Soil Bioavailability of Aminomethylphosphonic Acid:

A Metabolite of Glyphosate

Thesis

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ABSTRACT

Aminomethylphosphonic acid (AMPA) is a microbial degradation product of the herbicide glyphosate, and industrial phosphonates. In addition to possible negative effects on human health, AMPA may inhibit soil microbial growth and alter the soil microbial community composition. Strong soil adsorption causes AMPA to persist in the environment, slowing degradation, and making AMPA a possible long-term environmental contaminant.

Review of the literature as described in Chapter 1 revealed two main knowledge gaps. One was the effects of AMPA in isolation from glyphosate. Most studies apply glyphosate to soil, with AMPA formation and dissipation occurring simultaneously with dissipation of glyphosate, making isolation of effects difficult. The second gap was in the effects of AMPA in different soil types. Although there have been many studies on the behavior and effects of glyphosate in different soil types, there is scarce data that isolates the effects of AMPA.

Therefore, the research objectives were to (1) study the effects of AMPA on soil microorganisms, (2) investigate how soil type affects AMPA bioavailability, (3) determine if chemical extractability can be used to predict AMPA bioavailability, and (4) investigate AMPA in isolation from glyphosate. Based on the literature, the hypotheses were that (1) a higher concentration of AMPA would be found to have a greater effect on

soil microorganisms, and (2) bioavailability would be less in soils with high clay, high iron and aluminum oxides, and low pH.

Chapter 2 describes a 139-day incubation study on three diverse soils with no exposure to glyphosate. These soils included a sandy Granby soil, and two high clay soils with different mineralogy, Blount and Jory. Three field relevant concentrations of AMPA, including the control, were applied directly to soil, and the effects of AMPA on soil microbial respiration and phospholipid fatty acids were analyzed. Chapter 3 describes an investigation of AMPA bioavailability using chemical extraction, and correlations of extractable AMPA with microbial responses.

Total soil carbon and pH appeared to be the most important soil factors affecting response to AMPA. Based on PLFA results, AMPA was the least bioavailable in the sandy Granby soil, which was counter to the hypothesis. The effects of AMPA on PLFA were strong in Blount soil only on day 7 and 21, but effects were seen in Jory soil throughout the incubation. Results showed hormetic effects in metabolic quotient and PLFA abundance responses to AMPA concentration, with the lower concentration showing greater response than the higher concentration. The significance of this finding is that effects of a chemical at a low concentration cannot always be predicted based on test results using a higher concentration, which is frequently done in toxicological studies. There was no clear evidence that AMPA at field relevant concentrations negatively impacted soil health.

AMPA extractability was highest in sandy soil, and lowest in high clay, high iron and aluminum oxide soil, consistent with the hypothesis. Extractability results showed an

interaction effect between soil and AMPA concentration. The higher AMPA concentration was proportionately more extractable in the sandy soil than the lower concentration. Correlations between extractable AMPA and PLFA were weak and mostly negative, consistent with the expected increase in stress markers towards the end of the incubation. Results did not provide evidence that AMPA extractability using monopotassium phosphate reflected AMPA bioavailability.

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CHAPTER 1: AMINOMETHYLPHOSPHONIC ACID: A REVIEW

ABSTRACT

Aminomethylphosphonic acid (AMPA) is an intermediate metabolite that results from the degradation of the herbicide glyphosate. Glyphosate was classified in 2015 by the World Health Organization as a probable carcinogen, and AMPA is also a suspected carcinogen. Previous studies have shown that AMPA is susceptible to adsorption in soils and can accumulate in soils receiving glyphosate. Strong adsorption may reduce AMPA's bioavailability to soil microorganisms, slowing degradation, and making AMPA a possible long-term source of water contamination. However, few studies have focused on the effect of AMPA on the soil microbial community. This document presents a review of the research literature on AMPA with emphasis on its effects on soil microorganisms. Sources of AMPA, prevalence in the environment, processes of adsorption, degradation and leaching are discussed. Finally, effects on the environment and human health risks are presented. Conflicting results in the literature may be due to different soil types and environmental conditions, as well as differences in assumptions, methods, and analysis. Conclusions about the effects of toxins at lower concentrations cannot be based on the effects of toxins measured at higher concentrations, due to the possibility of thresholds, and non-monotonic, nonlinear responses. Also, although the conclusion of most studies is that concentrations of AMPA found in the environment are well below established regulatory limits, some authors have questioned the validity of regulatory studies.

AMPA SOURCES

Aminomethylphosphonic acid (AMPA) is the major metabolite of the herbicide glyphosate, [N-(Phosphonomethyl) glycine) (Rueppel et al., 1977). AMPA is also produced by the degradation of industrial and household phosphonates, used in detergents, water cleaning, flame retardants, insecticides, and many other uses (Studnik et al., 2015). AMPA has been discussed in the literature mostly in conjunction with glyphosate studies, so AMPA will be presented here similarly.

AMPA in the environment, is produced mainly through microbial degradation of glyphosate (Grandcoin et al., 2017), and is not subject to photodegradation (von Mérey et al., 2016). AMPA is typically detected at 13.3% to 50.1% of applied glyphosate (von Mérey et al., 2016). Because the use of glyphosate worldwide has increased over 14-fold since 1974 when Roundup was first sold commercially by Monsanto (Benbrook, 2016), AMPA in the environment has also increased.

In 1995, 28 million lbs of glyphosate were applied annually in the U.S. (Benbrook, 2016). According to Hawkins and Hanson (2019), 280 million lbs of glyphosate are now being applied annually to an average of 298 million acres in the U.S., a 10-fold increase since the introduction of glyphosate resistant crops by Monsanto in 1996. As of 2008, glyphosate made up 50% of the total herbicide usage in the U.S., followed by atrazine at 17%, acetochlor at 8%, metolachlor at 7%, 2,4-D at 4%, and 14% for all others (Fernandez-Cornejo et al., 2014).

Glyphosate is a broad-spectrum, post-emergence, foliar herbicide, and is translocated through the plant (Hawkins and Hanson, 2019). It is relatively low-cost ($3-

$5/acre per application for field crops), with low toxicity to both terrestrial and aquatic non-target organisms according to an ecological risk assessment of Roundup by Giesy et al., (2000). Use of glyphosate on glyphosate resistant crops, enables no-till and conservation tillage which increases soil health by conserving soil moisture, and reducing compaction and soil erosion (Hawkins and Hanson, 2019). Glyphosate is also widely used for non-glyphosate resistant crops for early season burndown, termination of cover crops, and as a harvest aid to ensure uniform crop maturity in cereals (Hawkins and Hanson, 2019). Although 84% of glyphosate is applied to corn, soybean, and cotton in agriculture, glyphosate is also widely used in orchards, vineyards, and vegetable crops, and in non-agricultural settings to control weeds in aquatic systems, pasture, forests, rights-of-way, and by homeowners and landscapers (Hawkins and Hanson, 2019). Glyphosate is also used as a plant growth regulator in sugarcane (Hawkins and Hanson, 2019).

The development of glyphosate resistant weeds has reduced the effectiveness of glyphosate use, particularly for glyphosate resistant crops (Heap and Duke, 2018). Seventeen glyphosate resistant weeds have been identified in the U.S., most of which are impacting glyphosate resistant crop systems (Heap and Duke, 2018). No new glyphosate-resistant crops are under development, and although glyphosate use has not yet decreased, glyphosate use has stabilized upon evidence of increasing glyphosate resistant weed populations (Duke, 2018).

AMPA OCCURRENCE IN THE ENVIRONMENT

According to von Mérey et al. (2016), is not volatile, and due to strong adsorption, significant losses as a result of leaching or runoff are not expected. However, according to Battaglin et al. (2014), AMPA and glyphosate are both detected frequently in soils and sediment, ditches and drains, precipitation, rivers and streams in the United States. AMPA has been detected more frequently and at similar or higher concentrations than glyphosate, especially at the outlets of wastewater treatment plants, in sediment, and large rivers (Grandcoin et al., 2017). In the US, both AMPA and glyphosate are found more frequently in surface waters than in groundwater (Scribner et al., 2007; Battaglin et al., 2014). In Argentina, AMPA and glyphosate have been found more frequently in suspended particulates and stream sediment than in surface waters (Aparicio et al., 2013).

Higher concentrations of AMPA (0.96 mg kg^{-1}) than glyphosate (0.48 mg kg^{-1}) have been found in agricultural soils sampled in the US (Scribner et al., 2007). This is also true in Argentina with 2.26 mg kg^{-1} AMPA found vs. 1.5 mg kg^{-1} of glyphosate found (Aparicio et al., 2013). In Europe, a study of 317 agricultural topsoils found the maximum for both glyphosate and AMPA concentration to be 2 mg kg^{-1} (Silva et al., 2018). Table 1.1 lists maximum concentrations of AMPA found in soils of the US, EU, and Argentina.

Leaching experiments by Okada et al. (2016) in different soil types showed that glyphosate and AMPA tended to be retained in the top 5 cm of the soil profile. The ratio of AMPA to glyphosate tends to increase in the deeper soil horizons (Rampazzo et al., 2013), where it may accumulate due to a reduction in degrading microorganisms

(Sviridov et al., 2015). No-till management results in higher concentrations of AMPA in soil than conventional tillage (Fomsgaard et al., 2003). Both AMPA and glyphosate have been found to be highest under permanent crops, and lowest under dry pulses and fodder crops (Silva et al., 2018). Glyphosate and AMPA are both found in plant debris, with approximately 2.6% of the initially applied glyphosate extractable mostly in the form of AMPA (Mamy et al., 2016).

GLYPHOSATE APPLICATION AND AMPA FORMATION

According to Hawkins and Hanson (2019), the average single application rate of glyphosate for field crops, such as corn, soybeans, cotton, and sugar beets in the U.S. ranges from 0.8 kg to 1.1 kg hectare^{-1}, with an average of 1 application per year.

Assuming this concentration falls on bare soil in a no-till system, with an interaction depth of 5 cm (Okada et al., 2016), and soil bulk density of 1.5 g cm^{-3} (von Mérey et al., 2016), this would result in a maximum concentration of 8.8 nmol g^{-1} glyphosate in the top 5 cm of soil. Since AMPA typically represents 13.3 to 50.1% of applied glyphosate (von Mérey et al., 2016), multiplying glyphosate by those percentages would result in a range of 1.2 to 4.4 nmol g^{-1} concentration of AMPA in the top 5 cm of soil.

The maximum allowed single and annual application rate for glyphosate in the U.S. is 9 kg hectare^{-1} for pasture, forestry, and non-food tree crops (U.S. Environmental Protection Agency [USEPA], 2020). Assuming the same interaction depth and soil bulk density, this would result in a maximum concentration of 71 nmol g^{-1} of applied

glyphosate, with an AMPA concentration range of 9.4 to 35 nmol g^{-1} AMPA in the top 5 cm of soil.

Due to persistence, AMPA can accumulate in the soil. The European Food Safety Authority [EFSA] (2015) calculated an AMPA concentration of 2 ug g^{-1} (18 nmol g^{-1}) after a maximum EU one time application of 4.3 kg ha^{-1}, tillage depth of 5 cm, bulk density of 1.5 g cm^{-3}, and AMPA formation rate of 53%. A predicted worst-case accumulation of AMPA after 10 years at the maximum rate, assuming a half-life of 633 days, would be 6.2 ug g^{-1} (56 nmol g^{-1}),

ADSORPTION

As phosphonates, both glyphosate and AMPA adsorb strongly to soil. According to Borggaard and Gimsing (2008), glyphosate adsorbs to variable-charge surfaces, mainly onto aluminum and iron oxides, allophane/imogolite, and organic carbon. Anion exchange capacity and positively charged surfaces on humus, iron and aluminum oxides and some silicate clays increase with a decrease in pH (Weil and Brady, 2017). Glyphosate and AMPA are anions, and according to Barja and Afonso (2005), the adsorption mechanisms of glyphosate and AMPA are similar. Both glyphosate and AMPA form monodentate or bidentate inner-sphere complexes with iron oxide through the phosphonate moiety, leaving the carboxylate and amino group non-coordinated to the iron oxide surface (Barja and Afonso, 2005).

Adsorption lessens mobility and bioavailability to degrading microorganisms (Al-Rajab and Schiavon, 2010). Bioavailability rather than total amount of a chemical in the soil determines how much effect a chemical can have on soil biota (Kelsey et al., 1997).

AMPA can also accumulate in soil because its generation from glyphosate is faster than its degradation (Simonsen et al., 2008; Okada et al., 2016). According to laboratory studies conducted by Sidoli et al. (2016), the main factors affecting adsorption of glyphosate and AMPA to soil are soil pH, phosphorus content, and concentration of amorphous iron and aluminum oxides, in that order. Zhang et al. (2015) also found pH to be the most important factor in degradation in a study of citrus orchard soil however Okada et al. (2016) found the adsorption of glyphosate increased with higher soil clay content and cation exchange capacity (CEC) and decreased with higher pH and phosphorus. After initial adsorption occurs, glyphosate and AMPA can age and become increasingly inaccessible over time by partitioning into organic matter or diffused into soil micropores (Kelsey et al., 1997). According to Alexander (2000), aging refers to the phenomenon in which bioavailability in relation to the total amount of chemical remaining in soil is reduced over time. In the process of aging, glyphosate and AMPA become inaccessible by forming covalent bonds with soil constituents and/or becoming physically sequestered into soil nanopores (Kelsey et al., 1997; Alexander, 2000).

Competition for sorption sites between phosphate and glyphosate is highly dependent on soil type, (Laitinen et al., 2009) and addition of phosphate significantly decreases adsorption in the soil (Simonsen et al., 2008; Kanissery et al., 2015).

DEGRADATION

As phosphonates, AMPA and glyphosate both have a stable C-P bond that is resistant to degradation (Grandcoin et al., 2017; McGrath et al., 2013). The main pathways of glyphosate degradation to AMPA and sarcosine are illustrated in Figure 1.1. AMPA ultimately degrades to inorganic phosphate, ammonium, and CO_2 which can increase the total phosphorus in aquatic systems (Borggaard and Gimsing, 2008). AMPA degrades most readily under oxic conditions (Grandcoin et al., 2017)

According to Rueppel et al. (1977), degradation to AMPA is the most prevalent pathway of glyphosate degradation. Most of the literature identifying microorganisms that degrade glyphosate and AMPA have determined that these microorganisms use glyphosate and/or AMPA as a phosphorus source (Zhan et al., 2018). Gram-positive bacteria *Arthrobacter atrocyaneus* ATCC 13752 are capable of degrading glyphosate to AMPA, then utilizing AMPA as the sole phosphorus source and converting the carbon in AMPA to CO_2 (Pipke and Amrhein, 1988). Gram-negative *Flavobacterium* sp. GD1 is able to degrade glyphosate to AMPA in the presence of inorganic phosphate, but is only able to mineralize AMPA to phosphate in the absence of inorganic phosphate (Balthazor and Hallas, 1986). *Agrobacterium radiobacter* SW9 is able to use glyphosate as the sole source of carbon, and to mineralize small amounts of AMPA even in the presence of phosphate (McAuliffe et al., 1990). *Pseudomonas sp*. LBr degrades glyphosate to AMPA but is only able to degrade small amounts of AMPA for phosphorus, excreting the rest (Jacob et al., 1988). Additional bacteria identified as able to degrade both glyphosate and AMPA as the sole source of phosphorus include *Bacillus megaterium* 2BLW and

Pseudomonas sp. 4ASW (Quinn et al., 1989). *E. coli* utilizes AMPA as the sole phosphorus source (Cordeiro et al., 1986). According to (Krzyśko-Łupicka and Orlik, 1997), fungal species *Mucor III* and *Penicillium II R* grow well using glyphosate as the sole phosphorus source, and AMPA is the main metabolite.

In studies of both glyphosate treated and untreated soils, microorganisms able to degrade AMPA were found more frequently than those able to degrade glyphosate (Dick and Quinn, 1995). However, microorganisms able to degrade glyphosate occur more abundantly in soils previously treated with glyphosate (Dick and Quinn, 1995).

The half-life of AMPA can be highly variable based on soil properties and environmental factors (Bai and Ogbourne, 2016; Nguyen et al., 2018). The soil half-life used by the European Food Safety Authority (EFSA) for calculating worst case AMPA accumulation was 633 days (EFSA, 2015). In field studies that included 8 U.S. sites and 3 Canadian sites, AMPA was estimated to have a soil half-life range of 76 to 240 days, with a median of 145 days (Oppenhuizen, 1993; Oppenhuizen and Goure, 1993; Gustafson and Bleeke, 2000), as cited by Giesy et al. (2000). AMPA half-life was found to be much shorter by Zhang et. al. (2015). Zhang found AMPA half-life ranging between 10 and 37 days in a field study conducted in three provinces of China (Table 1.2). In laboratory studies, AMPA soil half-life has been found to range from 25 to 98 days (Table 1.2). The aquatic half-life of AMPA has been reported to range from 7-14 days by Homer and Kunstman (1988) as cited by Giesy et al. (2000), similar to glyphosate.

Table 1.2 shows the soil half-life of AMPA compared to the half-life of glyphosate from a variety of studies conducted in the field and laboratory. Bergström et al. (2011) found that degradation of AMPA can be more rapid than glyphosate in lysimeter studies, and that more leaching of AMPA can occur from a clay soil than from a sandy soil due to colloid and particle facilitated transport. Conversely, Grunewald et al. (2001) found that AMPA degraded more slowly than glyphosate in field soils. Bento et al. (2016) found that cold, dry conditions slowed degradation, and that AMPA was more persistent than glyphosate.

LEACHING

Due to its polar nature, glyphosate is strongly sorbed to soil minerals, reducing probability of leaching (Borggaard and Gimsing, 2008), despite its high water solubility (Battaglin et al., 2014). Soil structure and rainfall are more important factors in leaching of glyphosate than adsorption and degradation (Borggaard and Gimsing, 2008). Although adsorption in sandy soils is low, field studies have shown minimal leaching in unstructured, uniform, sandy soils that lack macropores. However, leaching can be severe in uniform, but coarse-textured or gravelly soils, such as under railway embankments, where glyphosate is applied at high rates for weed control (Borggaard and Gimsing, 2008). A shallow groundwater table is at more risk of glyphosate or AMPA contamination than deeper groundwater (Borggaard and Gimsing, 2008; Van Stempvoort et al., 2014).

Degradation of glyphosate is most rapid in the soil with the lowest adsorption capacity, which reduces pollution of water by glyphosate, but increases pollution of water by AMPA. Conversely, when adsorption is high and degrading capabilities low, the authors hypothesize that there is greater risk of contamination of ground-water, due to slow remobilization of these residues (Al-Rajab and Schiavon, 2010).

Differences in tillage practice such as no-till vs. conventional, appear to have no effect on leaching (Fomsgaard et al., 2003; Okada et al., 2016). However, Simonsen et al. (2008) concluded that fertilizing soil with phosphate increases the risk of glyphosate leaching.

Transport to surface waters

Timing of rainfall after glyphosate application, and rainfall intensity are significant factors in transport of glyphosate and AMPA (Grandcoin et al., 2017). Poor soil structure, such as compaction and crusting, at the time of rainfall can significantly increase surface runoff of applied glyphosate (Todorovic et al., 2014). Particle facilitated transport is an important mechanism in the transport of glyphosate and AMPA to surface waters (Aparicio et al., 2013).

Transport in atmosphere

In the atmosphere, higher concentrations of glyphosate have been found than AMPA, since AMPA is a metabolite and can only enter the atmosphere through wind erosion, whereas glyphosate can enter the atmosphere through spray drift (Chang et al., 2011). Long range transport of glyphosate and AMPA through the atmosphere has not been studied (Grandcoin et al., 2017).

