converted to inorganic phosphate plus formaldehyde which then undergoes further processing downstream (Sviridov et al., 2014). This degradation pathway is considered to be less common in the bacterial community; therefore its rarity may be one reason why AMPA appears to persist and accumulate to a greater degree in the environment than GLY. It is important to note however that a small number of organisms such as *O. anthropi* GPK 3 and

Achromobacter sp. Kg 16 have been isolated that possess all the enzymes to degrade GLY and AMPA to simpler non-toxic products (Ermakova et al., 2008). This is an important microbial characteristic when identifying potential bioremediation organisms so as to avoid the buildup of toxic intermediates like AMPA and formaldehyde in the soil system.

PERSPECTIVES

GLY and AMPA have varying chemical properties and rates of degradation that can account for the greater accumulation of AMPA in the environment, especially in soils where it is strongly absorbed (Schnurer et al., 2006). Both GLY and AMPA have strong soil sorption properties, but still are mobile and found extensively in many aquatic and soil systems (Battaglin et al., 2014). Additionally, both have been found to be toxic to aquatic organisms, rodents, and humans, but only under very high doses (Acquavella et al. 2004; Alberdi et al. 1996; Battaglin, 2014; Chan and Mahlar, 1992). It seems likely that AMPA and GLY will further accumulate in the environment because agricultural application rates and GR cropping systems continue to increase.

The relatively short time for the widespread use of GLY in GR cropping limits the ability to fully understand the long-term impacts on ecosystems. Although AMPA in particular is accumulating in the environment, its toxicity to soil microorganisms under in situ conditions is largely uninvestigated. However, some studies have shown that microbial degradation is greater under oxic conditions (Grandcoin et al., 2017) and have developed efficient metabolic pathways for AMPA and GLY degradation and utilization (Ermakova et al. 2008; Sviridov et al. 2015). Additionally, it is well known that microorganisms have the capacity to quickly adapt to new substrates.

Table 1.1 Concentrations of glyphosate and AMPA in aquatic systems, soils, and precipitation.

Hydrologic Setting	Number of Samples	Glyphosate Detections†	AMPA Detections‡	Median Glyphosate	Maximum Glyphosate	Median AMPA	Maximum AMPA
		%		nmol L^{-1}			
All Sites	3,732	39 (1,470)	55 (2,052)	<0.12	2815	0.36	3575
Streams	1,508	52 (791)	72 (1,079)	0.18	432	1.8	252
Groundwater	1,171	6 (68)	14 (168)	<0.12	12	<0.18	44
Ditches and drains	374	71 (265)	81 (302)	1.18	2526	3.9	3575
Large rivers	318	53 (169)	89 (284)	0.18	18	2.0	40
Soil water	116	34 (40)	65 (76)	<0.12	5.9	0.54	17
Lakes, ponds, and wetlands	104	34 (35)	30 (31)	<0.12	1780	<0.18	369
Precipitation	85	71 (60)	72 (61)	0.65	14.8	0.36	4.3
WWTP outfall	11	9 (1)	82 (9)	<0.12	1.8	4.0	23
		%		nmol g^{-1}			
Soil and sediment	45	91 (41)	93 (42)	0.06	2.8	0.16	3.1

† The percentage of sites where GLY was detected, the number of sites is shown in parenthesis
‡ The percentage of sites where AMPA was detected, the number of sites is shown in parenthesis
Adapted from Battaglin et al. (2014)

Table 1.2 Half-life of glyphosate in soils.

Soil class	Glyphosate half-life (days)			
	Topsoil (0-30 cm)		Subsoil (30-80 cm)	
	Oxic	Anoxic	Oxic	
Silty clay loam	18	45		Kanissery et al. (2015)
	14.5			Al-Rajab and Hakami (2014)
	19			Al-Rajab and Schiavon (2010)
Silt loam	15	51		Kanissery et al. 2015
	18	42		Kanissery et al. 2015
Sand	16.9		36.5	Bergstrom et al. (2011)
Clay	110		151	Bergstrom et al. (2011)
Loam	9			Simonsen et al. (2008)
Loess	3.5			Yang et al. (2015)
	1.5-53.5			Bento et al. (2016)
Clay Loam	7.1			Mamy et al. (2016)
	10.6			Druart et al. (2011)
	4			Al-Rajab and Schiavon (2010)
Sandy loam	14.5			Al-Rajab and Schiavon (2010)

Adapted from Grandcoin et al. (2017)

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CHAPTER 2. LONG-TERM GLYPHOSATE EFFECTS ON ROUNDUP READY SOYBEAN RHIZOSPHERE MICROORGANISMS: A GREENHOUSE STUDY

ABSTRACT

The practice of glyphosate tolerant cropping has gained popularity following the introduction of glyphosate tolerant soybean cultivars in 1996. Since then glyphosate has become an extremely common form of agricultural weed control. However, after 10 or more years of glyphosate tolerant cropping, field observations including increased prevalence of soybean root rot and sudden death syndrome, and emerging research suggests that long-term glyphosate usage is having non-targeted and cumulative effects on soil microorganisms. To investigate these observations a multifactorial greenhouse experiment was conducted to simulate long-term glyphosate treatment in the field over a period of 8 cropping seasons. Two historical soil managements were used, one with, and one without a long-term history of glyphosate application, and three different glyphosate formulations, Monsanto Powermax®, Agrisolutions Cornerstone®, and Cornerstone® with Agro-Plus Grozyme® and AgSpectrum Glycure®. Intra-root rhizosphere soil was collected from glyphosate tolerant soybean rhizoboxes that allowed non-destructive soil sampling on days 1 and 7 after application. Rhizosphere samples were profiled for microbial functional groups using phospholipid fatty acid analysis, and probed for ^{13}C glyphosate incorporation using stable isotope probing. The objectives were to use phospholipid fatty acid analysis to investigate microbial community shifts between soil managements, investigate functional microbial group differences between glyphosate treatments, and identify potential glyphosate degrading functional microbial groups with ^{13}C stable isotope probing. Results showed a strong impact of soil type on maintaining the broad microbial composition based on PLFA profiling. Additionally, the microbial

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community in a soil that never received glyphosate does shift and likely adapts to use

glyphosate as a substrate, most notably by fungus for the Cornerstone® and

Cornerstone® with Agro-Plus Grozyme® and AgSpectrum Glycure® treatments. There

was also significant ^{13}C-glyphosate incorporation for the 18:1ω9 fungal biomarker in the

Cornerstone® treatment.

INTRODUCTION

Glyphosate (N-(phosphonomethyl) glycine) was first introduced in 1974 as a non-selective, broad spectrum, post-emergent herbicide to control weed competition in agricultural farming (Goldsborough and Brown, 1988). It gained large popularity and increased usage in 1996 with the introduction of transgenic glyphosate resistant (GR) soybean (*Glycine max*) cultivars and again in 1998 with GR corn (*Zea mays*) cultivars. These GR cultivars have led to a glyphosate (GLY) dominated herbicide market, which in the United States has seen an increase from 1.4 million kg of GLY added to soybeans in 1996 to 31.1 million kg in 2009 and a corresponding decrease of all other herbicides from 78.6 million kg to 50.0 million kg (Coupe and Capel, 2016). Additionally, a report for 2014 showed that in the United States, total usage of GLY for all crops in the farm sector was approximately 108.8 million kg (Myers et al., 2016) and data collected from the National Agricultural Statistical Service shows that in 2016 approximately 48.5 million kg of GLY active ingredient was applied to soybean crops alone.

In GR cultivars GLY is generally applied in the form of a foliar spray in which it is absorbed through the leaves and is systemically translocated throughout the plant before finally being expelled into the rhizosphere via root exudates. In GLY sensitive weeds, GLY's main mode of action is by prohibiting the action of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate acid pathway, which is essential for the synthesis of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan in both plants and some microorganisms (Duke et al., 2012). However, bacterial genes encoding GLY insensitive forms of EPSPS do exist which were

first isolated from bacterial species of *Agrobacterium spp*., *Achromobacter spp*., and *Pseudomonas spp*. strains. The *Agrobacterium spp*. EPSPS gene isolated from strain CP4 proved to have superior GLY tolerance and is the transgenic source for many of the GR cultivars today (Funke et al., 2006).

The efficacy of this transgene has led to the development of glyphosate tolerant cropping (GTC) systems, increased the practice of no till farming, and has dramatically reduced weed competition in the United States and worldwide. This is good news for erosion control and for aiding efforts to reduce the negative effects of herbicide and fertilizer run-off. However, the long-term sustainability of a GTC system is often called into question as there are still many unknowns regarding its long-term effects on rhizosphere microorganisms and the occurrence of GLY resistant weeds continues to rise. Common weed species like *Amaranthus palmeri*, *Conyza canadensis*, and *Ambrosia artemisiifolia* are known to be resistant to GLY and historical evidence shows that on average since 1996 there have been eight new reported cases of GLY resistant weed species identified each year (Heap, 2018). Increased resistance may necessitate increased GLY application frequency or dosage concentration in the future so as to maintain current levels of weed control and crop productivity. This will increase any long-term effects on the GR plants and their corresponding rhizosphere microbial communities. Therefore, it's important to fully understand microbial rhisosphere dynamics as they relate to community composition and GLY degradation in-order to develop alternate management practices which may mitigate the long-term detrimental effects.

Past research has shown that GLY treatments can lead to increases in microbial respiration and stimulate fungal growth in the rhizosphere, possibly by acting as a metabolic substrate for various saprophytic microorganisms (Haney et al., 2000; Kremer et al., 2005; Wardle and Parkinson, 1990). Some conflicting studies have also claimed that GLY can negatively affect plant immunity by binding tightly with essential cations which have importance as plant metabolic and disease suppressing co-factors (Duke et al., 2012; Johal and Huber, 2009). Similar research in this area has also shown that non-GR plants can develop depletions of zinc, boron, iron, and manganese in plant tissues, probably due to immobilization from GLY chelation (Cakmak, et al., 2009; Eker et al., 2006; Neumann et al., 2006). Additionally, it has long been demonstrated that GLY is a strong clay mineral chelator (Sprankle et al., 1975). Thus extensive chelation of plant immune mineral based co-factors resulting from long-term GLY application may deplete the soil's available nutrient stores resulting in nutrient deficiencies. This coupled with stimulated fungal activity could result in increased plant susceptibility to fungal diseases.

Many microorganisms have been shown to metabolize GLY, for instance multiple gram-negative organisms from the family Rhizobiaceae have been found to degrade GLY in-vitro (Liu et al., 1991; Mcauliffe et al., 1990), and research by Krzysko-Lupicka et al. (1997) demonstrated that fungal colonies are the main microbial degraders of GLY. Thus indicating that increased fungal degradation relative to other microbial functional groups could lead to a subsequent increase in fungal activity and biomass directly following GLY application. A genus of particular interest is the plant fungus *Fusarium spp.*, which is identified as the main culprit of sudden death syndrome and root

rot in soybeans (Keen et al., 1982; Martinelli et al., 2004). One study found that GLY application to GR soybeans increased the occurrence of sudden death syndrome and rhizosphere colonization by *Fusarium solani* and *Fusarium glycines* (Sanogo et al., 2000). Additionally, the growth of *Fusarium spp.* was found to be stimulated in-vitro in aqueous solutions of soil and GLY, and extended exposure led to the increased abundance of *Fusarium oxysporum* and *Fusarium solani* (Krzysko-Lupicka and Sudol, 2008). An increase in fungal abundance is concerning for both humans and plants because it can increases plant fungal stress and the prevalence of mycotoxins in the grain.

Additionally, it has been extensively shown that application of GLY can alter the microbial balance of beneficial and non-beneficial microorganisms. For example, Kremer et al. (2005) showed that root exudation of GLY by GR soybeans coincides with high exudation rates of amino acids and carbohydrates, and favors increased fungal colonization. Another study by Kremer and Means (2009) demonstrated higher root colonization by *Fusarium spp.* in GR soybean and corn treated with GLY compared to non-treated GR cultivars. Additionally, *Arbuscular mycorrhiza* (AMF), a beneficial filamentous fungus that acts to collect sequestered nutrients for plant cortical cell uptake was shown to reduce its colonization of roots in non-GR plants after GLY application (Wan et al., 1998). A microcosm study by Lancaster et al. (2009) found higher concentrations of gram-negative fatty acid methyl esters after repeated GLY application, indicating that gram-negative bacteria are also stimulated by GLY.

To address the concerns of long-term GLY effects on rhizosphere microorganisms, a simulated long-term greenhouse experiment was conducted. The

experiment consisted of GR soybean and corn plants grown under two soil managements, one with and one without a long-term history of GLY application. GLY treatments consisted of two common commercial GLY formulations, one GLY formulation mixed with two additional chemicals (to stimulate rhizosphere microorganisms), and a control treatment. The objectives were to use phospholipid fatty acid analysis (PLFA) to i) determine if long-term GLY application causes an overall microbial community shift in the soybean rhizosphere of the historically non-GLY-treated soil management, and if this shift is similar to the historically GLY-treated soil management, ii) investigate microbial functional group differences between GLY treatments, and iii) identify GLY degrading microbial functional groups using ^{13}C-PLFA stable isotope probing (SIP).

MATERIALS AND METHODS

Soils

Effort was made to collect two soils of similar chemical composition and taxonomic classification for this study so that the soils differed significantly only in their GLY application history. For the greenhouse study described below was collected from 0-39 cm depth soil pits in 1 cm depth increments from agricultural sites located in Knox and Delaware counties in Ohio. During the collection process the soil was stored in plastic bags and transported in coolers to the laboratory. Soils were stored at 4° C, and large rocks, roots, and organic matter were removed by sieving (2 mm) prior to placement into the rhizoboxes. Approximately 2500 g of soil was carefully placed into each of the sixteen rhizoboxes in 1cm increments starting with the 38-39th at the bottom

to reconstruct the field soil profile. There was approximately 62 g of soil for each 1 cm increment.

The management soil with a long-term history of GLY application (< 10 years) (conventional management), a Bennington silt loam (fine, illitic, mesic Aeric Epiaqualf) was collected from a farm in Knox county practicing a no-till GR corn and soybean rotation. Applications of GLY were made up to three times per season to the soybean rotation and one time per season to the corn rotation. The management soil with no history of GLY application (non-conventional management), a Blount silt loam (fine, illitic, mesic Aeric Epiaqualf) was collected from a farm managed without synthetic (no GLY) and organic inputs in Delaware county that had a diverse crop rotation. The previous five years was alfalfa-orchard grass, corn, oats-alfalfa-orchard, spelt-timothy-clover, and timothy-clover. Textural analysis (Table 2.1) for the two management soils showed that the conventional management soil had 4 % more clay and 1 % more sand than the non-conventional management soil. Total carbon content differed between the two management soils with 1.47 % in the non-conventional management soil and 2.46 % in the conventional management soil, pH was the same at 7.0.

Experimental Design

The experimental design was a completely randomized 2 X 4 factorial design with two soil treatments and four GLY treatments. The soil treatments were a conventional management soil and a non-conventional management soil, both with the same taxonomic soil type. The four GLY treatments were (1) Monsanto Powermax® (PM) (potassium salt carrier); (2) Agrisolutions Cornerstone® (CS) (isopropylamine salt

carrier); (3) a mixture of Agrisolutions Cornerstone®, Agro-Plus Grozyme® and AgSpectrum Glycure® (CS+); and (4) a non-treated control. Per manufacturer recommendation the treatments containing Agrisolutions Cornerstone® (CS, CS+) had an 80% non-ionic surfactant added to the mixture from Southern Ag (Rubonia, FL).

GR corn and soybean plants were grown in rhizoboxes and placed in secure wooden holders to maintain an upright position during each of the growth periods. The rhizoboxes (adapted from Bott et al., 2008) measured 400 x 200 x 20 mm and were fitted with a hinged acrylic or Plexiglas side panel for easy access to the root rhizosphere. Each of the two soils had a total of eight rhizoboxes with two replicates per treatment. The plants were grown for eight growth periods over three years, with each growth period being 58 days. A GLY burn down spray was applied on day 1 followed by planting on day 10. Seeds were hydrated on wet paper towels in petri dishes at 23° C prior to sowing to increase plant establishment. Two foliar applications of GLY were administered during growth stages V3 to V5, and V6 to V7 (approximately 30 and 51 days after planting) and applied at the recommended field application rates of 1,013 g ai ha^{-1} for PM, and at 964 g ai ha^{-1} for CS and CS+. Isotopically labeled treatments contained 99.9% pure ^{13}C glyphopsate added to the mixture at concentrations of 39.36µg ^{13}C kg^{-1} for the PM treatment, and 37.724µg ^{13}C kg^{-1} for the CS and CS+ treatments. A solution of 25 mL Peters® 20 / 20 / 20 Professional fertilizer was added at a concentration of 3.745 g L^{-1} to each rhizobox 30 and 50 days after planting to maintain plant nutrient status. Rotations of corn and soybeans were made after growth periods 3, 5, and 7. The experiment was

conducted under controlled greenhouse conditions that had 12 hours light and dark cycles at 23° C ambient temperature.

Rhizosphere Sample Collection

Rhizosphere samples for corn and soybean plants were collected exactly 1 and 7 days after each GLY application. However, only sample data collected from the soybean plants will be reported on in this study. Rhizosphere sampling was done by opening the face of the rhizobox while slanted to remove approximately 10 g of rhizosphere and intra-root soil. All samples were stored at -20° C for no more than two months in 50 mL falcon tubes before analysis. Three analytical replicates were collected for each rhizobox. Following each growth period, soil was removed from each rhizobox in sixteen 25 mm depth increments, organic root material was then removed by sieving (2 mm). Each soil increment was placed back into the rhizobox in order.

