THESIS

BIOCHAR EFFECTS ON SOIL MICROBIAL COMMUNITIES AND RESISTANCE OF ENZYMES TO STRESS

Submitted by

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ABSTRACT

BIOCHAR EFFECTS ON SOIL MICROBIAL COMMUNITIES AND RESISTANCE OF ENZYMES TO STRESS

Biochar, a product of the pyrolysis of organic material, has received wide attention as a means to improve soil fertility and crop productivity, absorb pollutants in soil, and sequester carbon to mitigate climate change. Little information exists on the short- and longer-term effects of biochar on soil microbial communities and enzyme activities, relative to other organic amendments such as manure. Therefore, the objectives of this study were to determine the short and longer terms effects of biochar amendment on soil microbial communities, arbuscular mycorrhizal (AM) fungi, and enzyme activities in a semi-arid soil. Secondly, due to the porosity and surface area of biochar, enzyme stabilization on biochar was assessed to determine if biochar could prohibit the loss of extracellular enzyme activity following a denaturing stress.

In a field study, a fast pyrolysis biochar (CQuest) derived from oak and hickory hardwood was applied to calcareous soil of replicate field plots in fall 2008 at a rate of 22.4 Mg ha^{-1} (dry wt.). Other plots received dairy manure (42 Mg ha^{-1} dry wt), a combination of biochar and manure at the aforementioned rates, or no amendment (control). Plots were annually cropped to corn (*Zea maize* L.). Surface soils (0-30 cm) were sampled directly under corn plants in late June 2009 and early August 2012, one and four years after treatment application, and assayed for microbial community fatty acid profiles and six extracellular enzyme activities involved in C, N, and P cycling in soil. In addition, AM fungal colonization was assayed in corn roots in 2012.

Relative to the manure treatment, biochar had no effect on microbial community biomass, community structure, extracellular enzyme activities, or root colonization of corn by AM fungi.

Manure amendment increased microbial biomass in 2009, when total FAME concentration was 2.3-fold and 2.6-fold greater in manure and biochar plus manure treatments, respectively, compared to non-amended soil. The concentration of the AM fungal FAME biomarker (16:1ω5c) was significantly reduced by the manure treatments in 2009 (P=0.014) but not in 2012. In 2009, principle components analysis (PCA) revealed shifts in the FAME structure of the soil microbial community in response to the manure treatments. However, the effects of manure on microbial biomass and community structure were short-lived, as no effects were observed in 2012.

A laboratory incubation study was conducted to determine whether biochar would stabilize extracellular enzymes in soil and prohibit the loss of potential enzyme activity following a denaturing stress such as microwaving. Soil was incubated in the presence of biochar (0, 1, 2, 5, or 10% by weight) and exposed to increasing levels of microwave stress. Results showed that extracellular enzymes responded differently to biochar rate, stress level and their interactions. The main effect of stress level was highly significant (P<0.0001) on the potential activities of β-glucosidase, β-D-cellobiosidase, N-acetyl-β-glucosaminidase, and phosphatase enzymes. Potential activity of leucine aminopeptidase was significantly affected by biochar rate (P=0.016), stress level (P<0.0001), and their interaction (P=0.0008). In addition, potential activity of β-xylosidase was marginally affected by biochar's interaction with stress level (P=0.066). The potential activity of these two enzymes were reduced after a 36-day incubation in the presence of biochar. For β-xylosidase, intermediate application rates (1 and 5 %) of biochar prevented a complete loss of this enzyme's potential activity after soil was exposed to 400 (1% biochar treatment) or 1600 (5% biochar treatment) J microwave energy g^{-1} soil. In conclusion, this study demonstrated that land application of biochar may not affect microbial community

biomass, potential activities of soil enzymes, or AM fungal biomass in soil, or alter community structure, presumably because of the type of biochar employed in this study. Both biochar and manure added carbon to soil, but microorganisms were responsive to manure rather than biochar. While biochar had no effect on potential activity of soil enzymes in the field study, the laboratory incubation study revealed that biochar has the potential to stabilize extracellular enzymes and prohibit the loss of potential enzyme activity in soil when exposed to a denaturing stress.

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CHAPTER 1

INTRODUCTION

Biochar is a form of black carbon (C) created by thermal degradation of organic material (e.g., wood, manure, leaves, etc.) in a low or zero oxygen environments (pyrolysis). It is distinguished from charcoal and similar materials by its use as a soil amendment (Lehmann and Joseph, 2009). Depending on the temperatures reached during pyrolysis and the initial properties of the feedstock used, biochar's chemical and physical properties may vary (Keech et al., 2005; Gundale and DeLuca, 2006). For example, high-temperature pyrolysis (>550°C) produces biochars that generally have high surface areas (> 400m^2 g^{-1}) (Downie et al., 2009; Keiluweit et al., 2010), are highly aromatic and therefore recalcitrant to decomposition (Singh and Cowie 2008), and are good adsorbents (Mizuta et al., 2004; Lima and Marshall, 2005). Low temperature pyrolysis (< 550°C), on the other hand, favors greater recovery of C and nutrients (e.g. N, K, and S) that are increasingly lost at higher temperatures (Keiluweit et al., 2010). Low-temperature biochars, which have a less-condensed C structure, are expected to have greater reactivity in soils than higher temperature biochars (Steinbeiss et al., 2009). Furthermore, when pyrolyzed, plant species with many large diameter cells in their stem tissues can lead to greater macropore quantities in biochar particles. Larger numbers of macropores can, for example, enhance the ability of biochar to adsorb larger molecules such as phenolic compounds (Keech et al., 2005).

Because of its macromolecular structure which may contain aromatic C, biochar is more recalcitrant to microbial decomposition than uncharred organic matter (Baldock and Smernik, 2002). Biochar is thought to have long mean residence times in soil, ranging from 1,000 to 10,000 years, with 5,000 years being a common estimate (Skjemstad et al., 1998; Swift, 2001; Krull et al., 2003). However, its recalcitrance and physical nature represent

significant obstacles to the quantification of long-term stability (Lehmann, 2007). Figure 1.1 shows the concept of pyrolysis of feedstock when biochar is produced and the heat and multitude of gaseous components that are captured to produce energy.

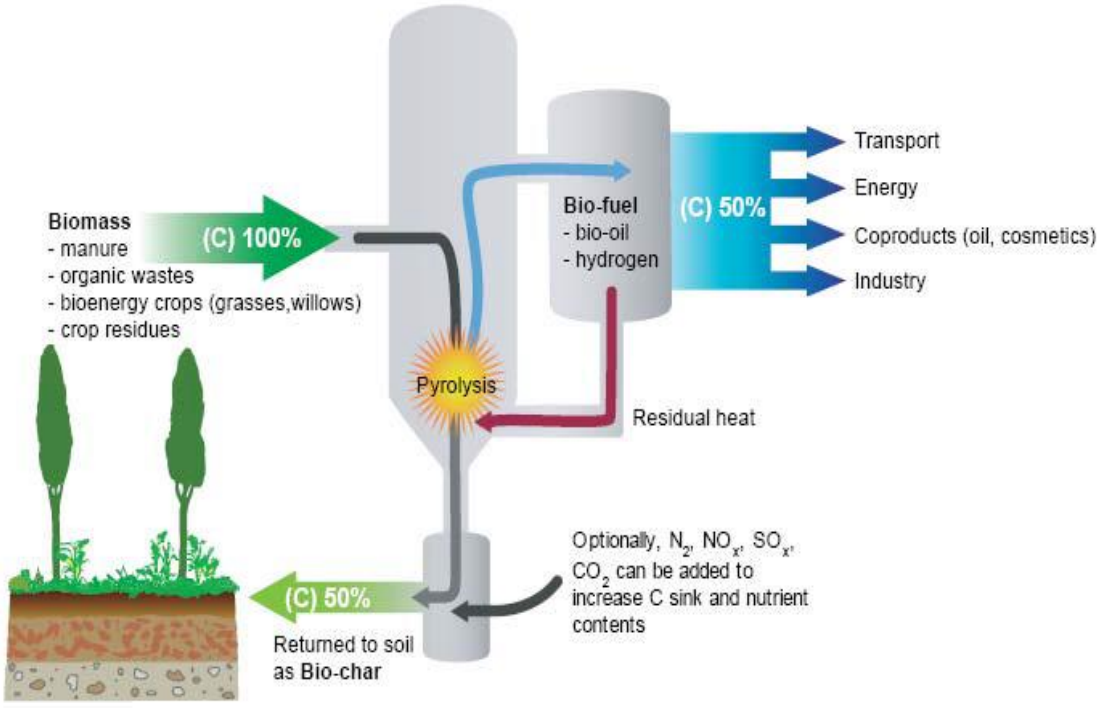

Figure 1.1 Schematic representation of pyrolysis processes of organic materials to produce biochar along with biogases (Lehmann 2007).

Recently, biochar application to soil is being considered as a mechanism for long-term storage of C and can play a key role in climate change mitigation by reducing atmospheric CO_2 concentrations (Lehmann et al., 2006). Biochar may also reduce soil greenhouse gas emissions, such as nitrous oxide (N_2O) or methane (CH_4). By trapping these gases in pores (Clough et al., 2010; Gaunt and Lehmann, 2008), biochar may contribute to the decrease or a slowing of the increase in global warming. Biochar is also being examined as a means to improve soil fertility

as observed in Terra Preta soils. These soils feature over 70 times more biochar than the surrounding soil and have a high level of sustained fertility (Glaser et al., 2001). Biochar application has been shown to improve soil fertility by increasing the pH of acid soils (Van Zwieten et al., 2010a), increasing water retention (Rondon et al., 2006), reducing nutrient leaching (Laird et al., 2010) or adsorbing cations and natural organic matter (Liang et al., 2006).

While biochar has been studied for its effects on soil chemical and physical properties, biochar's effects on soil microbial communities are understudied. In a one of the few published studies, Thies and Rillig (2009) explained that biochar could have a positive effect on microbial community biomass by providing a habitat, where bacteria and fungi could escape from predators, as well as providing substrates to meet many of their diverse C, energy, and nutrient needs. Also, some research has suggested that changes in soil microbial community composition may occur due to biochar as observed in Amazonian Dark Earths (Terra Preta). These soils have greater microbial biomass, and in some cases, greater diversity than the surrounding area (Kim et al., 2007).

The effects of biochar on soil fungi and especially mycorrhizal fungi have received greater attention. Pioneering studies, conducted primarily in Japan, provided evidence that biochar can have positive effects on the abundance of arbuscular mycorrhizal (AM) fungi (Ishii and Kadoya, 1994), and Warnock et al. (2007) found that AM and ectomycorrhizal (EM) fungi, the most commonly occurring types of mycrorrhizal fungi, were positively affected by biochar. However, positive effects are not universal as other have found that biochar can negatively affect AM fungi abundance (Gaur and Adholeya, 2000; Birk et al., 2009; Warnock et al., 2010).

