CHARACTERIZATION OF MICROBIAL LIFE COLONIZING BIOCHAR AND BIOCHAR-AMENDED SOILS

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Soil microorganisms and their activities are critical for soil function, thus, understanding how biochar soil amendment may affect soil microbial life is critical for assuring that soil quality and the integrity of the soil subsystem are maintained. In this study, I characterized microbial life colonizing biochar and biochar-amended soils sampled from the Cornell Musgrave Farm, Aurora, NY, to study the effects of biochar soil amendment on microbial abundance; basal respiration; metabolic quotient (qCO_2); carbon, nitrogen and phosphorus exoenzyme activities and locations; microbial community composition; and, the identity of the dominant fungi colonizing biochar. Microbial biomass carbon (MBC), measured by simultaneous chloroform fumigation extraction, was impaired by the adsorption of 47.5% more dissolved organic carbon (DOC) liberated from cells to 30 t ha^{-1} biochar-amended soils than to unamended control soils. Adjusted by use of the Freundlich model to correct for DOC adsorption, MBC increased by 18.5-37.5% with an increase in the biochar application rate from 12 to 30 t biochar ha^{-1}. Meanwhile, high biochar-amended soils had lower basal respiration, which resulted in lower values for qCO_2. These results indicate a possible increase in microbial carbon use efficiency and a decrease in C turnover in response to biochar addition. I found the 30 t ha^{-1} biochar-amended soils had 615.3% and 15.0% higher activities of alkaline phosphatase and aminopeptidase, but 81.3% and 82.2% lower activities of β-D-glucosidase and β-D-cellobiase, respectively, than those in the unamended control soils. This indicates a low demand for C substrate relative to cell needs for N or P in response to biochar addition. These results suggest that the

changes observed qCO_2 in biochar-amended soils may indeed be linked to increased microbial C use efficiency. We also localized the presence of active alkaline phosphatase and β-D-glucuronidase particularly on biochar particles using Enzyme-Labeled Fluorescence (ELF) and visualized bacterial cocci and bacilli and thread-like fungal hyphae either on or in the biochar porous structure using scanning electron microscopy. PCR-T-RFLP fingerprinting analyses revealed that both the bacterial and fungal community compositions were affected strongly by biochar addition and did respond differently to different biochar application rates and time since biochar was applied. Furthermore, sequenced fungal internal transcribed spacer (ITS) regions revealed a shift, from families of the *Basidiomycota* and *Ascomycota*, to families of the *Zygomycota* and *Glomeromycota* (arbuscular mycorrhizal fungi, AMF) in response to biochar addition, thus, I suggest that the adsorption of essential nutrients on biochar allows these fungi to colonize, produce exoenzymes and meet their mineral nutrient needs. The recalcitrance of biochar suggests that the septate fungi (mainly from families of the *Basidiomycota* and *Ascomycota*) may not be able to meet their carbon needs from biochar and thus are not encouraged to colonize. To conclude, our data suggest that profound changes in soil microbial communities are occurring in biochar amended soils that apparently lead to tighter cycling and reduced system loss of both nutrients and carbon. Biochar clearly influences the diversity of microbes colonizing its surfaces, their activities and their abundance, with a net result of the conservation of resources within the soil system.

BIOGRAPHICAL SKETCH

Hongyan Jin was born and grew up in a lovely town in Northern China. In 1998, she attended Jilin Agricultural University in Changchun, China, where she received her Bachelor of Science degree in Applied Chemistry with particularly interest in Soil Chemistry and Environmental Science. As an undergraduate student, she served as a student association officer at the Department of Resources and Environmental Sciences and received awards of "Outstanding Student", "Excellent Student Leader" and "Excellent Graduate". Proudly, she received Level-A scholarship for four consecutive years and the Syngenta Agriculture Education Fund award. She concluded her undergraduate degree with an Honors Thesis under the guidance of Dr. Yan Wang. In the last half year at university, she took an internship in the Sales Department of Dongya Seed Co., Ltd. She also spent time volunteering in a survey project sponsored by BASF International Chemistry Company and working on data collection and marketing.

In 2002, Hongyan began her Master's of Science degree at China Agricultural University under the guidance of Dr. Jingguo Wang. As her interest in soil biogeochemistry and environmental management increased, she initiated research on soil C and N cycling and management at six experimental stations located across three provinces in China. As a graduate student, she was a teaching assistant in the soil biogeochemistry graduate course, a mentor for several undergraduate students in research. She was appointed as the Vice President of the student association in China Agricultural University. She was recognized as an Outstanding Student Leader. She graduated with Distinction in Research in 2006.

By the time she finished her Master's of Science degree, she was awarded with a very competitive fellowship offered by the Department of Soil and Crop Sciences,

Cornell University, which she accepted, where she focused her research on the use of biochar in agriculture with professional interests in ameliorating problem soils and improving outcomes in small-holder agriculture. She worked as a research assistant and teaching assistant in Dr. Janice Thies' research group. She served as a Soil and Crop Sciences Graduate Student Association Officer and President and Treasurer of the Cornell Amber Show Troupe. She presented her research at three professional society meetings from 2006 to 2009. She received support from a Biology and Environmental Biochemistry (BEB) Small Grant and Graduate School Research and Conference Travel Grants.

She has enjoyed very much the time at Cornell and is very grateful for having many friends around her, especially those friends working closely alongside her. They have shown much love, care and support and made it possible for her to get through all the difficulties in life and study.

给我的挚爱

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LIST OF ABBREVIATIONS

AMF	Arbuscular Mycorrhizal Fungi
AMMI	Additive Main Effects with Multiplicative Interaction
ANOVA	Analysis of Variance
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
C, G, A, T	Nucleobase: Cytosine, Guanine, Adenine, Thymine
CEC	Cation Exchange Capacity
CTAB	Cetyl Trimethylammonium Bromide
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl Pyrocarbonate
DGGE	Denaturing Gradient Gel Electrophoresis
DI	Deionized
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphates
DOC	Dissolved Organic Carbon
EC	Electrical Conductivity
EDTA	Ethylene Diamine Tetraacetic Acid
ELF	Enzyme-Labeled Fluorescence
ELFA	Enzyme-Labeled Fluorescence Alcohol
ELF-G	ELF 97 β-D-glucuronide
ELF-P	ELF 97 Phosphate
EtBr	Ethidium Bromide
FISH	Fluorescent *in situ* Hybridization
IPC	Interaction Principal Components
IPCA	Interactive Principal Component Analysis
ITS	Internal Transcribed Spacer
LSU	Large Subunit
MBC	Microbial Biomass Carbon
MCA	7-amino-4-methylcoumarin
MUF	4-methylumbelliferone
MUF-G	4-methylumbelliferyl-β-D-glucuronide
MUF-P	4-methylumbelliferyl phosphate
NCBI	National Center for Biotechnology Information
ND	Not Detected
ODW	Oven Dried Weight
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction

PEG	Polyethylene Glycol
PLFA	Phospholipid Fatty Acid
qCO_2	Metabolic Quotient
rRNA	Ribosomal Ribonucleic Acid
sCFE	Simultaneous Chloroform Fumigation Extraction
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
SSE	Sum of Squares Error
SSU	Small Subunit
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TOC	Total Organic Carbon
TP	Terra Preta
T-RFLP	Terminal Restriction Fragment Length Polymorphism
T-RFs	Terminal Restriction Fragments
UV	Ultraviolet

CHAPTER 1

CHARACTERIZATION OF MICROBIAL LIFE COLONIZING BIOCHAR AND BIOCHAR-AMENDED SOILS

INTRODUCTION

The use of biochar as a soil amendment for improving soil quality and on-farm benefits has drawn researchers' attention and interest greatly (Thies and Suzuki, 2003; Young et al., 2005; Lehmann, 2007; Lehmann et al., 2007; Rondon et al., 2007; Laird, 2008; Thies and Rillig, 2009). When farmers and gardeners apply biochar to soils, they are attempting to follow in the footsteps of the "Terra Preta Legacy" in Western Amazonia (Glaser et al., 2001). The high sustained fertility of these soils is proposed to be the presence of black carbon. Biochar use today is meant to mimic this process; yet, we do not know how it can best be adapted for use in soils outside its region of origin. Much is left to discover on the effects of biochar on soil microbial communities. Particularly, the unique microbial ecology that is essential to soil fertility and nutrient dynamics in biochar-amended soils and the degree to which biochar is a benefit is heavily dependent on the microbial community in these soils, which have received very little attention.

LITERATURE REVIEW

Biochar as a Soil Amendment

Biochar amendment practices have a long history. It was an old practice in China to mix firewood ashes with soils and livestock dung followed by heating and aging for several months, before the mixture was added directly into the field as a fertilizer. Direct burning of plant residues in the field after harvest for land clearing is common all over the world. Recently, due to its possible contribution to C sequestration, crop

productivity, ability to remove organic contaminants and it is affinity for nutrients and water, biochar soil amendment has drawn soil scientists' attention leading to an increasing number of studies, expanding this field of research rapidly.

First, amending soils with biochar provides a direct input of black C into agricultural soil (Young et al., 2005; Skjemstad et al., 1996; Liping and Erda, 2001), which contributes to reducing the release of atmospheric greenhouse gases from soil. Conversion of biomass C to biochar C, followed by burying the biochar in soil may sequester about 50% of the initial C in soils compared to direct land application of unburned biomass (Lehmann et al., 2006). Carbon in the form of biochar is relatively recalcitrant, therefore, the long persistence of biochar in soil will help to mitigate climate change as the potential of soils to be a sink for atmospheric CO_2 increases.

Second, biochar can promote crop growth by supplying and retaining nutrients and by improving soil physical and biological properties (Lehmann et al. 2003; Lehmann and Rondon, 2006). Hoshi (2001) studied the feasibility of adding bamboo charcoal to soil to produce high-quality green tea with reduced fertilizer inputs. After three years of monitoring, the bamboo charcoal tended to retain the supplied fertilizers in the rhizosphere and kept the soil pH in a range that was suitable for the growth of tea trees. The height and volume of the tea trees in the plots in which the charcoal was used were, on average, 20% and 40% higher, respectively, than they were in the unamended control plots. The best tea tree growth was found in the plots treated with 100 g m^{-2} y^{-1} crushed bamboo charcoal. However, improved crop growth is not true for all crops or for all soils. Rondon et al. (2007) found that biomass growth of beans rose with biochar applied at 60 Mg C ha^{-1}; but fell to the same value as for control treatments when the biochar applied was increased to 90 Mg C ha^{-1}. Chan et al. (2007) conducted a pot trial to investigate the effect of biochar applications on radish yield.

Results suggested a significant biochar×nitrogen fertilizer interaction, which suggested a role for biochar in improving crop N fertilizer use efficiency. But in the absence of N fertilizer, application of biochar to the soil did not increase radish yield, even at the highest application rate used of 100 t ha^{-1}; also, a significant reduction in dry matter production of radish was observed when biochar was applied at the lowest rate of 10 t ha^{-1}. The effect of biochar on plant productivity depends strongly on the amount added. Lehmann et al. (2003) suggested that the initial beneficial effects of biochar additions on nutrient availability are due to high base cation (Ca, Cu, K, Zn) and P availability. However, nitrogen limitation resulting from the high C:N ratio of the biochar added may occur in some cases and result in decreased yields at high application rates (Lehmann et al., 2003).

Third, it has been well recognized that the presence of charcoal in soil could enhance the adsorption of contaminants, such as pesticides, heavy metals and toxic secondary metabolites. Wardle et al. (1998) investigated short-term ecological effects of charcoal on the Boreal forest plant-soil system. Their results showed that the charcoal present in the soil adsorbed secondary metabolites and phenolics, which are produced by decomposition of ericaceous vegetation. The presence of these metabolites commonly slows nutrient cycling and retards tree seedling growth. Thus, the growth of *B. pendula* was stimulated by adding charcoal. In addition, adding charcoal enhanced seedling shoot to root ratios of *P. sylvestris* and *B. pendula* tree species. This response was thought to indicate greater N uptake and greater efficiency of nutrient uptake in these trees. Wardle et al. (1998) thereafter identified that *B. pendula* trees took up 6.22 times more N when charcoal was added to the root zone. Mizuta et al. (2004) investigated the effectiveness of powdered bamboo charcoal as an adsorbent for removing NO_3^- from polluted soil. Their results showed that the powdered bamboo charcoal had high adsorption effectiveness and weak temperature

dependency, which qualified it as an attractive option for the *in situ* treatment by adsorption of nitrate-N-contaminated ground and surface waters. They also considered that the removal of chemicals, which depends on physical adsorption, is effective in bamboo charcoal carbonized at a temperature of 1000 ℃. Charcoal produced at this temperature had the largest specific surface area and pore volumes in the micro-pore range. Ohe et al. (2003) investigated the adsorption behavior of nitrate anions from an aqueous solution using activated C prepared from coconut shells and charcoal prepared from bamboo. Their results showed that the adsorption of nitrate anions on these adsorbents increased with an increase in the specific surface area of the adsorbent. Yang and Sheng (2003) showed that the residues produced from burning wheat and rice were 400-2500 times more effective than unamended soil in adsorbing diuron over the concentration range of 0-6 mg L^{-1} in water. They also reported that the diuron adsorption by charcoal-amended soils increased with increasing charcoal content. Yu et al. (2006) examined the role of charcoal in the sorption and desorption behaviors of diuron pesticide in soil. The results showed that the sorption-desorption hysteresis had a positive correlation with the micro-pore volume of the charcoal-amended soils. Their results also indicated that the presence in soil of small amounts of charcoal produced at high temperature can have a marked effect on the release behavior of organic compounds. Chun et al. (2004) and Sheng et al. (2005) reported that the charcoal derived from wheat was well-carbonized and had a relatively high surface area and low oxygen content, and had a high affinity for organic compounds. A study by Zhang (2005) showed that wheat-derived char incorporated in soil (1% g g^{-1}) resulted in a 10-fold increase in sorption and a significant decrease in solution-phase benzonitrile concentration in the char-amended soil slurry.

Fourth, possible changes in soil pH triggered by biochar amendment could affect many aspects of microbial activity. Under extremely acidic or alkaline conditions,

microbial activity is decreased. Neutral to alkaline pH levels are generally favorable for the microbial degradation of various compounds (Leahy and Colwell, 1990). Houot et al. (2000) examined the effect of soil chemical, physical and biological properties on accelerated microbial degradation of atrazine after repeated applications in 47 agricultural soils with pH values ranging from 5.5 to 8.3. Their results indicated that among the factors of soil pH, sorption coefficients, total microbial activity, organic C and clay content, soil pH was related most significantly to the rate of atrazine mineralization (r=0.83). Kastner et al. (1998) reported that a bacteria strain introduced into the soil did not exhibit activity due to unfavorable pH (5.2) of the soil. Neutralizing the soil pH to 7.0 resulted in a ten-fold increase in the soil respiration rate.

Fifth, biochar has been suggested to have the potential to reduce leaching of organic and inorganic contaminants from agricultural soils (Lehmann et al., 2006) because of the strong adsorption affinity of biochar for soluble nutrients such as ammonium and phosphate. However, it remains uncertain how effective biochar will be in mitigating offsite movement of nutrients in different soils under different climate regimes.

Little is known about charcoal preservation over time and the causes of its degradation once it is buried in soil. Charcoal is usually regarded as a relatively inert substance that is altered little by chemical or biochemical processes (Nichols et al., 2000). However, few studies have shown that this may not be the case. Frink (1992) and Bird et al. (2002) showed that charcoal found in an archeological site had either undergone diagenetic changes or had a decreased C content.

Interactions of Biochar with Soil Microorganisms and Microbial Exoenzymes

Microbial communities in soil can be very sensitive to ecosystem perturbations (Schloter et al., 2003), and biochar soil amendment is no exception. Biochar specific

properties make interactions between biochar and microorganisms quite complicated. On one hand, soil microbial activity, abundance and community composition may be affected by the quality and quantity of the biochar used to amend the soil. On the other hand, microorganisms may have a biodegrading effect on the quantity and properties of the biochar. Furthermore, the interactions between biochar and microorganisms will affect nutrient cycling and nutrient availability remarkably both to microorganisms and to plants grown in biochar-amended soils.

Microbial community activity, biomass and composition change in soils rich in biochar (Thies and Suzuki, 2003). Zackrisson et al. (1996) investigated the effects of artificially produced charcoal on soil microbial properties at six sites. Microbial biomass was consistently enhanced in humus when it was placed adjacent to charcoal particles. Decomposition of plant litter was sometimes also affected by being in the proximity of charcoal but the direction of these effects was unpredictable. Uvarov (2000) studied the respiration of the soil microbial community, the decomposition rate of soil organic matter and cotton strips, and herb seed germination in an area containing charcoal kilns, compared with control soil systems. The results indicated a significantly higher level of soil biological activity in the soil with charcoal versus the control sites. Rivera-Utrilla et al. (2001) showed that activated C adsorbs microorganisms strongly, and that this adsorption increases with higher hydrophobicity. Steiner et al. (2008) observed that basal respiration, microbial biomass, population growth and the microbe's assimilation efficiency (expressed by the metabolic quotient) increased linearly and significantly with increasing charcoal concentrations (50, 100 and 150 g kg^{-1} soil). Quite a few studies conducted in Japan provided strong evidence that the application of charcoal to soil can have positive effects on the abundance of arbuscular mycorrhizal fungi (AMF). Matsubara et al. (2002) found that the application of coconut shell derived black carbon to soil at a rate

of 30% by volume stimulated a 69% increase in plant pathogen resistance due to the increased colonization by AMF on *Asparagus officinalis*. Yamato et al. (2006) identified a 42% increase in root colonization by AMF that was attributed to *Acacia mangium* bark-derived charcoal application. Rondon et al. (2007) suggested that AMF colonization of N-fixing *Phaseolus vulgaris* roots increased by 16% when *Eucalyptus deglupta*-derived biochar was applied at a rate of 90 g kg^{-1} soil. A study on the effect of activated carbon on mycorrhizal colonization of *Quirks robur* seedlings showed a 624% acceleration in mycorrhiza formation; meanwhile, the rapid mycorrhiza colonization increased drought resistance in *Quirks robur* (Herrmann et al., 2004). Warnock et al. (2007) suggest five possible mechanisms by which biochar might influence AMF abundance: (1) alteration of soil physico-chemical properties; (2) indirect effects on mycorrhizae through effects on other soil microbes; (3) plant-fungus signaling; (4) interference and detoxification of allelochemicals on biochar; and (5) provision of refugia that protect fungi and bacteria from their grazers. Nishio and Okano (1991) reported that root infection by AMF fungi significantly increased alfalfa yield by 40 - 80% when 1 kg m^{-2} of biochar was added to an alfalfa field in a volcanic ash soil. Saito and Marumoto (2002) believed that charcoal particles acted as a micro-habitat for AMF and enabled them to survive, and meanwhile provided protection from saprophytes. Another insightful study done by O'Neill et al. (2009) adopted both culturing and molecular methods to characterize the microbial community in Terra Preta (TP) soil, the fertile Amazonian Dark Earths, which are valued as a historical inspiration for biochar use as a soil amendment benefiting crop growth and carbon storage in the modern age (Lehmann et al., 2006). By comparing the microbial communities in the TP sites and the background soils adjacent to them in the Brazilian Amazon, they found that culturable bacteria were more abundant in TP soils than in adjacent soils. A cluster analysis of restriction fragment length

polymorphisms of isolates cultured and the cloning and sequencing of bacterial DNA recovered from PCR-DGGE gels both indicated the presence of microbes that are uniquely associated with soils high in black carbon as compared to adjacent soils and that these organisms have greater phylogenetic similarity to each other across TP sites than in comparison to their corresponding adjacent soils.

As both biochar and microorganisms are hydrophobic and have a large surface area, it is quite possible that microorganisms are retained in biochar pores allowing them to create a suitable habitat for themselves. One study showed that biochar is able to serve as a habitat for extraradical fungal hyphae that sporulate in the microspores due to lower competition from saprophytes (Saito and Marumoto, 2002). However, the mechanism for this is not yet clear. Also, the porous structure of biochar and its strong affinity for nutrients could provide microorganisms with substrates and protect them from their natural predators. However, little is known about which particular microbial communities could be involved preferably. Another important possibility is that nutrient-containing substrates might be preferentially adsorbed by biochar, which could result in increased nutrient availability for microbial colonization (Smith et al., 1992).

Although exoenzymes secreted by microorganisms may slowly hydrolyze adsorbed substrates (Estermann and McLaren, 2006), low-molecular-weight substrates are generally believed to be degraded by intracellular enzymes. The latter require the compound to enter the cells of microorganisms in order to be metabolized. An adsorbed compound binding to solid biochar is not free to be transported into cells. Reactions catalyzed by such extracellular enzymes may be markedly affected by adsorption because the enzymes may lose their catalytic activity. This is further complicated by findings that some microorganisms have the ability to directly access

soil-adsorbed substrates and degrade them in the sorbed state (Feng et al., 2000; Park et al., 2002). Similarly, this suggests that bioavailability of adsorbed chemicals is affected by not only by the adsorptive mechanism but also the characteristics of the microorganisms in question. Guerin and Boyd (1997) observed that soil-sorbed naphthalene was wholly unavailable to one bacterial strain, but used directly by another, due to differences in the physiological characteristics of the organisms. Adsorption of both substrate and microorganisms to biochar surfaces may result in a higher concentration of substrate near the attached bacterial cells and, therefore, may increase substrate utilization (Ortega-Calvo and Seiz-Jimenez, 1998). Purines, amino acids, and peptides that enter the interlayer region of expanding clays, such as montmorillonite, may become physically protected from microbial degradation because the cells cannot access the substrate. Thus, whether a nutrient substrate in its adsorbed state can be directly degraded depends on the capacity of the microorganism to access and use the adsorbed substrate. This capacity relates to both physiological characteristics of binding the microorganisms and adsorptive mechanisms of the chemical to biochar. However, it is still not clear if charcoal's adsorption of compounds inhibits microbes, increases nutrient immobilization (Schimel et al., 1998), or provides microbes a protected site with adequate resources and away from predation (Pietikäinen et al., 2000).

Considering the complexity of interactions between biochar, nutrient cycling and microorganisms in soils, many questions still remain to be answered regarding microbial ecology in biochar-amended soils. The possible interactions between the soil microbial community and biochar are hypothesized to include: (1) Biochar is a porous substance with high water holding capacity and affinity for chemicals and nutrients, which cause the selection of microbial communties; (2) Biochar contains relatively recalcitrant carbon, this change in substrate availability will lead to changes in

microbial community composition; (3) Biochar provides the structural habitat needed for a rich microbial community to take hold; and (4) Microorganisms have degrading effects on biochar. Within this context, my objectives were (1) to assess the effect of corn stover biochar on important soil microbiological parameters, basal respiration, microbial biomass C and the metabolic quotient, (2) to investigate the significance of exoenzyme activity and resultant nutrient mineralization in response to biochar soil amendment, (3) to verify microbial colonization and exoenzymes adsorption on biochar particles, (4) to characterize the microbial community composition and particularly, the metabolic degradation potential of the fungal community in response to biochar soil amendment.

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CHAPTER 2

MICROBIAL BIOMASS CARBON AS AN INDICATOR OF MICROBIAL ABUNDANCE RESPONDS TO BIOCHAR SOIL AMENDMENT

ABSTRACT

Biochar is recommended as a soil conditioner, even though little information is available about its effects on soil ecology. In this study, we investigated the effects of biochar added to a temperate soil cropped to corn on microbial biomass. Microbial biomass carbon (MBC) as an indicator of microbial abundance was investigated in biochar-amended soils collected from a field experiment at the Cornell Musgrave Farm, Aurora, NY in 2007. In biochar-amended soils, the microbial biomass was determined by use of simultaneous chloroform fumigation extraction, which was impaired by the strong adsorption to biochar and soil constituents of up to 50.6% of the dissolved organic carbon (DOC) added. In contrast, only 34.3% of DOC added was adsorbed in unamended soil. All established methods for extracting DOC from soil proved to be inadequate for extracting DOC from biochar-amended soil; hence, we introduced a correction method that is based on DOC equilibrium adsorption isotherms. The Freundlich model was tested and yielded a better fit (r^2=0.795-0.881, *SSE*=0.12-0.20) for DOC adsorption in all biochar-amended soils than the Langmuir model (r^2=0.495-0.790, *SSE*=0.21-0.51). The data obtained was adjusted by DOC adsorption equilibrium isotherms and indicated that MBC increased by 18.5-37.5% with increasing biochar application rate from 12 to 30 t biochar ha^{-1}. The statistical significance of this increase in MBC depended highly on biochar quantity. There was no significant interaction between biochar and fertilizer applied (P=0.76) on MBC during the short, six month duration of the field experiment. Basal respiration and the metabolic quotient (qCO_2) decreased in soils amended with high rates of biochar (12

and 30 t biochar ha^{-1}). These results suggest that adding high amounts of biochar to soil increases microbial C use efficiency. This shift may have implications for soil C sequestration in that biochar application may positively influence soil organic C retention.

INTRODUCTION

The soil microbial community plays a critical role in regulating processes such as decomposition of organic matter, nutrient cycling and greenhouse gas emissions (Anderson and Domsch, 1986; Zeller et al., 2001; Garcia et al., 2002); hence, it is important to understand how agricultural management practices regulate microbial abundance and activity. The importance of soil microbial activity is emphasized by the fact that 80-90% of the processes in soil are reactions mediated by microorganisms (Nannipieri and Badalucco, 2003). Assessing the dynamics of soil microorganisms gives us insight into the response of soil ecosystems to environmental changes and other human impacts (Mader et al., 2002). Microbial biomass, respiration and the metabolic quotient (qCO_2) are used as indicators of changes in soil quality (Anderson and Domsch, 1990). Soil respiration and the metabolic quotient are used as indicators of microbial activity and of changes occurring in the soil due to the addition of plant and animal residues, changes in management or soil pollution (Anderson and Domsch, 1990; Wardle and Ghani, 1995) and, in this case, to evaluate the effects of biochar soil amendment on soil microbial community dynamics (Thies and Rillig, 2009).

Biochar (biomass-derived charcoal) is produced by pyrolyzing a variety of crop and forest residues, and carbon-based wastes from agriculture and construction (Walsh et al., 1999). The use of biochar as a soil amendment strategy for improving soil health and better using natural resources has drawn considerable attention and interest. Biochar soil amendments have been studied broadly due to their possible contribution

to increasing C sequestration (Young et al., 2005; Lehmann et al., 2006), adsorption capacity for organic contaminants and nutrients, and water (Wardle et al., 1998; Ohe et al., 2003; Yu et al., 2006), and its effect on crop productivity (Lehmann et al., 2003; Fu et al., 2004; Lehmann and Rondon, 2006; Rondon et al., 2007; Chan et al., 2007). An understanding of soil processes is the key to monitoring the influence of biochar soil amendment practices on the fertility and quality of soil, and thus, on environmental quality. Despite the obvious importance of the soil biological response to a biochar amendment, very little is known about the effects on soil microbial biomass and activity. Biochar soil amendments have been reported to increase the size of microbial biomass carbon (MBC) and rates of microbial activities (Zackrisson et al., 1996; Wardle et al., 2008). The application of biochar can also have positive effects on the abundance of mycorrhizal fungi (Matsubara et al., 2002; Yamato et al., 2006; Rondon et al., 2007; Warnock et al., 2007).

For the MBC analysis in this study, simultaneous chloroform fumigation extraction (sCFE) was used to measure microbial biomass (Witt et al., 2000). However, using the extractable organic C to calculate microbial biomass C from the additional C made extractable by the fumigation is still controversial; especially in soil samples that have received biochar. The main difficulty is that the extraction of DOC from lysed from microbial cells is incomplete due to the strong adsorption of DOC on biochar and soil constituents (Qualls, 2000; Guggenberger and Kaiser, 2003).Therefore, a correction factor is needed to account for the adsorbed microbial DOC released. Liang et al. (2010) used an isotope method to develop an extraction coefficient. However, this method is time-consuming and expensive to conduct. Here, we investigated the use of a much simpler approach, adsorption equilibrium isotherms of DOC, to correct for the microbial released DOC adsorbed to biochar and soil. The general method used to obtain sorption characteristics of a given chemical involves the

equilibration of different solute concentrations with the adsorbent under investigation and measuring the amount of the solute that has not been adsorbed and is still in solution. A variety of adsorption isotherm models have been studied (Kaiser and Zech, 1997; Michalzik et al., 2003; Ussiri and Johnson, 2004; Walton et al., 2005), however, model performance was not consistent between different studies due to the different characteristics of adsorbates and adsorbents studied. Here, we adopted a rapid experiment to generate two simple and widely used adsorption equilibrium isotherms, the Freundlich and Langmuir isotherms, to characterize our biochar-amended soils.

This comparative study was performed to determine changes in soil microbial biomass carbon and basal respiration in response to biochar soil amendment. The specific objectives of this study were to determine (1) the DOC adsorption kinetics of biochar-amended soils to correct for adsorption of lysed microbial DOC to biochar; (2) the most suitable model to describe DOC adsorption to biochar-amended soil; (3) the microbial biomass carbon estimates before and after the adjustment by the DOC adsorption isotherm model; (4) the effects of biochar soil amendment on microbial biomass both in bulk and rhizosphere soils; (5) the interactive effects of inorganic fertilization and biochar application on microbial biomass in both bulk and rhizosphere soils; and (6) the effects of biochar soil amendment on soil basal respiration and the metabolic quotient, qCO_2.

MATERIALS AND METHODS

Field Experimental Design

The field experiment was established at Cornell Musgrave Farm in Aurora, NY, in May, 2007. The biochar applied was obtained from BEST Energies, Inc. (56 Gindurra Road, Somersby, NSW 2250, Australia) and was produced by pyrolyzing corn stover. Corn stover biochar was incorporated into the soil at four rates (0, 1, 12,

30 t biochar ha^{-1}) before planting corn at a density of 32,000 seeds $acre^{-1}$. Nitrogen fertilizer (17% nitrate N and 17% ammoniacal N) was added at four different rates (67, 94, 121, 135 kg N ha^{-1}) for two rates (0 and 12 t biochar ha^{-1}) of biochar. There was an incomplete factorial arrangement of two treatment factors (N fertilizer, biochar) in 10 combinations. Treatments were replicated 3 times in a completely randomized design (Table 2.1).

Table 2.1 Experimental design showing treatments (T1-T2) assigned in an incomplete factorial design

Treatment No.		Biochar (t ha^{-1})			
		0	1	12	30
Fertilizer (kg ha^{-1})	67	T1		T5	
	94	T2		T6	
	121	T3	T9	T7	T10
	135	T4		T8	

Sampling and Sample Preparation

In November, 2007, seven bulk soil samples were taken from each plot from 0-15 cm depth along a random S shape across each plot using a soil auger (12 mm diam) and composited. Each composite soil sample was mixed well and obvious root material removed with forceps. The samples were zipped into sterile plastic bags and put in an ice chest immediately. Before moving to the next plot, the sampling probe was sterilized with bleach and rinsed three times with purified water in order to avoid cross-contamination. The samples were sieved (2 mm mesh), homogenized and stored at 4 ℃. These soil samples were used within 24 h after sampling for (1) method development and (2) soil microbial biomass carbon and basal respiration assays.

Development of a Method to Determine Microbial Biomass C in Biochar-amended Soils

I observed a strong adsorption of free DOC on biochar and soil constituents; hence, I initially determined the time required to reach adsorption equilibrium for DOC. A DOC stock was prepared by stirring 400 g soil samples (taken from soils adjacent to experimental plots) with 1000 ml deionized water, centrifuging, recovering the supernatants, air-evaporating the supernatants until the DOC concentration was over 500 μg ml^{-1}. The exact concentration of DOC stock was determined by a TOC analyzer (Shimadzu TOC-5000A Autoanalyzer, Columbia, MD, USA). A series of slurries was prepared for each treatment. In detail, 40 ml of 0.05 M K_2SO_4 was added to each 10 g (ODW) soil sample. After thorough shaking and centrifuging at 10,000 × g, the supernatant was removed. Then, another 40 ml of 0.05 M K_2SO_4 and DOC stock was added to each slurry after autoclaving the slurries at 121 ℃. Thirty-three replicates for each treatment were set and DOC was added to achieve a final concentration of 1 mg g^{-1} soil. At regular intervals between 5 and 360 min (at 5, 10, 15, 20, 30, 60, 90, 120, 150, 240, 360 min) after the DOC was added, a subset of three samples was centrifuged (5 min at 10,000×g), and the supernatants were transferred to fresh Eppendorf tubes. The concentration of free DOC in the supernatants was determined on a TOC analyzer (Shimadzu TOC-5000A Autoanalyzer). The amount of adsorbed DOC was calculated as the difference between the total amount added and that remaining in the supernatant.

The time required to reach adsorption equilibrium of DOC was determined by the time that DOC adsorption became relatively constant. To develop the DOC adsorption equilibrium isotherms for each biochar application rate, a series of sample slurries were prepared the same way as those used to determine contact time. Then,

DOC was added at nine concentrations (5, 10, 15, 20, 30, 40, 60, 80, and 100 μg ml^{-1}). After thorough shaking and centrifuging at 10,000 × g, I destructively sampled the supernatant from each DOC concentration series. DOC in the supernatant was detected on a TOC analyzer (Shimadzu TOC-5000A Autoanalyzer). The amount of adsorbed DOC was calculated as the difference between the total amount added and that detected in the supernatant.

At equilibrium, the amount of a substance adsorbed $\{S\}$(moles g^{-1} dry weight) depends on the concentration of the substance remaining in solution $\{Ce\}$(moles ml^{-1}). Two models were chosen to examine DOC equilibrium adsorption isotherms, the Freundlich equation (Kano et al., 2000) and the Langmuir equation (Langmuir, 1918).

(1) Freundlich isotherm: According to the Freundlich equation,

Equation 2.1 $S = K \cdot C_e^{\,n}$

where: *S*, is the amount adsorbed at equilibrium (μg g^{-1} soil); *Ce*, the equilibrium concentration of the adsorbate (DOC); and *K* and *n* are Freundlich constants, n giving an indication of how favorable the adsorption process is and *K* is the adsorption capacity of the adsorbent. *K* can be defined as the adsorption or affinity coefficient and represents the quantity of adsorbate adsorbed onto a carbon adsorbent for a unit equilibrium concentration. The slope, *n*, ranging between 0 and 1, is a measure of adsorption intensity or surface heterogeneity, becoming more heterogeneous as its value gets closer to zero (Haghseresht and Lu, 1998). A value of *n* below one indicates a normal isotherm while n above one is indicative of cooperative adsorption (Fytianos et al., 2000).

(2) Langmuir isotherm: According to the Langmuir equation,

Equation 2.2 $S = \dfrac{S_T \cdot C_e}{C + C_e}$

Where: S_T and C are the equilibrium constants, which are related to rate of adsorption and adsorption capacity, respectively; S is calculated from the total concentration of DOC added; C_T and the dry weight content of the sample slurry, D_{slurry} (g dry weight ml^{-1}).

Equation 2.3 $$S = \frac{C_T - C_e}{D_{slurry}}$$

The Freundlich equation was transformed into a linear model by taking the log of both sides. i.e., $\log\{S\} = \log K + n\log\{Ce\}$, then fitted to the data points using the modified linear regression model in JMP 6.0, which yielded the two parameters K and n. The Langmuir equation was transformed into a linear model by taking reciprocals. i.e., $1/S = C/S_T \times 1/Ce + 1/S_T$, then it was fitted to the data points using the transformed linear regression model in JMP 6.0, which yielded the two parameters C and S_T that are related to rate of adsorption and adsorption capacity, respectively.