ENVIRONMENTAL EFFECTS OF AMPA

Effects on Water Quality

Some bacteria and algae can use phosphonates as a phosphorus source, so the leaching of AMPA into surface waters may contribute to eutrophication (Studnik et al., 2015). AMPA ultimately degrades to inorganic phosphate, ammonium, and CO2, which can result in increased phosphorus load on waterways (Borggaard and Gimsing, 2008). Groundwater contaminated with levels of glyphosate or AMPA higher than the US drinking water MCL for glyphosate of 700 ug L^{-1} (4.14 x 10^3 nmol L^{-1} glyphosate, 6.31 x 10^3 nmol L^{-1} AMPA) have not been reported in the literature. However, a few samples have been reported to exceed the European threshold of 0.1 ug L^{-1} (0.6 nmol L^{-1} glyphosate, 0.9 nmol L^{-1} AMPA) (Borggaard and Gimsing, 2008).

Glyphosate Effects on Crop Health

The herbicidal effects of glyphosate are due to the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme from the shikimate pathway, which leads to prevention of the biosynthesis of the amino acids phenylalanine, tyrosine, and tryptophan (Gomes et al., 2014). Duke et al. (2012) concluded that there was no appreciable impact of glyphosate on plant health due to mineral deficiencies or decreased resistance to pathogens. In fact, ultra-low doses of glyphosate can stimulate plant growth in a phenomenon known as hormesis, and to be effective as a fungicide (Duke, 2018).

However, other studies have concluded that glyphosate could be impairing disease defenses of glyphosate resistant crops, increasing populations of soil and plant microbial pathogens, and reducing uptake of nutrients by crops (Kremer et al., 2005; Zobiole et al., 2011; Martinez et al., 2018). Depending on availability of organic carbon or phosphorus in soil, glyphosate can change the fungal population to favor strains that can use glyphosate as the sole source of phosphorus or carbon (Krzyśko-Łupicka and Orlik, 1997). Another negative impact has been the evolution of glyphosate resistant weeds (Duke, 2018).

AMPA Effects on Crop Health

Simonsen et al. (2008) concluded that AMPA soil residues can be taken up by plants, but pose no risk to crop yield. However, AMPA has been shown to be phytotoxic and high levels have been found in glyphosate resistant (GR) crops (Bohm et al., 2008; Duke, 2011). AMPA's exact mode of toxic action is unknown (Gomes et al., 2014). According to Reddy et al. (2008), the injuries to glyphosate resistant plants are due to AMPA formed from glyphosate degradation. AMPA affects chlorophyll biosynthesis and reduces plant growth (Reddy et al., 2004; Gomes et al., 2014). AMPA has been found to impair the processes of DNA reparation and mRNA synthesis in plants (Sviridov et al., 2015).

AMPA Effects on Aquatic Life

Levine et al. (2015) found no observed adverse effect on fathead minnow, *Pimephales promelas,* and *Daphnia magna*, in fish early-life cycle and fish full-life cycle studies, using AMPA concentrations at least 100 times higher than realistic environmental concentrations. They concluded that there was little or no threat to aquatic life from AMPA at realistic exposures.

However, Guilherme et al. (2014) concluded AMPA presents a genotoxic hazard to fish. They found chromosomal and DNA damage to the European eel, *Anguilla anguilla* L., in comet and erythrocytic nuclear abnormalities (ENA) assays, respectively, as a result of short-term exposure and realistic concentrations of AMPA found in aquatic environments. De Brito Rodriguez et al. (2019) also found genotoxic effects to zebrafish in the comet assay from AMPA, and glyphosate with LOEC of 1.7 mg L^{-1}, and LOEC of 0.4 mg L^{-1} for POEA, a surfactant in glyphosate-based herbicides.

Glyphosate and AMPA Effects on Soil Microorganisms

Few studies have investigated the specific effects of AMPA on soil microorganisms, but since AMPA is chemically similar to glyphosate, it might be expected to have similar effects on soil microorganisms.

A number of studies have found no evidence that glyphosate affects soil microbial activity, biomass, diversity, or community composition at recommended field applications rates (Busse et al., 2001; Duke et al., 2012; Zabaloy et al., 2016). Haney et

al. (2000) and von Mérey et al. (2016) concluded that glyphosate should have no adverse effect on soil microorganisms even at excessive rates.

However, a number of other studies have found that glyphosate causes an increase in fungi (Wardle and Parkinson, 1992; Krzyśko-Łupicka and Orlik, 1997), and an increase in fungal soil pathogens (Kremer et al., 2005), as well as a decrease in beneficial soil bacteria to plants (Zobiole et al., 2011; Newman et al., 2016). Gomez et al. (2009) and Schnurer et al. (2006) found glyphosate negatively affected soil microbial biomass, growth, respiration, and metabolic activity.

Von Mérey et al. (2016) tested the effects of glyphosate and AMPA on soil nitrogen transformation processes at concentrations over five times above predicted worst case concentrations, and found that soil nitrogen transformation was temporarily stimulated at the highest rates, but unaffected at lower rates that were still well above field relevant concentrations. They concluded that soil microorganisms were unlikely to be adversely affected by glyphosate or AMPA at field relevant concentrations.

Von Mérey et al. (2016) also studied the effects of AMPA on earthworms in artificial soil, using AMPA concentrations much higher than would normally be found in the field. They found no significant effects on earthworm mortality, biomass, or reproduction, and concluded there should be no negative impact at field relevant concentrations. However, in a similar earthworm test in artificial soil, but at field relevant AMPA concentrations (Domínguez et al., 2016) found significantly reduced juvenile earthworm biomass.

TOXICITY

Glyphosate is considered relatively non-toxic to humans and other mammals because the metabolic pathway causing herbicide effects only exists in plants and some microorganisms (Bai and Ogbourne, 2016). Compared to glyphosate, AMPA is considered less toxic or no more toxic (Giesy et al., 2000). Glyphosate-based herbicides such as Roundup are more toxic than glyphosate due to the surfactants used to increase penetration and coverage of the herbicide (Battaglin et al., 2014).

Mixtures of herbicides can be more toxic than herbicides used alone. AMPA and glyphosate are usually found in mixtures with other pesticides and the ecological toxicity of mixtures is largely unknown (Battaglin et al., 2014). In a study of herbicide mixtures, Roustan et al. (2014) found that AMPA was the most active single compound of four herbicides including glyphosate, atrazine, and their respective metabolites, AMPA and desethyl-atrazine (DEA). They found that cytogenetic toxicity of the four herbicides in a mixture, was 20-fold higher than the most active single compound, AMPA, and was 100-fold increased after light-irradiation. The authors suggest that oxidative stress is the probable cause of toxicity.

In a review of epidemiology studies of glyphosate and cancer, Mink et al. (2012) found no evidence of a causal relationship between glyphosate exposure and cancer in humans. Since then, several comprehensive reviews of glyphosate risks to human health have been conducted in the past few years by the European Food Safety Authority (EFSA), the US Environmental Protection Agency (USEPA), and the International Agency for Research on Cancer (IARC).

The IARC (2017) concluded that glyphosate should be classified as a Group 2A carcinogen, and "probably carcinogenic to humans." The review covered studies on exposure data, epidemiological studies of cancer in humans, in vitro studies on human cells, in vitro and in vivo studies on mice and rats. Several large case-controlled epidemiology studies from the US and Canada, showed a positive association between non-Hodgkin lymphoma and glyphosate exposure. They also found sufficient evidence in experimental animals for glyphosate carcinogenicity. Human in vitro cell studies indicated strong evidence that glyphosate, and glyphosate-based herbicides (GBH) cause genotoxicity. The evidence that AMPA causes genotoxicity was moderate. Strong evidence exists that glyphosate, GBH, and AMPA can induce oxidative stress (IARC, 2017).

The EFSA (2015) report states that AMPA presents a similar toxicological profile to glyphosate. They concluded that the active substance glyphosate, and its degradation products (including AMPA) were unlikely to pose a carcinogenic hazard to humans and the evidence did not support classification as a carcinogen. However, the EFSA (2015) report stated three areas of concern. (1) Glyphosate could not be ruled out as an endocrine disruptor, and there is a need for further testing. (2) Risk to wild non-target invertebrates could not be ruled out. (3) Eight out of 24 registrants submitted technical specifications with data gaps regarding impurities.

The USEPA (2016) study evaluated 23 epidemiological studies, 15 animal carcinogenicity studies, and nearly 90 genotoxicity studies for the active ingredient glyphosate. The conclusion was that available data did not support classifying

glyphosate as a carcinogen, and specifically an association between glyphosate and non-Hodgkin lymphoma could not be determined.

Benbrook (2019) compared the IARC and USEPA studies and determined three main reasons for the difference in conclusions. (1) Whereas the IARC relied mostly on peer-reviewed studies, 70% of which were positive, the USEPA relied mostly on registrant-commissioned, unpublished regulatory studies, 99% of which were negative. (2) The USEPA studies were based on technical glyphosate, whereas the IARC studies included glyphosate-based herbicides and AMPA. (3) The USEPA studies focused on general population exposures, whereas the IARC also considered occupational exposure, and other elevated exposure scenarios.

Regulatory agencies determine maximum concentration limit (MCL) of glyphosate in drinking water and acceptable daily intake (ADI) based on risks associated with glyphosate and AMPA exposure. In the US, there is no MCL for AMPA, but the MCL for glyphosate is 700 ug L^{-1} (6.31 x 10^3 nmol L^{-1}), and the reference dose (RfD) or dietary ADI is 1 mg kg^{-1} of body weight (equivalent to 5.9 nmol g^{-1} body weight). AMPA is generally considered to be no more toxic than glyphosate (Giesy et. al., 2000). To put environmental exposure to AMPA into perspective, a 60 kg adult drinking 2L of water per day containing the U.S. MCL of glyphosate would consume 1.4 x 10^3 ug (8.28 x 10^3 nmol) of glyphosate, well within their ADI of 6.0 x 10^4 ug (3.55 x 10^5 nmol) per day of glyphosate. In addition, in the largest and most comprehensive study of environmental occurrence of glyphosate in the U.S. conducted by Battaglin et. al. (2014), the maximum glyphosate found was less than 0.48 ug g^{-1} in soil or 0.43 ug mL^{-1} in any

water body sampled. In the US, in residential/non-occupational settings, children 1-2 years old are the subpopulation with the highest exposures to glyphosate, due to consumption of dust and soil through hand to mouth exposure, in addition to dietary intake. The high-end estimated exposure of children 1-2 years old is 0.47 mg kg^{-1} day^{-1} (USEPA, 2016), which is within the ADI.

The MCL levels and ADI vary significantly between different countries. In Europe the MCL is 0.1 ug L^{-1} for any individual contaminant, and 0.5 ug L^{-1} total MCL for all contaminants (Grandcoin et al., 2017). The MCL for glyphosate is 280 ug L^{--1} in Canada (Leyva-Soto et al., 2018), and 1000 ug L^{-1} in Australia (Bai and Ogbourne, 2016). The current ADI in Germany is 0.3 mg kg^{-1} day^{-1} but it has been proposed to increase it to 0.5 mg kg^{-1} day^{-1} (Mesnage et al., 2015).

Toxicology studies in the IARC (2017) report on AMPA include Mañas et al. (2009), who found chromosomal damage in human liver Hep-2 cells, DNA damage in human lymphocytes, and chromosomal damage in laboratory mice. Roustan et al. (2014) found chromosomal damage from AMPA in hamster ovary cells was increased with light irradiation. In an additional study on laboratory mice, Manas et al. (2013) confirmed DNA damage to liver and blood cells was induced after 14 days consumption of 100mg $kg^{-1}day^{-1}$ (901.0 nmol g^{-1} day^{-1} AMPA) via drinking water. Toxicology studies included in the IARC (2017) report are listed in Table 1.3.

In a subsequent epidemiology study of a population living next to agricultural fields in Sonora, Mexico., Leyva-Soto et al. (2018) found significantly higher exposure to AMPA than glyphosate from well and drainage water. They found a significant

correlation between self-reported consumption of contaminated water, and incidence of diabetes and hypertension. They also concluded that agricultural workers and brick makers were at potential health risk from exposure to AMPA.

Subsequent toxicology studies include Kwiatkowska et al. (2017) who found AMPA exposure at 5mM ($5x10^6$ nmol mL^{-1}) for 24h, decreased cell viability and ATP level in human peripheral blood mononuclear cells (PBMCs), but was less toxic that glyphosate. Woźniak et al. (2018) found DNA damage from 500 μM ($5x10^5$ nmol mL^{-1}) for 24h, from AMPA, glyphosate, and Roundup 360, with AMPA being least toxic and Roundup 360 being most toxic. Martinez and Ahmad (2019) concluded that AMPA could breach the blood brain barrier and cause neurological damage at high concentrations (100 μM or 1 x 10^5 nmol mL^{-1}), from a study on brain microvascular endothelial cells. Table 1.4 lists toxicology studies published after the IARC (2017) report.

DISCUSSION AND CONCLUSIONS

Based on most studies conducted, realistic environmental exposures to glyphosate and AMPA are well within acceptable regulatory limits established for glyphosate, so AMPA should not pose a health risk to humans. However, Mesnage et al. (2015) have questioned the way regulatory agencies establish regulatory limits, and Benbrook (2019) has questioned the way regulatory agencies have conducted risk assessment. Conflicting results found in different studies on glyphosate and AMPA effects may be due to several

reasons, including false assumptions, false conclusions, lack of methods for precise measurement, errors in method, and differences in materials and methods.

An example of an assumption that may be false is that AMPA is the primary metabolite of glyphosate (Borggaard and Gimsing, 2008; Bergström et al., 2011), which has been based on conclusions from studies which find mostly AMPA from glyphosate degradation (Rueppel et al., 1977), not taking into account that sarcosine may have degraded so rapidly it can't be measured.

Effects of glyphosate on organisms and environment are confounded with the effects of its metabolites, AMPA and sarcosine, because it is difficult to study them separately, and precisely measure their effects separately. Degradation of glyphosate to AMPA vs sarcosine can be dependent on the soil microorganisms present, or other soil conditions. Differences due to glyphosate degradation taking varying pathways may explain some of the difference in study results.

Results of studies can vary widely based on the materials (soils) used, and experimental conditions (Bento et al., 2016; Nguyen et al., 2018).

The assumption that testing effects using excessive concentrations of glyphosate or AMPA, covers testing at field relevant concentrations may not be valid, as shown by the striking difference in conclusions drawn in the earthworm studies by von Mérey et al. (2016) which found no effect at excessive concentrations vs. Domínguez et al. (2016) which found significant effects at field concentrations. Similarly, in effects on aquatic life, Levine et al. (2015) found no significant effects using high concentrations of AMPA, whereas Guilherme et al. (2014) found significant effects at field concentrations. This

shows that the possibility of hormesis must be taken into account when choosing test concentrations.

Controlled studies on the combined effects of herbicide mixtures are rare, but the study by Roustan et al. (2014) clearly showed that mixtures can be more toxic, and the likelihood of coexistence of mixtures in the environment is high, for example, glyphosate and dicamba.

Degradation dynamics of AMPA are rarely studied separately from glyphosate, and study duration is rarely long enough to measure the half-life of AMPA. Instead, the two processes of generation and degradation of AMPA are modelled, based on a few observation points, and the half-life of AMPA is calculated and projected well past any observation points, as was done in Bergström et al. (2011). While it is statistically valid to interpolate between observation points, it is not valid to extrapolate beyond observation points.

TABLES

Table 1.1 Maximum Concentrations of AMPA Found in Agricultural Soil

Reference	AMPA Concentration ug g^{-1}	AMPA Concentration nmol g^{-1}	Reference Description
Scribner et al. (2007)	0.96	8.65	Maximum AMPA concentration found in 193 samples of Indiana agricultural soil
EFSA (2015)	2.04	18.38	Worst case initial PEC (Predicted environmental concentration) from a single glyphosate application of 4.32 kg/ha to bare soil
Aparicio et al. (2013)	2.26	20.36	Maximum AMPA concentration found in 16 agricultural soils in Argentina
Silva et al. (2018)	2.00	18.01	Maximum AMPA concentration found in 317 European Union agricultural topsoils
EFSA (2015)	6.18	55.68	Worst case PEC concentration from 10 years of annual 4.32 kg/ha glyphosate applied to bare soil

Table 1.2 Predicted AMPA Half-Life across Diverse Soil Types and Environmental Conditions, after Glyphosate Application to Soil (adapted from Bai and Ogbourne, 2016).

Soil Type	Soil Depth	pH	Clay	Org OC	AMPA	Temp	Study Duration	References
	cm		-------%---		$t_{1/2}$ (days)	C°	(days)	
Loam	0-10	5.6	15.3	1.9	37	†	42	Zhang et al. (2015)
Loam	0-10	4.2	18.1	4.7	10	†	42	Zhang et al. (2015)
Sandy	0-2.5	6.5	13.3	2.7	32	14	810	Simonsen et al. (2008)
Loam	0-10	7.1	26.5	1.9	26	30	30	Bento et al. (2015)
Clay	0-30	7.2	46.5	4.4	35	20	64	Bergström et al. (2011)
Clay	30-60	7.4	56.1	0.0	98	20	64	Bergström et al. (2011)
Sandy	0-30	7.4	7.7	2.0	60	20	64	Bergström et al. (2011)
Sandy	30-60	6.4	0.0	1.0	93	20	64	Bergström et al. (2011)
Loam	0-10	8.2	9.3	20.0	25	28	140	Mamy et al. (2005)
Loam	0-10	8.2	37.7	1.7	34	28	140	Mamy et al. (2005)
Loam	0-10	7.6	23.5	1.0	75	28	140	Mamy et al. (2005)

†Field conditions in Zhejiang and Guangdong Province, China.

Table 1.3 Toxicology Studies on AMPA Exposure adapted from (IARC, 2017).

All studies on AMPA showed significant effects.

Species Tissue/Cells	End-Point / Test	Dose (LED or HID)	Comments	Reference
Human Liver Hep-2	Chromosomal Damage / DNA Strand Breaks, comet assay	4.5 x10^3 nmol/mL (500 ug/mL)	P<0.05 at 4.5 mM; P<0.01 at up to 7.5 mM Dose-response relationship (r>=0.90; P<0.05)	Mañas et al. (2009)
Human Lymphocytes	DNA Damage / Chromosomal Aberrations	1.8 x 10^3 nmol/mL (200 ug/mL)	P<0.05	Mañas et al. (2009)
Mouse,Balb C (M,F) Bone marrow (PCE)	Chromosomal damage / Micronucleus formation	200 mg/kg bw (1.8 x 10^3 nmol/g bw)	One injection per 24 h, 2x100 sampled 24 h after last injection P<0.01 at the lowest dose (200 mg/kg bw)	Mañas et al. (2009)
Hamster, Chinese CHO-K1 ovary cell line	Chromosomal damage / Micronucleus formation	0.01 ug/mL (0.09 nmol/mL)	P<=0.05, in the dark -S9 Highest increase was observed at very low dose (0.0005 ug/mL) -S9 but with light-irradiation (P<0.01	Roustan et al. (2014)

Table 1.4 Toxicology Studies on AMPA Exposure conducted after IARC (2017).