Chemical Analysis and Phospholipid Fatty Acid Extraction

Total carbon and nitrogen content was measured by dry combustion using an elemental analyzer (Carlo Erba CHN EA 1108, now Thermo Fisher Scientific, Waltham, MA). Soil pH was measured after 10 min of standing and gentle stirring at a 1:1 mixture of soil and deionized water (10 g soil:10 mL water) using a glass membrane electrode (Sparks et al., 1996).

Microbial functional group composition was quantified based on the PLFA extraction procedure described by Frostegard et al. (1993). In brief, phospholipids were extracted from approximately 2 g of rhizosphere soil using a single-phase mixture of chloroform, methanol and citrate buffer (Bligh and Dyer, 1959). The upper organic phase

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of this mixture was decanted and then fractionated into its neutral, glycolipid, and phospholipid components using silica acid columns. Conversion of the phospholipids to methyl-esters was then performed by alkaline methanolosis using 0.2 M methanolic KOH (Chowdhury and Dick, 2012). Fatty acids were analyzed on a gas chromatograph (Hewlett-Packard 5890 Series II; Ultra 2 column; carrier gas, helium; temperature ramping 120 to 260° C at a rate of 5° C per minute) with a flame ionization detector and controlled with MISystem software (MIDI Inc. Newark, DE). The same PLFA extract was analyzed for ^{13}C using an Agilent 6890 gas chromatograph coupled to a Delta V Advantage mass spectrometer (Thermo Fisher Scientific, Waltham, MA).

A total of 15 fatty acid biomarkers were consistently identified in each sample. The biomarkers were then summed into five corresponding microbial groups. Actinobacteria (Actino) is represented by the sum of the individual 16:0ME, 17:0ME, and 18:0ME biomarkers, gram-negative (Gram⁻) bacteria is represented by the sum of 16:1ω7, 17:0c, 19:0c ω8, and 18:1ω7, gram-positive (Gram⁺) bacteria is represented by the sum of 15:0i, 15:0a, 16:0i, 17:0i, and 17:0a, saprophytic fungus (Fungi) is represented by the sum of 18:2ω6 and 18:1ω9, the arbuscular mycorrizae fungus (AMF) are represented by the single 16:1ω5 biomarker, and all the individual biomarkers were summed to represent the complete biomass of the microbial community (Total). Additionally, the stress indicator ratios of the saturated to monounsaturated PLFAs (S:M), and the cyclopropyl to their monoenoic precursors (C:P) were also used as indicators of physiological status. The saprophytic fungal to bacterial ratio (F:B) was also used as an additional parameter of the microbial community composition, and was

calculated as total Fungi PLFAs over the sum of the Actino, Gram⁻, and Gram⁺ PLFAs (Moore-Kucera and Dick, 2008a). The biomarkers are named using the standard colon notation, with the total number of carbons in the chain before the colon, followed by the number of double bonds after the colon. In the case of a double bond, the location of the bond is identified by an omega (ω) symbol followed by the carbon number as counted from the omega end (opposite the carboxyl end) of the fatty acid. The structural confirmation indicators of iso (i), anteiso (a), and cyclo (c) will follow the double bond number. The location of a methyl group on the 10th carbon is indicated by ME. Absolute (nmol g^{-1}) and relative (mol. %) abundance were calculated according to Zelles, (1999).

^{13}C Analysis and Calculations

Using the mass balance equation as described by Pelz et al., (1997), the δ^{13}C values were corrected for the addition of the lone carbon atom introduced during methanol derivatization. Than the percentage of ^{13}C incorporated (%^{13}C-INCORP) into a given individual PLFA biomarker was calculated following the description by Moore-Kucera and Dick, (2008a):

Equation 1: $\quad R = \left(\left(\dfrac{\delta\frac{^{13}C}{^{14}C}}{1000} \right) + 1 \right) \times R_{PDB}$

Equation 1 is used to convert the delta values to the carbon isotope ratio (R = ^{12}C/^{13}C), where the Pee Dee Belemnite (PDB) ratio acts as the standard reference ratio, R_{PDB} = 0.0112.

Equation 2: $\quad F = \dfrac{R}{(R+1)}$

Equation 2 is used to calculate the fraction of ^{13}C in the labeled (F_{tx}) and unlabeled (F_{t0}) rhizoboxes.

Equation 3: $\%^{13}C\ INCORP = 100 \times \left(\frac{(F_{tx} - F_{t0}) \times [PLFA]}{[13C - added]} \right)$

Where, [PLFA] is the concentration (μg C kg^{-1} soil) of the individual PLFA biomarker, and [13C-added] is the concentration (μg ^{13}C kg^{-1}) of ^{13}C label added to the rhizobox. Additionally, the relative distribution of ^{13}C ($\%^{13}$C-DIST) incorporated into an individual biomarker is expressed as the proportion of ^{13}C incorporated in a given biomarker over the sum of ^{13}C incorporated in all biomarkers for a given treatment, expressed as a percentage.

Fusarium DGGE and qPCR Analysis

Denaturing gradient gel electrophoresis (DGGE) was performed on samples from growth period 8. Metagenomic DNA was extracted from soil samples using the PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA), followed by PCR-DGGE to examine the *Fusarium spp*. communities. A nested PCR was done to amplify the *Fusarium spp*. elongation factor 1α gene fragment using primers EF1 and EF2 (O'Donnell et al., 2009), then a second round of PCR was done using primers Alfie1 and Alfie2-GC, which have been shown to further discriminate the major *Fusarium* spp. (Urashima et al., 2012; Yergeau et al., 2005). DGGE analysis was performed using the PhorU system (Ingeny, Leiden, Netherlands), and images were captured using the AlphaImager® HP System (ProteinSimple, San Jose, CA). DGGE gel image data was analyzed using ImageJ2 software (Rueden et al., 2017). The resulting treatment DGGE

bands were compared against known reference *Fusarium spp.* bands using the same method.

Quantitative polymerase chain reaction (qPCR) was done on growth period 8 samples using a primer set specific to the *Fusarium spp.* internal transcribed spacer (ITS) region (Blum et al., 2004). The sample derived qPCR standard was obtained using the *Fusarium* specific ITS primers with the pooled DNA extracted from all samples as a template. A standard curve was then generated using serial dilutions of the sample derived ITS amplicons. PCR was performed using the Mx3000 real time PCR system (Stratagene, La Jolla, CA).

Statistics

All statistical analyses were performed using SAS version 9.4, with the exception of microbial community ordinations. The analytical reps from the first and second GLY applications were averaged for each rhizobox, and since no significant ($p < 0.05$) differences were detected between sampling days 1 and 7, the sampling day data was combined and used as replicates. For multivariate ordinations, non-metric multidimensional scaling was performed on the five summed microbial groups and the three stress indicator ratios using PcOrd version 6.22. A Euclidian distance measure was used with 250 runs of real data, and Monte Carlo simulations were conducted with 50 randomized runs and a stability criterion of 0.0001. For denaturing gradient gel electrophoresis (DGGE) and quantitative polymerase chain reaction (qPCR), samples were collected from growth period 8 only and means separation analyses were performed on qPCR data using tukey HSD.

For PLFA, all data was log transformed to achieve normality (Kolmogorov-Smirnov test), and the microbial functional groups were used for statistical analysis as opposed to the individual biomarkers as proposed by Zelles (1999) to provide a more robust measure of the microbial community composition of major functional groups. The PROC mixed command was used for designing a repeated measures split plot model to determine the overall main factor effects of soil type on the subplot factor of glyphosate treatment and growth period. Then means separation analysis was done on PLFA functional group data with the tukey HSD test within each growth period and soil management. PLFA data from growth period 3 was removed from the data set because it was found to be unreliable.

For % ^{13}C-INCORP and % ^{13}C-DIST, only six biomarkers gave delta values large enough to be clearly identifiable in all chromatograms. For this reason means separation on the microbial functional groups was not possible and instead was performed on these six biomarkers. Additionally, undetectable levels of ^{13}C label were found in greater than 40 % of the samples for growth periods 4 and 8 each. So in order to create a more complete data set, the analytical replicates from growth periods 4 and 8 were combined and analyzed as one. Treatment comparisons were made using Tukey HSD.

RESULTS

Overall Absolute PLFA Treatment Comparisons

An overall significant ($p < 0.05$) effect of treatment and soil management was detected for absolute PLFA abundance. For the soil management, the absolute PLFA

abundance was 150 to 200 % higher in the non-conventional management soil than the conventional management soil for all PLFA microbial groups.

The overall treatment comparisons for the non-conventional management soil showed the CS and CS+ treatments had significantly ($p < 0.05$) lower overall abundance for Gram$^+$ and Actino, compared to the control. Additionally, for the conventional management soil only the CS treatment had significantly ($p < 0.05$) lower overall abundance for the Gram$^-$ and Gram$^+$, compared to the control. The stress ratios for the non-conventional management soil had significantly ($p < 0.05$) higher overall ratios in the CS and CS+ treatments for the F:B and C:P ratios, compared to the control. For the conventional management soil there was a higher C:P ratio in the CS treatment, and higher F:B ratio for all treatments, compared to the control.

Absolute PLFA Treatment Comparisons within Growth Periods

Absolute PLFA microbial groups for each GLY treatment were compared within each soil management and within each growth period to the control. Results showed that in the conventional management soil (Table 2.2) no significant ($p < 0.05$) differences in any microbial groups were detected between any of the treatments for growth periods 2, 5, 7, or 8 compared to the control. However, in growth period 1 the PM treatment showed a significant ($p < 0.05$) decrease for Gram$^+$ and AMF of 15% and 21% compared to the control, respectively. In growth period 4, the PM treatment had a significant ($p < 0.05$) decrease for both the Fungi and AMF of 30% each compared to the control, and a significant ($p < 0.05$) decrease for Actino of 24% compared to the control. In contrast, growth period 6 for the PM treatment had a significant ($p < 0.05$) increase for all

microbial groups ranging from 71% in the Gram$^+$ to 98% in the Actino, compared to the control. The CS treatment in growth period 1 had a significant ($p < 0.05$) increase of 24% in the Gram$^+$ compared to the control, but in growth period 4 the CS treatment had a significant ($p < 0.05$) decrease of between 40 % to 69 % compared to the control across all groups. Conversely in growth period 6, the CS treatment had a significant ($p < 0.05$) increase of 30 % or greater compared to the control for all microbial groups, except AMF which was unaffected; additionally, the Fungi group had the greatest increase at 42 % compared to the control. The CS+ treatment in growth period 1 had a significant ($p < 0.05$) increase of 29 % in Gram$^+$ compared to the control, but in growth period 4 the CS+ treatment had significant ($p < 0.05$) decreases of 63 % and 43 % compared to the control for AMF and Fungi, respectively. However, in growth period 6 the Fungi increased by 104 % compared to the control while the Actino and F:B ratio increased compared to the control by 22 % and 75 %, respectively.

For the non-conventional management soil (Table 2.2), significant ($p < 0.05$) microbial group differences within each soil management and within each growth period showed that only growth period 2 had no significant ($p < 0.05$) differences for any treatment compared to the control. However, for the PM treatment in growth period 1, there was a significant ($p < 0.05$) decrease of 14 % in AMF compared to the control, but there were no significant ($p < 0.05$) differences for growth period 4 compared to the control. The PM treatment in growth period 5 had significant ($p < 0.05$) decreases for Gram$^-$ and Fungi of 29 % and 32 % compared to the control, respectively. However, in growth period 6 all groups significantly ($p < 0.05$) increased compared to the control,

ranging from 48 % in the Gram⁻ to 88 % in AMF. Significant ($p < 0.05$) increases

compared to the control for all groups except the Fungi continued into growth period 7,

ranging from 45 % in Actino to 88 % in AMF. For growth period 8, only the PM

treatment had a significant ($p < 0.05$) decrease of 14 % in AMF, and an increase of 26 %

in the F:B ratio compared to the control.

      Additionally, for the CS treatment in the non-conventional management soil

(Table 2.2) no significant ($p < 0.05$) differences were observed in growth periods 1 or 6

compared to the control, but growth period 4 had significant ($p < 0.05$) increases

compared to the control in Actino and Fungi of 23 % and 17 %, respectively. Growth

period 5 for the CS treatment had significant ($p < 0.05$) decreases compared to the control

in all microbial groups of 35 % or less, but a significant ($p < 0.05$) increase compared to

the control of 13 % was observed in the F:B ratio. For growth period 7, the CS treatment

F:B ratio increased to 38 % higher than the control. The CS treatment for growth period 8

only had an increase compared to the control in the AMF of 33 %. For the CS+ treatment,

in growth period 1 significant ($p < 0.05$) increases compared to the control of 14 %, 52 %

and 35 % were observed for the Gram⁻, Fungi, and F:B ratio, respectively. For the CS+

treatment in growth period 4, significant ($p < 0.05$) decreases for all groups were

observed ranging from 15 % in the Actino to 46 % in Gram⁺ compared to the control;

however, a 60 % increase compared to the control was observed in the F:B ratio. For

growth period 5, the CS+ treatment showed significant ($p < 0.05$) decreases compared to

the control in all groups ranging from 33 % to 44 %. Conversely, the CS+ treatment for

growth period 6 had significant ($p < 0.05$) increases compared to the control ranging from

20 % to 76 % for all microbial groups, relatively no significant ($p < 0.05$) glyphosate

treatment effects were found for growth periods 7 or 8 compared to the control.

Relative PLFA Abundance

Analysis of the overall relative PLFA abundance showed a significant ($p < 0.05$)

effect of soil management, but a significant ($p < 0.05$) time effect was only detected for

AMF and Fungi. GLY treatments were compared against the control for each growth

period separately and within each soil management (Fig. 2.1).

For the conventional management soil (Fig. 2.1 A), growth periods 1, 2, 5, and 7

had no significant ($p < 0.05$) treatment differences compared to the control. However, for

the PM treatment in growth period 8 there was a significant ($p < 0.05$) increase in Fungi

of 8.3% compared to the control. For the CS treatment in growth period 4, there was a

significant ($p < 0.05$) 2.2 % decrease in AMF compared to the control. The CS+

treatment in growth period 4 also had significant ($p < 0.05$) decreases compared to the

control of 2.5 % and 2.8 % for AMF and Fungi, respectively. Additionally, the CS+

treatment had significant ($p < 0.05$) 6.3 % and 8.7 % increases in Fungi compared to the

control for growth periods 6 and 8, respectively. However, there was a significant ($p <$

0.05) 1.3 % decrease in AMF compared to the control for growth period 6.

For the non-conventional management soil (Fig. 2.1 B), growth period 2 had no

significant ($p < 0.05$) treatment differences compared to the control. However, the PM

treatment in growth period 1 had a significant ($p < 0.05$) decrease in AMF of 1.0 %

compared to the control, but there were significant ($p < 0.05$) increases compared to the

control of 1.0 % and 1.5 % for growth periods 6 and 7, respectively. Also for the PM

treatment, Fungi was significantly ($p < 0.05$) higher than the control by 3.5 % in growth period 8. For the CS treatment, growth periods 5 and 7 had a significant ($p < 0.05$) increase in Fungi compared to the control of 2.0 % and 5.2 %, respectively. Also for the CS treatment, there was a significant ($p < 0.05$) increase of 1.2 % in AMF for growth period 6 compared to the control. For the CS+ treatment in growth periods 1, 4, and 8, there were significant ($p < 0.05$) Fungi increases compared to the control of 4.5 %, 5.4 %, and 1.2 %, respectively. Additionally, there was a significant ($p < 0.05$) decrease of 2.2 % in AMF for growth period 4 compared to the control. However, in growth period 6 AMF was significantly ($p < 0.05$) higher by 1.2 % compared to the control.

% ^{13}C Incorporation

The overall % ^{13}C-INCORP for the non-conventional management soil had significantly ($p < 0.05$) higher values than the conventional management soil for all biomarkers (Fig. 2.2). For example, the % ^{13}C-INCORP values ranged from 0.0 % to 0.051 % and 0.0 % to 0.14 % in the conventional and non-conventional management soils, respectively; also, the average total % ^{13}C-INCORP in all microbial biomarkers were higher in the non-conventional than the conventional management soil for the CS, CS+ and PM treatments by 71 %, 20 %, and 3.3 %, respectively. This trend corresponded to absolute PLFA abundance, which was also higher in the non-conventional management soil. Additionally, a higher number of treatment differences for the % ^{13}C-INCORP was also detected in the non-conventional management soil, this also corresponds to that observed for the absolute and relative PLFA abundance.

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Treatment effects for the conventional management soil (Fig. 2.2 A) showed that the CS treatment had significantly ($p < 0.05$) higher %[13]C-INCORP for the 18:1ω9 biomarker compared to the CS+ or PM treatments, but no significant ($p < 0.05$) differences were detected for the other five biomarkers. However, the CS+ treatment did have significantly ($p < 0.05$) higher total %[13]C-INCROP (0.023 %) for all six biomarkers, compared to the PM (0.015 %) and CS (0.011 %) treatments.

For the non-conventional management soil (Fig. 2.2 B), the CS treatment showed significantly ($p < 0.05$) higher %[13]C-INCROP than the PM treatment for the 16:1ω7, 17:0iso, and 18:1ω9 biomarkers at 0.002 %, 0.004 % and 0.006 % higher, respectively. The CS treatment was also significantly ($p < 0.05$) higher than the CS+ treatment for the 16:0ME biomarker at 0.002 % higher; however, this was reversed for the 18:0ME biomarker where the CS+ treatment was significantly ($p < 0.05$) higher than the CS treatment at 0.002 % higher.