The total concentration of DOC, C_T, during the DOC assays was calculated from the equilibrium concentration of DOC in the supernatants, Ce, and D_{slurry} and from the values of model parameters:

For the Freundlich equation: **Equation 2.4** $$C_T = C_e + D_{slurry} \cdot K \cdot C_e^{\,n}$$

For the Langmuir equation: **Equation 2.5** $$C_T = C_e + D_{slurry} \cdot \frac{S_T \cdot C_e}{C + C_e}$$

Examining equilibrium adsorption isotherms of DOC allowed us to obtain the adjusted $E_{C1}'$and E_{C0}', and thus the adjusted microbial biomass carbon. A comparison was made between the unadjusted and adjusted methods. In sCFE,

Equation 2.6 $$Biomass C = E_{C1}' - E_{C0}'$$

where E_{C1}'= adjusted organic C extracted from fumigated soil; E_{C0}'=adjusted organic C extracted from non-fumigated soil.

Briefly, moist soils were incubated with distilled $CHCl_3$ overnight. The soil was then extracted with 0.5 M K_2SO_4; a non-fumigated control was extracted under the same conditions at the time fumigation commenced. The flush of extractable DOC due to fumigation was calculated as the difference between fumigated and non-fumigated samples (Vance et al., 1987; Witt et al., 2000). DOC in the extracts was determined on a TOC analyzer (Shimadzu TOC-5000A Autoanalyzer) using persulfate oxidation.

Soil water content was determined by weighing soil samples before and after drying for 24 or more hours at a temperature of 105 ℃ until the soils had reached a constant weight.

Soil Basal Respiration and Metabolic Quotient

Soil basal respiration is the biological oxidation of organic matter to CO_2 by either aerobic or anaerobic organisms, and is positively correlated with microbial activity (Alef, 1995). In this study, soil basal respiration was determined by measuring the change in the electrical conductivity of an alkali trap once a week and comparing it to a standard curve (Zibilske, 1994). Specifically, 20 g (ODW equivalent) of soil from each biochar treatment was weighed into a 100 ml beaker and put into a 1 L airtight jar. The jar, together with another 50 ml vial containing 40 ml, 0.5 M KOH (the appropriate volume was determined from a preliminary test) was placed into the 1 L airtight jar and incubated at 25 ℃ for 8 weeks (WK). Five ml of DI water was added to the bottom of each jar to maintain high humidity. During the incubation, the electrical conductivity (EC) of the KOH trap was measured once a week. The CO_2 respired was calculated using the equation below,

Equation 2.7 $$CO_2(absorbed) = 22 \times V_{trap} \times C_{initial} \frac{EC_{initialKOH} - x}{EC_{initialKOH} - EC_{1/2K_2CO_3}}$$

where $EC_{initialKOH}$ is the EC reading of the initial KOH trap (0.5 M); $EC_{1/2K_2CO_3}$

is the EC reading of the fully CO_2-saturated state of the KOH trap, K_2CO_3 solution (0.25 M); V_{trap} is the volume of the KOH trap; $C_{initial}$ is the initial concentration of the KOH trap. The metabolic quotient was calculated as the ratio of basal respiration to microbial biomass C (Anderson and Domsch, 1990).

RESULTS AND DISCUSSION

Adsorption Kinetics

Initially, I determined the time course for the adsorption of DOC in the unamended control and soil amended with biochar at 30 t biochar ha^{-1}. The experimental results of the adsorption of DOC to the soil and biochar matrix at a final concentration of 100 μg DOC ml^{-1} soil with varying contact times are shown in Fig. 2.1. Percent adsorption dramatically increased with increasing contact time in the first 30 min. Then, the adsorption of DOC gradually reached equilibrium after approximate 1.5 h of incubation (Fig. 2.1). During adsorption of DOC, DOC molecules reach the boundary layer initially; then, they diffuse onto the adsorbent surface; and finally, they diffuse into the porous structure of the adsorbent. This phenomenon takes a relatively long contact time. Data shown in Fig. 2.1 reveal that the curves are single, smooth, and continuous, leading to saturation. This suggests a monolayer coverage of DOC on the biochar and/or soil surface. At the adsorption equilibrium, 50.6% of the initial DOC added to slurries of soil that received 30 t biochar ha^{-1} had disappeared from the dissolved phase, compared with only 34.3% of the initial DOC adsorbed to unamended soil slurries (Fig. 2.1). Thus, there was about 50% more of the initial DOC adsorbed to soil that received 30 t biochar ha^{-1} than to the unamended soil. This indicates that biochar is a strong sorbent for DOC. This raised concern about possible differential DOC extraction efficiency in biochar-amended compared to unamended soil when the sCFE method is adopted to determine soil microbial biomass C.

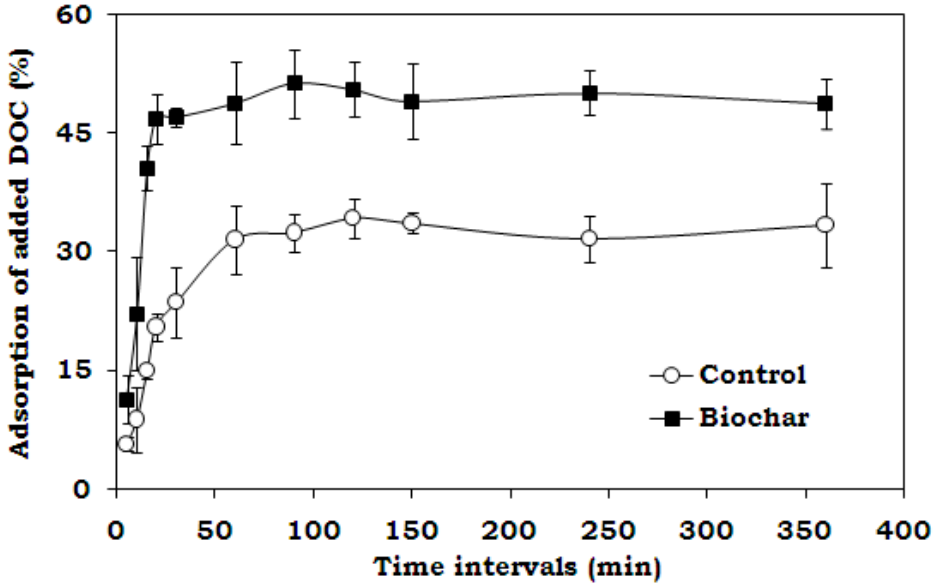

Figure 2.1 Time course of DOC adsorption in slurries of soil samples amended with biochar at a rate of 30 t ha^{-1} and unamended soil. For both treatments, the adsorption kinetics of DOC (final concentration, 100 µg DOC ml^{-1}) is shown. The error bars indicate standard deviations (n=3).

Effect of Biochar Application Rates

The adsorption of DOC to unamended soil and those amended with 1 t biochar ha^{-1} was significantly lower than the absorption to soils amended with 12 and 30 t biochar ha^{-1}. Soil receiving high rates of biochar added adsorbed more DOC than the soil matrix alone. The detectable DOC (*Ce*) increased with increasing concentration of DOC added (C_T) while it decreased as the rate of biochar amendment decreased (Fig. 2.2). Compared to unamended control soils, the average adsorption of DOC in soils with 30 t ha^{-1} biochar added increased by 27, 14, 19, 21, 12, 12, 11, 8, to 5% for the initial DOC concentrations given above, respectively. This can be attributed to increased biochar surface area and availability of more adsorption sites in the high biochar-amended soils. Also, it was observed that the adsorption percentage of initial DOC for all treatments had a decreasing trend with increasing initial concentration of DOC added, indicating that the adsorption capacity of biochar and soil constituents on

DOC does not increase infinitely with increasing concentrations of initial DOC under the condition that the adsorption sites remain constant. On the contrary, biochar and soil constituents tend to reach their maximum DOC adsorption capacity with the increasing concentration of initial DOC. Similar observations were reported by Cheng et al. (2005) where adsorption of dissolved natural organic matter onto modified granular activated carbon was observed.

Equilibrium Adsorption Isotherms of DOC

Conformation of the experimental data into both the Freundlich and the Langmuir isotherm models was done separately for individual biochar treatments. The experimental data analyzed according to the linear form of the Freundlich isotherm is shown in Fig. 2.3. The Freundlich equation gives a better fit to the experimental data for all treatments with varing biochar application rates than the Langmuir equation. The adsorption of DOC to unamended control soil was significantly lower than the adsorption to soils amended with 12 and 30 t ha^{-1} biochar (Figs. 2.2 and 2.3).

The biochar-amended soils adsorb more DOC under field conditions than the unamended control soils, thus the model application should overcome the underestimation of DOC adsorption, especially in soils receiving high rates of biochar. The Freundlich model used in this study showed significantly higher DOC concentration values in extracts ranging from 50-78% after model adjustment.

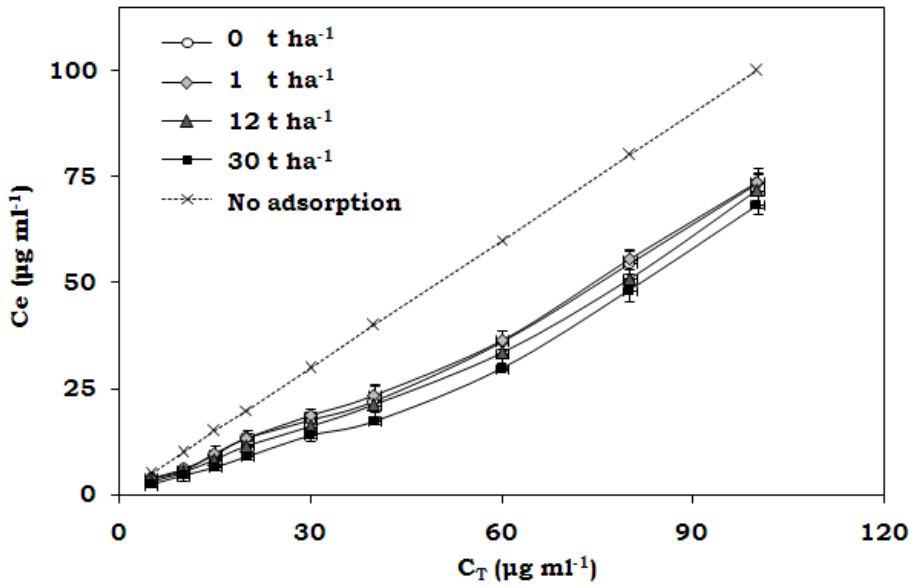

Figure 2.2 Concentrations of DOC in soil slurries with 0, 1, 12, and 30 t biochar ha^{-1} applied. The error bars indicate standard deviations. The concentrations of free DOC expected in the absence of adsorption are indicated by the dotted line.

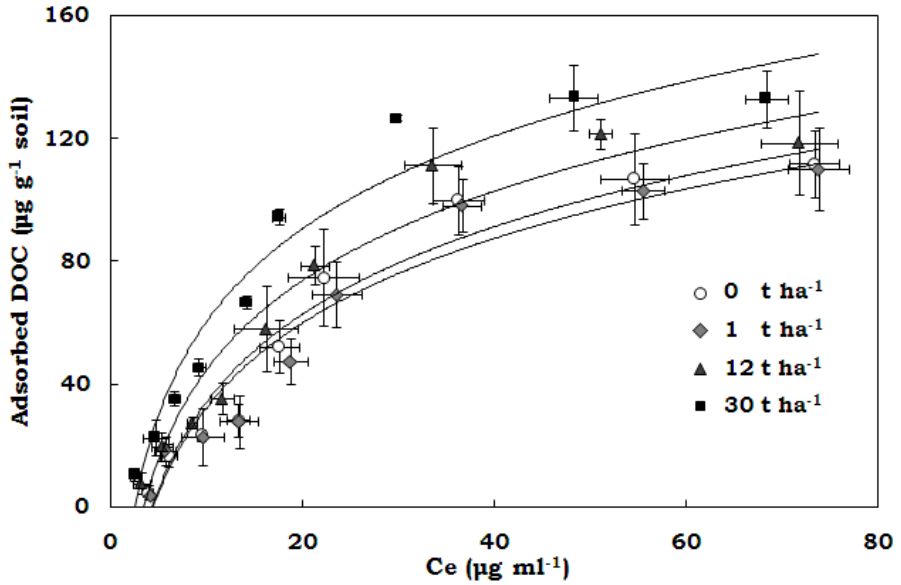

Figure 2.3 Equilibrium adsorption isotherms of DOC for 0, 1, 12, 30 t biochar ha^{-1} applied fitted to the Freundlich equation (lines). The error bars indicate standard deviations (n=3).

Test of Kinetics Models

The values of the parameters of the two isotherm models and the related correlation coefficients are given in Table 2.2. The Freundlich model yielded a somewhat better fit (r^2 ranging from 0.80 to 0.88) than the Langmuir model (r^2 ranging from 0.50 to 0.79). Similar observations were made by Vandenbruwane et al. (2007), where both the Freundlich and Langmuir models had similar goodness-of-fit and parameter estimates for the adsorption of DOC onto mineral soils. The value of *n* in the Freundlich model is closer to zero for 12 and 30 t ha^{-1} biochar-amended soils than for the adsorbents soil only or soil with 1 t biochar ha^{-1}. This indicated that more DOC adsorption surface heterogeneity was caused by the high biochar amendment. The value of *n* decreased with increasing rate of biochar applied. This indicated favorable adsorption for DOC on added biochar particles (Kano et al., 2000; Ng et al., 2002). When data were fitted to the Freundlich adsorption model, the adsorptive affinity, *K*, of DOC to soils amended with 30 t biochar ha^{-1} ranged from 6.52 to 8.94, about three times that of soils amended with 12 t biochar ha^{-1}, and about three times higher than that of soils amended with 1 t biochar ha^{-1} and unamended soils. However, no distinct difference in the adsorptive affinity of DOC was found between the unamended soils and those soils amended with 1 t biochar ha^{-1} (Table 2.2).

Table 2.2 Parameters of the Freundlich and Langmuir equilibrium adsorption isotherms for DOC and adsorbent soils amended with varying amount of biochar

Treatment (t biochar ha^{-1})	Freundlich				Langmuir			
	n	*K*	r^2	*SSE (%)*	S_T	*C*	r^2	*SSE (%)*
0	0.91-1.09	1.74-2.88	0.841	0.16	–35.05 - –388.0	–40.56 - –54.82	0.641	0.36
1	0.91-1.11	1.50-2.79	0.795	0.20	–27.90 - –273.5	–37.02 - –51.22	0.607	0.39
12	0.78-0.94	3.44-5.37	0.837	0.16	–116.2 - 86.64	188.5 - 284.0	0.495	0.51
30	0.70-0.82	6.52-8.94	0.881	0.12	–890.7 - 163.9	71.70 - 88.19	0.790	0.21

The applicability of both kinetic models was verified through the value of r^2 and the sum of squared error (*SSE*, %). *SSE* is generally used to determine the validity of each model. The higher the value of r^2 and the lower the value of *SSE*, the better the goodness of fit is considered to be. The adsorption of DOC to biochar-amended soils was better described by the Freundlich isotherm model than by the Langmuir isotherm model when the value of both r^2 and *SSE* are compared. Our results conflict with those of Hameed et al. (2007), where the adsorption of methylene blue onto bamboo-based activated carbon was found to fit he Langmuir isotherm model better than Freundlich model, under soil-free conditions.

Despite a number of assumptions and simplifications of isotherm models, the fits of the isotherm models based on our results of adsorption equilibrium isotherm experiments describe well the DOC concentrations in the soil extracts. The adsorption isotherms in our study were obtained using an initial DOC solution which was extracted from the same field soils we work on and concentrated, therefore, the same sources of DOC for building the adsorption model and for follow-on microbial biomass assays guaranteed the consistency and accuracy of the detection of DOC liberated from microbial cells given the fact that the chemical composition of DOC species remains consistent as a result of the use of the same source soils (Kaiser and Zech, 2000), as do the adsorption characteristics.

Effect of Biochar Amendment on Soil Microbial Biomass C

The highest MBC was measured in bulk soils amended with 30 t biochar ha^{-1}, the lowest MBC was measured in bulk unamended soils (Fig. 2.4). This increased trend with increasing biochar addition indicated a positive response of the microbial biomass to biochar application. The MBC in rhizosphere soils showed a similar trend. The 1 t biochar ha^{-1} treatment did not significant affect MBC. Meanwhile, bulk soil

MBC showed a close correlation with rhizosphere soil MBC. Microbial biomass carbon for 30 t ha^{-1} biochar-amended bulk soils before adjustment ranged from 69.7-74.5 μg C g^{-1} soil (mean value of 73.9 μg C g^{-1} soil) of DOC, and higher than these for 12 t ha^{-1} biochar-amended soils, although the difference was not statistically significant. After the adjustment by DOC adsorption equilibrium isotherms, the difference in MBC for these two treatments became statistically significant (Fig. 2.4). Examining DOC adsorption equilibrium isotherms made it possible to better estimate the amount of total microbial liberated C in biochar-amended soils.

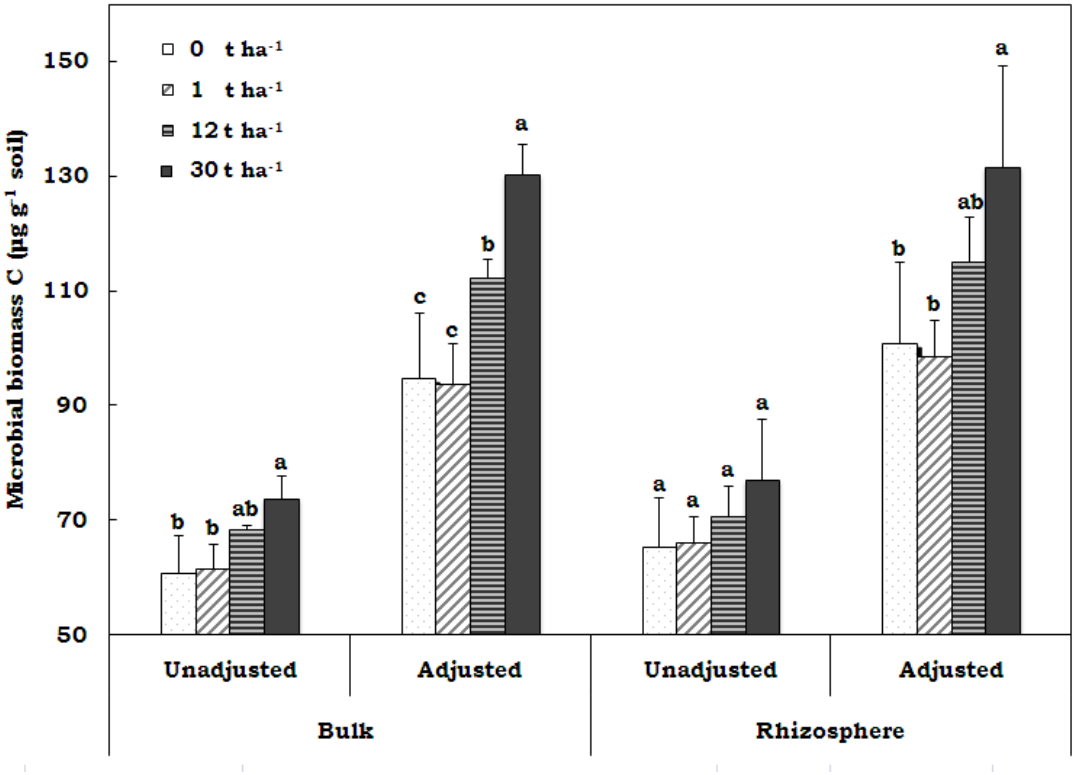

Figure 2.4 MBC in both bulk and rhizosphere soil affected by biochar amendment. A comparison of MBC before and after adjustment by use of the Freundlich isotherm model is also shown. Variations of MBC for each treatment are presented as error bars.

Our finding of increased MBC in biochar-amended soils was consistent with the study reported by Zackrisson et al. (1996), who investigated the effects of artificially produced charcoal on soil microbial properties at six sites and found that microbial biomass was enhanced when it was placed adjacent to charcoal particles; and by Pietikainen et al. (2000), who observed higher specific growth rates of microbial communities in charcoal enriched soil layers than in the unburned humus enriched soil layer. The microbial biomass was affected not only by the quantity, but also by the quality of the carbon input as observed by others (Srivastava and Singh, 1991; Guggenberger and Zech, 1999). MBC, by definition, reflects the degree of immobilization of C in microbial biomass. Decreased soil microbial biomass lowers the capacity of the soil to hold in nutrients; while increased microbial biomass may lead to temporary immobilization of nutrients in biomass (McGill et al., 1986).

Interaction of Biochar Amendment and Fertilization on Soil Microbial Biomass C

Addition of higher rates of N fertilizer (121, 135 kg ha^{-1}) resulted in higher MBC in both bulk and rhizosphere soil when no biochar was added (Fig. 2.5). Increases in soil MBC with rising fertility levels are consistent with the observed increase in microbial activity in a study by Steiner et al. (2004). For each N fertilizer amendment rate, the addition of biochar to plots resulted in increased MBC. Particularly, this positive effect was statistically significant in bulk soil at the lowest rate of N additions, although data variation was rather high. ANOVA showed there was a significant effect of sampling position ($P<0.01$), biochar application ($P<0.01$) and N application ($P<0.01$) on MBC, indicating that the microbial biomass is a sensitive indicator for soil biological changes as a response to these management practices. However, no statistically significant interaction effect of biochar and fertilizer on MBC was found ($P=0.76$).

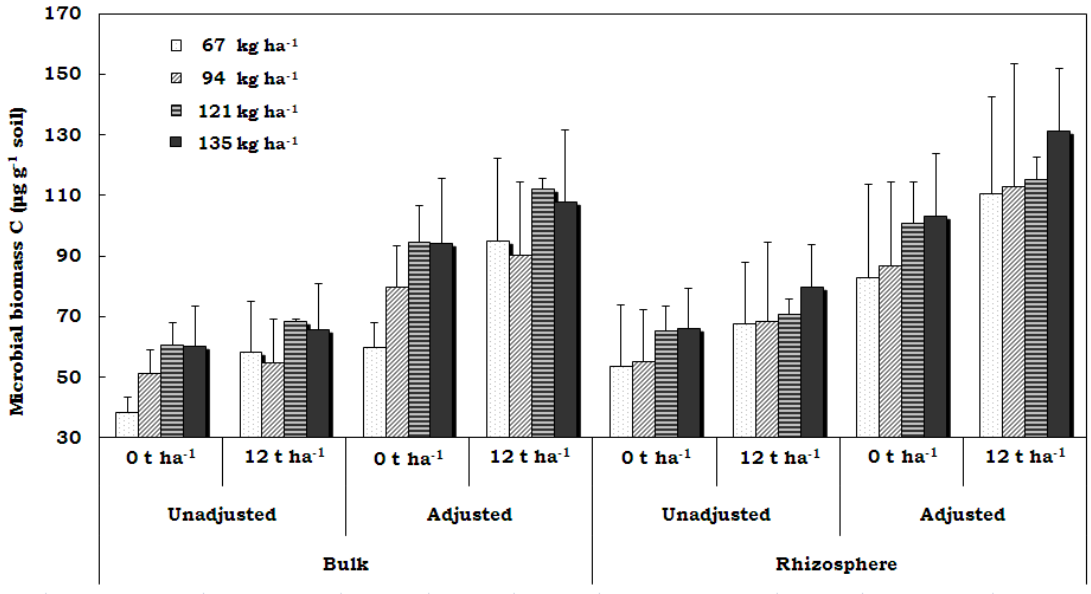

Figure 2.5 MBC in both bulk and rhizosphere soil affected by both biochar amendment (0 and 12 t ha^{-1}) and N fertilization (67, 94, 121 and 135 kg ha^{-1}). The adjusted MBC for different adsorbents were also compared to the unadjusted MBC. Variations of MBC for each treatment are presented as error bars.

Compared with bulk soil MBC in control soils (receiving 67 kg ha^{-1} N fertilizer only), the bulk soil MBC increased by 57.5% on plots receiving 135 kg ha^{-1} N, by 58.6% on plots receiving biochar, and by 80.1% on plots where both amendments were added. Compared with rhizosphere soil MBC in the control plots, the rhizosphere soil MBC increased by 24.5% on plots receiving 135 kg ha^{-1} N, by 33.2% on plots receiving biochar, and by 58.3% on plots where both amendments were added.

We can clearly see the synergistic effects in both bulk and rhizosphere soil, although these were not statistically significant. A similar observation was reported by Steiner et al. (2004, 2007) who studied the synergistic effects of mineral fertilizer and charcoal application on crop yield on a highly weathered Central Amazonian upland soil. We assumed that once biochar particles were incorporated into soils, they could serve as a porous medium binding N from fertilizer and other plant nutrients, creating a nutrient rich micro-environment that is an ideal habitat for microbial growth. This

could help explain the increased soil microbial abundance with biochar and N amendments. Adding biochar may in some cases increase the C:N ratio of soils, thus enhanced competition between crops and microbes could occur (Lehmann et al., 2003). Microbes have a high N demand and a greater potential for N immobilization than plants (Friedel et al., 2001). This potential crop N stress could be overcome by N applications. This further suggested that not only microbial abundance, but also microbial immobilization and mineralization have a close relationship with C and N inputs to soil.

Effect of Biochar Amendment on Soil Basal Respiration and Metabolic Quotient (qCO_2)

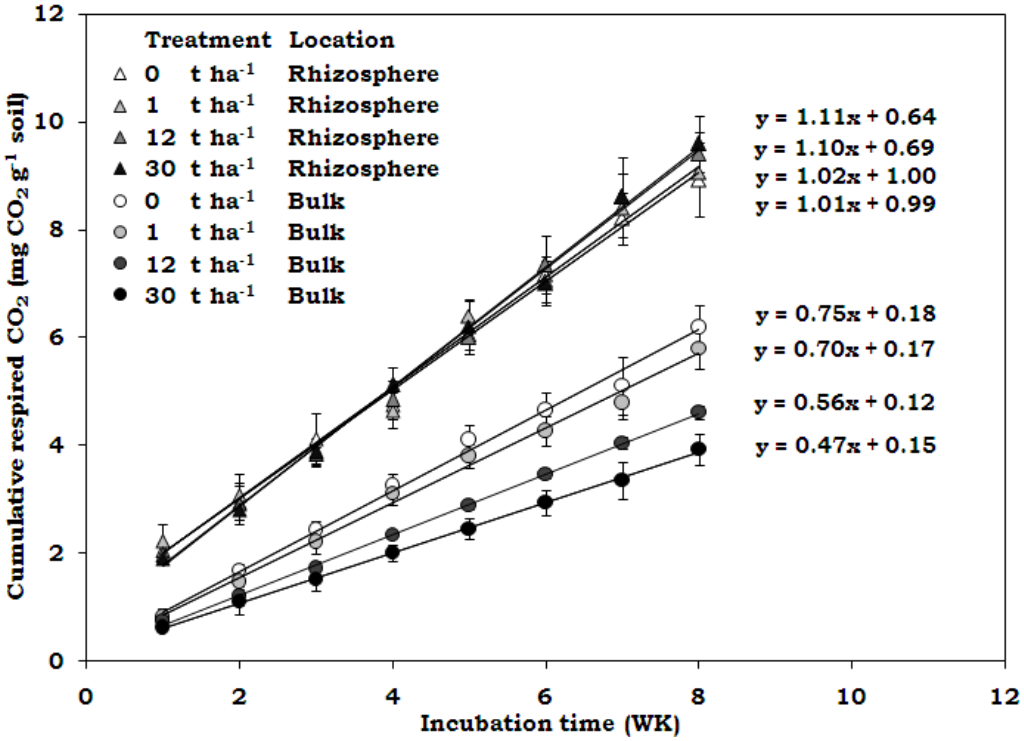

Figure 2.6 Cumulative respired CO_2 for biochar-amended and unamended bulk and rhizosphere soils over an 8 week incubation. Data were fitted by linear regression (lines and equations).

We monitored the kinetics of soil basal respiration to gain insight into changes in microbial activity associated with biochar soil amendment. For purposes of comparison, the cumulative curves of CO_2 evolved were developed as shown in Fig. 2.6. Bulk soil basal respiration decreased dramatically with increasing biochar application rate, while no significant difference of rhizosphere soil basal respiration was found between different biochar treatments (Fig. 2.6). Regardless of the biochar application rate, respired CO_2 was much higher in rhizosphere soils than in the bulk soils, this fact could be attributed to the higher microbial activity and abundance in rhizosphere soils where there is more labile C and nutrients and biological activities are more intense than in bulk soils (Paul, 2007). In rhizosphere soils, the release of organic substances from corn roots is a key process influencing soil basal respiration (Grayston et al., 1996) relative to the addition of biochar. The significance of changes in CO_2 evolution from bulk soils in response to biochar soil amendment was analyzed using repeated measures (n=6). Regression analysis indicated that bulk soil respiration varied significantly ($P < 0.05$) as a function of biochar application rate. The mean respiration rate of the 30 t ha^{-1} biochar-amended bulk soils was the lowest of all the treatments (3.09 μg h^{-1} g^{-1}), while that of the unamended bulk soils was the highest (4.73 μg h^{-1} g^{-1}). In contrast, there was no significant difference in rhizosphere soil respiration as a function of biochar application rate seen from the close slope values of the regression lines (Fig. 2.6). The mean respiration rate of biochar-amended and unamended rhizosphere soils was 7.98-8.91 μg h^{-1} g^{-1}. This indicated that the effect of biochar addition on the basal respiration was more pronounced in bulk soil than in rhizosphere soil, where the rhizosphere soil had more intense respiration activity overall. The finding of decreased basal respiration in biochar-amended soil agrees with those of Wardle et al. (2008), who observed that soil respiration was lower in the soil, where pure charcoal was applied, as compared to the control without charcoal.

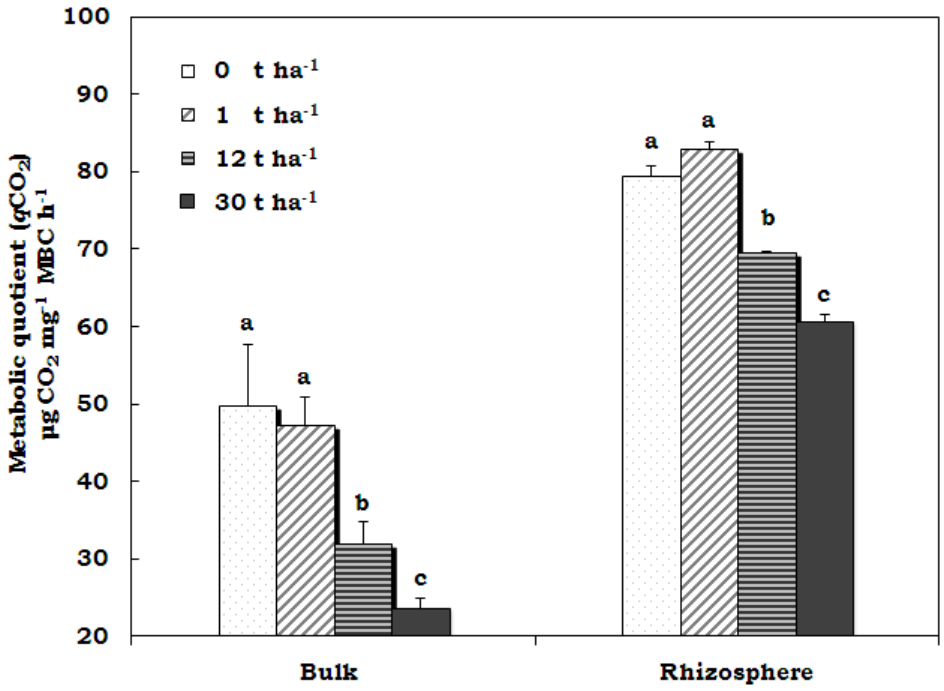

Figure 2.7 Metabolic quotient (qCO_2) at bulk and rhizosphere soils receiving 0, 1, 12 or 30 t biochar ha^{-1}.

The respiration rate per unit of microbial biomass, or metabolic quotient (qCO_2) is easier to interpret. The qCO_2 is a reflection of "microbial efficiency", since it is a measure of the energy necessary to maintain metabolic activity in relation to the energy necessary for synthesizing biomass (Bardgett and Saggar, 1994). In this study, qCO_2 decreased as the biochar added to the soil increased (Fig. 2.7), this was consistent with the study done in high black carbon-containing Anthrosols from the central Amazon, Brazil, where the Anthrosols were observed to have 61-80% lower ($P < 0.05$) CO_2 evolution per unit C over 532 days compared to their respective adjacent soils with low black carbon contents (Liang et al., 2008). The lowest *q*CO2 values were measured in soils that received the highest biochar applied (30 t ha^{-1}), differing statistically from all the other treatments. The difference was more distinguishable in bulk soils than in rhizosphere soils. Disturbances, i.e., rapidly changing environmental conditions, are known to cause increased values of qCO_2, also qCO_2 has been shown

to decline during ecosystem development during succession (Wardle and Ghani, 1995). Anderson and Domsch (1990) viewed the basal respiration as reflecting the activity of the whole microbial community, including dormant as well as active stages. Most likely, dormant stages make up the larger part of the biomass, but the small active part contributes dramatically to the respiration.

Biochar Addition Increases Microbial C Use Efficiency

The increased microbial biomass C, but decreased microbial respiration found in biochar-amended soil suggsts that the microbes in the high biochar-amended soils likely produced more cell mass per unit of C degraded than those in unamended soil. In other words, soil microbes degraded less C and tended to immobilize C as illustrated by the increase in microbial biomass in response to biochar addition, i.e., biochar addition increased microbial C use efficiency.

Substrate quality is one of the most important factors influencing the degradation activity of soil microbes (Cheshire and Chapman, 1996; Paul and Clark, 1996). In this study, the addition of biochar brings recalcitrant and relatively stable carbon into soil, the high C:N ratio of the biochar might decrease the turnover of carbonaceous compounds mediated by the relevant microbial processes. Under optimal laboratory conditions, a microbial growth efficiency of 60% is usually considered realistic for the decomposition of labile C compounds, such as glucose, while more recalcitrant constituents decompose slowly resulting in a lower efficiency factor. Lignin has the lowest efficiency factor and thus the highest residence time (Paul and Clark, 1996).

Another possibility is that C use efficiency increased as a result of an increase in the ratio of fungal to bacterial activity because of the greater growth efficiency of fungi and the accumulation of carbon in the less decomposable fungal biomass. Fungi, with their extensive hyphal networks, may be able to form hyphal bridges on biochar

between microbes and host plants, allowing use of C resources from plants instead of C degradation. Once biochar is incorporated into the soil, soil nutrients, exoenzymes and microbes are in intimate contact with it. This co-location effect provides a more favorable environment for fungal growth relative to bacterial growth. Also, fungi are more tolerant of the alkaline soil environment of the biochar than bacteria (Griffin, 1972), the corn stover biochar applied in our study had a pH of 8-10, thus, the alkaline conditions resulting from biochar addition may favor fungal growth over bacterial growth. Additionally, increased fungal decomposition may aid in organic matter retention by two mechanisms. First, reported fungal carbon assimilation efficiencies tend to be significantly higher than those for bacteria (Adu and Oades, 1978), i.e., a higher proportion of the carbon metabolized by fungi is retained in biomass instead of respired as CO_2. Where fungal carbon assimilation efficiencies range from 30 to 70%, bacterial carbon assimilation efficiencies range from 20 to 40% in the stationary growth phase (Elliott et al., 1983). Second, fungal decomposition may aid in the retention of soil organic matter by producing more recalcitrant metabolites than those produced by bacteria. Fungal biomass has a higher proportion of cell-wall material than bacterial biomass. Cell-wall components decompose more slowly and the decomposed material is stabilized as biomass end products more quickly than cytoplasmic material (Kassim et al., 1981).