Microbially-produced extracellular enzymes are important for organic matter decomposition and nutrient cycling for microbial as well as plant uptake. Some of these enzymes

are thought to be short-lived unless they are protected from proteolysis (Burns 1982; Nannipieri et al., 2002). Biochar, with its capacity to absorb a wide range of organic and inorganic molecules, may provide a mechanism to protect these enzymes (Bailey et al., 2010; Jin, 2010; Lehmann et al., 2011), but in general, there is a poor understanding of the possible effects of biochar on these enzymes. Currently, few studies have been conducted to examine the relationship between biochar and soil enzyme activity. Bailey et al. (2010) studied the effects of fast pyrolysis switchgrass biochar on four soil enzymes (β-glucosidase, N-acetyl-β-glucosaminidase, lipase, and leucine aminopeptidase) to determine if biochar would consistently modify soil enzyme activities. Their results showed that biochar had inconsistent and unpredictable effects on soil enzymes depending on the enzyme and the method they used. Jin (2010) showed that the activity of two C cycling enzymes (β-D-glucosidase and β-D-cellobiosidase) decreased after biochar addition to soil.

My thesis addressed the effects of biochar amendment on soil microbial communities and enzymes involved in C, N and P cycling. Furthermore, because biochar has the potential to sorb enzymes, my thesis focused on the effect of biochar on enzyme stabilization when soils are subsequently exposed to a denaturing stress (i.e., microwave stress). Field and laboratory studies were conducted to examine the main objectives of my thesis. The field study addressed the first objective, which was to 1) determine the short- and longer-term effects of biochar amendment on soil microbial communities, AM fungi, and enzyme activities. The laboratory study addressed the second objective, which was to 2) assess the potential for biochar to stabilize soil enzymes and increase enzyme resistance to microwave stress. Because biochar is a carbon source and its physical structure provides microbial habitats, I hypothesized that biochar would increase soil microbial biomass and shift microbial community structure towards greater relative abundances

of AM fungi. I also hypothesized that biochar would decrease extracellular enzyme activities in soil because of its ability to absorb these enzymes, but that enzyme activities would be resistant to stress disturbance in the future due to the stabilizing effect of biochar. I tested these hypotheses by analysis of variance tests with an α level of 0.05.

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CHAPTER 2

SHORT AND LONGER- TERM EFFECTS OF BIOCHAR AND MANURE AMENDMENT ON SOIL MICROBIAL COMMUNITIES, AM FUNGI, AND ENZYME ACTIVITIES

INTRODUCTION

Biochar is a form of black carbon (C) created by thermal degradation of organic material (e.g., wood, manure, leaves, etc.) in a low or zero oxygen environment (pyrolysis). It is distinguished from charcoal and similar materials by its use as a soil amendment (Lehmann and Joseph, 2009). As compared to higher temperatures (> 500^{o}C), when organic material undergoes pyrolysis at relatively low temperature (< 550°C), the resulting biochar has a greater recovery of C and nutrients (e.g. N, K, and S) that potentially can increase soil fertility when land applied (Steinbeiss et al., 2009). Biochars also have reactive surfaces that can sorb and exchange nutrients and native organic matter (Liang et al., 2006). Biochar's ability to enhance soil fertility has been demonstrated in tropical soils, where long-term biochar inputs have helpedcreate highly fertile soil known as Terra Preta, or Amazonian Dark Earth (Sombroek, 1966; Glaser et al., 2001). Yet, the fertility aspects of biochar land application are less understood in temperate climates, and especially semi-arid temperate climates.

Only a few studies have examined the effects of biochar amendment on temperate, semi-arid soils. Lentz and Ippolito (2012) studied the comparative effects of biochar vs. manure amendment on the chemical properties of calcareous soil in semi-arid temperate climate. The authors applied either 22.4 Mg ha^{-1} biochar or 42 Mg ha^{-1} manure and observed decreases in soil extractable Cu, Zn, P, K, Mg, Na, and NO_3-N with biochar compared to manure application. However, no data were collected on the response of soil microbial communities or enzymes in this field study.

Thies and Rillig (2009) hypothesized that biochar could have a positive effect on the biomass of microbial communities, by providing a habitat where bacteria and fungi could escape from predators as well as find substrates to meet many of their diverse C, energy, and mineral nutrient needs. Also, some research has suggested that changes in soil microbial community composition may occur due to biochar as observed in Terra Preta soil. Terra Preta soils have greater microbial biomass, and in some cases, greater diversity than the surrounding area (Kim et al., 2007). More specifically, pioneering studies have provided evidence that biochar can have positive effects on the abundance of arbuscular mycorrhizal (AM) fungi (Ishii and Kadoya 1994). In another study, both AM and ectomycorrhizal fungi were positively affected by biochar presence (Warnock et al., 2007). It has also been shown that AM colonization of wheat roots increased to 20-40% two years after Eucalyptus wood additions of 0.6-6 Mg ha^{-1}, while the colonization rate was 5-20% in controls (Solaiman et al., 2010). However, positive effects are not universal as other have found biochar additions to have negative effects on the abundance of AM fungi (Gaur and Adholeya, 2004; Birk et al., 2009; Warnock et al., 2010).

Equally as important to potential shifts in microbial community structure and function, the effect of biochar on extracellular enzymes is not well understood. Microbially-produced extracellular enzymes are important for decomposition of organic matter and cycling of nutrients for microbial as well as plant uptake. Biochar, with its capacity to absorb a wide range of organic and inorganic molecules, may affect enzymes by sorbing them and/or their substrates (Bailey et al., 2010; Jin, 2010; Lehmann et al., 2011). Currently, limited studies have been conducted to examine the relationship between biochar and soil enzyme activity. Bailey et al. (2010) studied the effects of biochar made from fast pyrolysis of switchgrass on four soil enzymes (β-glucosidase, N-acetyl-β-glucosaminidase, lipase, and leucine aminopeptidase) to determine if

biochar would consistently modify soil biological activities. Their results showed that biochar had inconsistent and unpredictable effects on soil enzymes.

It is important that biochar effects on soil biological properties be quantified, as microbial communities provide important supporting, regulating and provisioning soil ecosystem services (Comerford et al., 2013). In addition, microbial properties and enzyme activities are dynamic and highly sensitive to environmental change (Nannipieri et al., 2003), and thus changes in these properties might indicate potential long-term effects of biochar on soil nutrient cycling processes. Therefore, my objective was to determine the short- and longer-term effects of biochar amendment on soil microbial communities, AM fungi, and enzyme activities. My hypothesis was that biochar would increase soil microbial biomass and shift microbial community structure towards greater relative abundances of AM fungi, and that biochar would decrease extracellular enzyme activities in soil because of its ability to sorb these enzymes and/or their substrates.

MATERIAL AND METHODS

Study site, soil, and amendments

A long-term field study was established in fall 2008 near Kimberly, Idaho (42°31′N, 114°22′ W, elevation of 1190 m) to quantify the effects of a single biochar or manure application on crop productivity and soil quality. The soil was a Portneuf silt loam (coarse-silty, mixed superactive, mesic Durinodic Xeric Haplocalcids), pH 7.6, containing 20 % clay, 56% silt, 24% sand, 1.2% organic carbon, and having an 8.8% calcium carbonate equivalency. For 33 years prior to this study, the site was cropped to an alfalfa–corn–bean–grain rotation, and no manure had been applied since 1986. Additional details of the study site are described in Lentz and Ippolito (2012).

Manure and biochar chemical characteristics are presented in Table 1. Dairy cattle (*Bos* species) solid manure was obtained from unconfined piles from a local dairy. The material contained little or no straw bedding and comprised 55.3% solids at time of application. The biochar material was provided by Dynamotive Energy Systems (West Lorne, Ontario, Canada) and was marketed under the name CQuest. It was derived from oak and hickory hardwood sawdust and created by fast pyrolysis at 500°C. The biochar had an ash content of 14%, which was determined by using ASTM methods for wood charcoal (600°C). The biochar had an oxygen:carbon ratio of 0.22, a surface area of 0.75 m^2 g^{-1}, and its pH was 6.8. Additional details regarding the manure and biochar treatments are provided in Lentz and Ippolito (2012).

Experimental Design

The experimental design was a randomized complete block design with three replicates and four treatments (control, biochar, manure, and biochar plus manure). Plots were 4.6 m wide and 5.2 m long and included eight planted rows. Each plot was separated by a 1.5 m-wide. Due to limited biochar availability, it was not possible to enlarge the plots or add additional blocks. Treatments were applied once, in November 2008. Details of the field operations are provided in Lentz and Ippolito (2012) but in brief, the field was prepared by growing spring barley (*Hordeum vulgare* L.) in 2008 and moldboard plowing to a 20-cm depth after barley harvest. Solid manure was hand-applied to the soil surface on Nov. 21, 2008, at a rate of 42 Mg dry wt ha^{-1}. Three days later, biochar was hand-applied to appropriate plots at a rate of 22.4 Mg dry wt ha^{-1}, immediately after which all plots were rototilled to a depth of 15 cm. The field was roller harrowed on April 21, 2009, and Round-Up ready silage corn (*Zea mays* L.) (Monsanto, St. Louis, MO) was planted annually in May and harvested in October during the 2009-2012 study. Corn was managed with standard, conventional methods, which included spring applications of

urea N fertilizer and herbicides to control weeds, and sprinkler irrigation every 7 to 14 days to meet crop evapotranspiration requirements (Lentz and Ippolito, 2012).

Table 2.1 Selected chemical properties of biochar and manure applied to the experimental plots in November 2008. Data are from Lentz and Ippolito (2012).

Property	Units	Biochar	Manure
pH		6.8	8.8
EC	dS m^{-1}	0.7	13.4
Ash	%	14	ND[†]
Total C	%	66.2	26.4
Total N	%	0.32	2.15
Organic N	%	0.32	2.12
NO_3-N	mg kg^{-1}	1.5	80.6
NH_4-N	mg kg^{-1}	1.2	220
K	mg kg^{-1}	3400	13500
Ca	mg kg^{-1}	3700	22000
Mg	mg kg^{-1}	1500	8230
Na	mg kg^{-1}	200	3750
P	mg kg^{-1}	300	4080

[†]ND: Not Determined

Soil Sampling

Soils were sampled in late June 2009 and again in early August 2012. The 2012 sampling occurred at the R1/silking stage. In 2009, four cores (0-30 cm deep) were collected from each plot and composited into one bag. In 2012, two cores (0-30 cm deep) were collected from one plant that was in the 5th row of the plot and 2 meters into the plot. The two cores were collected directly under the plant, one core on each side of the plant, in order to collect roots along with soil. Samples were stored on ice and transported in ice chests to the laboratory for analysis. Soils from 2009 were cryopreserved at -80°C. Soils from 2012 were sorted by hand to remove roots,

which were stored at 4°C for staining of AM fungi. Soil from 2012 was then divided and either stored at -20°C for microbial community and enzyme analyses or air-dried and stored at room temperature for chemical analyses.

Soil Chemical Analyses

Soil pH was determined using the method of Thomas (1996) using a 1:1 soil:deionized water extract. Total C and N were determined by dry combustion (Nelson and Sommers, 1996; Thermo-Finnigan FlashEA1112; CE Elantech Inc., Lakewood, NJ). A 2M KCl extract (Mulvaney, 1996) method was used to determine NO_3-N and NH_4-N content. Inorganic C analysis using a modified pressure-calcimeter method (Sherrod et al., 2002) and then total organic C was determined by difference between total and inorganic C.

