

**Characterization of Soil Microbial Communities in Agricultural and  
Forest Ecosystems**

by

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A dissertation submitted to the Graduate Faculty of  
Auburn University  
in partial fulfillment of the  
requirements for the Degree of  
Doctor of Philosophy

Auburn, Alabama  
December 8, 2012

Keywords: soil microbial community, ARISA, PLFA.

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## Abstract

Soil microbial community plays a critical role in nutrient cycling. The overall objective of this work was to determine responses of soil microbial communities to agricultural and forest management practices. Field experiments were conducted in one forestry site and two field crop sites. Soil microbial community structure was determined using phospholipid fatty acid (PLFA) analysis and automated ribosomal intergenic spacer analysis (ARISA).

The objective of the first field experiment was to determine effects of fertigation on soil microbial biomass, activity and microbial community structure in a loblolly pine plantation. This experiment was established in 2000 on a Blanton sandy soil near Aiken, South Carolina. The experiment was arranged in a randomized complete block design of three blocks with fertilization and irrigation as factors. Soil samples were collected in November of 2006 and May and September of 2007. The soil organic carbon was significantly higher for fertilized and fertigated treatments. Soil pH was higher in the irrigated treatment compared to the control and fertilized treatments. PLFA profiles showed that fertigation as well as sampling time influenced changes in the soil microbial community. Bacterial and fungal ARISA profiles revealed changes in soil microbial community associated with fertigation. The PLFA and fungal ARISA profiles showed fertigation treatment and sampling time influenced soil microbial community structure. Soil organic carbon for fertigation treatment was significantly higher than in the control and positively correlated with fungal biomarker. These results indicate that fertigation can influence

soil microbial community structure and activity along with soil chemical and biochemical properties in soil.

The objective of the second field experiment was to examine effects of nitrogen sources and soil pH on soil microbial communities in a long-term crop rotation system. The long-term soil fertility experiment, Cullars Rotation, consisting of a three-year rotation of cotton, corn, wheat, soybean and clover, was established in 1911 on Marvyn sandy loam soil. Soil samples were collected in June and October of 2008 and February of 2009 at two depths (0-5 and 5-15 cm). Soil pH values for no input and no lime plots were lower than other treatment plots. Fungal biomarker (18:2 $\omega$ 6,9) concentration was lower in the surface soil and subsurface soil for no lime treatment. The arbuscular myccorrhizal biomarker (16:1 $\omega$ 5) concentration was lower in the surface soil and subsurface soil for no input treatment. Bacterial biomarkers, fungal biomarker (18:2 $\omega$ 6,9) and arbuscular myccorrhizal biomarker (16:1 $\omega$ 5) were positively correlated to soil organic carbon content. Multivariate analyses of PLFA and ARISA profiles showed that changes in soil microbial communities were associated with soil pH and nitrogen source (inorganic fertilizer vs legumes).

The objective of the third field experiment was to examine effects of tillage practices on soil microbial communities. This experiment, located in Belle Mina, AL, was arranged in a randomized complete block factorial design with four replications on a Decatur silt loam soil. Tillage treatments included conventional tillage and no tillage in a continuous corn production system. Soil samples were taken at depths of 0-5 and 5-15 cm in April of 2008. The long-term no-tillage treatment resulted in higher soil carbon and nitrogen contents, viable microbial biomass, and phosphatase activities at the 0-5 cm depth than the conventional tillage treatment. Soil microbial community structure, assessed using phospholipid fatty acid (PLFA) analysis and

automated ribosomal intergenic spacer analysis (ARISA), varied by tillage practice and soil depth. The abundance of PLFAs indicative of fungi, bacteria, arbuscular mycorrhizal fungi, and actinobacteria was consistently higher in the no-till surface soil. Results of principal components analysis based on soil physicochemical and enzyme variables were in agreement with those based on PLFA and ARISA profiles. Soil organic carbon was positively correlated with most of the PLFA biomarkers. These results indicate that tillage practice and soil depth were two important factors affecting soil microbial community structure and activity, and conservation tillage practices improve both physicochemical and microbiological properties of soil.

Overall, these results indicate that changes in soil microbial community structure were influenced by changes in soil properties due to management practices, such as fertigation, lime application, nitrogen fertilization, crop rotation with winter legumes and conservation tillage.

## Acknowledgments

I would like to express my deep sense of gratitude to my major advisor, Dr. Yucheng Feng, for her guidance, support and valuable suggestions during this dissertation work. I would also like to thank committee members Dr. Charles Mitchell, Dr. Lisa Samuelson, Dr. Mark R. Liles, and Dr. Gobena Huluka for their valuable guidance, suggestions for proper execution of my research and readiness to help me at all times throughout the program. I would like to thank Dr. Zhanjiang (John) Liu for the use of LI-COR 4300 sequencer in the Fish Molecular Genetics and Biotechnology Laboratory at Auburn University and Dr. Edzard Van Santen for helping with statistical analysis. I would like to thank John McInroy for helping with fatty acid analyses. I would also like to thank Dr. Kathy Lawrence who in a short notice, agreed for serving as university reader.

I am deeply indebted to my parents, the late P.K. Mathen and Mrs. Annamma Mathen for their constant support in all fronts of my life. I would like to thank my wife Simi Susan Iype and son George for the constant support during my doctoral program. I would like to thank my brothers Kurian Mathew and Jijo Mathew for their moral support. I would also like to thank Father Dr. Mathew Koshy and family for their moral support. I am equally thankful to all my lab partners, Mrs. Michelle Owsley, Mrs. Rasanthi Udenika Wijsinghe and my other friends in the department, Anthony Kumi and Thomas Antony, for their help.

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## List of Abbreviations

ANOSIM	Analysis of Similarity
ARDRA	Amplified rDNA Restriction Analysis
ARISA	Automated Ribosomal Intergenic Spacer Analysis
DGGE	Denaturing Gradient Gel Electrophoresis
FAME	Fatty Acid Methyl Ester
IGS	Intergenic Spacer
NMDS	Non-metric Multidimensional Scaling
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
PLFA	Phospholipid Fatty Acid
PLFA-SIP	Phospholipid Fatty Acid-Stable Isotope Probing
OTU	Operational Taxonomic Unit
Taq	<i>Thermus aquaticus</i>
TGGE	Temperature Gradient Gel Electrophoresis
T-RFLP	Terminal Fragment Length Polymorphism
UV	Ultraviolet

## **Chapter I. Introduction and Literature Review**

### **Introduction**

Soil is one of the complex ecosystems that contains diverse groups of microorganisms such as archaea, bacteria, fungi and viruses. Soil microbial communities play a critical role in organic matter decomposition and nutrient cycling as well as the productivity and sustainability of agricultural and forest ecosystems. Among the soil microbial organisms, bacteria and fungi serve as major source and sink of plant nutrients. Bacteria are more abundant in number and can contain up to one billion individuals per gram of soil while fungi makes up most of the soil microbial biomass. Most of these microorganisms cannot be cultured using conventional techniques. In addition, these soil microorganisms are highly sensitive to changes introduced by soil management practices and pollution. Management practices such as crop rotation, fertilization, irrigation, lime application and tillage can cause changes in soil microbial community structure, due to changes in soil chemical and physical properties. Soil factors such as particle size distribution, organic matter content and cation exchange capacity as well as environmental factors such as temperature, rainfall and vegetation can also influence soil microbial community structure. Long-term soil productivity and sustainability depends on recycling of nutrients mediated by soil microorganisms. Studying soil microbial communities using culture independent methods has recently gained interest in the area of sustainable agriculture and forest productions.

Recent development of polymerase chain reaction-based culture independent methods such as PCR-DGGE (Polymerase chain reaction-denaturing gradient gel electrophoresis) and ARISA (Automated ribosomal intergenic spacer analysis) has significantly improved the ability to study microorganisms in soil. Culture independent methods using nucleic acids are widely used for soil microbial community analyses such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal fragment length polymorphism (T-RFLP), amplified rDNA restriction analysis, automated ribosomal intergenic spacer analysis (ARISA), cloning and sequencing, microarrays, quantitative PCR (qPCR) and pyrosequencing (Buee et al., 2009; Cardinale et al., 2004; Kielak et al., 2009; Muyzer, 1999; Wessén et al., 2010). These molecular methods provide better resolution and a highly reproducible way of estimating microbial community structure and diversity compared to culture dependent methods. Also, a polyphasic approach that includes phospholipid fatty acid analysis along with PCR-based methods have helped in identifying changes in soil microbial communities (Ramsey et al., 2006).

## **Objectives**

The overall objective of this work was to determine responses of soil microbial communities to various agricultural and forest management practices. Specifically, this study assessed changes in soil microbial community structure due to different management practices in agricultural and forest ecosystems using soil microbial community analysis. To accomplish this, three field experiments were conducted. The objective of the first field experiment was to determine the effects of fertigation on soil microbial biomass, activity and microbial community structure in a loblolly pine plantation. The second field experiment examined the effect of nitrogen sources and soil pH on soil microbial communities in a long-term crop rotation system.

The objective of the third field experiment was to examine effects of tillage practices on soil microbial communities. The culture independent soil microbial community analyses used in this study are a biochemical method involving phospholipid fatty acid analysis (PLFA) and a DNA finger printing technique involving automated ribosomal intergenic spacer analysis (ARISA). PLFAs are lipids present in the cell membrane of prokaryotic and eukaryotic microorganisms and serves as indicators of viable microbial biomass and community structure. The DNA fingerprinting tool, ARISA, utilizes length polymorphism of the intergenic spacer region between 16S and 23S subunit rRNA genes for bacteria and 18S and 28S for subunit rRNA genes for fungi.

## **Literature Review**

### ***1) The biodiversity concept***

Biological diversity is the variation of organisms at different levels of biological organization, such as communities, genes, and operational taxonomic unit (OTU). Ecologists are often interested in studying factors that regulate community diversity across temporal and spatial scales. In order to do so, they have developed a range of diversity indices to document and analyze environmental diversity patterns. Most of these diversity indices were originally used for examining species diversity in ecology but they have been adapted to microbial ecology for studying microbial diversity in soil.

Diversity indices are widely used for studying diversity in a set of samples or within habitats and can also be used to study across spatial scales. The indices are categorized into inventory and differentiation diversity (Whittaker, 1972). Inventory diversity can be classified into point diversity,  $\alpha$  diversity,  $\gamma$  diversity and  $\epsilon$  diversity. Point diversity is the diversity of a single sample, while  $\alpha$  (alpha) diversity represents diversity of a set of samples or within-habitat

diversity.  $\gamma$  (gamma) diversity is the diversity of a landscape and  $\epsilon$  (epsilon) diversity represents diversity of a biogeographical area. Similarly, differentiation diversity can be classified into pattern diversity,  $\beta$  diversity and  $\delta$  diversity. Pattern diversity is the diversity in variation of samples (point diversity) taken within a relatively homogenous habitat.  $\beta$  diversity is the measure of between-habitat diversity, while  $\delta$  diversity represents changes in species composition and abundance that occurs among landscapes within a biogeographical area.

Diversity consists of two components: richness and evenness. The first component measures number of species or OTU compared with the total number of individual organisms in a given area; evenness indicates variability in species or OTU abundance. Estimates of species richness and evenness are used to calculate a diversity index. The indices of diversity that measure richness and evenness together is called a heterogeneity index (Peet, 1984). The diversity indices widely used in the field of ecology are Shannon and Simpson indices (Magurran, 2004). The first diversity index is the Simpson diversity index, which calculates the probability of two individuals drawn at random from a finite community belong to same species or OTU (Simpson, 1949). The Shannon diversity index was originally developed for information theory and later applied to ecological diversity studies to determine the evenness of species distribution at a given location (Shannon and Weaver, 1949).