CONCLUSIONS

The contrasting patterns of CO_2 release and microbial biomass production in response to biochar addition suggest that the total microbial community in biochar-amended soil became more efficient at utilizing carbon for cell growth than in unamended soil. Part of the biochar effect may be explained by an altered microbial composition in response to biochar addition; possibly, the increased ratio of fungal to

bacterial biomass. Meanwhile, biochar soil amendment may conserve organic matter because the decomposer community present in biochar-amended soil may have a higher proportion of fungi, and hence a higher microbial C use efficiency, than that present in unamended soil. The active microbial populations in biochar-amended soil may also turn over more slowly, resulting in higher steady state levels of organic matter, given similar input rates. Knowledge of how changes in microbial community structure can alter nutrient dynamics is important for understanding how microbial ecology responds to biochar soil amendment.

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CHAPTER 3

COMPARISON OF MICROBIAL COMMUNITY COMPOSITION IN SOIL AMENDED WITH BIOCHAR USING TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

ABSTRACT

PCR-T-RFLP fingerprinting was used to investigate changes in microbial community composition in rhizosphere and bulk soils resulting from biochar soil amendment. Soil samples were taken from a biochar amendment experiment installed at the Cornell Musgrave Farm, Aurora, NY. Due to the difficulties I found in recovering DNA from biochar-rich soil samples, three widely adopted DNA extraction protocols were compared and found to have varied influences on the yield and purity of DNA extracted from biochar-amended soils. However, applying different extraction methods did not affect the soil microbial community analysis as tested by T-RFLP profiling on both the bacterial and fungal communities. Results demonstrated that the Powersoil^{TM} kit (MoBio) protocol was capable of reliably extracting PCR-amplifiable genomic DNA from biochar-amended soils and was chosen for further T-RFLP studies. Interactive Principal Component Analysis (IPCA) of T-RFLP data obtained by digesting the ITS and 16S rRNA gene amplicons with the restriction enzymes HhaI and Sau96I suggested a strong divergence in the structural composition of both soil bacterial and fungal communities in response to both biochar incorporation and sampling location (bulk and rhizosphere). I concluded that the microbial community composition was mainly affected by biochar addition and responded differently to different biochar application rates and time since biochar application. Knowledge of the microbial community composition represents a first step toward understanding how soil function might change in response to biochar soil amendment.

INTRODUCTION

The use of biochar as a soil amendment strategy for improving soil health and better using natural resources has greatly drawn our attention and interest (Lehmann and Joseph, 2009). Studies on biochar as a soil amendment practice have become interdisciplinary because of the many and varied influences it may have on soil microbial processes through changes in the soil structure, the availability of water and nutrients, the global C cycle, atmospheric chemistry, and the environmental fate of organic contaminants (Lehmann and Rondon, 2006; Lehmann, 2007; Laird, 2008; Steiner et al., 2008; O'Neill et al., 2009). Biochar-specific properties make interactions between biochar and microorganisms quite complicated. On one hand, soil microbial activity, abundance and community composition may be affected by the quality and quantity of the biochar used to amend the soil. On the other hand, microorganisms may have a biodegrading effect on the quantity (Shneour, 1966) and properties of the biochar (Cheng et al., 2006). Reports regarding the effects of biochar soil amendment on soil microbial communities have received increasing attention. For example, Uvarov (2000) studied the respiration of the soil microbial community, the decomposition rate of soil organic matter and cotton strips, and herb seed germination in an area containing charcoal kilns, compared with control soil systems. The results indicated a significantly higher level of soil biological activity in the soil with charcoal versus the control sites. Rivera-Utrilla et al. (2001) showed that activated C adsorbs microorganisms strongly, and that this adsorption increases with higher hydrophobicity. Quite a few studies conducted in Japan provided strong evidence that the application of charcoal to soil can have positive effects on the abundance of mycorrhizal fungi. Yamato et al. (2006) reported a 42% increase in root colonization by AMF in response to *Acacia mangium* bark-derived charcoal application. Rondon et al. (2007) suggested that AMF colonization of N-fixing *Phaseolus vulgaris* roots

increased by 16% where *Eucalyptus deglupta*-derived biochar was applied at a rate of 90 g kg^{-1} soil. However, the factors governing the composition and functions of the soil microbial community in biochar-amended soils are by no means fully understood (Thies and Rillig, 2009).

Recently, the approaches for studying soil microbes have moved from biochemical and microbiological determinations, such as enzyme activities, microbial biomass and respiration coefficients, towards the investigation of community composition using molecular methods. Although methods such as the Biolog assay and phospholipid fatty acid (PLFA) analysis are useful, their low level of resolution does not provide a very detailed or fine-scale resolution of microbial community structure. These limitations have been overcome to some extent by use of rRNA gene analysis in studies of microbial community composition. The amplification by PCR of rRNA genes from soil DNA samples, combined with fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) (Smalla et al., 2001), terminal restriction fragment length polymorphism (T-RFLP) (Thies, 2007), amplified rDNA restriction analysis (ARDRA) (Dang and Lovell, 2000), and cloning and sequencing, provide detailed information about the species composition of whole communities (Torsvik and Ovreas, 2002). These techniques, especially T-RFLP, are the most extensively used molecular methods for monitoring changes in microbial community composition and diversity (Dunbar et al., 2000; Lukow et al., 2000; Edel-Hermann et al., 2004; Edwards et al., 2004; Thies, 2007).

Terminal restriction fragment length polymorphism (T-RFLP) pattern analysis, a DNA-based profiling technique has become a widely used and informative tool for studying microbial communities (Anderson and Cairney, 2004; Thies, 2007). This technique involves the use of a fluorescently labeled oligonucleotide primer for PCR

amplification of rRNA gene fragments and the digestion of the PCR products with one or more restriction enzymes, generating labeled terminal restriction fragments (T-RFs) of different lengths according to the DNA sequence of the organisms present in the soil community. The T-RFs are separated by high-resolution electrophoresis on an automated DNA sequencer, allowing the simultaneous characterization of microbial communities in numerous environmental samples. Organisms in a community are thus differentiated based on sequence variation that results in T-RFs of different lengths, which in turn create a pattern unique to that community. The resulting patterns can be used to make inferences about environmental effects on community composition or evaluate community-level dynamics.

In most biochar soil amendment studies, the focus has been on monitoring agronomic parameters, such as soil physical and chemical properties, plant yield and greenhouse gas emissions. However, without an understanding of the links between the observed phenomena and the basic composition of the soil microbial community, the influence of the biotic component on biochemical processes in biochar-amended soils will remain in a "black box". The objective of this study was to compare the microbial community composition in soil amended with biochar using T-RFLP analysis of bacterial 16S rRNA genes and the fungal internal transcribed spacer (ITS) region.

MATERIALS AND METHODS

Field Experimental Design and Treatments

A field experiment was established at the Cornell Musgrave Farm, Aurora, NY, in May, 2007. Each plot was 7.5 m long ×4.5 m wide with 1-2 m wide alley in between the plots. Corn stover biochar was incorporated into the soil at rates of 0, 1, 12, 30 t biochar ha^{-1}, to each plot before planting corn seeds at a density of 32,000 seeds per

acre in the field. Treatments were replicated 3 times in a completely randomized design.

Sample Collection

Soil samples were collected from each biochar field treatment in October, 2007, and October, 2008. In detail, seven bulk soil cores were taken randomly from 0-15 cm depth from each plot and composited. Three rhizosphere soil cores were taken from three randomly selected corn plants by gently shaking soil off the roots and compositing samples within plots, respectively. Before moving to the next plot, the sampling probe was sterilized with bleach and rinsed three times with purified water in order to avoid contamination between plots. Each composite soil sample was mixed well and obvious root material removed with forceps. The samples were zipped into plastic bags and shipped on ice within 48 h to the laboratory at Cornell University, NY. The samples were sieved (2 mm mesh), homogenized and stored at 4 ℃ and a sub-sample of each sieved soil was stored at -80 ℃ for downstream molecular analyses.

Soil DNA Extraction

Molecular profiling analysis of the microbial communities in biochar-amended soil requires efficient and unbiased DNA extraction and purification methods (Ogram 2000; LaMontagne et al., 2002; Thies and Suzuki, 2003). Because of the variety of microbial species and strong adsorption properties of biochar to the phosphate-backbone of the DNA molecule, extracting and purifying high-quality microbial DNA from biochar-amended soil is much more difficult than that from other environmental samples (Wilson 1997; Thies and Suzuki, 2003). To evaluate whether different DNA extraction protocols affect estimates of bacterial and fungal community composition from biochar-amended soils based on the T-RFLP profiles, we compared three

protocols to extract soil DNA based on DNA yield, purity, PCR amplifiability and T-RFLP patterns.

To find an efficient protocol for DNA extraction from biochar-rich environmental samples, we compared three widely adopted DNA extraction protocols for their ability to extract genomic DNA from 0, 1, 12, 30 t ha^{-1} biochar-amended soils. All three methods were based on the direct lysis of cells in the sample, with subsequent recovery and purification of nucleic acids.

Method I: This protocol was modified from LaMontagne et al. (2002). Before extraction, all solutions were rendered DNase-free by treatment with 0.1% diethyl pyrocarbonate (DEPC).

---Pre-wash

Soil of 500 mg and 4 ml phosphate buffer (0.12 M, pH 8) (LaMontagne et al., 2002) were added to micro-centrifuge tubes (Eppendorf , Germany) and shaken at room temperature on an orbital shaker for 5 min at 150 rpm. After centrifugation at 4 ℃ for 10 min at 6,000 ×g, the pellets were washed once again.

---Dispersion

The pellets were suspended in 1 ml of cetyl trimethylammonium bromide (CTAB) extraction buffer (Griffiths et al., 2000) and 0.5 ml of phenol–chloroform–isoamyl alcohol (25:24:1; pH 8.0) in micro-centrifuge tubes (Eppendorf, Germany) containing 250 mg of zirconia/silica beads (0.1 mm; Biospec Products, Bartlesville, OK, USA). The extraction mixture was beaten at 1000 rpm for 2 min.

--- Lysis

After the addition of 500 µl of lysis buffer (50 µM Tris–HCl [pH 8]; 40 µM ethylene diamine tetraacetic acid [EDTA; pH 8]), 20 µl of lysozyme (10 mg ml^{-1};

Sigma-Aldrich, Germany) and 5 μg poly-dIdC (Barton et al., 2006), mixtures were vortexed briefly (30 s) and incubated at 37 ℃ for 30 min. Sodium dodecyl sulphate (SDS) was added to a final concentration of 2%; the samples were again vortexed and then incubated at 70°C for 1 h. After this, 6 μl of proteinase K (Sigma-Aldrich, Germany) were added. Samples were then vortexed and incubated at 50 ℃ for a further 30 min followed by centrifugation for 15 min at 10,000 ×g.

---Precipitation

The supernatants were transferred to fresh micro-centrifuge tubes, and the aqueous phase, containing the nucleic acids, was extracted by mixing an equal volume of chloroform–isoamyl alcohol (24:1) and shaking gently by hand, followed by homogenization at 2,800 rpm for 10 min and centrifugation (10,000 ×g) for 10 min. The upper layers were transferred to fresh tubes and 0.5 ml sterile deionized water was added into the former tubes to wash the pellets. The upper layers were mixed and centrifuged at 12,000 ×g for 5 min, then were treated with 0.5 vol of 50% (w v^{-1}) PEG8000 and 0.1 vol of 5 M NaCl (Yang et al., 2007). The samples were mixed by inverting gently, incubated for more than 1 h or overnight at 4 ℃ and centrifugated (10,000 ×g) for 15 min to precipitate the nucleic acids.

---Wash

The pelletted nucleic acids were washed twice in 70% (v v^{-1}) ice-cold ethanol and air dried before re-suspending in 50 μl DEPC-treated water (Griffiths et al., 2000).

Method II: The MoBio Powersoil™ DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA). DNA was extracted from 500 mg of soil according to the manufacturer's instructions. In Method II, a bead matrix and lysis buffer were used to pulverize cells by horizontal shaking on a vortex mixer, followed by precipitation of the organic contaminantes, and adsorption of DNA to a spin filter, a wash step, and the

elution of DNA in buffer. The protocol was followed per the manufacturer's instructions.

Method III: The MoBio UltraClean™ soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA). DNA was extracted from 500 mg of soil according to the manufacturer's instructions.

DNA Quantification

To check DNA yield, crude DNA extracts (5 µl) were agarose (1.5%) gel electrophoresed in TBE buffer. The gel was stained with 0.5 µg ml^{-1} ethidium bromide in advance. Then, the gel loaded with DNA extracts was photographed by a transilluminator using the filter specified for ethidium bromide staining to check if sample DNA extracts had a positive DNA band with the right size. To accurately quantify DNA concentrations, the quantity of extracted DNA was estimated against a calf thymus DNA standard curve in an ethidium bromide (EtBr) solution using a EC3™ Fluorescence BioImager (UVP, LLC, Upland, CA, USA) and the accompanying Quantity One™ software (Bio-Rad, Hercules, CA, USA). Standards were prepared with a series of dilutions of calf thymus DNA (Bio-Rad). Each standard dilution and each sample DNA extract was mixed thoroughly with 100 µl, 0.2 µl ml^{-1} EtBr/TBE quantification buffer in each well of a UV-transparent quantification plate. The loaded plate was visualized and photographed by a UV spectrophotometer. The acquired image was analyzed and quantified using the Quantity One software which calculates the sample DNA concentrations by using the standard curve of 10-100 ng of calf thymus DNA versus intensity.

DNA Purity Evaluation

Co-extracted humic acids and proteins are two major contaminants when DNA is extracted from environmental samples. Humic acids and proteins absorb at 230 nm

and 280 nm, respectively; whereas, DNA absorbs at 260 nm. In this study, the purity of DNA was assessed spectrophotometrically by calculating A260/A230 and A260/A280 ratios for humic acid contamination and protein impurities, respectively. A260/A230 ratios greater than 2 and A260/A280 ratios greater than 1.7 indicate high purity DNA, while low ratios indicate humic acid or protein contamination, respectively (Ning et al., 2009).

PCR Conditions

For PCR of the bacterial community, the quantified DNA was used as the template for amplifying 16S rRNA genes. This was performed by using the fluorescence-labeled forward primer 27f (5'-[6FAM] AGA GTT TGA TCC TGG CTC AG-3') and the unlabeled reverse primer 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') (Fig. 3.1). Reactions were carried out with the following reagents in 50 µl reactions: 5 µl, 1-3 ng μl^{-1} template DNA; 5.0 µl, 10×PCR Buffer; 1.0 µl, 10 mM dNTPs; 0.5 µl, 10 mg ml^{-1} bovine serum albumin (BSA); $MgCl_2$, 2 mM; 0.5 µl, 10 µM forward primer 27f; 0.5 µl, 10 µM reverse primer 1492r; 0.5 µl, 5U μl^{-1} *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA); 33.0 µl nuclease-free sterile water. Positive controls used DNA from pure laboratory cultures of *E. coli* for bacteria. Negative controls used sterilized distilled water in place of template DNA.

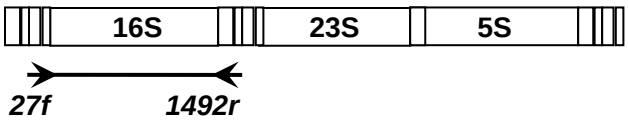


Figure 3.1 A 16S rRNA gene map indicating the location of primers used in this study. Primers 27f and1492r were used to target the 16S rRNA gene region. The arrows indicate the direction of primer extension.

For PCR of the fungal community, the quantified DNA was used as template targeting the internal transcribed spacer (ITS) region of the rRNA gene cluster, which has taxonomic significance (White et al., 1990; Lord et al., 2002; Borneman and

Hartin, 2000). Primers ITS1f (5'-[6FAM] CTT GGT CAT TTA GAG GAA GTA A-3') and the unlabeled reverse primer ITS4r (5'-TCC TCC GCT TAT TGA TAT GC-3') were used to target the entire ITS region (Fig. 3.2). Reactions were carried out with the following reagents in 50 µl reactions: 10 µl 1-3 ng μl^{-1} template DNA; 5.0 µl, 10×PCR Buffer; 3.0 µl, 10 mM dNTPs; 0.5 µl, 10 mg ml^{-1} BSA; 6.0 µl, 25 mM $MgCl_2$; 1.0 µl, 10 µM forward primer ITS1Ff; 1.0 µl, 10 µM reverse primer ITS4r; 1.0 µl, 5 U μl^{-1} *Taq* DNA polymerase (Applied Biosystems); 22.5 µl nuclease-free sterile water. Positive controls used DNA from pure laboratory cultures of *Glomeromycetes* for fungi. Negative controls used sterile distilled water in place of template DNA. In the PCR-T-RFLP procedure, BSA was added to all PCR reaction mixtures in order to reduce any PCR inhibitory effects from any contaminants that may have been carried over after extraction.

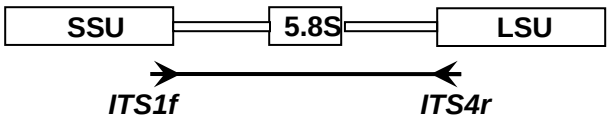


Figure 3.2 ITS primer map indicating the target of primers used in this study. Primers ITS1f and ITS4r were used to amplify the entire ITS region. The arrows indicate the direction of primer extension. SSU: Small Subunit; LSU: Large Subunit.

In PCR temperature cycling, the PTC-100 programmable thermal cycler (M.J. Research, Watertown, MA) was programmed for an initial denaturing step at 94 ℃ for 5 min followed by 27 cycles of 94 ℃ for 45 s, 56 ℃ for 45 s and 72 ℃ for 1 min with a final extension step at 72 ℃ for 10 min. Cycling parameters were tested starting with 18 through 36 cycles at nine-cycle increments to address the bias often associated with amplification of mixed templates. Twenty-seven cycles were found to be optimal for reproducible and consistent amplification of the representative communities without kinetic or template biases becoming large factors.

We used three independent replicates derived from separate DNA extractions, PCR amplifications and restriction digestions. This ensured sufficient concentration of PCR products and avoided template sampling variation in pipetting community DNA when constructing the PCR reaction mixture.

T-RFLP Analysis

After amplification, DNA was checked on 1.5% agarose gels, then replicate reactions were combined and the DNA concentration determined by UV spectrophotometry. The quantified DNA was dried and resuspended in nuclease-free water to a concentration of approximately 20 ng μl^{-1}. The restriction enzymes HhaI (Promega, Madison, WI) recognizing the site of 5'-GCG$^{\blacktriangledown}$C-3' and Sau96I (New England Biolabs, Ipswich, MA) recognizing the site of 5'-G$^{\blacktriangledown}$GNCC-3' were used to digest amplified sample DNA, respectively. Two, separate, 30 µl restriction enzyme digest reactions were prepared per sample which contained 0.5 µl enzyme (either HhaI or Sau96I, New England Biolabs, Beverly, MA, USA); 3.0 µl of the manufacturer's recommended 10×buffer; 0.3 µl, 10 mg ml^{-1} BSA; 11.2 µl nuclease-free water and 15 µl approximate 20 ng μl^{-1} amplified sample DNA. Restriction digests were carried out in an MJ Research PTC 100 thermal cycler held at 37 ℃ for 4.5 h with a final step of 70 ℃ for 15 min to stop the reaction. Complete digestion of the DNA was verified by inspecting digested products run on a 1.5% agarose gel and visualized using a UV spectrophotometer. T-RFLP digests were purified using a PERFORMA DTR Edge Plates (Edge BioSystems, Gaithersburg, MD), dried in an evaporation vacuum and then resuspended in a 10 µl mix containing 9.85 µl of formamide and 0.15 µl of Liz 500 size standard (Applied Biosystems). All samples were denatured at 94 ℃ for 4 min and chilled on ice until loaded. Subsequently, terminal restriction digests were run on a 3730XL ABI electrophoretic capillary sequencer (Applied Biosystems). T-RF

sizing was performed on electropherogram output using Genemapper (Applied Biosystems) fragment analysis software.

T-RFLP data were analyzed by the Additive Main Effects with Multiplicative Interaction (AMMI) model using MATMODEL^{TM} software (Microcomputer Power, Ithaca, NY, USA) (Gauch, 1992). AMMI constructs interaction principal components (IPC) from the interaction between the main effects (treatments vs. terminal restriction fragments, T-RFs) so that the differential responses of T-RFs to treatments can be assessed.

RESULTS

Effect of Biochar on Extraction of Genomic DNA from Soil

To examine microbial genomic DNA recovery from biochar-rich samples, I developed test samples that contained 0.5 g of pure biochar particles, to which I added 10 μg of purified bacterial genomic DNA. I then compared the recovery of this DNA by using the Powersoil^{TM} soil DNA extraction protocol and meanwhile tested the adsorption strength of DNA to biochar-rich samples.

As shown in Fig. 3.3, DNA recovery decreased dramatically when biochar was added. Approximately 30.6% of added DNA was recovered in the absence of biochar, while the amount of DNA recovered was 2.7% in the presence of biochar. This indicated that biochar was effectively adsorbing DNA. Given the high initial load of genomic DNA (10 μg), this experiment clearly demonstrated the difficulty in recovering DNA from samples that contain biochar. I suggest that biochar has a high affinity for the phosphate backbone of DNA. These results are consistent with studies done by Rapaport et al. (1981) and Gani et al. (1999), where genomic DNA was found to have a high binding activity on charcoal.

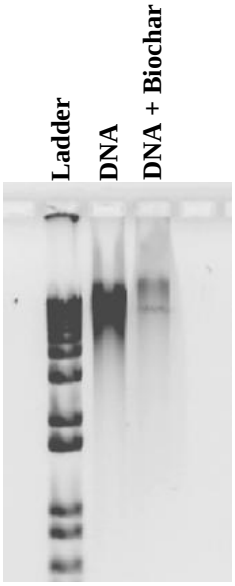


Figure 3.3 Microbial genomic DNA extracted using the Powersoil™ soil DNA kit with and without biochar added. Lane 1 = fragment size ladder (1kb). Lane 2 = 10 µg of purified bacterial DNA subjected to the Powersoil™ soil DNA extraction protocol. Lane 3 = the same amount of purified bacterial DNA with the addition of 0.5 g of biochar subjected to the Powersoil™ soil DNA extraction protocol.

Comparison of DNA Yield and Purity, PCR Amplifiability and T-RFLP Profile among Extraction Methods

(1) DNA yield

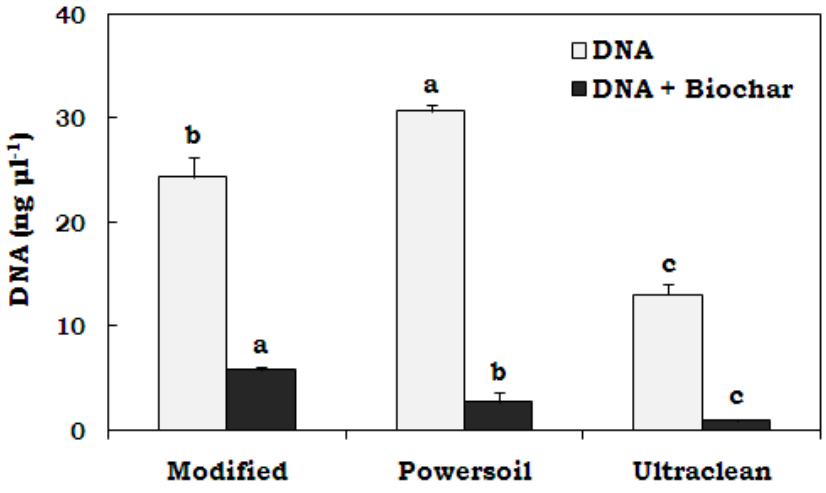


Figure 3.4 DNA recovery in the presence and absence of biochar using three extraction protocols.

DNA concentrations obtained using the three extraction procedures are shown in Fig. 3.4. The DNA recovery rate in the presence of biochar obtained with the modified protocol (LaMontagne et al., 2002) ranged from 5.4% to 6.1%. The Powersoil™ protocol recovered 1.6% to 3.3% of the DNA in the presence of biochar. DNA recovered using the Ultraclean™ protocol was the least and was significantly less than ($P<0.01$) those obtained using the modified protocol and the Powersoil™ protocol either in the presence or absence of biochar. Because the crude DNA yields relate to cell lysis and extraction efficiencies, DNA recovery efficiency was found to vary depending on the extraction method used. The modified protocol and the Powersoil™ protocol recovered significantly higher amounts of DNA than the Ultraclean™ protocol (Fig. 3.4). Regardless of the extraction method, biochar addition significantly decreased DNA recovery efficiency. Results also showed clearly that, in the presence of biochar, the modified protocol and the Powersoil™ protocol were more efficient in extracting DNA than the Ultraclean ™ protocol. The strong affinity of DNA for biochar found in this study is consistent with a previous study that reported strong adsorption of DNA on the solid surfaces of charcoal powder (Gani et al., 1999).

My results provide a comparison between the three extraction methods in their DNA extraction efficiency in the presence of biochar; however, they do not test how uniformly the three extraction protocols extract diverse community members from biochar-amended soils. Therefore, I assessed the ability of the three extraction methods to extract high quantity and quality DNA from 0, 1, 12, 30 t ha^{-1} biochar-amended soils.

Regardless of the extraction method, the amount of DNA extracted decreased with increasing biochar application rate from 0 t ha^{-1}, 1 t ha^{-1}, 12 t ha^{-1} to 30 t biochar ha^{-1} (Fig. 3.4). The amount of DNA obtained using the Ultraclean™ protocol was

significantly affected by the rate of biochar additions. However, DNA yield obtained by the Powersoil™ protocol was significantly lowered by high biochar application rate, and DNA yield obtained by the modified protocol did not show a significant difference among different biochar application rates. In the high biochar applied soils, DNA yielded by the modified protocol and the Powersoil™ protocol was 53.4% and 18.4% higher than that yielded by the Ultraclean™ protocol. These results confirm that all three methods successfully extract DNA from the three biochar-amended soils tested (Table 3.1). However, differences were apparent with respect to the quantity and quality of DNA extracted using the three methods. That is, the Powersoil™ protocol and the modified protocol were more efficient in extracting DNA from biochar-amended soils than the Ultraclean™ protocol. This finding is consistent with the study done by Hilyard et al. (2008), where the Powersoil ™ protocol was found to be efficient in extracting microbial DNA from sediment samples, whereas the Ultraclean™ protocol was found to be efficient in extracting microbial DNA from cultured isolates.

When a two-factor ANOVA was carried out, the biochar application rate was found to have a significant effect on the DNA yield, with DNA extraction most reduced by the 30 t ha^{-1} biochar application ($P<0.05$). Choice of extraction method also had a significant effect, where the modified protocol and the Powersoil™ protocol were most efficient in extracting DNA, while the Ultraclean™ kit was least successful. The modified protocol and the Powersoil™ protocol were very similar with regard to their efficiency in extracting DNA from 0, 1 and 12 t ha^{-1} biochar-amended soil, and consequently, a further one-factor ANOVA was carried out. No significant difference was noted, however, between the modified protocol and the Powersoil™ protocol with respect to DNA yield from 0, 1 and 12 t ha^{-1} biochar-amended soil ($P>0.05$).

Table 3.1 DNA yield, purity and ability to amplify using three DNA extraction protocols

Protocols	Treatments t ha^{-1}	DNA yield	Purity		PCR results	
			A260 / A230	A260 / A280	Bacteria	Fungi
Modified	0	31.01±1.85	1.14±0.01	1.10±0.28	(+)	(++)
	1	29.78±8.68	1.26±0.09	1.06±0.13	(+)	(++)
	12	25.15±1.88	0.66±0.05	1.01±0.07	(+)	(++)
	30	20.09±3.21	0.30±0.02	0.49±0.08	(+)	(++)
Powersoil	0	31.19±4.89	2.05±0.05	2.04±0.07	(++)	(++)
	1	30.07±3.81	2.18±0.12	1.92±0.10	(++)	(++)
	12	25.42±3.20	2.15±0.26	1.87±0.21	(++)	(++)
	30	17.31±0.15	2.14±0.13	1.98±0.28	(++)	(++)
Ultraclean	0	22.03±2.76	2.07±0.03	1.87±0.04	(++)	(++)
	1	22.03±0.83	2.07±0.11	1.77±0.05	(++)	(++)
	12	13.75±2.91	2.16±0.13	1.48±0.08	(++)	(++)
	30	7.93±1.09	1.80±0.06	1.84±0.13	(++)	(++)

(2) DNA purity

Humic acid contamination denoted by the A260/A230 ratio of the DNA extracts varied among the three extraction protocols. Regardless of the biochar application rate, the modified protocol gave significantly lower A260/A230 ratio for DNA extracts than the other two extraction protocols. This indicated that the modified protocol was least effective in obtaining high quality DNA among the three methods we compared. Increasing biochar amendment decreased the purity of DNA extracts obtained by the modified protocol with respect to humic acid contamination, as shown in Table 3.1, where a significant decrease in A260/A230 ratio was found in the 12 and 30 t ha^{-1} biochar-amended soils. However, biochar amendment didn't have a significant effect on humic acid contamination of DNA extracts obtained by the modified protocol or the Powersoil™ protocol. Given the rule of thumb, A260/A230 > 2 indicates pure DNA with low humic acid contamination (Ning et al., 2009), the Powersoil™ protocol and the Ultraclean™ protocol performed better than the modified protocol in reducing humic acid contamination of the DNA extracts.

Extraction method had a significant effect on the ratio of A260/A280, but no significant effect of biochar application rate was found. Given the rule of thumb, A260/A280>1.7 indicates pure DNA with low protein contamination (Ning et al., 2009), the data shown in Table 3.1 showed the Powersoil™ and the Ultraclean™ protocol had lower yields of co-extracted protein than the modified method regardless biochar application rate. Our results suggested that the Powersoil™ and the Ultraclean™ protocol were highly efficient in removing humic acids and proteins.

Thus, the three extraction methods we tested did demonstrate significant differences in the yield and purity of crude DNA extracts obtained from biochar-amended soils. The yield of crude DNA extracts decreased with increasing biochar

application rate, which is likely due to the high adsoption of DNA molecules on biochar. The Powersoil^{TM} protocol was found to be effective in obtaining not only high yield DNA, but also high quality DNA.

(3) Ability to amplify DNA extracts by PCR

The purity of the crude DNA was also checked by the ability to amplify a region of bacterial 16S rRNA gene of bacteria by PCR with primers of 27f and 1492r and fungal ITS region with primers of ITS1F and ITS4R. Five µl aliquots of PCR product were run on Tris-borate-EDTA (TBE) agarose gels (1.5%) containing ethidium bromide (1 ng ml^{-1}; Maniatis et al., 1982) for PCR product staining and visualization. Gel images were captured using a UV transilluminator.

The gel image indicated that the PCR amplified bacterial 16S rRNA and fungal ITS region for use in downstream analyses to be between 1,430-1,500 bp and 620-650 bp in size, respectively (Table 3.1), consistent with the expected length of PCR products. Additionally, nonspecific PCR amplification was not detected in any lanes. Although DNA extracts obtained from the three methods were all amplifiable, PCR products amplified from bacterial DNA extracts obtained by the other two extraction methods were purer and of better quality than those obtained by the modified protocol (Table 3.1).

(4) T-RFLP fingerprinting of bacterial and fungal community composition

I evaluated whether the different DNA extraction procedures resulted in a different microbial community composition. To do this, T-RFLP data were analyzed by the AMMI model which combines the additive elements of ANOVA with the multiplicative elements of PCA based on a binary matrix according to the presence or absence of aligned fragments instead of peak heights within each electropherogram. Thus, it allows us to test the similarity among restriction fragment composition

obtained using different extraction procedures. Duplicates were run as a means of confirming the reproducibility of each method.

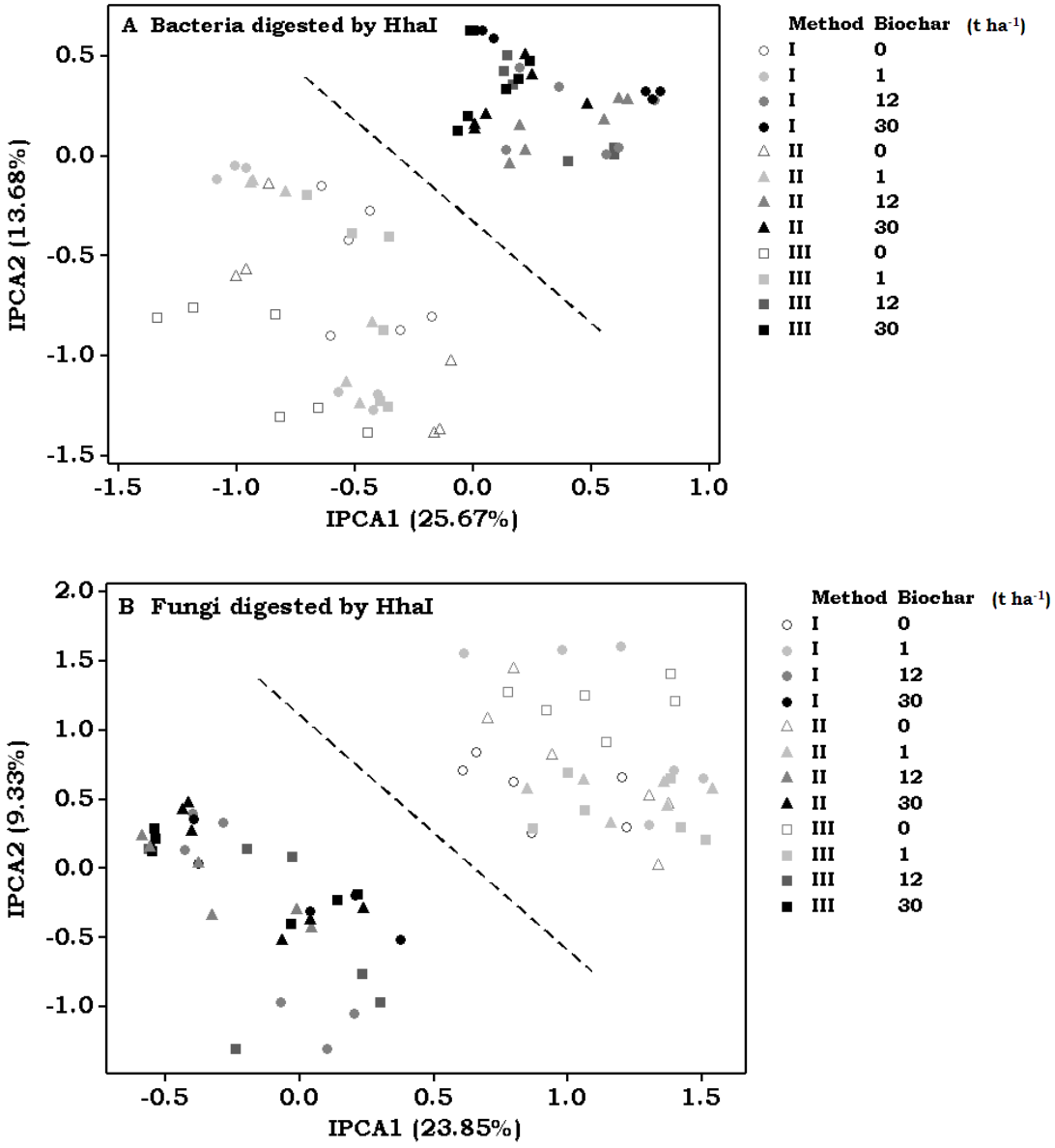


Figure 3.5 AMMI plots of T-RFLP fingerprints of the bacterial (A) and fungi (B) community composition in biochar-amended and unamended soils. Each point represents one separately analyzed replicate sample. Points are gradiently-colored according to biochar application rates and in shape according to extraction methods.