Soil Enzymes

Potential soil enzyme activities were analyzed according to the florescence enzyme protocols described in Steinweg et al. (2013) and Bell et al. (2013). The six enzymes assayed were three C-cycling enzymes (β-D-cellobiosidase, β-glucosidase, and β-xylosidase), 1 C/N cycling enzyme (N-acetyl-β-glucosaminidase), 1 N cycling enzyme (leucine aminopeptidase), and 1 P cycling enzyme (phosphatase).

All assays included appropriate blanks, where soil suspensions were incubated in the absence of enzyme substrate. To correct for quenching of fluorescence signals by soil, biochar, or manure, standard curves were prepared for each replicate plot soil sample by incubating soil suspensions in the presence of increasing concentration of 4-methylumbelliferone (MUB) or 7-amino-4-methylcoumarin (MUC) standard. Incubations were conducted at 25ºC. Fluorescence measurements of the plates were read on a Tecan Infinite® M200 microplate (Tecan, Mannedorf, Switzerland) at 365 nm excitation and 450 nm emission wavelengths.

Fatty Acid Methyl Ester (FAME) Extractions

Fatty acids were extracted from soil samples using the ester-linked FAME method (Schutter and Dick, 2000). In brief, 3 g soil was extracted with 0.2 M methanolic KOH during a 37°C, 1-h incubation with periodic mixing followed by pH neutralization with 1.0 M acetic acid. Hexane was then added to divide the FAMEs into an organic phase, followed by centrifugation (480×g for 10 min). The hexane layer was transferred to a clean tube and each tube was placed under a gentle stream of N_2 to evaporate of hexane. Finally, each sample was redissolved in hexane and transferred to a gas chromatograph (GC) vial and 20 μg of internal standards (13:0 and 19:0) were added before the hexane solvent was completely evaporated.

Samples were then sent to the University of Delaware, where FAMEs were dissolved in 1:1 hexane: methyl-tert-butyl ether and analyzed on a HP 6890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) equipped with a 25 m×0.2 m fused silica capillary column (5% diphenyl-95% dimethylpolysiloxane) and a flame ionization detector. FAMEs were identified and their relative peak areas determined by the MIS Aerobe method of the MIDI system (Microbial ID, Newark, DE).

AM Fungal Root Colonization

Arbuscular mycorrhizal fungal colonization of corn roots were quantified in 2012 using the magnified gridline intersect method detailed in McGonigle et al. (1990). Fine, fibrous roots were hand-picked from soil samples and washed in water to remove all particulates. Root staining followed the method outlined by the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) (http://invam.wvu.edu/methods/mycorrhizae/staining-roots). Roots were placed in rectangular plastic cassettes with 0.9 mm holes, and cleared in hot 10% KOH to remove cytoplasmic contents from cells. To minimize agitation, we heated KOH in a large beaker over a Bunsen burner until boiling, turned off the burner, and immediately added

cassettes for a 10-minutes soak period. Afterwards the roots were washed five times in water and then immersed in 2% HCl for 20 minutes. Next, roots were stained with trypan blue, rinsed with five changes in water and stored at 4^{o} C. Roots were mounted on glass slides and for each sample, 100 intersects were examined under a microscope at 400x magnification for AM fungal hyphae, arbuscules, and vesicles.

Statistical Analysis

Univariate data were analyzed by one-way analysis of variance (ANOVA) tests for a randomized complete block design in SAS (ver. 9.3, SAS Institute, Cary, North Carolina). Mean effects were separated using LSD at the α=0.05 level. For microbial community analysis, FAME data were converted from nmol g^{-1} soil to relative percent basis. Data were then analyzed by principal components analysis (PCA) with the PC-ORD statistical package (MjM software, Gleneden Beach, OR, 1999).

RESULTS

Soil Chemical Properties

Treatment effects on soil chemical properties in 2009 are shown in Table 2.2). Manure and biochar + manure treatments increased total N 1.2- and 1.4-fold, respectively, compared to the control, while adding biochar alone did not change total N in soil. The biochar + manure treatment contained the greatest quantity of organic C (1.86%) as compared to all other treatments. When applied individually, biochar or manure increased organic C 1.6- fold, or 1.5-fold, respectively, over control. Relative to the control, biochar + manure increased extractable P 6-fold, while manure alone produced a 4-fold increase. Manure and biochar + manure treatments more than doubled soil NO_3-N levels. Adding biochar alone had no influence on soil extractable

P, NO_3-N, or NH_4-N. In 2012, nearly four years after treatment applications, soil chemical properties were unaffected by biochar, manure, or biochar + manure (Table 2.3).

Table 2.2 Soil chemical properties under corn in June 2009 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3).

Treatment	Total N	Organic C	pH	Ext.P	NH_4-N	NO_3-N
	%	%		--------	mg kg $^{-1}$	---------
Manure	0.11a†	1.14bc	7.49a	1.67a	2.48a	48.1a
Biochar	0.09b	1.21b	7.59a	0.37b	1.38a	16.2b
Biochar + Manure	0.13a	1.86a	7.60a	2.37a	2.47a	49.9a
Control	0.09b	0.77c	7.60a	0.40b	1.37a	16.3b
LSD	0.02	0.38	ns‡	1.19	ns	17.6
$P_r > F$	0.0078	0.0023	0.65	0.015	0.11	0.0043

†Within columns, means followed by different letters are significantly different at $\alpha = 0.05$.
‡ns = not significant.

Table 2.3 Soil chemical properties under corn in August 2012 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3).

Treatment	Total N	Organic C	pH	Ext.P	NH_4-N	NO_3-N
	%	%		----------	mg kg^{-1}	----------
Manure	0.13a[†]	0.81a	7.76a	9.67a	1.70a	4.50a
Biochar	0.12a	0.77a	7.73a	8.33a	1.40a	5.56a
Biochar + Manure	0.12a	0.81a	7.76a	10.7a	2.10a	4.50a
Control	0.13a	0.78a	7.80a	8.50a	1.40a	3.40a
LSD	ns[‡]	ns	ns	ns	ns	ns
Pr > F	0.93	0.13	0.65	0.38	0.23	0.19

[†]Within columns, means followed by different letters are significantly different at $\alpha = 0.05$.
[‡]ns = not significant.

Soil Enzymes

The majority of soil enzyme potential activities were not affected by any of the soil amendments in 2009 or 2012 (Tables 2.4 and 2.5). The only enzyme whose potential activity was strongly and significantly affected was β-xylosidase in 2009, where manure and biochar + manure increased the potential activity of β-xylosidase 4.7-fold and 5.6-fold, respectively, compared to control (Fig. 2.1). By 2012, the effect of manure and biochar + manure on the potential activity of β-xylosidase was no longer significant (Fig. 2.2).

Table 2.4 Potential soil enzyme activities under corn in June 2009 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3).

Treatment	β-glucosidase	β-D-cellobiosidase	N-acetyl-β-glucosaminidase	Phosphatase	Leucine aminopetidase
	nmol product g^{-1} dry soil h^{-1}				
Manure	113a[†]	46.2a	26.7a	141a	170a
Biochar	87.1a	24.2a	15.1a	138a	417a
Biochar + Manure	112a	35.1a	22.2a	168a	501a
Control	83.1a	35.4a	23.2a	104a	358a
LSD	ns[‡]	Ns	ns	ns	Ns
Pr > F	0.91	0.74	0.80	0.79	0.25

[†] Within columns, means followed by different letters are significantly different at α = 0.05.
[‡]ns = not significant.

Table 2.5 Potential soil enzyme activities under corn in August 2012 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3).

Treatment	β-glucosidase	β-D-cellobiosidase	N-acetyl-β-glucosaminidase	Phosphatase	Leucin aminopetidase
	nmol product g^{-1} dry soil				
Manure	72.3a[†]	44.1a	14.2a	164a	222a
Biochar	112a	51.0a	17.4a	174a	223a
Biochar + Manure	117a	48.5a	19.9a	163a	254a
Control	67.5a	21.3a	10.1a	152a	214a
LSD	ns[‡]	Ns	ns	ns	Ns
Pr > F	0.12	0.37	0.47	0.52	0.58

[†] Within columns, means followed by different letters are significantly different at α = 0.05.
[‡]ns = not significant.

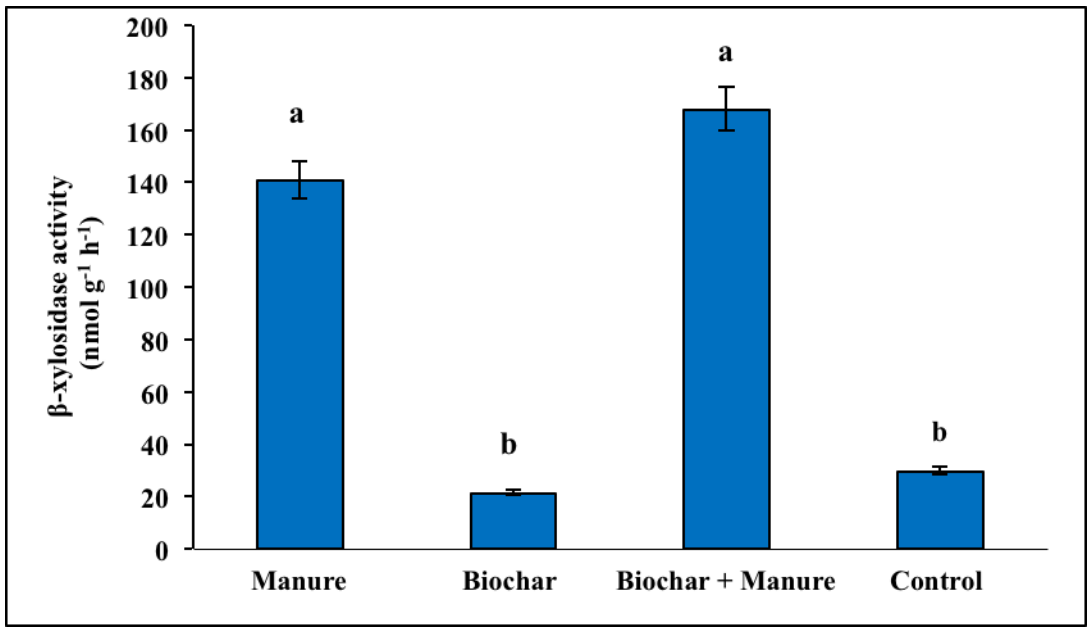

Figure 2.1 Potential activity of β-xylosidase under corn in June 2009 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3). Histogram bars labeled by the same letter are not significantly different (α = 0.05).