On a smaller scale, microbial diversity indices can be used to describe changes of microbial communities (Kennedy and Smith, 1995). They can function as bio-indicators for studying ecological dynamics and the impact of stress on a microbial community. A soil microbial diversity index is important in understanding biogeochemical and nutrient cycling influenced by a diverse group of microorganisms. The roles of bacteria, archaea, fungi, and viruses are extremely diverse and the beneficial functions include nitrogen transformation,

organic matter decomposition, mycorrhizal relationship, stabilization of aggregates and nutrient cycling (Kennedy and Smith, 1995; Kennedy and Gewin, 1997). Therefore, soil microbial diversity can be used for assessing soil management practices that are important in maintaining long-term sustainability and productivity in agricultural ecosystems.

In addition to the needs for sustainable and productive agricultural ecosystems, the demand of forest products are rapidly increasing due to the exponential growth in the world's population. Loblolly pine (*Pinus taeda* L.) is one of the most important commercial species, occupying more than 13 million ha in southern United States (Schultz, 1997). Sustainability of these intensively managed forests depends on soil microbial diversity and management practices. Intensive management practices in loblolly pine plantation include fertilization, physical and chemical site preparation and use of genetically improved root stocks (Jokela et al., 2004). These management practices sometimes adversely affect the long-term sustainability and productivity in forests. Thus, soil microbial diversity can be used for understanding the role of soil microbial communities in forest ecosystems to maintain productivity and sustainability.

## ***2) Methods for studying soil microbial communities***

Soil microbial communities can be studied using various techniques, including traditional plate counts and direct counts as well as biochemical based fatty acid analysis and molecular based methods. These methods can be broadly classified into culture dependant, biochemical and molecular methods.

### *a) Culture dependant methods*

#### *i) Plate counts*

Soil microbial communities were traditionally studied by culture dependant methods using selective plating and direct counts. The plate count method is fast, inexpensive and can

provide information about the active microbial population (Tabacchioni et al., 2000). However, current estimates suggest that less than 1 % of bacteria found in soil can be cultured in growth medium. About 1.5 million species of fungi exist in the world, but there are limitations in using current standard laboratory culture methods to culture many fungi found in soil (van Elsas et al., 2000). Another limitation of the plate count method is that it favors fast growing microorganisms that prefer growth conditions such as optimum temperature, pH, and light as well as the existence of a carbon substrate in the culture medium.

*ii) Community-level physiological profiling and sole carbon source utilization patterns*

The culture dependant method for accessing microbial diversity in soil is based on differences in the nutritional requirement of microorganisms. Biolog plates consisting of 96-well microtitre plate with 95 different carbon substrates are available to assess the diversity through sole carbon source utilization (Garland and Mills, 1991). Biolog ([www.biolog.com](http://www.biolog.com)) also has an Eco-plate consisting of 31 different environmentally relevant carbon sources. This is a relatively quick and inexpensive method, but the main limitation is that it cannot detect uncultivable microorganism and has bias towards fast-growing cultivable bacterial species and fungal species that produce large quantities of spores.

More specifically the limitations of community level physiological profiling are as follows: it (1) can be used for only cultivable population under experimental conditions, (2) favors fast growing organisms, (3) favors growth of organisms that prefer the carbon substrate used in the growth media, and (4) gives only potential metabolic diversity (Garland and Mills, 1991). In studying soil microorganisms the carbon sources used in community level physiological profiling may not be representative of those present in soil (Yao et al., 2000).

However, even with these limitations, this method is useful in studying functional diversity and valuable tool when used in conjunction with other culture independent methods.

*b) Culture independent methods*

The culture independent method for assessing microbial community profiles involves the extraction of biomarker compounds from the community of organisms being studied. The important characteristics of microbial community biomarkers are that (1) they should be present in relatively stable amounts in a group of microorganisms, (2) the proportion of the marker should not vary with growth or environment, and (3) they should degrade rapidly after the deaths of microorganisms. The biomarkers most widely used in soil microbial community studies are deoxyribonucleic acids (DNA) and phospholipid fatty acids (PLFA).

Culture independent methods for studying microbial community profiles can be subdivided into biochemical and molecular based methods

*i) Biochemical based methods*

*(1) Phospholipid fatty acid analysis*

The culture independent biochemical-based method using phospholipid fatty acid analysis (PLFA) is based on the variability of fatty acids present in cell membranes of different microorganisms. Phospholipid fatty acids serves as biomarker for viable microbial biomass and community structure (White et al., 2009). This lipid biomarker present in viable prokaryotic and eukaryotic microorganisms which serves in membrane fluidity and degrades rapidly on cell death makes it an ideal biomarker for studying microbial biomass and community structure (Allison and Miller, 2005; Kennedy and Gewin, 1997). The primary advantage of the PLFA method is the ability to fingerprint whole, viable soil microbial communities. PLFA technique uses extraction of lipids from soil using organic solvents using Bligh and Dyer's protocol,

followed by separation of phospholipids using solid phase extraction. Phospholipids fatty acids are then converted to fatty acid methyl esters (FAMES) and analyzed by gas chromatography. This method is useful for studying bacteria and eukaryotes, which have membranes composed of unbranched fatty acid chains attached to glycerol by ester linkages; thus the fatty acids can be extracted. However, archaea have membranes composed of branched hydrocarbon chains attached to glycerol by ether linkages and these membrane lipids cannot be extracted using this method.

Phospholipid fatty acids degrade in soils upon cell death, and total PLFA correlate with microbial biomass C and N in forest soil (Leckie et al., 2004b; Zelles et al., 1992). The PLFA makes up a relatively constant proportion of the cell biomass and signature biomarker fatty acids exist that can be used for differentiating major taxonomic groups (Findlay, 2004; Kirk et al., 2004). PLFA profiles of soil and environmental samples can be analyzed using multivariate methods in order to detect changes in composition of bacterial and fungal community profile. However, the primary limitations of this method are (1) it cannot be used to characterize microorganisms at species level, (2) determination of biomarker PLFAs for specific microorganisms requires their isolation in a pure culture and (3) microorganisms can produce varying amounts and types of PLFA due to growth conditions and environmental stress.

A variation of PLFA analysis, PLFA based stable isotope probing (PLFA-SIP), can provide quantitative and chemotaxonomic information on soil microbial communities utilizing an isotope labeled substrate (Chen et al., 2008; Evershed et al., 2006). The quantitative and chemotaxonomic information acquired from PLFA-SIP is highly complementary to that obtained by gene-probed based method (Evershed et al., 2006). PLFA-SIP can be used to study structural as well as functional diversity of soil microorganisms.

*ii) Molecular based methods*

There are a number of molecular methods that have been developed to study microbial diversity. These include but are not limited to nucleic acid reassociation and hybridization and PCR- based methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction length fragment polymorphism (T-RFLP), automated ribosomal intergenic spacer analysis (ARISA), microarrays, metagenomics, quantitative PCR and pyrosequencing.

*(1) Nucleic acid reassociation*

DNA reassociation is used to measure genetic complexity and estimate diversity of microbial communities (Torsvik et al., 1990; Torsvik et al., 1998). To do so, the total DNA from soil is extracted, purified, denature and allowed to reanneal. The rate of hybridization or reassociation will depend on the sequence similarity as well as decrease with an increase in the complexity or diversity of DNA sequence. The time needed for half of the DNA to reassociate ( $C_{0t_{1/2}}$ ) can be used as diversity index as it takes into account both the amount and distribution of reassociation. In one study, DNA reassociation was used to study bacterial communities in pristine soil and sediments and found that soil can contain up to 10,000 different bacterial types, and that the diversity of the total soil community was at least 200 times more than the bacterial isolate (Torsvik et al., 1998). However, the limitation of this method is that it depends on lysis and extraction efficiency of nucleic acids from soil.

*(2) PCR-based methods*

*(a) Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE)*

The PCR-based method was first used in studying bacterial communities (Muyzer et al., 1993). This method involves extracting of total community's DNA from soil, amplifying a short region of 16S rRNA gene that differs in sequence among organisms in the community, and resolving the mixture of gene fragments (200-700 bp) based on differential DNA dissociation or melting behavior. The amplified fragment lengths from different organisms are equal in length; the melting behavior is related to sequence composition. This melting behavior depends on the length of the PCR product, GC content and the nucleotide sequence. The fragments migrate through a gel matrix with electric potential and along a gradient of increasing concentration of DNA denaturant (urea and formamide). When the DNA fragment reaches a sufficient denaturation concentration, the double strands separate and cause the fragment to stop moving through the gradient. The resulting fingerprint is comprised of different fragments migrating to different points in the gel. A chemical denaturation gradient using urea or formamide is used in DGGE, while temperature denaturation gradient is used in TGGE.

The community level fingerprints from the DGGE/TGGE banding pattern can be used for diversity studies based on the number and intensity of DNA bands as well as similarity between treatment effects (Kirk et al., 2004). The specific DGGE/TGGE bands can be excised from gels, reamplified and sequenced to provide more information about structural and functional diversity (Theron and Cloete, 2000). Sequencing of these bands can identify specific taxonomic groups of microorganisms in the community.

A study conducted in two different agricultural soils-one organic and another sandy soil found out that differences between the two agricultural soils were significantly higher when the total bacterial population was analyzed using reassociation analysis, amplified rDNA restriction analysis (ARDRA) and DGGE analysis when compared to cultivable bacterial population

(Ovreas and Torsvik, 1998). Total bacterial diversity was determined using reassociation analysis of bacterial DNA combined with amplified rDNA restriction analysis (ARDRA) and denaturing gradient gel electrophoresis.

*(b) Restriction fragment length polymorphism (RFLP) and terminal restriction length fragment polymorphism (T-RFLP)*

A PCR-based culture independent tool used to study microbial diversity using DNA polymorphism is also known as amplified ribosomal DNA restriction analysis (ARDRA). In study by Liu et al. (1997) analyzing microbial communities, PCR-amplified ribosomal DNA was digested with a 4-base pair cutting restriction enzyme. The differences in fragment lengths are detected using agarose or non-denaturing polyacrylamide gel electrophoresis (Liu et al., 1997; Tiedje et al., 1999). This method is used in detecting changes in the structure of microbial diversity. In diverse microbial communities, the band patterns become too complex to analyze since a single species can have four to six restriction fragments (Tiedje et al., 1999). The resolution of this method can be increased by using a six- base cutting restriction enzymes by reducing the number of restriction fragments per species.

T-RFLP can be a useful tool in studying microbial diversity in an environment (Liu et al., 1997; Osborn et al., 2000). This method uses the same principle as RFLP except one of the PCR primers is labeled using fluorescent dye, allowing the detection of only the labeled restriction fragment (Liu et al., 1997). The banding pattern obtained the from T-RFLP can be used for analyzing complex communities and providing information on diversity of each of visible band representing as a single OTU (Tiedje et al., 1999). The banding pattern can also be used to measure similarities between samples as well as to measure species richness and evenness (Liu et al., 1997).