% [13]C Distribution

For the conventional management soil (Fig. 2.3 A), the CS treatment had significantly ($p < 0.05$) higher %[13]C-DIST than the CS+ treatment for both the 16:1ω7 and 18:1ω9 biomarkers, at 14 % and 13 % higher, respectively; however, the CS treatment was significantly ($p < 0.05$) lower than the CS+ and PM treatments for the 18:0ME biomarker, at 16 % and 10 % lower, respectively.

For the non-conventional management soil (Fig. 2.3 B), the PM treatment for the 16:1ω7 biomarker was significantly ($p < 0.05$) higher than the CS and CS+ treatments at 19 % and 17 % higher, respectively. However, the PM treatment in the non-conventional

management soil was significantly ($p < 0.05$) lower than the CS and CS+ treatments for the 17:0a biomarker at 6 % and 9 % lower, respectively. The only other significant ($p < 0.05$) treatment effect for the non-conventional management soil was for the CS treatment, which was 11 % and 19 % higher than the CS+ and PM treatments for the 18:1ω9 biomarker, respectively.

When comparing the soil management effects for %[13]C-DIST, the only biomarker that was consistently higher in both soil managements and the same treatment was the 18:1ω9 biomarker for the CS treatment. It also had the greatest average %[13]C-DIST than any other biomarker in any treatment for both soil managements, at 28 % and 25 % for the non-conventional and conventional management soils, respectively (Fig. 2.3 B).

Microbial Community Ordinations

Ordinations using absolute PLFA concentrations for growth periods 1, 4, and 8 showed there was no distinct treatment shifts overall or within any growth period. However, there was a shift over time, which was shown by a distinct shift in microbial community groupings from growth periods 1 to 8 for both soil managements (Fig. 2.4). A slight grouping overlap between growth periods 1, 4, and 8 was observed for the conventional management soil (Fig. 2.4 A), but this overlap was not observed for the non-conventional management soil (Fig. 2.4 B).

Ordinations using absolute PLFA concentrations were also used to detect conventional and non-conventional management soil effects. They showed that the microbial community soil groupings remained distinctly separate from growth period 1 to 8 (Fig. 2.5). Additionally, a joint overlay plot (the angle and length of a line indicate the

direction and strength of the relationship for that ratio) showed that both the C:P and S:M

stress indicator ratios increased dramatically with time (toward growth period 8) and do

not appear to be affected by a particular treatment or soil management, indicating that

time is the driving factor for microbial nutrient and physiological stress.

Fusarium **DGGE and qPCR Analysis**

The DGGE gel showed banding for all growth period 8 treatments and 8 known

pathogenic *Fusarium spp.* (Fig. 2.6). ImageJ analysis showed multiple bands which did

not appear in the control samples (Fig. 2.7). However, all 4 of the control treatment bands

for the conventional management soil appeared in the PM and CS+ treatments, and 4 of

the 7 bands in the non-conventional management soil control appeared in all of the non-

conventional management soil treatments. Most interesting was the conventional

management soil CS treatment which showed two distinctly separate bands that did not

appear in the control treatment, but which corresponded to the *Fusarium semitectum*

reference bands (Fig. 2.8).

Interestingly, the qPCR results of the *Fusarium spp.* specific ITS gene copies

were similar between the conventional management soil CS treatment and the control

(Fig. 2.9). Additionally, the qPCR analysis also showed that the PM and CS+ treatments

for both soil managements were significantly ($p < 0.05$) higher than the control treatment,

with the CS+ treatment being significantly ($p < 0.05$) higher than the PM treatment for

only the conventional management soil, at 56 % higher on average (Fig. 2.9).

DISCUSSION

Soil Management Effects

This study found no significant ($p < 0.05$) differences in rhizosphere microbial composition based on PLFA profiling between sampling days one and seven after GLY application. GLY's absorption speed can vary depending on leaf characteristics, humidity, and the physiochemical properties of the adjuvants used (Leaper and Holloway, 2000). Previous research by Singh and Singh (2008) and Haderlie (1977) have found that the majority of GLY is absorbed and translocated within the first 7 days after application, but only 1 to 10 % is translocated within the first day. Thus the results from the greenhouse study suggest that < 10 % of applied GLY reaches the rhizosphere microbial communities within one day after GLY application and is sufficient enough to cause shifts in functional microbial groups.

The microbial community shifted much more in the non-conventional management soil than the conventional management soil due to GLY application. For example, absolute PLFA abundance in the non-conventional management soil had a total of 40 significant ($p < 0.05$) treatment differences for all microbial groups compared to the control, whereas only 19 differences were detected in the conventional management soil (Table 2.1). The same pattern was observed for the $\%^{13}C$-INCORP data, where only 2 significant ($p < 0.05$) treatment differences were detected in the conventional management soil, and 4 in the non-conventional management soil (Fig. 2.2). Similarly, the relative PLFA and $\%^{13}C$-DIST data had the same pattern (Fig. 2.1, 2.3).

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These findings would suggest that the conventional management soil microbial community was well adapted to use GLY as a substrate. In contrast the non-conventional management soil which had never received GLY had much more dramatic microbial community shifts, indicating the microbial community was adjusting to degrade GLY. Indeed the non-conventional management soil PLFA markers generally increased in comparison to the control, indicating the microbial community was responding to GLY as a carbon substrate rather than causing chronic toxicity.

A study by Lane et al. (2012) applied GLY to the same non-conventional management soil and found severe suppression of respiration compared to the same conventional management soil, which was stimulated by GLY. However, this was a short-term study which only had GLY applied once, and it seems could not have had the time needed for the microbial community to degrade and detoxify GLY. Conversely, Zabaloy et al. (2012) found that non-conventionally treated soils resulted in increased microbial carbon respiration when exposed to GLY, and a lesser response was seen in the conventionally treated GLY soils. Another study by Allegrini et al. (2015) similarly studied historically GLY treated and untreated soils and showed using a pollution community tolerance assay that microbial tolerance to GLY was not consistent with the history of GLY exposure, however they did not specifically use rhizosphere soil in the study.

The ordinations for the conventional and non-conventional management soil's microbial communities clustered separately throughout the experiment (Fig. 2.5), indicating that the microbial communities remained distinctly different. Additionally the

absolute PLFA abundance in the non-conventional management soil was consistently 150 % to 200 % higher overall even though the two soil managements were very similar in soil type and texture (Table 2.1). However, differences in the history of crop rotation remained which may account for these differences. For example the, conventional management soil was collected from a farm which was practicing a simple no-till corn and soybean rotation for the last five years, whereas the non-conventional management soil was tilled and had a diverse rotation in the last five years that included corn, alfalfa, oats, and timothy clover. The diversity in crop rotation for the non-conventional management soil could diversify the microbial community more than a simple corn soybean rotation, increasing its abundance and allowing it to be more responsive to inputs.

However, soil type has much stronger control in maintaining microbial structure against the impact of soil management (Cavigelli et al., 2005; Dequiedt et al., 2011). Additionally, there may be sub populations that were affected but PLFA profiling may not have the resolution to detect such shifts. Indeed Newman et al. (2016) found with deep sequencing both negative and positive microbial responses of operating taxonomic units at the genus level due to GLY, on these same soils.

Microbial Community Treatment Effects

The AMF and Fungi in the conventional management soil had a significant ($p < 0.05$) decrease of absolute PLFA abundance for all treatments in growth period 4, but interestingly a significant ($p < 0.05$) increase in these microbial groups was observed for growth period 6, and no differences were observed for any other growth period (Table

2.2). These results also corresponded to changes in the relative PLFA abundance data

except that growth period 8 also had the relative Fungi abundance significantly ($p < 0.05$)

increasing for the PM and CS+ treatments over the control (Fig. 2.1 A). These changes

were observed across most GLY treatments and only in growth periods 4, 6 and 8, which

were growth periods that had crop rotations. While this was reflected in both the relative

and absolute PLFA values for AMF and Fungi, it was also true for the absolute PLFA

values of Actino, Gram$^-$, and Gram$^+$ for the conventional management soil (Table 2.2).

Suggesting that in a conventionally managed soil the presence of corn or soybean plants

may cause a shift in the rhizosphere microbial communities due to the particular plant

species, which could result in differential responses to GLY when the plant is re-

introduced. This seemed to be the case for AMF and Fungi PLFA abundance. However,

it should be noted that this rotational effect only occurred in the conventional

management soil which had a long history of GR corn and soybean rotation, indicating

that long-term GLY application results in fungal and AMF communities that have an

adaptive interaction with the rhizosphere of GR corn or soybean plants.

This same rotational effect was not observed in the non-conventional management

soil for either absolute or relative PLFA. However, there were far more treatment

differences detected for both relative and absolute PLFA across all treatments in the non-

conventional management soil (Table 2.2 and Fig. 2.1 B). For example, relative PLFA for

either the CS or the CS+ treatment had significantly ($p < 0.05$) higher Fungi abundance

for all growth periods except growth period 2, compared to the control (Fig. 2.1 B). The

PM treatment often did not show such effects. This indicates that GLY induces greater

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changes in the microbial groups for the non-conventional management soil, specifically

for the CS and CS+ fungal community. This agrees with previous research which shows

that repeated GLY application can increase fungal abundance and colonization in the

rhizosphere (Kremer and Means, 2009; Lancaster et al., 2009; Rosenbaum et al., 2014).

Additionally, some decreases in relative AMF abundance for the CS and CS+

treatments were observed in the conventional management soil for growth periods 4 and

6 (Fig. 2.1 A) and also in the non-conventional management soil for growth periods 4 and

7 (Fig. 2.1 B). One possible explanation for this decrease is that the isopropylamine salt

carrier used in the CS and CS+ formulations has a more negative effect on AMF

communities than the potassium salt carrier used in the PM treatment. This agrees with a

field and greenhouse study by Feng et al. (2005) which showed decreased prevalence of

soybean rust disease caused by the fungus *Phakopsora pachyrhizi* when treated with a

GLY isopropylamine salt. It was suggested that the reduced presence of the fungal

pathogen (*Phakopsora pachyrhizi*) was because it had a GLY isopropylamine sensitive

form of the EPSPS enzyme.

Many AMF species may also have a GLY isopropylamine sensitive EPSPS

enzyme. This is of concern because AMF form beneficial interactions with plants that

increase plant growth and nutrient uptake and decrease plant diseases (Verbruggen and

Kiers, 2010). A compounding factor is that AMF has a close connection with plant

cortical cells via a network of hyphae, and in some species even penetrate into the root

cells for the exchange of nutrients. This action further increases their exposure to GLY

containing root exudates, likely more so than other non-endophyte microorganisms.

The growth period 8 DGGE gel showed two unique bands for the conventional management soil CS treatment that matched the *Fusarium semitectum* reference (Fig. 2.8). *Fusarium semitectum* has been widely isolated from soil and soybean pods, and is considered to be moderately pathogenic to soybean seeds (Ivic, 2014; Lori and Sarandon, 1989; Arias et al., 2013). Interestingly though the conventional management soil CS treatment was not significantly different than the control for the qPCR *Fusarium spp*. ITS copies (Fig. 2.9), indicating the CS treatment did not have much change in *Fusarium* DNA abundance. This situation is reversed for the CS+ and PM treatments, where no unique bands matched with any reference, but significant ($p < 0.05$) increases were detected in the quantity of *Fusarium spp*. ITS copies compared to the control (Fig. 2.9). This could be due to long-term use of the CS treatment negatively interacting with beneficial *Fusarium spp*. by decreasing their abundance and creating the opportunity for pathogenic colonization by *Fusarium semitectum* without affecting overall abundance compared to the control. In contrast, the CS+ and PM treatments appeared to stimulate *Fusarium spp*. compared to the control, which may mean there was greater diversity and beneficial *Fusarium spp.* available to suppress pathogenic colonization by *Fusarium semitectum*.

The DGGE, qPCR, and PLFA results suggest that long-term GLY treatment is affecting the GR soybean rhizosphere fungal communities especially for the CS and CS+ treatments, with a more dramatic effect in the non-conventional management soil. These results agree with other studies which show increased *Fusarium spp*. colonization following GLY application (Hanson and Fernandez, 2003; Magdalena et al., 2013;

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Zobiole et al., 2010). Furthermore, Mekwatanakarn and Sivasithamparam (1987) compared herbicide types and found that only GLY increased saprophytic fungal abundance and decreased beneficial bacterial abundance; whereas other herbicides only increased fungal abundance.

Additionally, for both soil management systems absolute PLFA abundance of AMF decreased from the first 1 to 4 growth periods to the last 5 to 8 growth periods by 64 % on average, and the Fungi decreased by 49 %. However, the relative Fungi abundance increased by 21 % on average, but the relative AMF abundance decreased by 30 % over the same time frame. This occurred in all treatments including the control, indicating that the experimental conditions over time caused a shift towards a saprophytic dominated fungal composition and suppression of AMF. This highlights the limitations of a greenhouse study, and the importance of conducting future field studies under in situ conditions.

^{13}C Incorporation

The %^{13}C-INCORP and %^{13}C-DIST data suggested that the fungal communities for the CS treatment are incorporating more ^{13}C-GLY than the other treatments regardless of soil management history. This is shown in Figs. 2.2 and 2.3 where significantly ($p <$ 0.05) greater incorporation was observed in the 18:1ω9 biomarker for the CS treatment of both management soils. This pattern was partially matched in the relative PLFA data, where the Fungi in the CS treatment for the non-conventional management soil recorded significantly ($p < 0.05$) higher values than the control for three of the seven measured growth periods (Fig. 2.1 B), more than any other treatment. It must be noted that the

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results do not show one specific biomarker is incorporating ^{13}C-GLY significantly more than any other biomarker. It is only the CS treatment where there was consistently significantly more 18:1ω9 ^{13}C-GLY incorporation than the other treatments.

A possibility for the increased ^{13}C incorporation in the 18:1ω9 biomarker for the CS non-conventional management soil could be attributed to salt carrier differences between the CS and PM treatments. The CS treatment utilizes an isopropylamine salt carrier, while the PM treatment has a potassium salt carrier. In both cases the salt is weakly bonded to GLY by an ionic attraction. GLY salts vary widely in commercial GLY formulations and are mainly used as a method to increase GLY penetration into plant tissue by increasing water solubility. In a study by Nalewaja et al. (1996) an isopropylamine GLY formulation was tested against the pure GLY acid, and an ammonium, sodium, and calcium salt carrier. The results showed that isopropylamine GLY caused the greatest absorption in wheat plants compared to pure GLY or other GLY salts. Additionally, many studies have shown that GLY can stimulate fungal communities (Haney et al., 2000; Kremer et al., 2005; Wardle and Parkinson, 1990). Therefore, greater absorption of the CS treatment GLY formulation could result in greater fungal stimulation, and greater 18:1ω9 fungal biomarker incorporation.

CONCLUSION

The first objective of this study was to determine if long-term GLY treatment causes an overall shift in the non-conventional management soil such that the microbial communities become similar to a conventional management soil. The multivariate ordination analysis showed separate clustering of the management soils at growth period 1 which did not merge by growth period 8, based on PLFA profiling. This supports the conclusion that there is a strong impact of soil type on maintaining the broad microbial composition based on PLFA profiling, but leaves open the possibility that longer term GLY applications may be needed to significantly shift microbial populations. Also, it is possible that sub populations did shift but this was beyond the resolution sensitivity of PLFA biomarkers to detect.

The second objective of this study was to investigate the effects of long-term GLY treatments on the rhizosphere microbial functional groups within soil management systems. The study showed that the majority of GLY treatment effects on microbial PLFA composition and ^{13}C-GLY incorporation occurred in the non-conventional management soil. This indicates that the conventional management soil had adapted to better tolerate and degrade GLY. Also, the study showed that in a conventional management soil, crop type determined if there was a shift in the microbial community due to GLY treatment. Additionally, the results also supported previous research that showed GLY can stimulate fungal communities. However, significant fungal increases were small in abundance, and GLY also appeared to decrease the PLFA AMF biomarker at times.

The third objective was to determine the fate of ^{13}C-GLY among PLFA functional groups. The results showed increased % ^{13}C-INCORP and % ^{13}C-DIST in the CS treatment for the fungal 18:1ω9 biomarker relative to the other treatments. Also, a unique DGGE banding pattern was observed in the CS treatment which corresponded to the *Fusarium semitectum* fungal organism.

It is important to consider that this was a greenhouse study, and that in situ trials could yield different results. However, the major conclusion is that the microbial community in a soil that never received GLY does shift and likely adapts to use GLY as a substrate, most notably by fungus. However, the results also showed some negative effects of GLY with suppression of AMF in both soil managements, and stimulation of one *Fusarium spp.* pathogen in the conventional management soil. Lastly there was evidence that the isopropylamine GLY carrier caused greater treatment effects than when potassium was used as a carrier. Whether this was a direct effect of isopropylamine or a synergistic effect with GLY cannot be ascertained from this study.

TABLES

Table 2.1 Soil chemical and physical properties

Soil Type	Management	pH	AMPA	GLY	C	Soil Texture		
						Clay	Silt	Sand
			nmol g^{-1} soil			%		
Bennington Silt Loam	Conventional	7.0	747.5	0.0	2.46	45	43	12
Blount Silt Loam	Non-Conventional	7.0	0.0	0.0	1.47	41	48	11

Table 2.2 Absolute PLFA concentrations for all growth periods.