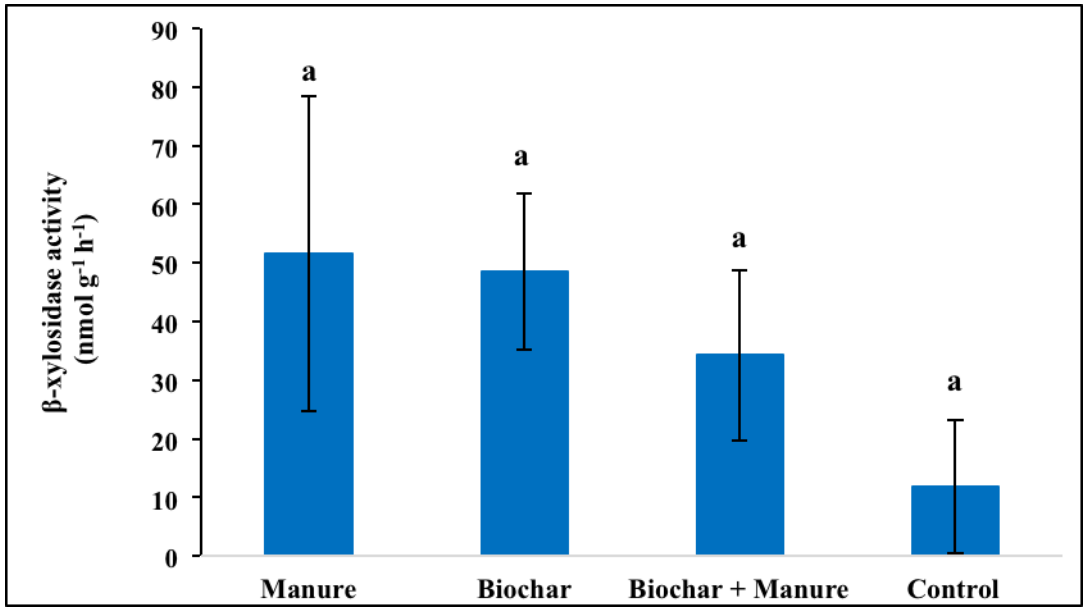

Figure 2.2 Potential activity of β-xylosidase under corn in August 2012 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3). Histogram bars labeled by the same letter not significantly different (α = 0.05).

Microbial Community FAME Structure

Microbial biomass in 2009, as estimated by total concentration of FAMEs, was significantly affected by the amendment applied (Fig. 2.3). Total FAME concentration was greater in manure and biochar + manure treatments, which increased microbial FAME biomass 2.3-fold and 2.6-fold, respectively, as compared to the control. Adding biochar alone, however, did not increase microbial biomass. In 2012, no significant difference in total FAME biomass was detected among the treatments (Fig 2.4).

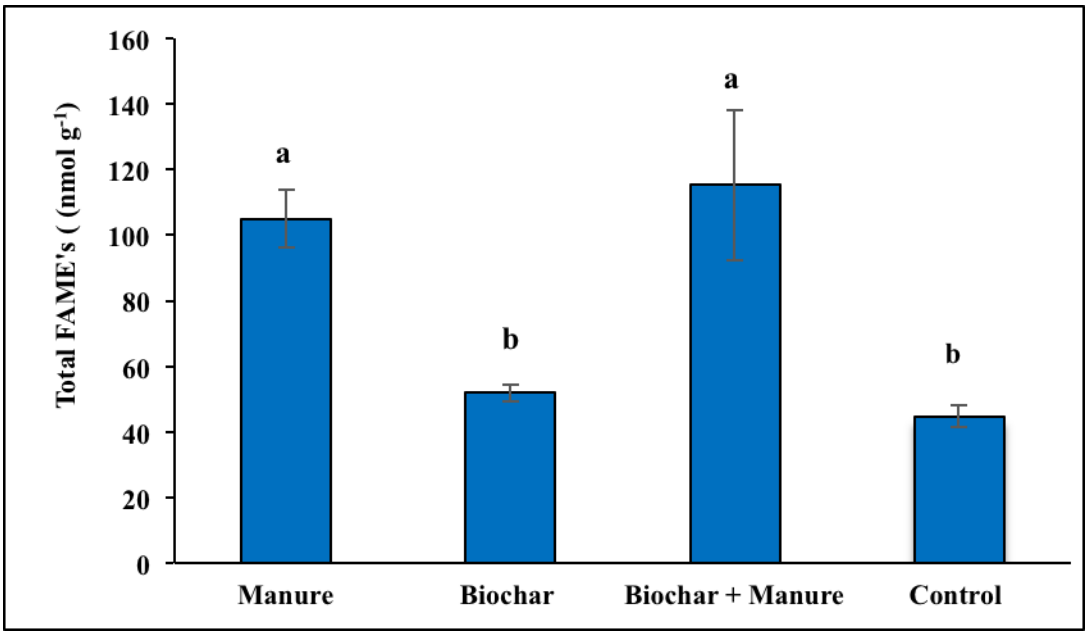

Figure 2.3 Total FAME concentration under corn in June 2009 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3). Histogram bars labeled by the same letter are not significantly different ($\alpha = 0.05$).

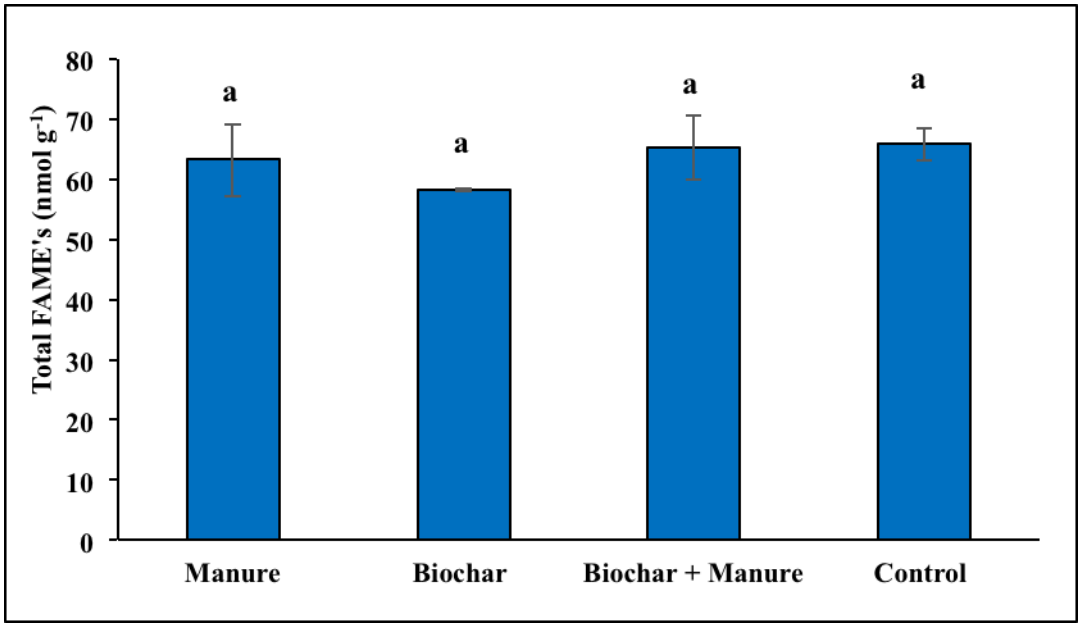

Figure 2.4 Total FAME concentration under corn in August 2012 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3). Histogram bars labeled by the same letter are not significantly different ($\alpha = 0.05$).

The FAME biomarker for AM fungi (16:1ω5c) was significantly affected by treatments in 2009. The concentration of 16:1ω5c was significantly lower in manure and biochar + manure treatments than in soil receiving biochar alone or no amendment (Fig. 2.5). In 2012, all soil communities contained similar amount of 16:1ω5c, and there were no significant effects of the soil amendments (Fig. 2.6).

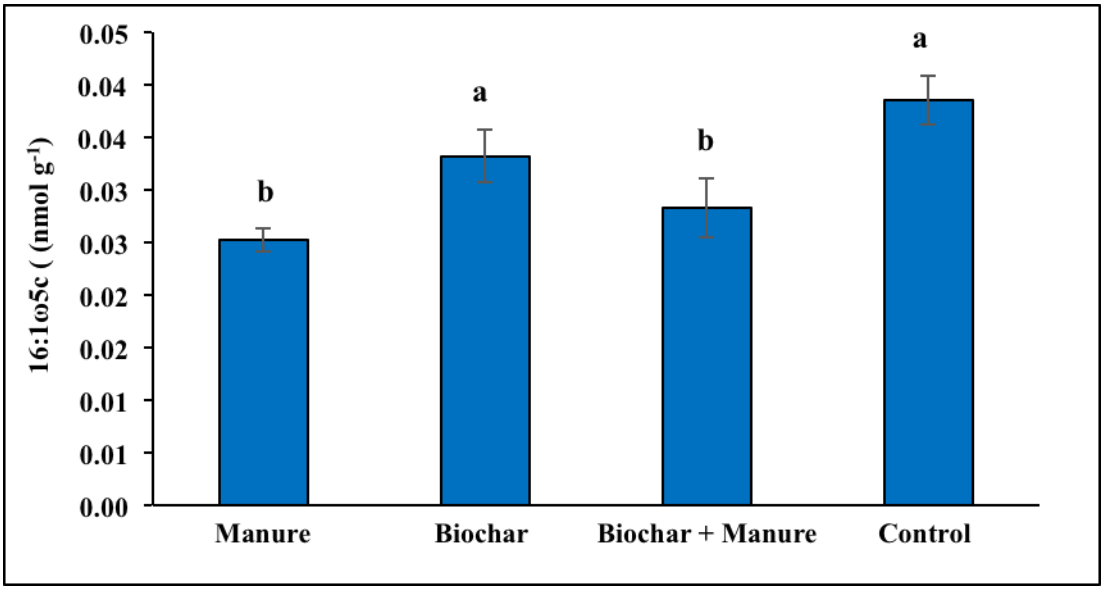

Figure 2.5 Concentration of FAME biomarker for AM fungi (16:1ω5c) under corn in June 2009 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3). Histogram bars labeled by the same letter are not significantly different (α = 0.05).

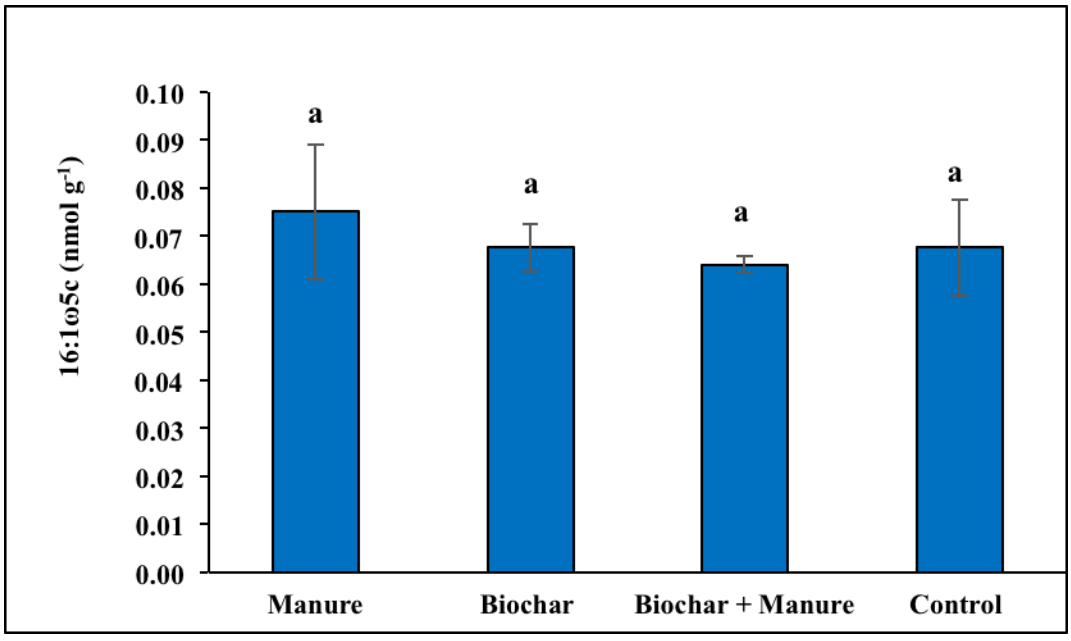

Figure 2.6 Concentration of FAME biomarker for AM fungi (16:1ω5c) under corn in August 2012 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3). Histogram bars labeled by the same letter are not significantly different (α = 0.05).