*(c) Automated ribosomal intergenic spacer analysis (ARISA) and Ribosomal intergenic spacer analysis (RISA)*

The intergenic spacer region is between 16S and 23S ribosomal subunits for bacteria; and 18S and 28S ribosomal subunits for fungi are amplified by PCR, denatured and separated on a polyacrylamide gel under denaturing conditions. This amplified region code for tRNAs is widely used for differentiating between bacterial strains and closely related species because of the intergenic spacer length and sequence. This method was first used to study microbial diversity of three fresh water environments in Wisconsin (Fisher and Triplett, 1999). In RISA, the sequence polymorphisms are detected using silver stain, while in ARISA one of the primers is fluorescently labeled and is automatically detected. Both methods give highly reproducible bacterial community profiles, but RISA requires large quantities of DNA and the resolution is low. ARISA increases the sensitivity of the method and is less time-consuming than RISA. ARISA has been used to study specific bacterial and fungal community patterns to differentiate soil types (Ranjard et al., 2001). Another study used this method to find the genetic structure of the bacterial and fungal community in different soil zones, which were compared between residue type and location in each soil zone (Nicolardot et al., 2007).

*(d) Metagenomics (clone library and sequencing)*

Metagenomics, defined as genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms (Handelsman, 2004), can be used to survey genetic and functional diversity of uncultured soil microorganisms. DNA is extracted by direct lysis of cells within soil or by separation of cells from soil particles followed by cell lysis and extraction. The 16S genes of the soil microbial community are amplified from extracted total soil DNA using PCR. The PCR products are cloned into cosmid, BAC or other vectors and

sequenced. These sequences of the soil microbial community genome can be compared with ribosomal database and then classified phylogenetically. The metagenomic approach depends on extraction of high-purity DNA from microorganisms in soil or other environmental sample and construction of metagenomics library in a suitable vector and host (Kakirde et al., 2010).

The metagenomic approach was used to study bacterial phylum Verrucomicrobia found in soil, water and sediments, which has been recalcitrant to cultivation methods (Kielak et al., 2009). Another study conducted to survey soil microbial diversity in clover-grass pasture used the metagenomic approach by sequence analysis of a universal clone library of genes coding for small-subunit rRNA (Borneman et al., 1996).

*(e) Microarrays*

Microarrays are used in monitoring the composition, structure, activity and dynamics of microbial populations. This method is used for assessing the diversity of soil microorganisms using DNA-DNA hybridization together with DNA microarrays (Greene and Voordouw, 2003). DNA microarrays can be used for bacterial diversity studies since a single array can contain thousands of sequences with high specificity. Also, microarrays can be used for detecting specific genes in specific gene targets such as nitrogenase, nitrate reductase or ammonia monooxygenase to provide functional diversity information. Using RNA instead of genomic DNA for microarray analysis can indicate genes of active microorganisms. Limitations of this method include dependence on prior knowledge of DNA sequences and inability to identify unknown microbial groups. In one study, DNA microarray using Geochip 2, which can detect more than 10,000 genes and 150 functional groups, was used to study the number and diversity of genes involved in organic carbon decomposition in forest soil (Zhang et al., 2007). They found

that the number of functional genes and the gene diversity index were correlated with increasing soil organic carbon content.

(f) *Quantitative PCR*

The advent of polymerase chain reaction (PCR) has accelerated the progress of molecular biology (Mullis et al., 1994). Quantitative or real-time PCR is used in studying changes in soil microbial community structures as well as quantifying the changes in various bacterial or fungal groups. Quantitative PCR is based on semi-conservative replication of DNA to enable exponential amplification of the target sequence and produce more than billion copies after 30-35 cycles of DNA synthesis and quantified using fluorescently labeled probes and dyes. A soil microbial community was analyzed using taxon-specific primers for bacteria and fungi in three distinct soils using quantitative PCR (Fierer et al., 2005). Another soil study used quantitative PCR to find changes in soil bacterial and archeal groups due to different management practices such as tillage (Wessén et al., 2010). The abundance of specific microbial groups such as ammonia oxidizing bacteria were quantified using *amoA* and ribosomal 16S genes (Mendum et al., 1999).

The advantages of using quantitative PCR are (1) high throughput and reproducibility, (2) high specificity, (3) a low detection limit, and (4) accurate quantification of genes and transcripts. Limitations of this method are that it (1) depends on the specificity of the primers, and (2) involves a higher cost of reagents and specialized thermocycler.

Sequenced-based approaches can survey the microbial community structure in an extensive manner. Sequence-based approaches using quantitative PCR based on amplification of 16S and 18S gene was used to concurrently analyze bacterial and fungal communities in different land-use types such as hardwood and pine forests as well as cultivated and livestock

pasture lands (Lauber et al., 2008). The sequence-based approach gives detailed phylogenetic information and assessment of biogeographical pattern exhibited by soil microbial communities.

*(g) High-throughput or pyrosequencing*

High-throughput or pyrosequencing is a culture independent molecular method widely used in studying microbial diversity that characterizes microbial communities using 16S rRNA by processing hundreds of thousands of sequences simultaneously (Liu et al., 2007; Roesch et al., 2007). The tag-encoded pyrosequencing can be used to simultaneously analyze samples in a single run (Acosta-Martinez et al., 2008). The tag or bar code containing 8-12 bp sequence adapter attached to one of the primers is used in a PCR reaction prior to pyrosequencing. Pyrosequencing used to estimate bacterial species in four soil types from agricultural and forest soils found that the number of bacterial species did not exceed 52,000 (Roesch et al., 2007). In addition, the tag-encoded pyrosequencing was used to study different soil types from North and South America to study the structure and diversity of bacterial communities in these regions (Lauber et al., 2009). However, limitations of this method include the assumptions that all prokaryotes possess some regions of 16S rRNA gene sequences that are homologous to the primers used for the PCR amplification step. Additionally, this method also cannot detect less abundant groups of prokaryotes in soil or sediments.

*c) Data analysis*

Microbial community analysis using molecular and biochemical methods depend on multivariate statistical analysis. Several multivariate analyses methods are widely used in studying microbial community analysis including dimensional techniques (principal component analysis and multidimensional scaling), discriminant analysis (canonical discriminant analysis) and cluster analysis. Multivariate analyses using exploratory approaches are used to identify

patterns in large datasets, but these analyses do not directly explain the reasons for these existing patterns.

Of the multivariate analyses used in studying microbial communities, principal components analysis (PCA) is the oldest and most widely used multivariate technique of exploratory data analysis (Hotelling, 1936; Pearson, 1901). Commonly used in studying changes in microbial community structure, this multivariate analysis uses dimensionality reduction technique. The PCA procedure calculates new synthetic variables called principal components, which are linear combinations of original variables that account for most of the variance in the original data. The principal components are such that most of the information, measured in terms of total variance, is preserved in the first few components. When all the variables are in the same unit and scale, variance-covariance matrix is used in PCA. On the other hand, a correlation matrix is used when variables are in different units and scale. The main aim of this multivariate analysis is to represent the relative position of objects and magnitude of variation between variables in a reduced space. The PCA results are displayed in biplot when most of variances are accounted by first two or three principal components. This method is used for character data and not for fingerprint data. The fingerprint must be converted to a band matching table before analyzing. Previously, PCA was used to analyze various fingerprinting techniques such as automated ribosomal intergenic spacer analysis (ARISA), terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE). In addition, bacterial and fungal community structure of an ARISA fingerprint was analyzed using PCA to find out changes in soil microbial structure due to plant residues in different soils (Nicolardot et al., 2007).

Non-metric multidimensional scaling (NMDS) is another multivariate technique used to identify patterns when analyzing a molecular fingerprint. The NMDS algorithm ranks distances between objects, and uses these ranks to map the objects on a two-dimensional ordination space. Several iterations of the NMDS procedure are done to obtain the lowest stress value (best goodness of fit) based on different random positions of objects. Previously, NMDS was used to compare diversity patterns of microbial communities using length heterogeneity-PCR of 16S rRNA gene from soil samples undergoing different land management practices (Mills et al., 2006). Another study conducted to find within and between-lake variable of bacterioplankton communities used the NMDS technique to fingerprint ARISA data (Yannarell and Triplett, 2004).

Multivariate data analysis procedure using discriminant analysis has been successfully used to assess changes in microbial community structure due to treatment and seasonal effects (Drijber et al., 2000; Spedding et al., 2004). Canonical discriminant analysis, similar to PCA, is a multivariate exploratory data analysis procedure used in studying changes in the soil microbial community structure. This method is based on a large number of possibly correlated characteristics on which measurements are taken, and the analysis obtains a few new variables that can help in describing differences between various populations. These new variables obtained as linear combinations of original measurements are called canonical variables. In SAS, before running the PROC CANDISC procedure, a stepwise discriminant analysis (PROC STEPDISC) was done for selecting variables with STEPDISC procedure to select a subset of the quantitative variables for use in discriminating among the classes. The set of variables that make up each class is assumed to be multivariate normal with a common covariance matrix. The canonical discriminant analysis using CANDISC procedure was then used to evaluate the

discriminatory model. The number of dimensions that is used to distinguish among different treatments was determined by the number of canonical discriminant functions, which are linear combinations of variables.

Cluster analysis is a multivariate analysis that represents similarity between objects (e.g. sampling sites or soil samples) based on values of multiple variables, so that similar objects are depicted near to each other and dissimilar objects are found further apart from each other. The main aim of cluster analysis is to reduce within-group variation and maximize between-group variation to categorize objects and thereby reduce the dimensions of the dataset. There are several linkage rules for conducting a cluster analysis, such as nearest neighbor (distance between two clusters is the distance between their closest neighboring points), furthest neighbor (distance between two clusters is the distance between their two furthest objects) and UPGMA (distance between two clusters is the average distance between all intercluster pairs).

A final multivariate method used to study soil microbial diversity, rarefaction is a method used to compare observed richness among environments that have been unequally sampled or among samples that have been unequally sub-sampled. The main limitations of rarefaction analyses occur when comparing rarefaction curves from different environments because the technique assumes that rarefaction curves do not cross at a higher sample size and error bars in rarefaction curves describes variation of sub-samples rather than the precision of the observed richness. Parametric or non-parametric analysis of variance tests using estimates of richness derived from rarefaction analysis of multiple samples are used to determine the statistical significance of richness when comparing two environments.

*d) Limitations in studying soil microbial communities*

The phenotypic and genetic diversity of microorganisms in soil are very high (Torsvik and Ovreas, 2002), but about 90-99% of bacteria observed under microscope cannot be cultured

using common laboratory techniques (Trevors, 1998). This is also the case with fungal species that also cannot be cultured using laboratory culture media. Therefore, culture independent methods, especially molecular methods, are widely used in studying microbial diversity in soil.

Molecular methods based on PCR have been widely used to overcome limitations of culture-based methods, but the culture independent molecular method depends on the quality of extracted DNA from soil and PCR amplification. In DNA extraction, lysis efficiency of cells and fungal structure varies for different microbial groups (Prosser, 2002). For example, Gram negative bacteria will be easily lysed when compared to Gram positive bacteria. If the method of DNA extraction is too harsh, both Gram positive and Gram negative bacteria may be lysed but the process can cause shearing of DNA (Wintzingerode et al., 1997). Lysis efficiency also varies for different fungal cells. The spores will lyse differently when compared to mycelial structures. The variation in lysis of bacterial cells and fungal structures can lead to bias in molecular-based methods.