Species Tissue/Cells	End-Point / Test	Results	Dose (LED or HID)	Comments	Reference
Human peripheral blood mononuclear cells (PBMCs).	Cell viability and ATP level / calcein-AM/ propidum iodide (PI) viability test	Decreased viability and ATP level but only at high concentrations	5mM at 24 h (5.0×10^5 nmol/mL)	P<0.001; 24 h incubation Glyphosate more toxic than AMPA	Kwiatkowska et al. (2016)
Human PBMCs	DNA damage / DNA strand breaks; comet assay	DNA damage associated with oxidative stress	500 uM at 24 h (5.0×10^8 nmol/mL)	AMPA less toxic than glyphosate (250uM) and much less toxic than Roundup 360 Plus(5uM).	Woźniak et al. (2018)
Human BMECs derived from induced pluripotent stem cells	Blood Brain Barrier permeability	Disruption of Blood Brain Barrier. Changes in neuronal cell metabolic activity and glucose uptake	100 uM (1.0×10^8 nmol/mL)	Neurological damage may result from high concentration exposure	Martinez et al. (2018)

FIGURES

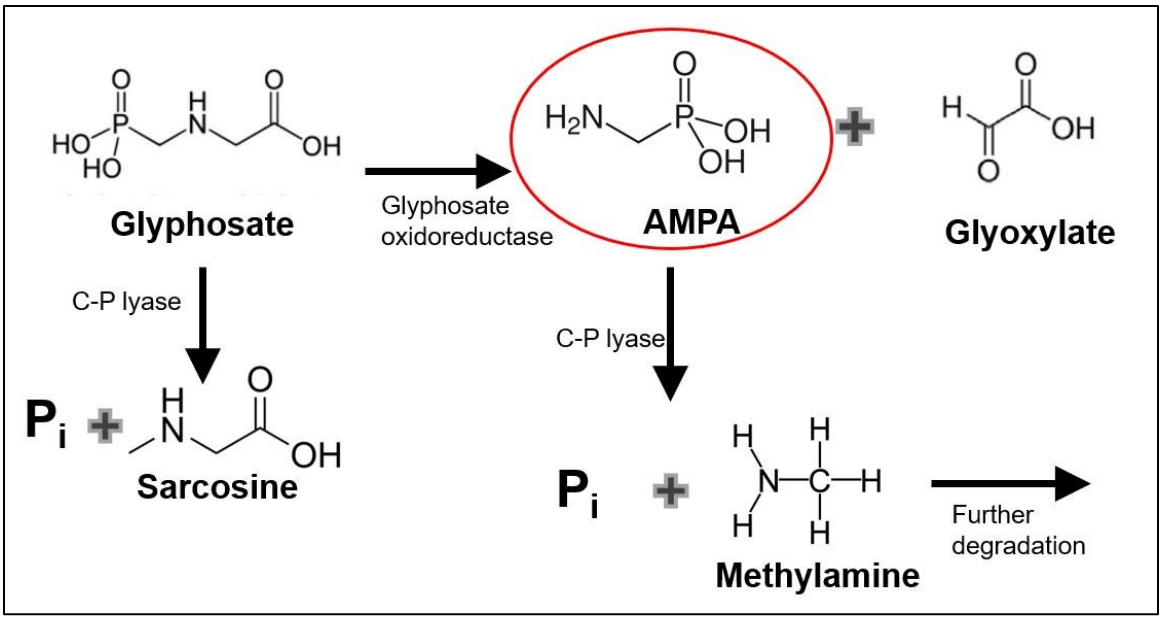

Figure 1.1 Main Glyphosate Biodegradation Pathways in the Environment

Adapted from (Sviridov et al., 2015; Grandcoin et al., 2017).

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CHAPTER 2: RESPIRATION AND PHOSPHOLIPID FATTY ACID ANALYSIS

ABSTRACT

Aminomethylphosphonic acid (AMPA) is an intermediate metabolite that results from the degradation of the herbicide glyphosate, and industrial phosphonates. In addition to negative impacts on human health, and non-target plant species, AMPA metabolized from glyphosate may play a role in inhibiting growth of some soil microorganisms and altering the soil microbial community composition. Although several studies indicate that use of glyphosate increases soil pathogens and reduces beneficial soil microorganisms, few studies have focused on the effect of AMPA, in isolation from glyphosate, on the soil microbial community. The objective of this study was to determine the effects of AMPA on soil microorganisms, and how AMPA bioavailability is affected by soil type and time. The experimental design was a 3 X 3 factorial that had the following treatments: three diverse soil types (Jory silty clay loam, Blount silt loam, or Granby loamy sand) and three AMPA rates of 0, 1, or 2 μg AMPA g^{-1} (0.0, 9.01, or 18.01 nmol g^{-1}, respectively) which reflect in situ rates. The soils had no known history of exposure to glyphosate. Destructive sampling took place at days 0, 7, 21, 42, 70, and 139, and phospholipid fatty acid analysis on those samples was conducted. Expired CO^2C was measured at approximately 14-day intervals, throughout the incubation period using NaOH traps. Respiration rate and metabolic quotient were calculated. AMPA resulted in hormetic effects in metabolic quotient and PLFA abundance results for total biomass, Gram-positive bacteria, protozoa, and Gram-positive/Gram-negative ratio, where the 9.01 nmol g^{-1} (1X) concentration showed greater effects than the 18.01 nmol g^{-}

[1] (2X) concentration, contrary to expectations. Soil properties were a major controller of PLFA responses to AMPA, with total soil carbon and possibly pH appearing to be the most important factors. There was no clear evidence that AMPA negatively impacted soil health using a 20% effect size threshold, at field relevant concentrations, or that could be predicted based on an average US one-time glyphosate application. There was a significant and lasting increase in the Gram-positive to Gram-negative bacterial ratio in Blount soil, but that is not an indication of negative soil health impact. However, the maximum concentration used in this study was only one third of the predicted worst-case concentration from 10 years of AMPA accumulation following repeated glyphosate application. Further study would be required to rule out negative soil health impacts of AMPA from long-term glyphosate use.

INTRODUCTION

Aminomethylphosphonic acid (AMPA) is the major metabolite of glyphosate (Rueppel et al., 1977), the active ingredient in the herbicide Roundup©, resulting from microbial degradation in soils (Borggaard and Gimsing, 2008). AMPA is also produced by degradation of industrial and household phosphonates, including detergents, water cleaning agents, flame retardants and anti-corrosives (Studnik et al., 2015). Because of the dramatic increase in use of glyphosate worldwide, since the introduction of glyphosate tolerant crops by Monsanto in 1997 (Benbrook, 2016), the presence of AMPA in the environment has also significantly increased. AMPA has been detected frequently in soils, sediment, ditches, drains, precipitation, and streams in the United States (Battaglin et al., 2014), along with glyphosate. AMPA is commonly found at the outlets of wastewater treatment plants, due to the increased use of industrial and household phosphonates (Grandcoin et al., 2017).

The ubiquity of AMPA in the environment is of concern due to its possible effects on human health. The International Agency for Research (IARC) on Cancer report (IARC, 2017) which concluded that glyphosate was a probable carcinogen, also found strong evidence that AMPA induces oxidative stress. AMPA has been found to cause chromosomal and DNA damage in human cells (Mañas et al., 2009; Woźniak et al., 2018). Other toxicology studies have found that AMPA can cause neurological damage (Martinez and Al-Ahmad, 2019), and decrease cell viability of human blood cells (Kwiatkowska et al., 2017).

AMPA was found to have the greatest cytogenetic toxicity in a study of the herbicides glyphosate and atrazine, and their respective metabolites, AMPA and desethyl-atrazine (DEA) (Roustan et al., 2014). Although the levels of AMPA and glyphosate found in the US environment are well-below the U.S. Environmental Protection Agency (USEPA) glyphosate MCL of 700 ug L^{-1} (6.31 x 10^3 nmol L^{-1}), these herbicides are usually found in mixtures in the environment, and the interaction effects of mixtures are largely unknown (Battaglin et al., 2014). Roustan et al.(2014) found that mixtures of glyphosate, atrazine, AMPA, and DEA were 20 times more toxic than the single most active compound, AMPA, alone, and that light irradiation increased toxicity of the mixture 100 times.

In addition to human health impacts, AMPA has been shown to have negative environmental impacts. AMPA affects chlorophyll biosynthesis in plants (Reddy et al., 2004; Gomes et al., 2014), and impedes repair of DNA and mRNA synthesis (Sviridov et al., 2015). In aquatic life, AMPA had been found to cause genotoxic effects on fish (Guilherme et al., 2014; de Brito Rodrigues et al., 2019). Domínguez et al. (2016) found that field relevant concentrations of AMPA significantly reduced earthworm biomass.

There is relatively little information available on the effects of AMPA on soil microorganisms and soil health, in isolation from glyphosate, and at field relevant concentrations. However, glyphosate has been found to change the competitive relationship of fungal species (Wardle and Parkinson, 1992; Krzyśko-Łupicka and Orlik, 1997), and increase fungal soil pathogens (Kremer et al., 2005), as well as decrease plant beneficial soil bacteria (Ermakova et al., 2010; Zobiole et al., 2011; Hadi et al., 2013;

Newman et al., 2016). Quinn et al. (1988) found that glyphosate application inhibited microbial growth, and changed the community composition. Schnurer et al. (2006) found glyphosate application inhibited soil microbial growth and respiration, and stated that this effect could have been caused by a glyphosate metabolite such as AMPA.

One study of AMPA effects on soil microorganisms, in isolation from glyphosate, was conducted by von Mérey et al. (2016). This study measured soil nitrogen transformation processes, after application of an AMPA concentration at 5 times the predicted worst case. Results showed transitory stimulatory effects on soil nitrogen transformation processes, and no effects at lower concentrations that were still well above field relevant concentrations. This study concluded that soil microorganisms were unlikely to be adversely affected by AMPA at field relevant concentrations. Interestingly, Grossbard (1985) also found that glyphosate application increased nitrate formation, but simultaneously inhibited actinomycetes growth and O_2 uptake. This indicates that AMPA could have both stimulatory and inhibitory effects depending on the endpoint (microbial growth, respiration, nitrogen transformation) being measured.

Glyphosate's mode of toxic action is to hinder the EPSP synthase enzyme in the shikimate pathway which suppresses synthesis of proteins and secondary metabolites in plants and soil microorganisms (Sviridov et al., 2015; Bai and Ogbourne, 2016). AMPA's exact mode of toxic action is unknown (Gomes et al., 2014).

While photocatalytic techniques are effective at removing glyphosate and AMPA in wastewater treatment plants, bioremediation techniques are most promising for removal of these contaminants in the environment (Zhan et al., 2018). Degradation of

glyphosate and AMPA is largely performed by microorganisms (Rueppel et al., 1977; Borggaard and Gimsing, 2008). Knowledge of the specific organisms capable of degrading phosphonates, and soil characteristics that affect degradation are needed to develop effective bioremediation methods (Ermakova et al., 2008; Zhan et al., 2018).

Phosphonates, including glyphosate and AMPA, have a stable C-P bond that is resistant to microbial degradation (McGrath et al., 2013). Microorganisms capable of degrading phosphonates, must have the specific enzymes necessary to break the C-P bond (Quinn et al., 1989).

The degradation of glyphosate can follow two main pathways, utilizing different sequences of enzymes, producing either AMPA or sarcosine. In the AMPA pathway, glyphosate is degraded to AMPA and glyoxylate. AMPA is then ultimately degraded to ammonium, phosphate, and CO_2 (Borggaard and Gimsing, 2008; Sviridov et al., 2015). Organisms that degrade glyphosate to AMPA cannot always degrade AMPA, so it is excreted to the environment (Balthazor and Hallas, 1986; Jacob et al., 1988).

According to Rueppel et al. (1977), degradation to AMPA is the most prevalent pathway of glyphosate degradation. Most of the literature identifying microorganisms that degrade glyphosate and AMPA have determined that these microorganisms use glyphosate and/or AMPA as a phosphorus source

In studies of both glyphosate treated and untreated soils, microorganisms able to degrade AMPA were found more frequently than those able to degrade glyphosate (Dick and Quinn, 1995). In addition, microorganisms able to degrade glyphosate occur more abundantly in soils previously treated with glyphosate (Dick and Quinn, 1995).

The divergent effects of AMPA on soil microbial communities, are likely due to differences in environmental conditions (Nguyen et al., 2018). Both glyphosate and AMPA are strongly adsorbed in soil, which decreases bioavailability and microbial degradation (Schnurer et al., 2006; Al-Rajab and Schiavon, 2010; Sviridov et al., 2015). AMPA adsorption is increased in soils with high clay content, aluminum, iron, and organic carbon (Borggaard and Gimsing, 2008; Zhang et al., 2015; Okada et al., 2016; Sidoli et al., 2016). According to Sidoli et al. (2016) and Zhang et al. (2015), soil pH has been found to be the most important factor in determining AMPA adsorption, and that low pH increases adsorption. AMPA degrades most readily under oxic conditions (Grandcoin et al., 2017).

Most research on AMPA has been in combination with glyphosate. Furthermore, there is little information on how soil type affects microbial responses to AMPA. Therefore, the objective was to investigate the bioavailability of AMPA at field relevant concentrations to soil microorganisms, and any negative impacts soil on health.

MATERIALS AND METHODS

Soils

Three soil types were chosen to obtain a range of textures and mineralogy. The Blount silt loam (fine, illitic, mesic Aeric Epiaqualf) soil was obtained from a farm in Delaware County, Ohio, that had been organically managed since 2003. Soil was sampled in mid-May, 2018, from an area maintained as sod (mixed grass/legume) that

had seen very little agricultural activity in the past 30 years other than being mowed or hayed and was wet at least a quarter of the year. Prior to 1990, the area had been conventionally farmed with corn and soybeans (J. Dickinson, personal communication, January 22, 2020). The second soil was a Granby (sandy, mixed, mesic Typic Endoaquaoll) from a hardwood forest in Henry County, Ohio, sampled in early July 2018. The third soil was a Jory silty clay loam (fine, mixed, active mesic Xeric Palehumult) from Douglas Fir forest in Corvallis, Oregon, sampled in March 2018.

All the three soils are typically used for field crop agriculture (USDA National Agricultural Statistics Service Cropland Data Layer, 2008-2019; Soil Survey Staff, 2020) and in general are soils where glyphosate could be expected to be applied. However, none of the sites that were sampled likely had received any glyphosate. The Jory and Granby soils came from unmanaged forest that has never been cultivated as far as is known. The Blount soil site did have conventional crop management until 1990 (after which it was under organic management that used no chemicals), but that was before the widespread implementation of glyphosate tolerant cropping that started in the mid-1990s. Further evidence for non-exposure to glyphosate is that both extractable glyphosate and AMPA were undetectable in these soils.

Soil sampling sites were chosen to avoid previous glyphosate exposure. Approximately 15 samples were taken from each site at approximately a 10-cm depth and composited. The samples were passed through a 2-mm sieve and stored at 4°C.

Experimental Design

The experiment was a laboratory incubation that had 3 replications and a 3 x 3 factorial design with three soil types (Blount, Granby, Jory), and field relevant AMPA concentrations: 0 (0x), 1 (1X), or 2 (2X) µg AMPA g^{-1} (0.0, 9.01, or 18.01 nmol g^{-1}, respectively). The 139-day duration exceeded the half-life of AMPA reported in most of the literature on laboratory studies (Mamy et al., 2005; Bergström et al., 2011; Zhang et al., 2015; Bento et al., 2016). The AMPA concentrations were based on the maximum concentrations found in studies in agricultural soils (Scribner et al., 2007, Aparacio et al., 2013; Silva et al., 2018). These concentrations are about halfway between the 1.2 and 35 nmol g^{-1} of AMPA that could be expected in soil from a U.S. average one-time glyphosate application of 1.1 kg $hectare^{-1}$ versus a U.S. maximum one-time glyphosate application of 9 kg $hectare^{-1}$ (Hawkins and Hanson, 2019), concentration of glyphosate in the upper 5 cm of soil (Okada et al., 2016), and AMPA representing 13.1 to 50.1% of the applied glyphosate (von Mérey et al., 2016).

Field moist soils were mixed with the appropriate solution of AMPA and deionized water to attain 0, 9.01, 18.01 nmol AMPA g^{-1} soil and 67% field moisture capacity in each soil treatment. A period of 24 hours was allowed for the AMPA to adsorb at 20°C. For each experimental unit, 40g (dry weight basis) of spiked soil was placed into a 55 mm diameter Wheaton jar.

The experimental units were placed into air-tight 1L Mason jars along with a beaker of sodium hydroxide solution to trap carbon dioxide, and incubated at 20°C. At

days 0, 7, 21, 42, 70, and 139, three replicates of each treatment were removed from incubation, and soil samples were stored at -20°C, until PLFA and AMPA extraction analysis could be performed. At day 7, 14, 21,32, 42, 56, 70, 83, 98, 113, 125, or 139 the sodium hydroxide traps were replaced, and the traps were titrated with 0.01 M HCl to determine CO_2-C.

Soil Chemical Analyses

Soil properties are shown in Table 2.1. Soil pH was determined on 1 to 1 soil:deionized water ratio and measuring with a glass membrane electrode (Sparks et al., 1996). Total carbon and nitrogen content were measured on an elemental analyzer (Carlo Erba CHN EA 1108, now Thermo Fisher Scientific, Waltham, MA). Field capacity of the soil was determined by saturating soil, weighing, then oven drying samples at 105°C, and re-weighing. To ensure that the soils contained no glyphosate or AMPA, 3 replicate samples of each soil were analyzed prior to the experiment for glyphosate and AMPA using a procedure adapted from Miles and Moye (1988). Briefly, 3 g of soil was shaken for 15 min in 12 mL of a 0.1 M extraction solution of monopotassium phosphate, then centrifuged at 3000 rpm for 30 minutes. The supernatant was filtered through 0.30 µm filter paper. This procedure was repeated two more times, combining the supernatant. 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. Concentrations of AMPA and

glyphosate were reported in ppb and confirmed that the samples contained no detectable glyphosate or AMPA.

Sand, silt and clay percentages were referenced from previous studies of the same sites (Lee et al., 2007; Nye et al., 2014; Dick et al., 2018). The average iron and aluminum oxide content from soil pedons sampled as Blount, Granby, and Jory soil series was obtained from the National Cooperative Soil Survey (2020). The Soil Characterization Database contains data from two extraction methods: 1) the dithionite citrate extraction method, which represents both the crystalline and amorphous iron and aluminum oxide content, and 2) the ammonium oxalate extraction method which extracts only amorphous iron and aluminum oxide, according to McKeague and Day (1965).

Respiration Analysis

Respiration CO_2-C in each jar was measured by adding $BaCl_2$ to the NaOH trap and titrating to a pH of 7 with 0.01M HCL solution, then calculating evolved CO_2-C from the difference in HCL solution required between the blanks and the sample (Horwath and Paul,1994). The rate of respiration on each sampling day was calculated by dividing the evolved CO2-C by the number of days since last replacing the NaOH trap.

Microbial Analyses

Phospholipid fatty acid analysis (PLFA) is a culture independent technique widely used to determine microbial biomass, community composition, and physiological status (Willers et al., 2015). However, disparities exist over the interpretation of fatty acids

between different studies (Willers et al., 2015). In particular, ratios interpreted as stress markers, such as saturated to monounsaturated and cyclopropyl to monoeic precursors may be due to change in species composition not associated with stress (Frostegård et al., 2011). Contradictory results have also been found in different studies in interpretation of the Gram-positive to Gram-negative ratio (Willers et al., 2015). A brief review of stress marker interpretation is presented below.