In 2009, principle components analysis (PCA) revealed shifts in the FAME structure of soil microbial communities in response to soil amendments (Fig. 2.7). Communities separated along Principle Component 1 (PC1) according to whether they had received manure (either alone or in combination with biochar) or not. Communities from biochar and control soils grouped along the negative regions of both PC1 and PC2, and clearly separated from manure and biochar + manure plots. According to multi-response permutation tests for a blocked design, marginally significant differences between treatments were found for manure versus biochar (P=0.062), manure versus control (P = 0.064), and biochar + manure vs. biochar (P=0.073). The AM fungal biomarker, (16:1ω5c), was negatively correlated with PC 1 (r=-0.72) and positively with PC 2 (r=0.43). In 2012, clear differences in soil microbial community structures due to treatments were not as evident as was observed in 2009 (Fig. 2.8). Furthermore, MRBP analysis showed no significant differences among treatments (P=0.77).

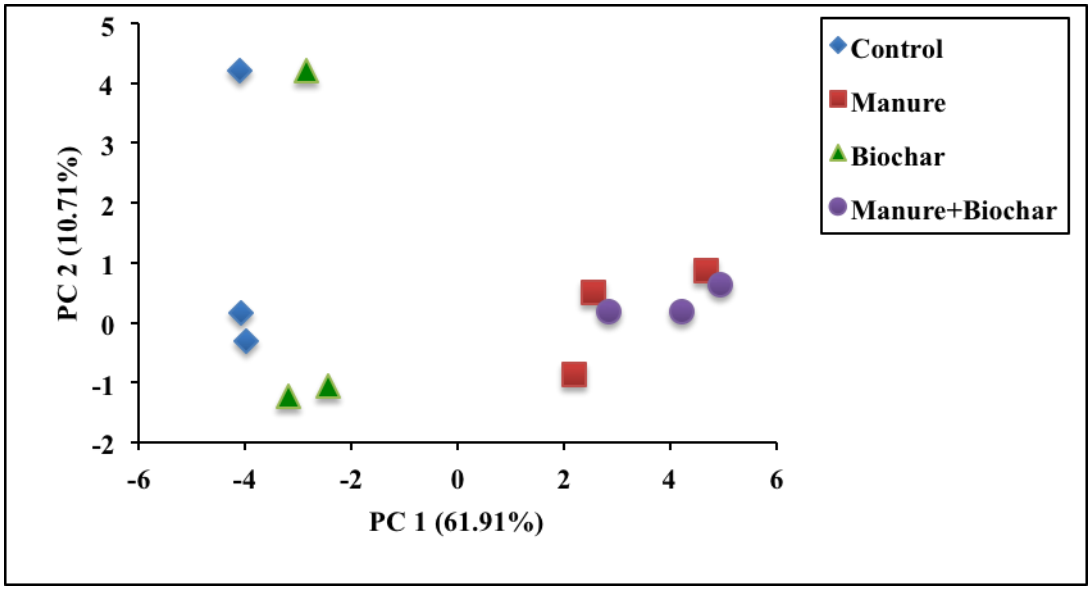

Figure 2.7 Principle components analysis (PCA) of microbial community fatty acid methyl esters (FAMEs) under corn in June 2009 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3).

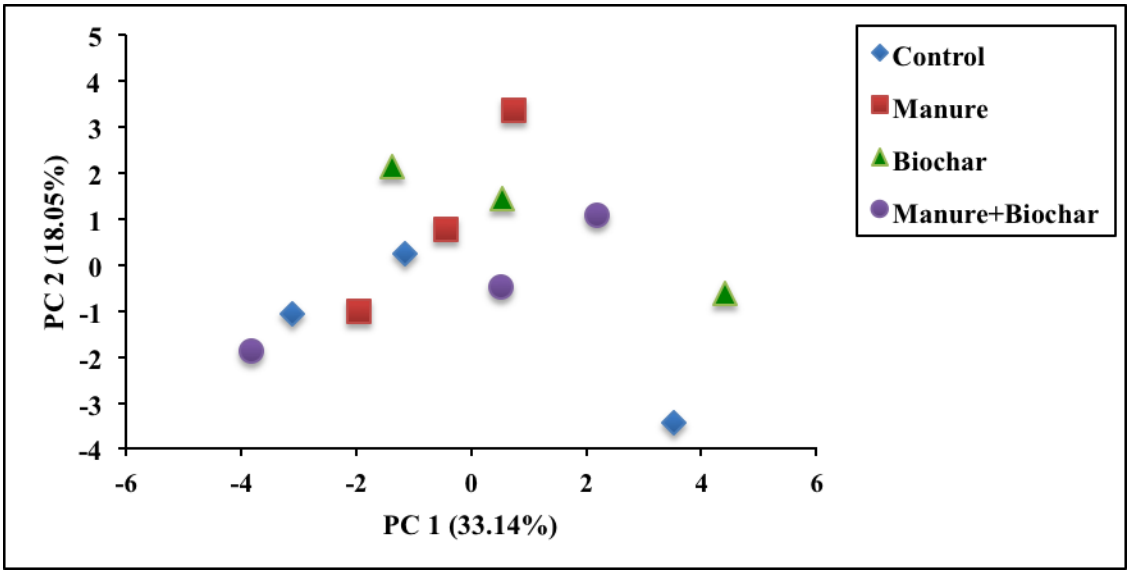

Figure 2.8 Principle component analysis (PCA) of microbial community fatty acid methyl esters (FAMEs) under corn in August 2012 (0-30 cm depth), after a November 2008 application of biochar, manure, or biochar plus manure to experimental research plots (n=3).

In 2012, the percentage of mycorrhizal colonization in corn roots was analyzed. Data were expressed by summing occurrences of hyphae, arbuscules and vesicles. Manure application decreased mycorrhizal colonization 27% relative to roots from control plots. Root colonization was also lower, at 17%, in biochar + manure application. Biochar did not impact root colonization, with levels that were similar to control (Fig 2.9).

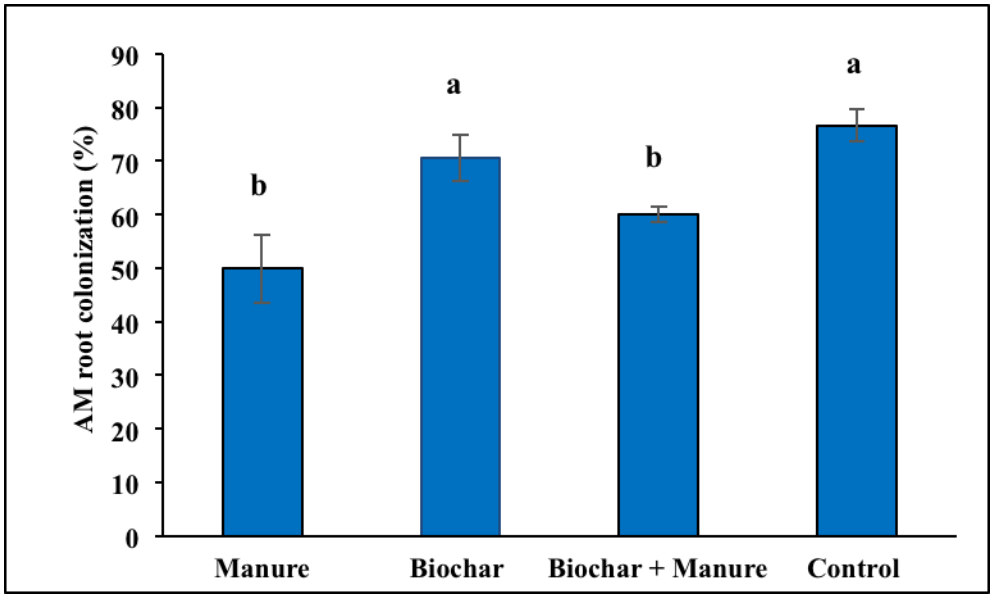

Figure 2.9 Percent colonization of corn roots by AM fungi in 2012 plots receiving manure, biochar, or both, three years after application (n=3). Histogram bars labeled by the same letter are not significantly different ($\alpha = 0.05$).

DISCUSSION

The purpose of this study was to determine the short and longer-term effects of a biochar amendment on soil properties, in comparison to a common organic soil amendment (manure). We found that biochar had relatively few effects on soil chemical and microbial properties, relative to manure, and that regardless of the treatment, effects were mainly temporary and did not extend to three years post-application. In this study, microbial community biomass and structure were largely affected by manure in the short-term, but not biochar. Both biochar and

manure increased soil organic C levels to similar amounts, and even more so when applied together. Increases in organic C were likely the result of biochar and manure C input since those compounds contain relatively high amounts of organic C. Similar observations were found by Rogovska et al. (2011), Bolan et al. (2012), and Yang et al. (2013). However, the lack of microbial biomass response to biochar indicated that little of the biochar C was available for microbial degradation. In addition, biochar did not enhance total N, NH_4-N, NO_3-N, as well as available P in soil, indicating that the biochar used and/or the rate at which it was applied, was not as effective at improving nutrient availability as was manure (Lentz and Ippolito, 2012).

Other researchers have suggested that biochar benefits microbial communities by enhancing the physical and chemical characteristics of the soil (Lehmann and Joseph 2009; Atkinson et al., 2010; Jindo et al., 2012), providing suitable habitats for microorganisms that protect them from predators (Pietikäinen et al., 2000), supplying labile C substrates for degradation (Thies and Rillig, 2009; Smith et al., 2010), enhancing the availability of macro-nutrients such as N and P (Atkinson et al., 2010; Lammirato et al., 2011), or sorbing compounds that would otherwise inhibit microbial growth (Kasozi et al.,2010). To date, these mechanisms have been poorly studied and are mainly discussed in terms as possible explanations. The results of this study show that biochar has no effect on microbial communities compared to manure. An inconsistent effect of biochar on microbial communities suggests that biochar effects are likely biochar-specific, related to the rate applied to soil, or related to site and soil characteristics. For example, others have found no effect of biochar on microbial communities when the biochar does not affect the pH of an already neutral or alkaline soil (Meynet et al., 2012), or when biochar does not provide enough labile C substrates (high pyrolysis temperature) or nitrogen

(hardwood biochar) to stimulate microbes (Bruun et al., 2011; Luo et al., 2011; Novak et al., 2012).