In addition to DNA quality and PCR amplification, characterization of microbial communities in soil depends on the quantity of DNA extracted from environment. A study conducted to find bias involved in DNA extraction using commercial DNA extraction kit found that complete characterization of bacterial diversity cannot be done by multiple extraction of DNA from the soil sample (Feinstein et al., 2009).

The PCR amplification efficiency can be affected due to (1) inhibition of PCR amplification due to co-extracted contaminants, (2) differential amplification due to mixture of homologous molecules as the template, (3) formation of artefactual PCR products such as chimeras and heteroduplex, (4) contamination in extracted DNA, and (5) 16S rDNA variation due to *rrn* operon heterogeneity.

One contaminant, humic acid or humic substances co-extracted with soil DNA, can strongly inhibit PCR. The PCR efficiency can be improved by removing these co-extracted substances by purification procedures or by addition of non-acetylated BSA and T4 gene 32 protein (gp32) (Kreader, 1996). Also, The PCR efficiency due to differential amplification caused by a homologous DNA template can be improved by increasing the specificity of the primers.

PCR artifact products include chimeras, heteroduplex, deletion mutants due to stable secondary structures and point mutants due to misincorporation by DNA polymerases. Chimeras are a PCR artifact formed between two different DNA molecules with high sequence similarity by annealing of an incompletely extended primer and template switching during DNA synthesis (Odelberg et al., 1995; Pääbo et al., 1990). The formation of chimeras can be avoided by limiting the number of PCR cycles and reducing highly fragmented DNA as a template. Similarly, the formation of a heteroduplex can be avoided by limiting the number of PCR cycles. Furthermore, PCR templates containing stable secondary structures often yield low amplification efficiency or cause mutation due to deletion. PCR efficiency due to stable secondary structures can be improved by using *E. coli* single-stranded binding protein in PCR reactions (Chou, 1992). The formation of point mutation can be caused by mis-incorporation of nucleotides during strand synthesis by *Taq* DNA polymerase. In this case, PCR amplification can be improved using proof reading DNA polymerase from hyperthermophilic archaeon *Pyrococcus furiosus* (Pfu), thereby reducing the mis-incorporation rate.

Contamination of DNA can be due to tube-to-tube contaminants or contaminated reagents. The tube-to-tube contaminant can be removed by UV treatment and pre-PCR uracil DNA glycosylase digestion. Another limitation in study soil microbial diversity is that 16S rRNA

genes of some bacteria and archaea have a variable number of copies of *rrn* operon and sequence heterogeneity between operons (Crosby and Criddle, 2003). These differences can interfere with gel electrophoresis patterns and analysis of 16S rDNA clone libraries as it is not clear whether one 16S rDNA sequence represents one organism or is one representative gene of the entire 16S rRNA operon of a microorganism (Wintzingerode et al., 1997).

### ***3) Influence of agricultural management practices on soil microbial biomass and activity***

Agricultural management practices such as fertilizer application, tillage and crop rotation can cause changes in soil microbial biomass and activity. A study conducted in a no-till cropped to corn using organic and inorganic N fertilizer found that organic manures cause an increase in the accumulation of organic carbon in soil (Peacock et al., 2001). This in turn resulted in an increase in microbial biomass and caused changes in soil microbial community structure. There was significant increase in soil C, N and microbial biomass in the topsoil for an organically fertilized plot when compared to inorganically fertilized plots. Another study analyzed the influence of site fertility on soil microbial biomass and activity on long-term fertilized soil, finding that nitrogen fertilization using ammonium nitrate caused 20-30% reduction in microbial biomass and respiration (Fisk and Fahey, 2001). Gross nitrification and nitrate immobilization both were increased by fertilization, and the nitrate played an important role in microbial N cycling in fertilized forest soil.

Li et al. (2008) studied the effect of long-term fertilizer application using N and P fertilizers, and organic manure on soil microbial biomass C and bacterial community cropped to corn. The soil microbial biomass was significantly higher in manure treated along with N and P fertilized treatments when compared to other treatment plots. This study showed that the addition of organic and inorganic fertilizers as well as the crop growth stage and soil chemical conditions

influenced soil microbial characteristics (Li et al., 2008). A long-term fertilization study in a grassland ecosystem found that fertilizer management impacts the size of microbial biomass in soil (O'Donnell et al., 2001). The microbial biomass C was significantly higher in organic manure plots when compared to mineral fertilized plots. A long-term experiment in a wheat-fallow cropping system found that inorganic fertilizers have an effect on soil biochemical process (Dick et al., 1988). The study compared different fertilizers such as green manure, animal manure and inorganic fertilizer. Soil enzymatic activity increased significantly in organically fertilized plots when compared to plots fertilized with inorganic fertilizer.

Tillage is widely used in annual cropping systems and involves mixing the top 15-25 cm of soil in preparation of planting. Tillage practices help in periodic disturbance of soil environment and decomposition of organic matter but it also causes altered pore volume and pore structure, reduced vertical stratification, destruction of biopores from past roots and hyphae and dispersal of microbial communities and fungal hyphae.

A study was conducted to identify the changes in microbial biomass and activity due to zero tillage and conventional tillage using moldboard plough in a wheat-fallow system (Carter, 1986). The surface soil had significantly higher microbial biomass C and N as well as activity measured by microbial respiration compared to conventionally tilled soil. The same parameters were significantly higher in the conventionally tilled sub-surface. In a long-term study conducted to find the effect of tillage on microbial biomass found that no-tillage treatment had a significant increase in microbial biomass C and N in the top soil when compared to conventional tillage (Balota et al., 2003). The lack of disturbance in no-till plots provided a steady source of organic carbon to the microbial community, while disturbance in soil due to conventional till results in loss of carbon due to a temporary flush of CO<sub>2</sub>. In Brazil, a study was conducted to find the

effect of tillage on maize, wheat and soybean rotation (Silva et al., 2010). No-till plots had significantly higher microbial biomass C and N when compared to conventionally tilled plots and was highly correlated with grain yield. The study concluded that microbial biomass C and N were sensitive indicators for changes in soil and crop management regimes.

A study conducted in a long-term wheat growing system with green manure crops as a source of nitrogen found that the soil enzyme activity and microbial biomass were significantly higher than the conventionally fertilized plots (Bolton Jr et al., 1985). Another study using wheat-oat-barley-forage rotation found that five year rotation had 4% more soil N and 12% more microbial biomass N than two year rotation (McGill et al., 1986). The organic manure applied plots had significantly higher microbial biomass N when compared to inorganically fertilized plots. In a study conducted in a grassland ecosystem comparing unfertilized and intensively managed pasture found that microbial respiration showed seasonal variation with a high during winter and spring and a low during summer and autumn (Bardgett et al., 1999). Overall, these studies conclude that agricultural management practices such as nitrogen fertilization, tillage, and crop rotation influence soil microbial biomass and activity.

#### ***4) Influence of forest management practices on soil microbial biomass and activity***

Soil microbial biomass performs critical functions for forestry ecosystem by serving as labile source and sink of C, N, S, P and other elements. Relatively rapid assessment of soil microbial biomass has been possible based on physiological, biochemical and chemical techniques including chloroform fumigation and incubation and chloroform fumigation extraction. Microbial biomass is also closely associated with above ground plant productivity and microbes depend directly on plant carbon inputs (e.g., root exudates) in different ecosystems (Zak et al., 1994).

Microbial biomass and nitrogen transformation are important components that respond to variation in nutrient availability. Forest management practices include nitrogen fertilization, site preparation and use of genetically improved planting materials. Studies conducted on forest soil found positive, negative, and neutral influence on soil microbial biomass and activity due to nitrogen fertilization (Allen and Schlesinger, 2004; Blazier et al., 2005; Gallardo and Schlesinger, 1994). Inconsistencies in changes in soil microbial biomass and activity, especially of fertilization, can be due to rate of fertilization, formulation and type of fertilizer, soil moisture, temperature, soil properties such as pH, CEC and texture, as well as stand age (Thirukkumaran and Parkinson, 2000). The effect of inorganic nitrogen in forest soils has been attributed to increase of osmotic potential to toxic levels, lowering of soil pH, inhibition of fungal lignolytic enzyme production and a decrease in the level of soil enzymes that degrade soil organic matter (Vance and Chapin, 2001).

A study conducted in warm temperate forest soil concluded that N fertilization increased microbial biomass in the forest floor, but the mineral soil microbial biomass responded only to P fertilization (Gallardo and Schlesinger, 1994). Soil respiration and microbial activity increased with N and P addition. This study also found seasonal variation in soil respiration and microbial biomass with a summer maximum and winter minimum.

A long-term fertilization study in hardwood forest soil using nitrogenous fertilizer found that microbial biomass and respiration decreased in fertilized plots by 20-30% when compared to unfertilized plots (Fisk and Fahey, 2001). Another study conducted in a 7-year-old cottonwood and loblolly pine plantation on a well-drained sandy loam soil found that nitrogen fertilization had significant negative effect on soil respiration in cottonwood but no effect on the loblolly pine plantation (Lee and Jose, 2003). Microbial biomass was significantly reduced in plots

planted with both tree species and microbial activity was highest at soil pH 6.0. The major factors influencing soil respiration were microbial biomass, soil organic matter and soil pH in cottonwood plots, while fine root production and soil organic matter influenced loblolly pine plots.

A study conducted in Norway spruce forest soil found that nitrogen fertilization decreased microbial biomass by 40% and microbial activity by 30% (Demoling et al., 2008). The decrease in microbial biomass and activity was due to carbon limitation in soil. Another study conducted in a shinnery-oak ecosystem found that microbial biomass C and N exhibited rapid response to water and nitrogen addition (Qishui and Zak, 1998). The addition of nitrogen increased microbial biomass C, N mineralization rate and root growth over a period of four months. This study also found that ectomycorrhizal colonization decreased due to nitrogen fertilization.

Blazier et.al (2005) conducted a study to investigate the influence of fertilization on soil microbial biomass and activity, finding that nitrogen addition decreased microbial biomass and dehydrogenase activity. The availability of labile carbon substrate as root exudates was reduced due to nitrogen fertilization. The results from this study showed that in an intensively managed pine plantation, labile C substrates containing carbohydrates, amino acids and fatty acids as root exudates can influence microbial biomass and activity and may be a predominant determinant of soil N availability.

Nitrogen fertilization application in temperate coniferous forest to increase tree growth had variable effects on soil microbial biomass. Most studies have reported decreases in microbial biomass and activity due to fertilization (Ohtonen et al., 1992; Thirukkumaran and Parkinson, 2000). The formulation and type of nitrogenous fertilizers have different effects on microbes,

since some fertilizers such as urea can become carbon as well as nitrogen sources and also results in change to the soil pH.

Annual fertilization in a loblolly pine plantation using N, P, K and micronutrients decreased soil microbial biomass C and N, and soil pH in four different types of soil (Rifai et al., 2010). Microbial biomass C showed a seasonal effect, but this was not the case for microbial biomass N. The C/N ratio was lower in the summer months and peaked during the spring. The soil carbon and nitrogen content were influenced by fertilizer treatment. Microbial biomass C increased in surface mineral soil due to organic manure application in Norway spruce forest (Borken et al., 2002). The study found surface application of organic manure increases microbial biomass and activity in the surface horizon of spruce forest. Basal respiration and microbial biomass C was significantly correlated in plots treated with no manure as well as organic manure applied plots.

The above literature showed changes in soil microbial biomass and activity due to nitrogen fertilization has a positive, negative and neutral influence. These results can be due to many interacting factors such as soil physiochemical properties and intensive management practices in forest soil.