Kieft et al. (1997) has found that bacteria change their cell volume and morphology and fatty acid composition in response to nutrient stress. According to Kieft, nutrient stressed Gram-negative bacteria show increases in saturated to monounsaturated ratios, and cyclopropyl to precursor ratios, whereas nutrient stressed Gram-positive bacteria change from rod to coccus shaped, but do not change membrane lipids. Cyclopropyl fatty acids are associated with slow growth, and a decrease in substrate availability (Bossio and Scow, 1998). Branched fatty acids representative of Gram-positive bacteria tend to decrease in response to high substrate availability (Bossio and Scow, 1998). An increase in monounsaturated to saturated fatty acids ratio may indicate increased aerobic conditions or an increase in available carbon substrate availability (Bossio and Scow, 1998).

In a study comparing virgin prairie, agricultural, and restored prairie soils, McKinley et al. (2005) found that increased prairie age correlated with higher total biomass, and lower protozoa and fungal biomass. This study also found that as prairie age increased, Gram-positive and Gram-negative bacteria both increased, but Gram-positive increased at a higher proportion of total biomass. In addition, monounsaturated and

Gram-negative bacteria increased in proportion to increased amounts and diversity of carbon sources.

Frostegård et al. (1993) found in a comparison of arable vs forest soils with metal contamination, that both Gram+ and Gram- are negatively impacted, saturated fatty acids increased in arable soil, and cyclopropyl fatty acids increased in both forest and arable soils. Zelles et al. (1994) found that Cu contamination was correlated with increased abundance of Gram-negative bacteria and decreased abundance of Gram-positive bacteria.

In this study, phospholipid fatty acid methyl esters were determined by the method described in Frostegård et al. (1993). Briefly, phospholipids were extracted from approximately 2 g of soil using a one-phase chloroform, methanol and citrate buffer extractant (Bligh and Dyer, 1959). The upper organic phase was then fractionated into neutral, glycolipid and phospholipid components using silica acid columns. Alkaline methanolysis using 0.2M methanolic KOH was performed to convert the phospholipids to methyl-esters (Chowdhury and Dick, 2012). Nonadecanoic methyl ester (19:0) was added as an analytical standard to allow GC peak areas to be converted to a molar basis. Fatty acids were analyzed on an HP 5890 gas chromatograph with peak identification software (MIDI Inc., Sherlock MIS).

Table 2.2 shows the phospholipid fatty acids that were used for PLFA analysis. A total of 65 fatty acids that were present in at least 5% of samples were summed to represent total microbial biomass. Nineteen fatty acid biomarkers that were documented in the literature as distinctly belonging to a given taxonomic group, were categorized as

Gram-positive bacteria, Gram-negative bacteria, actinomycetes, and saprophytic fungi. The fungal to total bacterial ratio was calculated as saprophytic fungi divided by the sum of Gram-positive bacteria, Gram-negative bacteria, and actinomycetes. Three ratios were calculated as stress markers: the ratio of total monounsaturated to total saturated fatty acids, the ratio of total cyclopropyl fatty acids to total monounsaturated precursors, and the ratio of Gram-positive to Gram-negative bacteria.

Nomenclature used for the fatty acids is the total number of carbon atoms, followed by a colon, followed by the number of double bonds. For Gram-positive bacteria, the number of double bonds is followed by "a" or "i" which refer to anteiso and iso branching. For other taxonomic groups, the number of double carbon bonds is followed by "w" to indicate the methyl or "ω" end of the molecule, then the position of the double bond from the methyl end of the molecule. The suffix "c" indicates *cis* geometry. "ME" indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule, and "cyclo" indicates cyclopropyl fatty acids.

Metabolic quotient was calculated as the respiration rate (nmol CO_2-C g^{-1} soil g^{-1} day) divided by total microbial biomass PLFA absolute abundance (nmol g^{-1} soil g^{-1}) (Anderson and Domsch, 1993).

Statistical Analyses

Repeated measures ANOVA, multiple comparisons using Tukey's Honestly Significant Difference (HSD) correction ($P<0.05$), were conducted in R (version 3.5.1). Summary statistics including mean responses and standard error were compiled using R packages.

For each of the PLFA taxonomic groups and stress-markers in Table 2.2, statistical analyses were conducted on the absolute concentration (nmol $g^{-1}C$) of PLFA. Metabolic quotient was calculated using respiration and PLFA from the same samples, and respiration rate at the time of destructive sampling. Statistical analysis on respiration rate was conducted on samples destructively sampled on the last day of incubation.

Non-metric multidimensional scaling ordination (NMS) was conducted using PC-ORD, version 6, to analyze the multivariate response of the taxonomic groups and stress markers. For NMS analyses, the absolute concentration (nmol g^{-1} C) of PLFA data was first relativized as nmol percent of microbial biomass, then transformed using the sqrt function to create a more normally distributed data set and to reduce the coefficient of variation among PLFAs. The Sorensen distance measure was used, forty runs with real data were conducted, and Monte Carlo simulations were conducted using 50 randomized runs and a stability criterion of 0.00001.

To determine the relationship between taxonomic groups based on NMS scores, a joint plot was created using a second overlay matrix containing the transformed values for the taxonomic groups and stress indicators listed in Table 2.2. The angle and length of a line indicate the direction and strength of the relationship (Peck, 2010). The NMS scores from each axis were exported from PC-ORD, joined to sample factor data (soil, concentration, sampling time), and the matrix read into R for ANOVA and plotting of means. Repeated measures ANOVA was conducted on the NMS scores to determine if soil type, AMPA concentration, sampling time, or their interaction had significantly different effects ($P<=0.05$) along a given axis.

Analysis of Soil Health Impact

An approach based on Kvas et al. (2017) was used to evaluate possible negative soil health impacts of AMPA. Briefly, if there was a difference between the control and an AMPA treatment that was inhibitory, statistically significant at $P<0.05$, with an effect size greater than 20%, and not transitory (still evident at the end of the 139-day incubation), the effect was considered to have potential negative impacts to soil health. Kvas et al. (2017) stressed that a single indicator should not be used to judge soil health on its own, and that multiple soil health indicators should be evaluated at the same time. The test suite developed by Kvas et al. (2017) to evaluate petroleum hydrocarbon contaminated sites included eight indicators. In this study on AMPA effects, respiration and total biomass were the only commonly used soil health indicators. In addition, metabolic quotient, which is the ratio of respiration to total biomass, was evaluated using this approach.

RESULTS

Respiration

Results of respiration in each soil type for each AMPA concentration are shown in Figure 2.1.

Three-way repeated measures ANOVA showed that AMPA concentration overall was not a significant effect on respiration response when averaged across soils and time,

varying only 9.02 and 7.95 nmol CO_2-C g^{-1} soil day^{-1} higher for the 1X and 2X than the control at 620.84 nmol CO_2-C g^{-1} soil day^{-1}. There were no significant overall interaction effects of concentration with soil type or day of sampling. The main effect of soil type on respiration was highly significant at $P<7.19 \times 10^{-10}$, with respiration for Blount, Granby and Jory soil averaging 698, 601, and 580 nmol CO_2-C g^{-1} soil day^{-1}, respectively. Sampling day was only significant as a main effect at $P< 0.06$, with respiration generally declining at a slope of -3.48 nmol CO_2-C g^{-1} soil day^{-1} over time for all treatments. Within each sample, respiration response had highly significant differences by day at $P<1x10^{-16}$, and soil x day interaction effects were significant at $P<0.05$.

Even though the main effect of AMPA concentration on respiration was not significant, multiple comparisons of concentration effects within each soil type and each day did show some significant differences between $P<0.1$ and $P<0.01$. For Blount soil on day 21, the respiration rate for the 2X concentration was 16.14% higher than the control ($P<0.05$), and 13.89% higher than for the 1X concentration ($P<0.1$). On day 32, respiration rate for both the 1X and 2X concentration was higher than the control at 9.5% ($P<0.05$), and 7.84% ($P<0.05$) respectively. On day 70, respiration rate for the 2X concentration was 5.66% higher than the control ($P<0.01$), and the 1X respiration rate was 4.94% higher than for 2X ($P<0.05$).

For Granby soil, multiple comparisons of concentration effects showed differences on days 21 and 113 only at the $P<0.1$ level. On day 21, the 2X concentration was 15 % higher than the 1X concentration ($P<0.07$). On day 113, the 2X concentration was 17 % lower than the control ($P<0.1$).

For Jory soil, multiple comparisons of concentration effects showed significant differences only on day 7. The 1X concentration was 5.6 % lower than the control ($P<0.03$). The 2X concentration was 4.2 % higher than the control ($P<0.1$), and the 2X concentration was 10 % higher than 1X ($P<0.01$).

Metabolic Quotient

Metabolic quotient can be used as a measure of physiological stress, since a higher metabolic quotient indicates that more energy is being expended to maintain biomass (Anderson and Domsch, 1993).

Three-way repeated measures ANOVA showed significant main effects of AMPA concentration for metabolic quotient ($P<0.05$). Metabolic quotient for the 1X concentration was 23 % lower than the control on average, whereas metabolic quotient for the 2X concentration was 7.2 % lower than the control. ANOVA did not show any significant interaction effects between AMPA concentration and soil type or day. Soil type was a significant main effect ($P< 0.001$) with metabolic quotient for Granby and Jory both lower than Blount at 35 % and 41 % respectively.

Multiple comparisons of AMPA concentration effect on metabolic quotient averaged over soils on each day is shown in Figure 2.2. There is only one significant difference on day 21, where the 1X concentration is 36 % lower ($P<0.05$) than the control.

Soil type had a stronger main effect than AMPA concentration. Multiple comparisons of soil type main effect on metabolic quotient shows that Blount soil had

significantly higher metabolic quotient than Granby and Jory soils on days 21, 42, 70, and 139 in Figure 2.3.

Multiple comparisons of AMPA concentration effects within each soil type and each day showed significant differences in all three soils. Results for metabolic quotient comparing AMPA concentration effects in each soil type on each sampling day are shown in Figure 2.4. For Blount soil on day 21, the metabolic quotient of the 1X concentration was 51 % lower than the control ($p<0.05$). For Granby soil, the metabolic quotient on day 70 for the 2X concentration was 54 % higher than the control ($p<0.05$), and 63 % higher than for 1X ($P<0.01$). For Jory soil, on day 7, the 2X concentration was 47 % higher than the control $P<0.05$). On day 21, both the 1X and 2X concentrations were lower than the control at 42% lower ($P<0.05$), and 43 % lower ($P<0.05$), respectively.

AMPA Concentration and PLFA Abundances

The effect of AMPA concentration averaged over soils and sampling days is shown in Figure 2.5. Repeated measures ANOVA indicated that significant differences ($P<0.05$) in response to AMPA concentration alone were seen in microbial biomass, Gram-positive bacteria, protozoa, and the Gram-positive/Gram-negative ratio. There were no other significant differences of AMPA concentration on taxonomic groups or stress markers. For all groups with significant differences, the 1X AMPA concentration had the highest PLFA response, and the control 0X concentration had the lowest.

Day showed significant main effects ($P<0.001$) for stress marker ratios SAT/MONO and CY/PRE, and community composition ratios FB and Gram-positive/Gram-negative ratios, with ratios increasing as the incubation progressed. Soil type showed highly significant main effects ($P<0.001$) for all taxonomic groups and stress markers except for Gram-positive bacteria, where it was not at all significant. There was only one two-way interaction effect between soil and concentration for Gram-positive/Gram-negative ratio with significant differences when multiple comparisons were done. In Blount, 1X was significantly higher than 0X ($P<0.001$) and higher than 2X ($P<0.01$).

Repeated measures ANOVA showed total biomass, Gram-positive bacteria, actinomycetes had three-way interaction effects at the $P<0.1$ level. Gram-negative bacteria, total bacteria, and Gram-positive/Gram-negative ratio had three-way interaction effects at ($P<0.05$). There were no other significant three-way interactions. Table 2.3 shows multiple comparisons of AMPA treatment effect on each sampling day in each soil revealed additional differences in effect of the three AMPA treatments. In Blount soil, for the taxonomic groups (actinomycetes, Gram-positive bacteria, Gram-negative bacteria, protozoa, saprophytic fungi, and microbial biomass), significant effects of AMPA concentration were clustered on days 7, and 21, and all six groups were affected on both days, except for protozoa, which only showed effect on day 21. Granby soil showed the least overall effect of AMPA concentration on the taxonomic groups. Gram-positive bacteria showed effects on day 7 only, whereas Gram-negative bacteria, microbial biomass, and protozoa showed effects on day 70 only. In Jory soil, AMPA

concentration effects were spread out over all taxonomic groups and all sampling days, except for day 139.

For the stress markers and ratios, AMPA concentration appears to have a peak effect on day 7 with eight soil stress-marker effects, then a second peak effect on days 42 and 70, with five and six soil stress-marker effects, respectively. At the end of the incubation, on day 139, the Gram-positive/Gram-negative ratio in Blount, and CY/PRE ratio in Jory showed significant differences.

Soil Microbial Community Structure

Differences in the soil microbial community (SMC) structure due to soil type, AMPA concentration, and sampling day, are shown in Figure 2.6. The means of NMS scores along each ordination Axis were plotted for soils, AMPA concentration, and sampling day. ANOVA of NMS scores indicated that all three soils were significantly different from each other at $P<0.001$, along Axis 1. Along Axis 2, both Granby and Jory were significantly different from Blount at $P<0.001$, but Jory and Granby were not significantly different.

Along Axis 1, there were no significant differences between the three AMPA concentrations. Along Axis 2, the 1X concentration were significantly different from 0X at $P<0.001$, and different from 2X at $P<0.01$. Along Axis 1, all sampling days were significantly different from each other at $P<0.001$, except for day 21 which was not significantly from day 42 and day 70, and day 42 which was not significantly different from day 70. Along Axis 2, there were no significant differences between day 0 and 7,

between day 7 and day 21, or between days 42, 70 and 139. Otherwise, along Axis 2, all sampling days were significantly different at $P<0.001$.

A comparison of the NMS joint plot and the plot of soil means suggests that Jory soil tended to be higher in fungi, and the stress markers SAT/MONO ratio and the CY/PRE ratio, than Blount and Granby soils, whereas Blount tended to be higher in Gram-positive bacteria, and Granby tended to be higher in actinomycetes, and protozoa. A comparison of the joint plot with the means of AMPA concentration plot, suggests that concentration 1X tended to be slightly higher in Gram-positive bacteria, than soils with 0X AMPA which tended to be slightly higher in Gram-negative bacteria. Comparing the joint plot with the plot of sampling day means indicates that day 139 tended to be higher in the stress markers SAT/MONO and CY/PRE than day 0.

Repeated measures ANOVA indicated that the interactions between soil and sampling day, and concentration and sampling day along both axes were highly significant ($P<0.001$). Along Axis 2 only, there was significant three-way interaction ($P<0.01$) between soil, concentration and sampling day.

Since total biomass is a soil health indicator, total biomass was examined for any instances where AMPA treatments resulted in biomass significantly lower than the control. There were five instances, (1) in Blount soil on day 7 the 1X treatment was 14.2% lower than the control, (2) in Blount soil on day 7 the 2X treatment was 24 % lower than the control (3) in Granby soil on day 70 the 2X treatment was 33 % lower, (4) in Jory soil on day 0 the 2X treatment was 31 % lower, and (5) in Jory soil on day 7 the 2X treatment was 31% lower than the control. However, all these effects were transitory.

There were no significant inhibitory effects in Jory or Blount past day 7. In Granby, there were no inhibitory effects on total biomass at the end of the incubation on day 139, or any sampling day before day 70.

DISCUSSION

Respiration

A question of ecological relevance regarding the effects of AMPA on soil microorganisms, is whether AMPA had a negative impact on soil health. Soil respiration is one of the indicators used to quantify soil health, and a large decrease in soil respiration would indicate a negative impact on soil health. However, an increase in soil respiration would not indicate a negative impact on soil health (Kvas et al., 2017). In Blount soil, AMPA was consistently stimulatory of respiration. Differences between AMPA effects in Jory and Granby soils also showed that AMPA was mostly stimulatory. There were two cases where respiration for the AMPA treatment was lower than the control, (1) on day 113 in Granby soil the 2X concentration was 17 % lower but only at significance of $P<0.1$, and (2) on day 7 in Jory, the 1X concentration was 5.6 % lower with $P<0.5$. In both cases these effects were transitory. To address case 1, following Kvas et al. (2017), negative impact to respiration as a soil health indicator should not be interpreted unless the magnitude of the difference is greater than 20%. In both cases the magnitude of difference was less than 20% and the effects were transitory. Therefore, it can be concluded that there were no negative soil health effects of AMPA on respiration beyond day 7.

Metabolic Quotient

An increased metabolic quotient is an indicator of physiological stress, since more energy is being expended to maintain biomass. As shown in Figure 2.2, the AMPA treatments had an overall effect on day 21 of decreasing metabolic quotient. Also, even though not statistically significant, the 1X treatment tended to have the lowest metabolic quotient throughout the incubation. Metabolic quotient in Granby soil was higher in the AMPA treatments than in the control on day 70, but this was a transient effect. Since the control treatment overall was showing a higher metabolic quotient than the AMPA treatments, it can be interpreted that AMPA was reducing stress for the soil microorganisms.

A possible explanation for this is that AMPA provided an additional source of carbon and phosphorus. No new substrate was added to the soils during the incubation, and most of the labile organic carbon would have been degraded early in the incubation. The graphs of respiration in Figure 2.4 showing a sharp decrease in respiration rate between day 7 and 21, then a slower decline during the remaining incubation, support the idea that labile carbon was consumed at a rapid pace early in the incubation. This would then require the soil microorganisms to switch to consuming more complex organic molecules, including AMPA. The graph comparing metabolic quotient in the three soils in Figure 2.3 also supports the idea that metabolic quotient was highly dependent on available organic carbon. Figure 2.3 shows that Blount soil had a much higher metabolic quotient than Granby and Jory, which is consistent with the lower organic carbon content of Blount soil (2.1%) compared to Granby (4.3%) and Jory (4.5%). It can therefore be

concluded, from the results on metabolic quotient, that AMPA overall decreased physiological stress of soil microorganisms, possibly due to providing a source of organic carbon, and had no negative impact on soil health.

PLFA Abundance

Overall, AMPA appeared to have a stimulatory effect on total biomass, Gram-positive bacteria, and protozoa, and increased the Gram-positive/ Gram-negative ratio. As shown in Figure 2.5, when AMPA concentrations were averaged across all soils and all sampling days, the 1X concentration showed the highest PLFA response for these taxonomic groups and the 0X control soil had the lowest response. The fact that the 1X concentration had a higher response than the 2X concentration, is an example of hormesis (Calabrese et al., 1999), and indicates that the response to AMPA is not linear.

The term hormesis describes an effect in which a low dose of a toxin can be biologically stimulatory, while causing toxic effects above a threshold (Calabrese et al., 1999). The mechanism of hormesis may be an adaptive response to environmental stressors (Calabrese et al., 1999). A hormetic effect has been shown for glyphosate by Kryzsko-Lupicka and Sudol (2008) who found that lower doses (1.0 and 1.5 mM) stimulated *Fusarium* fungi significantly, whereas a dose of 2.0 mM was inhibitory. Although a common biological phenomenon that has been observed across microbial, plant, and animal taxa, the hormetic effect is not frequently reported in toxicology studies because these studies are usually conducted at very high doses above the level where hormesis would be evident (Calabrese et al., 1999).

Another possible explanation for the higher positive response to the 1X than the 2X concentration, would be a threshold where the toxic effects of AMPA to one group of microorganisms start to exceed AMPA's value as a nutrient source to another group of microorganisms. According to Frostegård et al. (2011), detection and interpretation of a decrease in fatty acid abundance can be difficult, since a toxic substance may inhibit enzymes that degrade PLFA.