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	Conventional							Non-Conventional						
	Actino	Gram-	Gram+	AMF	Fungi	Total	F:B	Actino	Gram-	Gram+	AMF	Fungi	Total	F:B
	nmol g^{-1}													
Growth Period 1														
PM	17.8a	31.3a	20.9b	4.0b	12.8a	86.8b	0.18a	26.7a	50.0b	37.4b	9.7b	22.1b	145.9b	0.19b
CS	20.1a	41.4a	30.7a	5.4ab	16.1a	113.6ab	0.17a	28.9a	58.3ab	45.1a	11.7a	23.9b	168.0ab	0.18b
CS+	19.6a	39.3a	31.8a	5.5a	18.5a	114.7a	0.20a	30.7a	59.7a	43.2ab	11.2ab	31.3a	176.1a	0.23a
Control	20.0a	38.8a	24.7ab	5.1ab	15.0a	103.7ab	0.18a	28.2a	52.5ab	42.7ab	11.3a	20.6b	155.4ab	0.17b
Growth Period 2														
PM	15.7a	27.5a	23.7a	3.3a	9.6a	79.8a	0.14a	29.9a	57.5a	49.6a	9.5a	25.0a	171.5a	0.18a
CS	19.5a	34.5a	29.5a	4.2a	12.9a	100.7a	0.15a	25.0a	47.8a	39.2a	8.9a	19.7a	140.6a	0.17a
CS+	16.3a	31.1a	23.1a	3.6a	11.5a	85.6a	0.16a	27.4a	47.5a	39.0a	8.9a	20.2a	142.9a	0.18a
Control	29.7a	64.5a	50.8a	3.0a	23.8a	171.8a	0.15a	32.0a	55.8a	44.6a	10.3a	25.6a	168.4a	0.19a
Growth Period 4														
PM	7.9ab	15.2a	12.9a	1.4ab	4.2b	41.5b	0.12ab	13.9ab	21.7a	22.1a	5.2a	7.9ab	70.7a	0.13b
CS	5.6b	10.8b	7.6b	0.73b	2.9b	27.7c	0.13a	15.5a	24.6a	24.0a	5.9a	9.9a	79.9a	0.15b
CS+	9.5a	14.4a	14.3a	0.89b	3.4b	42.5ab	0.10b	10.7b	16.0b	12.3b	2.9b	6.4b	48.4b	0.24a
Control	10.4a	18.3a	17.7a	2.4a	6.0a	54.8a	0.13ab	12.6ab	21.2a	22.6a	5.2a	8.4ab	70.0a	0.15b
Growth Period 5														
PM	7.2a	15.0a	12.2a	1.5a	9.6a	45.5a	0.27a	11.6ab	21.6b	19ab	4.4ab	11.1b	67.7b	0.21b
CS	8.4a	16.7a	13.2a	1.6a	9.6a	49.6a	0.24a	11.7b	22.1b	16.1b	3.9b	12.6ab	66.3b	0.26a
CS+	6.9a	15.5a	11.7a	1.5a	6.6a	42.1a	0.19a	10.9b	20.4b	13.8b	3.5b	10.2b	58.8b	0.23ab
Control	9.0a	19.0a	13.7a	1.6a	8.9a	52.3a	0.20a	16.3a	30.6a	24.7a	5.9a	16.4a	94.0a	0.23ab

Values in a column within a growth period followed by the same lower case letter are not significantly (p<0.05) different.

Table 2.2 Continued

	Conventional							Non-Conventional						
	Actino	Gram-	Gram+	AMF	Fungi	Total	F:B	Actino	Gram-	Gram+	AMF	Fungi	Total	F:B
							nmol g^{-1}							
						Growth Period 6								
PM	6.6a	12.3a	12.1a	1.1a	3.8a	35.9a	0.12b	10.8a	18.0a	14.5a	3.4a	7.4a	54.1a	0.17ab
CS	4.4b	9.2ab	9.1b	0.87ab	3.1ab	26.8b	0.14b	5.5b	11.8b	9.3b	1.9b	5.4b	34.0b	0.20a
CS+	4.1bc	9.3b	7.1c	0.54b	4.4a	25.5bc	0.21a	10.4a	15.6a	13.0a	2.7a	6.0ab	47.6a	0.16b
Control	3.3c	7.0b	7.1c	0.60ab	2.2b	20.2c	0.12b	5.9b	12.0b	8.8b	1.8b	5.0b	33.5b	0.19ab
						Growth Period 7								
PM	5.7a	10.8a	10.2a	0.93a	5.5a	33.2a	0.2a	10.3a	17.1a	15.4a	2.7a	7.6a	53.1a	0.18b
CS	5.2a	10.6a	8.6a	1.0a	4.7a	30.2a	0.19a	5.8b	12ab	6.8b	1.2b	6.8a	32.7b	0.29a
CS+	4.8a	9.5a	7.5a	0.69a	4.3a	26.8a	0.19a	7.6ab	13.1ab	8.0ab	1.8ab	5.4a	35.9ab	0.19b
Control	4.2a	10.7a	8.5a	0.73a	4.4a	28.6a	0.16a	7.1b	11.2b	8.9b	1.5b	5.8a	34.5b	0.21b
						Growth Period 8								
PM	4.3a	7.2a	5.3a	0.43a	3.9a	21.0a	0.32a	10.3a	14.9a	12.9a	1.5b	9.1a	48.7a	0.24a
CS	5.0a	9.7a	8.4a	0.39a	4.1a	27.7a	0.18a	10.8a	17.0a	15.0a	2.4a	8.1a	53.3a	0.19b
CS+	4.3a	8.8a	7.3a	0.26a	4.5a	25.1a	0.31a	10.4a	16.8a	15.9a	2.3a	8.9a	54.3a	0.21ab
Control	4.0a	10.2a	9.1a	0.36a	3.7a	27.4a	0.16a	9.1a	14.3a	12.9a	1.8ab	6.6a	44.7a	0.19b

Figure 2.1 Relative abundance of the AMF and Fungi PLFA biomarkers for the conventional (A) and the non-conventional (B) management soils.

Bars within a growth period with the same upper case letter are not significantly different at $p < 0.05$ for the Fungi PLFA biomarkers, and bars within a growth period with the same lower case letter are not significantly different at $p < 0.05$ for the AMF PLFA biomarker. Standard error is show

Figure 2.1
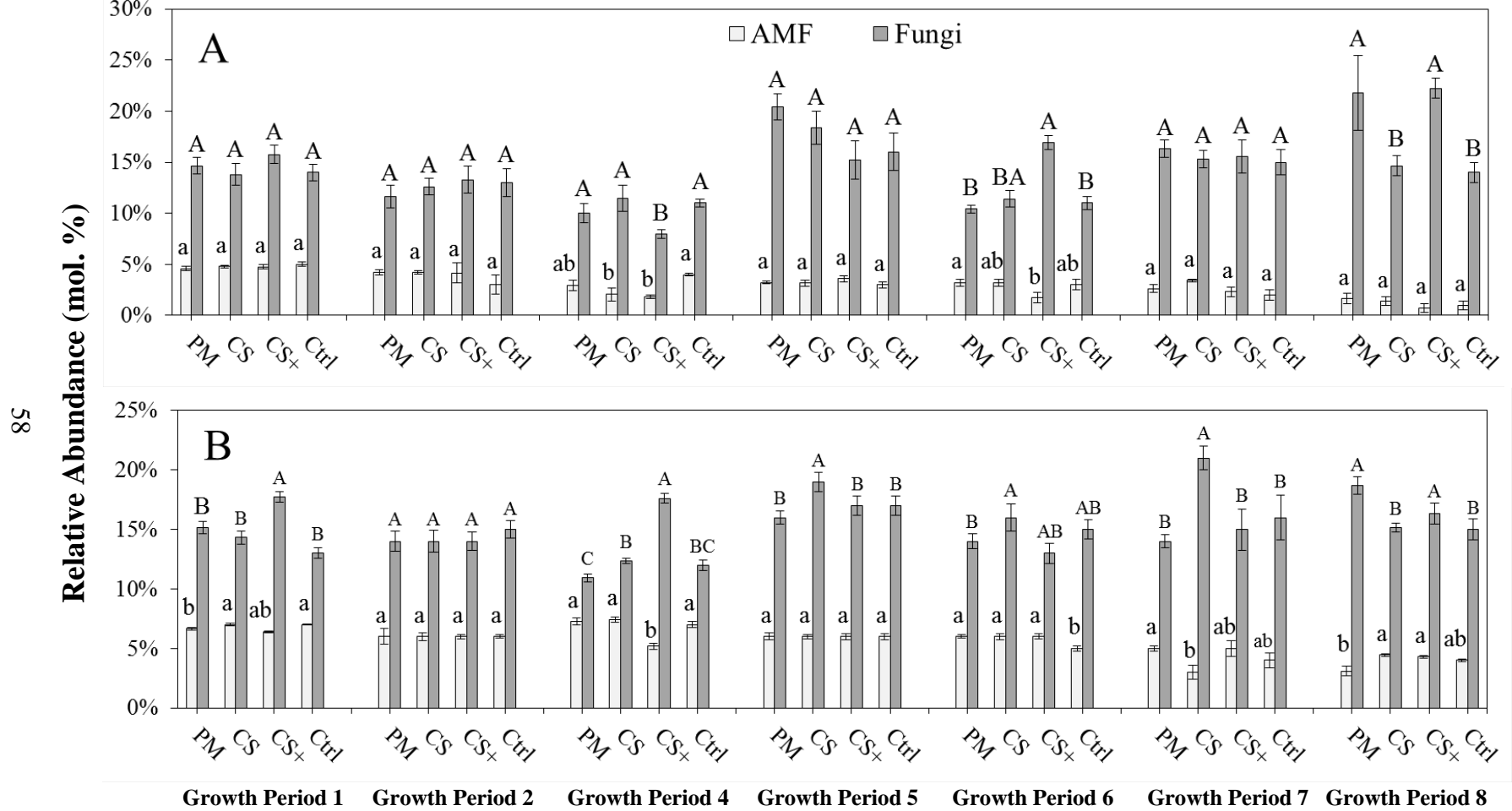
Figure 2.2 %^{13}C-INCORP in PLFAs for the conventional (A) and non-conventional (B) management soils.
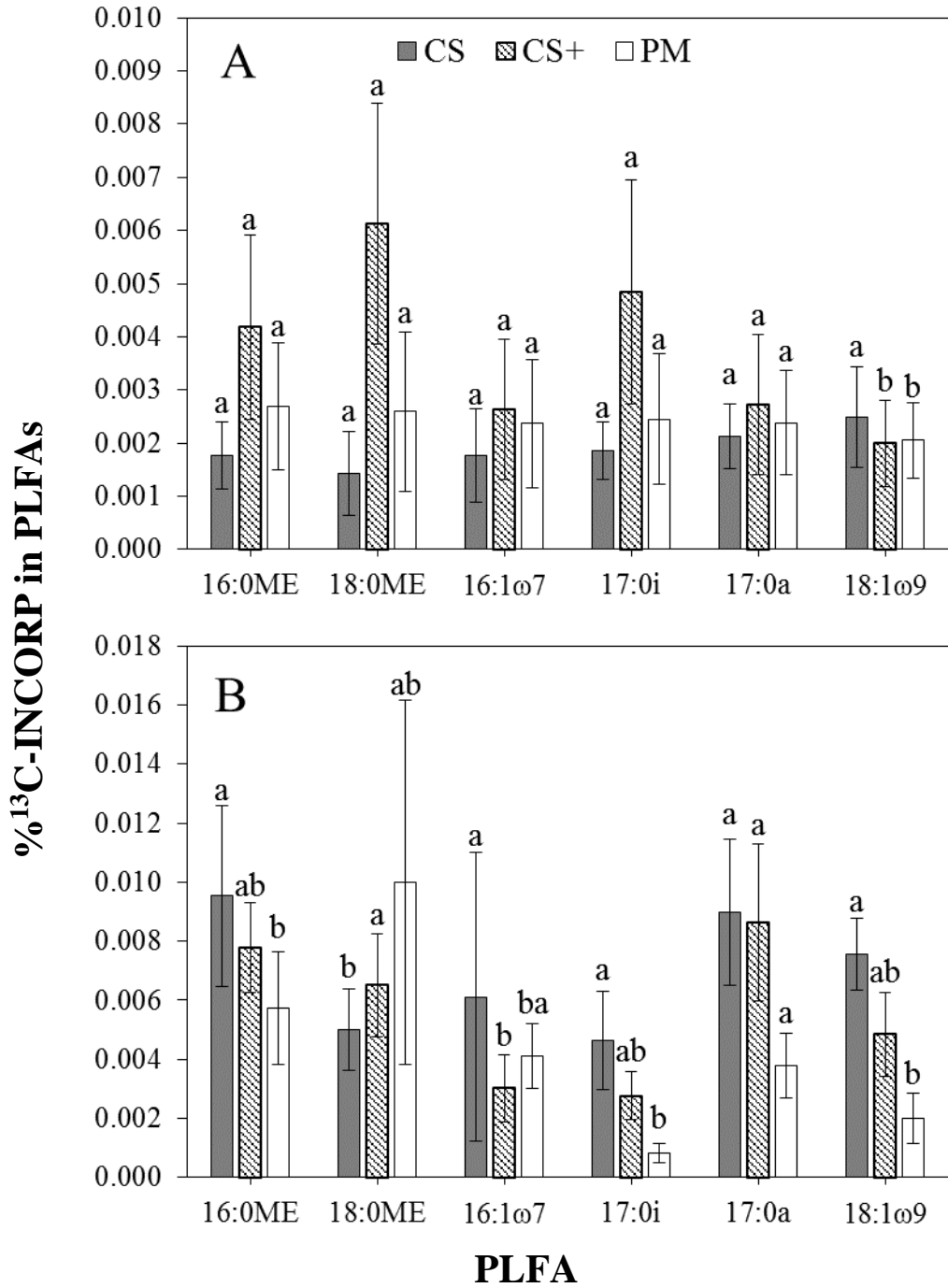
Bars for a given biomarker with the same lower case letter are not significantly (p<0.05) different. Standard error is shown.

Figure 2.3 %^{13}C-DIST in PLFAs for the conventional (A) and non-conventional (B) management soils.
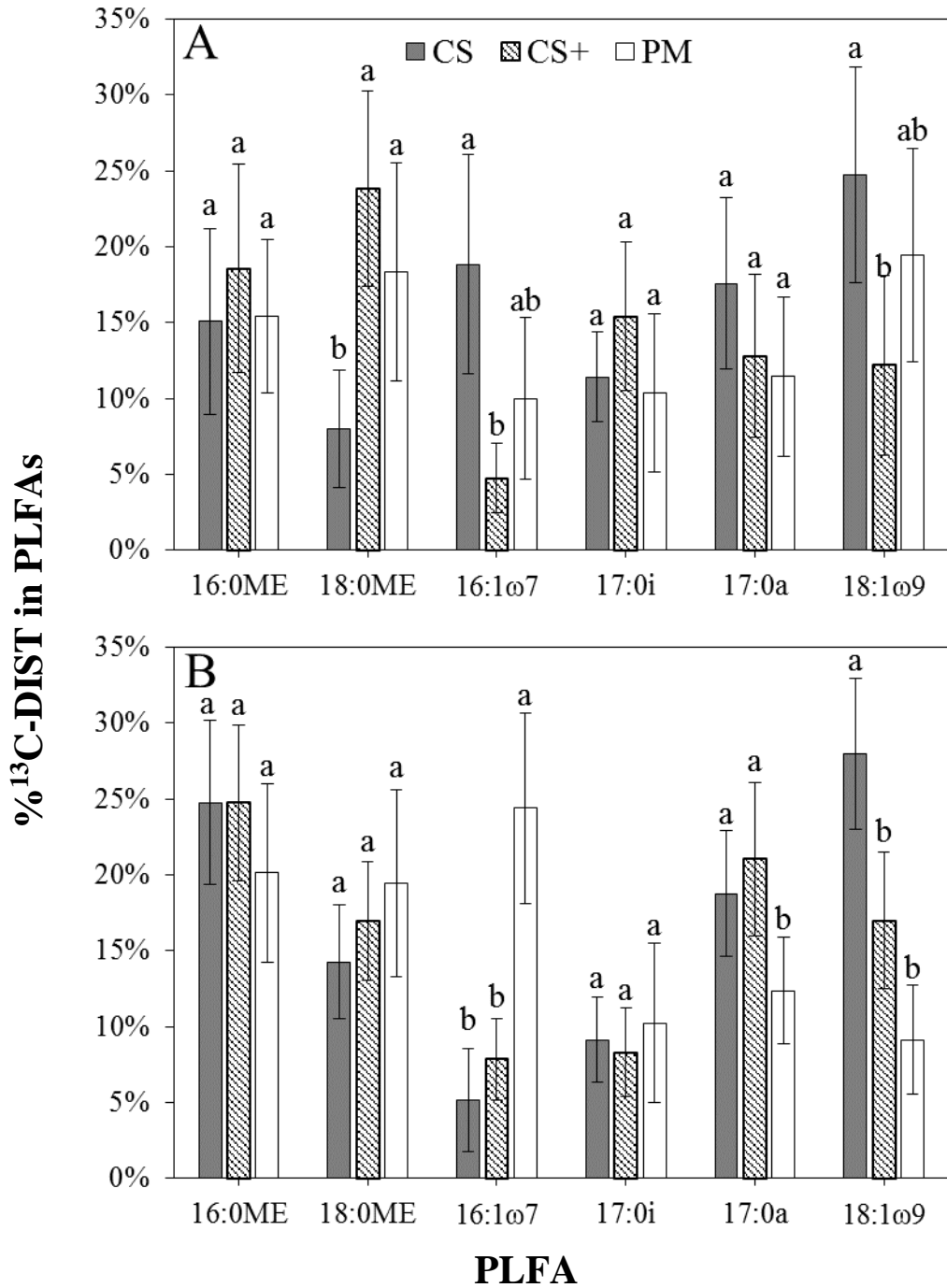
Bars for a given biomarker with the same lower case letter are not significantly (p<0.05) different. Standard error is shown.