##### ***5) Factors influencing changes in soil microbial community structure and diversity***

Environmental and management factors mainly influence microbial diversity in soil. The environmental factors include soil type, parent material, landscape, organic matter and climatic variables, while the management factors include crop, fertilizers, crop rotation pattern, tillage, and pesticides (Kennedy and Gewin, 1997). These environmental factors can influence soil physiochemical properties such as soil pH, soil carbon, nitrogen and phosphorous.

Bacterial and fungal communities were strongly correlated with soil properties in a study conducted in the southeastern US across four land use types that included cultivated fields, pasture land, pine forest plantation and mixed hardwood forest (Lauber et al., 2008). Soil pH was the most influential edaphic factor for bacterial community composition while soil C: N ratio and extractable P was the influential edaphic factor for fungal community composition. This study showed that specific changes in soil properties best predicts shifts in soil microbial community composition across landscapes. Another study in the Hoosfield acid strip, at Rothamstead Research, UK, found that soil pH had a significant influence on soil microbial community composition (Rousk et al., 2010). The changes in soil microbial community measured using PLFA analysis found that relative concentration of monosaturated PLFAs (16:1 $\omega$ 5, 16:1 $\omega$ 7c and 18:1 $\omega$ 7) increased with soil pH. Another study by the same authors using PLFA analysis found that a pH change from 4.5 to 8.3 increased bacterial biomass (PLFA) and decreased fungal biomass (ergosterol and fungal biomarker, 18:2 $\omega$ 6,9) (Rousk et al., 2009). The fungal to bacterial growth ratio was significantly higher in plots with soil pH 4.5 when compared to plots with a pH of 8.3.

A study conducted across North and South America found soil pH as the best predictor of bacterial community structure using pyrosequencing (Lauber et al., 2009). Soil pH influenced the relative abundance of bacterial groups such as Acidobacteria, Actinobacteria and Bacteroidetes. The overall bacterial phylogenetic diversity was positively correlated with pH with peak diversity in soil with a near-neutral pH. A study using T-RFLP analysis to assess changes in microbial community structure found that soil pH and nitrogen were important factors that influence bacterial community structure (Kennedy et al., 2004).

Changes in microbial diversity due to agricultural management practices have been studied extensively (Birkhofer et al., 2008; Esperschütz et al., 2007; Marschner et al., 2003). Several studies showed that nitrogen fertilization influence microbial activity (Birkhofer et al., 2008; Kandeler et al., 1999), microbial biomass (Marschner et al., 2003) and microbial community structure (Hamer et al., 2008; Hartmann et al., 2006; van Diepeningen et al., 2006). A study was conducted in upland acidic grassland soil to find the influence of plant and chemical factors on bacterial community structure (Kennedy et al., 2004). This study showed that lime and nitrogen amendment had significant effect on microbial biomass, activity and bacterial ribotype. Liming increased soil pH, microbial activity and ribotype number while nitrogen addition decreased soil pH, microbial activity and ribotype number. Addition of lime along with nitrogen had a significant effect on bacterial community structure and was influenced by soil pH.

The effect of long-term addition of organic and inorganic fertilizer amendments on soil chemical and biological properties was examined in a long-term study (Marschner et al., 2003). The soil microbial community structure of bacteria and eukaryotes were analyzed using DGGE banding patterns. The bacterial community was affected by fertilizer treatments, in contrast to eukaryotes. Both bacterial and eukaryotic community structure were significantly influenced by organic carbon and C: N ratio. The ratios of Gram positive to Gram negative bacteria and bacteria to fungal ratio determined that signature phospholipid fatty acids were higher in organically fertilized soil when compared to inorganically fertilized soil.

Bacterial and fungal diversity were studied by comparing the effects of precision farming to conventional management in soil cropped to corn (Schloter et al., 2003). Microbial biomass decreased in summer months due to higher temperature while the microbial community structure changed in late spring due to application of fertilizer and higher amount of root exudates in the

rhizosphere. Changes in soil microbial community structure of bacteria and fungi based on PLFA analysis were associated with soil water content, temperature and time of fertilization.

Several studies used PLFA concentration to calculate microbial diversity indices in different ecosystems (Asuming-Brempong et al., 2008; Bossio et al., 1998; Yao et al., 2006). A study analyzing organic, low input and conventional fertilizer management of soil also found no change in the microbial diversity index due to the selected management practices (Bossio et al., 1998). Asuming-Brempong et al. (2008) used PLFA profile data from soil in different fallow management practices to calculate richness, dominance and evenness microbial diversity indices. The study found no significant difference in Shannon diversity indices between treatments. Yao et al. (2006) used PLFA concentration for different land use patterns (pine versus turf), particularly turf grass age and soil depth to calculate the Shannon diversity index. The study found no significant changes in surface 0-5 cm for land use pattern (pine versus turf) and turf grass age, but there was a significant difference in diversity index for subsurface depth (5-15 cm) for the two youngest turf grass age. In a review article Frostegard et al. (2011) cautioned against use of PLFA data to calculate diversity indices. The diversity indices are calculated by using each PLFA as a “species” and the size or area of chromatogram peak as equivalent to frequency of species. Only few different PLFAs (10 or less) can be isolated from a soil sample even if there were thousand different fungal species in that soil sample. In addition, bacterial species have more variation in PLFAs compared to fungi. In the case of bacteria, there are some bacterial species with a few dominant PLFAs, while other bacterial species have different PLFAs in their membrane. So apparent changes in diversity reported using PLFA data are likely due to different amounts of PLFA in different samples and not due to changes in species richness or evenness.

Another study on the structure of soil microbial communities on a long-term experimental plot over two years found that experimental plots with similar agricultural management practices were significantly different from plots that were left fallow for over 50 years (Buckley and Schmidt, 2003). Microbial community structure was examined by monitoring the relative abundance of rRNA. Environmental factors, soil moisture and soil depth were found to have a greater influence on bacterial division Verrucomicrobia (Buckley and Schmidt, 2001b). The relative abundance of rRNA of Verrucomicrobia had significant impact on sampling time while plant composition in different sampled fields did not cause any significant change. Multivariate analysis of PLFA profiles showed that changes in soil microbial community structure occurred due to organic manure compared to inorganic amendments, and these changes were explained mostly by Gram negative bacterial biomarkers.

In a grassland ecosystem, fungi play more significant role in soil biogeochemical process of low input, unfertilized plots when compared to intensively managed system (Bardgett et al., 1999). The total PLFA profile showed a seasonal variation with a spring maximum and autumn minimum. These seasonal variations in soil PLFA pattern was significantly correlated to soil nitrogen and moisture content. Microbial biomass C and N also showed seasonal variation with summer maxima and winter minima. The soil microbial biomass C and N and total PLFA were higher in unfertilized when compared to fertilized soils. The ratio of soil fungal to bacterial ratio was also significantly higher in unfertilized plots and lowest in fertilized grassland.

Study conducted in long-term grassland management using PLFA analysis and PCR-DGGE analysis found N fertilizer and soil drainage had significant impact on soil microbial community structure (Clegg et al., 2003). PCR-DGGE analysis revealed a significant impact of N fertilizer on eubacterial and actinobacteria community structure while soil drainage had a

significant impact on actinobacteria and pseudomonads. Multivariate analysis of the PLFA profile showed that nitrogen fertilization and soil drainage together had a significant impact on soil microbial community structure. A study conducted in long-term grassland and agricultural soil using plate counts with different substrates as well as microbial biomass and enzyme activity (Kennedy and Smith, 1995) showed that the microbial biomass C and enzyme activity were higher in prairie soil while inorganic N was higher in cultivated agricultural soil with no significant difference in pH for the two soils.

Tillage can also cause changes in soil microbial community structure. Several studies reported the effect of tillage on soil microbial community structure and diversity. The effect of tillage was used to study changes in soil microbial community and diversity in agricultural soil (Ibekwe et al., 2002). The biomasses estimated from extractable PLFA were significantly higher in no-till when compared to conventional-tillage treatments. Quantitative assessment of DGGE band profile of 16S rRNA also showed differences due to tillage. The analyses of *amoA* (ammonia monooxygenase) genes showed that ammonia oxidizing bacteria from no-till soil were more diverse compared to conventionally tilled soil. Another study conducted in a long-term wheat fallow system assessing soil microbial communities using fatty acid methyl esters (FAME) found that microbial biomass was higher in no-till plots compared to conventionally tilled treatments (Drijber et al., 2000). The multivariate analysis of FAME differentiated wheat and fallow system by different tillage systems. The cropped plots had significantly higher microbial biomass compared to fallow plots. The effect of conventional tillage and no-tillage practices under long-term continuous cotton cropping systems found that changes in soil microbial community during growing season are influenced by soil conditions responding to cotton growth (Feng et al., 2003). The impact of tillage practices varied with season and depth.

Environmental variables such as moisture and temperature influenced microbial community composition associated with tillage practices during fallow or prior to crop establishment. A study conducted on soil microbial community structure in active and abandoned agricultural fields using terminal restriction fragment length polymorphism Buckley and Schmidt (2001a) found that microbial community structure was similar among plots that shared a long-term history of agricultural management despite differences in plant community composition. Microbial community structure differed significantly among plots that shared a long-term history of agricultural management such as no-till and conventional tillage and fields that had never been cultivated. The total amount of 16S rRNA gene for alpha Proteobacteria, beta Proteobacteria and Actinobacteria were significantly higher in fields that were never cultivated than in the fields with a history of cultivation. The effect of tillage on soil microbial community structure using PLFA profiles was studied in a vegetable cropping system (Calderon et al., 2001), finding that tillage caused changes in PLFA profiles, and eubacterial biomarker (18:1  $\omega$ 7t) decreased in tilled soil. The tilled soil also had a high efflux of CO<sub>2</sub> immediately after tillage operation.

The effect of nitrogen fertilization on bacterial diversity was studied in a century old manure-treated agro-ecosystem (Sun et al., 2004). The soil fertilized with N and N-P fertilizer had similar bacterial community profiles when compared to manure treated plots. Significantly higher number of bacterial ribotypes was observed in long-term manure treated plots. Another study conducted to measure changes in soil microbial community due to nitrogen fertilizers as well as stubble addition (Wakelin et al., 2007) found that the fungal community structure changed with stubble addition. The quantitative PCR showed *nifH* (nitrogen fixation) and *napA* (denitrification) gene abundance increased with stubble addition while *amoA* (ammonia

monooxygenase) gene increased with nitrogen addition. The changes in microbial community structure in this study were significantly correlated to total C, N, K and Na.

A study conducted to find factors influencing soil microbial community structure in tomato cropping system found that cover cropping and polyethylene mulching had significant influence on soil microbial communities compared to soil temperature, moisture, pH and texture (Buyer et al., 2010). Microbial biomass measured using total PLFA significantly increased for cover crop treatments. The vetch cover crop increased the amount and proportion of Gram-negative bacteria, fungi and arbuscular mycorrhizal fungi.

Demoling et al. (2008) conducted a study to find the effect of nitrogen fertilization on soil microbial community structure in forest soil. Fungal biomass decreased more than bacterial biomass due to fertilization. The microbial community structure also changed due to fertilization when compared to unfertilized plots. Also, the bacterial growth rate was negatively affected by fertilization when compared to fungal growth rate (Demoling et al., 2008). Another study conducted in forest soil found that effect of fertilization on microbial community was strongly influenced by growth of tree species planted and litter input (Leckie et al., 2004a).