Total biomass is another indicator used to quantify soil health (Kvas et al., 2017). The effect of AMPA on total biomass in each soil on each sampling day indicated only four instances where AMPA was inhibitory at a magnitude greater than 20%, all at the 2X concentration, and all prior to day 21. Since these few inhibitory effects of AMPA on total biomass were transitory, it can be concluded that AMPA did not negatively impact this soil health indicator.

PLFA Stress Markers

ANOVA indicated that the overall effect of AMPA on stress markers CY/PRE and SAT/MONO was not significant, and therefore does not provide evidence for a negative impact on soil health. Taking a closer look at multiple comparisons on each sampling day of AMPA treatment effect in each soil, the stress markers do not show a clear pattern of significant differences and for the most part are not evident by the end of the incubation.

There was only one instance in which the CY/PRE ratio was elevated in Jory soil, on the final day of incubation, day 139. However, the magnitude of difference was less

than 10%, so according to the 20% threshold (Kvas et al., 2017) this stress marker elevation by itself would not indicate negative impact to soil health.

PLFA Microbial Community Responses

In Blount soil, the 1X treatment showed elevated Gram-positive/Gram-negative ratios above 20% at $P<0.05$, on days 7, 21, 42, and 139. An examination of the difference between the 1X treatment and the control for each of these taxonomic groups, showed that from day 21 onward, both Gram-negative and Gram-positive bacteria were stimulated by the 1X AMPA treatment, but the magnitude of Gram-positive stimulation was greater. Interestingly, this finding of greater proportional increase of Gram-positive to Gram-negative bacteria is similar to the finding of McKinley et al. (2005), where increased prairie age, which could be expected to increase soil health, showed a proportionately higher increase of Gram-positive to Gram-negative bacteria.

The results show that the effect of AMPA on PLFA taxonomic biomarkers varied depending on the combination of soil type, and AMPA concentration, and was not linear over time. By day 139 however, there were no significant effects of AMPA on any PLFA biomarkers, with the exceptions of the Gram-positive/Gram-negative ratio in Blount soil, and the CY/PRE stress marker in Jory soil. As shown in Table 2.3, Blount and Jory soils both had early differential shifts in PLFA response due to different AMPA concentrations across multiple taxonomic groups on days 7 and 21, with Blount more clearly showing this pattern.

Although one property Blount and Jory soils have in common is high clay content, it is not likely that this property caused the similarity of response. High clay content should have increased AMPA adsorption early in the incubation, making AMPA less bioavailable, and reducing differences in PLFA response due to AMPA early in the incubation. However, this was not the early response of Blount and Jory soils. In addition, the minimum response to AMPA occurred in the Granby soil, with a high sand and low clay content. If clay content was the most important factor, the Granby soil could be expected to have the lowest adsorption, highest AMPA bioavailability, and greatest PLFA difference in response to different AMPA concentrations. However, this was also not the case. Therefore, it seems that other factors besides high clay content was more important in determining the similarity of the early PLFA response in Blount and Jory soils.

The other factors to consider are pH, organic carbon, and aluminum and iron oxide contents, which would all have affected the variable charges, and anion exchange capacity. Blount soil had the highest pH (6.29), the lowest organic carbon (2.1%), and lower aluminum and iron oxide contents (44% amorphous Fe and 15% amorphous Al of Jory soil). These properties would cause AMPA adsorption to be less in Blount soil than in Jory soil. This would make AMPA more bioavailable in Blount soil than in Jory soil, and could explain the more pronounced early PLFA response shown in Blount soil than in Jory soil. However, Granby soil had a pH (5.67) slightly higher than Jory (5.34), and also lower Fe and Al oxides (49% and 15% of Jory), which should have reduced AMPA adsorption and increased AMPA bioavailability as compared to Jory. On the other hand,

Granby had a slightly higher carbon content (4.5%) than Jory at (4.3%), which would have increased AMPA adsorption in Granby, and reduced AMPA bioavailability. If carbon content was a more important factor than Fe and Al oxides in determining AMPA bioavailability, that could explain why Granby soil showed the least overall difference in PLFA response to differences in AMPA concentration. It is also consistent with the finding that Blount soil, with the lowest carbon content, had the most consistent early PLFA response across all taxonomic groups to different concentrations of AMPA. However, it would be counter to the conclusion of Sidoli et al. (2016) and (Zhang et al. (2011), who found pH to be the most important factor in determining AMPA adsorption. It should be noted that Sidoli et al. (2016) did not investigate any variation in organic carbon content of soils in their study, due to previously contradictory findings, and thus could not conclude that pH was more important the organic carbon.

Another factor to consider is the inherent abundance of the specific organisms most tolerant of AMPA and/or most effective at degrading AMPA in the different soil types. However, this study was limited to PLFA and identifying abundances of PLFA in taxonomic groups, not specific organisms.

The effect of AMPA on the stress markers SAT/MONO and CY/PRE also depended on soil type, AMPA concentration and time. The CY/PRE ratio, where differences occurred, showed that the effect of the 1X AMPA concentration was to decrease CY/PRE, and the effect of 2X concentration was to increase CY/PRE. This effect on the CY/PRE ratio indicates there may have been a threshold between the 1X

and 2X concentrations, below which microbial stress was reduced, and above which stress was increased.

Results of this study in Figure 2.6 and Table 2.3, show the effect of AMPA changed over time between inhibitory and stimulatory, and was not always monotonic. For example, in Blount soil on day 7, the 0X concentration had the highest PLFA abundance for all taxonomic groups, actinomycetes, Gram-positive, Gram-negative, and fungi, with the exception of protozoa. By day 21, that had changed to 1X having the highest PLFA abundance, for all taxonomic groups including protozoa, and 0X having the lowest abundance.

For both Granby and Jory soils on day 7, the Gram-positive/Gram-negative ratio was highest for 1X, and 0X and 2X were lowest. By day 42 in Granby soil, that had reversed so that 1X was the lowest, and 0X and 2X were highest. By day 70 in Jory soil, that had reversed so that 1X was lowest, 2X was highest and 0X was in between.

The non-monotonic effects of AMPA could be explained by the previously discussed phenomenon of hormesis (Calabrese et al., 1999). Hormesis could also explain why the response fluctuated from inhibitory to stimulatory over time. Because AMPA is being degraded, its bioavailability could pass the threshold over time.

The 9.01 nmol g^{-1} (1X) and 18.01 nmol g^{-1} (2X) AMPA concentrations used in this study represent the maximum concentrations empirically found in several field studies (Scribner et al., 2007, Aparacio et al., 2013; Silva et al., 2018). They are consistent with average estimates of AMPA that might be found in soil, assuming US average and maximum allowed one-time applications of glyphosate, and a no-till or

permanent cropping system where glyphosate will remain in the upper few centimeters of soil (Hawkins and Hanson, 2019, Okada et al., 2016, von Mérey et al., 2016).

However, the 18.01 nmol g^{-1} 2X AMPA rate is only half of the possible maximum 35.44 nmol g^{-1} estimate based on the calculation assumptions. In addition, the maximum 2X AMPA concentration used in this study was only 32% of the predicted worst-case AMPA accumulation of 55.68 nmol g^{-1} after 10 years of maximum application rates (EFSA, 2015). Therefore, it is possible that testing at this higher rate could have revealed stronger evidence of negative soil health impacts.

CONCLUSIONS

For respiration, the 2X treatment did not have a greater effect than the 1X treatment. For metabolic quotient overall, the 1X treatment had a larger effect on metabolic quotient than the 2X concentration which is counter to the expectation that the 2X concentration would have a greater effect. For PLFA abundance, most of the effects of AMPA were transitory and by day 139, no longer significant. Though effects were transitory, AMPA showed a significant stimulatory effect on microbial biomass, Gram-positive bacteria, protozoa, and the Gram-positive/Gram-negative ratio. These effects largely occurred at the 1X AMPA rate not the 2X rate, which is counter to the expectation that the higher concentration would have a more significant effect. These results show that due to hormesis, AMPA effect at a higher concentration cannot be predicted from test results at a lower concentration and vice versa.

No conclusions can be drawn about the effects of soil properties on bioavailability of AMPA based on respiration or metabolic quotient results. There were no statistically significant interaction effects between soil and AMPA treatment in these results. However, results for PLFA showed that soil properties were a major controller of microbial responses to AMPA. Total soil carbon appeared to be a more important factor in determining the bioavailability of AMPA, than iron and aluminum oxide content, or texture and clay content. Blount soil, with a higher pH and lower carbon content, showed a significantly different response than Jory and Granby soil to AMPA concentration, with a substantially greater increase in Gram-positive/Gram-negative ratio.

For respiration overall, AMPA was slightly stimulatory to respiration in all three soils, and the few cases where AMPA treatments appeared inhibitory were transitory. It can therefore be concluded that AMPA had no negative impacts on soil health based on respiration. Metabolic quotient results indicated that AMPA did not have negative impacts on soil health. The few inhibitory effects of AMPA on total biomass were transitory, indicating that AMPA did not negatively impact microbial abundance. Although the 1X treatment in Blount soil showed a substantially higher Gram-positive/Gram-negative ratio than the control throughout the incubation, this was due to greater comparative stimulation of Gram-positive bacteria by the AMPA treatment, not an inhibition of Gram-negative bacteria. This study found several other negative impacts of AMPA on PLFA total biomass and SAT/MONO and CY/PRE stress indicators that were transitory and/or below a 20% effect size threshold, that by themselves did not represent clear evidence of negative soil health impact.

TABLES

Table 2.1 Soil Chemical Properties

Soil	Site/ Mgmt.	pH	Total C	Total N	Sand	Silt	Clay	Total Fe†	Total Al†	Amorphous Fe†	Amorphous Al†
			----------%----------								
Blount	Organic farm	6.29	2.1	0.2	11.0	48.0	41.0	1.48	0.16	0.42	0.11
Granby	Woodlot	5.67	4.5	0.4	86.0	10.9	3.1	0.25	0.13	0.47	0.11
Jory	Douglas Fir Forest	5.34	4.3	0.3	14.0	34.0	52.0	6.10	0.78	0.95	0.72

†Adapted from National Cooperative Soil Survey (2020)

Table 2.2 Fatty Acids and Taxonomic Groups for PLFA Analysis

Taxonomic Group	Specific PLFA Markers	References
PLFA Biomarkers		
Gram-Positive Bacteria	15:0i, 15:0a, 16:0i, 17:0i, 17:0a	(Vestal and White, 1989); (Willers et al., 2015)
Gram-negative Bacteria	16:1 w7c, 17:0 cyclo w7c, 19:0 cyclo w7c, 18:1 w7c	(Zelles et al., 1997)
Actinomycetes	16:0ME, 17:0ME, 18:0ME	(Vestal and White, 1989), (Federle et al., 1986)
Fungi (Saprotrophic)	18:2w6c, 18:3 w6c,	(Zelles et al., 1997), (Vestal and White, 1989), (Frostegård et al., 2011)
Protozoa	20:4 w6c, 20:3 w6c	(Moore-Kucera and Dick, 2008), (Vestal and White, 1989)
Microbial Stress Indicators		
SAT/MONO Ratio	Sum of Saturated / Sum of Monounsaturated	(Bossio and Scow, 1998)
Saturated PLFAs	14:0, 15:0, 17:0, 16:0, 18:0, 20:0	(Willers et al., 2015), (Zelles et al., 1997)
Monounsaturated PLFAs	16:1 w7c, 18:1 w7c, 18:1 w9c, 17:1 w8c, 18:1 w5c, 20:1 w9c, 16:1 w9c, 16:1 w5c	(Bossio and Scow, 1998)
CY/PRE Ratio	(cy17:0+cy19:0)/(16:1w7c+18:1w7c)	(Kieft et al., 1997), (Moore-Kucera and Dick, 2008)
Total Microbial Biomass	Sum of all extractable PLFAs (65)	(Frostegård et al., 1991), (McKinley et al., 2005)
Total Bacteria	Sum of Gram-Positive, Gram-Negative, Actinomycetes, 15:0, 17:0	(Moore-Kucera and Dick, 2008)
FB Ratio	Fungi/Total Bacteria	(Federle et al., 1986), (Frostegård and Bååth, 1996)
Gram-positive/Gram-negative Ratio		(Willers et al., 2015)

Table 2.3 PLFA Microbial Biomarker Profiles over Time in Soils Amended with AMPA.

		BLOUNT SOIL									
Day	Conc	Actino	Gram+	Gram-	Protozoa	Fungi	Biomass	FB	G+/G-	SM	CP
		nmol g^{-1}									
0	0X	9.5	10.2	20.7	0.3	2.0	69.7	0.05	0.48	0.45	0.54
0	1X	8.8	11.5	18.5	0.3	1.8	72.0	0.04	0.62	0.47	0.52
0	2X	11.0	12.9	24.2	0.3	1.4	80.4	0.03	0.54	0.46	0.52
7	0X	10.2a†	11.6a	22.3a	0.3	1.4a	76.4a	0.03ab	0.52b	0.49	0.57b
7	1X	8.0b	10.4ab	16.6b	0.1	1.3ab	65.6ab	0.04a	0.62a	0.49	0.57b
7	2X	8.1b	8.6b	18.3ab	0.1	0.9b	58.1b	0.03b	0.47b	0.48	0.60a
21	0X	5.8b	5.2b	12.3b	0.0b	0.6b	39.1b	0.03	0.42b	0.46	0.65
21	1X	9.7a	13.1a	20.2a	0.5a	1.4a	78.7a	0.03	0.65a	0.52	0.61
21	2X	7.6ab	8.1ab	16.4ab	0.3ab	0.8b	54.9ab	0.02	0.48ab	0.50	0.65
42	0X	6.7	8.0	13.9	0.3	1.1	49.5	0.04	0.56	0.54	0.65
42	1X	8.8	11.9	17.6	0.3	1.1	70.2	0.03	0.68	0.56	0.65
42	2X	7.1	9.5	14.7	0.2	0.9	54.7	0.03	0.61	0.57	0.65
70	0X	7.1	9.4	14.0	0.2	0.7	52.0	0.02b	0.68	0.60ab	0.64
70	1X	7.8	9.9	15.7	0.5	1.1	63.7	0.03a	0.63	0.55b	0.68
70	2X	7.4	10.5	15.1	0.3	0.8	57.3	0.02b	0.69	0.62a	0.68
139	0X	4.7	4.7	9.1	0.2	0.5	31.9	0.03	0.50b	0.60	0.75
139	1X	7.2	10.4	12.3	0.3	0.9	54.9	0.03	0.83a	0.69	0.73
139	2X	5.2	5.3	9.9	0.4	0.5	35.3	0.02	0.50b	0.63	0.76

Continued

Table 2.3 Continued

		GRANBY SOIL									
Day	Conc	Actino	Gram+	Gram-	Protozoa	Fungi	Biomass	FB	G+/G-	SM	CP
		nmol g-1									
0	0X	8.1	11.2	24.4	0.3	1.2	74.6	0.03	0.45	0.46	0.47
0	1X	8.8	11.2	24.8	0.3	1.1	79.3	0.02	0.45	0.46	0.47
0	2X	8.3	9.8	21.9	0.3	1.0	67.6	0.02	0.45	0.47	0.48
7	0X	6.2	6.7b	18.0	0.1	0.8	50.5	0.02ab	0.37ab	0.44b	0.51ab
7	1X	8.8	11.1a	24.9	0.4	1.0	78.8	0.02b	0.45a	0.48a	0.48b
7	2X	8.2	7.5ab	24.4	0.3	1.1	67.8	0.03a	0.32b	0.43b	0.53a
21	0X	8.1	10.7	23.7	0.3	1.1	73.6	0.03	0.45	0.50	0.52b
21	1X	9.2	12.2	26.4	0.4	1.1	86.4	0.02	0.46	0.52	0.49c
21	2X	8.3	9.5	24.8	0.3	1.2	74.6	0.03	0.39	0.50	0.55a
42	0X	8.4	12.3	22.8	0.3	0.9	74.2	0.02	0.54a	0.53	0.51ab
42	1X	8.1	10.5	23.3	0.4	0.8	76.7	0.02	0.45b	0.54	0.50b
42	2X	8.8	12.9	24.1	0.4	1.2	80.8	0.03	0.53a	0.53	0.52a
70	0X	7.9	11.0	22.0ab	0.3a	0.9	70.6ab	0.02	0.50	0.55b	0.52
70	1X	8.4	11.6	24.0a	0.4a	1.0	81.7a	0.02	0.48	0.57a	0.51
70	2X	5.4	7.9	15.0b	0.0b	0.6	47.7b	0.02	0.53	0.57ab	0.52
139	0X	6.3	8.9	18.0	0.3	0.9	58.2	0.02	0.50	0.58	0.53
139	1X	7.0	8.5	19.7	0.4	0.8	65.0	0.02	0.43	0.57	0.57
139	2X	7.0	10.0	19.5	0.4	0.7	64.4	0.02	0.52	0.62	0.55

Continued

Table 2.3 Continued

		JORY SOIL									
Day	Conc	Actino	Gram+	Gram-	Protozoa	Fungi	Biomass	FB	G+/G-	SM	CP
		nmol g-1									
0	0X	4.9	5.7ab	22.9	0.6	1.5ab	55.8ab	0.05	0.25b	0.46b	0.77
0	1X	6.4	11.6a	23.5	0.3	1.9a	78.8a	0.04	0.49a	0.63a	0.67
0	2X	3.4	4.8b	15.5	0.0	0.7b	38.3b	0.03	0.29b	0.52b	0.65
7	0X	5.9a	10.9a	25.7a	0.1	1.7	73.2a	0.04	0.43	0.61	0.73b
7	1X	5.7ab	9.7ab	23.5ab	0.1	1.6	73.1a	0.04	0.41	0.62	0.77ab
7	2X	4.4b	6.6b	18.6b	0.0	1.2	50.7b	0.04	0.35	0.59	0.77a
21	0X	4.6b	6.6b	19.6	0.0	1.3	53.3b	0.04	0.33	0.59	0.81
21	1X	7.3a	13.0a	30.0	0.2	1.9	95.1a	0.04	0.43	0.66	0.82
21	2X	6.9ab	12.1a	28.1	0.1	1.8	85.9ab	0.04	0.44	0.68	0.82
42	0X	7.6	13.0	30.0	0.1b	2.5a	99.6	0.05a	0.43	0.68	0.82b
42	1X	6.6	11.5	27.0	0.3a	1.6b	86.2	0.04b	0.43	0.67	0.84ab
42	2X	7.3	12.0	28.7	0.1ab	2.4a	96.5	0.05a	0.42	0.71	0.86a
70	0X	5.6	10.7	22.5	0.0b	1.4	72.4	0.04	0.48b	0.73a	0.79b
70	1X	6.1	10.6	24.8	0.3a	1.6	78.7	0.04	0.43c	0.68b	0.86a
70	2X	7.0	15.0	28.8	0.1b	2.0	98.6	0.04	0.52a	0.76a	0.83a
139	0X	6.0	10.1	26.5	0.2	1.6	78.2	0.04	0.37	0.70	0.89b
139	1X	5.6	9.5	22.5	0.0	1.2	71.8	0.03	0.41	0.72	0.95a
139	2X	6.9	12.4	31.8	0.1	1.9	91.4	0.04	0.40	0.74	0.93a

†Values in a column followed by the same letter are not significantly different at P<0.05 with in a sampling day and taxonomic group (FB = fungal/bacterial ratio, G+/G- = Gram-positive/Gram-negative ratio, SM = SAT/MONO, CP=CY/PRE).