Analysis of T-RFLP profiles, generated by restriction digestion of PCR products, demonstrated that the choice of the DNA extraction method did not significantly

influence either the bacterial (Fig. 3.5A) or fungal community profiles generated (Fig. 3.5B). This was reflected in the clustering of points detected from each sample in the AMMI plot (Fig. 3.5). However, microbial community composition in the unamended and 1 t ha^{-1} biochar-amended soils was found to be significantly different from those in the 12 and 30 t ha^{-1} biochar-amended soils. In addition, these results demonstrated that the Powersoil™ protocol was capable of reliably extracting PCR-amplifiable genomic DNA from biochar-amended and unamended soils. Surprisingly, humic acid/protein co-extraction was not problematic for biochar-amended soils, regardless of extraction method employed, likely due to non-selective DNA adsorption on the biochar.

Although the three DNA extraction methods we tested were found to have varied influences on DNA yield and purity, applying different extraction methods did not affect the soil microbial community profile analysis. This observation disagreed with previous studies reporting affects on microbial community profiles when analyzed by denaturing gradient gel elecrophoresis (DGGE), where different DNA extraction protocols did affect the final analysis (De Lipthay et al. 2004; Carrigg et al., 2007). However, the finding in this study agreed with the study done by Ning et al. (2009), where the microbial community DGGE profiles showed consistency between the Ultraclean™ kit DNA extraction protocol and the Powersoil™ kit DNA extraction protocol. This indicates that the effect of DNA extraction on microbial community profiling analysis varies with different environmental samples DNA is extracted from and different molecular profiling methods. Thus, in this study, I have confidence in using the Powersoil™ soil DNA kit as the DNA extraction method of choice for further T-RFLP studies, since it yielded a high quantity and good purity of DNA.

Bacterial and Fungal Community Composition Affected by Biochar Soil Amendment

PCR-T-RFLP analysis was done on both bulk and rhizosphere field soils sampled from experimental fields at the Cornell Musgrave Farm in Aurora, NY, after one year (Sampled in Oct., 2008). T-RFLP profiles showed that the restriction enzyme HhaI was able to differentiate the bacterial (Fig. 3.6A) and fungal (Fig. 3.6B) community in the 0 and 1 t ha^{-1} biochar-amended soils from those in 12 and 30 t ha^{-1} biochar-amended soils. Also, distinct shifts in the bacterial community composition were detected between bulk soils and rhizosphere soils (See separation by circles and triangles in Fig. 3.6A). But this separation was only significant for the 12 and 30 t ha^{-1} biochar-amended soils for fungal community composition (Fig. 3.6B). Our finding was consistent with the study done by Otsuka et al. (2008), where the bacterial community composition was found to be significantly different in tropical rainforest soils contain high-charcoal caused by heavy forest fires from soils without fire damage when analyzed using PCR-DGGE profiling analysis.

The restriction enzyme Sau96I was also used to access the AMMI analysis of T-RFs, the operational taxonomic units, within the bacterial (Fig. 3.6C) and fungal (Fig. 3.6D) community of biochar-amended soils. Use of Sau96I as the restriction enzyme showed a significant discrimination between 0, 1 t ha^{-1} biochar-amended soils and 12, 30 t ha^{-1} biochar-amended soils, regardless of the bacterial or fungal community. As can be seen in Fig. 3.6, discrimination between the microbial community in bulk soils and that in rhizosphere soils was only significant for the 12 and 30 t ha^{-1} biochar-amended soils.

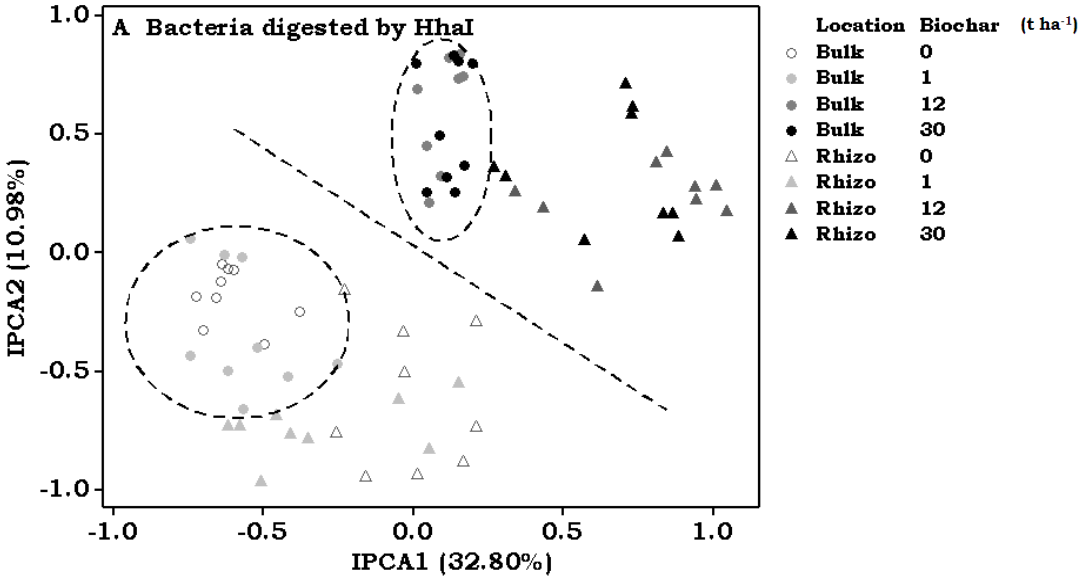
The comparative analysis revealed a shift in both the bacterial and fungal community structures between 0 or 1 t ha^{-1} biochar treatment and 12 or 30 t ha^{-1} biochar treatment, which indicated that the shift in the community compositions were

related to the rate of biochar applied. Finally, the shifts between biochar application rates were mainly explained by the first dimension of the IPCA plot, indicating that biochar had a stronger affect on the microbial community composition than sampling location (bulk vs. rhizosphere).

T-RFLP fingerprinting is not used to identify each member in a community; rather, it is used to produce a profile from which comparisons can be made. T-RFLP fingerprinting analysis is used to produce an overall pattern of the community, not to identify each individual species or genus in that community. The individual peaks displayed become the units, or phylotypes, used in the monitoring process.

The electropherogram for bacterial T-RFs showed clearly that both Hha1 and Sau96I were able to differentiate bacterial community composition between 0 and 30 t ha^{-1} biochar-amended soils, which was consistent with the finding obtained by the AMMI analysis shown in Fig. 3.6. As shown in the electropherogram, HhaI generated several major T-RFs present only in unamended soils. For instance, T-RF 108, 313 and 465 bp as indicated by arrows, were found mainly in unamended soils, and were hardly detected in 30 t ha^{-1} biochar-amended soils (Fig. 3.7A). Sau96I generated several major T-RFs present only in unamended soils. For instance, T-RF 143, 262bp as indicated by arrows, were mainly found in unamended soils, and were hardly detected in 30 t ha^{-1} biochar-amended soils (Fig. 3.7C). The application of biochar to soil also induced considerable changes in fungal community composition, as illustrated in Fig. 3.7B and C. These changes corresponded to not only an increased or decreased abundance of existing peaks in the unamended soils, but also the total number of peaks per T-RF pattern between unamended and 30 t ha^{-1} biochar-amended soil samples. The T-RF pattern shown in Fig. 3.7A-D illustrated slightly if not significantly more T-RFs peaks present in unamended soils than those in biochar-

Figure 3.6 AMMI analysis of bacterial and fungal community composition generated by HhaI (A, B) and Sau96I (C, D) restriction enzymes (Sampled in Oct. 2008).


A Bacteria digested by HhaI
IPCA2 (10.98%)
IPCA1 (32.80%)
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0
Location Biochar (t ha-1)
Bulk 0
Bulk 1
Bulk 12
Bulk 30
Rhizo 0
Rhizo 1
Rhizo 12
Rhizo 30

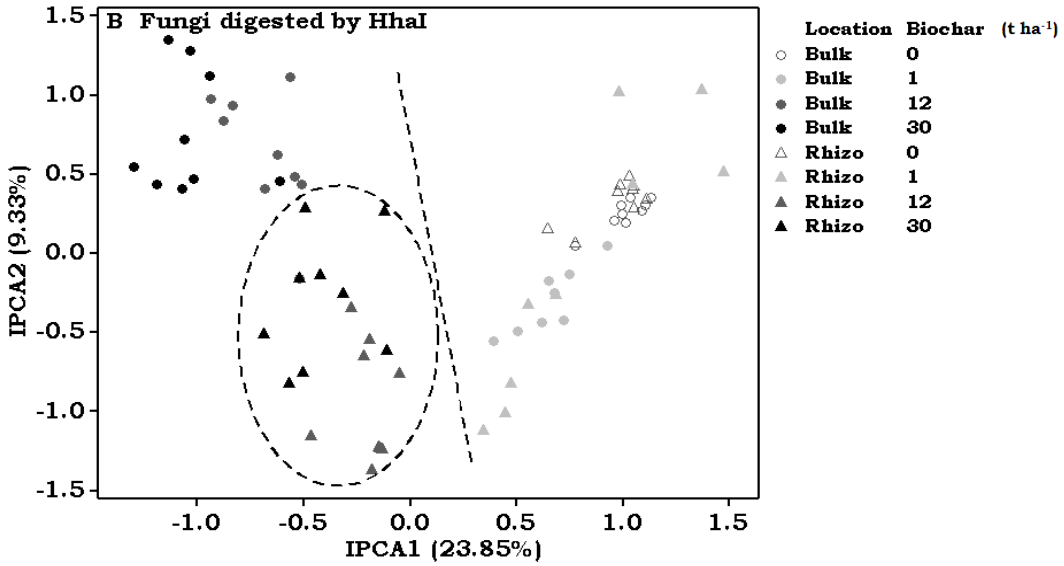
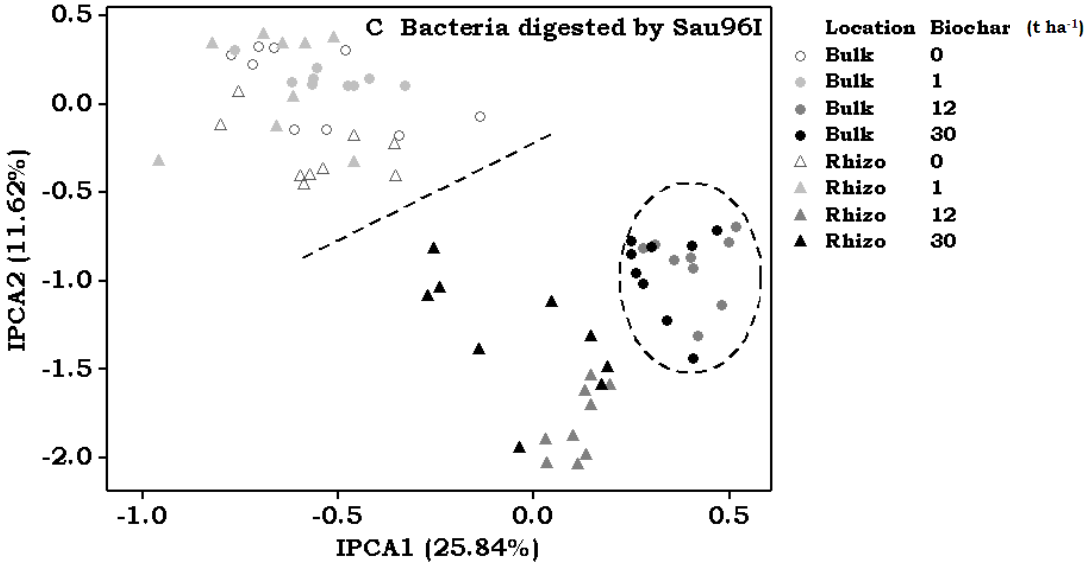

B Fungi digested by HhaI
IPCA2 (9.33%)
IPCA1 (23.85%)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Location Biochar (t ha-1)
Bulk 0
Bulk 1
Bulk 12
Bulk 30
Rhizo 0
Rhizo 1
Rhizo 12
Rhizo 30

Figure 3.6 (Continued)



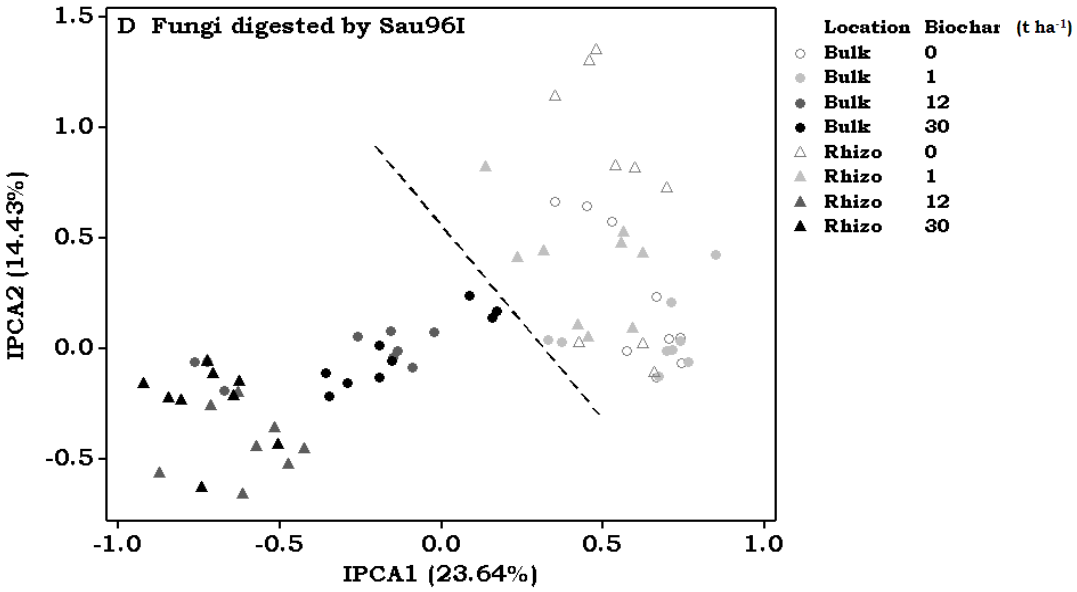
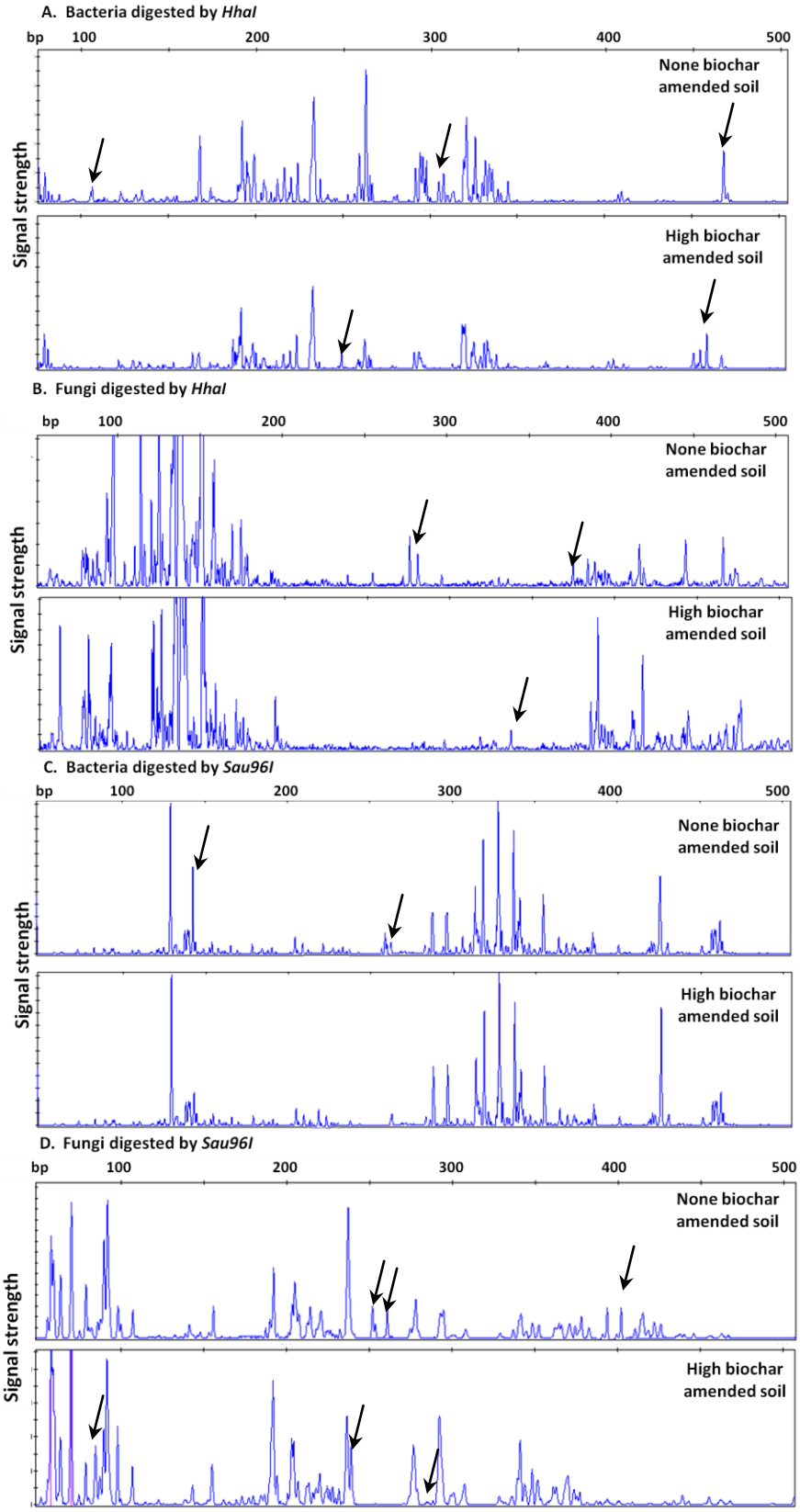


Figure 3.7 T-RFLP profiles of bacterial and fungal communities generated from PCRs from 0 and 30 t biochar ha^{-1} treatment (only bulk soils), and digested by Hha1 and Sau96I. Fragment size in bp. The major T-RF bands with a different location are indicated by arrows.


A. Bacteria digested by HhaI
bp
100
200
300
400
500
None biochar amended soil
High biochar amended soil
Signal strength
B. Fungi digested by HhaI
bp
100
200
300
400
500
None biochar amended soil
High biochar amended soil
Signal strength
C. Bacteria digested by Sau96I
bp
100
200
300
400
500
None biochar amended soil
High biochar amended soil
Signal strength
D. Fungi digested by Sau96I
bp
100
200
300
400
500
None biochar amended soil
High biochar amended soil
Signal strength

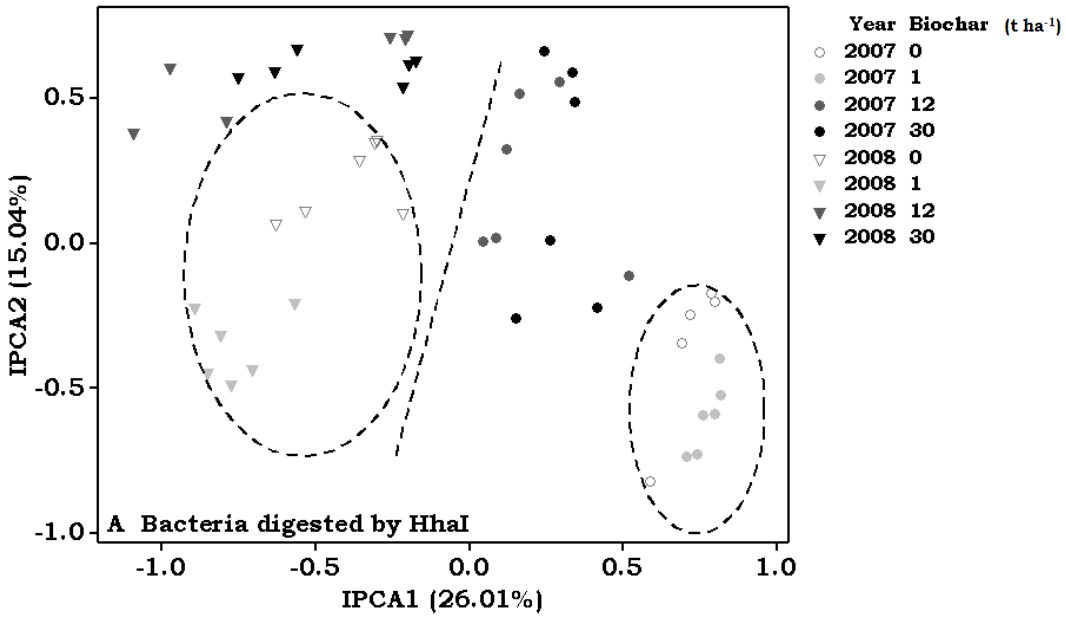
amended soils. These results are consistent with the idea that biochar addition decreases microbial diversity by altering substrate composition, i.e., the addition of biochar selected for populations whose metabolism would be benefited. However, the evidence here was not very clear. Adopting multiple approaches to analyze soil microbial diversity is needed to validate these observations.

It is unclear at present which specific bacterial or fungal groups contribute to the polymorphisms seen in T-RFLP fingerprints from biochar-amended soils; therefore, further cloning and sequencing analysis of microbial genomic DNA was performed in order to reveal specific bacterial and fungal groups that preferentially appear in biochar-amended soils.

Comparison of Different Biochar-amended Soil Microbial Community Composition Changes One Year after Biochar Addition

The application of biochar induced considerable changes in both bacterial and fungal community composition, as shown in Fig. 3.8A-D. The AMMI analysis revealed an evident shift in the community composition between 0, 1 t ha^{-1} biochar-amended soils and 12, 30 t ha^{-1} biochar-amended soils. In addition, the microbial community compositions from 2007 and 2008 were differentiated from each other regardless of the restriction enzymes used, indicating that the shifts in the community structures were likely related to duration of biochar in soil. Finally, the shifts of biochar-amended soil microbial communities were mainly explained by the first and the second IPCA dimensions, respectively, indicating that time since biochar addition produced a stronger alteration of bacterial and fungal community structure than the biochar addition alone. This finding is consistent with the study done by Campbell et al. (2008), where a distinctly different microbial community composition was measured in soils burned for 2 and 4 years by using phospholipid fatty acids (PLFAs).

Figure 3.8 AMMI analysis of bacterial and fungal communities generated by HhaI (A, B) and Sau96I (C, D) restriction enzymes (Oct., 2007 vs Oct., 2008).


Year Biochar (t ha-1)
2007 0
2007 1
2007 12
2007 30
2008 0
2008 1
2008 12
2008 30
A Bacteria digested by HhaI
IPCA2 (15.04%)
IPCA1 (26.01%)
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0

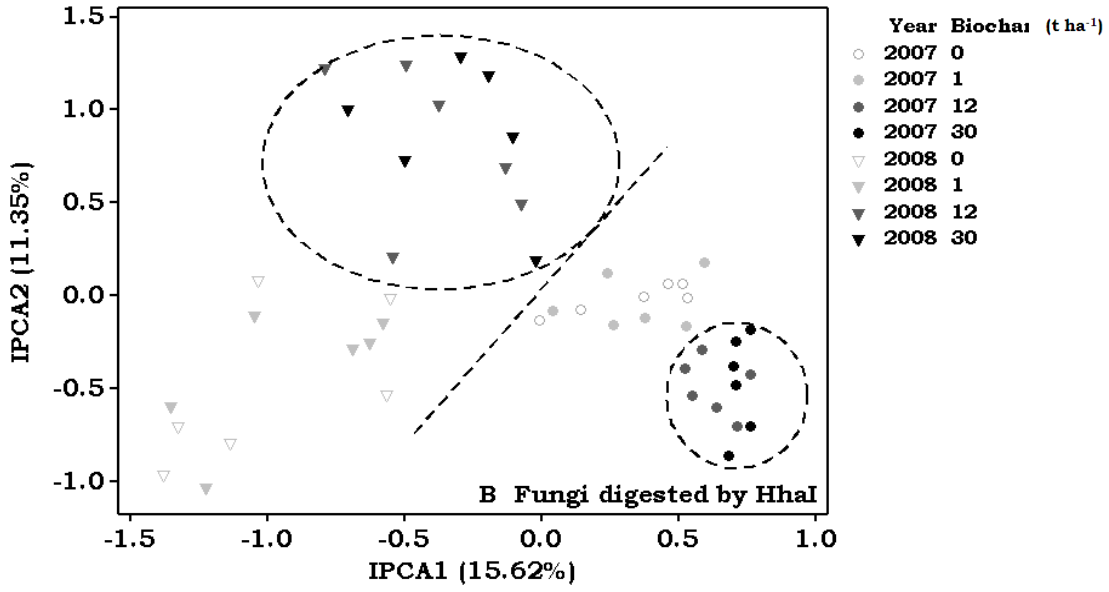
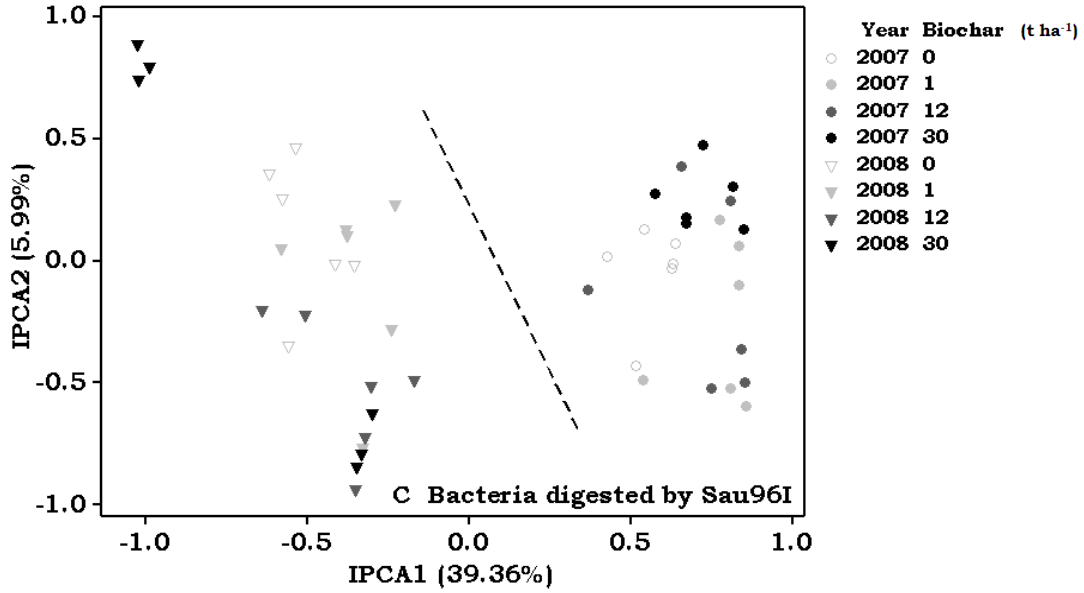
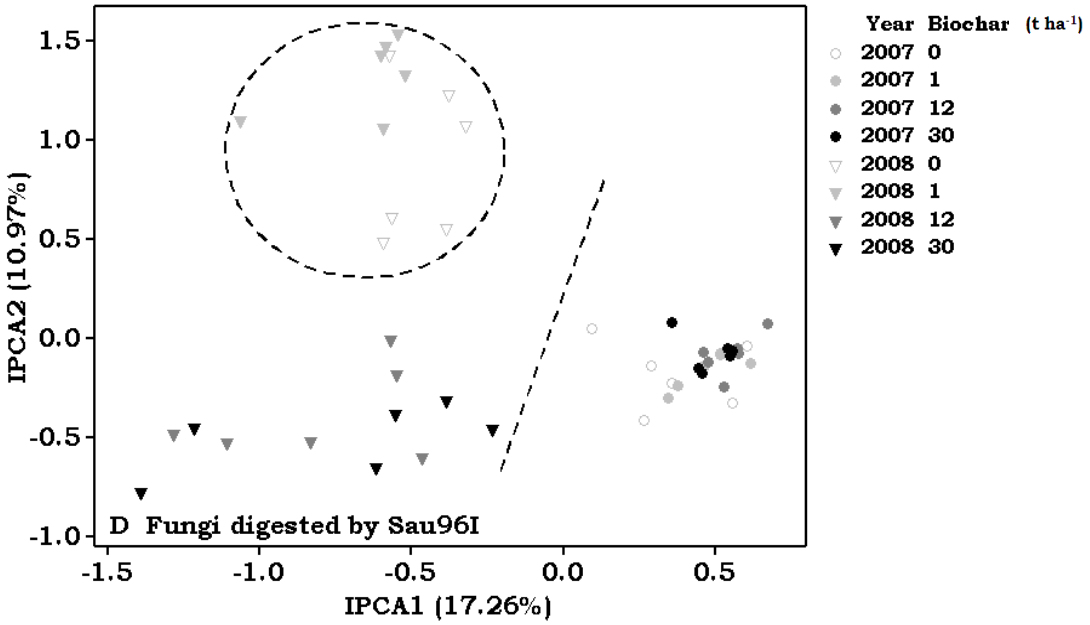

Year Biochar (t ha-1)
2007 0
2007 1
2007 12
2007 30
2008 0
2008 1
2008 12
2008 30
B Fungi digested by HhaI
IPCA2 (11.35%)
IPCA1 (15.62%)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-1.0
-0.5
0.0
0.5
1.0

Figure 3.8 (Continued)





DISCUSSION

Both the bacterial and fungal community compositions evaluated in this study shift significantly after biochar application at a rate higher than 12 t ha^{-1}, and over one year after biochar application. Microbial community composition varied depending on the biochar application rate. The height of the peaks revealed by the T-RF profiles indicated the relative abundance of microbial groups harboring identical terminal restriction fragments. Biochar itself is not a source of nutrients usable by microorganisms except for those that contain neglectable ash minerals (Lehmann and Joseph, 2009). Therefore, the microbial community compositions detected are less likely due to the biochar material itself, but more likely due to the effect of biochar on soil physical and chemical properties, such as retention of water and adsorption of nutrients, altered pH, CEC, porosity, aeration, etc.

Several studies reported a higher microbial biomass in biochar-enriched soils (Zackrisson et al., 1996; Steiner et al., 2008), these are consistent with the increased microbial biomass C we found in biochar-amended soils (Chapter 2). Results presented here clearly demonstrated that not only the abundance, but also microbial community compositions, were significantly affected by different biochar application rates. The higher biomass/abundance in biochar-amended soils could be attributed to a change in diversity rather than a change in colonization by particular species.

It has been suggested that biochar particles provide a protective habitat for microorganisms through pore size exclusion of predators (protozoa) (Thies and Rillig, 2009). Recently, it was demonstrated that the predation regimen could act as a major structuring force for the bacterial community composition in an aquatic system (Jürgens et al., 1999). Furthermore, partial protection of *Rhizobium leguminosarum* from protozoan grazing in soil due to the addition of bentonite clay was observed

(Heynen et al., 1988). In my study, the possible protective effect of biochar particles for microorganisms may have represented a selective pressure on the community composition in high biochar-amended soils. Alternatively, altered nutrient availability by the adsorptive effect of biochar may have caused changed bacterial and fungal diversities.

According to Van Gestel et al. (1996), the vicinity between microbes, organic matter, and clay is required for the survival of microbes, in which organic matter and clay particles provide substrates and nutrients. The non-organic matter of biochar itself (Lehmann and Joseph, 2009) and the strong adsorptive effect of biochar on substrates and nutrients (Chapter 2) in high biochar-amended soils seem to favor the formation of biochar-nutrient aggregates and thus change the vicinity between microbes and substrates and nutrients. Therefore, T-RFs found in the high biochar-amended soils may represent microbial species better adapted to either limited or more accessible nutrients and substrates. The development of biofilms in soils consisting of a dense of clay aggregates was previously found to contain one or more bacteria and grains of iron oxides (Lünsdorf et al., 2000). The biochar particles are also capable of forming aggregates that serve as housing for microbes which may at least partly explain the altered microbial diversity in soils contain high biochar particles.

Corn stover biochar used in this study has a pH of 8.2-10.0, which was probably responsible for some community changes, especially for fungi, which have a high tolerance to extreme pH conditions. The presence or absence of some T-RFs detected in high biochar-amended soils may indicate that either some bacteria may not have been able to withstand the alkaline environment or some fungi may become dominant due to their preference in relatively alkaline conditions.

PCR-T-RFLP profiling analysis has to be treated with caution due to biases inherent to low DNA recovery rates and varied PCR amplification in high biochar-amended soils, although the three DNA extraction protocols tested in our study did not affect the T-RFLP profiling analysis. PCR-T-RFLP profiling microbial community represents the community composition difference of the whole soil microbial community but not the specific presence, abundance and activity of its functional groups. As a consequence, these results did not necessary imply a microbial metabolic function change in response to high biochar application. Therefore, a cloning and sequencing strategy for the identification of microorganisms living in association with biochar is needed.

CONCLUSIONS

In this study, the microbial composition was mainly affected by biochar addition and did respond differently to different biochar application rates and time since biochar application. These results demonstrate specific microbe-biochar associations in biochar-amended soils. Knowledge of the microbial community structure represents a first step toward understanding soil function in response to biochar soil amendment. In addition to community composition, the analysis of microbial metabolic function within a given population will greatly increase our comprehension of the role of bacteria and fungi in soil processes important for geochemical dynamics of elements, specifically carbon, nitrogen, and phosphorus. An integrated multi-technique approach where physiological, biochemical and cloning and sequencing methods are combined is recommended. This will allow us to identify microorganisms stimulated in biochar-amended soils better than the use of a single T-RFLP fingerprinting method. I also suggest studies on water and nutrient availability, pH and porosity in biochar-amended soils in conjunction with the study of microbial community compositions, because this

knowledge will help to identify the specific physical or chemical properties affected by biochar addition that are the dominant factors altering microbial community compositions.