The effect of irrigation and tillage on soil microbial diversity was conducted in different landscapes following different irrigation and tillage practices such as native sagebrush vegetation (NSB), irrigated moldboard plowed crops (IMP), irrigated conservation – chisel –tilled crops (ICT) and irrigated pasture systems (IP) (Entry et al., 2008). The active bacterial, fungal and microbial biomass correlated with soil C, with the soil bacterial diversity highest in the subsurface soil of NSB and lowest in IMP soil. The lower diversity indices were found in soil with highest soil C such as ICT, IP and surface soil of NSB.

The above mentioned studies show microbial community structure are influenced by wide variety of factors including soil type, moisture, soil pH, soil texture and temperature along with management factors such as cover cropping, fertilizers, organic amendments and crop rotation. Many of these factors influence structure and composition of microbial community directly or indirectly.

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## **Chapter II. Response of Soil Microbial Community to Fertilization and Irrigation in a Loblolly Pine Plantation**

### **Abstract**

Nitrogen is often the limiting nutrient for loblolly pine growth in southeastern USA. Intensive management practices such as fertilization and irrigation can improve productivity and sustainability of forest soil. Furthermore, soil microbial communities are known to respond to different management practices. The objective of this study was to determine effects of fertigation on soil microbial biomass, activity and microbial community structure. The field experiment was arranged in randomized complete block factorial design with fertilization and irrigation as factors. Soil samples were collected from March 2006 to October 2007 for soil microbial biomass and activity, with the exception of December 2006 and February 2007. Soil samples collected in November 2006, May 2007 and September 2007 were used for soil microbial community analyses. Changes in soil microbial community structure were analyzed using phospholipid fatty acid (PLFA) analysis and automated ribosomal intergenic spacer analysis (ARISA). Soil microbial biomass was significantly higher for fertilized, irrigated and fertigation treatments than the controls. Basal respiration for fertilized plots was significantly higher than for the control plots. The FDA hydrolysis and basal respiration were significantly higher for fertilized as well as fertigation treatments. Soil organic carbon for fertigation treatment was significantly higher than the control and positively correlated to the fungal biomarker. The PLFA profiles showed that fertigation and sampling time influenced changes in the soil

microbial community structure. Bacterial and fungal ARISA profiles also revealed changes associated with fertigation and sampling time in the soil microbial community. These results indicate that fertigation was an important factor affecting soil microbial community structure and activity, leading to the conclusion that intensive management practices like fertigation can improve both chemical and microbiological properties in soil.

## **Introduction**

Forests cover about one-third of earth's land surface. Soil is an integral part of the forest ecosystem, providing nutrients and water for trees and other vegetation. It is estimated that one gram of soil can contain up to 10 billion bacteria and 200 m of fungal hyphae (Leake *et al.*, 2004; Roesch *et al.*, 2007; Trevors, 2010). Microorganisms in soil, especially bacteria and fungi, play an important role in nutrient cycling of C, N, S, P and other elements. In addition, soil microorganisms play a vital role in nutrient poor ecosystems for the acquisition of nutrients. Mycorrhizal fungi and nitrogen fixing bacteria are responsible for 80% of all nitrogen and up to 75% of phosphorous that is acquired by trees in temperate and boreal forests (van der Heijden *et al.*, 2008). Mycorrhizal fungi supply a range of limiting nutrients like N, P, Cu, Fe and Zn to plants in exchange for carbon. The arbuscular mycorrhizal fungi (AMF) and ecto-mycorrhizal fungi are abundant in temperate and boreal forests as well as in tropical forests. The ecto-mycorrhizal fungi can acquire N from forest litter through extensive hyphal networks that forage for nutrients. Because soil bacteria and fungi can respond relatively quickly to environmental changes, these microorganisms can be used to assess the effect of intensive management practices such as fertilization and irrigation. Soil microbial biomass is closely associated with above ground plant productivity and heterotrophic microorganisms depend directly on plant C inputs (e.g., root exudates) (Zak *et al.*, 1994).

Loblolly pine (*Pinus taeda L.*) forest covers about 14 million ha in the southeastern USA and are widely used for forest products such as pulp and sawn-log products. Productivity of loblolly pine plantation is limited due to low fertility of soils in the southeastern USA. In forest soil, nitrogen is often the most limiting nutrient for tree growth. Intensively managed pine plantations help in producing the wood and fiber required to satisfy the demands of growing populations. Practices like fertilization and irrigation have been widely used to increase forest productivity.

Forest productivity depends on the supply of nutrients and water. Fertigation involves application of fertilizer through irrigation water, which can reduce fertilizer usage and minimize ground water pollution due to nitrate leaching. Fertigation through drip irrigation can reduce overall fertilizer application rates and minimize adverse environmental impacts of agricultural and forest production. This technique is widely used in intensively managed vegetable crops ( e.g., tomato) as well as in high density fruit orchards (Mahajan and Singh, 2006; Neilsen et al., 1998). Fertilization of forest plantations has become increasingly important as an intensive management practice in recent years (Fox, 2000). Below ground response to intensive management practices such as fertilization and irrigation is critical in assessing soil carbon dynamics and long-term sustainability of forest plantation soil.

Soil water availability is important not only for plant growth but also for microbial activity. It plays a critical role in transport of nutrients and energy, cellular metabolism, and osmotic potential. Changes in water status can impact the physiology and structure of soil microbial communities; different groups of microorganism are affected differently by water potential changes (Drenovsky et al., 2004; Griffiths et al., 2003). An increase in soil water availability increased the ratio of bacterial to fungal biomass as well as increased fungal PLFA

in a tall grass prairie soil (Williams and Rice, 2007). PLFA analyses conducted in forest soils across different climatic zones showed that soil moisture and organic matter contents influenced microbial community composition (Brockett et al., 2012). A study conducted by Leckie et al. (2004) found soil organic carbon was correlated to microbial community structure. Another study conducted by Lauber et al. (2008) across different land-use types found that bacterial community composition was influenced by soil pH, while fungal community composition was associated with changes in soil nutrient status.

Soil microbial community analyses using culture-independent and community-level approaches have been widely used to study microbial response to environmental changes and soil management practices. In the current study, effects of fertilization and irrigation on soil microbial communities were investigated in a seven-year-old loblolly pine plantation. The specific objectives were to (1) study the effect of fertigation on soil microbial biomass and activity, and (2) characterize soil microbial communities in a loblolly pine plantation using culture independent techniques, i.e., PLFA analysis and ARISA.

## **Materials and Methods**

### ***Study Site***

The field experiment was located in the U.S. Department of Energy Savannah River Site, a National Environmental Research Park, near Aiken, South Carolina. The soil is a loamy siliceous, thermic Grossarenic Paleudult (Blanton series). Annual precipitation during the study period was 1034 mm in 2006 and 988 mm in 2007. The site was farmed until the 1950s and then converted to longleaf pine (*Pinus palustris* Mill.) and loblolly pine stands that were harvested in 1999. The 0.22 ha experimental plots were planted in February 2000 with loblolly pine seedlings at a spacing of 2.5 m x 3 m (1333 trees ha<sup>-1</sup>). The experiment was arranged in randomized

complete block factorial design with three blocks and irrigation and fertilization as factors. In irrigated treatment plots, water was supplied using drip irrigation lines every year since planting from April through September. During this study 501 mm and 832 mm of irrigation water was supplemented in 2006 and 2007, respectively, to meet evaporative demand and to provide favorable soil moisture for tree growth. Fertilizer was applied through the drip irrigation system at a rate of 120 kg N ha<sup>-1</sup> yr<sup>-1</sup> in 26 weekly split applications between April 1 and September 30 (Samuelson et al., 2009). A concentrated liquid nitrogen fertilizer solution of 7:0:7 (N: P: K) plus micronutrients (0.225% B, 0.01% Cu, 0.05 % Mn, 0.001% Mo and 0.03% Zn) was used in irrigation water. Phosphorous was applied separately through the drip irrigation system at a rate of 53 kg ha<sup>-1</sup> yr<sup>-1</sup>. The non-irrigated plots (control and fertilized plots) received only the minimum amount of water (130 mm annually) needed to apply liquid fertilizer and flush fertilizer lines. Chemical weed control was performed on all plots as needed. Air temperature and precipitation were measured using an on-site standard weather station (Dynamet, Dynamax, Houston, TX). A detailed description on the field experiment can be found in Coleman et al. (2004) and Samuelson et al. (2009).

#### ***Soil sampling and chemical analysis***

Soil samples were collected to a depth of 15 cm from March 2006 through October 2007 with the exception of December 2006 and February 2007 for a total of 18 sampling months. Detailed sampling procedures were described by Samuelson et al. (2009). Briefly, soil samples were collected at three positions 0.75 m apart along a 3-m transect randomly located within each plot. The center position of a transect was located within a row equidistant between trees and was closer to the drip irrigation line than the north and south sampling positions. Soil samples were transported to the laboratory on ice. Tree roots were removed by hand before determination

of moisture content and microbiological analyses. Sub-samples of soil collected at the beginning and end of the sampling period were air dried for total carbon and nitrogen analysis using a TruSpec CN analyzer (Leco Corp., St. Joseph, MI) and for soil pH determination using 1:1 soil/water suspension. Chemical analyses were performed in duplicate. Detailed sampling procedures are described by Samuelson et al. (2009).

#### ***Soil microbial biomass C***

Soil microbial biomass C was determined using the chloroform fumigation-incubation method (Horwath and Paul, 1994). Briefly, a soil sample (25 g dry weight equivalent of moist soil) adjusted to 50% of water holding capacity were placed in 150 ml beakers and pre-incubated for 5 days, fumigated with chloroform for 24 hours, and then incubated in closed Mason jars for additional 10 days at  $23 \pm 1^\circ\text{C}$ . Carbon dioxide produced during the incubation period was trapped in 1 M NaOH solution (5 ml) and quantified by titration using 0.25 M HCl.

#### ***Soil microbial activities***

Basal respiration was measured by static incubation-titrimetric determination (Alef and Nannipieri, 1995). Soil samples (25 g dry weight equivalent of moist soil) adjusted to 50 % of their water holding capacities were placed in 150 ml beakers preincubated for five days and then incubated for 10 more days in closed Mason jars at  $23 \pm 1^\circ\text{C}$ . Carbon dioxide produced during the incubation period was trapped in 1 M NaOH solutions (5 ml) and quantified by titration with 0.25 M HCl.

Fluorescein diacetate (FDA) hydrolysis was used to indicate overall soil microbial activity. A procedure modified following Dick et al. (1996) was used to quantify the FDA hydrolysis product. Briefly, 1 g of moist soil and 20 ml of 60 mM phosphate buffer (pH 7.6) were pre-incubated on a rotary shaker at  $23 \pm 1^\circ\text{C}$  for 15 min. Then 100  $\mu\text{l}$  of FDA solutions (4.8

mM) were added and the suspension was shaken for an additional 2 hours. The reaction was stopped by the addition of 20 ml of acetone. The suspension was centrifuged and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured using a Shimadzu UV-160 spectrophotometer at 490 nm.

### *Soil microbial community analyses*

The soil samples collected in November 2006, May 2007 and September 2007 from control and fertigation treatments were used for extractions of lipids and DNA for PLFA and ARISA analyses. One portion of each sample was frozen at -20°C and used for DNA extraction. The remaining soil samples were stored at 4°C for no more than 2 weeks before lipid extraction.