The results of this study did not support our hypothesis that biochar amendment would negatively affect soil enzyme potential activities. This hypothesis was based on previous studies that reported substrate sorption by biochar that may inhibit the enzyme- substrate reaction by blocking reaction sites. Biochar sorption capacity is likely affected by the porosity and cation exchange capacity of this material (Thies and Rilling, 2009; Jindo et al., 2012). Because of different behaviors of biochar in soil, different adsorption behaviors and biological activities may be observed due to widely varying pH, surface area, pore size distribution, and charge properties (Brewer et al., 2009; Gaskin et al., 2009). In one study where 0 or 2% biochar (w/w) was added to three soil types, Bailey et al. (2010) observed varied effects of biochar on soil enzymes and attributed this to either stimulation of the microbial activity or blocking or sorption of the substrates.

The expected benefit of biochar on the AM fungi biomarker (16:1ω5c) in soil was not confirmed in this study. Our study differed from a recent study conducted by Ameloot at el. (2012), who found a remarkable increase in the 16:1ω5c AM fungal marker in a low temperature biochar treatments compared to the control treatment. Similarly, Warnock et al. (2007) reported that biochar enhanced AM fungal populations in soil by several mechanisms including: (1) changes in chemical and physical properties, (2) indirect effects on mycorrhizae through effects on other microbes, (3) plant–fungal signaling interference, (4) sorption of inhibitory chemicals on biochar, and (5) protection from fungal grazers.

In this study, biochar had neutral effects on soil AM fungal biomass and corn root colonization. Greater differences in AM fungal biomass and root colonization were observed

with the manure treatment; both were negatively impacted by manure application in 2009. This was likely due to increased soil fertility following manure application. When P and other nutrients are abundant (such as when following manure addition), plants rely less on AM fungi to supply nutrients and root colonization and AM fungal biomass in soil is reduced (Corbin et al., 2003; Covacevich et al., 2006; Gryndler et al., 2006).

CONCLUSIONS

The aim of this study was to evaluate the effects of biochar on the soil microbial community, AM fungi, and potential soil enzyme activities relative to a common organic soil amendment (manure). This study demonstrated that additions of a hardwood-derived, fast-pyrolysis biochar did not affect microbial community biomass, structure, soil enzyme activities, AM fungal biomass in soil, or AM fungal colonization of corn roots, in a calcareous soil when applied at 22.4 Mg dry wt ha^{-1}. Therefore, this study demonstrated that biochar additions do not always affect soil microbial communities. Land disposal of biochar may be an effective means to sequester C, but if growers wish to apply a carbon-based soil amendment to enhance microbial growth and activity, manure rather than biochar would likely be more effective in the short-term.

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CHAPTER 3

STABILIZING EFFECT OF BIOCHAR ON SOIL EXTRACELLULAR ENZYMES AFTER A DENATURING STRESS

INTRODUCTION

Extracellular enzymes are the primary means by which soil bacteria and fungi degrade insoluble macromolecules, including soil organic matter (SOM) and detritus, into smaller soluble molecules that can be microbially assimilated (Dick, 2002). Extracellular enzymes allow microbes to access unavailable carbon and nutrients in SOM by catalyzing the first step of decomposition and nutrient mineralization, i.e., depolymerization of complex carbon substrates too large to enter microbial cells. Plant components such as cellulose, hemicellulose, and lignin, and microbial cell wall materials are among the more abundant soil organic compounds that are degraded enzymatically. However, extracellular enzymes may be found in different soil locations; they may be associated with biotic components such as proliferating and non-proliferating cells or with dead cells and cell debris, or sorbed to clay minerals or soil colloids (Burns, 1982). Extracellular enzymes associated with humic colloids and clay minerals may have a relatively long half-life (compared to enzymes in the soil aqueous phase), with these associations likely the best form of protection from the environment (Burns, 1982). Ladd (1978) demonstrated that many enzymes are capable of binding to humic material, giving the enzymes a persistence they would not otherwise display in the hostile extracellular environment of the soil.

Enzyme stabilization may maintain enzymatic activity and also protect against proteolysis and other denaturing events (Skujins, 1976; Nannipieri et al., 1996; Nannipieri et al., 1988). Yet, we are still at the beginning of practical applications to manipulate stabilized enzymes for beneficial ecosystem services such as bioremediation, C sequestration, and plant

growth promotion. Jastrow et al. (2007), for example, proposed that by modifying the soil physicochemical environment, fungal growth and their extracellular enzymes could be promoted for C sequestration. Amonette et al. (2009) purposely tested the ability of four alkaline fly ashes to stabilize tyrosinase enzymes, finding that enzyme activity was protected in the presence of fly ash, although the mechanism of stabilization was not elucidated. Another material, biochar, with its potential capacity to sorb a wide range of organic and inorganic molecules, may affect enzymes (and inherently their activity) by sorbing them and/or their substrates (Bailey et al., 2010; Jin, 2010). In general, however, there is a poor understanding of the possible biochar effects on soil extracellular enzymes in biochar-amended soil.

Currently, there is interest in biochar creation and land application for the purposes of biogas production, C sequestration, and increasing soil fertility (Lehmann et al., 2006). Because of its porous nature, researchers have speculated that biochar can provide habitats for bacteria and fungi (Thies and Rillig, 2009). If biochar can attract soil microbes and sorb extracellular enzymes, it is possible that biochar could stabilize enzymes and protect enzymes from degradation or denaturation during environmental stress. Biochar could thus be a useful material in cases where enzyme stabilization is desired. Therefore, the objective of this study was to determine the stabilizing effect of biochar on enzyme activities exposed to a denaturing stress, in this case microwaving.

MATERIAL AND METHODS

Soil and Biochar

Biochar-free soil (0-30 cm depth) was collected November 2012 from the border of a research field located near Kimberly, Idaho (42°31′N, 114°22′ W, elevation of 1190 m). The soil was a Portneuf silt loam (coarse-silty, mixed superactive, mesic Durinodic Xeric Haplocalcids)

with 20% clay, 56% silt, and 24% sand, 1.2% organic C, and an 8.8% calcium carbonate equivalency. The electrical conductivity (EC) of the soil was 0.50 dS m^{-1} and its pH was 7.6 (saturated paste; Thomas, 1996; Rhoades, 1996). Prior to the study, the soil was air-dried and passed through a 2-mm mesh sieve.

Biochar was provided by Dynamotive Energy Systems (West Lorne, Ontario, Canada) and was marketed under the name CQuest. It was derived from oak and hickory hardwood sawdust and created by fast pyrolysis at 500°C. As described by Lentz and Ippolito (2012), the biochar had an ash content of 14%, an oxygen:carbon ratio of 0.22, a surface area of 0.75 m^2 g^{-1}, and a pH of 6.8. Additional details regarding biochar chemical properties are listed in Table 3.1

Table 3.1. Selected chemical properties of a fast pyrolysis, hardwood-derived biochar (CQuest) used in the laboratory incubation study. Data are from Lentz and Ippolito (2012).

Property	Units	Biochar
Surface Area	m^2 g^{-1}	0.75
Ph		6.8
EC	dS m^{-1}	0.7
Ash	%	14
Total C	%	66.2
Total N	%	0.32
Organic N	%	0.32
NO_3-N	mg kg^{-1}	1.5
NH_4-N	mg kg^{-1}	1.2
K	mg kg^{-1}	3400
Ca	mg kg^{-1}	3700
Mg	mg kg^{-1}	1500
Na	mg kg^{-1}	200
P	mg kg^{-1}	300

Incubation Experiment

The incubation experiment was conducted with five biochar treatments (0%, 1%, 2%, 5%, and 10% biochar in soil, wt:wt), each replicated 20 times in glass scintillation vials. Final dry weight of soil plus biochar in each vial was 10.0 g. Soil and biochar were mixed by placing the vials on their sides and gently rolling the vials until biochar was mixed thoroughly with soil. After mixing, 1.8 ml of distilled water was added to each vial to achieve a moisture content of 18%, which was equivalent to 60% of the soil's water holding capacity. All vials were weighed, and then they were loosely capped and incubated for 36 days at 25^{o}C. The length of the incubation was arbitrarily selected to allow microbial biomass to recover from being air-dried and rewetted and to allow enzyme production. Water was added to each vial every two or three days to maintain constant water content.

Microwave irradiation

Following the incubation period, soils were subjected to microwave (MW) stress using a 650 Watt household-type MW oven. Microwave (MW) stress was selected because of its denaturing ability through heat, as well as moisture reduction. Others have successfully employed this strategy to inhibit microbial activity and examine extracellular enzyme stability (Knight and Dick, 2004). The power output of the MW was determined according to Neas and Collins (1988), by measuring the rise in temperature of 1000 mL of distilled water (initial temperature 21^{o}C) in a 1-L beaker after microwaving at full power for two minutes. The power output was calculated as:

$P = C_p K \Delta T\ m/t$

where P is the apparent power absorbed by the water sample (J s^{-1}), Cp is the heat capacity of water (J ml^{-1} $^{o}K^{-1}$), K is a factor (4.184) to convert thermal chemical cal ml^{-1} $^{o}K^{-1}$ to watts (J s^{-1}),

ΔT (^oC) is the difference between final temperature and initial temperature of water, m is the mass of the water (g); and t is the duration (s) of MW energy application. Using this equation, the MW oven output was calculated as 675 W (J s^{-1}).

The 20 replicates of each biochar treatment were divided into 5 stress levels and microwaved for different lengths of time to achieve microwave energy "stress" levels of 0, 400, 800, 1600, and 3200 J g^{-1} soil. Random vials were immediately measured for soil temperature, and a subsample of each vial was placed in an oven at 105°C for 24 hours for gravimetric water content determination.

Enzyme Assays

Dehydrogenase activity was measured immediately following the microwave stress according to the method of Trevors (1984). As an intracellular enzyme, this enzyme was employed as an indicator of microbial activity and its response to microwaving. The potential activities of six extracellular enzymes were quantified according to fluorescence enzyme protocols as described in Steinweg et al. (2013) and Bell et al. (2013) The six enzymes included three C-cycling enzymes (β-D-cellobiosidase, β-glucosidase, and β-xylosidase), 1 C and N cycling enzyme (N-acetyl-β-glucosaminidase), 1 N cycling enzyme (leucine aminopeptidase), and 1 P cycling enzyme (phosphatase).

All assays included appropriate blanks, where soil suspensions were incubated in the absence of enzyme substrate. To correct for quenching of fluorescence signals by soil and biochar, standard curves were prepared for each sample by incubating soil suspensions in the presence of increasing concentration of 4-methylumbelliferone (MUB) or 7-amino-4-methylcoumarin (MUC) standard. Incubations were conducted at 25º C. Fluorescence

measurements of the plates were read on a Tecan Infinite® M200 microplate (Tecan, Mannedorf, Switzerland) at 365 nm excitation and 450 nm emission wavelengths.

Statistical Analysis

Statistical analyses of the data were performed with SAS version 9.3 (SAS Institute, Cary, North Carolina) using the Proc Mixed procedure. Two-way factorial analysis of variance (ANOVA) tests were performed to determine the effect of biochar rate, stress level, and their interaction on enzyme activities (α=0.05).