FIGURES

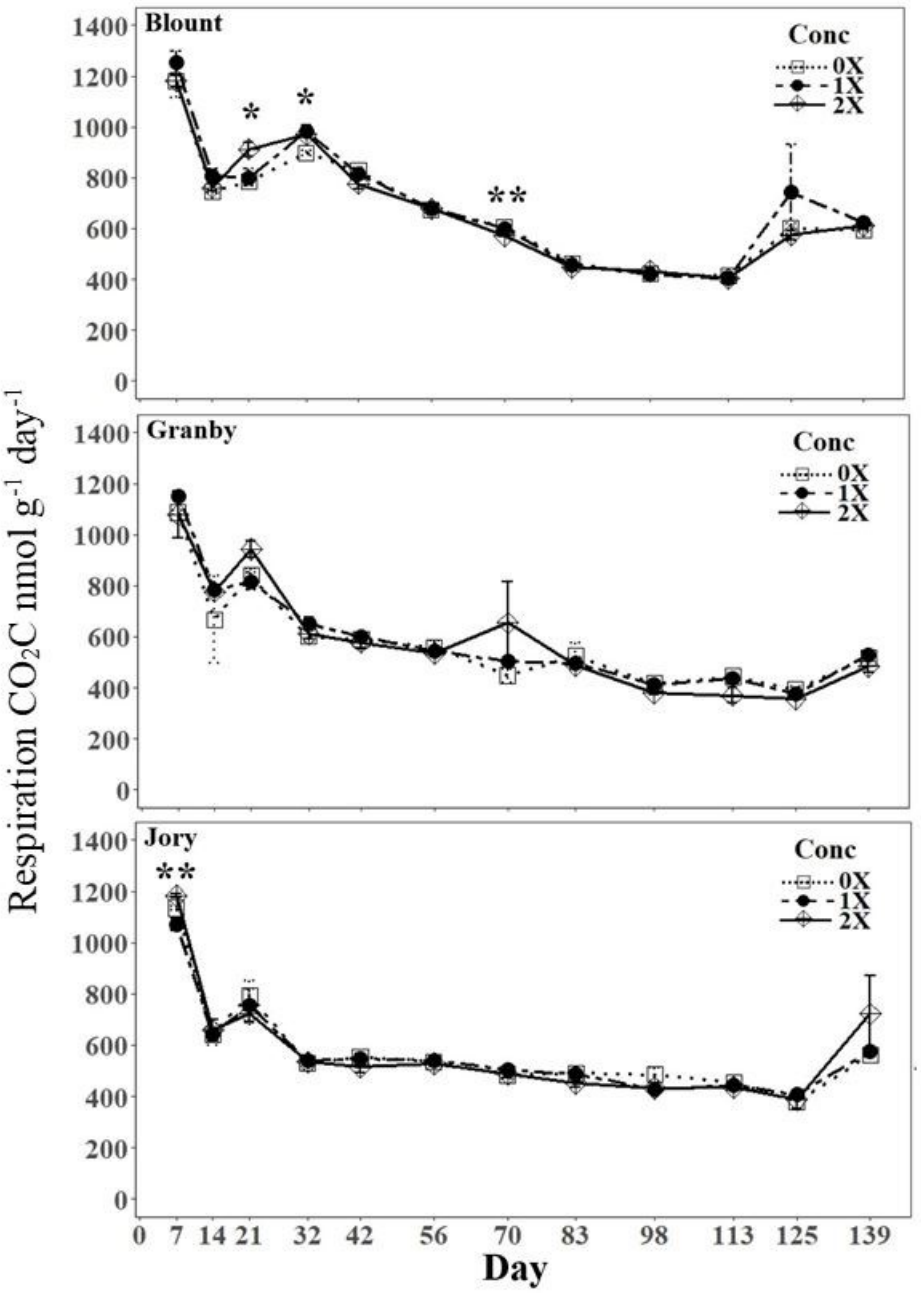

Figure 2.1 Effect of AMPA Concentration on Respiration over Time.

Error bars indicate standard error. (* P<0.05, ** P<0.01)

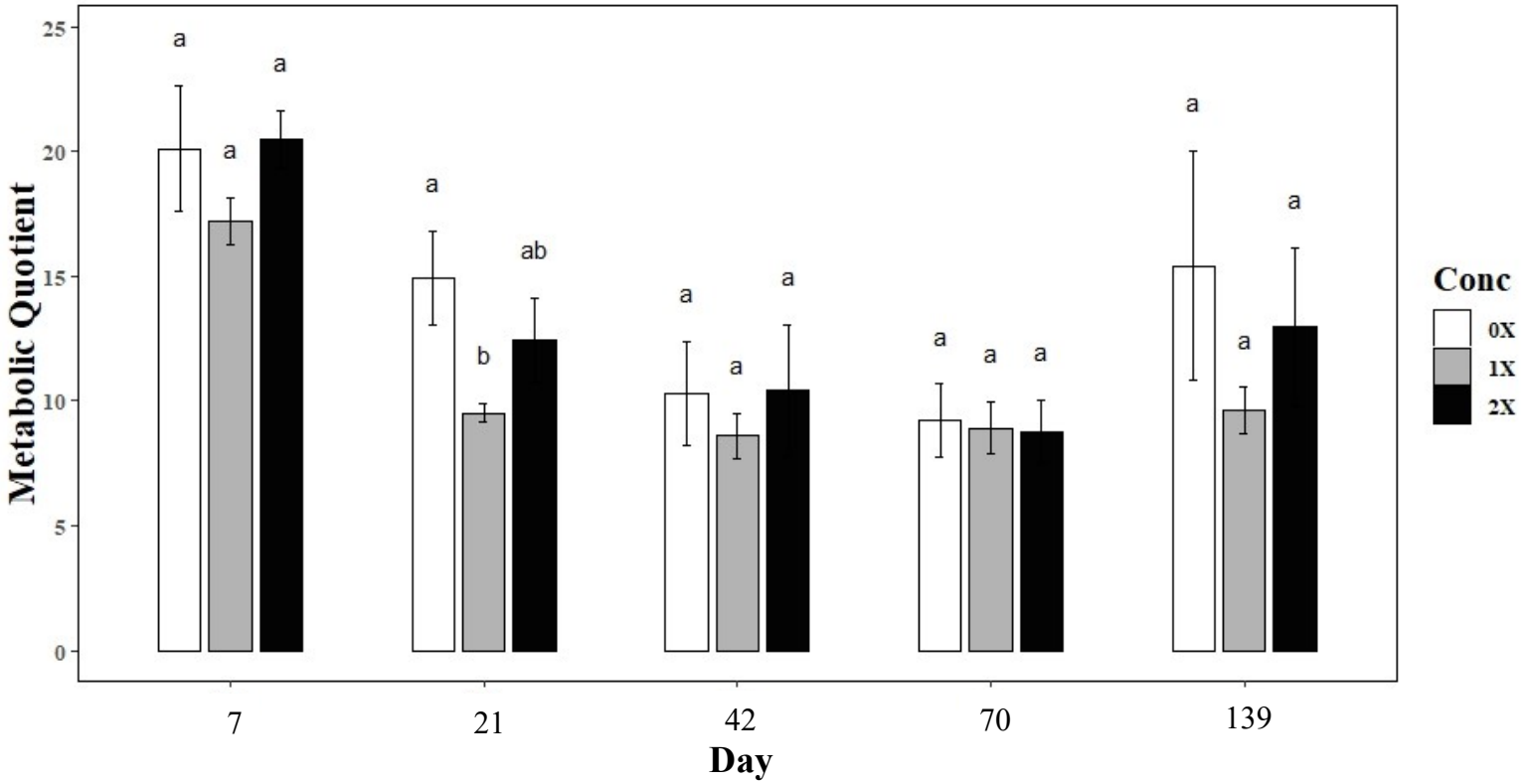

Figure 2.2 Effect of AMPA Concentration on Metabolic Quotients.

Error bars indicate standard error. Bars with the same letter within a sampling day are not significantly different at $P<0.05$.

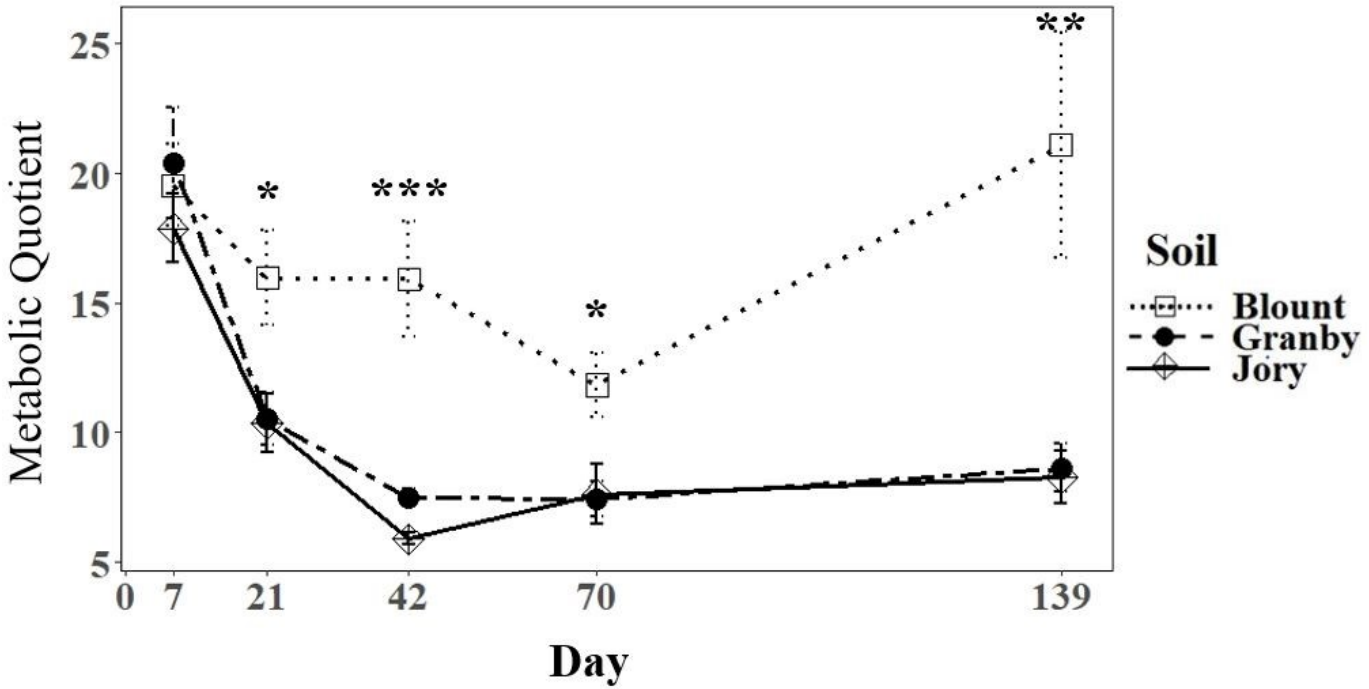

Figure 2.3 Effect of Soil Type. Averaged across AMPA Concentration, on Metabolic Quotient over Time. (* P<0.05, ** P<0.01, *** P<0.001). Error bars indicate standard error.

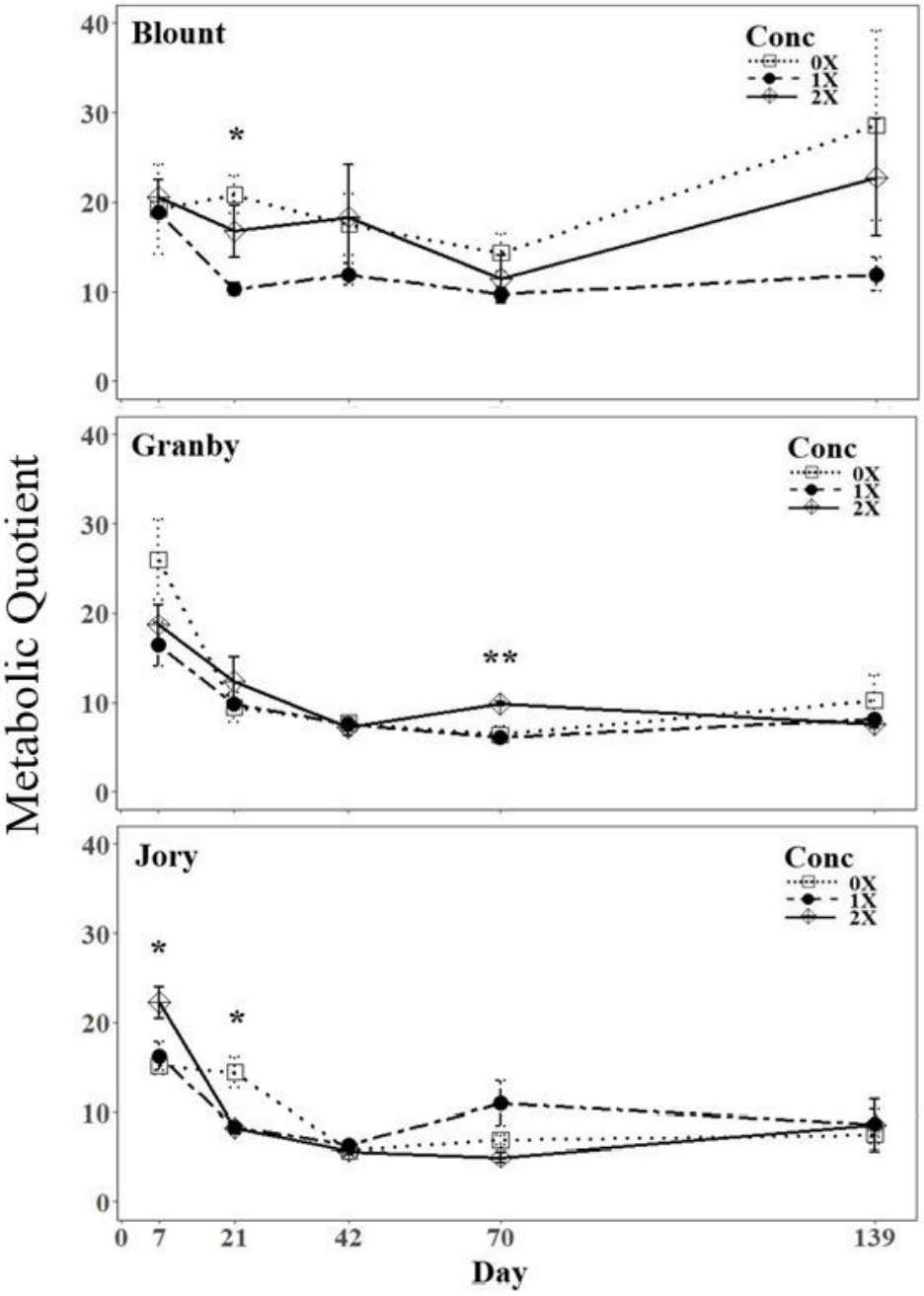

Figure 2.4 Effect of AMPA Concentration on Metabolic Quotient for Soils over Time. (*P<0.05, ** P<0.01). Error bars indicate standard error.

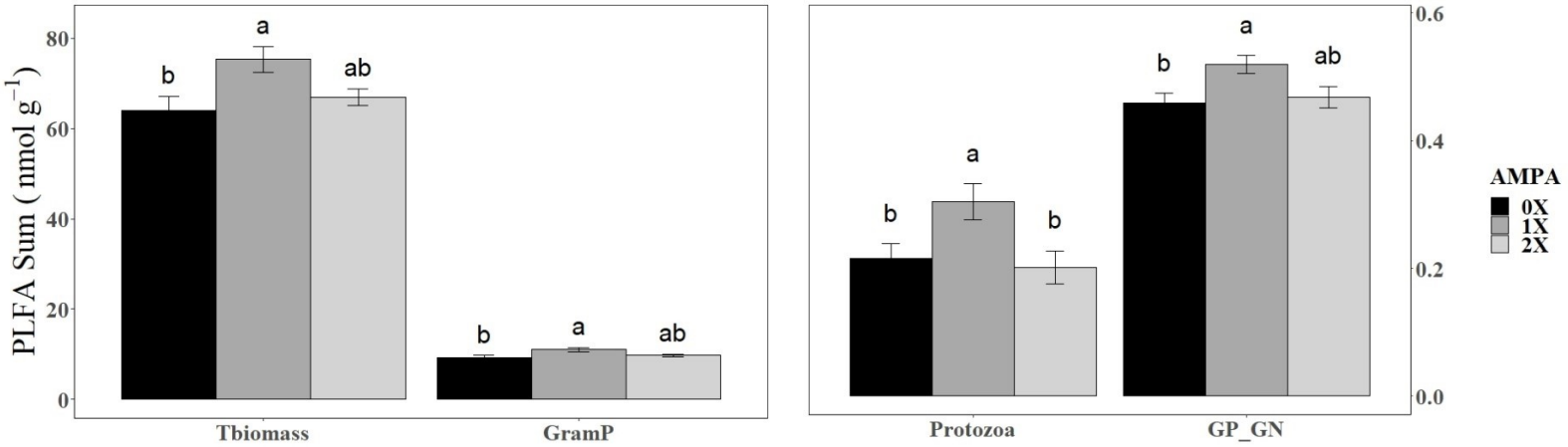

Figure 2.5 Effect of AMPA Concentration on PLFA Taxonomic Biomarkers.

Bars with the same letters within a taxonomic group are not significantly different at $P<0.05$.

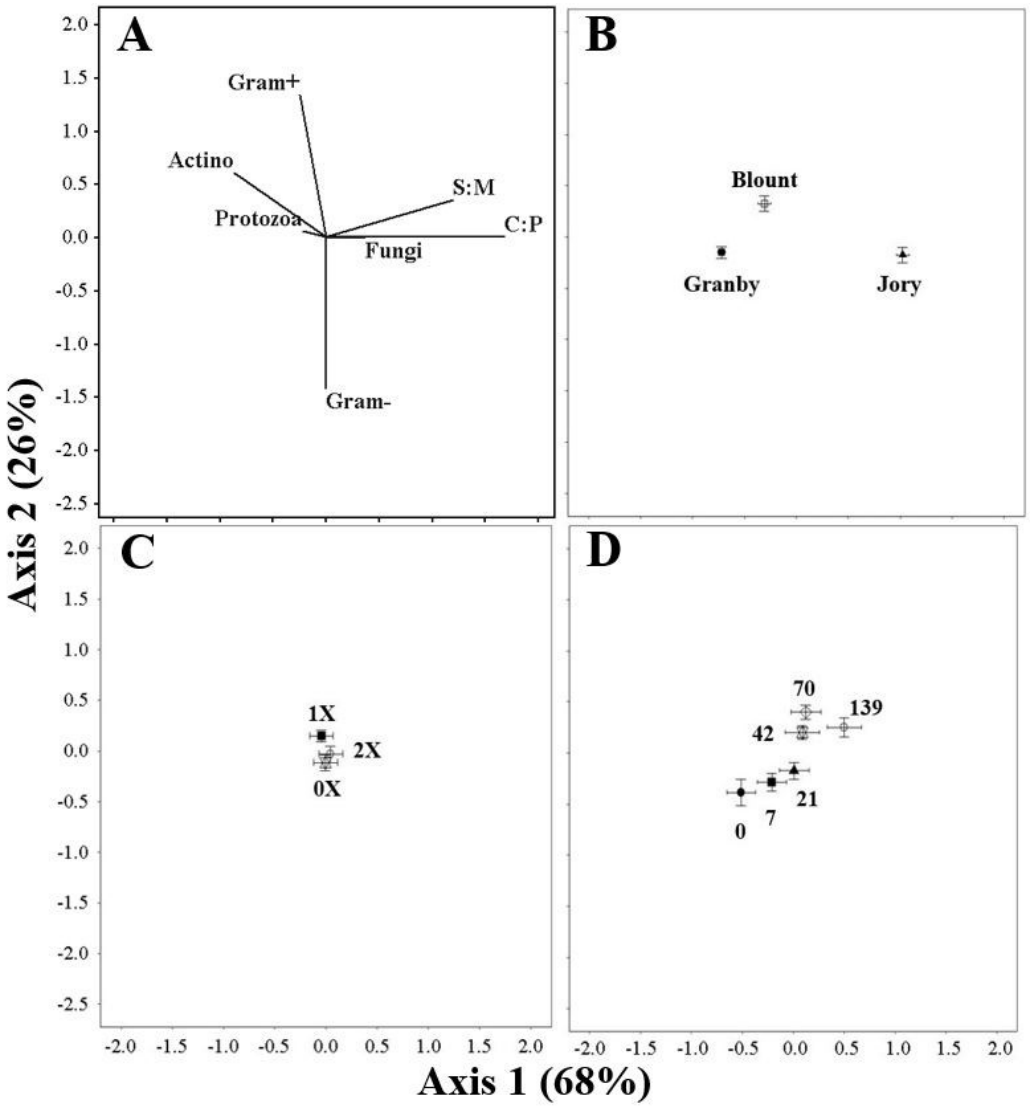

Figure 2.6 Non-metric Multidimensional Scaling (NMS) Joint Plot and Means of NMS Scores, for Soil, AMPA Concentration, and Sampling Day.