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CHAPTER 4

AMINOPEPTIDASE AND PHOSPHATASE EXOENZYME ACTIVITIES ARE INCREASED RELATIVE TO β-D-GLUCOSIDASE, β-D-CELLOBIASE IN AGRICULTURAL SOILS AMENDED WITH CORN STOVER BIOCHAR

ABSTRACT

The increasing use of biochar as an agricultural soil amendment has raised concerns about its possible effects on soil microbial communities. Soil hydrolytic exoenzyme activity, as an indicator of soil microbial activities and its metabolic requirements and available nutrients, was investigated by sampling soils from a corn field amended with varying rates of corn stover biochar in Aurora, NY. Activity of fluorescently labeled substrate analogues for β-D-glucosidase, β-D-cellobiase, aminopeptidase and phosphatase in all treatments were monitored for varying incubation times and statistically analyzed by fitting results to the Michaelis-Menten model. The determination of exoenzyme activity with fluorescently labeled substrate analogues was impaired by the strong adsorption of 66.7% more of the enzymatically liberated fluorophores 4-methylumbelliferone (MUF) and 34.6% more of the fluorophores 7-amino-4-methylcoumarin (MCA) adsorbed to the high biochar-amended soils than those adsorbed to the unamended control soils. I overcame this limitation by measuring the equilibrium adsorption isotherms for both MUF and MCA compounds and using correction models. Using this new approach, it was found that the biochar-amended soils had 615.3% and 15.0% higher activities of alkaline phosphatase and aminopeptidase, but 81.3% and 82.2% lower activities of β-D-glucosidase, β-D-cellobiase respectively. These changes in enzyme activities suggest that phosphorus (P) and nitrogen (N) use is increased relative to carbon (C) mineralized.

Biochar is recalcitrant and therefore unlikely a labile C source to meet microbial C needs, rather, an alternative mechanism of assimilating C could occur in biochar-amended soils (e.g., symbionts obtaining C from their host or trapping respired CO_2 to form carbonate as an alternative C source). Overall, I suggest that profound changes in microbial nutrient needs are occurring in biochar amended soils that apparently lead to tighter C cycling, and contribute to C conservation within soil systems high in black carbon (e.g., Terra Preta soils of Brazil).

INTRODUCTION

Enzymes mediate the activities performed by microorganisms in soil, therefore, are indicators of important soil biological processes (Tabatabai and Dick, 2002; Caldwell, 2005; Paul, 2007; Thies and Grossman, 2006). Some enzymes are active only in living cells (Frankenberger and Dick, 1983), whereas enzymes involved in the splitting of macromolecules must be transported out of the cells in order to achieve close contact with their substrates (Sinsabaugh et al., 2008). From an ecological point of view, microbial hydrolytic exoenzymes are of particular interest because they catalyse the rate-limiting steps of important metabolic processes such as organic matter degradation, mineralization and nutrient cycling. Hydrolytic exoenzymes involved in carbon (C), nitrogen (N) and phosphorus (P) cycling have received particular attention due to their capacity to catalyze processes such as the cleavage of C containing polymers such as cellulose, starch or hemicellulose into smaller molecules, the breakdown of complex protein polymers into amino acids and the release of organically bound phosphate into forms available for soil microbial community and plant uptake (Tomme et al., 1995; Klose et al., 1999; Hayes et al., 2000).

The activities of microbial exoenzymes are largely regulated by soil moisture,

temperature, pH and substrate (nutrient) availability (Nannipieri et al., 1983; Thies and Grossman, 2006), but can also be used as indicators of microbial response to different treatments. Several studies have shown that the enzymes respond rapidly to changes in the environment. Among these studies, changes in plant species composition (Grierson and Adams, 2000), seasonal variation (Wallenstein et al., 2009) and organic amendments (Garc á-Gil et al., 1999) all influence in the activity of soil exoenzymes. Even single enzyme or enzyme groups have been shown to have value as indicators of the state of the soil microbial community (Bastida et al., 2008). Mele and Crowley (2008) suggest that the study of enzyme diversity and their associated activities provides an effective approach to examining functional diversity in soils. Furthermore, exoenzyme responsiveness to environmental changes makes them a potential indicator of soil biological quality.

With the increasing interest in using biochar for promoting soil fertility and improving soil health, a number of recent studies have emphasized the benefit of using biochar as a soil amendment to improve physical and chemical properties of soils and of its stimulating effect on soil microorganisms (Lehmann, 2007a; Lehmann, 2007b; Ogawa et al., 2006; Laird, 2008; Novak et al., 2009). The beneficial effects of biochar are higher potassium (K), P, and zinc (Zn) availability, and to a lesser extent, calcium (Ca) and copper (Cu) have been observed in soils enriched with biochar (Lehmann et al., 2003a; Lehmann, 2007b; Steiner et al., 2007; Warnock et al., 2007). An increase in biological nitrogen fixation by common beans was found in biochar-amended soils (Rondon et al., 2007). Biochar was found to be responsible for maintaining high levels of soil organic matter (SOM) and available nutrients in anthropogenic soils of Terra Preta soils of the Brazilian Amazon (Fearnside et al., 2007). In addition, biochar-amended soils can create unique habitats exhibiting higher adsorptive internal surface area and a higher CEC (Liang et al., 2006). Lower greenhouse gas emissions were

found in biochar-amended soils (Rondon et al., 2007; Gaunt and Lehmann, 2008; Rogovska et al., 2008; Kuzyakov et al., 2009; Spokas et al., 2009; Liang et al., 2010). Studies on the response of the soil microbial community to biochar soil amendment have focused mainly on monitoring changes in microbial abundance, activity and community composition (Zackrisson et al., 1996; Uvarov, 2000; Jin et al., 2008; Rogovska et al., 2008; Steiner et al., 2008; O'Neill et al., 2009; Liang et al., 2010). Steiner et al. (2008) reported increased microbial activity in soils enriched in biochar. Thies and Jin (2009) found higher microbial biomass, but lower microbial respiration in biochar-amended soils than in unamended soils, which suggested an increase in microbial C use efficiency, possibly associated with a change of metabolic function in the microbial community. It has been suggested that the porous structure of biochar particles can be a good habitat for microorganisms (Thies and Rillig, 2009). Studies have suggested that bacterial cells and fungal hyphae colonizing corn stover biochar particles and indicated the microorganisms could be protected from predation and nourished by C substrates and other mineral nutrients adsorbed on biochar particle surfaces (Saito and Muramoto, 2002; Warnock et al., 2007). There have been several studies that reported stimulation of indigenous arbuscular mycorrhizal fungi by biochar (Nishio, 1996; Ishii and Kadoya, 1994), which was reflected in increased plant growth.

Besides these studies, the way in which biochar affects soil microorganisms and microbial exoenzymes may be distinct from other soil amendment strategies because the porous structure and adsorptive properties of biochar make its interaction with microorganisms and microbial exoenzymes complex. Soil exoenzymes can form complexes with organic and inorganic materials (Tabatabai and Fu, 1992) that stabilizes or reduces of their catabolic activity (Gianfreda and Bollag, 1994; Ra and Gianfreda, 2000; Rao et al., 2000; Rosas et al., 2008). The binding characteristics of

exoenzymes to organic and inorganic materials can alter soil exoenzyme activities (Bonmati et al., 1998; Gianfreda and Ruggiero, 2006). I assumed that the activity of exoenzymes could be substantially affected by the potential interactions of these proteins and the biochar surfaces. If adsorption of enzymes on biochar alters the structure of the enzyme functional group or active site, it may inhibit its metabolic function, and result in a decreased enzyme activity. Conversely, if the adsorption does not affect the enzyme functional group or active site, or increases the contact of enzyme and adsorbed substrates on biochar, enhanced enzyme activity may occur. With this, I also assumed that the effect of adsorption on enzyme activity would differ among the different enzyme classes based on the similarities or differences of their molecular composition and structure, and their adsorption characteristics on biochar.

In the context of soil productivity, the impact of biochar amendments on microbial exoenzyme activities is critical. Nonetheless, there is still poor knowledge about how biochar affects the activity of microbial exoenzymes in C mineralization, nutrient availability, greenhouse gas emissions and other associated soil biochemical processes. In this paper, I describe a comprehensive analysis of four key hydrolytic exoenzyme activities in corn stover biochar-amended soils sampled from a field experiment conducted at Cornell Musgrave Research Farm in Aurora, NY. The objectives were to: (1) study the kinetics of fluorophore formation in biochar-amended and unamended soils as a prerequisite for estimating soil exoenzyme activity using a fluorogenic model substrate-based method; (2) evaluate the Michaelis-Menten kinetics of soil alkaline phosphatase, β-D-glucosidase, β-D-cellobiosidase and aminopeptidase in response to biochar soil amendment; (3) test the sensitivity of the fluorogenic model substrate method using fluorophores, 4-methylumbelliferone (MUF) and 7-amino-4-methylcoumarin (MCA), to determine exoenzyme kinetics in soils with different amounts of biochar added; (4) evaluate the potential application of this technique for

evaluating the physiological activity of the soil microbial community.

MATERIALS AND METHODS

A field experiment was initiated at Cornell Musgrave Research Farm, Aurora, NY, in May, 2007. Each plot was 7.5 m long×4.5 m wide with 1-2 m wide alley in between the plots. Corn stover charcoal was incorporated into the soil at rates of 0, 1, 12, 30 t ha^{-1}, to each plot before planting corn seeds at a density of 32,000 seeds per acre in the field. Soil samples were obtained from each plot in May, 2008. In detail, seven soil cores were taken randomly from 0-15 cm depth from each plot and composited. In order to collect samples aseptically, before moving to the next plot, the sampling probe was sterilized with bleach and rinsed three times with purified water in order to avoid contamination between plots. Each composite soil sample was mixed well and obvious root material removed with forceps. The samples were zipped into plastic bags, shipped on ice and analyzed within 48 h at Cornell University, Ithaca, NY.

Choice of Method

A number of protocols have been developed to measure soil exoenzyme activity; they differ in the nature of the substrate, assay conditions, incubation time and detection methods such as colorimetric, fluorimetric or radiolabelled (Marx et al., 2001; Grosjean et al., 2007; Butterfield et al., 2008). Enzyme activity is normally described by the kinetic parameters V_{max} and K_m, which relate reaction rates to substrate concentration following the Michaelis-Menten model (Michaelis and Menten, 1913). We adopted the fluorimetric method using fluorogenic model substrates. The artificial fluorophores involved are MUF and MCA, which are highly fluorescent and therefore small quantities of hydrolyzed substrate can be easily detected, allowing assays to be carried out at low substrate concentrations. Besides,

the measurement of enzyme activity using MUF and MCA substrates has a close relationship with naturally occurring processes such as the turnover of macromolecular substrate containing glucosides, glucosaminides, organic phosphorous and sulphurous compounds (Freeman et al., 1995; Hoppe, 1983). However, this method involves a procedure for extracting and purifying the fluorophore product from the biochar treated soils prior to analysis. Therefore, the recovery of the enzymatically released fluorophores can be dramatically reduced by adsorption to not only soils, but the biochar in soils as it has a strong adsorptive affinity to organic and inorganic molecules (Thies and Rillig, 2009); consequently, enzyme activities are likely to be underestimated. To correct the recovery of the enzymatically released fluorophore, a novel approach based on fluorophore adsorption kinetics and adsorption equilibrium isotherms was used that allowed accurate estimation of the follow-on enzyme activities.

Correction Method for Determining Exoenzyme Activities

The distribution of fluorophores between the liquid phase and the solid adsorbent phase is a measure of the equilibrium in the adsorption process and can be expressed by the more popular isotherm models (Limousin et al., 2007). The adsorption isotherm indicates how the adsorption molecules are distributed between the liquid and the solid phases when the adsorption process reaches an equilibrium state (Limousin et al., 2007). The analysis of the isotherm data by fitting them to Langmuir and Freundlich isotherm models is an important step toward finding a suitable model that can be used for correction purposes. Adsorption isotherms are important to describe how the MUF and MCA fluorophores interact with soil and biochar particles, and is critical in correcting the total fluorophore concentration in soil slurries.

Because I observed a strong adsorption of free fluorophores, I initially

determined the time required to reach adsorption equilibrium for the two fluorophores, MUF and MCA. A series of soil slurries was prepared for each biochar treatment (0, 1, 12, 30 t biochar ha^{-1}) by adding 5 g (ODW) soil to 40 ml of 50 mM, pH 5.0, acetate buffer, the headspace of each slurry was immediately flushed with N_2 for 3 min in order to create an anoxic atmosphere. The samples were stored at 4 ℃ until exoenzyme activities were measured. MUF or MCA was added to a final concentration of 40 or 10 μM, respectively. At regular intervals between 5 and 600 min after substrate addition, a subset of three samples was centrifuged (5 min at 10,000 ×*g*), and the supernatants were transferred to fresh Eppendorf tubes. The pH of samples containing MUF was increased to 11.0 by the addition of NaOH (final concentration, 40 mM). Precipitation of carbonates was prevented by the addition of Na_4EDTA (1.7 M; final concentration, 0.1 M). The concentrations of free fluorophores in the supernatant were determined by a SLM 8000 spectrofluorometer (Olis, Bogart, GA, USA) at an excitation wavelength of 370 nm and an emission wavelength of 450 nm. For calibration, MUF and MCA standards were prepared in methanol at concentrations of 0.1 to 0.7 μM. The amount of adsorbed fluorophores was calculated as the difference between the total amount added and the amount remaining in the supernatant. The time required to reach adsorption equilibrium for the two fluorophores, MUF and MCA, were determined by examining the relationship between the incubation time and the adsorbed fluorophores.

Equilibrium adsorption isotherms were determined by adding seven concentrations of the fluorophores, 5, 10, 20, 40, 70, 110, 160 μM for MUF and 5,10, 15, 20, 30, 40, 50 μM for MCA, to a set of soil slurries from the different field soil treatments. At equilibrium, the amount of a substrate adsorbed (*S* nmole g [dry weight]$^{-1}$) depends on the concentration of the fluorophore remaining in solution (*Ce* nmole ml^{-1}) according to the Freundlich equation:

Equation 4.1 $S = K \cdot C_e^{\,n}$

Here, **Equation 4.2** $S = \frac{C_T - C_e}{D_{slurry}}$

where K is the affinity coefficient (ml g[dry weight]$^{-1}$); n is the adsorption capacity of the adsorbent, an indication of the favorability of the adsorption process; C_T (nmole ml^{-1}) is fluorophore added; D_{Slurry} (g [dry weight] ml^{-1}) is the dry weight of soil in the slurry. The Langmuir isotherm model assumes monolayer adsorption onto a surface containing a finite number of adsorption sites of uniform strategies of adsorption with no transmigration of adsorbate in adsorption surface (Weber and Chakkravorti, 1974).

And the Langmuir equation:

Equation 4.3 $S = \frac{S_T \cdot C_e}{C + C_e}$

Here, $S = \frac{C_T - C_e}{D_{slurry}}$ (Eq. 4.2), where S_T and C are the Langmuir constants related to adsorption capacity and rate of adsorption, respectively.

After the adsorption equilibrium is reached (incubation time was determined at the first step), the concentrations of free fluorophores were measured in the supernatants, and the amount of fluorophores adsorbed per gram (dry weight) of soil was calculated. Equations 1 and 2 were then fitted to the data points respectively, using the nonlinear-regression tool of JMP 6.0, which yielded the two parameters, K and n for the Freundlich model and S_T and C for the Langmuir model.

The total concentration of fluorophore, C_T, liberated during the exoenzyme assays could be calculated from the equilibrium concentrations (*Ce*) of MUF or MCA in the supernatants of the soil slurries and from the values of the parameters determined for each biochar amendment treatment:

For the Freundlich model: **Equation 4.4** $C_T = C_e + D_{slurry} \cdot K \cdot C_e^{\,n}$

For the Langmuir model: **Equation 4.5** $C_T = C_e + D_{slurry} \cdot \frac{S_T \cdot C_e}{C + C_e}$

Michaelis-Menten Kinetics of Exoenzymes in Biochar-amended Soils

The correction method of fitting data in adsorption models was used to determine the Michaelis-Menten kinetics of the hydrolytic exoenzymes alkaline phosphatase (EC 3.1.3.1), β-D-glucosidase (EC 3.2.1.21), β-D-cellobiase (EC 3.2.1.91) and leucine aminopeptidase (EC 3.4.11.1) (Saiya-Cork et al., 2002; Margon and Fornasier, 2008; Li et al., 2009). Alkaline phosphatase, β-D-glucosidase and β-D-cellobiase were assayed with MUF-phosphate (Sigma, Saint Louis, MO, USA), MUF-β-D-glucoside (ANASpec, Fremont, CA, USA) and MUF- β-D- cellobioside (Gold Biotechnology, Saint Louis, MO, USA). Aminopeptidase activity was measured with MCA-labeled leucine (Gold Biotechnology, Saint Louis, MO, USA). Duplicate 4 ml aliquots of the soil slurry were transferred to autoclaved 10 ml serum vials containing stirring bars. The enzymatic reaction was started by the addition of 1 ml of substrate analogue solution to yield final concentrations of 5, 10, 25, 60, and 120 μM. Each vial was sealed with a sterile butyl rubber stopper and flushed with N_2 for 1 min. During incubation, all slurries were stirred at 200 rpm and incubated at a temperature of 17 ℃ (the *in situ* temperature). Considering the different significance of varying enzyme activities, slurries were incubated in parallel for three time intervals, 30 min, 2 h and 8 h for each enzyme assay to determine an optimal incubation time. In addition, for each concentration, three different blanks (B1, B2, and B3) were incubated in parallel. One blank (B1), used to assess nonenzymatic hydrolytic cleavage of the substrate analogues, was boiled for 30 min prior to incubation in order to inhibit the enzyme. This method of inactivation was chosen because of the adsorptive capacities of the biochar-amended soils. In order to correct for the fluorescent compounds released

from the soil matrix during boiling, a second blank (B2) was incubated without substrate analogues. The fluorescence caused by the compounds extracted from the soils without boiling was determined in a third blank (B3) which was also incubated without substrate analogues. After incubation, the slurries were transferred to microcentrifuge tubes and centrifuged for 5 min at 10,000 ×*g*, and the concentrations of free dissolved fluorophores were determined fluorometrically as given above. The detection limit for the concentration of free fluorophores was estimated using this new approach.

Each enzyme measurement generated a large amount of raw data and to make the most effective use of this for comparisons between samples, a standardized way of processing data was established. A calibration curve was concentrated to convert the resulting rates (in relative units of fluorescence $cm^{-3}\ h^{-1}$) into nM of fluorescence $cm^{-3}\ h^{-1}$ according to the specific standard. V_{max} and K_m were empirically estimated values obtained by fitting the experimental data to the Michaelis-Menten equation. Then, the maximum enzyme activity, V_{max} (nM cm^{-3} soil h^{-1}), and $K_m + S_n$ (the sum of the half saturation constant plus the concentration of natural substrate [μM]) were estimated:

Equation 4.6
$$\frac{A \cdot t_{inc}}{A_{hydrol}} = \frac{K_m + S_n}{V_{max}} + \frac{1}{V_{max}} \cdot A$$

Here, t_{inc} is the incubation time (in hours) and A_{hydrol} is the concentration of substrate analogue enzymatically hydrolyzed during the incubation; A denotes the total concentration of substrate analogue added.

RESULTS AND DISCUSSION

Adsorption Kinetics

The adsorption of both fluorophores MUF and MCA began to reach saturation

after 2 h of incubation (Fig. 4.1A and B). Thus the incubation time necessary for the fluorophores, MUF and MCA with initial concentrations of 40 and 10 μM respectively, to reach equilibrium was 2 h. The amount of fluorophore adsorbed in biochar-amended soils increased with time and, at some point in time, reached a constant value beyond which no additional fluorophores were removed from solution. At this point, the amount of fluorophore desorbing from the adsorbent, soil and biochar matrix, is in a state of dynamic equilibrium with the amount of fluorophore being adsorbed onto the soil and biochar matrix. The time required to attain this state of equilibrium was termed the equilibrium time, and the amount of fluorophore adsorbed at the equilibrium time reflects the maximum adsorption capacity of the adsorbent under those operating conditions. Three consecutive fluorophore transport steps should be associated with the adsorption of the fluorophore from solution by the adsorbent (Faust and Aly, 1987), soil and biochar particles in our case. First, the fluorophore migrates through the solution, i.e., film diffusion, followed by fluorophore movement from the particle surface into interior sites by diffusion and finally the fluorophore is adsorbed onto the active sites on the surface of soil and biochar particles. This phenomenon takes a relatively long contact time. After 2 h of incubation, about 54.0% of the MUF and 61.8% of the MCA which had been added to the 30 t ha^{-1} biochar-amended soils had disappeared from the solution phase. In contrast, only 32.4% of the MUF and 45.9% of the MCA adsorbed to unamended control soils (Fig. 4.1). That is, there was 66.7% more of the MUF and 34.6% more of the MCA adsorbed to the high biochar-amended soils than those adsorbed to the unamended control soils. Thus, the measurement of exoenzyme activities in biochar-amended soils requires an incubation time of at least 2 h when the major fraction of the liberated fluorophores were adsorbed to the soil and biochar matrix concomitant with their enzymatic liberation from the substrate analogues.

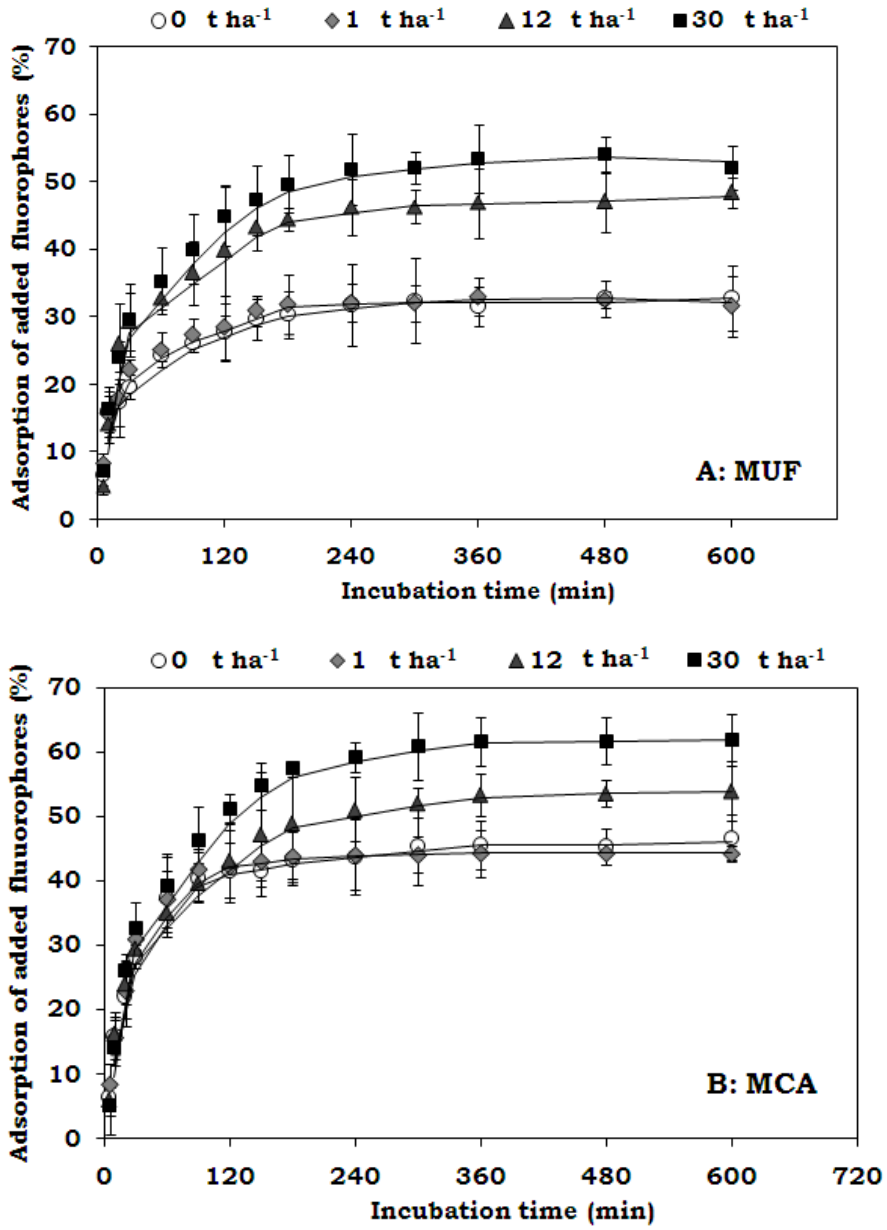

Figure 4.1 Time course of fluorophore adsorption in biochar-amended soil slurries. For all four biochar amendment treatments, the adsorption kinetics of MUF (A, 40 μM) and of MCA (B, 10 μM) are shown. The error bars indicate standard deviations (n=3).

Effect of Concentration of Fluorophores on Adsorption Kinetics

The proportion of adsorbed fluorophores increased dramatically with increasing equilibrium concentrations then decreased gradually as the initial concentration of MUF was increased (Fig. 4.2A and B). This can be explained by the fact that all

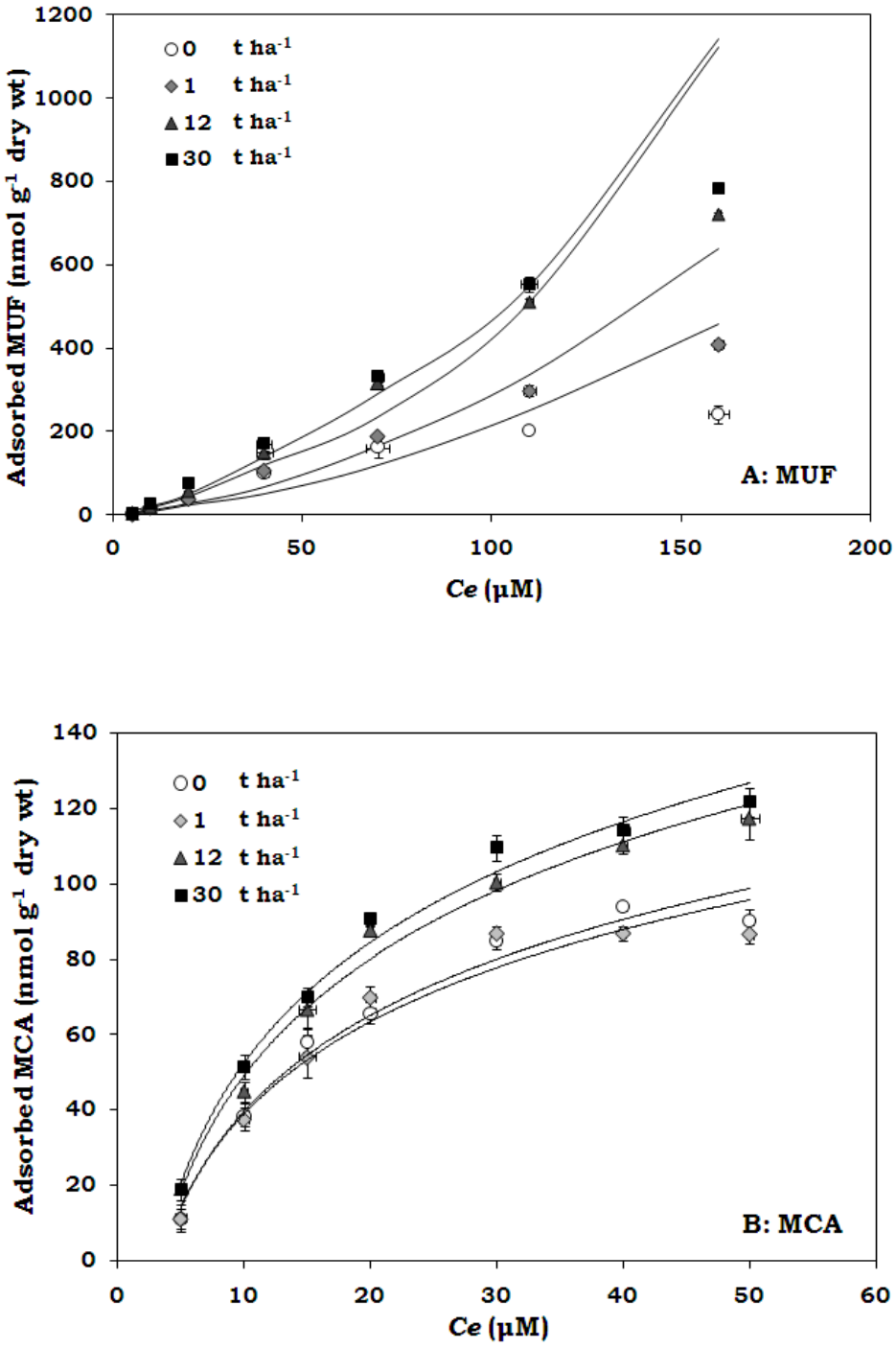

Figure 4.2 Equilibrium adsorption isotherms of MUF (A) and MCA (B) for each biochar amending treatment fitted to the Freundlich equation (lines). The error bars indicate standard deviations (n=3).

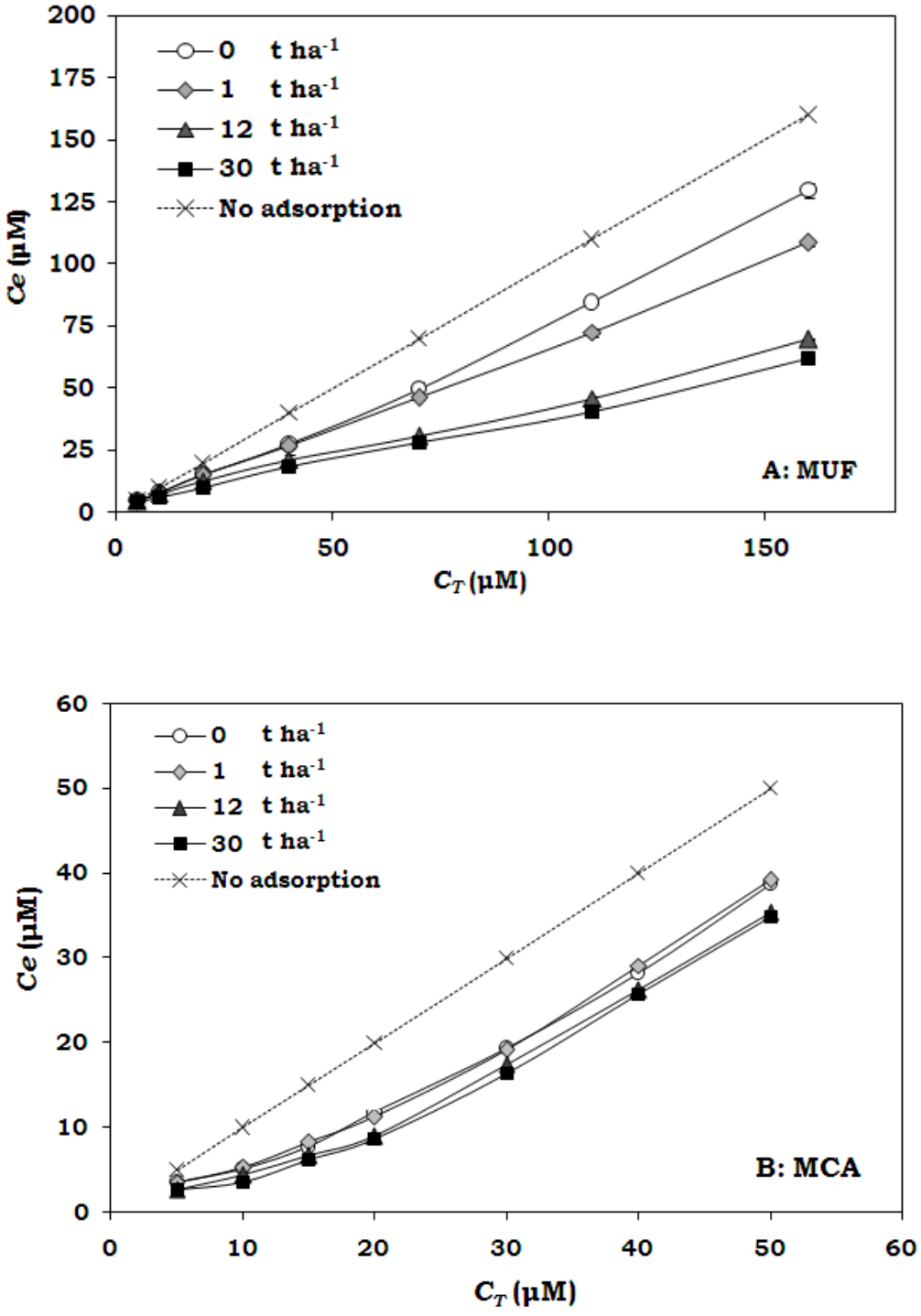

Figure 4.3 Concentrations of dissolved fluorophores MUF (A) and MCA (B) in biochar-amended soil slurries after 6 h of incubation. The concentrations of free fluorophores expected in the absence of adsorption are indicated by the dotted line.

biochar-amended soil samples had a limited number of active sites, which would have become saturated above a certain concentration. The adsorption capacity for MUF at equilibrium increased from 1.42 to 242.26 nmol g^{-1} in unamended soils, from 5.47 to 782.54 nmol g^{-1} in 30 t ha^{-1} biochar-amended soils with an increase in the initial MUF concentration from 5 to 160 μM. Similarly, the adsorption capacity for MCA at equilibrium increased from 11.29 to 90.16 nmol g^{-1} in unamended soils, from 18.83 to 121.63 nmol g^{-1} in 30 t ha^{-1} biochar-amended soils with an increase in the initial MCA concentration from 5 to 50 μM (Fig. 4.2A, B).

It is evident that both the unamended control soils and the biochar-amended soils have high capacity to adsorb the fluorophore molecules, MUF and MCA, from solution, the process attaining equilibrium gradually. The significantly higher adsorption found in high biochar amended soils is likely due to the porous structure of the corn stover biochar, which has a high internal surface area that contributes to the high hydrophobic attraction (Cheng et al., 2005). In addition, I observed that the adsorption of the fluorophores MUF and MCA on biochar-amended soils depended on their concentrations in a nonlinear relationship (Fig. 4.2 and 4.3). Therefore, equilibrium adsorption isotherms had to be adopted prior to measuring exoenzyme activities instead of using a linear correction for adsorption.

Adsorption Isotherms

From the adsorption measurements, the corn stover biochar was found to have a remarkably high adsorbing capacity for the fluorophores, with an addition rate of 12 or 30 t biochar ha^{-1} (Fig. 4.2 and 4.3). This characteristic could be attributed to the high surface area of the corn stover biochar, similar to the strong adsorption capability of other activated carbons for butane, methane and other molecules (Walton et al., 2004). Fig. 2 typically shows the adsorption isotherms of fluorophores at room temperature

for different biochar amended soils. Adsorption isotherms were analyzed using two well-known isotherm models, Langmuir and Freundlich. The constants were calculated for these two models and were given in Table 4.1. Besides the value of r^2, the applicability of both kinetic models were verified through the sum of squares for error (*SSE*, %). The higher the value of r^2 and the lower the value of *SSE*, the better the goodness of fit will be. By comparing the correlation coefficients (r^2) and the sum of squares for error (*SSE*, %) (Table 4.1), the Freundlich isotherm equation yielded a somewhat better fit than the Langmuir isotherm equation. The adsorptive affinity, *K*, of MUF to the 30 t ha^{-1} biochar treatment was 0.87 ml g^{-1}, higher than that to the 0 and 1 t ha^{-1} biochar treatments. The affinity of the high biochar treatment for MCA was 18.85, higher than that for MUF. It is important to note that model parameters (Table 4.1) allow the prediction of adsorption data and can be further used to predict the total fluorophore concentration (C_T) in soil slurry by using the detectable concentration in the equilibrium solution (*Ce*) (Walton et al., 2004). This is very convenient for use in the follow-on enzyme activity assays where the enzymatically released fluorophores need to be corrected using the isotherm model.