### *Phospholipid fatty acid (PLFA) analysis*

PLFA analysis, performed as described by Feng et al. (2003). It involved extraction of total lipids from soil, fractionation of total lipids, derivatization of fatty acids to form FAME, and analysis of FAMES. Briefly, duplicate field moist soil samples (8 g dry weight) were used for extracting total lipids using a single-phase citrate buffer-chloroform-methanol solution (1:2:0.8 v/v/v, pH 4). The phospholipids were separated from neutral lipids and glycolipids using silicic acid column chromatography. The phospholipids were then subjected to a mild alkaline methanolysis, and resulting FAMES were extracted using hexane and dried under nitrogen gas. The FAMES containing 19:0 methyl ester as an internal standard were analyzed using a Hewlett Packard 5890 gas chromatograph with a 25-m HP Ultra 2 capillary column and a flame ionization detector. FAME peaks were identified using the MIDI peak identification software (MIDI, Inc., Newark, DE) and quantified based on the internal standard added. The nomenclature for fatty acids used here was described by Feng et al. (2003) .

### *Automated ribosomal intergenic spacer analysis (ARISA)*

ARISA involves total community DNA extraction from soil, with PCR amplification using fluorescence-tagged oligonucleotide primers targeting intergenic transcribed spacer region, automated electrophoresis, laser detection of fluorescent DNA fragments, and analysis of ARISA peak banding patterns. Total soil DNA was extracted from 10 g of moist soil samples using a PowerMax™ Soil DNA Kit (MoBio Labs Inc., Carlsbad, CA) following the manufacturer's instructions. The extracted DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and stored at -80°C until use. Both bacterial and fungal-ARISA were performed to determine soil microbial community structure.

The bacterial primers used in the PCR reactions were ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale et al., 2004). The reaction mixture contained 12.5 µL of 2X GoTaq colorless master mix (Promega, Madison, WI), 25 µg of bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO), 0.2 µM of ITSF primer, 0.2 µM of ITSF primer labeled with IRD800 fluorochrome (LI-COR, Lincoln, Nebraska), 0.4 µM of ITSReub primer, 5 µL of template DNA (~20 ng) and nuclease free water to make the final volume 25 µL. Amplification was performed on a Biometra T-Gradient thermo cycler (Whatmann, Goettingen, Germany) using the following cycling parameters: 3 min at 94°C, 30 cycles of 60 s at 94°C, 30 s at 55°C and 60 s at 72°C, and a final 5 min at 72°C (Ranjard et al., 2000).

The fungal automated intergenic spacer analyses were performed using ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3') (Nicolardot et al., 2007; Ranjard et al., 2006). The reaction mixture (25 µL) consisted of 12.5 µL of 2X GoTaq colorless master mix, 25 µg of bovine serum albumin, 0.3 µM of ITS1F primer, 0.1 µM of ITS1F primer labeled with IRD800 fluorochrome, 0.4 µM of 3126T primer, and 5 µL of

template DNA (~20 ng). The thermocycling conditions were as follows: 4 min at 95°C, 35 cycles of 60 s at 95°C, 30 s at 53°C and 60 s at 72°C, and a final 7 min at 72°C (Kennedy et al., 2005a; Kennedy et al., 2005b).

A total of 5 µL amplified PCR products (2.5 µL from each replicate) was mixed with 2.5 µL of stop buffer (LI-COR Blue Stop Solution), denatured at 95°C for 2 min, and then placed on ice. The denatured PCR products (0.8-1 µL) were loaded on 6% polyacrylamide gel along with 0.8 µL of the IRD800 50-700 bp sizing standard (LI-COR). ARISA fragments were resolved under denaturing conditions for 9 hours at 1,500 V using the LI-COR 4300 sequencer. The laser scanned banding pattern image from the LI-COR sequencer was converted to 8-bit TIFF using Kodak 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY).

### ***Data analysis***

Microbial biomass C and soil microbial activity data were analyzed with SAS software (Version 9.1.3) using PROC MIXED, PROC GLIMMIX and correlation procedures. The mole percent distribution of PLFAs was analyzed by multivariate analyses using PROC PRINCOMP (principal component analysis), PROC STEPDISC (stepwise discriminatory analysis), and PROC CANDISC (canonical discriminant analysis). Analysis of PLFA profiles was performed using a set of 36 fatty acids that were present in most of the samples. Total bacterial PLFA were calculated by sum of 14:0, 15:0, a15:0, i15:0, i16:0, 16:1 $\omega$ 5, 16:1 $\omega$ 7, 16:1 $\omega$ 9, 17:0, a17:0, i17:0, 18:0 18:1 $\omega$ 7, and cy19:0. The ectomycorrhizal fungi were assessed using 18:2 $\omega$ 6,9 (Hogberg et al., 2010; Kaiser et al., 2010; Nilsson et al., 2005). The physiological stress was indicated by the ratio cy19:0/18:1 $\omega$ 7 (Jackson et al., 2003; Moore-Kucera and Dick, 2008). The PLFA 18:1 $\omega$ 7c is the precursor molecule of cy19:0, and the ratio of these two fatty acids are used as a stress

indicator ratio. The arbuscular mycorrhizal fungi were assessed using the fatty acid 16:1 $\omega$ 5 (Joergensen and Wichern, 2008; Madan et al., 2002).

ARISA banding pattern images were processed using the software BIONUMERICS ver. 5.0 (Applied Maths, Belgium). Each image was normalized using the 50-700 bp sizing standard as the external reference standard, which allowed for comparison of multiple gels. Levels of similarity between DNA fingerprints were compared using a densitometric curve-based method with the Cosine similarity coefficient after the conversion, normalization, and background subtraction with mathematical algorithms of banding patterns. Dendrograms were developed using cluster analysis performed with the Cosine similarity coefficient and unweighted pair-group method using average linkages (UPGMA). The position tolerance was set at an optimization of 0.5%, and band comparison was made using a position tolerance of 1%. Principal components analysis was used to determine distribution of fingerprint patterns according to different treatments.

An Analysis of Similarity (ANOSIM) was used to test whether observed separation among treatment groups based on the ARISA profile was significant (Clarke, 1993; Edenborn et al., 2011; Kent et al., 2007). This procedure uses the Bray-Curtis similarity matrix to calculate the test statistic R (Clarke, 1993). ANOSIM is analogous to a one-way ANOVA based on multi-group data and generates an R value that ranges from -1 to +1, with values greater than 0 indicating greater dissimilarity between treatment groups than among samples and P value indicating level of significance. Generally, R lies between 0 and +1; values smaller than zero were considered unlikely because they would indicate dissimilarity among replicates within treatments rather than between treatments (Chapman and Underwood, 1999). The magnitude of

R indicates the degree of separation between treatments, with score of +1 indicating complete separation and 0 indicating no separation between treatments.

## Results

### *Effect of fertilization and irrigation on soil microbial biomass and activity*

Soil organic carbon contents for fertilization and fertigation treatments were significantly higher than the control (Table 2.1). The soil pH was significantly higher for the irrigation treatment than control and fertilization treatments (Table 2.1). Daily average soil temperature and daily rainfall from March 2006 to October 2007 are shown in Figure 2.1. The average soil temperature in November 2006, May 2007 and September 2007 was 13.5, 21.5 and 23.9°C while cumulative rainfall during these three months was 11, 3.9 and 7.8 mm, respectively. Microbial biomass C ranged from 107 to 190  $\mu\text{g C g}^{-1}$ , basal respiration ranged from 33.8 to 55.7  $\mu\text{g g}^{-1}$  and microbial activity measured using FDA hydrolysis ranged from 12.7 to 36.8  $\mu\text{g g}^{-1} \text{ hr}^{-1}$  for all treatments and sampling months. Microbial biomass C for fertigation, fertilization and irrigation treatments were 14%, 9% and 14% higher than the control treatment respectively (Table 2.1). Basal respiration was the highest in the fertilization treatment.

The analysis of the variance table for microbial biomass C and activity from March 2006 to October 2007 showed that the main effects (i.e., treatment, position and month) were significant at  $P \leq 0.05$  except for treatment effect on FDA hydrolysis (Table 2.2). Basal respiration had a significant two-way interaction between treatment and position (Table 2.2). Basal respiration for the fertilization and fertigation treatments at the center sampling position was significantly higher than other treatments (Fig. 2.2 A). The microbial activity measured using FDA hydrolysis had significant two-way interactions among treatment, month and

position. FDA hydrolysis was higher for all treatments in the center sampling position (Fig. 2.2 B).

The microbial biomass C, basal respiration and FDA hydrolysis averaged for fertigation and control treatments showed variation across the 18 month sampling period (Fig. 2.3). Microbial biomass C for control treatment was higher during October 2006 and September 2007 compared to fertigation treatment, and the lowest values were observed during March 2006 and January 2007 for both treatments. Basal respiration was also higher in November 2006, March, June, July and September 2007 for control treatment when compared to fertigation treatment. The FDA hydrolysis was higher for control treatment when compared to fertigation treatment November 2006, March and September 2007, and lowest values were observed during March 2006, and July 2006. Soil moisture content was higher for fertigated treatment in April 2006, April, May and October 2007 compared to control treatment.

Microbial biomass C for the control and fertigation treatments did not change significantly for the three sampling periods when microbial community analyses were performed (Table 2.3). The basal respiration for the control treatment in September 2007 was significantly higher compared to May 2007, but it did not change significantly in the three sampling periods for the fertigation treatments. The FDA hydrolysis for the control and fertigation treatments did not change significantly for the three sampling periods (Table 2.3). Soil moisture for the control treatment in September 2007 was higher than the other two sampling months.

#### ***Interactions between soil microbial biomass and activity variables***

Correlation analysis for different treatments showed that basal respiration was significantly correlated with microbial biomass C for all treatments with correlation coefficients ranging from 0.41 to 0.61 (Table 2.4). The FDA hydrolysis was significantly correlated with

microbial biomass C for control, irrigation, and fertigation treatments. Correlations between basal respiration and FDA hydrolysis were significant for all treatments with correlation coefficients ranging from 0.34 to 0.48. Microbial biomass C was correlated with soil moisture for fertigation treatment ( $r = 0.21$ ).

### ***Soil microbial community structure by PLFA analysis***

Soil microbial community analyses were performed on soil samples collected from the control and fertigation treatments in November 2006 and May and September 2007. PLFA analysis identified a total of 36 fatty acids that were consistently present in the samples for the three sampling times; they were used for data analysis. These fatty acids ranging in carbon length from C12 to C20 consisted of saturated, mono and polyunsaturated, methyl-branched and cyclopropane fatty acids.

Principal components analysis of PLFA profiles showed that 80% of the total sample variation was explained by the first three principal components (PCs). The first and third principal components (PC 1 and PC 3) accounted for 57% and 10 % of the total variation, respectively (Fig. 2.4). PC 1 separated out the May 2007 sampling period. PC 3 separated the treatment effect in May and September 2007. Fertigation treatment as well as sampling time influenced changes in soil microbial community structure. These changes were driven by bacterial population as indicated by influential fatty acids with highest loadings such as 18:0, cy19:0 and 18:1 $\omega$ 7c for the first and third principal components (Table 2.5). The PLFAs 16:0 have not been associated with any specific group of microorganisms. The PLFAs 18:0, cy19:0 and 18:1 $\omega$ 7c are biomarker fatty acids for bacteria (Findlay, 2004; Paul and Clark, 1996).