Figure 2.4 Absolute PLFA microbial group ordinations of growth periods 1, 4 and 8 for the conventional (A) and non-conventional (B) management soils.
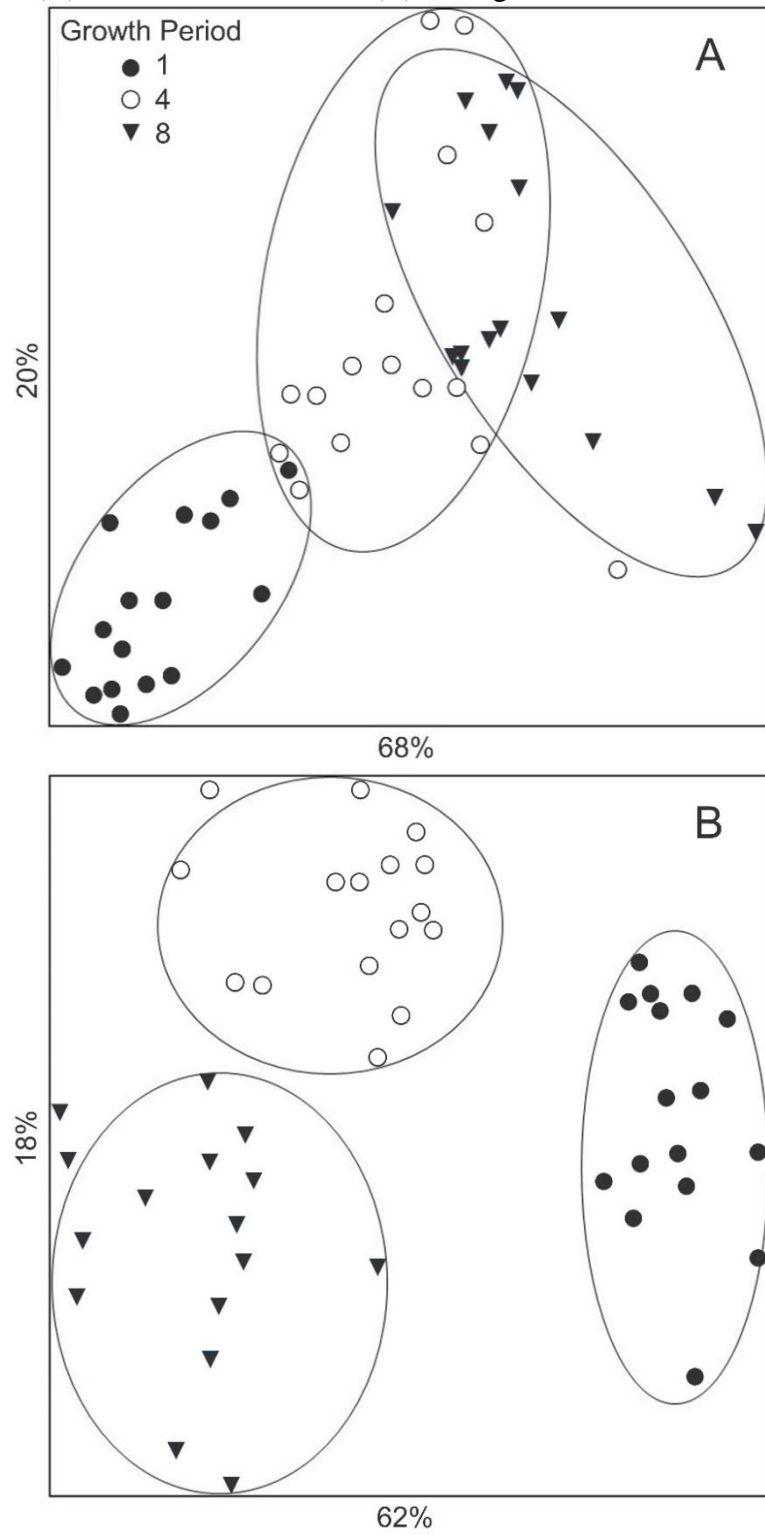
Figure 2.5 Absolute PLFA microbial group ordination of growth periods 1 and 8.
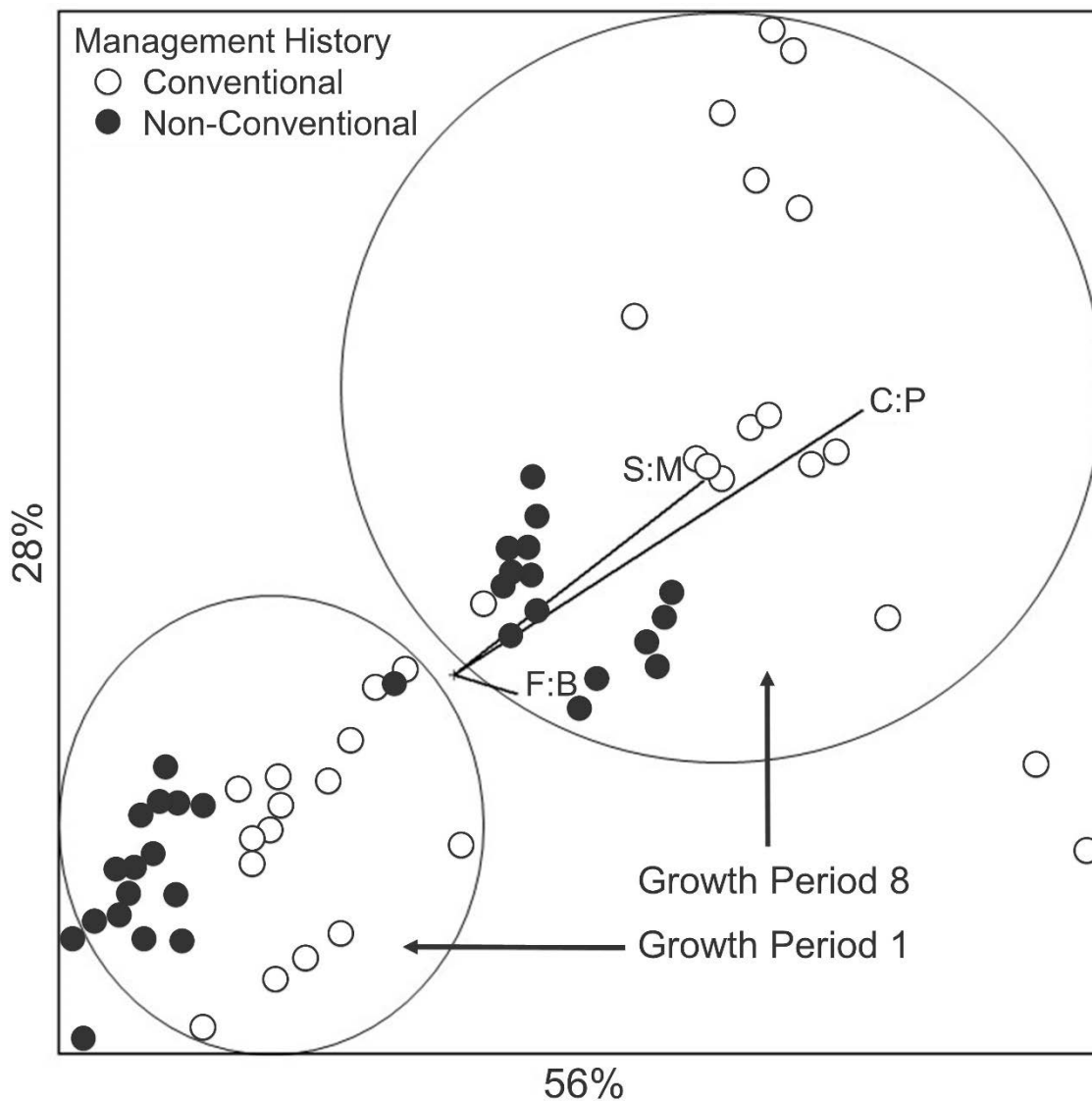
A joint overlay plot shows the S:M, C:P, and F:B ratios, the angle and length of a line indicate the direction and strength of the relationship for a given ratio.

Figure 2.6 DGGE gel showing separation of amplicons from the *Fusarium spp.* for the growth period 8 treatments and pure culture references.
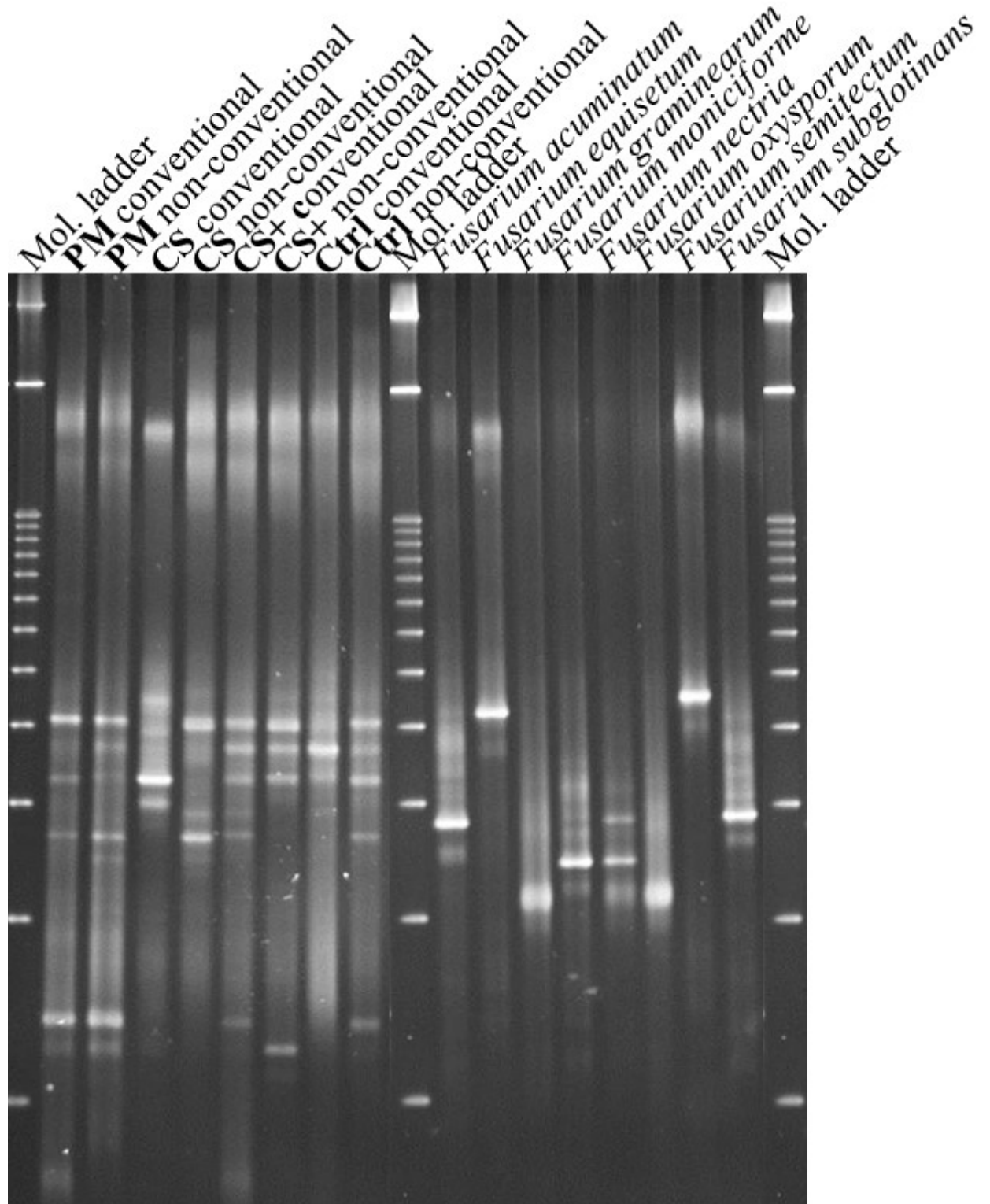
Figure 2.7 DGGE gel showing glyphosate treatment lanes from Fig. 2.6, and their banding pattern analysis.
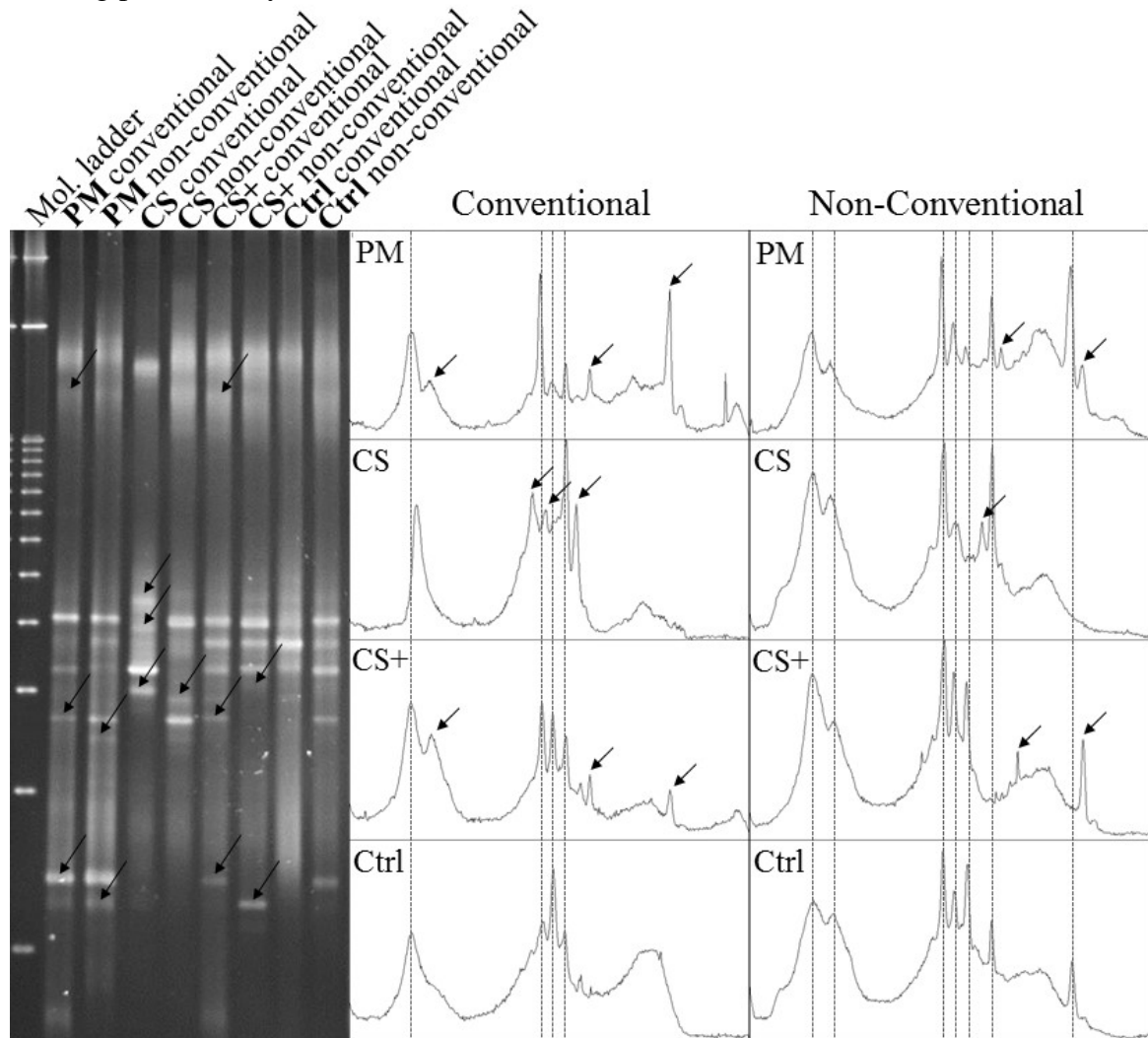
The dotted lines show bands present in the control (Ctrl) treatment and the arrows show unique bands in the glyphosate treatments which do not appear in the control treatment.