Michaelis-Menten Kinetics of the Four Exoenzymes

For the four exoenzymes, the maximum rates and half saturation constants were estimated after correction for adsorption and for background fluorescence of the soils (Table 4.2). The activity of alkaline phosphatase could only be determined after 0.5 h incubation regardless of the initial concentration of substrate analogue added. Given that we added the same series of concentrations of substrate analogue, this could indicate that the activity of alkaline phosphatase was significantly higher in all soil slurries than that of β-D-glucosidase, β-D-cellobiase and leucine aminopeptidase which, in contrast, could be determined after 2 h and 2 h and 8 h respectively.

Table 4.1 Constants of the Freundlich and Langmuir equilibrium adsorption isotherms for adsorption of fluorophores on different biochar treatments

Fluorophores	Biochar added (t ha^{-1})	Freundlich Model				Langmuir Model			
		n	*K*	r^2	*SSE*	S_T	*C*	r^2	*SSE*
MUF	0	1.398	0.510	0.850	1.745	-10.597	-33.481	0.730	0.362
	1	1.592	0.365	0.912	1.107	-11.901	-32.767	0.553	0.613
	12	1.891	0.367	0.970	0.359	-25.510	-27.940	0.870	0.018
	30	1.739	0.868	0.936	0.678	-33.377	-24.672	0.735	0.023
MCA	0	0.728	8.677	0.701	0.605	-276.855	-73.262	0.603	0.008
	1	0.723	8.514	0.703	0.585	-237.925	-65.550	0.684	0.005
	12	0.620	16.029	0.821	0.260	643.128	76.609	0.900	0.000
	30	0.588	18.847	0.749	0.380	517.464	53.986	0.717	0.001

Likewise, the fact that the activity of leucine aminopeptidase was obtained even after 8 h incubation indicated the relatively lower activity of leucine aminopeptidase in all soil slurries than that of the other three exoenzymes.

Alkaline phosphatase in high biochar-amended soils exhibited a $K_m + S_n$ of 244.63 μM, which is about double that in 12 t ha^{-1} biochar treatment, and threefold higher than that in the unamended control and 1 t ha^{-1} biochar treatments (Table 4.2). Highest alkaline phosphatase activities were detected in the high biochar-amended soils, exhibiting a V_{max} of 296.13 nmol cm^{-3} h^{-1}, which were about threefold higher than that in the unamended control and 1 t ha^{-1} biochar treatments.

Nevertheless, the activities of β-D-glucosidase, β-D-cellobiase were significantly higher within the soils amended with no or low biochar than in the soils amended with medium or high biochar. In the 12 or 30 t ha^{-1} biochar treatment, β-D-glucosidase activity was 3-4 times lower than that in the 0 or 1 t ha^{-1} biochar treatment. The activity of β-D-cellobiase followed a similar trend with high activity in unamended control and 1 t ha^{-1} biochar-amended soils, and low activity in the 12 and 30 t ha^{-1} biochar-amended soils.

Leucine aminopeptidase had the lowest activity of any of the enzymes tested in our study, with a mean activity of less than 47, 12 and 3 nmol cm^{-3} h^{-1} detected after 0.5, 2 and 8 h incubations respectively (Table 4.2). Particularly, in none or low biochar amended soils and leucine aminopeptidase activity did not exceed 2.2 nmol cm^{-3} h^{-1} when the activity was measured after 8 h incubation (Table 4.2). The leucine aminopeptidase activity in the 12 and 30 t ha^{-1} biochar-amended soils was about double those in 0 and 1 t ha^{-1} biochar-amended soils. Leucine aminopeptidase was not significantly affected by the low rate of biochar addition (Table 4.2). However, its activity was significantly higher in soils amended with more than12 t ha^{-1}. The

relatively high aminopeptidase activity was measured after 30 min incubation, and it declined rapidly when it was measured after 2 h and 8 h incubation. The incubation time significantly influenced the activity assay. Interestingly, even though the exoenzyme activity assay did not remain constant using different incubation times, a consistent trend of exoenzyme activity change with rate biochar added was observed (Table 4.2).

With a detection limit for free fluorophores of 0.01 μM MUF/MCA, the minimum detectable alkaline phosphatase activity after correction for adsorption and for background fluorescence was below the limit after 2 h incubation. For β-D-glucosidase and β-D-cellobiase activity, the activity became undetectable after 8 h of incubation, while for leucine aminopeptidase, the activity was still detectable after 8 h incubation. With this detection limit, no significant activity was determined for alkaline phosphatase after 2 h incubation, for β-D-glucosidase and β-D-cellobiase after 8 h incubation. In the fluorogenic substrate-based method we adopted to determine exoenzyme activities in biochar-amended soil slurries, the introduction of substrate analogues into the soil slurry has been proposed to yield more realistic values of enzyme activities (Meyer-Reil, 1986).

Table 4.2 Estimate of the half saturation constant, *Km + Sn*, and of the maximum velocity, *Vmax*, of the four hydrolytic enzymes determined for the different biochar treatments.

Incubation time	Biochar added (t ha^{-1})	Alkaline phosphatase		β-D-Glucosidase		β-D-Cellobiosidase		L-Leucine aminopeptidase	
		Km+Sn (µM)	*Vmax* (nmol cm^{-3} h^{-1})	*Km+Sn* (µM)	*Vmax* (nmol cm^{-3} h^{-1})	*Km+Sn* (µM)	*Vmax* (nmol cm^{-3} h^{-1})	*Km+Sn* (µM)	*Vmax* (nmol cm^{-3} h^{-1})
30 min	0	50.36	41.40	270.77	152.18	276.49	166.89	70.16	27.19
	1	60.05	45.13	176.96	102.83	316.17	179.71	130.18	38.15
	12	102.20	111.88	52.40	21.44	125.12	38.58	88.68	53.74
	30	244.63	296.13	69.43	28.47	78.08	29.64	97.78	68.81
2 h	0	ND	ND	482.02	102.84	727.79	191.41	45.27	7.94
	1	ND	ND	392.05	74.46	458.33	113.84	44.26	7.98
	12	ND	ND	115.64	37.45	289.11	62.56	47.52	14.76
	30	ND	ND	140.84	38.58	148.22	33.05	43.70	16.18
8 h	0	ND	ND	ND	ND	ND	ND	26.42	1.95
	1	ND	ND	ND	ND	ND	ND	28.23	2.13
	12	ND	ND	ND	ND	ND	ND	33.60	3.58
	30	ND	ND	ND	ND	ND	ND	30.25	4.05

Exoenzymes as Indicators of the Metabolic Potential of Soils

First, the significantly increased alkaline phosphatase activities found in high biochar-amended soils indicated an increase in P use with increasing biochar additions. Phosphatases (alkaline or acid) hydrolyze phosphomonoesters and, in some cases, phosphodiesters releasing plant available phosphate (Turner et al., 2002; Toor et al., 2003). Studies on the relationship between soil available P and phosphatase activity have had controversial results. Harrison (1983) concluded that there was a positive relationship between soil available P and phosphatase activity. Similarly, Garg and Bahl (2008) concluded that phosphatase activity was higher in soil with higher Olsen extractable P. With increased inorganic P, the release of phosphatase also increased. There is, however, a contradictory evidence from Kiss et al. (1974) who described a linear inverse relationship between phosphate concentration in soil solution and phosphatase activity. Mccallister et al. (2002) and Leinweber (2008) reported that phosphatase activity, although related to P availability, is not a straight forward measurement of P status. The stimulation of phosphatase activity is largely due to increased microbial numbers in the soils, which, with time, causes a build up of enzyme levels (Feder et al., 1973). My finding of an increased phosphatase activity in high biochar-amended soils is more likely due to an increased microbial abundance (Chapter 2), and therefore, increased enzyme levels in response to biochar addition; or, to a shift to autotrophic or mutualistic organisms that obtain needed C by other mechanisms. Phosphatase transform P in organic form to an inorganic form, as these enzymes are responsible for soil organic phosphorus mineralization and the release of inorganic phosphorus needed by microorganisms and plants (Sarapatka, 2003). Thus, my results indicated a higher P availability for microbe and plant use due to increased phosphatase activity in response to biochar addition. This is consistent with the increased available phosphorous in soils enriched with biochar found by Lehmann et

al. (2003; 2007b) and Steiner et al. (2007). I suggest that increased phosphatase activity could be attributed to the beneficial colonization of bacterial cells and fungal hyphae on, in and between biochar particles (Jin and Thies, 2009), whose exoenzyme activities release plant available phosphate in the biochar-amended soils. It is also likely that the co-location of phosphatase and phosphorous substrates to the biochar allows for increased contact of phosphatase with the substrates. Biochar particles are porous, they have a high surface area; they provide unique micro-habitats for closely associated microorganisms and their substrates in soils. I hypothesize that the enzymes produced by soil microorganisms in these protected sites, i.e., biochar particles, did not have to diffuse towards sites with high availability of substrates. Therefore, not only enzyme producers, but also soil protozoa and other animals that do not produce any extracellular enzymes, could profit from hydrolysis of organic P containing compounds (Allison, 2006).

Second, the 12 and 30 t ha^{-1} biochar-amended soils exhibited lower rates of β-D-glucosidase and β-D-cellobiase activity than the low or no biochar-amended soils. Since glucose and cellulose are liberated by β-glucosidase and β-cellobiase, respectively, this finding showed that the introduction of biochar into soils significantly reduced β-D-glucosidase and β-D-cellobiase activity, possibly explaining the reduced C mineralization in soils amended with high rates of biochar found by my earlier study (Chapter 2) and others (Spokas et al., 2009; Liang et al., 2010). The reduction of β-D-glucosidase and β-D-cellobiase activity in soils amended with high rates of biochar could reflect a lower need for their corresponding products, glucose and cellulose. This could be due to the increased access of available C to microbes by co-locating microbes and complex C substrates on biochar particles. Another possible reasoning might be that the recalcitrance of biochar is unlikely a C source to meet microbial C needs, rather, an alternative mechanism of assimilating C could occur in

biochar-amended soils (e.g., symbionts obtaining C from their host or trapping respired CO_2 to form carbonate as an alternative C source). I suggest that the decreased C mineralization in biochar-enriched soils contributes to the observed long-term preservation of labile C and high levels of SOM retained in other soils with high black carbon contents (e.g., terra preta soils of Brazil) (Solomon et al., 2007; Liang et al., 2010).

Third, aminopeptidases catalyze the cleavage of amino acids from polypeptide substrates. There are other classes of aminopeptidases in addition to leucine aminopeptidase, but assays of environmental samples generally show the greatest activities towards leucine- and alanine-linked substrates, so leucine aminopeptidase activity is used broadly as an indicator of peptidase potential and organic N acquisition activity from amino acids (Stursova et al., 2006). In this study, N acquisition activity from peptide degradation evidenced by an increase in aminopeptidase activity, increased with increases in the rate of biochar applied originally to the soil. I suggest here that the increased N use is likely due to an increase in microbial abundance (Chapter 2), thus, an increase in aminopeptidase production. This would result in an increase in the breakdown of N-rich substrates, but not necessarily due to increased degradation of crop residues or litters, which contain a high proportion of low-N constituents, such as glucose and cellulose. The increased degradation of N-containing compounds could increase the available N supply to microbes and plants in response to biochar addition.

CONCLUSIONS

Taken together, these results indicate that phosphate and peptide decomposition were induced by high rates of biochar amendment, while glucose and cellulose breakdown were not. These results suggest that biochar amendment increases the need

for microbial P and N acquisition relative to C acquisition. The high adsorption capacity of biochar for structurally different substrates, enzymes or other compounds, and the decreased activity of C mineralizing enzymes likely contribute to the observed long-term preservation of labile C in biochar-amended soils. However, whether our finding of increased microbial P and N needs relative to C need in response to biochar soil amendment is due to (1) the increased presence of mutualists who receive C from their hosts but have metabolic needs for nutrients other than C; or, (2) fewer exoenzymes associated with C cycling produced; or, (3) the adsorption of these exoenzymes on biochar and their resultant inactivation remains unknown. Thus, further investigation on the functional state of microorganisms and the interaction of exoenzymes and biochar in soils amended with high rates of biochar is needed to verify our findings.

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CHAPTER 5

ALKALINE PHOSPHATASE AND β-D-GLUCURONIDASE ARE ACTIVE ON BIOCHAR PARTICLES FROM AMENDED SOILS

ABSTRACT

We localized the presence of active alkaline phosphatase and β-D-glucuronidase on biochar particles from biochar-amended soil slurries using ELF (Enzyme-Labeled Fluorescence), a high-resolution, fluorescence-based enzyme imaging method. Unamended field soils were collected from experimental fields in Aurora, NY. Corn stover biochar was added to the field soils at 0.2% (g g^{-1}) or left unamended. Soils were incubated for four weeks on benches in a glasshouse prior to subsampling and analysis. Use of this protocol allowed us to observe the presence of exoenzymes and quantify their activity in sampled soils. We determined the kinetics of ELFA precipitation in biochar-amended and unamended soils over time and in relation to varying concentrations of initial substrate added. In addition, the suitability of ELF 97 phosphate (ELF-P) and ELF 97 β-D-glucuronide (ELF-G) for measuring exoenzyme activities in biochar-amended soils was tested by comparing the ELF results to classical measurements of bulk alkaline phosphatase activity using the fluorogenic substrates, 4-methylumbelliferyl phosphate (MUF-P) and 4-methylumbelliferyl-β-D-glucuronide (MUF-G). We observed activities of alkaline phosphatase and β-D-glucuronidase in both control and biochar-amended soil slurries, with the highest activity observed on the surfaces of the biochar particles. Biochar is a strong adsorbent, thus it is likely that either the substrates or the enzymes or both are being adsorbed on the biochar. The time-course of ELFA formation exhibited a lag period followed by a finite period of linear increase and then reached a plateau. We highlighted very subtle but important differences between the ELF and MUF methods

in determining the alkaline phosphatase and β-D-glucuronidase activities in biochar-amended soils. Both ELF-P and MUF-P hydrolysis rates increased with increasing concentrations of added substrates (ELF-P and MUF-P, respectively) with the exception of MUF-P hydrolysis rates in biochar-amended soil slurries at high initial MUF-P concentrations. The use of the ELF method allowed us to detect increased activity of alkaline phosphatase, but decreased activity of β-D-glucuronidase when the soil slurries contained biochar. The MUF method tended to underestimate the activity of alkaline phosphatase and β-D-glucuronidase in biochar-amended soil slurries when the initial MUF-P concentration was high. A correction strategy is needed when using a spectrofluorometric approach, such as MUF, to calculate exoenzyme activities, especially in strongly sorbing samples, such as those containing biochar.

INTRODUCTION

Intensive studies have been done on the use of biochar as a soil amendment strategy for improving soil health and better using natural resources (Thies and Suzuki, 2003; Young et al., 2005; Lehmann, 2007; Lehmann et al., 2007; Rondon et al., 2007; Laird, 2008; Thies and Rillig, 2009). Despite the potential for biochar to enhance soil fertilizer use efficiency, increase agricultural productivity and ecosystem stability (Asai et al., 2009; Bruun et al., 2009; Lehmann et al., 2009; Novak et al., 2009), surprisingly little is known about its effects on soil ecology, particularly on soil exoenzyme activities, which contribute substantially to the decomposition and nutrient cycling of complex substrates and associated nutrient elements (Gil-Sotres et al., 2005; Rejmánková and Sirova, 2007; Kuzyakov et al., 2009). Identifying the mechanisms involved in any changes in soil exoenzyme activity caused by amending soils with biochar may lead to a better understanding of any functional changes in the soil microbial community and associated nutrient transformation processes in response to

biochar soil amendment (Schimel and Weintraub, 2003; Allison and Vitousek, 2005; Enowashu et al., 2009). However, the heterogeneity of soil coupled with the strong adsorptive capacity of biochar for water, nutrients (Jackson and Barak, 2005; Amonette and Joseph, 2009), and even the microorganisms themselves (Jin and Thies, 2009), ensures that this will not be an easy task.

Reactions catalyzed by exoenzymes may be affected markedly by the adsorptive capacity of biochar. Enzymes sorbed to biochar surfaces may lose their catalytic activity or increase their efficiency if substrates are also sorbed and accessible. This is further complicated by findings that some microorganisms have the ability to access soil-adsorbed substrates directly and degrade them in the sorbed state (Feng et al., 2000; Park et al., 2002). Similarly, this suggests that bioavailability of adsorbed substrates is affected not only by their sorption but also by the characteristics of the exoenzymes or microbial cells in question.

Adapting techniques for use in localizing alkaline phosphatase and β-D-glucuronidase in biochar-amended soil slurries was a challenge. The presence of the exoenzymes phosphatase and β-glucuronidase in biochar-amended soils was determined using ELF technology, where weakly blue-fluorescent substrates form a bright yellow fluorescent precipitate upon enzymatic cleavage right at the site of activity (Nielsen et al., 2002; Kragelund et al., 2005; Kragelund et al., 2008). One of the greatest strengths of the ELF technology is that it allows us to directly mark the sites of enzyme action. The other remarkable strength of the ELF technology is that the high intensity and photo-stability of ELF alcohol precipitates overcome most of the difficulties associated with background autofluorescence from cells (Cox and Singer, 1999; Štrojsivá and Vrba, 2006; Štrojsová and Vrba, 2007). Other molecular methods, such as fluorescent *in situ* hybridization or immuno-fluorescence, require

many reaction steps. A biochemical indicator, such as the enzyme assay approach, is very straightforward and convenient, and comprises only one reaction step and a short staining time compared to immuno-labeling. In the ELF method, the novel soluble substrates, ELF 97 phosphate (ELF-P) and ELF 97 β-D-glucuronide (ELF-G) are available commercially. Upon hydrolysis by the corresponding enzyme, phosphatase and β-D-glucuronidase, respectively, the non-fluorescent substrate converts to fluorescent ELF 97 alcohol (ELFA), which is water-insoluble and forms a yellow-green precipitate at the site of enzymatic activity (Gonz âez-Gil et al., 1998; Xia et al., 2008), thus allowing for cellular or subcellular localization of the exoenzyme activity. The products of enzymatic cleavage can be counted for the intensity of ELFA positive dots or screened for the presence of ELFA fluorescence using epifluorescence (Gonz âez-Gil et al., 1998) or confocal (Dyhrman and Palenik, 1999) microscopy. Most frequently, the ELF method has been used to determine nutrient status in aquatic ecosystems, but has rarely been used in soils (Dyhrman and Palenik, 1999; Rengefors et al., 2001; Cao et al., 2005; Van Wambeke et al., 2008). On the other hand, ELFA precipitation is a complex process which may not necessarily be linear with time (Huang et al., 1992; Van Wambeke et al., 2008). Time-course information on ELFA fluorescence development is therefore crucial. Studies on the kinetics of ELFA-labeling are needed to obtain a linear time-course in product formation, which is generally required for reliable interpretation of data in terms of enzyme activities and to make sample comparisons more relevant.

The ability to localize active exoenzymes and microbial cells is important for understanding whether the enzymes phosphatase and β-D-glucuronidase are adsorbed to the biochar and soil matrix, and whether the adsorbed exoenzymes or cells are still active in cleaving substrates. However, no studies have yet been made to visualize the exoenzyme activity *in situ* in soil slurries. I aimed to:

(1) Localize the position of active alkaline phosphatase and β-D-glucuronidase enzymes in biochar-amended and unamended soil slurries; and

(2) Estimate changes in the activity of alkaline phosphatase and β-D-glucuronidase in response to amending soils with biochar.

MATERIALS AND METHODS

Sample Preparation

Soil was sampled from a corn field located at the Cornell Musgrave Farm, Aurora, NY, in April, 2009. This field was on Honeoye Silt Loam and had been in a corn-alfalfa rotation for past last three years. Soils are high-pH glacial tills (pH=8.0), and are representative of the highly productive soils of New York (with 3.0% organic matter). The sampled soil was air-dried and sieved through a 3 mm mesh screen. Then, part of the soil was mixed with corn stover biochar particles (BEST Energies, Inc., Somersby, NSW, Australia) at a rate of 0.2% (g g^{-1}), the remaining soil was left unamended. Pots (18 cm deep and 12 cm diam) were filled with unamended soil or the soil/biochar mixture to a weight of about 600 g. The bottoms of the pots were covered by nylon nets to allow drainage. Three replicates were set for each treatment. Two to three corn (Hybrid M1821, Cornell University) seedlings were transferred into each pot. Each pot was moistened and incubated in a glasshouse at Cornell University (Ithaca, NY, USA) where a 14 h photoperiod per 24 h was maintained. Temperatures were 20 ℃ during the light phase and 18 ℃ when dark, plants were grown for four weeks. Deionized water was added to each pot as needed during the course of the incubation. Three samples were collected from each pot with a sterile spatula in early May, 2009, and composited. The samples were transported to the laboratory immediately after sampling. Slurries were prepared from the soil samples by adding 60 ml distilled water to 3 g soil.

ELF Enzyme Assays

The ELF protocol was used to detect and localize ELFA-labeling of alkaline phosphatase and β-D-glucuronidase activity as described previously by Štrousiva and Vrba (2007). Each substrate (ELF-G: 2 mM; ELF-P: 5 mM, Invitrogen, Carlsbad, CA) was diluted with detection buffer to obtain concentrations of 50, 125, 312.5, 625, and 1000 μM and filtered through a 0.2 μm membrane filter. Four microliters of the filtered solutions were added, individually, to 50 μl of soil slurry in Eppendorf tubes, which yielded initial concentrations of 4, 10, 25, 50, and 80 μM of substrate, respectively. The experiment was run in triplicate for each initial substrate concentration and soil/enzyme combination. Incubation times were 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0 and 8.0 h for the initial substrate concentration of 10 μM for each substrate to determine an optimal incubation time that avoided insufficient ELFA labeling or over-ELFA labeling of background particles. The samples were incubated at 17 ℃ (the *in situ* temperature) for the incubation times given above. The incubation was terminated by adding 10 μl, 0.45 M phosphate-buffered saline (pH 6), containing formaldehyde solution (4% final concentration), to the reaction slurry. Five μl of the sample mix was spread out on a glass microscope slide and the characteristic patterns of ELFA labeling were visualized to localize the enzyme activity using a fluorescence microscope (Olympus BX61, Lombard, IL, USA) in the Life Sciences Core Laboratories Center, Cornell University, Ithaca, NY. The MetaMorph software package (Universal Imaging Corporation, Downingtown, PA, USA) was used to control image acquisition, processing and analysis. Enzyme hydrolysis of the ELF substrates results in the formation of a brightly fluorescent precipitate of ELFA where enzyme activity has occurred. I documented the locations and characteristic patterns of ELFA labeling by use of a digital camera (Olympus, Center Valley, PA) mounted onto the microscope and connected to a PC-based image analysis system (MetaMorph) [for

a detailed description see Nedoma et al. (2003)]; and counted the ELFA precipitate dots (total number of ELFA precipitate dots= number of ELFA precipitate dots per slide ×average of 200 views) for each soil/substrate combination at each incubation time.

MUF Enzyme Assays

Alkaline phosphatase and β-D-glucuronidase in sample soils were assayed fluorometrically using the substrates, 4-methylumbelliferyl phosphate (MUF-P, Sigma, St. Louis, MO) and 4-methylumbelliferyl- β-D-glucuronide (MUF-G, Gold Biotechnology, St. Louis, MO), according to the method described by Max et al. (2005). Sample suspensions were prepared by adding 5 g (ODW) soil in 40 ml of 50 mM, pH 5.0, acetate buffer and homogenizing for 1 min. Duplicate 4 ml aliquots of the soil slurry were transferred to autoclaved 10 ml serum vials containing stirring bars. The enzymatic reaction was started by adding 1 ml of substrate analogue solution to each slurry to yield final concentrations of 4, 10, 25, 50, and 80 μM for each substrate. Ten incubation time intervals were set, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0 and 8.0 h. During incubation, all slurries were stirred at 200 rpm and incubated at a temperature of 17 ℃ (the *in situ* temperature) for the incubation times given above. To measure the fluorescence caused by compounds coextracted from the soils, control and biochar-amended soils were incubated with no substrate added. Following incubation, samples were transferred to microcentrifuge tubes and centrifuged for 5 min at 10,000 ×g to remove soil and biochar particles, and 0.1 ml of 1.0 M NaOH was added to the soil-free supernatant to halt enzymatic activity and facilitate fluorescence detection. The concentrations of free dissolved fluorophores were determined by a SLM 8000 spectrofluorometer (Olis, Bogart, GA, USA) at an excitation wavelength of 370 nm and an emission wavelength of 450 nm. MUF standards in methanol at

concentrations of 0.1 to 0.7 μM were used for calibration and final calculations.

RESULTS

Localization of Enzyme Activities

We observed the activities of alkaline phosphatase and β-D-glucuronidase in biochar-amended soil slurries, particularly on the surfaces of biochar particles (Fig. 5.1A, B, D, E, I). Moreover, both enzymes investigated were also observed in the unamended soil slurries (Fig. 5.1C, F), where the soil particles were not always as recognizable as the biochar particles. Alkaline phosphatase activity was detected in both biochar-amended and unamended soil slurries. In addition, ELFA labeling was reproducible in both biochar-amended and unamended soil samples. Alkaline phosphatase activity was localized predominatly in association with biochar particle surfaces. This indicates that the alkaline phosphatase enzymatic reaction was most likely occurring in biofilms covering the biochar particle surfaces, or, directly on the biochar surface. Either the substrates or the enzymes or both are likely being adsorbed onto the biochar particles or are being held in the biofilm. In the control unamended samples, both alkaline phosphatase and β-D-glucuronidase activity were rarely detected (Fig. 5.1C, F).

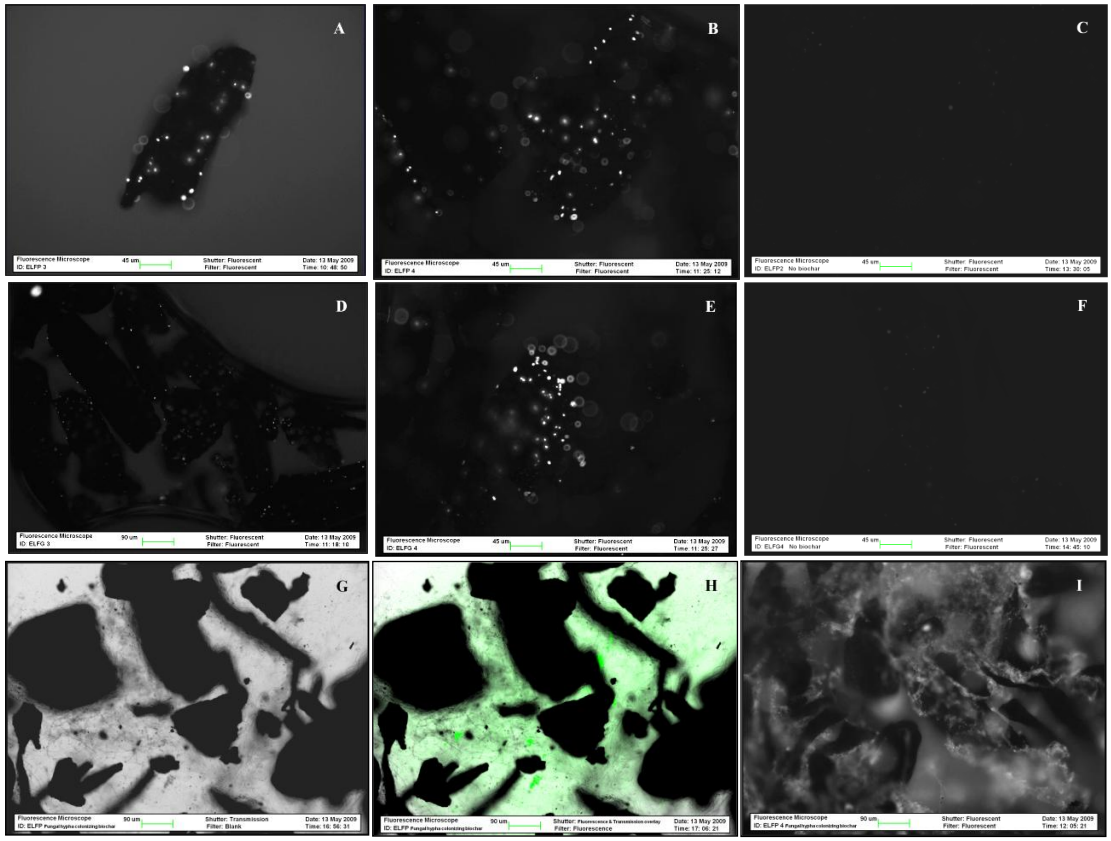

Figure 5.1 Enzyme activity on biochar particles in biochar-amended soils visualized by fluorescence microscopy. The images were taken by use of MetaMorph software. Scale bars for the respective fluorescence images are located at the bottom of the image. The bright dots in panels A, B, C, D, E, F, I indicate fluorescence of ELF alcohol precipitates (enzyme activity sites). The green color in panel H indicates fluorescence of ELF alcohol precipitates, which were obtained by overlaying the image using transmission shutter G and the image using the fluorescence shutter. Figures 1A-C show alkaline phosphatase activity sites: (A) bright-field image of alkaline phosphatase activity sites in biochar-amended soil slurries after incubating for 3 h; (B) bright-field image of alkaline phosphatase activity sites in biochar-amended soil slurries after incubating 4 h; (C) bright-field image of phosphatase activity sites in control soil slurries after incubating 2 h. Figure 1D, E, F localization of β-D-glucuronidase activity sites: (D) β-D-glucuronidase activity sites in biochar-amended soil slurries after incubating 3 h; (E) β-D-glucuronidase activity sites in biochar-amended soil slurries after incubating 4 h; (F) β-D-glucuronidase activity sites in control soil slurries after 4 h incubation. Panel G contains a fungal hypha colonizing biochar. Panel 1H shows the phosphatase sites demonstrated in green color using overlaying panel G with the fluorescence shutter. Panel 1I shows a fungal hypha colonizing biochar with bright field of phosphatase activity sites in the mycorhizosphere (surrounding biofilm).

Time Course of ELFA Labeling

Time-courses of ELFA formation exhibited a lag period followed by a finite period of linear increase and plateaued or decreased thereafter (Fig. 5.2A, B). The intensity of ELFA labeling in the soil slurries varied with incubation time. Incubations of <1.5 h were insufficient in most cases for detecting ELFA labeling. Incubations >6 h led to an increase in ELFA-labeled background particles that impeded proper localization of the enzymes in the biochar-amended soil slurries. Phosphatase and β-D-glucuronidase activities in the biochar-amended soils were detected primarily on the biochar particle surfaces (Fig. 5.1). No significant ELFA labeling was measured in the substrate minus controls. For both substrates, ELF-P and ELF-G, ELFA formation reached a maximum after about 6 h incubation.

For phosphatase activity, the intensity of positive ELFA-labeling in control soil slurries was low after 1.5 h incubation but increased with longer incubation times. In contrast, the intensity of positive ELFA-labeling in the biochar-amended soil slurries was higher than in control soil slurries after 1.5 h incubation but did not increase significantly in the following 2 h incubation (Fig. 5.2A).

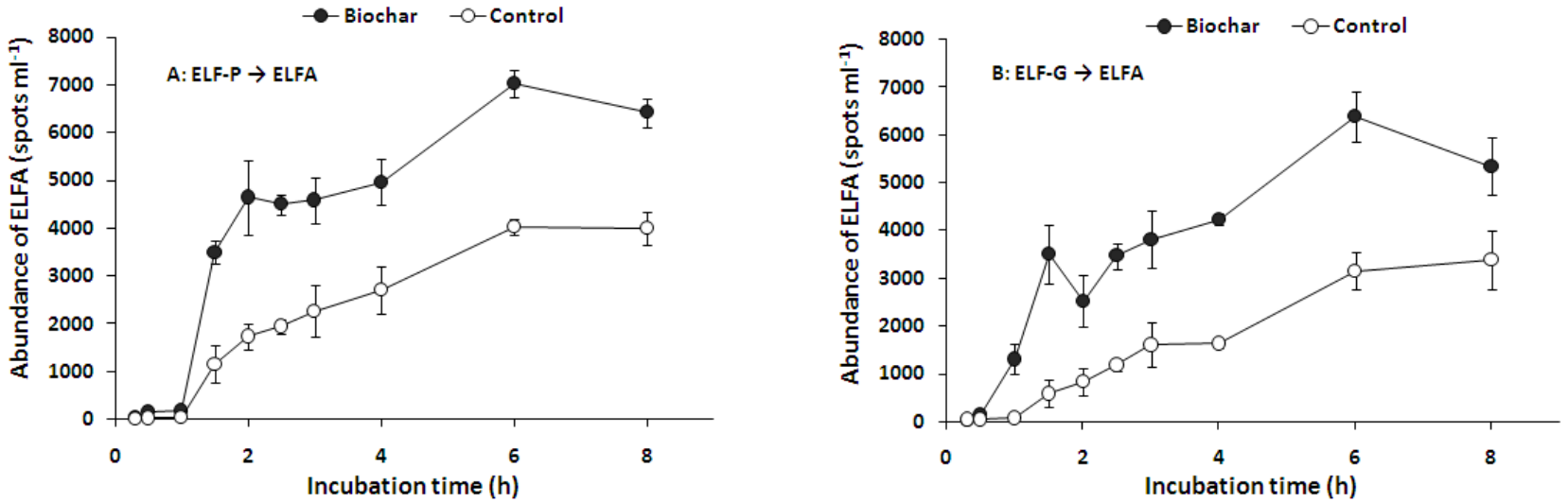

Figure 5.2 (A) Increased phosphatase activity and (B) β-D-glucuronidase activity in biochar-amended compared to unamended soil. Error bars represent ± standard deviation (n=3).

Time development of ELFA labeling varied similarly for β-D-glucuronidase activity, which was detected in all treatments (Fig. 5.2B). Control treatments had a lower intensity of enzyme-positive ELFA dots after the 1.5 h incubation, whereas higher intensity was observed after the 2 and 3 h incubations. Biochar-amended soil slurries had a different time course of ELFA labeling compared to that of the unamended treatments (Fig. 5.2B) in that biochar-amended soils had a faster increase during the linear phase of ELFA labeling than in control soil slurries. After 6 h incubation, β-D-glucuronidase positive ELFA labeling declined in biochar-amended soil slurries; a higher intensity of ELFA labeling was observed on biochar and/or soil particles than was free in the solution.

Comparison of Time Courses for ELF and MUF Formation

Similar kinetics of MUF formation over incubation time were observed in both biochar-amended and control soil slurries (Fig. 5.3A, B). Time-courses of MUF formation also exhibited lag periods when the change in MUF fluorescence was small or none, followed by periods of linear increases. Incubations of <1 h were, in most cases, insufficient to detect released MUF. We did not find a significantly different trend in MUF formation between the control and biochar-amended soil slurries within the first 3 h of incubation. After 4 h incubation, we found significantly higher MUF formation in the control soil slurry than in the biochar-amended soil slurry (Fig. 5.3A, B) and for longer incubation times (>4 h), the formation of MUF tended to be steady. This indicates that the substrate hydrolysis rates can be determined between 1.5-4 h incubations, e.g., the period of linear increase.