RESULTS

Stress Effects on Soil Temperature, Moisture, and Microbial Activity

Microwaving provided stress through heat and loss of soil water, and the effect of microwave stress on soil water loss was influenced by biochar treatment. At the lowest stress level (400 J g^{-1} soil), loss of soil water was significantly reduced in soil amended with 10% biochar, compared to soil amended with 0%, 1%, or 2% biochar (P=0.012) (Fig. 3.1). The attenuation of moisture loss by the 5% biochar treatment was marginally significant (P=0.058) at this stress level. A dramatic loss of soil water occurred when MW energy was 800 J g^{-1} soil, at which more than 80% of the total soil moisture was lost in soil with 0% and 1% biochar (Fig. 3.1). Soil moisture content was slightly higher at 2%, 5%, and 10%, biochar but the differences were not statistically significant. At this stress level, soil temperature rose to approximately 70°C in all soils. All soils reached 0% water content when MW irradiation applied was 1600 J g^{-1} soil. Soil temperature reached 100°C with energy applied at 1600 or 3200 J g^{-1} soil.

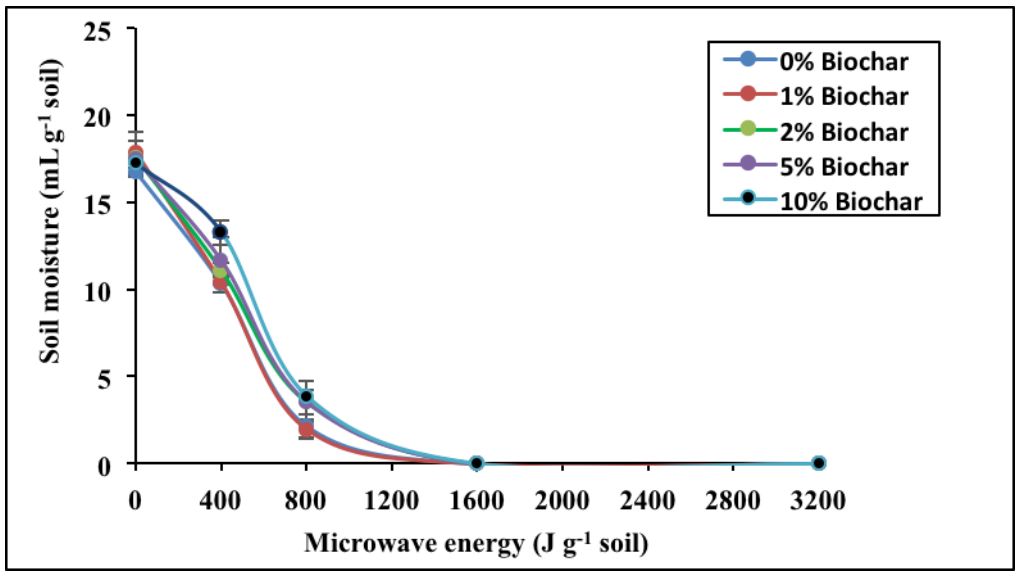

Figure 3.1 The effect of microwave energy stress on the moisture content of soil after 36 days of incubation with either 0, 1, 2, 5, or 10% biochar amendment to soil (wt:wt). Error bars represent the standard error of the mean (n=4).

Microbial activity, as indicated by dehydrogenase activity, decreased with increasing MW energy up to 800 J g^{-1} but then increased at higher energy levels (Fig. 3.2). Dehydrogenase activity was significantly lower after exposure to 800 J g^{-1} soil than after exposure to 0, 1600, or 3200 J g^{-1}. At 3200 J g^{-1} of microwave energy, dehydrogenase activity was at a level that was significantly greater than the other MW energies (P=0.01).

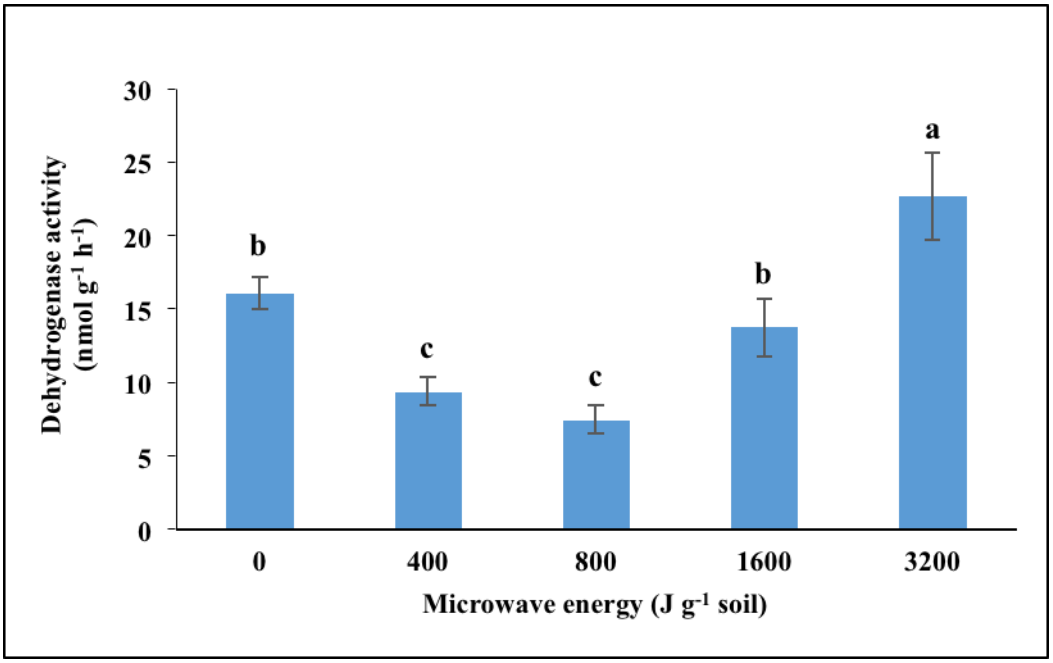

Figure 3.2 The effect of microwave energy stress on dehydrogenase activity in soil after 36 days of incubation with either 0, 1, 2, 5, or 10% biochar amendment to soil (wt:wt). Histogram bars labeled with different letters are significantly different (α=0.05)

Extracellular Soil Enzymes

In this study, extracellular enzyme activities were differentially affected by biochar rate, stress level, and their interaction. Main effect of stress level (but not biochar) was highly significant ($P<0.0001$) on the activities of β-glucosidase, β-D-cellobiosidase, N-acetyl-β-glucosaminidase, and phosphatase. The potential activity of these enzymes decreased dramatically with increasing MW energy, regardless of biochar rate. Potential activities of β-D-cellobiosidase and N-acetyl-β-glucosaminidase fell below detection limits when MW energy was applied at 3200 J g^{-1}, whereas β-glucosidase and phosphatase potential activity were at relatively low activity levels (Table 3.2).

Table 3.2 The effects of microwave energy stress on the mean (± 1 standard error) of β-glucosidase (BG), β-D-cellobiosidase (CB) N-acetyl-β-glucosaminidase (NAG), and phosphatase (PHOS) potential activity in soil (nmol g^{-1} soil h^{-1}), averaged across biochar treatments.

	Microwave energy (J g^{-1} soil)				
Enzyme	0	400	800	1600	3200
BG	36.0 ± 5.76a	5.70 ± 1.57b	2.76 ± 0.75b	0.27 ± 0.23b	0.15 ± 0.14b
CB	4.11 ± 0.80a	1.10 ± 0.37a	0.38 ± 0.20a	2.16 ± 0.21a	0.00 ± 0.00a
NAG	3.69 ± 0.41a	0.80 ± 0.27b	0.37 ± 0.22b	0.41 ± 0.29b	0.00 ± 0.00b
PHOS	60.0 ± 8.08a	23.6 ± 2.27b	8.98 ± 0.89bc	0.95 ± 0.41c	0.21 ± 0.15c

[†]Within rows, means followed by different letters are significantly different at $\alpha = 0.05$.

In contrast, a different pattern was observed for leucine aminopeptidase potential activity, which was significantly affected by biochar rate (P=0.016), stress level (P<0.0001), and their interaction (P=0.0008). Prior to stress exposure, leucine aminopeptidase potential activity was significantly reduced in soils receiving 1%, 2% or 5% biochar compared to control soil and soil receiving 10% biochar (Fig. 3.3). When soils were exposed to 400 or more J g^{-1}, potential activity declined in all soils to the point where no difference among biochar treatments existed. Cessation of the potential enzyme activity was observed in all soils when MW energy was applied at 1600 or 3200 J g^{-1} soil.

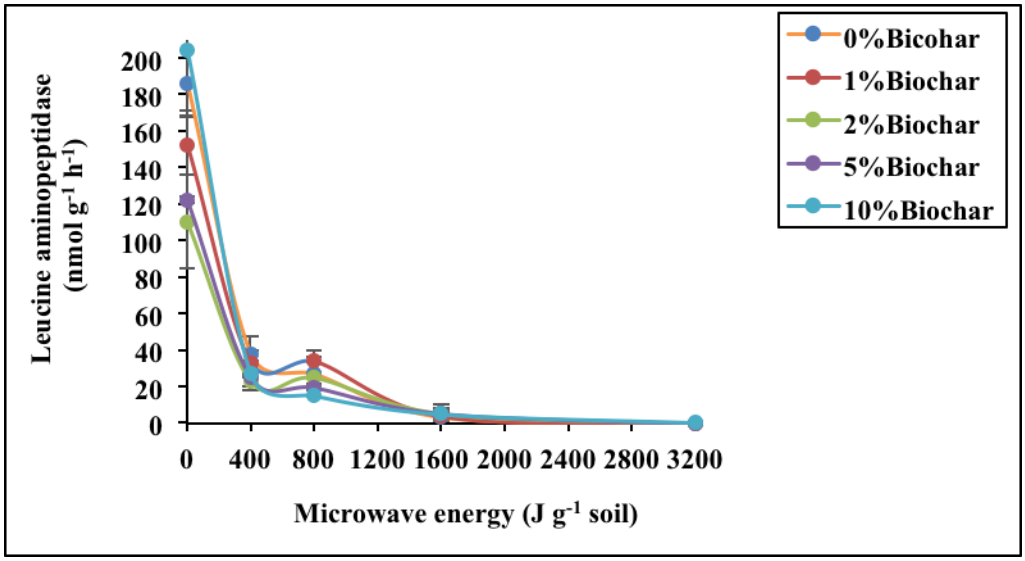

Figure 3.3 The effect of microwave energy stress on leucine aminopeptidase potential activity in soil after 36 days of incubation with either 0, 1, 2, 5, or 10% biochar amendment to soil (wt:wt). Error bars represent the standard error of the mean (n=4).

For β-xylosidase potential activity, the interaction effect of biochar and stress level was marginally significant (*P*=0.066). Without any microwave stress, biochar reduced potential activity of this enzyme regardless of application rate (Fig. 3.4). When exposed to a stress of 400 J g^{-1}, potential activity remained steady in soil receiving 1% biochar, whereas potential activity declined in all other treatments. After a stress exposure of 1600 J g^{-1}, β-xylosidase potential activity increased in 5% biochar amended soil and was significantly greater than the activities of the other soils.