Figure 2.8 DGGE gel showing the CS conventional treatment and *Fusarium spp.* reference lanes from Fig. 2.6, and their banding pattern analysis.
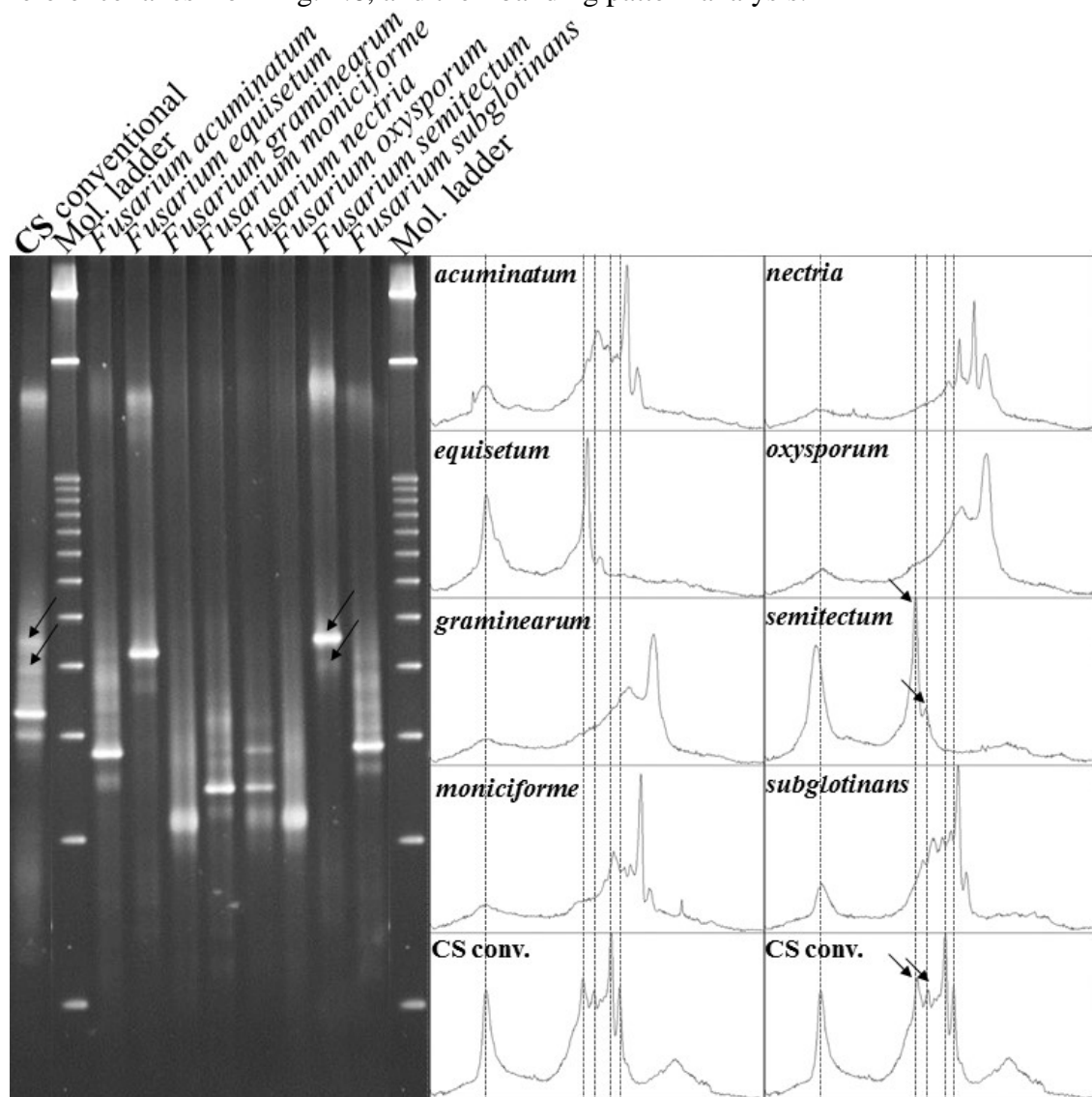
The dotted lines show bands present in the conventional management soil CS treatment, and the arrows show bands which are similar between the conventional management soil CS treatment and the *Fusarium semitectum* reference.

Figure 2.9 qPCR for *Fusarium spp.* specific ITS gene copies for growth period 8.
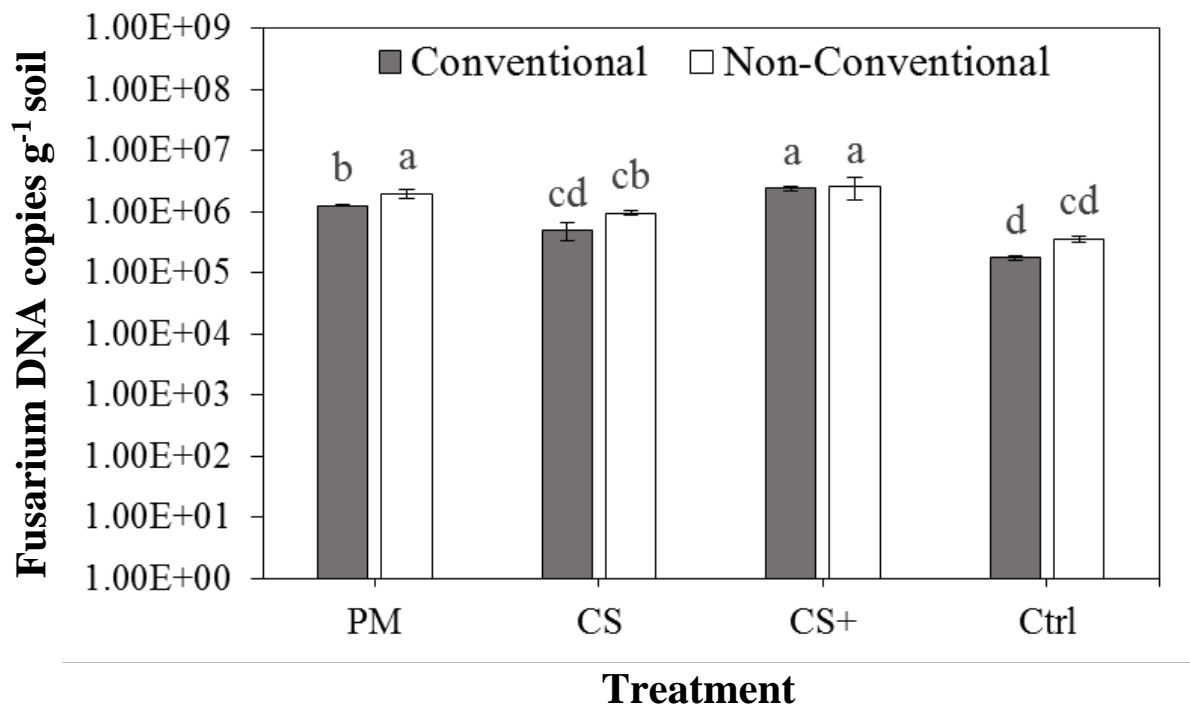
Bars with the same lower case letter are not significantly (p<0.05) different.

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CHAPTER 3. ACCUMULATION OF GLYPHOSATE AND AMPA IN THE RHIZOSPHERE AND BULK SOIL OF A LONG-TERM CORN AND SOYBEAN GREENHOUSE STUDY

ABSTRACT

The environmental accumulation of herbicides is of long-term ecological importance. For the past 40 years glyphosate (GLY) has been the most widely used agricultural herbicide, and is generally considered to have low environmental impacts. Its most common metabolite aminomethylphosphonic acid (AMPA) is found widely in agricultural soils and has nearly twice the half-life of GLY. For both GLY and AMPA little information is available on their rates of accumulation in bulk or rhizosphere soil under long-term GLY tolerant cropping. Also, there is limited information from long-term studies on microbial responses in soils with crops treated with GLY. Therefore, a long-term (8 cropping periods) GLY tolerant cropping greenhouse experiment was conducted, which had a corn-soybean rotation, three GLY treatments, and two soil managements (no GLY vs >10 years GLY). Rhizosphere soil samples were collected at cropping periods 4, 5, and 8, and bulk soil samples were collected before the start of the experiment and at cropping period 8 to be tested for extractable GLY and AMPA. Microbial fatty-acid biomarkers were profiled in both the rhizosphere and bulk soil at cropping period 8. Correlations were made between fatty-acid biomarkers and extractable GLY and AMPA within the rhizosphere and bulk soils. Results suggest that long-term GLY application leads to the accumulation of GLY, and especially AMPA within the bulk soil. They also suggest that greater microbial biomass in the rhizosphere is associated with low levels of GLY and AMPA. Additionally, the abundance of actinobacteria and fungal microbial phospholipid biomarkers increased with GLY application and were significantly correlated in the bulk soil to bulk soil GLY

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concentrations for the management soil that had a previous history of long-term GLY exposure.

INTRODUCTION

Glyphosate (GLY) is a widely used herbicide since the introduction of glyphosate tolerant cropping (GTC) that is an effective and economical weed control system. Because of its widespread use, it is important to determine its environmental mobility and degradation potential and that of its metabolites. The most commonly detected metabolite is aminomethylphosphonic acid (AMPA) (Grandcoin et al., 2017; Barrett and McBride, 2005). Both GLY and AMPA have a high chelation potential and compete most commonly with phosphate for sorption sites on clay minerals and organic matter (Borggaard and Gimsing, 2008).

Despite their high ability to chelate within the soil both GLY and AMPA still have been shown to be mobile in the environment. Runoff studies in agricultural systems have shown that GLY and AMPA bound to eroded soil can leach into shallow groundwater and bind to the underlying soil sediment, this is especially likely if high amounts of competing phosphates are present in the soil and groundwater (Grandcoin et al., 2017). Additionally, the water solubility of GLY and AMPA is about $6.2x10^7$ and $5.0x10^8$ nmol L^{-1} at 20° C, respectively (Mérey et al., 2000). This is higher than other commonly used herbicides such as simazine ($9.9x10^3$ nmol L^{-1}), dicamba ($2.0x10^7$ nmol L^{-1}), and treflan ($5.6x10^2$ nmol L^{-1}) (Shaner, 2014). There is increasing evidence that GLY is reaching the groundwater (Borggaard and Gimsing, 2008; Gomes et al., 2016; Kwiatkowska et al., 2014). Therefore, since microorganisms can degrade GLY and AMPA, it is very important to understand the fate of these compounds in soils and the mechanisms and conditions that control degradation. This information is needed as a

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basis for management systems that promote microbial degradation of GLY and AMPA before they reach our streams and lakes.

The rate at which GLY and AMPA are degraded in agricultural systems directly affects their accumulation and is mostly controlled by two main factors. First, by plant mediated degradation in GTC, in this case degradation occurs shortly after plant absorption. A GLY oxidase gene inserted into these plants degrades GLY inside the plant into its AMPA and glyoxylate metabolites before ever reaching the soil (Alves Corrêa et al., 2016; Van Burggen et al., 2018). The second and most dominant factor is degradation mediated by soil microbial activity, which occurs both in the bulk and rhizosphere soil. The rate of GLY degradation depends on microbial community composition, microbial abundance, soil chemistry, and nutrient availability (Borggaard and Gimsing, 2008; Duke et al., 2011; Mamy et al., 2016; Singh and Singh, 2016; Zobiole et al., 2011). In GTC systems both of these degradation processes occur simultaneously, which further increases the speed at which metabolites such as AMPA are produced and accumulated in the soil system.

The first place to detect GLY and AMPA degradation and accumulation is in the soil rhizosphere, which has been characterized as a zone of intense microbial activity and abundance (Berendsen et al., 2012; Nannipieri et al., 2003). Exudates, sloughing of cells, and root hair turnover by plant roots in this zone promote microbial functions, including degradation of herbicides (Brimecombe et al., 2001). Many herbicides are well known to be degraded in this soil zone by microorganisms (Singh and Singh, 2016). Therefore,

known microbial degraders such as saprophytic fungi would be the most likely group of microorganisms to degrade these foreign chemicals.

The bulk soil outside the influence of roots can degrade herbicides, but likely at lower rates than rhizosphere soil because there is lower levels of carbon substrates and nutrients driving microbial activity (Lareen et al., 2016). Therefore, in the bulk soil it would be expected that a lower microbial abundance and activity would result in increased half-life of GLY and AMPA. Evidence for this is a report by Aparicio et al. (2013) in Argentina, where GLY and AMPA concentrations were measured in agricultural soil basins from 16 farms that used GLY extensively for periods from 4 to 19 years. They found concentrations ranging from 0.2 - 8.9 and 2.7 – 20.3 nmol g^{-1} for GLY and AMPA, respectively. Additionally, an in depth review by Battaglin et al. (2014) found GLY to have a half-life of 2 to 215 days and AMPA of 60 to 240 days in agricultural soils, which correlated to microbial activity.

Concerns of long-term carryover effects are also well founded. A study looking at potato seed plants showed that initial GLY application had minimal effect on the mother crop, but the emergence of daughter crops the following year resulted in erratic and slow emergence, malformed leaves, and multiple and enlarged shoots. (Hutchinson et al., 2014). Furthermore, a greenhouse study by Blackshaw and Harker (2016) in Canada found detectable levels of GLY and AMPA in non-GMO pea (*Pisum sativum*), canola (*Brassica napus*), and wheat (*Triticum monococcum*) crops following GLY application in previous years. The findings revealed that increasing concentrations of GLY and AMPA reduced overall plant biomass by 20% starting at a dose of 473 and 360 nmol g^{-1} soil for

GLY and AMPA, respectively. Although this is much higher concentrations than field rates, the authors noted that they may reflect long-term GLY and AMPA applications with on-going and increased usage.

Unfortunately most studies do not distinguish between GLY and AMPA concentration in the rhizosphere and bulk soil fractions. This is important because of differences in microbial community composition and abundance within the plant rhizosphere and the corresponding bulk soil that could affect degradation of GLY and AMPA. To address this, the first objective of this study was to determine under greenhouse conditions the effects of long-term GTC on accumulation of GLY and AMPA in the corn (*Zea mays*) and soybean (*Glycine max*) rhizosphere and bulk soil of two soils, one that had previously received long-term field applications and another soil of same soil type that had never received GLY. The second objective was to determine the relationship between microbial community composition in the rhizosphere and bulk soil based on fatty-acid microbial biomarkers and accumulation of GLY or AMPA after 8 growth periods of GTC.

MATERIALS AND METHODS

Soils

Effort was made to collect two soils of similar chemical composition and taxonomic classification for this study so that the soils differed significantly only in their GLY application history. For the greenhouse study described below soil was collected from 0-39 cm depth soil pits in 1 cm depth increments from agricultural sites located in Knox and Delaware counties in Ohio. During the collection process the soil was stored in

plastic bags and transported in coolers to the laboratory. Soils were stored at 4° C, and large rocks, roots, and organic matter were removed by sieving (2 mm) prior to placement into the rhizoboxes. Approximately 2500 g of soil was carefully placed into each of the sixteen rhizoboxes in 1cm increments starting with the 38-39th at the bottom to reconstruct the field soil profile. There was approximately 62 g of soil for each 1 cm increment.

The soil management with a history of GLY application (> 10 years) (conventional management), a Bennington silt loam (fine, illitic, mesic Aeric Epiaqualf) was collected from a farm in Knox county practicing a no-till GR corn and soybean rotation. Applications of GLY were made up to three times per season to the soybean rotation and one time per season to the corn rotation. The soil management with no history of GLY application (non-conventional management), a Blount silt loam (fine, illitic, mesic Aeric Epiaqualf) was collected from a farm managed without synthetic (no GLY) and organic inputs in Delaware county that had a diverse crop rotation which for the previous five years was alfalfa-orchard grass, corn, oats-alfalfa-orchard, spelt-timothy-clover, and timothy-clover. Textural analysis (Table 2.1) for the two soil managements were analyzed and found that the conventional management soil had 4 % more clay and 1 % more sand than the non-conventional management soil. Total carbon content differed between the two managements with 1.47 % in the non-conventional management soil and 2.46 % in the conventional management soil, pH was the same at 7.0.

Experimental Design

The experimental design was a completely randomized 2 X 4 factorial design with two soil treatments and four GLY treatments. The soil treatments were a conventional management soil and a non-conventional management soil, both with the same taxonomic soil type. The four GLY treatments were (1) Monsanto Powermax® (PM) (potassium salt carrier); (2) Agrisolutions Cornerstone® (CS) (isopropylamine salt carrier); (3) a mixture of Agrisolutions Cornerstone®, Agro-Plus Grozyme® and AgSpectrum Glycure® (CS+); and (4) a non-treated control. Per manufacturer recommendation the treatments containing Agrisolutions Cornerstone® (CS, CS+) had an 80% non-ionic surfactant added to the mixture from Southern Ag (Rubonia, FL).

GR corn and soybean plants were grown in rhizoboxes and placed in secure wooden holders to maintain an upright position during each of the growth periods. The rhizoboxes (adapted from Bott et al., 2008) measured 400 x 200 x 20 mm and were fitted with a hinged acrylic or Plexiglas side panel for easy access to the root rhizosphere. Each of the two soils had a total of eight rhizoboxes with two replicates per treatment. The plants were grown for eight growth periods over three years, with each growth period being 58 days. A GLY burn down spray was applied on day 1 followed by planting on day 10. Seeds were hydrated on wet paper towels in petri dishes at 23° C prior to sowing to increase plant establishment. Two foliar applications of GLY were administered during growth stages V3 to V5, and V6 to V7 (approximately 30 and 51 days after planting) and applied at the recommended field application rates of 1,013 g ai ha^{-1} for PM, and at 964 g ai ha^{-1} for CS and CS+. A solution of 25 mL Peters® 20 / 20 / 20 Professional fertilizer

was added at a concentration of 3.745 g L^{-1} to each rhizobox 30 and 50 days after planting to maintain plant nutrient status. Rotations of corn and soybeans were made after growth periods 3, 5, and 7. The experiment was conducted under controlled greenhouse conditions that had 12 hours light and dark cycles at 23° C ambient temperature.

Rhizosphere and Bulk Soil Sample Collection

Rhizosphere samples were collected for growth periods 4, 5, and 8 at exactly 1 and 7 days after each GLY application. Rhizosphere sampling was done by opening the face of the rhizobox while slanted to remove approximately 10 g of rhizosphere and intra-root soil. 50 g of bulk soil was collected at two time points, before the initial placement of soil into the rhizoboxes (time zero), and at the conclusion of growth period 8. All samples were stored at -20° C in 50 mL falcon tubes until analysis. Three analytical replicates were collected for each rhizobox. Following each growth period, soil was removed from each rhizobox in sixteen 25 mm depth increments, organic root material was then removed by sieving (2 mm). Each soil increment was placed back into the rhizobox in order.

Laboratory Analyses

Extractable GLY and AMPA was analyzed using a scaled procedure adapted from Miles and Moye (1998). Briefly, 3 g of soil was shaken for 15 min in 12 ml of a 0.1 M extraction solution of monopotassium phosphate. This was repeated 2 more times, and the supernatants were combined and filtered through 0.45 micron filter paper. The solution was analyzed according to the US EPA 547 method using a Waters Alliance 2695 High Performance Liquid Chromatography instrument (Waters Corp., Milford,

MA) controlled with Empower Pro 2005 software. Raw concentrations of AMPA and GLY were reported in ppm and converted nmol g^{-1}.