(A) Joint plot overlay shows vectors based on relative abundances of taxonomic groups and stress markers. (B), (C), and (D) show means and standard error of NMS scores for soils, AMPA concentration, and sampling days, respectively.

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CHAPTER 3: EXTRACTABLE AMPA IN RELATION TO MICROBIAL RESPONSES

ABSTRACT

Aminomethylphosphonic acid (AMPA) is an intermediate metabolite of the widely used herbicide glyphosate. Few studies have focused on the effects of AMPA in isolation from glyphosate on soil microorganisms. The negative effects on soil microorganisms found in glyphosate studies may be due in part to AMPA. These effects include increased fungal pathogens, decreased beneficial bacteria, shifts in community composition, and decreased microbial growth and biomass. Soil type has been found to have a significant impact on glyphosate bioavailability to soil microorganisms, and persistence in soil. The objective was to determine AMPA's extractability in diverse soils over time in tandem with the microbial response after amending soils with AMPA in the absence of glyphosate. The experimental design was a 3 X 3 factorial that had the following treatments: three soil types (Jory silty clay loam, Blount silt loam, or Granby loamy sand) and three AMPA rates 0.0 nmol g^{-1}, 9.01nmol g^{-1}, or 18.01 nmol g^{-1}; 0X, 1X, or 2X of typical in situ rates, respectively. The soils had no known history of exposure to glyphosate. Destructive sampling took place at Days 0, 7, 21, 42, 70, and 139, followed by phospholipid fatty acid (PLFA) analysis. Respiration was measured at approximately 14-day intervals. Using 0.1 M KH_2PO_4 as a chemical extractant, this study found that low pH, high organic carbon, high Fe/Al oxides, and high clay reduce extractability. Extraction efficiency was highest in the Granby 2X treatment (30%) and lowest in the Jory 1X treatment (8.5%). The results of this study do not provide evidence that KH_2PO_4 extractability correlates with AMPA bioavailability consistently enough to

use extractability as an estimate of bioavailability. No significant correlations were found between extractable AMPA and respiration or metabolic quotient. Contrary to expectations, Jory soil showed a greater number and more significant PLFA correlations than either Granby or Blount soils, despite lower extractability. Only weak to moderate negative correlations were found in Jory soil with Gram+ bacteria, fungi, sulfate-reducing bacteria, protozoa, the fungal/bacterial ratio, and the CY/PRE ratio, and it is likely these correlations were due to the progression of incubation time not AMPA bioavailability.

INTRODUCTION

Aminomethylphosphonic acid (AMPA) is an intermediate metabolite produced by degradation of glyphosate (Rueppel et al., 1977), and industrial and household phosphonates (Studnik et al., 2015). The presence of AMPA in the environment has significantly increased, due to the dramatically increased use of glyphosate since 1997, with introduction of glyphosate tolerant cropping by Monsanto (Benbrook, 2016). AMPA has been detected frequently in soils, sediment, ditches, drains, precipitation, and streams in the United States (Battaglin et al., 2014), along with glyphosate, and is commonly found at the outlets of wastewater treatment plants (Grandcoin et al., 2017).

In addition to the many studies indicating negative impacts on human health by glyphosate (Bai and Ogbourne, 2016), some research indicates that AMPA can also negatively impact human health (Mañas et al., 2009; Kwiatkowska et al., 2017; Woźniak et al., 2018), aquatic organisms (Guilherme et al., 2014; de Brito Rodrigues et al., 2019), and earthworms (Domínguez et al., 2016).

There is relatively little information available on the effects of AMPA in the absence of glyphosate on soil microorganisms. von Mérey et al. (2016) found that AMPA temporarily stimulated soil N transformations at very high concentrations, but no effect at lower concentrations. The conclusion was that AMPA should have no negative effects on soil microorganisms.

Simonsen et al. (2008) suggests that the negative effects on soil microorganisms found in glyphosate studies may be due in part to AMPA. These effects include

increased fungal soil pathogens (Kremer et al., 2005), decreased plant beneficial soil bacteria (Zobiole et al., 2011; Newman et al., 2016), and shifts in community composition towards increased fungi (Wardle and Parkinson, 1992; Krzyśko-Łupicka and Orlik, 1997). Gomez et al. (2009) and Schnurer et al. (2006) found application of glyphosate negatively affected soil microbial biomass, growth, respiration, and metabolic activity.

Since AMPA adsorbs strongly to soil, particle facilitated transport and preferential flow are the main mechanisms by which AMPA can leach or runoff from soil into the aquatic environment (Bergström et al., 2011).

AMPA adsorption is increased in soils with high clay content (Okada et al., 2016), high amounts of aluminum and iron oxides, high amounts of organic carbon (Borggaard and Gimsing, 2008), and low pH (Zhang et al., 2015; Okada et al., 2016; Sidoli et al., 2016). Barja and Alfonso (2005) report that AMPA forms inner-sphere complexes with iron oxide through the phosphonate moiety. Soil pH has been found to be the most important factor in determining AMPA adsorption (Sidoli et al., 2016; Zhang et al., 2015).

In environmental science, bioavailability is defined as the availability of a chemical for biological assimilation (Alexander, 2000). A counteracting factor is strong adsorption, which decreases the bioavailability and microbial degradation of AMPA (Schnurer et al., 2006; Al-Rajab and Schiavon, 2010; Sviridov et al., 2015). In addition, due to the process of aging, where covalent bonds are formed with soil constituents and sequestered into soil nanopores, contaminants become less bioavailable to soil

microorganisms over time (Kelsey et al., 1997; Alexander, 2000). Decreased bioavailability increases AMPA persistence in the environment where it can become a long-term source of water contamination (Grandcoin et al., 2017).

For AMPA to be biologically available, it must either be in soil solution or easily desorbed (Alexander, 2000). Desorption may occur from changes in soil chemistry, such as rainfall after phosphate fertilizer application, that causes competition with AMPA for sorption sites (Simonsen et al., 2008). In addition, soil microorganisms have various mechanisms for facilitating the desorption of organic chemicals from soil surfaces (Alexander, 2000). Microorganisms that solubilize inorganic phosphate exist that can release inorganic phosphorus (P) (Sato and Comerford, 2006; Walter Osorio, 2011). P solubilizing microorganisms excrete organic acids and anions which desorb phosphorus from soil surfaces (Walter Osorio and Habte, 2013). AMPA binds to soil surfaces via the phosphate moiety, forming both monodendate and bidendate inner sphere complexes with metal oxides. (Barja and Afonso, 2005). Monodendate complexes are somewhat reversible by ligand exchange, whereas bidendate complexes are not reversible (Basta, 2017). P solubilizing organisms could drive desorption of AMPA via ligand exchange, or there could be other mechanisms for facilitating desorption. *Bacillus megaterium* is a known P solubilizing organism (Walter Osorio, 2011), and a known AMPA degrading microorganism (Quinn et al., 1989).

AMPA degrading microorganisms, must have the specific enzymes necessary to break the stable C-P bond (Quinn et al., 1989; McGrath et al., 2013). The specific C-P

lyase enzyme used to break the C-P bond in glyphosate and AMPA may be different (Jacob et al., 1988).

The few studies that exist on AMPA persistence and degradation differ widely on the half-life of AMPA (Bai and Ogbourne, 2016). This is likely due to differences in soil types and environmental conditions used for these studies (Bai and Ogbourne, 2016; Nguyen et al., 2018). Table 3.1 lists half-lives determined by various field and laboratory studies. Additionally, Giesy et al. (2000) found the median half-life of AMPA in 8 US and 3 Canada sites was 145 days, and according to Battaglin et al. (2014) the range of half-life is between 60 and 240 days.

Microbial bioremediation techniques could be effective for removal of AMPA from the environment (Zhan et al., 2018). Knowledge of the specific organisms capable of degrading phosphonates, and soil characteristics that affect bioavailability and degradation would be useful in developing effective bioremediation methods (Ermakova et al., 2008; Zhan et al., 2018).

Vigorous chemical extraction techniques are commonly required by government regulations to assess risk of a chemical contaminant in soil (Kelsey et al., 1997). However, this vigorous extraction does not simulate what is actually bioavailable in soil (Kelsey et al., 1997; Simonsen et al., 2008).

Bioavailability tests for soil pollutants can be expensive, time consuming, and lack precision, and a chemical procedure that predicts bioavailability would therefore be useful (Kelsey et al., 1997). Results by Kelsey et al. (1997) suggest that it is possible to predict bioavailability of an organic compound to a specific organism in a specific soil by

selecting a chemical extractant that approximates the uptake of the organic compound by the specific organism. However, extraction by a single solvent is unlikely to predict bioavailability of multiple species, in different soils (Kelsey et al., 1997).

Most research on AMPA has been in combination with glyphosate. Furthermore, there is little information on how soil type affects AMPA bioavailability to microorganisms. Therefore, the objectives were to determine: 1) the chemical extractability of AMPA in different soil types; and 2) the potential of extractable AMPA to reflect its bioavailability for microorganisms.

MATERIALS AND METHODS

Soils

Three diverse soils that have been used for agricultural purposes, were chosen to obtain a range of textures and mineralogy. The Blount silt loam (fine, illitic, mesic Aeric Epiaqualf) was obtained from a farm in Delaware County, Ohio, that had been organically managed since 2003. Soil was sampled in mid-May 2018, from an area maintained as sod (mixed grass/legume) that has had very little agricultural activity in the past 30 years other than being mowed or hayed. Prior to 1990, the area had been conventionally farmed with corn and soybeans (J. Dickinson, personal communication, January 22, 2020). The second soil was a Granby (sandy, mixed, mesic Typic Endoaquaoll) from a hardwood forest in Henry County, Ohio, sampled in early July 2018. The third soil was a Jory silty clay loam (fine, mixed, active mesic Xeric Palehumult) from Douglas Fir forest near Corvallis, Oregon, sampled in March 2018.

All three soils are typically used for field crop agriculture (USDA National Agricultural Statistics Service Cropland Data Layer, 2008-2019; Soil Survey Staff, 2020) and in general are soils where glyphosate could be expected to be applied. However, none of the sites that were sampled likely had received any glyphosate. The Jory and Granby soils came from unmanaged forest that has never been cultivated as far as is known. The Blount soil site did have conventional crop management until 1990 (after which it was under organic management that used no chemicals), but that was before the widespread implementation of glyphosate tolerant cropping that started in the mid-1990s. Further evidence for non-exposure to glyphosate is that that both extractable glyphosate and AMPA were undetectable in these soils.

Soil sampling sites were chosen to avoid previous glyphosate exposure. Approximately 15 samples were taken from each site at a 10-cm depth and composited. The samples were passed through a 2-mm sieve and stored at 4°C.

Experimental Design

The experiment was a laboratory incubation that had 3 replications and a 3 x 3 factorial design with three soil types (Blount, Granby, Jory), and field relevant AMPA concentrations: 0 (0x), 1 (1X), or 2 (2X) μg AMPA g^{-1} (0.0, 9.01, or 18.01 nmol g^{-1}, respectively) . The duration exceeded the half-life of AMPA reported in most of the literature on laboratory studies (Mamy et al., 2005; Bergström et al., 2011; Zhang et al., 2015; Bento et al., 2016). The AMPA concentrations were based on field relevant

concentrations found in studies in agricultural soils (Scribner et al., 2007, Aparacio et al., 2013; Silva et al., 2018).

Field moist soils were mixed with the appropriate solution of AMPA and deionized water to attain 0, 9.01, 18.01 nmol AMPA g^{-1} soil and 67% field moisture capacity in each soil treatment. A period of 24 hours was allowed for the AMPA to adsorb at 20°C. For each experimental unit, 40 g (dry weight basis) of spiked soil was placed into a 55 mm diameter Wheaton jar.

The experimental units were placed into air-tight 1 L Mason jars along with a beaker of sodium hydroxide solution to trap carbon dioxide, and incubated at 20°C. At days 0, 7, 21, 42, 70, and 139, three replicates of each treatment were removed from incubation, and soil samples were stored at -20°C, until PLFA and AMPA extraction analysis could be performed. At day 7, 14, 21,32, 42, 56, 70, 83, 98, 113, 125, and 139 the sodium hydroxide traps were replaced, and the traps were titrated with 0.01 M HCl to determine CO_2-C.

Soil Physical and Chemical Analyses

Soil pH was determined on 1 to 1 soil:deionized water ratio and measuring with a glass membrane electrode (Sparks et al., 1996). Total carbon and nitrogen content were measured on an elemental analyzer (Carlo Erba CHN EA 1108, now Thermo Fisher Scientific, Waltham, MA). Field capacity of the soil was determined by saturating soil, weighing, then oven drying samples at 105°C, and re-weighing. Sand, silt, and clay percentages were referenced from previous studies of the same sites (Lee et al., 2007; Nye et al., 2014; Dick et al., 2018). The average iron and aluminum oxide content from

soil pedons sampled as Blount, Granby, and Jory soil series was obtained from the National Cooperative Soil Survey (2020). The Soil Characterization Database contains data from two extraction methods: 1) the dithionite citrate extraction method, which represents both the crystalline and amorphous iron and aluminum oxide content, and 2) the ammonium oxalate extraction method which extracts only amorphous iron and aluminum oxide, according to McKeague and Day (1965).

Monopotassium phosphate (KH_2PO_4) was chosen as the extractant based on its ability to displace phosphate (Miles and Moye, 1988), and potentially simulate AMPA bioavailability in soil (Simonsen et al., 2008). Alkaline extractants such as KOH, NaOH, and borate have higher recovery rates in clay soil and organic matter than KH_2PO_4, but do not simulate bioavailability (Simonsen et al., 2008). They can also extract humic substances which interfere with HPLC analysis (Miles and Moye, 1988; Aubin and Smith, 1992; Todorovic et al., 2013). Extraction using deionized water would more closely reflect what would be bioavailable in a natural system (Simonsen et al., 2008). However, deionized water was not used as an extractant in this study based on previous lab results which indicated that AMPA recoveries using deionized water were below the limit of detection (0.5 ppb).

Extractable AMPA was determined by a procedure adapted from Miles and Moye (1988). In brief, the procedure was to shake 3 g of soil for 15 min in 12 mL of a 0.1 M solution of monopotassium phosphate (KH_2PO_4), then centrifuge at 3000 rpm for 30 minutes. The supernatant was filtered through 0.30 µm filter paper.

This procedure was repeated 3 times, and the supernatants combined to analyze soil for the presence of glyphosate or AMPA, prior to the experiments. To ensure that the control soils contained no glyphosate or AMPA, 3 replicate samples of each soil were analyzed prior to the experiment, and analysis confirmed that the samples contained no detectable glyphosate or AMPA.

For the incubation study samples, the extraction procedure was performed a single time. The rationale for using a single extraction was to 1) reduce variance, 2) more closely model what might be bioavailable to microorganisms, 3) use a simpler, less time-consuming method that would be more feasible for a commercial laboratory to use.

The supernatant from extraction 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. Concentrations of AMPA were reported in ppb.

Respiration and PLFA Analyses

CO_2-C respiration in each jar was measured by adding $BaCl_2$ to the NaOH trap and titrating to a pH of 7 with 0.01M HCL solution, then calculating evolved CO_2-C from the difference in HCL solution required between the blanks and the sample, according to the method described in Horwath and Paul (1994). The rate of respiration on each sampling day was calculated by dividing the evolved CO2-C by the number of days since last replacing the NaOH trap.

Phospholipid fatty acid methyl esters (PLFA) were determined by the method described in Frostegård et al, (1993). Briefly, phospholipids were extracted from approximately 2 g of soil using a one-phase chloroform, methanol and citrate buffer extractant (Bligh and Dyer, 1959). The upper organic phase was then fractionated into neutral, glycolipid and phospholipid components using silica acid columns. Alkaline methanolysis using 0.2M methanolic KOH was performed to convert the phospholipids to methyl-esters (Chowdhury and Dick, 2012). Nonadecanoic methyl ester (19:0) was added as an analytical standard to allow GC peak areas to be converted to a molar basis. Fatty acids were analyzed on an HP 5890 gas chromatograph with peak identification software (MIDI Inc., Sherlock MIS).

Table 3.2 shows the phospholipid fatty acids that were used for PLFA analysis. A total of 65 fatty acids that were present in at least 5% of samples were summed to represent total microbial biomass. Nineteen fatty acid biomarkers that were documented in the literature as distinctly belonging to a given taxonomic group, were categorized as Gram-positive bacteria, Gram-negative bacteria, actinomycetes, and saprophytic fungi. The fungal to total bacterial ratio was calculated as saprophytic fungi divided by the sum of Gram-positive bacteria, Gram-negative bacteria, and actinomycetes. Three ratios were calculated as stress markers: the ratio of total monounsaturated to total saturated fatty acids, the ratio of total cyclopropyl fatty acids to total monounsaturated precursors, and the ratio of Gram-positive to Gram-negative bacteria.

Nomenclature used for the fatty acids is the total number of carbon atoms, followed by a colon, followed by the number of double bonds. For Gram-positive

bacteria, the number of double bonds is followed by "a" or "i" which refer to anteiso and iso branching. For other taxonomic groups, the number of double carbon bonds is followed by "w" to indicate the methyl or "ω" end of the molecule, then the position of the double bond from the methyl end of the molecule. The suffix "c" indicates *cis* geometry. "ME" indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule, and "cyclo" indicates cyclopropyl fatty acids.

Metabolic quotient was calculated as the respiration rate (nmol CO_2-C g^{-1} soil g^{-1} day) divided by total microbial biomass PLFA absolute abundance (nmol g^{-1} soil g^{-1}).

Statistical Analyses

Extraction efficiency was calculated using day 0 extractable AMPA as percent of applied AMPA. Applied AMPA was 9.01 nmol g^{-1} for the 1X treatment, and 18.01 nmol g^{-1} for the 2X treatment.

Data analysis was conducted using R v. 3.5.1. Repeated measures ANOVA and multiple comparisons using Tukey's Honestly Significant Difference (HSD) correction ($P<0.05$) and simple correlations were conducted in R (version 3.5.1).