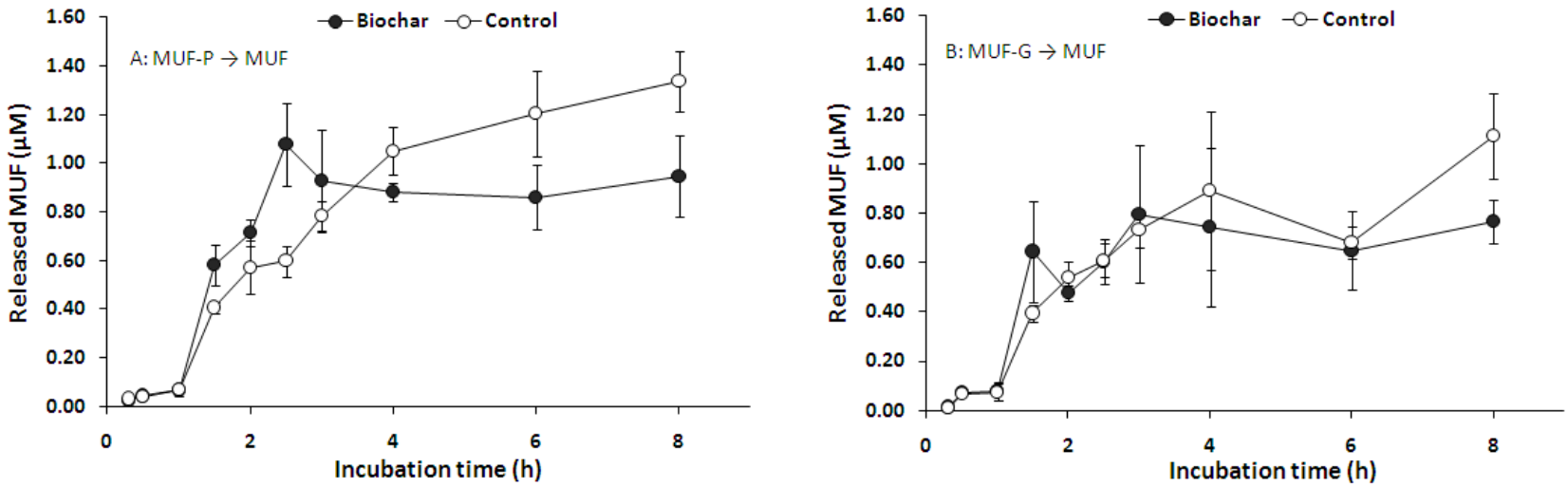

Figure 5.3 Increases in enzymatically released MUF over time for (A) phosphatase activity and (B) β-D-glucuronidase activity. Error bars represent ±standard deviation (n=3).

Phosphatase activity could not be reliably detected in biochar-amended soils using MUF-P as a substrate as no detectable increase in fluorescence occurred in the 3 h after MUF-P was added (Fig. 5.3A). In contrast, time courses of MUF formation were easily measured in control soils across incubation times (Fig. 5.3A). Time-courses of MUF formation resembled those measured for ELFA formation using the ELF method. Lag periods varied from 0 to 60 min (Fig. 5.2A and 5.3A). Periods of linear increase lasted 60-120 min (Fig. 5.2A and 5.3A).

ELF-based Alkaline Phosphatase and β-D-glucuronidase Activity Assays

The results of time course development of ELF labeling confirmed that 1.5-4 h incubation was generally sufficient to optimize ELFA labeling. Several incubation times, between 0.5 and 6 h, were used. Usually, no ELFA labeling developed in incubations <1.5 h; whereas, incubations >4 h led to an increase in ELFA-labeled background particles, which prevented proper localization of the enzyme activities in the soil slurries. Thus, we set the incubation time from 2 to 4 h in the linear increase phase and varied the concentration of initial substrates added. At each initial concentration of added substrate, the slope of the increase during the linear phase was used to calculate the ELF-P and ELF-G hydrolysis rates.

We found the exoenzyme hydrolysis rates varied with different initial concentrations of added substrates. Both ELF-P and ELF-G hydrolysis rates showed slightly increasing trends with the increasing concentration of added ELF-P and ELF-G, respectively. Variation around each curve was rather large, suggesting high variability from one sample to another. The range of concentrations tested was not always sufficient to obtain maximum hydrolysis rates (Nedoma and Vrba, 2006).

When initial concentrations of added ELF-P were larger than 25 μM, the ELF-P hydrolysis rates in both treatments were similiar (See solid lines in Fig. 5.4); whereas,

ELF-G hydrolysis rates were significantly higher in biochar-amended soils than in control soils regardless the initial concentration of ELF-G added. ELF-based exoenzyme β-D-glucuronidase activity was relatively stable in biochar-amended soil slurries when the initial concentration of added ELF-G ranged between 10-80 μM (See solid lines in Fig. 5.5).

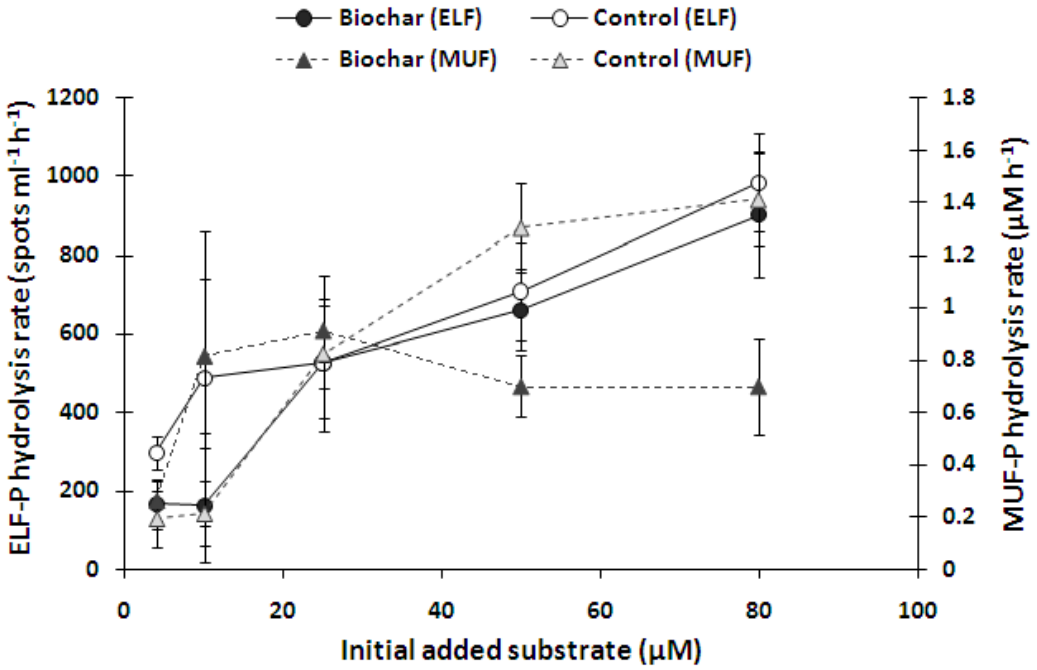

Figure 5.4 Enzyme activity curves showing the effect of the initial concentration of substrate added on ELF-P and MUF-P hydrolysis rates. ELF-P (counts of positive ELFA labeling over time) and MUF-P hydrolysis rate (detected by spectrofluorometry) were measurable in both biochar-amended and unamended soils. Error bars are ±standard deviation (n=3).

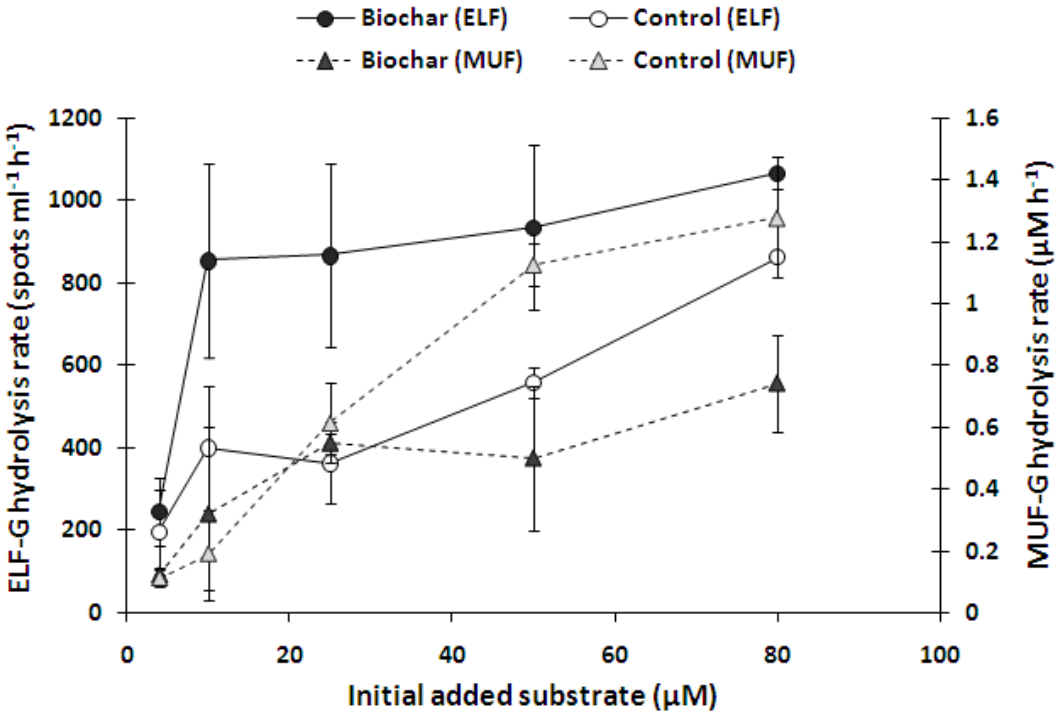

Figure 5.5 Enzyme activity curves showing the effect of the initial concentration of substrate added on ELF-G and MUF-G hydrolysis rates. ELF-G (counts of positive ELFA labeling over time) and MUF-G hydrolysis rates (detected by spectrofluorometry) were measurable in both biochar and unamended soils. Error bars are ±standard deviation (n=3).

Comparison of ELF-based and MUF-based Assays for Estimating Phosphatase and β-D-glucuronidase Activity

We compared phosphatase and β-D-glucuronidase activity measured by use of the ELF assay with the commonly used spectrofluorometric method employing 4-methyllumbelliferyl phosphate (4-MUP; Sigma) and 4-methyllumbelliferyl β-D-glucuronide, respectively, as the substrates and 4-methyllumbelliferon as the standard.

Phosphatase activity, calculated from the linear phase, was shown to vary from 0.1 to 1.7 µM h^{-1} (See dotted lines in Fig. 5.4). The phosphatase activity in biochar-amended soils obtained using MUF-P as substrate was not consistent with the data obtained using ELF-P as substrate. The spectrofluorometric method did not detect any increasing trend with the increasing initial substrate concentrations. This was probably

due to the underestimation of the MUF being released in biochar-amended soils wherein the MUF molecule was likely sorbed strongly on biochar particles (Chapter 4). A correction strategy will be needed for more accurate spectrofluorometric determinations of phosphatase activity, especially in samples that have strong adsorption properties, such as biochar.

The β-D-glucuronidase activity in biochar-amended soils was higher than that in the control soils detected using the ELF counting method but when MUF-G was used as a substrate in parallel incubations, control soils exhibited higher counts (Fig. 5.5). Therefore, the activity of β-D-glucuronidase in biochar-amended soils was significantly underestimated using the MUF method. However, for the β-D-glucuronidase activity in control soils, the effect of substrate concentration on MUF-G hydrolysis rate followed a trend similar to that obtained using ELF-G as a substrate.

Using β-D-glucuronidase ELF-labeling, a much higher hydrolysis rate was observed in biochar-amended soil slurries compared to the control soils; however, the MUF-based assay yielded much lower MUF-labeling intensity in the biochar-amended soils than in the control soils. This suggests that the MUF method underestimated MUF-G hydrolysis in the biochar-amended soils.

DISCUSSION

Localization of Alkaline Phosphatase and β-D-glucuronidase Activities

A representative view of the localization of ELFA precipitates on biochar particles and across a fungal hypha colonizing biochar particles are shown in Fig. 5.1. The microscope image indicated that alkaline phosphatase and β-D-glucuronidase activities were localized on biochar particles. This suggests that either the enzymes are adsorbed to biochar surfaces while maintaining their catalytic activity; or, substrates

are co-located on biochar particles, which increased enzyme access to the substrates. I found that both the the alkaline phosphatase and β-D-glucuronidase activities occurred mainly on biochar particles, which are also shown to have significant fungal colonization. This indicates that biochar surfaces may be the main site of P and C transformation via fungal colonization, as has been suggested previously (Warnock et al., 2007), in that biochar has a stimulating effect on fungal colonization. Production and activity of exoenzymes in the soils is likely community specific, as suggested by previous studies on natural populations of plankton, where exoenzyme activities in slurries were detected for only a few species (Rengefors et al., 2003; Cao et al., 2005; Van Aarle et al., 2007). ELFA labeling in the soil slurries likely results from enzyme activity associated with live microbial cells because: (1) microbial cells are colonizing biofilms on biochar porous structures; and, (2) the bound cells could be still be actively hydrolyzing substrates. The assumption that biochar is a good habitat for microbes was confirmed by the high intensity of ELFA labeling found particularly on biochar particles in the majority of experiments. Most frequently, the ELF method has been used to localize exoenzyme activity in aquatic ecosystems or plant tissues or fungi in pure culture, but has rarely been used in soils (Dyhrman and Palenik, 1999; Rengefors et al., 2001; Van Aarle et al., 2002; Cao et al., 2005; Van Wambeke et al., 2008). My study showed that it is also possible to use the ELF method to visualize the exoenzyme activity *in situ* in soil slurries and on biochar particles.

Effect of Biochar on Alkaline Phosphatase and β-D-glucuronidase Activities

We detected alkaline phosphatase and β-D-glucuronidase activity in unamended control and biochar-amended soils, particularly on biochar particles, but less observed activity in the unamended soils. Changes in ELFA spot abundances over time in the incubations for both ELF-P and ELF-G hydrolysis confirmed the presence of lag,

linear and plateau phases that were apparent in both biochar-amended and unamended soils (Fig. 5.2A, B). This lag time was also reported for freshwater phytoplankton samples (Nedoma et al., 2003; Dignum et al., 2004; Duhamel et al., 2009).

The role of enzyme activity, particularly phosphatase activity in soil slurries has been well studied (Sardans et al., 2008; Ge et al., 2009) and the enzymes are produced under particular stimulating conditions, such as when P supply is limited. Pioneering studies showed microorganisms can change their enzyme-secreting behavior under different conditions (Nannipieri et al., 2002; Schimel and Weintraub, 2003). In the present case, for the assay on phosphatase activity, I showed that ELFA labeling was higher in biochar-amended soil slurries than that in unamended soil slurries. This suggests that, in response to adding fresh biochar to soil, (1) microbial demand for P increased or, (2) soil P availability became more limiting during the four week incubation, or a combination thereof. A fungal hypha colonizing biochar was observed to have a cloud of active phosphatase activity sites in the biofilm surrounding it, suggesting that demand for P may be increased in the biochar surface environment and that biochar had a stimulating effect on fungal colonization as suggested by Warnock et al. (2007). Furthermore, the ELF-based β-D-glucuronidase activity was significantly increased in biochar-amended soils. This suggests that biochar amendment increased microbial demand for simple carbon and nitrogen containing substrates. Soil enzyme analyses to detect alkaline phosphatase and β-D-glucuronidase activity provided information about the nutrient status of the environment and the increased microbial nutrient demand with respect to inorganic phosphate and labile carbon and nitrogen in response to amending soil with biochar.

Considering the different initial substrate concentrations, it is important to say that there were no fundamental differences in the two exoenzyme activities in

response to biochar addition. With respect to initial substrate concentrations, there were changes in the enzyme activities. This observation supports the supposition that microbes adapt to different levels of substrates during their growth by changing the number of exoenzymes they produce. In other words, microbial populations selectively express substrate-sensitive enzymes in response to the metabolic demand for nutrients relative to their availability in the soil solution.

The range of initial substrate concentrations (5-80 μM) tested was not always sufficient to reach maximum hydrolysis rates, or multiphasic kinetics were present (Hoppe, 2003; Nedoma and Vrba, 2006; Kim et al., 2007; Senjarini et al., 2009). In the MUF-based assay, longer incubation times did not result in higher intensities of MUF released in the biochar–amended soil slurries. On the other hand, the intensity of MUF released in the control soil slurries increased with longer incubation times. The decrease in MUF detectable over time in the biochar-amended soil slurry was most likely due to a lower equlibrium concentration of MUF in the solution phase due to the strong adsorptive properties of biochar.

ELF-based vs. MUF-based Enzyme Activity Assays

In this study, alkaline phosphatase and β-D-glucuronidase activities were determined by both conventional (MUF-based) and a new *in situ* technique (ELF-based). However, the results from the two methods were not consistent with each other, particulary for the β-D-glucuronidase assay (Fig. 5.3). This is likely due to the different limitations of the two methods. I found that the MUF method tended to underestimate enzyme activities likely due to adsorption of the fluorophores to biochar, thus lowering their extraction efficiency. In the ELF-based method, enzyme activity sites may become covered by either biochar particles or soil constituents and thus remain uncounted in the two-dimentional view. But, because the ELF-based

method showed enzyme activities to mainly be distributed on the surface of biochar, the frequency of the accessible activity sites on biochar particles represents an approximation of the overall changes in enzyme activity in the presence of biochar. Van Aarle and Plassard (2010) quantified phosphatase activity of an ectomycorrhizal fungus in association with its host plant. Their results showed the vast majority of phosphatase activity was detected on the surface-bound to plant roots. This is consistent with my finding that enzyme activities are mainly distributed on the surface of biochar.

In contrast to previous studies (Dignum et al., 2004; Van Wambeke et al., 2008), the sample slurries were not concentrated prior to the enzyme assay, which was an improvement in the present work. The concentration step was necessary in the previous studies to obtain a sufficiently high concentration of the enzyme targeted for ELFA labeling in the slurry sample; however, this could affect enzyme-secreting behavior because both some classes of phosphatase and β-D-glucuronidase are inducible and their activity can be discouraged in the presence of high substrate concentrations. Surprisingly, in our study, the enzyme activity in soil slurry samples was sufficiently high for ELFA-labeling to occur without a pre-concentration step. Many tools for assessing soil exoenzyme activities have been used (Gerlach et al., 2006; Vahl et al., 2008; Creamer et al., 2009), but most of these standard methods are likely to be limited by the strong adsorption of extractants to biochar and soil. The ELF technique does not have these limitations. My results showed that the ELF method is suitable for the direct localization and detection of soil exoenzymes and offers new possibilities for further research on the fate of biochar added to soils.

CONCLUSIONS

Alkaline phosphatase and β-D-glucuronidase were active in biochar-amended soils, particularly, on the biochar particles. There is a need for more research on the available P, labile C and physiological conditions in biochar-amended and unamended soils. This will allow us to link enzyme activities more closely with substrate availability in response to biochar amendment. I found an increase in microbial P and C demand in biochar-amended soil using the ELF-based approach. Apparent P demand was high, relative to C demand, when cell requirements for C relative to P are considered. Yet, the higher β-D-glucuronidase activity observed in this study was not consistent with the findings on enzyme activities reported in Chapter 4. This is most likely related to the fact that the current experiment was carried out using fresh instead of aged biochar and incubated for only four weeks in a glasshouse. Fresh biochar will likely stimulate the activity of C mineralizing exoenzymes in the first few weeks after being added to soil as any bio-oils deposited on the biochar surface during pyrolysis are metabolized. Changes in enzyme activities as biochar ages in soil deserve further investigation. We also found that the classical measurements of exoenzyme activity based on MUF analysis and ELF-based analysis have their strengths and limitations. Thus, we suggest that it may be possible to use the MUF-based protocol, if it is corrected for the effect of biochar adsorption of the fluorophores on activities measured. Use of the ELF-based protocol is best to localize in situ enzyme activity, especially in samples containing adsorbents.

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CHAPTER 6

MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF FUNGAL COMMUNITIES COLONIZING BIOCHAR-AMENDED SOILS

ABSTRACT

We examined fungal communities living in close association with biochar particles in biochar-amended soils. Soils sampled from a corn field at the Cornell Musgrave Farm, Aurora, NY, were mixed with corn stover biochar particles at a rate of 0.2% (g g^{-1}), and incubated in a glasshouse for four weeks. Under a scanning electron microscope (SEM), microorganisms were observed to form biofilms with diverse, visible structures. Bacterial cocci and bacilli and thread-like fungal hyphae were visible either on or in the biochar porous structure. Cloning and sequencing of the fungal ITS region amplified from soil community DNA extracted from biochar-amended soil samples revealed the presence of a complex fungal community. Clones were assigned to 19 classes and 8 phyla with high sequence similarity (average > 95%) to known sequences in the NCBI database; in addition, there was a substantial number of unidentified fungal clones. Over 70% of the sequences obtained were classified as *Ascomycota*, *Basidiomycota* or *Zygomycota*. However, the relative gene frequency of the main phylotypes detected differed between biochar-amended and unamended soils; with a less genetically diverse community found in the biochar-amended soils. Biochar-amended soils had 98.8% more fungi classified as *Zygomycota* and nine-fold more unidentified fungal clones, while also having 31.2% lower abundance of *Basidiomycota* and 37.2% lower abundance of *Ascomycota* than unamended control soils. Lack of available carbon in and around biochar particles may be discouraging colonization by higher order saprophytic fungi. These findings suggest that fungal communities living in association with biochar are likely

functionally as well as taxonomically different from those dominating in unamended soil. Changes we observed in the structure of fungal populations in biochar-amended soils may help to explain the observed long-term preservation of C in other soils high in black carbon (e.g., Terra Preta soils of Brazil). How observed changes in fungal communities may alter soil function needs further study.

INTRODUCTION

Soil microbial community response to biochar amendment is an area of active study (Steiner et al., 2004; Warnock et al., 2007; O'Neill et al., 2009; Thies and Rillig, 2009). Fungal communities, in particular, appear to be influenced strongly by the presence of biochar in soil (Chapter 3). Soil is a complex environment in which biological activity is governed primarily by microorganisms. The microbial community in soil is normally very complex. It consists of hydrolytic, fermenting, respiring and syntrophic microorganisms that have diverse functions in organic matter turnover and nutrient transformations (Nannipieri et al., 2003). The beneficial effects of soil microorganisms, particularly fungi, include decomposition of organic matter and agrochemicals, and enhancing the bioavailability of nitrates, sulfates, phosphates and essential metals; thus, the activities of soil fungi are fundamental to proper functioning of the soil ecosystem (Rillig and Mummey, 2006; Paul, 2007). Knowledge of the structural interactions of fungi with biochar and changes induced in fungal communities by the presence of biochar are needed to better understand the how fungal roles might change in biochar-amended soils and how this might influence important soil functions.

Biochar-induced changes in organic matter turnover, nutrient transformations, carbon (C) sequestration and greenhouse gas (GHG) emissions are accompanied by clear changes in the microbial community composition and activity (Chapter 2-5).

Amending soils with biochar results in changes in soil nutrient availability (Glaser et al., 2002; Lehmann et al., 2003), cation exchange capacity (CEC, Liang et al., 2006) and C retention. It also enhances nitrogen fixation (Rondon et al., 2007) and mycorrhizal associations (Warnock et al., 2007) and engenders gross changes in bacterial and fungal communities (Chapter 3). Several factors could influence fungal species diversity in biochar-amended soils, as species differ in sensitivity to environmental factors such as pH, mineral solubility, soil moisture and organic matter content, or predation (Toberman et al., 2008; Rousk et al., 2009; Pena et al., 2010; Theuerl and Buscot, 2010).

Biochar particles have an extremely high surface area, a highly aromatic, recalcitrant structure, and their surfaces undergo decomposition and oxidation slowly over time (Lehmann et al., 2009). Some fungi are physiologically adapted to degrade carbon-rich, recalcitrant materials (Rinnan and Baath, 2009) and their branching morphology may allow them to ramify through the porous structure of biochar thus increasing surface nutrient exchange. With its increased surface area and microporous structure, biochar itself may serve as an ideal habitat for microbes (Thies and Rillig, 2009). For filamentous fungi, both mycelial growth and sporulation could be stimulated by adding biochar to soil. Plant roots also proliferate in the presence of biochar, possibly enhancing the interaction between roots and important fungal symbionts, such as arbuscular mycorrhizal fungi (AMF; Warnock et al., 2007). Together with differences in soil texture and the resulting effects on soil moisture, the distinct spatial heterogeneity in the biochar-amended soil may alter microbial community composition. The relative abundance of nutrients and the high C:N ratio indicate that N dynamics in biochar-amended soil will likely differ significantly from those in unamended control soils. Indeed, biochar introduced into soil has been shown to increase nitrification and biological nitrogen fixation (Rondon et al., 2007; DeLuca

et al., 2009). The presence of biochar and resultant edaphic effects likely change the factors driving competition in biochar-amended soil microbial communities. Recently, biochar-enriched Anthrosols (terra preta) were characterized by comparative sequence analysis of 16S rRNA genes of bacteria cultured from these soils (O'Neill et al., 2009). This analysis demonstrated that the Anthrosols contained a higher number of culturable bacteria and a greater diversity of isolates compared to adjacent, unmodified soils. However, microbial community structure and diversity changes caused by amending soils with biochar still remain poorly understood. This is particularly true for fungal communities, whose population structure and diversity is crucially important to nutrient cycling and C stability, particularly in agricultural soils.

I used scanning electron microscopy (SEM) in this study to visualize the association of microbial cells with biochar surfaces. Use of fluorescence staining and microscopy to examine microbes colonizing biochar is difficult due to the strong affinity of biochar for biological strains (Li et al., 2004). SEM allowed us to examine microbes associated with biochar surfaces after incubating biochar particles in soil. The main advantage of the SEM technique is that the material is observed in an immediately frozen state and does not involve chemical fixation and thus gives us a view of colonizing microorganisms with as little distortion as possible (Wang et al., 2009). Use of SEM has provided new understanding about the growth and activities of microorganisms in soil and has been employed successfully in studying associations between clay and microbial polymers in soil (Horath et al., 2006; Priester et al., 2007; Chenu and Tessier, 1995). However, care must be taken when preparing the samples, especially during sublimation. If this step is carried out for too long, freeze-drying of the sample occurs that results in shrinkage.

Cultivation methods have contributed substantially to our present knowledge of

the biochemistry and functional significance of soil microorganisms. However, it is well established that a majority of soil organisms are difficult or impossible to grow on laboratory media (Paul, 2007); i.e., only a small fraction of microorganisms have been cultivated by standard methods. Thus, cultivation methods provide a biased view of soil microbial abundance, diversity and community composition. The limitations of culturing methods can be overcome by the use of molecular approaches, which have greatly facilitated the identification of an increasing number of soil organisms (Mahmood et al., 2005; Singh et al., 2006; Thies, 2007). The analysis of 16S or 18S rRNA genes amplified from environmental DNA has expanded our view of microbial diversity in soil in recent years and has proven to be a powerful tool for investigating the microbial diversity in a wide range of environmental samples (Dedysh et al., 2006; Morales et al., 2009; Peay et al., 2009). Active soil fungi have clear functional significance in soil (Paul, 2007). The soil fungal community often better reflects substrate quality and availability than analyses of the bacterial community. Fungal community diversity in biochar-amended soils should provide significant information on the potential ecological roles of different fungal species or functional groups in biochar-amended soils.

In this work, the fungal community living in close association with biochar in biochar-amended soils was characterized. I visualized the microorganisms colonizing biochar particles and assessed the community structure and diversity of fungi present in biochar-amended compared to unamended soils.

MATERIALS AND METHODS

Collection and Preparation of Samples

Soils were sampled from a corn field on the Cornell Musgrave Farm, Aurora, NY, in April, 2009. Part of each soil sample was mixed with corn stover biochar at a

rate of 0.2% (g g^{-1}). The biochar was produced by carbonizing corn stover at 600 ℃ under slow pyrolysis (BEST Energies, Inc., Sommersby, NSW, Australia). The remaining soil was left unamended. Three replicates were set for each treatment. Soil was placed in pots and the moisture content adjusted to field capacity. Two or three corn seeds were sown in each pot. Pots were incubated in a glasshouse at Cornell University, Ithaca, NY, for four weeks. Water was added to the pots during the course of incubation as needed. Mixed samples from the incubated pots were collected with a sterile spatula in early May, 2009. The samples were transported to the laboratory immediately after sampling. Multiple biochar particles were hand-picked from the biochar-amended soil using forceps.

Scanning Electron Microscopy

In order to preserve the original structure of the sample, I used a scanning electron microscope equipped with a low temperature system (Chenu et al., 2001). A segment of the picked biochar aggregate (about 0.5×0.5×0.5 cm volume) was gently mounted on the metal stubs of a sample holder using forceps. The sample was then freeze-dried by immersion in a liquid nitrogen slush to bring the temperature down to -210 ℃. The sample was then transferred under vacuum to the preservation chamber. After freeze-drying, a small amount of colloidal silver adhesive paste (Electron Microscopy Sciences, PA, USA) was introduced from near the bottom of the sample to bind the sample to the sample holder, but was not allowed to reach the sample surface. Samples were then sputter-coated with a thin layer of gold and the biochar surface examined using LEO 1550 FESEM (Keck SEM) Scanning Electron Microscope (LEO Electronic Microscopy Inc., Thornwood, NY) at the Cornell Center for Materials Research, Cornell University, Ithaca, NY, USA.

DNA Extraction and PCR Amplification

The MoBio Powersoil^{TM} soil DNA extraction kit (Cambio, Cambridge, UK) was used to extract genomic DNA from biochar-amended and unamended soil samples. DNA was extracted from 500 mg of soil according to the manufacturer's instructions. Extracted DNA was either secondarily purified using a PowerClean^{TM} DNA Clean-Up Kit (Qiagen, Valencia, CA) or purified on a 1.0% (w v^{-1}) agarose gel followed by DNA recovery and elution (Qiagen Gel Purification kit). Purified DNA was used as a template for the PCR amplification of the fungal ITS region with the primers ITS1 and ITS2 (Kumar and Shukla, 2005). The ITS region lies between the 18S and 25S rRNA genes (250 bp of DNA comprising ITS1, the 5.8S rRNA gene, and ITS2) (Fig. 6.1). The oligonucleotide primer set was synthesized by Integrated DNA Technologies (IDT, IA, USA). The primer sequences and target are given in Table 6.1.

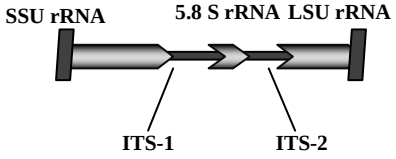


Figure 6.1 Fungal primers ITS 1 and ITS 2 cover the ITS region.

Table 6.1 Primer sets used in PCR amplification of the fungal ITS region (Kumar and Shukla, 2005)

Primer Pairs	Sequence 5'->3'	Target Gene	Amplicon size (bp)
ITS1	TCCGTAGGTGAACCTGCGG	ITS1, 5.8S rRNA, ITS2	~ 250
ITS2	GCTGCGTTCTTCATCGATGC		

Sample DNA (30 to 50 ng) was amplified in a 50 µl reaction cocktail containing (final concentrations), 1×*Pfx* buffer (Invitrogen, San Diego, CA, USA), 1.5 mM $MgSO_4$, 300 µM each dNTP (deoxynucleotide triphosphate), 0.3 µM each of the forward and reverse primers, and 1.0 U of *Pfx* polymerase (Invitrogen). Amplification

was performed in a thermal cycler (PTC-100, MJ Research, Watertown, MA, USA, programmed for an initial denaturing step at 94 ℃ for 5 min followed by 40 cycles of 94 ℃ for 30 s, 50 ℃ for 1min and 68 ℃ for 1 min with a final extension step at 68 ℃ for 3 min. Gradient PCR was adopted to empirically determine the optimum annealing temperature for the fungal ITS primer sets. Cycling parameters were tested starting with 30 through 45 cycles at 5-cycle increments to address the bias often associated with amplification of mixed templates. Forty cycles were found to be optimal in order to reproducibly and consistently amplify the representative communities. Since the proofreading activity of *Pfx* DNA polymerase degrades the A overhangs, PCR products were reacted with 3'A to create the blunt ends needed for TA cloning.

Clone Library Construction and Sequencing

Amplified PCR products with blunt ends were purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The purified PCR products were then cloned using a TOPO TA cloning kit (Invitrogen) in accordance with the manufacturer's instructions. The presence of inserts of the expected size was analyzed by direct PCR screening of 300 to 400 transformants. A small part of each transformed colony was amplified by PCR using the plasmid-specific primers M13F and M13R. The size of the inserts was checked by electrophoresis on a 1% (w v^{-1}) agarose gel. About 200 randomly selected colonies with the right sized insert were chosen for sequencing. ExoSAP-IT (USB, Cleveland, OH, USA) was used to clean the DNA amplicons from the chosen colonies. The sequences were determined on an ABI 3730X1 Automated Sequencer (Applied Biosystems, Foster City, CA, USA) at the Cornell Life Sciences Support Center, Ithaca, NY, USA.

Nucleotide Sequence Accession Numbers

The ITS region gene sequences of fungal clones were deposited in the GenBank nucleotide sequence database under accession numbers GU461322 to GU461586.

Phylogenetic Analyses

Retrieved sequences were submitted to the CHECK_CHIMERA program at the Ribosomal Database Project II (RDPII) (Cole et al., 2003) to detect the presence of chimeric artifacts. Sequence alignment, as well as phylogenetic and molecular evolutionary analyses, were carried out using MEGA version 3.1 (Kumar et al., 2004) with the NCBI database, using the Jukes–Cantor neighbor-joining algorithm. High similarity (average > 95%) of the cloned ITS region DNA sequences with those from environmental and/or cultivated members of the fungi deposited in NCBI database were used to infer the phylogenetic position of the sequences retrieved from biochar-amended and unamended soils. Distance matrices were constructed by the DNADIST program in PHYLIP (Felsenstein, 1989). Relatedness of clone libraries between paired soils were compared using ∫-LIBSHUFF (Schloss and Handelsman, 2005) and rarefaction curves, diversity indices, and lineage through time were evaluated using DOTUR (Schloss and Handelsman, 2005).

RESULTS

SEM Imaging of Bacterial Cells and Fungal Hyphae on Biochar Particles Picked from Biochar-amended Soils

Scanning electron microscopy was performed on biochar particles picked out of the biochar-amended soils. The biochar particle surfaces, viewed by SEM under varying magnification, are shown in Fig. 6.2.