Microbial fatty acid and functional group abundance was quantified based on the phospholipid fatty acid (PLFA) extraction procedure described by Frostegard et al. (1993). In brief, phospholipids were extracted from approximately 2 g of rhizosphere soil using a single-phase mixture of chloroform, methanol and citrate buffer (Bligh and Dyer, 1959). The upper organic phase of this mixture was decanted and then fractionated into its neutral, glycolipid, and phospholipid components using silica acid columns. Conversion of the phospholipids to fatty acid methyl-esters was then performed by alkaline methanolosis using 0.2 M methanolic KOH (Chowdhury and Dick, 2012). Fatty acids were analyzed on a gas chromatograph (Hewlett-Packard 5890 Series II; Ultra 2 column; carrier gas, helium; temperature ramping 120 to 260° C at a rate of 5° C per minute) with a flame ionization detector and controlled with MISystem software (MIDI Inc. Newark, DE).

A total of 15 fatty-acid biomarkers were consistently identified in each replicate sample. The biomarkers were then summed into five corresponding microbial groups. Actinobacteria (Actino) is represented by the sum of the individual 16:0ME, 17:0ME, and 18:0ME biomarkers, gram-negative (Gram$^-$) bacteria is represented by the sum of 16:1ω7, 17:0c, 19:0c ω8, and 18:1ω7, gram-positive (Gram$^+$) bacteria is represented by the sum of 15:0i, 15:0a, 16:0i, 17:0i, and 17:0a, saprophytic fungi (Fungi) is represented by the sum of 18:2ω6 and 18:1ω9, the arbuscular mycorrizae fungi (AMF) are represented by the single 16:1ω5 biomarker, and all the individual biomarkers were

summed to represent the complete biomass of the microbial community (Total).

Additionally, the stress indicator ratios of the saturated to monounsaturated PLFAs

(S:M), and the cyclopropyl to their monoenoic precursors (C:P) were also used as

indicators of physiological status. The saprophytic fungal to bacterial ratio (F:B) was

calculated as total Fungi PLFAs over the sum of the Actino, Gram⁻, and Gram⁺ PLFAs

(Moore-Kucera and Dick, 2008a). The biomarkers are named using the standard colon

notation, with the total number of carbons in the chain before the colon, followed by the

number of double bonds after the colon. In the case of a double bond, the location of the

bond is identified by an omega (ω) symbol followed by the carbon number as counted

from the omega end (opposite the carboxyl end) of the fatty acid. The structural

confirmation indicators of iso (i), anteiso (a), and cyclo (c) followed the double bond

location. The location of a methyl group on the 10th carbon is indicated by ME. Absolute

abundance concentrations (nmol g⁻¹) were calculated according to Zelles, (1999).

Total carbon and nitrogen content was measured by dry combustion using an

elemental analyzer (Carlo Erba CHN EA 1108, now Thermo Fisher Scientific, Waltham,

MA). Soil pH was measured after 10 min of standing and gentle stirring at a 1:1 mixture

of soil and deionized water (10 g soil:10 mL water) using a glass membrane electrode

(Sparks et al., 1996).

Statistical Analysis

All data was analyzed using SAS (version 9.4) and was tested for normal

distribution (Kolmogorow-Smirnov test). The analytical reps from the first and second

GLY applications were averaged for each rhizobox, as no significant ($p < 0.05$)

differences were detected between sampling days 1 and 7.

Extractable concentrations of GLY and AMPA were normally distributed. Means

separations were made using the Tukey (HSD) test. PLFA data in the bulk and

rhizsosphere soil were not normally distributed and non-transformable to produce a

normally distributed data set. Therefore, the plant, treatment, and soil management

effects were determined using the non-parametric Kruskal-Wallis H test, and all

correlations involving the microbial community data utilized the non-parametric

spearman's rank correlation coefficient. For all PLFA data, there was no effect of plant or

treatment.

<div align="center">

RESULTS

</div>

Extractable Glyphosate and AMPA

Extractable concentrations of GLY and AMPA in the rhizosphere soil often

resulted in no detection or very low levels (data not shown). Some exceptions were in

growth period 4, which had detectable concentrations for only the PM treatment of

$2.4\mathrm{x}10^{-5}$ and $3.1\mathrm{x}10^{-5}$ nmol g^{-1} in the conventional management soil, and $8.4\mathrm{x}10^{-5}$ and

$7.8\mathrm{x}10^{-5}$ nmol g^{-1} in the non-conventional management soil for GLY and AMPA,

respectively. The rest of the exceptions were in growth period 8, with GLY

concentrations of 220, 335, and 387 nmol g^{-1} in the conventional management soil for the

PM, CS and CS+ treatments, respectively. Additionally, in growth period 8 GLY

measurements of 730, and 257 nmol g^{-1} were detected in the non-conventional

management soil for the PM and CS treatments, respectively. There was no detectable

GLY or AMPA for any treatment in growth period 5.

Extractable bulk soil concentrations of GLY and AMPA ranged from 268 to 510

and 1390 to 2170 nmol g^{-1} in the conventional management soil, respectively (Fig. 3.1

A). Extractable bulk soil concentrations of GLY and AMPA ranged from 220 to 406 and

671 to 1674 nmol g^{-1} in the non-conventional management soil, respectively (Fig. 3.1 B).

In the bulk soil at time zero AMPA was detected at 747 nmol g^{-1} in the conventional

management soil, but none was detected in the non-conventional management soil (Table

2.1). For the bulk soil in the growth period 8, AMPA averaged 5.3 times greater

concentration than GLY across all GLY treatments and management soils (Fig. 3.1).

Additionally, for the bulk soil in growth period 8 GLY and AMPA averaged 2.0 and 1.6

times greater concentration in the conventional management soil (Fig. 3.1 A) than the

non-conventional management soil (Fig. 3.1 B) across all GLY treatments, respectively.

The only treatment difference was detected in the conventional management soil for the

CS+ treatment which measured 1.4 and 1.3 times higher concentration of GLY on

average compared to the PM and CS treatments, respectively (Fig. 3.1 A).

Correlations between Bulk Soil Biomarkers and Glyphosate and AMPA

Correlations were performed comparing the individual microbial fatty acid

biomarkers and the summed PLFA microbial groups in the bulk soil against the

extractable GLY and AMPA concentrations in the bulk soil (Table 3.1). In the

conventional management soil, weak but significant ($p < 0.05$) correlations were found

between GLY and the 17:0ME, 18:0ME, 16:1ω7, 15:0a, or 18:1ω9 biomarkers as well as

the Fungi group, but there were no significant ($p < 0.05$) correlations with AMPA for any of these markers. In contrast, the non-conventional management soil showed no correlations for GLY, but did show weak but significant ($p < 0.05$) correlations between AMPA and the 19:0c ω8 or 18:1ω9 biomarkers, respectively. The 18:1ω9 was the only biomarker to show significant ($p < 0.05$) correlations with GLY or AMPA in both soils. Correlations made from this analysis could be an indication that GLY or AMPA is having an associated effect on a specific microbial biomarker or microbial group in the bulk soil.

Correlations between Rhizosphere and Bulk Soil Biomarkers

Correlations were also performed comparing the PLFA biomarkers and the summed microbial PLFA groups of the bulk soil against the same PLFA biomarkers and groups in the rhizosphere soil (Table 3.2). Correlations for the conventional management soil show weak significant ($p < 0.05$) correlations between the bulk and rhizosphere soil for the 18:0ME, 16:1ω7, 15:0a, 18:2ω6 biomarkers or the Fungi group; as well as strong significant ($p < 0.01$) correlations for the 17:0ME, 18:1ω9 biomarkers, or the Actino group. In the non-conventional management soil far fewer and weaker correlations were observed overall, with only the 18:0ME bulk soil biomarker having a weak but significant ($p < 0.05$) correlation with its counterpart in the rhizosphere, no other significant correlation were observed. Correlations made from this analyses may be an indication that there are microbial community associations that can be made between the bulk and rhizosphere soil fractions for a particular soil management.

Bulk and Rhizosphere Soil PLFA

For PLFA analysis, no overall GLY treatment effects were detected within either soil management, therefore GLY treatments were treated as additional replicates and the overall averages are reported for the bulk and rhizosphere soil fractions by microbial group (Fig. 3.2), and by microbial biomarker in the rhizosphere (Table 3.3) and bulk soil (Table 3.4) for each soil management separately. Regardless of soil management, the sum total of all PLFA microbial biomarkers for the bulk and rhizosphere soil fractions ranged from 19.5 to 55.5 nmol g^{-1} and 16.4 to 99.1 nmol g^{-1}, respectively (data not shown). Regardless of soil fraction the sum total of all PLFA microbial biomarkers for the conventional and non-conventional management soils ranged from 16.4 to 84.1 nmol g^{-1} and 29.8 to 99.1 nmol g^{-1}, respectively (data not shown).

The PLFA microbial groups in the conventional management soil ranged from 0.6 to 10.7 nmol g^{-1} and 0.6 to 16.3 nmol g^{-1} for the bulk (Fig. 3.2 C) and rhizosphere (Fig. 3.2 A) soil, respectively. In the non-conventional management soil the PLFA microbial groups ranged from 1.1 to 15.4 nmol g^{-1} and 1.7 to 24.5 nmol g^{-1} for the bulk (Fig. 3.2 D) and rhizosphere (Fig. 3.2 B) fractions, respectively. Overall, the bulk soil fraction had more significant ($p < 0.05$) PLFA differences in the non-conventional management soil than the conventional management soil, compared to the control. However, the opposite was observed for the rhizosphere soil.

PLFA abundance for the conventional management rhizosphere soil had significantly ($p < 0.05$) higher abundance than the control for all biomarkers except 17:0c, 18:2ω6, F:B and C:P, while the 18:0ME, 16:1ω7, 16:0i, 17:0i, 18:1ω9 and Total

were very highly significant ($p < 0.001$) (Table 3.3). Additionally, for the conventional management rhizosphere soil all microbial groups were significantly ($p < 0.05$) higher than the control, while the Actino and Gram$^+$ groups were very highly significant ($p < 0.001$) (Fig. 3.2 A). The non-conventional management rhizosphere soil only had significantly ($p < 0.05$) higher abundance than the control for 16:0ME, 17:0ME, and 19:0c ω8, while the 15:0a biomarker was highly significant ($p < 0.01$) (Table 3.3). Additionally, for the non-conventional management rhizosphere soil only the Actino microbial group was significantly ($p < 0.05$) higher than the control (Fig. 3.2 B).

PLFA abundance in the conventional management bulk soil had significantly ($p < 0.05$) higher abundance than the control for the 18:0ME, 16:1ω7, 15:0a, and 18:1ω9 biomarkers (Table 3.4). Additionally, for the conventional management bulk soil only the Actino microbial group was significantly ($p < 0.05$) higher than the control (Fig. 3.2 C). The non-conventional management bulk soil had significantly ($p < 0.05$) higher abundance than the control for all biomarkers except the 17:0ME, 17:0i, 18:2ω6, while the 19:0c ω8 and 18:1ω9 were highly significant ($p < 0.01$). Additionally, for the non-conventional management bulk soil all five microbial groups had significantly ($p < 0.05$) higher abundance than the control (Fig. 3.2 D).

DISCUSSION

Glyphosate and AMPA Accumulation

At time zero, detectable levels of AMPA were found in the conventional management bulk soil, but not in the non-conventional management bulk soil (Table 2.1). Additionally, in growth period 8 bulk soil AMPA was found at much higher

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concentrations than GLY (> 5X). Assuming extraction efficiency is similar between these two compounds, the results suggest that GLY is more readily degraded and/or less protected than AMPA. Indeed, Simonsen et al. (2008) found that biological stability of AMPA is greater than GLY, probably resulting from greater initial binding to clay minerals.

This agrees with a study by Al-Rajab and Schiavon (2010) which showed that GLY and AMPA recovery in a field soil of similar texture to that used in the current study was 22 % and 77 % at 40 days after GLY application, respectively. This aligns with the current study, given that AMPA was detected at 5.3X higher concentration than GLY in the bulk soil (Fig 3.1) and the longest interval between applications and sampling was approximately 30 days. Additionally, Cheah et al. (1998) showed the half-life of GLY to be around 19 days in a field soil of similar texture when using KH_2PO_4 as an extractant, and AMPA concentrations increased rapidly upon GLY degradation. Thus, indicating that AMPA can quickly accumulate in such soils.

Extractable GLY and AMPA was undetectable or very low in the rhizosphere soil. Detectable levels were found for growth periods 4 and 8 with the PM treatment having the most detections, and GLY being detected more often than AMPA. Compared to the bulk soil, the rhizosphere soil had much lower levels of GLY and higher PLFA concentrations, regardless of soil management. This is likely due to the microbial activity that is expected in the rhizosphere, which promotes microbial properties with exudates and sloughing of cellular debris (Sylvia et al. 2005). This would drive higher rates of GLY and AMPA degradation and increase microbial biomass. This is supported by the

significantly (p < 0.05) higher rhizosphere biomarker PLFA concentrations compared to the control treatment, indicating increased microbial biomass in the presence of GLY application (Table 3.3 and Figs. 3.2 A, B). Even though the non-conventional management rhizosphere soil does not show as many significant differences, every biomarker and group is still higher, compared to the control (Table 3.3). Compared to the bulk soil this level of significance is not seen (Table 3.4, Figs. 3.2 C, D).

Therefore, the greater microbial biomass in the rhizosphere is associated with the low levels of GLY and AMPA. However, the interesting result is the low levels or near absence of GLY in the rhizosphere soils, even in the conventional management soil with its higher bulk soil AMPA levels. This would suggest that previously accumulated GLY or AMPA can be degraded. None-the-less, in the bulk soil or the larger soil volume, AMPA will continue to accumulate with repeated inputs of GLY.

Bulk Soil Glyphosate and PLFA Correlations

The GLY concentrations in the conventional management bulk soil were correlated with the conventional management bulk soil PLFA concentrations. They showed significant (p < 0.05) correlations with the 17:0ME, 18:0ME, 16:1ω7, 15:0a, 18:1ω9, and Fungi biomarkers (Table 3.1). Coincidentally, the conventional management bulk soil PLFA concentrations of these same biomarkers were significantly (p < 0.05) higher, compared to the control, with the exception of 17:0ME (Table 3.4). Furthermore, the Actino and Fungi groups that these biomarkers represent were also higher, though the Fungi was not significant, compared to the control (Fig. 3.2 C). This could be an

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indication that the Fungi and Actino microbial groups are key GLY degraders in the bulk soil fraction.

This is consistent with other literature, which showed that fungal and actinobacteria microorganisms are responsible for the degradation of many organophosphorus herbicides such as GLY (Duke, 2012; Klimek et al., 2001; Obojska et al., 2002). Many fungal species including *Penicillium citrium*, *Penicillium natatum*, *Penicillium chrysogenum*, *Trichoderma viridae*, *Scopulariopsis spand*, *Aspergillus niger*, and *Alternaria alternata* have been documented to degrade GLY; as well as *Arthrobacter atrocyaneus*, an actinobacteria (Singh and Walker, 2006).

In contrast, there were no significant correlations of GLY to any PLFA biomarker in the non-conventional management bulk soil (Table 3.1). However, the non-conventional management bulk soil PLFA concentrations had many biomarkers that were significantly ($p < 0.05$) higher, compared to the control (Table 3.4). Thus, indicating that GLY application does affect the microbial community in the non-conventional management bulk soil, but that no association can be made with GLY.

Bulk Soil AMPA and PLFA Correlations

Extractable AMPA concentrations in the conventional management bulk soil did not significantly ($p < 0.05$) correlate with any conventional management bulk soil PLFA biomarker (Table 3.1). However, for the non-conventional management bulk soil AMPA concentrations there were significant ($p < 0.05$) correlations with the 19:0c ω8, and 18:1ω9 biomarkers (Table 3.1). Additionally, the non-conventional management bulk soil

PLFA concentrations for these two biomarkers were highly significant ($p < 0.01$), where the other biomarkers were not, compared to the control (Table 3.4).

These two biomarkers are found in the Gram⁻ and Fungi microbial groups, which agrees with a review by Duke et al., (2011) who noted that organisms such as the Gram - *Pseudomonas spp.*, and *Rhizobium spp.*, have a C-P lyase enzyme which is necessary for utilizing AMPA as a phosphate source. However, GLY has been found to be toxic to these organisms. For example, Zobiole et al., (2011) demonstrated that the presence of GLY decreases the populations of *Pseudomonas spp* in the rhizosphere. Likewise Kremer et al., (2005) also documented reduced growth of some fungal strains in the presence of GLY. Based on these findings and the results from the current study, it is possible that the higher concentrations of GLY in the conventional management soil (Fig. 3.1) suppress Fungi and Gram⁻ C-P lyase activity. Additionally, the reason why there are significant ($p < 0.05$) correlations of AMPA with the 19:0c ω8 and 18:1ω9 biomarkers in the non-conventional management soil (Table 3.1) is because the toxic threshold for C-P lyase enzyme had not been reached, resulting in continued AMPA degradation.

However, overall extractable bulk soil AMPA poorly correlated with bulk soil PLFA biomarkers in both the conventional and non-conventional management soils (Table 3.1). Assuming that correlations reflect microbial degradation potential, then this indicates that the microbial communities were poorly adapted to degrade AMPA. This agrees with past research that shows AMPA is more resistant to microbial degradation than GLY, and it persists anywhere from 31 to 238 days longer in soils (Battaglin et al., 2014; Grandcoin et al., 2017).