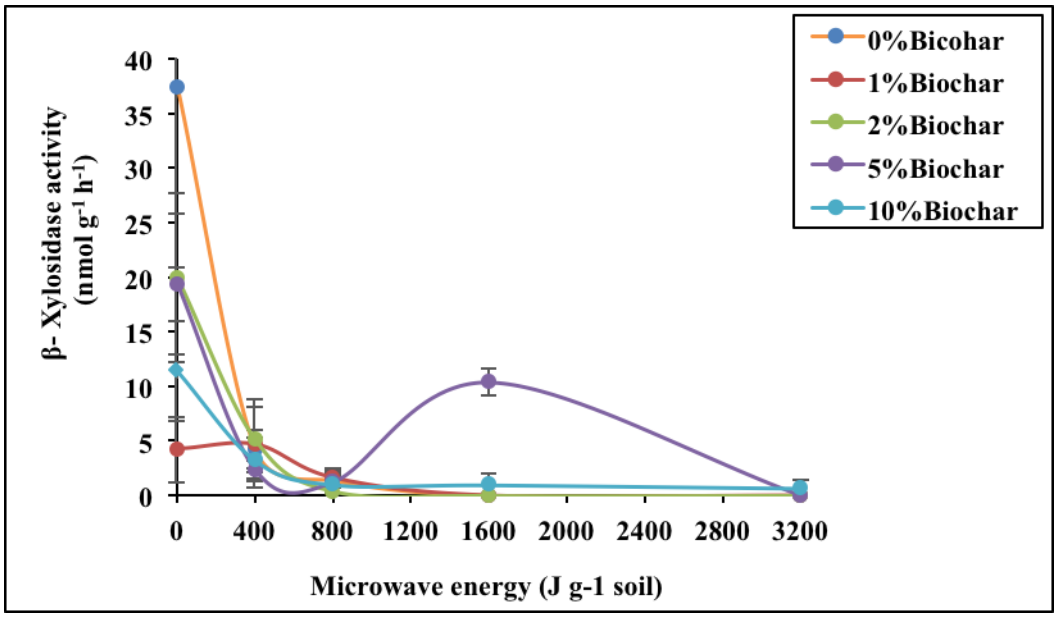

Figure 3.4 The effects of microwave energy stress on β-xylosidase activity in soil after 36 days of incubation with either 0, 1, 2, 5, or 10% biochar amendment to soil (wt:wt). Error bars represent the standard error of the mean (n=4).

DISCUSSION

In this study, dehydrogenase activity, an enzyme that only functions inside the cell, decreased with increasing MW irradiation up to 800 J g^{-1} and then increased with increasing MW irradiation up to 3200 J g^{-1} (Fig 3.2). The measurement of this enzyme was used to confirm that the stress applied was strong enough to kill living microbes and denature their intracellular enzymes, but not necessary affect stabilized extracellular enzymes. We observed that soil temperature rose to approximately 100°C when MW stress applied was ≥ 1600 J g^{-1}, and this might have stimulated the abiotic reduction of tetrazolium salt utilized in the assay and subsequently affected the colorimetric measurement of dehydrogenase activity. A similar result was found by Ciardi (1998) when dehydrogenase activity was measured at high temperature. The author found that dehydrogenase activity was still measurable at high temperature, and activity was even greater at 200°C that at 150°C in both fresh and air-dried soils. It is likely that with

temperatures of 100°C or more, the measured activities may be mainly driven by abiotic reactions such as hydrolysis, oxidation, and reduction that are masked at lower temperature. Such results demonstrate the need to develop a new method that can accurately evaluate dehydrogenase activity in soil affected by high temperature stress.

Soil enzymes are active in different soil locations. Burns (1982) named 10 categories of soil enzyme location. Enzymes might be associated with biotic components such as proliferating and non-proliferating cells (spores, cysts, etc.) or with dead cells and cell debris, or stabilized on clay minerals and humic colloids. Possibly stabilized enzyme on soil colloids can maintain their activity for extended periods of time (Burns, 1982; Nannipieri et al., 1996; Knight and Dick, 2004). In the present study, we tested if stabilized enzymes would be more resistant to microwave denaturation after enzymes were incubated in the presence of biochar. The results demonstrated that the ability of biochar to stabilize enzymes was dependent on the biochar application rate and the enzyme itself.

After a 36-day incubation period, biochar amendment did not affect potential activities of β-glucosidase, β-D-cellobiosidase, N-acetyl-β-glucosaminidase, or phosphatase, suggesting that biochar did not sorb these enzymes or their substrates/products during the enzyme assay. This contrasts with findings of Bailey et al. (2010) and Jin (2010), who noted reduction of enzyme activities in biochar-amended soil. Jin (2010) examined the effect of corn stalk biochar (slow pyrolysis at 550ºC) at rate of 0, 1, 12, and 30 Mg ha^{-1} on potential activity of two carbohydrate enzymes (β-D-glucosidase and β-D-cellobiosidase), and found that activities decreased after biochar additions to soils. Bailey et al. (2010) tested the effects of fast-pyrolysis biochar produced from swithchgrass on the potential activity of purified enzymes, and observed decreases in glucosidase potential activity. In the current study, biochar-induced reduction of

enzyme activities occurred only for leucine aminopeptidase and β-xylosidase. Leucine aminopeptidase is an enzyme that preferentially catalyzes the hydrolysis of leucine residues at the N-terminus of peptides and proteins (Rawling and Barrett, 2004; Matsui et al., 2006). B-xylosidase is essential for the complete breakdown of xylans (the major hemicellulose component in plant cell walls) and is produced by plant, animals and microbes (Poutanen and Puls, 1988; Nanmori et al., 1990; Saha, 2002). This study showed that all biochar treatments reduced potential activity of xylosidase enzyme in non-stressed soil, whereas intermediate rates (1-5%) of biochar amendment reduced the potential activity of leucine aminopeptidase. The reductions are likely related to the sorption or masking of enzymes, rather than sorption of substrates or products (as the assay corrected for quenching), presumably due to biochar porosity and reactive surface area (Thies and Rilling, 2009; Jindo et al., 2012). In contrast, the highest biochar application rate (10%) resulted in leucine aminopeptidase potential activity equivalent to the control treatment, which suggests that at high enough rates, biochar might stimulate enzyme production or protect enzymes from degradation so that higher activities are detected.

Previous studies have showed that the enzymes in soils are resistant to denaturation by heat and other stresses when associated with abiotic fraction such as soil colloids and clay minerals (Hayano and Katami, 1977; Deng and Tabatabai, 1997 Miller and Dick, 1995; Nannipieri et al., 1996). To date, this is the first study we are aware of that examines the potential application of biochar for the purposeful stabilization of extracellular enzymes in soil. This study found that biochar had variable effects on soil enzymes in terms of protecting enzymes from a denaturing stress. When exposed to microwave stress, leucine aminopeptidase potential activity declined with increasing stress levels, although at 800 J g^{-1}, potential activity was somewhat (but not significantly) maintained to a greater degree in soil amended with 1%

biochar compared to the other treatments. Interestingly, the 1% biochar treatment also maintained β-xylosidase potential activity when soil was exposed to 400 J g^{-1} of microwave stress, while a greater concentration of biochar (5%) protected β-xylosidase potential activity upon exposure to an even greater stress level (800 J g^{-1}).

Very little is known about the stabilization of extracellular enzymes interacting with biochar. It is likely that the size matching between pore size of biochar and the molecular diameter of enzymes will play a key role in achieving high enzymatic stability (Klibanov, 1983). Many other factors such as the temperature stability range and isoelectric point might also play an important role in enzyme stability, especially at high temperature. Both leucine aminopeptidase and β-xylosidase are relatively small in size (28-400 kDa and 20-120 kDa, respectively) and have a wide temperature stability range (25-100°C and 30-95°C, respectively) (Schomburg et al., 2013). Leucine aminipeptidase also has a high isoelectric point (8.2), meaning that this enzyme carries a net positive surface charge in soils below pH of 8.2, including the soil employed in this study (pH=7.6). In comparison, β-D-cellobiosidase and β-N-acetylglucosaminidase have relatively lower ranges of temperature stability (40-80°C and 20-37°C, respectively) and lower isoelectric points (3.8 and 4.6, respectively) (Schomburg et al., 2013). These enzymes would carry net negative surface charge, which might affect their ability to be stabilized on biochar. Others have found that enzyme size was important for enzyme stabilization on nanostructures such as mesoporous silica (Diaz and Balkus, 1996), but further research is necessary to understand the mechanisms of enzyme stabilization on biochar.

CONCLUSION

Extracellular enzymes are very important for decomposition of organic material and nutrient cycling. The aim of this study was to determine if biochar added to soil would stabilize

soil extracellular enzymes so that enzymes would retain potential activity after a denaturing stress. At the rates applied, biochar showed different effects on the enzyme studied. While most of the enzymes were not stabilized by biochar, β-xylosidase and leucine aminopeptidase were stabilized and protected to some degree from microwave stress by intermediate rates of biochar application. This study found that biochar's ability to stabilize enzymes appears to be enzyme specific as well as biochar rate specific. More research is needed to understand the mechanism(s) by which biochar stabilizes some extracellular enzymes but not others, and how stabilization is affected by different biochars and biochar concentrations in soil.

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CHAPTER 4

CONCLUSIONS

The aim of this thesis work was to investigate the short and longer-term effects of biochar on soil microbial community, potential enzyme activity, and AM fungi root colonization in a semi-arid soil. It also sought to determine if adding higher rates of biochar would stabilize soil extracellular enzymes and increase enzyme resistance to a denaturing stress (i.e., microwaving).

This study demonstrated that additions of a hardwood-derived, fast-pyrolysis biochar did not affect microbial community biomass, structure, soil enzyme activities, AM fungal biomass in soil, or AM fungal colonization of corn roots, when applied at 22.4 Mg dry wt ha^{-1}. In contrast to most published studies, soil enzyme activities were not depressed in the biochar-amended soil relative to control soil. Therefore, this study demonstrated that biochar additions do not always affect soil microbial communities. Land disposal of biochar may be an effective means to sequester C, but if growers wish to apply a carbon-based soil amendment to enhance microbial growth and activity, manure rather than biochar would likely be more effective in the short-term.

An inconsistent effect of biochar on microbial communities suggests that biochar effects are likely biochar-specific, related to the rate applied to soil, or related to site and soil characteristics. For example, others have found no effect of biochar on microbial communities when the biochar does not affect the pH of an already neutral or alkaline soil (Meynet et al., 2014), or when biochar does not provide enough labile C substrates (high pyrolysis temperature) or nitrogen (hardwood biochar) to stimulate microbes (Bruun et al., 2011; Luo et al., 2011; Novak et al., 2012).

At the rates applied in incubation study, biochar showed different effects on enzyme stabilization and resistance to denaturing stress. While most of the enzymes were not stabilized

by biochar, β-xylosidase and leucine aminopeptidase were stabilized and protected to some degree from microwave stress by intermediate rates of biochar application (1 and 5%). This study found that biochar's ability to stabilize enzymes is enzyme specific as well as rate specific. More research is needed to understand the mechanism(s) by which biochar stabilizes some extracellular enzymes but not others, and how stabilization is affected by different biochar concentrations in soil.