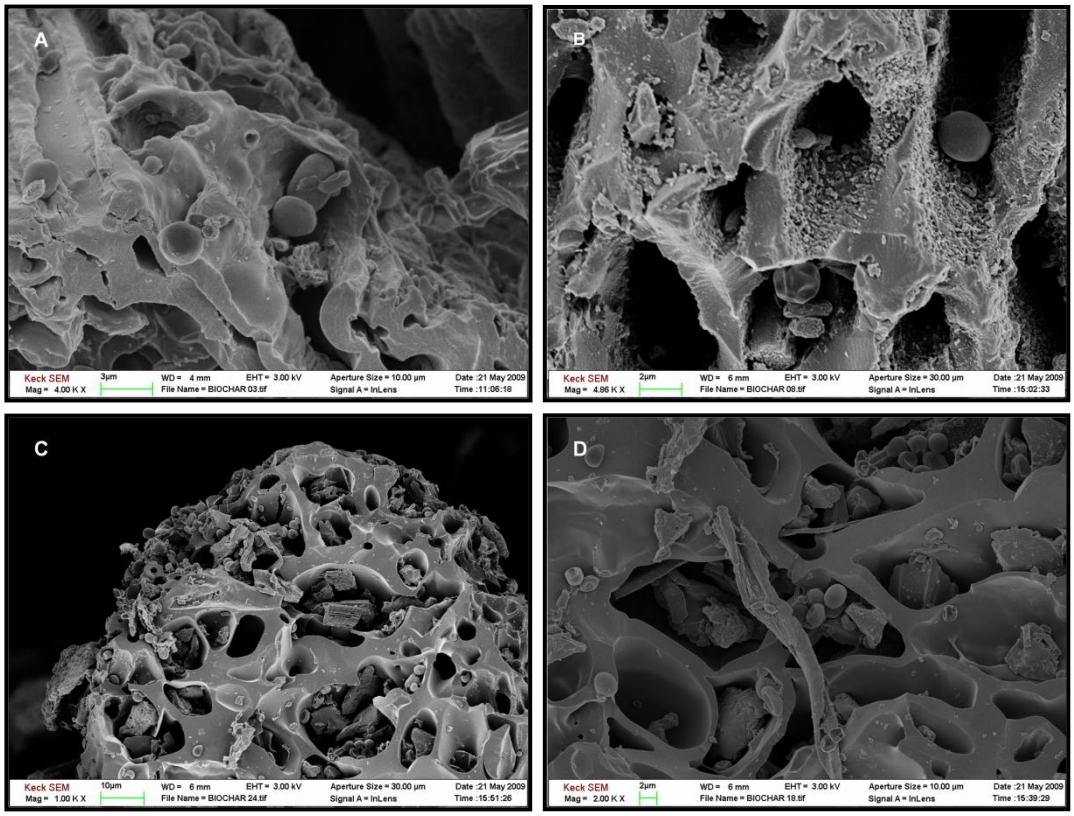


Figure 6.2 Scanning electron micrograph A-D. Microbial cells present on the biochar surface and in biochar pores, with overview in panel C, and detailed views in panels A, B, and D. Scale bars are shown underneath each image.

Most of the bacteria observed were cocci or bacilli that varied in size. Many cell aggregates, single-celled bacteria or archaea and possibly fungal hyphae were observed on biochar particles (Fig. 6.2). Cells were located either on the biochar surface or scattered in the biochar pores. Biofilms were not observed likely because of their dispersion during sample preparation.

DNA Extraction and Amplification

Total community genomic DNA was extracted successfully from control and biochar-amended soil samples. Due to the complexity of the soil samples, however, DNA extracts could not be amplified using the high fidelity *Pfx* polymerase; and, therefore, double purification of extracted DNA was needed to successfully PCR amplify the fungal ITS region.

Table 6.2 Results of sequence analysis and presumptive phylogenetic affiliations of fungal clones

Taxon	No. of clones in soil sample					
	Control			Biochar		
	Class sum	Phylum sum	Phylum %	Class sum	Phylum sum	Phylum %
Ascomycota › Mitosporic Ascomycota	19	86	48.9	2	27	30.7
Ascomycota › Pezizomycotina	11			3		
Ascomycota › Sordariomycetes	5			4		
Ascomycota › Dothideomycetes	27			8		
Ascomycota › Eurotiomycetes	17			10		
Ascomycota › Leotiomycetes	4			0		
Ascomycota › Dokmaia	1			0		
Ascomycota › Unidentified ascomycetes	2			0		
Basidiomycota › Agaricomycotina	34	35	19.9	11	11	12.5
Basidiomycota › Pucciniomycotina	1			0		
Zygomycota › unclassified zygomycetes	4	14	8.0	1	14	15.9
Zygomycota › Entomophthoromycotina	2			0		
Zygomycota › Mucoromycotina	8			13		
Glomeromycota › Glomeromycetes	1	1	0.6	3	3	3.4
Blastocladiomycota › Blastocladiomycetes	1	1	0.6	0	0	0.0
Chytridiomycota › Chytridiomycetes	0	0	0.0	5	5	5.7
Neocallimastigomycota › Neocallimastigomycetes	0	0	0.0	2	2	2.3
Unidentified soil fungus clone	4	4	2.3	21	21	23.9
Viridiplantae › Streptophyta	8	35	19.9	4	5	5.7
Viridiplantae › Chlorophyta	27			1		

Cloning and Phylogenetic Grouping of Cloned Sequences

Fungal ITS region sequences were obtained after the construction of clone libraries. All colonies were checked for the presence of inserts by PCR amplification of the ITS region followed by agarose gel electrophoresis. Sequencing of roughly 200 clones from each soil sample resulted in 176 successful sequencing results which contained inserts of the correct size from the unamended control soils and 88 from the biochar-amended soils. Phylogenetic analysis of fungal ITS sequences revealed the presence of a complex fungal population structure (Table 6.2). Based on these short sequences (approximately 250 bp), clones were assigned to fungal phylotypes with high similarity (average > 95%) by BLAST searching in the NCBI database. The high similarity of the cloned sequences to those in the NCBI database enabled identification to the class level in many cases. After BLAST searching, clones were assigned to 19 classes and 8 phyla with high sequence similarity (average >95%) to known sequences in the database (Table 6.2). In addition, there was a high proportion of unidentified fungal clones (23.9% and 2.3% in biochar-amended and unamended control soils, respectively; Table 6.2). Of the 19 fungal classes identified, 17 were represented in the cloned sequences from the control soil compared to only 13 classes represented in the cloned sequences from the biochar-amended soils, with 11 classes occurring in both soils. Over 70% of the sequences obtained were classified as *Ascomycota*, *Basidiomycota* or *Zygomycota* (Fig. 6.3A). There were 4 cloned sequences from the unamended soils and 21 cloned sequences from biochar-amended soils that did not group with any known phyla in the NCBI database.

Pattern of Distribution of Cloned Sequences

The gene frequency of the major phylotypes (*Ascomycota*, *Basidiomycota* and *Zygomycota*) and the minor phylotypes detected differed between unamended control

and biochar-amended soils. The proportional representation of the *Ascomycota* and *Basidiomycota* was 1.6 times higher in control soils; whereas, the proportional representation of the *Zygomycota* in biochar-amended soils, was twice that recovered from the unamended soils (Fig. 6.3). *Ascomycota* dominated the fungal populations identified in both control and biochar-amended soils. *Chytridiomycetes* and *Neocallimastigomycetes* were detected only in biochar-amended soils; whereas, *Leotiomycetes*, *Dokmaia*, *Pucciniomycotina* and *Blastocladiomycetes* were detected only in unamended control soils (Fig. 6.3).

The unidentified fungal ITS sequences did not show extensive similarity to any identified ITS gene sequences deposited in the NCBI database. There were 10.5 times more unidentified fungi clones from the biochar-amended soils as compared to unamended soils (Fig. 6.3). However, BLAST searching confirmed that these sequences did have high similarity to sequences from uncultured fungi deposited in the database by other researchers.

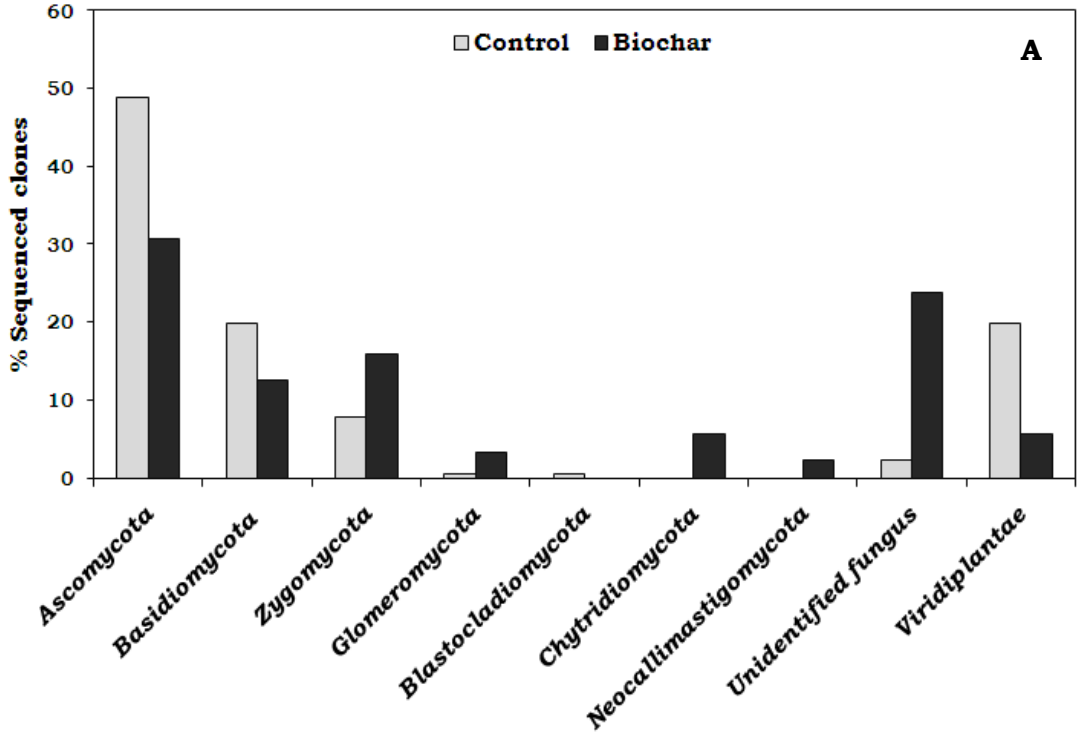
Statistical Comparison of Clone Libraries

Rarefaction is used to estimate the number of species expected in a random sample of individuals taken from a collection (Foote, 1992). I applied rarefaction analysis to the two clone libraries. The analysis of 30 clones drawn from each library appeared to be sufficient to detect the divergence of the number of the operational taxonomic units (OTUs) between the two soil samples (Fig. 6.3B) ($p<0.05$). Comparison of the two clone libraries was carried out using ∫-LIBSHUFF to measure genetic distances in library coverage. In this analysis, a library, C_X, is analyzed for singleton sequences across the evolutionary distance contained within the ITS region and the change in coverage, C_{XY}, is compared to a paired library. The reciprocal comparison is then made using the other library, C_Y, and the change in coverage, C_{YX}.

P values derived by comparing the libraries from biochar-amended and unamended soils were significantly different across the same genetic distance, showing distinct compositions of clones from each soil (Fig. 6.3C). Finally, in lineage through time plots (Fig. 6.3D), phylogenies as a function of time were analyzed to compare divergent lineages between the libraries (Bohannan and Hughes, 2003). The greater concavity observed in the lineage from biochar-amended soil clones indicated a higher abundance of closely related species, compared to the control soil clones.

Based on ITS region sequences, clone libraries from the different soils differed significantly both for species rarity and coverage (Fig. 6.3B, C), with a clear phylogenetic divergence between clone libraries from each soil type (Fig. 6.3D).

Figure 6.3 (A) Distribution of clone sequences among the major soil fungal phyla, (B) rarefaction curves with 95% confidence interval error bars, (C) ∫-LIBSHUFF analysis, and (D) lineage through time plot for ITS region gene libraries.


Control
Biochar
A
% Sequenced clones
60
50
40
30
20
10
0
Ascomycota
Basidiomycota
Zygomycota
Glomeromycota
Blastocladiomycota
Chytridiomycota
Neocallimastigomycota
Unidentified fungus
Viridiplantae

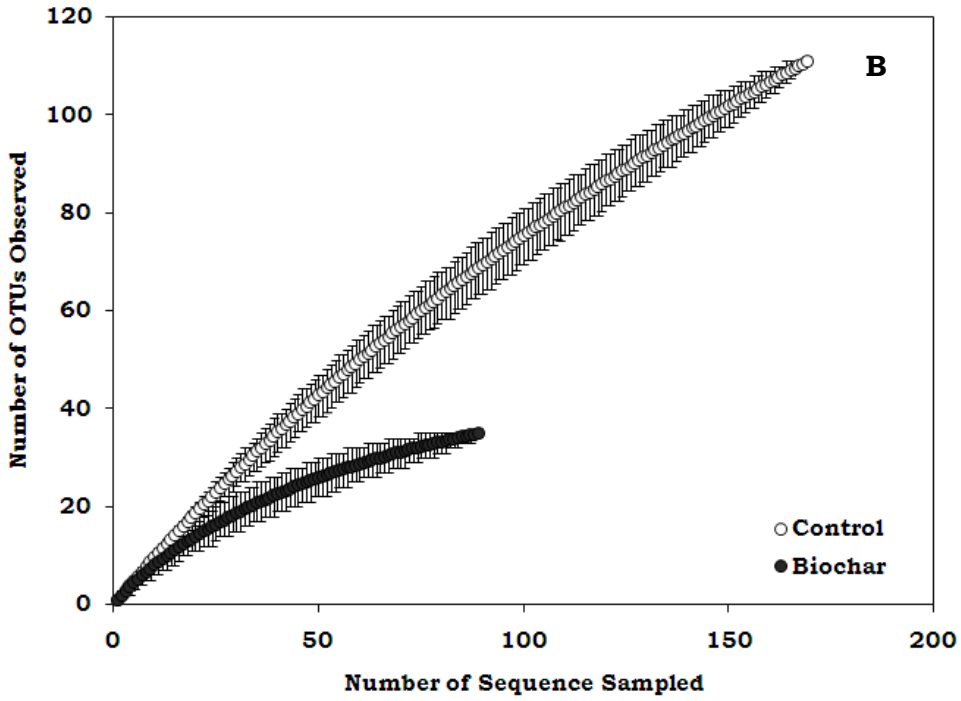
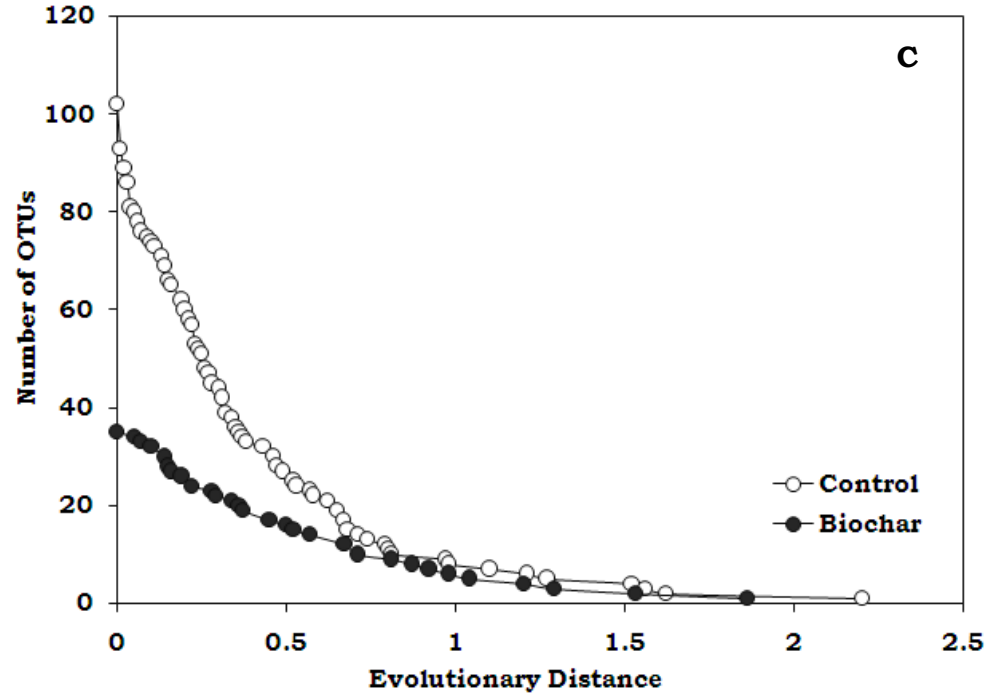
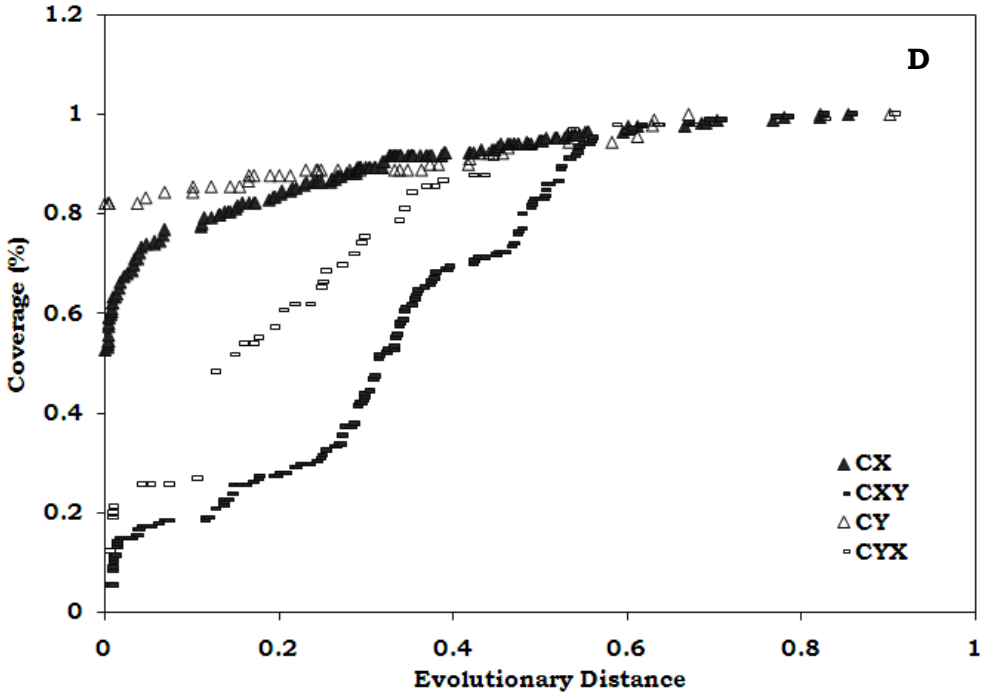

B
Number of OTUs Observed
120
100
80
60
40
20
0
0
50
100
150
200
Number of Sequence Sampled
Control
Biochar

Figure 6.3 (Continued)





DISCUSSION

SEM Imaging of Bacterial Cells and Fungal Hyphae on Biochar Particles Picked from Biochar-amended Soils

We observed bacterial cells and fungal hyphae located either on the surface or scattered in the pores in biochar particles (Fig. 6.2). These observations confirm the results of other researchers who have demonstrated that biochar has the capacity to support adsorbed bacteria (Pietikäinen et al., 2000), actinomycetes (Thies and Rillig, 2009), and saprophytic and mycorrhizal fungi (Zackrisson et al, 1996; Matsubara et al, 2002;Yamato et al, 2006; DeLuca et al., 2009). We observed that bacterial cells colonized the interior of the biochar pores primarily. The small neck size of these pores would protect cells from predation (Thies and Rillig, 2007); but, would also limit gas exchange, favoring colonization by microaerophiles, facultative aerobes or anaerobes.

Many organic substances, clay minerals, microbial cells and their constituents, and exoenzymes adsorb strongly to porous materials (Miura et al., 2007; Steiner et al., 2008; Matysik et al., 2009; Miura et al., 2009; Chapters 2-5). These are all likely to also be found in close association with biochar particles. However, soil constituents vary based on soil type and location; thus, the ecological significance of the adsorptive effects of biochar on microorganisms and their activities remains unclear. Bacteria and some fungi secrete polymeric substances, such as extracellular polysaccharide (EPS), and establish biofilms on the surfaces they colonize; and, quite likely, on biochar as well. Biofilms were observed when measuring enzyme activities associated with biochar particles (Chapters 4 and 5), but were not observed under SEM. This is likely due to their loss during sample preparation. Biofilms allow cells to remain attached to particles, remain hydrated longer and secrete hydrolytic enzymes without their

immediate loss by gravitational water movement. Biofilms form on all manner of hard surfaces and are typically hot-spots for microbial activity, making the biochar surface a likely hot-spot as well. Biochar, as a surface, is similar to mineral, metal and plastic surfaces in that it does not contain significant amounts of readily metabolizable C (Das et al., 2008); at least not after initial surface bio-oils have been metabolized. Thus, microbes forming biofilms associated with these surfaces must rely on dissolved organic C or inorganic C to meet their energy and cell C requirements. Low complex C availability would favor microbes with high C use efficiency or various autotrophs. The biochar biofilm habitat may exhibit similar patterns of CO_2 recycling via the carbonate cycle as has been observed in other biofilms communities (Stoodley et al., 1997; Davey and O'Toole, 2000; Branda et al., 2005). This may help to explain the lower rates of microbial respiration and increased stabilization of labile C observed commonly in biochar-amended soils.

Fungal ITS Region Sequencing

The high quality of PCR products and high rates of successful insertion in clones guaranteed the successful sequencing of the fungal ITS region. The primers used were designed to amplify the ITS region of all major fungal phyla, i.e., *Basidiomycota*, *Ascomycota*, *Zygomycota*, and *Glomeromycota;* our cloning and sequencing results support this specificity. Similar findings were reported in other studies by sequencing the ITS region of various eukaryotes from soils (Viaud et al., 2000; Ranjard et al., 2001; Meyer, 2004).

Unidentified Soil Fungal Groups and the Viridiplantae Sequences Obtained

We obtained a high number of unidentified fungi clones (23.9%) from the biochar-amended soils that were difficult to affiliate to a specific fungal class in the phylogenetic analysis. Similar problems have been reported in other soil community

diversity studies targeting the fungal community. Smit et al. (1999) analyzed 18S rDNA sequences and recovered 86-92% unidentified fungi from the wheat rhizosphere. Viaud et al. (2000) used ITS region sequence analysis, as was used here, and found that 86% of the sequences recovered were unidentified fungi. Compared to these studies, our recovery of 23.9% unidentified fungi clones was not remarkable. As molecular inquires of soil communities continue and the databases become more robust, more light will be shed on the seemingly high number of fungi not yet known.

We chose to amplify fungal ITS region sequences with fungal universal primers (Kumar and Shukla, 2005) in order to detect fungi belonging to all the main phyla. As a consequence, in addition to true fungi, the ITS sequences of the phylum *Viridiplantae* (assigned to classes, *Streptophyta* and *Chlorophyta*), also known as the green algae and green land plants, were also amplified (Table 6.2). To examine diversity in more specific groups of fungi such as in each main phylum, more specific primers could be used (Gardes and Bruns, 1993; Bruns et al., 1998; Carter and Gordon, 2007; Krüger et al., 2009).

Fungal Communities Change When Soils are Amended with Biochar

The comparison of presumptive phylogenetic affiliations of fungal clones showed that biochar-amended soils had a lower overall diversity, higher relative abundance of *Zygomycota*, *Glomeromycota* and *Neocallimastigomycota*, and a lower relative abundance of *Basidiomycota* and *Ascomycota* compared to unamended soils (Table 6.2 and Fig. 6.3). My findings showed that fungal taxa having a wide range of functional capabilities responded positively to the addition of the recalcitrant substrate, biochar.

Fungi in the *Zygomycota* are recognized as sucrose and cellulose degraders, the so-called “sugar fungi”; whereas, many *Basidiomycota* and *Ascomycota* are lignin-

degraders or "wood rot fungi" (Garrett, 1951). The higher relative abundance of *Zygomycota* and the lower relative abundance of *Basidiomycota* and *Ascomycota* detected in biochar-amended soils likely reflect a change in substrate availability and lability. Labile organic matter associated with biochar particles may stimulate exploration by the coenocytic hyphae of the *Zygomycota*. The absence of lignin or other complex organic matter associated with biochar likely discouraged colonization by the septate fungi. Along the same lines, exoenzymes appear adsorbed to biochar, suspended within biofilms (Chapter 5). Hydrolysis products will also likely remain associated with these biofilms. Thus, fungal hyphae in physical contact with biochar will likely absorb simple substrates while the availability of recalcitrant substrates is reduced as fungal exoenzymes become adsorbed to biochar, thus lowering their physical contact with the substrates.

Another possible explanation for this change in relative abundance of different fungal taxa could be the differential stimulation of various fungi by biochar additions to soil. When biochar is first added, r-strategists responds quickly, soluble or labile molecules, including inorganic nutrients, small amino acids, and simple carbohydrates, are targeted first (Robinson et al., 2005), which causes the relative abundance of labile substrate degraders to increase, most of these degraders comprise one of the most functionally diverse groups of saprotrophic microfungi. Once labile substrates disappear, fungal communities will undergo progressive changes, i.e., labile substrate decomposition is followed by a slow, progressive breakdown of more complex, recalcitrant substrates after a certain period of time by a complex assemblage of *Basidiomycota* and *Ascomycota* (Kjoller and Struwe, 2002). Our findings on the changes in fungal community structure associated with the change in dominant substrate (biochar organo-mineral complexes vs SOM) supports findings from other studies on the varying resource utilization capabilities exhibited by different fungal

decomposer species (Schmidt et al., 2007; Visser and Parkinson, 2009; McGuire et al., 2010). A third possible explanation is that biochar provides more colonizing sites for fungal hyphae, among which *Zygomycota* are benefited the most because many *Zygomycota* are considered primary colonizers in the decomposition of organic matter (Kjoller and Struwe, 2002) and some members of *Zygomycota* phyla may also be mycorrhiza-forming (Hanson et al., 2008).

Most *Basidiomycota* and some *Ascomycota*, including members of *Agaricomycotina*, are widely recognized polymer decomposers and producers of the enzymes responsible for lignin degradation (Lynch and Hobbie 1988; Read and Perez-Moreno, 2003; Deacon et al., 2006). *Ascomycota* were also found to be surprisingly efficient at mineralizing spruce wood and were particularly effective at producing enzymes, β-glucosidase and N-acetyl-glucosaminidase (Kanerva et al., 2006; Allison et al., 2009). The lower relative abundance of *Basidiomycota* in biochar-amended soils suggested a potentially decreased degrading capability of recalcitrant C in biochar-rich soils. Lack of available C in and around biochar particles may discourage colonization by higher order saprophytic fungi. We did observe a higher relative abundance of *Zygomycota*; however, because *Basidiomycota* have been shown to produce more C degrading enzymes and catalyze greater substrate mineralization than the *Zygomycota* or *Ascomycota* fungi (Hanson et al., 2008), the overall degrading capability in biochar-amended soils would be predicted to decrease. Changes we observed in the structure of fungal populations in biochar-amended soils may help to explain the frequently observed long-term preservation of C in other soils high in black carbon (e.g., Terra Preta soils of Brazil).

With the reclassification of the Oomycetes into the *Chrysophyta*, the *Chytridiomycota* is now the only phylum of fungi that has flagellated cells during part

of their life-cycle (James et al., 2006). The higher relative abundance of *Chytridiomycota* observed in biochar-amended soils suggested that the presence of biochar creates a more conducive environment for colonization by fungi that have flagellated cells during part of their life history. Biochar is known to increase the water holding capacity of soil and retain water in small pores to very low moisture tensions. Because the flagellated zoospores of the *Chytridiomycota* require water for dispersal (Powell, 1993), the ability of biochar to retain water, may provide the aquatic habitat necessary for the *Chytridiomycota* to thrive.

All populations in the *Neocallimastigomycota* phylum studied are obligate anaerobes (Gleason et al., 2007). The more anaerobic micro-environment around or in biochar pores could account for the higher relative abundance of *Neocallimastigomycota* in biochar-amended soils.

CONCLUSIONS

We observed microorganisms living in close association with biochar in biochar-amended soils under SEM. A less diverse fungal community, lower relative abundance of *Ascomycota* and *Basidiomycota,* and higher relative abundance of *Zygomycota* and *Glomeromycota* were identified in biochar-amended soils. Because soil fungal community members did not respond equally to the biochar amendment in our sequencing study, we further suggest that the unique properties of biochar, such as its strong adsorptive capacity and its high recalcitrant C content, may be important mechanisms for structuring the soil fungal community in natural environments. The loss or gain of certain fungal taxa may result in changes in carbon and nutrient cycling in response to biochar soil amendment. A focus of future work will be to elucidate the ecological significance of changes in fungal community structure in response to amending soils with biochar. Due to the difficulty in characterizing the ecological

functions of specific fungal taxa (Zak and Visser 1996; Torsvik and Ovreas, 2002), an attempt to link both a molecular approach and a cultivation-dependent approach to investigate the range of substrates consumed by various fungi (Hanson et al., 2008) could help to classify fungal taxa in relation to specific metabolic functions, thus help to explore the putative link between shifts in microbial molecular diversity and soil ecological functions in biochar-amended soils.

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CHAPTER 7

KEY FINDINGS AND FUTURE RESEARCH

KEY FINDINGS

In this study, I have addressed five key research questions in order to characterize the microbial life colonizing biochar and biochar-amended soils and set the groundwork for further work on how soil microbial ecology is affected by biochar soil amendment.

First, I investigated the effects of biochar amendment on microbial biomass carbon (MBC), basal respiration and the microbial quotient (qCO_2). The results indicated MBC was increased by 18.5-37.5% with an increase in the biochar application rate from 12 to 30 t biochar ha^{-1}. This positive response in MBC depended highly on the rate of biochar addition. A synergistic effect of biochar and inorganic N fertilizer on soil MBC was found. Meanwhile, the basal respiration and the qCO_2 were found to be significantly decreased with increasing rate of biochar addition. The increased microbial biomass and decreased basal respiration suggest biochar may increase microbial C use efficiency. Part of this effect could be explained by changes in microbial community composition, and likely, an increased ratio of fungal to bacterial biomass in soils amended with high rates of biochar.

Second, I investigated changes in microbial community composition in response to biochar addition by adopting PCR-T-RFLP fingerprinting. In order to obtain a high quality and quantity microbial genomic DNA from the environmental samples, three DNA extraction protocols were tested among which the Powersoil™ Soil DNA extraction kit protocol was demonstrated to reliably extract PCR-amplifiable genomic DNA from biochar-amended soils and was chosen for further T-RFLP studies.

Interactive Principal Component Analysis (IPCA) of the T-RFLP data obtained by digesting the PCR amplicons from microbial genomic DNA using restriction enzymes HhaI and Sau96I suggested a distinctly discriminative effect of both biochar addition and sampling location (bulk and rhizosphere) on the structural composition of both the bacterial and fungal communities. In addition, I also identified a further shift in both bacterial and fungal community composition one year after biochar was incorporated into the field soil. These observations support the findings reported in Chapter 2 and provide a explanation for the observed long-term fertility of other soils high in black carbon (biochar); that is, a fundamental change in the dominant microbial communities and their activities in biochar-amended soils.

Third, with an aim to explore the microbial metabolic potentials in C, N, P cycling in biochar-amended soils, I investigated the activity of soil exoenzymes, β-D-glucosidase, β-D-cellobiase, aminopeptidase and phosphatase, which are involved in C, N, and P biochemical cycling, respectively, in biochar-amended soils. Results showed that the biochar-amended soils had 615.3% and 15.0% higher activities of alkaline phosphatase and aminopeptidase, but 81.3% and 82.2% lower activities of β-D-glucosidase, β-D-cellobiase respectively. These changes in enzyme activities suggested that P and N use is increased relative to C mineralized in response to biochar addition. The decreased activity of C mineralizing enzymes likely contributes to the stability of labile C in biochar-amended soils. The increased need for microbial P and N acquisition relative to C in response to biochar application suggested a shift in microbial community composition in biochar-amended soils, one such possibility is an increase in the presence of AMF that form mutualistic symbioses with plant roots and metabolize C from their hosts in exchange for other nutrient elements, such as N and P from soil.

Fourth, after confirming the strong adsorptive effect of biochar in the exoenzyme activity assays reported in Chapter 4, I localized the presence of active alkaline phosphatase and β-D-glucuronidase specifically on biochar particles from biochar-amended soil (0.2% g g^{-1}, incubated for four weeks) using ELF (Enzyme-Labeled Fluorescence). I observed activities of alkaline phosphatase and β-D-glucuronidase in biochar-amended soil slurries, particularly on surfaces of the biochar particles. This suggested that either the substrates or the enzymes or both are being adsorbed on the biochar. I highlighted very subtle, but important, differences between the ELF-based and MUF-based methods in determining alkaline phosphatase and β-D-glucuronidase activities. The ELF-based method was prized for its ability to count the fluorescent signal directly and avoid the bias introduced by the adsorption properties of biochar that occurs when the MUF-based method is used. However, it could not be used to estimate rates as the signal intensity could not be measured directly. Thus, a solid correction method needs to be applied in the MUF-based method to analyze the activity of exoenzymes in biochar-amended soils.

Finally, considering the significant role of the fungal community colonizing biochar, I examined the fungal community living in close association with biochar particles in biochar-amended soils (0.2% g g^{-1}, incubated for four weeks). Under a scanning electron microscope (SEM), microorganisms were observed to form biofilms with diverse, visible structures. Bacterial cocci and bacilli and thread-like fungal hyphae were visible either on or in the biochar porous structure. Cloning and sequencing of the fungal ITS region from community DNA extracted from biochar-amended soil samples revealed the presence of a complex fungal community. Over 70% of the sequences obtained were classified as *Ascomycota*, *Basidiomycota* or *Zygomycota*. Compared to unamended soils, biochar-amended soils showed a much lower genetic diversity, a higher relative abundance of *Zygomycota, Glomeromycota*

and *Neocallimastigomycota* and a lower relative abundance of *Basidiomycota* and *Ascomycota*. Given that members of *Glomeromycota* form AMF, which obtain C from their host instead of from soil, the lowered metabolic quotient in high biochar-amended soils reported in Chapter 2 and the lowered C mineralization relative to increased P and N needs in high biochar-amended soils reported in Chapter 4 could be explained by a shift in microbial community composition, such as a relative increase in the abundance of *Glomeromycota,* whose C needs are met by the host but mobilize other nutrient elements needed. Another possibility might be that the lack of available carbon in and around biochar particles are discouraging colonization by higher order saprotrophic fungi.

In addition to these key findings, I also found that assays such as microbial biomass C using the simultaneous chloroform fumigation extraction method, exoenzyme activity using the MUF-based method and microbial genomic DNA extraction were impaired by the strong adsorption of extractants to biochar in amended soils. I overcame this limitation by measuring the equilibrium adsorption isotherms for extractants and building adsorption models for correction purposes. My approach could provide a useful tool to obtain accurate measurements for assays that involve extraction from or purification of samples that contain strong adsorbents.

FUTURE RESEARCH

The loss or gain of certain microbial taxa may result in shifts in carbon and nutrient cycling in response to biochar soil amendment. However, my findings only suggest that fungal communities living in association with biochar are likely functionally as well as taxonomically different. How observed changes in fungal communities may alter soil function needs further study. Due to the difficulty in characterizing the ecological functions of specific microbial taxa, I suggest an

integrated multi-technique approach where physiological, biochemical, stable isotope probing and molecular methods are combined that can yield results that will allow us to identify specific microbial groups involved in the shift of microbial community composition in biochar-amended soils. This could help to identify changes in microbial taxa and specific metabolic functions, thus allow us to explore the putative link between microbial diversity and community composition shifts in response to amending soils with biochar.