The difference between the control (0X) treatment and the AMPA 1X and 2X treatments were calculated for respiration rate as nmol CO_2-C g^{-1} day^{-1} and PLFA absolute concentration data as nmol g^{-1}. These data were used in correlation tests with extractable AMPA (nmol g^{-1}).

Correlation tests between respiration rate, PLFA and extractable AMPA were performed using Pearson's correlation. Input data was checked for normality using the Shapiro-Wilk test.

RESULTS

Soil Properties

Soil properties are shown in Table 3.3. Blount soil, from an organically managed farm, had the highest pH at 6.29 and the lowest total carbon and total nitrogen at 2.1% and 0.2% respectively. Granby soil had the highest total carbon and total nitrogen at 4.5% and 0.4% respectively, and a pH of 5.67. Jory soil was similar to Granby in pH (5.34), total carbon (4.3%), and total nitrogen (0.3%). Both Granby and Jory were from unmanaged wooded areas. Both Blount and Jory soils were high clay at 41% and 52% respectively, and low in sand at 11% and 14% respectively. Granby had high sand at 86% and low clay at 3.1%.

Extractable AMPA

AMPA extraction efficiency at time zero as percent of applied AMPA is presented in Table 3.4. Recovery ranged from a low of 8.5% for the Jory 1X treatment, to a high of 30% for the Granby 2X treatment. AMPA recovery was slightly higher for the 2X concentration than the 1X concentration in each of the three soils, and for both concentrations was lowest in Jory soil and highest in Granby soil.

Figure 3.1 shows the extractable AMPA (nmol g^{-1} dry soil) for each of the six treatments. For the 2X concentration, there were significant differences at each sampling day between the three soils in extractable AMPA, except for Granby and Blount on day 0. Granby soil consistently had the highest levels, and Jory soil had the lowest levels.

For the 1X concentration, the Blount and Granby soil were not significantly different at $P<0.05$, except for days 70 and 139. There was a slight increase in extractable AMPA for Granby soil on day 139, but it was not significantly different than day 70 at $P<0.05$. Jory 1X soil was significantly less than Granby 1X on all sampling days, and significantly less than Blount 1X, on all sampling days except day 139.

Correlation of Extractable AMPA with Microbial Properties

There was a weak correlation between extractable AMPA and the metabolic quotient in the Blount soil ($r=0.36$; $P<0.1$). There were no significant correlations between extractable AMPA and respiration or metabolic quotient overall, or for each AMPA concentration.

Table 3.5 shows the correlation for each soil/AMPA concentration treatment, versus respiration rate and metabolic quotient. There were no significant correlations between extractable AMPA and respiration rate or metabolic quotient. The highest correlation ($r=0.39$) was on respiration for the Jory 2X treatment but was not significant at $P<0.1$.

Although 46 PLFAs were tested for correlation, only correlations that had at least a weak correlation ($r>0.3$, $P<0.1$) between certain PLFAs and extractable AMPA

concentrations are presented in Table 3.6. These included Gram-positive bacteria, monounsaturated fatty acids, saprophytic fungi, protozoa, sulfate-reducing bacteria, and all four stress ratios, FB, SAT/MONO, CY/PRE, and Gram-positive/Gram-negative. The strongest correlation with extractable AMPA (-0.60, $P<0.01$) was for the 17:1 w8c fatty acid, indicative of sulfate-reducing bacteria in the Jory 2X treatment. There was also a weak to moderate negative correlation (-0.48, $P<0.05$) in the Jory 1X treatment with sulfate-reducing bacteria, and a weak to moderate negative correlation (-0.52, $P<0.05$) in the Jory 2X treatment with the CY/PRE stress ratio. The remaining 14 correlations were weak, and except for three instances in Jory 1X, they were negative correlations. There were no correlations in Blount 1X or Granby 1X treatments. Correlations were most prevalent in the Jory soil treatments. There were no significant correlations when averaging across all soils or averaging across all AMPA concentrations.

DISCUSSION

AMPA overall extraction efficiency was low (8.5-30%) in comparison to other studies using stronger extractants. R^2 values for the best fit exponential curves were low, especially for the sandy Granby soil, due to the variability of sample measurements. The highest R^2 values for the best fit curves were in the high clay Blount and Jory soils, at the higher 2X treatment, due to lower variability in these measurements.

Vigorous chemical extraction using alkaline extractants such as NaOH, KOH, or borate used in the Table 3.1 studies could have increased recovery % of AMPA.

However, these recoveries would not have been indicative of how much AMPA was bioavailable (Simonsen et al 2008).

Soil properties that promote AMPA adsorption are low pH (Sidoli et al., 2016; Zhang et al., 2015; Okada et al., 2016), high organic carbon (Borggaard and Gimsing, 2008), high clay (Okada et al., 2016), and high iron and aluminum oxides (Borggaard and Gimsing, 2008; Barja and Alfonzo, 2005). However, since the soils used did not have a singular change among these factors, it is difficult to identify the relative importance of these factors in controlling AMPA adsorption. For example, in the study by Sidoli et al. (2016), pH was found to be the most important factor, but variations in organic carbon were not tested due to variable results found in the literature.

Comparing the results of extractable AMPA at the 2X concentration, the sandy, low clay Granby soil showed the highest levels of extractable AMPA, and the Jory soil showed the lowest levels of extractable AMPA. This is consistent with the literature, since Jory had high levels of all properties (low pH, high organic carbon, Fe/Al oxides, clay) that are expected to increase adsorption. Since AMPA in Granby soil was more extractable than AMPA in Blount soil, this would indicate that clay content was what differentiated between the extractability of AMPA in Blount and Granby soils at the 2X concentration.

However, when comparing extractable AMPA results for the 1X concentration, there was no significant difference between Blount and Granby soil, while Jory again showed the lowest extractability. A possible reason for this difference is that anion

exchange sites in organic carbon in the Granby soil might have become fully saturated in the 2X treatment, making extraction in the Granby 2X treatment easier.

If bioavailability correlates with extractability of AMPA, that means that AMPA should have been the most biologically available in the Granby soil at the 2X concentration, and equally bioavailable in Blount and Granby at the 1X concentration, while least bioavailable in the Jory soil. The soil with the lowest bioavailable AMPA should manifest the lowest negative or positive microbial responses. However, the opposite appeared to be true, when examining correlations between extractable AMPA and various PLFAs, the Jory soil in both AMPA concentration treatments showed a greater number and more significant PLFA correlations than either Granby or Blount soils.

The negative correlations found with Gram+ bacteria, fungi, sulfate-reducing bacteria, protozoa, the fungal/bacterial ratio, and the CY/PRE ratio, indicate that as AMPA extractability decreased, these taxonomic groups increased. However, all of these groups could be expected to have relative increases as the incubation progressed due to decreased carbon availability. Anaerobic conditions that tend to increase as incubations progress could explain the increase in sulfate-reducing bacteria. The correlation could therefore be an association due to time, and not due to AMPA bioavailability.

Another possible reason for more prevalent correlations in Jory soil could be differences in the prevalence of microorganisms that solubilize inorganic phosphate in the three soils. Environmental conditions such as phosphate deficiency can trigger the production of organic acids and anions produced by these organisms to release phosphate

(Walter Osorio and Habte, 2013). Phosphorus content was not measured, but old, weathered soils such as Jory tend to be lower in available phosphorus than younger soils such as Blount and Granby (Walker and Syers, 1976; Turner et al., 2007), and could have resulted in a higher prevalence of inorganic phosphate solubilizing microorganisms.

CONCLUSIONS

The findings of this study are consistent with the literature, that low pH, high organic carbon, high Fe/Al oxides, and high clay reduce AMPA extractability, and therefore increase adsorption.

However, the results of this study do not provide evidence that KH_2PO_4 extractability reflects AMPA bioavailability. No significant correlations were found between extractable AMPA and respiration or metabolic quotient across all soils. Based on extractability results, AMPA should have been the most biologically available in the Granby soil at the 2X concentration, and equally available in Blount and Granby at the 1X concentration, while least bioavailable in the Jory soil. However, based on correlations between extractable AMPA and PLFA, the opposite of what was expected about bioavailability appeared to be true. Although most correlations were weak, Jory soil in both treatments showed a greater number and more significant PLFA correlations than either Granby or Blount soils. Weak to moderate negative correlations were found between AMPA extractability and Gram-positive bacteria, fungi, sulfate-reducing bacteria, protozoa, the fungal/bacterial ratio, or the CY/PRE ratio. However, all of these taxonomic groups could be expected to have relative increases as the incubation

progressed due to decreased carbon availability, and it is most likely that the correlations were an association with time, and not due to AMPA bioavailability.

TABLES

Table 3.1 Predicted AMPA Half-Life across Diverse Soil Types and Environmental Conditions after Glyphosate Application to Soil (adapted from Bai and Ogbourne, 2016).

Soil Type	Soil Depth	pH	Clay	Org C	AMPA	Temp	Study Duration	References
	cm		-----%----		$t_{1/2}$ (days)	C°	(days)	
Loam	0-10	5.6	15.3	1.91	36.9	†	42	Zhang et al.,(2015)
Loam	0-10	4.2	18.1	4.69	10	†	42	Zhang et al., (2015)
Sandy	0-2.5	6.5	13.3	2.7	32	14.3	810	Simonsen et al., (2008)
Loam	0-10	7.1	26.5	1.9	26	30	30	Bento et al., (2016)
Clay	0-30	7.2	46.5	4.4	34.9	20	64	Bergström et al., (2011)
Clay	30-60	7.4	56.1	0.0	97.6	20	64	Bergström et al., (2011)
Sandy	0-30	7.4	7.7	2.0	60.4	20	64	Bergström et al., (2011)
Sandy	30-60	6.4	0.0	1.0	93.1	20	64	Bergström et al., (2011)
Loam	0-10	8.2	9.3	20	25	28	140	Mamy et al., (2005)
Loam	0-10	8.2	37.7	1.65	34	28	140	Mamy et al., (2005)
Loam	0-10	7.6	23.5	0.95	75	28	140	Mamy et al., (2005)

†Field conditions in Zhejiang and Guangdong Province, China.

Table 3.2 Fatty Acids and Microbial Taxonomy and Stress Markers.

Taxonomic Group	Specific PLFA Markers	References
PLFA Biomarkers		
Gram-Positive Bacteria	15:0i, 15:0a, 16:0i, 17:0i, 17:0a	(Vestal and White, 1989); (Willers et al., 2015)
Gram-negative Bacteria	16:1 w7c, 17:0 cyclo w7c, 19:0 cyclo w7c, 18:1 w7c	(Zelles et al., 1997)
Actinomycetes	16:0ME, 17:0ME, 18:0ME	(Vestal and White, 1989), (Federle et al., 1986)
Fungi (Saprotrophic)	18:2w6c, 18:3 w6c,	(Zelles et al., 1997), (Vestal and White, 1989), (Frostegård et al., 2011)
Protozoa	20:4 w6c, 20:3 w6c	(Moore-Kucera and Dick, 2008), (Vestal and White, 1989)
Sulfate-Reducing	17:1 w8c	(Willers et al., 2015)
Microbial Stress Indicators		
SAT/MONO Ratio	Sum of Saturated / Sum of Monounsaturated	(Bossio and Scow, 1998)
Saturated PLFAs	14:0, 15:0, 17:0, 16:0, 18:0, 20:0	(Willers et al., 2015), (Zelles et al., 1997)
Monounsaturated PLFAs	16:1 w7c, 18:1 w7c, 18:1 w9c, 17:1 w8c, 18:1 w5c, 20:1 w9c, 16:1 w9c, 16:1 w5c	(Bossio and Scow, 1998)
CY/PRE Ratio	(cy17:0+cy19:0)/(16:1w7c+18:1w7c)	(Kieft et al., 1997), (Moore-Kucera and Dick, 2008)
Total Microbial Biomass	Sum of all extractable PLFAs (65)	(Frostegård et al., 1991), (McKinley et al., 2005)
Total Bacteria	Sum of Gram-Positive, Gram-Negative, Actinomycetes, 15:0, 17:0	(Moore-Kucera and Dick, 2008)
Fungal/Bacterial Ratio	Fungi/Total Bacteria	(Federle et al., 1986), (Frostegård and Bååth, 1996)
Gram-Positive/Gram-negative Bacterial Ratio		(Willers et al., 2015)

Table 3.3 Soil Chemical Properties.

Soil	Site/ Management.	pH	Total C	Total N	Sand	Silt	Clay	Total Fe†	Total Al†	Amorphous Fe†	Amorphous Al†
			----------%----------								
Blount	Organic farm	6.29	2.1	0.2	11.0	48.0	41.0	1.48	0.16	0.42	0.11
Granby	Woodlot	5.67	4.5	0.4	86.0	10.9	3.1	0.25	0.13	0.47	0.11
Jory	Douglas Fir Forest	5.34	4.3	0.3	14.0	34.0	52.0	6.10	0.78	0.95	0.72

†. Adapted from National Cooperative Soil Survey (2020)

Table 3.4 Extraction Efficiency of AMPA Immediately after Amending Soils with AMPA on Day 0 of the Incubation (0.1M KH_2PO^4).

Soil	AMPA Concentration	Recovery	Standard Error
		----------%----------	
Blount	1X	23.88	0.59
Blount	2X	26.22	1.32
Granby	1X	27.96	2.38
Granby	2X	30.06	1.44
Jory	1X	8.49	1.47
Jory	2X	9.60	0.54

Table 3.5 Correlation Coefficients (r-value) between Extractable AMPA (nmol g^{-1}) and Respiration (nmol g^{-1} day^{-1}) or Metabolic Quotient for each Treatment across all Sampling Days (no significant correlations at $P<0.1$).

Microbial Property	Blount 1X	Blount 2X	Granby 1X	Granby 2X	Jory 1X	Jory 2X
Respiration Rate	-0.23	0.00	-0.12	-0.12	0.04	0.39
Metabolic Quotient	-0.20	-0.31	0.12	0.23	0.02	0.05

Table 3.6 Correlations (r-value) between Extractable AMPA for Individual PLFAs, Sums, or Ratios within a Soil and AMPA Concentration Treatment.†

PLFA†	Blount 1X	Blount 2X	Granby 1X	Granby 2X	Jory 1X	Jory 2X
18:0 iso (Gram+)	0.27 ns ††	-0.25 ns	-0.22 ns	0.05 ns	0.14 ns	-0.44 *
20.1 w9c (Mono)	0.07 ns	-0.07 ns	0.23 ns	0.35 ns	0.40 *	0.14 ns
18.2 w6c (Fungal)	-0.13 ns	-0.06 ns	-0.14 ns	0.10 ns	-0.02 ns	-0.46 *
18.3 w6c (Fungal)	-0.11 ns	-0.43 *	0.06 ns	0.20 ns	0.42 *	0.09 ns
17.1 w8c (Sulfate-Reducer)	-0.15 ns	-0.33 ns	0.14 ns	0.16 ns	-0.48 **	-0.60 ***
Fungi	-0.19 ns	-0.44 *	-0.07 ns	0.18 ns	0.23 ns	-0.38 ns
Protozoa	0.03 ns	-0.25 ns	0.05 ns	0.26 ns	-0.43 *	-0.41 *
Fungal/Bacterial	-0.20 ns	-0.49 **	-0.27 ns	0.13 ns	0.29 ns	-0.43 *
SAT/MONO	0.03 ns	-0.19 ns	-0.23 ns	-0.49 **	0.40 *	0.13 ns
CY/PRE	-0.21 ns	-0.31 ns	-0.29 ns	0.23 ns	-0.40 *	-0.52 **
Gram+/Gram-	-0.01 ns	0.08 ns	0.32 ns	-0.42 *	0.32 ns	0.07 ns

†Only PLFAs that had at least one correlation with P<0.1, are shown out of a total of 30 individual FAMEs, 11 taxonomic groups, or 5 stress marker/ratios were tested.

††Not significant at P<0.1

*P< 0.1

**P < 0.05

***P< 0.01

FIGURES

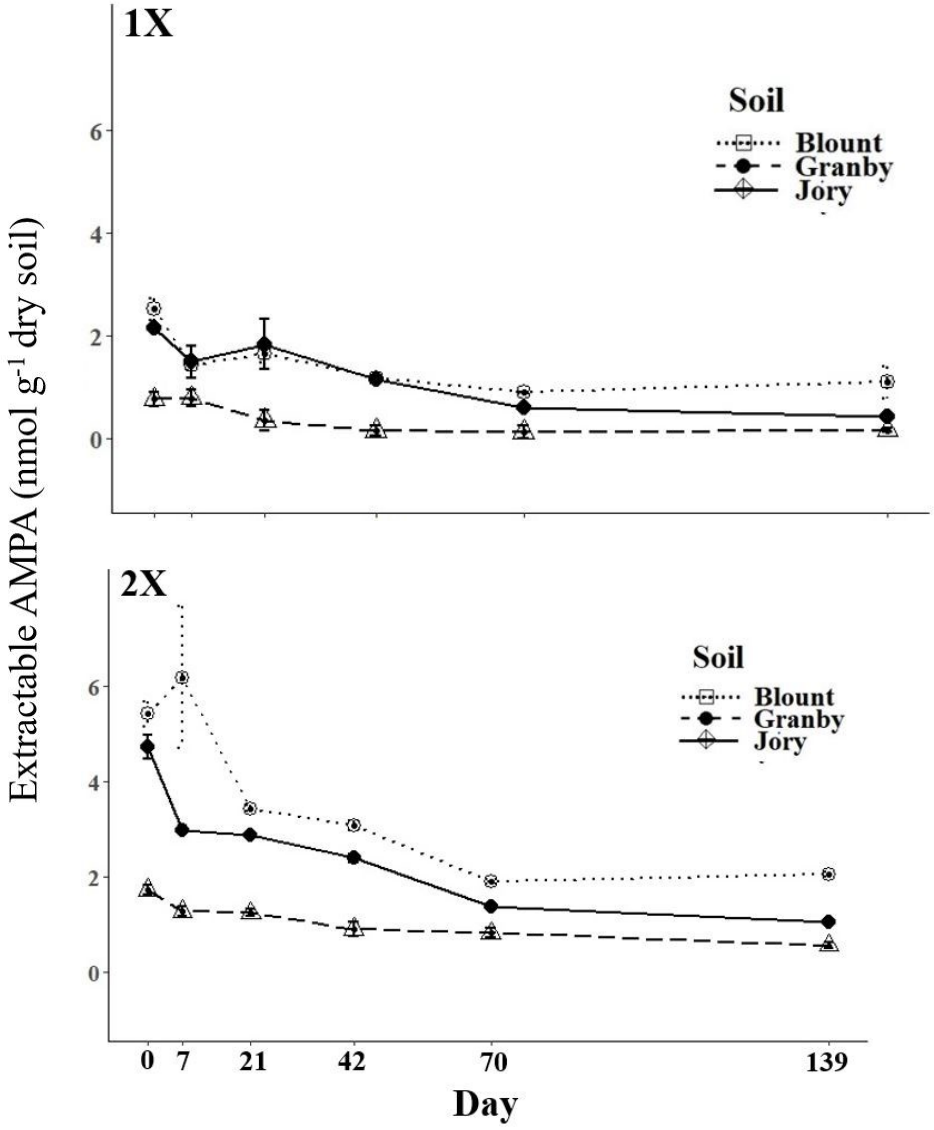

Figure 3.1 Comparison of KH_2PO^4 Extractable AMPA between soils, for the 1X and 2X AMPA Concentrations over Time (error bars = standard error).

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