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Bulk and Rhizosphere Microbial Community Correlations

Correlations of PLFAs for the conventional management soil showed a strong relationship between the PLFAs in the bulk soil and the PLFAs in the rhizosphere soil for many of the Actino and Fungi biomarkers. This was especially true for the 17:0ME, 18:0ME, 18:2ω6 and 18:1ω9 biomarkers, and the Actino and Fungi groups (Table 3.2). This is interesting because many of these same biomarkers also correlated well with the GLY concentrations in the conventional management bulk soil (Table 3.1). Likewise it was found that most of these biomarkers are significantly ($p < 0.05$) higher in both the bulk and rhizosphere of the conventional management soil, compared to the control (Tables 3.3, 3.4). The same correlations were not observed in the non-conventional management soil, only the 18:0ME biomarker in the non-conventional management bulk soil correlated significantly ($p < 0.05$) with its counterpart in the rhizosphere soil (Table 3.2). Additionally, for the non-conventional management soil very few significant ($p < 0.05$) correlations were found with GLY and AMPA concentrations (Table 3.1) even though abundance was higher for many biomarkers, compared to the control (Table 3.4).

The significant correlation of these PLFA biomarkers in the rhizosphere to those in the bulk soil, and with extractable GLY in the bulk soil would suggest that the most effective situation for GLY degradation in the bulk soil is when the microbial community is similar between the rhizosphere and bulk soil, for the conventional management soil. This points to a new avenue of research for a unique response of rhizosphere microbial communities to GLY and AMPA. This has not been shown in previous studies because the rhizosphere and bulk soil are not compared in the same study (Al-Rajab and

Schiavon, 2010; Gimsing et al., 2004; Kryuchkova et al., 2014). However, these conclusions are based on PLFA profiling which represent very broad groups and does not detect shifts in sub populations.

CONCLUSION

In conclusion the results of this study suggest that long-term GLY application leads to accumulation of GLY, and especially AMPA in the bulk soil. AMPA was seen to have poor correlation with bulk soil PLFA biomarkers. Indicating that microbial communities are poorly adapted to its degradation in the bulk soil. At the same time GLY had significant correlations to Actino and fungal biomarkers in the conventional management bulk soil. Indicating that greater AMPA accumulation may be a result of GLY being more easily degraded or less protected.

The implications of GLY and AMPA accumulation are potential toxicity to animals (Battaglin et al., 2014; Chan and Mahlar, 1992; Giesy et al., 2000), humans (Acquavella et al., 2004; Arbuckle et al., 2001), increased prevalence in soils (Grandcoin et al., 2017; Aparicio et al., 2013) and increased prevalence in aquatic systems (Battaglin et al. 2014). Therefore, it may be necessary to develop management practices to prevent accumulation in agricultural soils.

In the rhizosphere soil, greater microbial biomass is associated with low levels of GLY and AMPA. This is likely from increased microbial properties resulting from root exudates and the sloughing of cellular debris in the rhizosphere. Additionally, for the conventional management soil significant correlation of the Actino and fungal PLFA biomarkers in the rhizosphere to those PLFA biomarkers in the bulk soil suggest that the most effective situation for GLY degradation in the bulk soil is when the microbial community is similar between the rhizosphere and bulk soil. These findings justify future, more in-depth studies on differential microbial responses between rhizosphere and bulk

soil communities relative to the degradation of GLY or AMPA, and how this affects

long-term accumulation of these compounds.

Table 3.1 Correlations (r) of bulk soil PLFAs against extractable glyphosate and AMPA in the bulk soil.

	Conventional		Non-Conventional	
	GLY	AMPA	GLY	AMPA
16:0ME	0.41^{ns}	0.38^{ns}	-0.09^{ns}	0.32^{ns}
17:0ME	0.50^{*}	0.36^{ns}	-0.21^{ns}	-0.06^{ns}
18:0ME	0.61^{*}	0.45^{ns}	0.22^{ns}	0.40^{ns}
16:1ω7	0.51^{*}	0.40^{ns}	-0.13^{ns}	0.23^{ns}
17:0c	0.30^{ns}	0.24^{ns}	0.02^{ns}	0.44^{ns}
19:0c ω8	0.26^{ns}	0.33^{ns}	0.08^{ns}	0.54^{*}
18:1ω7	0.27^{ns}	0.20^{ns}	-0.13^{ns}	0.34^{ns}
15:0i	0.47^{ns}	0.43^{ns}	0.01^{ns}	0.30^{ns}
15:0a	0.53^{*}	0.45^{ns}	0.16^{ns}	0.43^{ns}
16:0i	0.44^{ns}	0.29^{ns}	-0.05^{ns}	0.21^{ns}
17:0i	0.20^{ns}	0.17^{ns}	-0.16^{ns}	0.10^{ns}
17:0a	0.34^{ns}	0.23^{ns}	-0.03^{ns}	0.27^{ns}
18:2ω6	0.39^{ns}	0.27^{ns}	0.23^{ns}	0.21^{ns}
18:1ω9	0.60^{*}	0.47^{ns}	0.17^{ns}	0.52^{*}
16:1ω5	0.35^{ns}	0.36^{ns}	-0.12^{ns}	0.19^{ns}
Actino	0.49^{ns}	0.42^{ns}	-0.06^{ns}	0.32^{ns}
Gram-	0.47^{ns}	0.37^{ns}	-0.07^{ns}	0.39^{ns}
Gram+	0.46^{ns}	0.37^{ns}	-0.04^{ns}	0.24^{ns}
AMF	0.35^{ns}	0.36^{ns}	-0.12^{ns}	0.19^{ns}
Fungi	0.54^{*}	0.44^{ns}	0.30^{ns}	0.48^{ns}

^{ns}Not significant at $p<0.05$
$^{*}p<0.05$
$^{**}p<0.01$
$^{***}p<0.001$

Table 3.2 Correlations (r) of bulk soil PLFAs against rhizosphere soil PLFAs.

	Conventional	Non-Conventional
16:0ME	0.41^{ns}	0.38^{ns}
17:0ME	0.64**	0.46^{ns}
18:0ME	0.59*	0.54*
16:1ω7	0.61*	0.27^{ns}
17:0c	0.14^{ns}	0.17^{ns}
19:0c ω8	0.18^{ns}	0.46^{ns}
18:1 ω7	0.19^{ns}	0.40^{ns}
15:0i	0.48^{ns}	0.39^{ns}
15:0a	0.55*	0.34^{ns}
16:0i	0.32^{ns}	0.09^{ns}
17:0i	0.20^{ns}	0.09^{ns}
17:0a	0.11^{ns}	0.17^{ns}
18:2ω6	0.59*	0.09^{ns}
18:1ω9	0.66**	0.10^{ns}
16:1ω5	0.30^{ns}	0.40^{ns}
Actino	0.64**	0.41^{ns}
Gram-	0.20^{ns}	0.34^{ns}
Gram+	0.41^{ns}	0.31^{ns}
AMF	0.30^{ns}	0.40^{ns}
Fungi	0.59*	0.06^{ns}

^{ns}Not significant at $p < 0.05$
*$p < 0.05$
**$p < 0.01$
***$p < 0.001$

Table 3.3 Absolute PLFA concentrations in the rhizosphere soil.

	Conventional		Non-Conventional	
	GLY	Control	GLY	Control
		nmol g^{-1} soil		
16:0ME	4.47**	3.61	7.91*	6.84
17:0ME	0.43*	0.15	0.59*	0.25
18:0ME	3.03***	1.67	3.75	3.38
16:1ω7	4.45***	2.18	4.71	4.19
17:0c	2.77	1.93	2.34	2.23
19:0c ω8	5.59**	4.44	5.43*	4.66
18:1ω7	4.77**	2.92	6.59	5.92
15:0i	4.26**	3.00	5.60**	4.73
15:0a	2.98**	2.10	3.56*	3.05
16:0i	3.82***	2.92	3.72	3.55
17:0i	1.82***	1.19	2.17	1.93
17:0a	1.76**	1.13	2.29	2.12
18:2ω6	3.83	2.63	4.95	4.28
18:1ω9	5.57***	3.48	6.86	6.40
16:1ω5	0.84**	0.26	2.52	2.35
Total	50.4***	33.6	62.2	55.9
F:B	0.24	0.22	0.23	0.24
C:P	1.11	1.35	0.70	0.67
S:M	0.96***	1.16	0.72	0.77

*$p < 0.05$
**$p < 0.01$
***$p < 0.001$

The overall average for all glyphosate treatments (GLY) is reported. Significant differences are relative to the control treatment for a given soil management.

Table 3.4 Absolute PLFA concentrations in the bulk soil.

|	Conventional		Non-Conventional	
	GLY	Control	GLY	Control
		nmol g^{-1} soil		
16:0ME	3.08	2.67	5.41*	4.37
17:0ME	0.52	0.44	0.65	0.59
18:0ME	1.50*	1.31	2.23*	1.70
16:1ω7	1.94*	1.62	3.15*	2.52
17:0c	1.56	1.49	1.59*	1.29
19:0c ω8	3.87	3.78	4.49**	3.17
18:1 ω7	2.46	2.40	4.94*	3.92
15:0i	2.30	1.92	3.36*	2.50
15:0a	1.62*	1.29	2.13*	1.56
16:0i	2.18	1.95	2.20*	1.82
17:0i	1.08	1.05	1.34	1.09
17:0a	1.14	1.07	1.47*	1.22
18:2ω6	1.52	1.40	2.74	2.55
18:1ω9	3.26*	2.90	4.83**	3.90
16:1ω5	0.77	0.66	1.60*	1.18
Total	28.8	26.0	42.1*	33.4
F:B	0.20	0.21	0.23	0.25
C:P	1.25	1.31	0.74	0.69
S:M	1.00*	1.08	0.77	0.80

*p<0.05
**p<0.01
***p<0.001

The overall average for all glyphosate treatments (GLY) is reported. Significant differences are relative to the control treatment for a given soil management.

Figure 3.1 Growth period 8 extractable GLY and AMPA for the conventional (A) and non-conventional (B) management bulk soil.
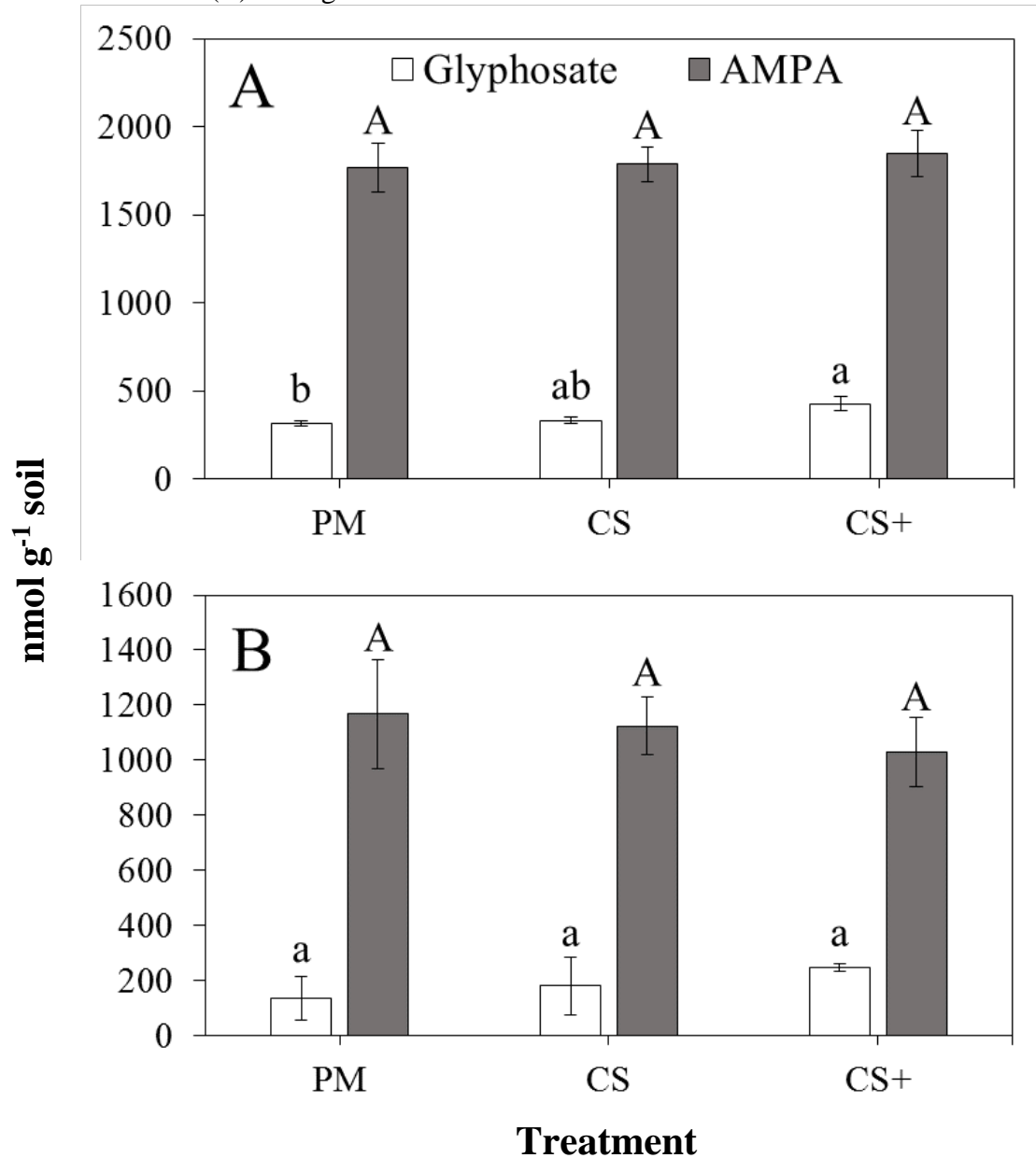
AMPA bars with the same upper case letter are not significantly ($p<0.05$) different, and GLY bars with the same lower case letter are not significantly ($p<0.05$) different. Standard error is shown.

Figure 3.2 Absolute PLFA concentrations for the conventional management rhizosphere soil (A), non-conventional management rhizosphere soil (B), conventional management bulk soil (C), and non-conventional management bulk soil (D).
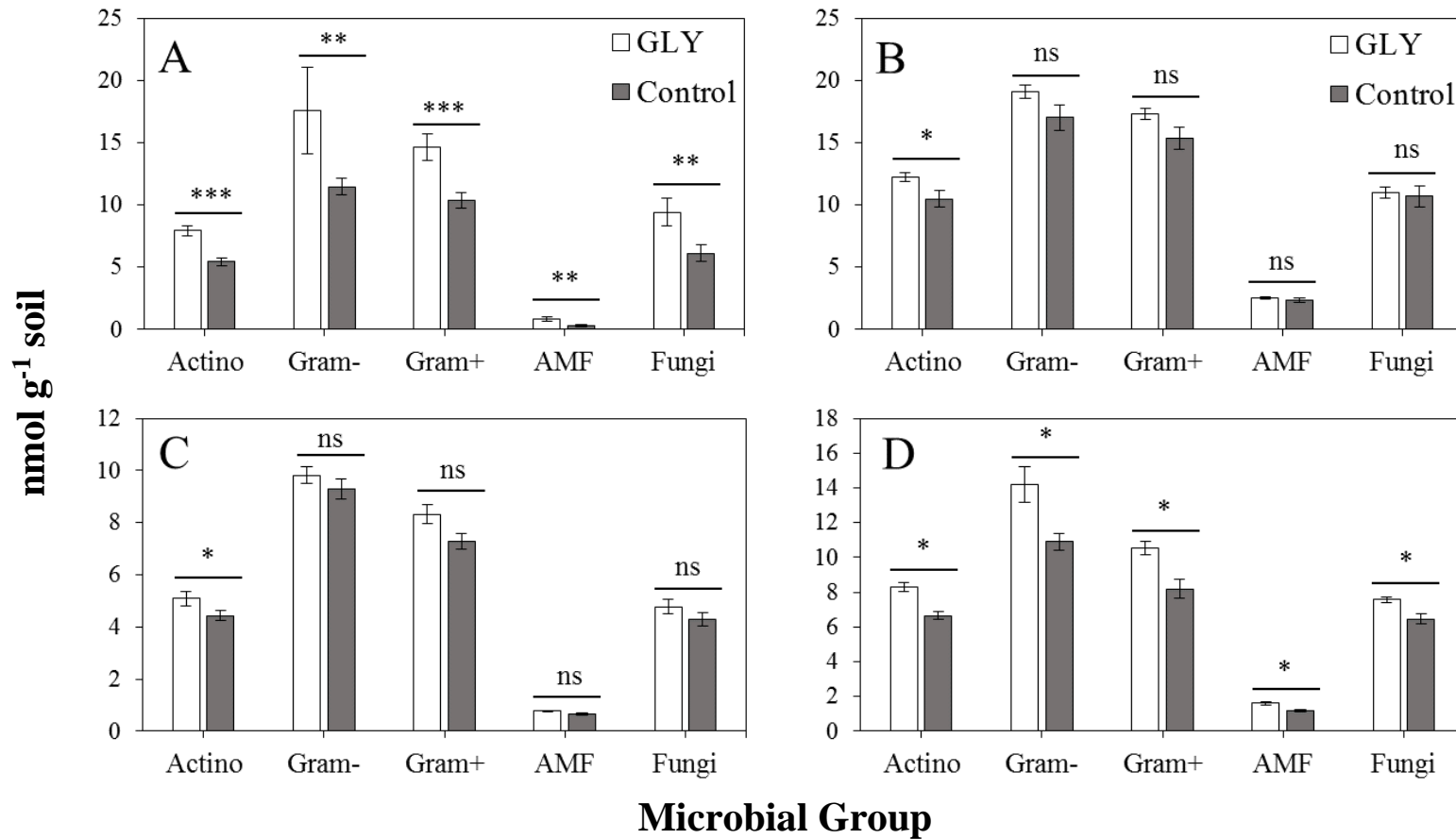
Significant differences are relative to the control, standard error is shown. *p<0.05, **p<0.01, ***p<0.001, [ns]Not significant at p<0.05.

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