PLFA profiles were also analyzed by canonical discriminant analysis. The first two canonical axes of this analysis explained 78% of total sample variation (Fig. 2.5). The first

canonical axis (Can 1) explained 64% of the total variation, and the second axis (Can 2) explained 14% of the total variation. The canonical discriminant analysis plot showed separate clusters by sampling time. Data points for fertigation and control treatments formed separate clusters except for September. The bacterial biomarkers (18:0 and 10Me16:0), and fungal biomarkers (18:3 $\omega$ 6 and 20:1 $\omega$ 9) were influential for the first canonical axis (Table 2.6). The influential fatty acids for the second canonical axis were bacterial biomarker (i15:0) and fungal biomarker (18:3 $\omega$ 6) (Table 2.6). The principal components analysis of PLFA profile revealed better separation of treatment effect except for the November sampling time in which there was overlapping of data points for fertigation and control treatments. The canonical discriminant analysis showed a better separation of the three sampling times compared to PCA. The data points for North sampling position in September 2007 were not within the cluster for control and fertigation treatments.

Bacterial biomarkers in November 2006 and May 2007 were higher for the fertigation treatment for all three sampling positions compared to September 2007 (Table 2.7). The fungal biomarker (18:2 $\omega$ 6,9) in November 2006 for the fertigation treatment was higher in the Center sampling position compared to other sampling positions. The fungal biomarker in May 2007 for fertigation treatment was higher in the South sampling position compared to other sampling positions. The fungal to bacterial ratio in May 2007 was higher in the South position for the control treatment compared to the North sampling position. The fungal to bacterial ratio in November 2006 and September 2007 were higher in the Center position for the fertigation treatment (Table 2.7).

The arbuscular mycorrhizal fungal biomarker in November 2006 for the South sampling position was higher compared to September 2007 in both control and fertigation treatments. The

stress indicator ratio (cy19:0/ precursor ratio) in November 2006 for the Center sampling position was higher for the fertigation treatment compared to the control treatment. The stress indicator ratio in the Center sampling position was higher for fertigation in November 2006 (Table 2.7). Total PLFA in November 2006 for fertigation treatment was higher in the Center sampling position compared to the other two sampling positions (Table 2.7).

Correlation analysis between PLFA biomarkers and soil properties revealed that bacteria ( $r= 0.31$ ), AMF ( $r= 0.26$ ), stress indicator ratio ( $r= 0.31$ ), and total PLFA ( $r= 0.27$ ) were significantly correlated with microbial biomass C (Table 2.8). The basal respiration was only significantly correlated with stress indicator ratio ( $r= 0.32$ ). The FDA hydrolysis was significantly correlated with bacteria ( $r= 0.46$ ), fungi ( $r= 0.31$ ), AMF ( $r= 0.30$ ), stress indicator ratio ( $r=0.53$ ), and total PLFA ( $r= 0.42$ ). The soil moisture content was negatively correlated with bacteria ( $r= -0.24$ ), fungi ( $r=-0.18$ ) and total PLFA ( $r=-0.25$ ). The soil organic carbon was only positively correlated with fungal biomarker, 18:2 $\omega$ 6 ( $r=0.29$ ).

#### ***Soil microbial community structure by ARISA analysis***

The DNA concentration extracted from the loblolly pine plantation soil ranged from 3.6 to 16.5  $\mu\text{g g}^{-1}$  dry soil. The number of bacterial ARISA bands ranged from 52 to 156 for November 2006, from 102 to 147 for May 2007 and from 44 to 87 for September 2007. The bacterial ARISA bands for the control treatment ranged from 44 to 156, and, for the fertigation treatment, from 58 to 131. The number of fungal ARISA bands ranged from 66 to 102 for November 2006, from 65 to 110 for May 2007 and from 48 to 133 for September 2007. The fungal ARISA bands for the control treatment ranged from 66 to 133 and, for the fertigation treatment, from 48 to 116.

PCA was used to analyze bacterial and fungal ARISA profiles (Fig. 2.6). The first and second principal components (PC1 and PC 2) explained 60% of the total sample variance for bacterial ARISA and 54 % for fungal ARISA. PC 1 for bacterial ARISA separated the data points by sampling time while the PC 2 separated the data points by treatments. The bacterial ARISA profile showed that data points for fertigation and control treatments for the three sampling times formed their own individual clusters. The fungal ARISA profile showed that PC 1 for fungal ARISA clearly separated the fertigation and control treatments while PC 2 separated the data points for the control treatment by sampling time. Fungal ARISA profiles showed a better separation of the treatment effect and sampling time than bacterial ARISA profiles.

The analysis of similarity for bacterial ARISA showed significant separation for sampling time ( $R= 0.32$ ,  $P\leq 0.05$ ), but no significant differences were observed for treatments and sampling position (Table 2.9). Significant differences were found between fertigation and control treatments for fungal ARISA analysis of similarity( $R= 0.20$ ,  $P\leq 0.05$ ), but no significant differences were observed for sampling time and position.

## **Discussion**

Soil microbial biomass C and activity increased due to fertigation when compared to the control treatment. Fertilization increased basal respiration, microbial biomass C, and microbial activity in loblolly pine plantation soil (Samuelson et al., 2009). This study found that fertilization can influence basal respiration due to wide variety of factors including changes in microbial biomass, microbial diversity, and belowground litter substrate quantity and quality. This observation was consistent with previous studies conducted in warm-temperate hard wood forest soil and forest soil planted with loblolly pine (Allen and Schlesinger, 2004; Gallardo and Schlesinger, 1994). The increase in microbial biomass C can be due to increase in availability

carbon substrate as root exudates or increase in litter due to increase in net primary productivity of pine trees. Another study conducted in a 7-year old loblolly pine plantation found that nitrogen significantly reduced microbial biomass (Lee and Jose, 2003). However that study used a maximum nitrogen fertilization rate twice that of this study, and the soil organic carbon content of soil was higher compared to this study.

Microbial biomass C determined by chloroform-fumigation incubation was significantly correlated with total PLFAs that are considered as viable microbial biomass. The observation in this study is consistent with the previous reports of significant correlation between total PLFAs and microbial biomass C (Feng et al., 2003; Zelles et al., 1995).

The fungal to bacterial PLFA ratio ranged from 0.02 to 0.11, which is consistent with fungal to bacterial ratio found in other studies conducted using forest soil (Frostegard and Baath, 1996; Leckie, 2005). The influential biomarker fatty acids for PCA were bacterial biomarker 18:0, aerobic bacterial biomarker 18:1 $\omega$ 7 and anaerobic bacterial biomarker cy19:0. The cy19:0 to precursor ratio decreased due to fertigation and irrigation. These results support the finding that PLFA stress indicator ratio decreases as water availability increases (Williams and Rice, 2007). The stress indicator ratio measured using cy19:0 to precursor ratio was higher for fertigation treatment compared to the control in November 2006. This increase in the stress indicator ratio can be due to starvation of bacteria due to low carbon, low oxygen, higher temperature or an increase in pH (Ratledge and Wilkinson, 1988). Kaiser et al. (2010) found fungal biomarkers 18:2 $\omega$ 6,9 and 18:1 $\omega$ 9 in forest soil and these fungal biomarkers, especially 18:2 $\omega$ 6,9, are associated to ectomycorrhizal fungi. These results support the finding that forest soil can contain higher concentration of fungal biomarkers especially 18:2 $\omega$ 6,9. The arbuscular mycorrhizal fungal (AMF) biomarker (16:1 $\omega$ 5) was also detected in this study. Results of this

study support the finding that this biomarker is associated with plant roots for absorption of plant nutrients (Allison and Miller, 2005). The fungal biomarker (18:2 $\omega$ 6,9) can be used as indicator for saprophytic and ectomycorrhizal fungi in forest soil (Joergensen and Wichern, 2008). The fungal biomarker (18:2 $\omega$ 6,9) was positively correlated to soil organic carbon, which agrees with a study conducted in forest soils containing oak, beech, spruce-fir-beech and pine (Hackl et al., 2005).

A similar microbial community study conducted in forest soil by Leckie et al. (2004) using PLFA and RISA found that soil organic carbon content influences microbial community structure. PLFA and fungal ARISA profiles showed better separation of treatment effect and sampling time than bacterial ARISA. In this study PLFA and fungal ARISA profiles showed that fertigation and sampling time influenced soil microbial community structure. These results showed that polyphasic studies using PLFA and PCR-based methods with different degrees of resolution can help identify changes in soil microbial community (Ramsey et al., 2006).

The culture independent molecular method- bacterial and fungal ARISA has been used to study changes in microbial community due to influence of leaf litter, tree species, carbon amendments and ecosystem restoration in forest soil (Banning et al., 2011; Khodadad et al., 2010; Lejon et al., 2005; Prevost-Boure et al., 2011) . The results from the current study show that soil microbial community analyses like PLFA and ARISA can be used to detected changes in soil microbial community due to fertigation. Shifts in soil microbial community composition can influence changes in nutrient cycling and thereby influence soil productivity.

## **Conclusions**

Results from the study indicate that fertigation caused significant changes in microbial biomass C. There was significant interaction between treatments and sampling position for basal

respiration with the Center sampling position significantly higher for fertilization and fertigation treatments. The FDA hydrolysis for the Center position was significantly higher for all treatments. The soil organic carbon was significantly higher for fertilized and fertigation treatments. Soil pH was higher in the irrigated treatment compared to the control and fertilized treatments. PLFA profiles showed that fertigation as well as sampling time influenced changes in the soil microbial community. Bacterial and fungal ARISA profiles revealed changes in soil microbial community associated with fertigation. The PLFA and fungal ARISA profiles showed that fertigation and sampling time influenced soil microbial community structure. Soil organic carbon for fertigation treatment was significantly higher than the control and positively correlated with the fungal biomarker. These results indicate that fertigation had a positive effect on soil microbial community structure and activity along with soil chemical and biochemical properties in soil.

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**Table 2.1 Soil chemical parameters, microbial biomass C, and basal respiration for different treatments during 18 months study period**

<b>Treatment</b>	<b>Soil organic carbon (mg g<sup>-1</sup> of soil)</b>	<b>Soil pH<sub>water</sub></b>	<b>Microbial biomass C (µg C g<sup>-1</sup> soil)</b>	<b>Basal respiration (µg CO<sub>2</sub>-C g<sup>-1</sup> soil)</b>
Control	5.9	5.6	133.0	46.4
Fertilization	7.5	5.6	151.7	52.7
Irrigation	6.5	6.0	145.5	41.9
Fertigation	7.5	5.8	152.1	45.4
LSD <sub>(0.05)</sub>	1.5	0.4	8.5	5.4

**Table 2.2 Probability values for effects of treatment, sampling position, and month on microbial biomass and activity**

<b>Source of variation</b>	<b>Microbial biomass C</b>	<b>Basal respiration</b>	<b>FDA hydrolysis</b>
Treatment	0.015	0.005	NS*
Position	0.015	<0.001	<0.001
Month	<0.001	<0.001	<0.001
Treatment x Position	NS*	0.001	0.001
Month x Treatment	NS*	NS*	0.001
Month x Position	NS*	NS*	<0.001
Treatment x Position x Month	NS*	NS*	NS*

\* Not significant at  $P \geq 0.05$

**Table 2.3 Microbial biomass C, basal respiration, FDA hydrolysis and soil moisture by sampling time**

Month	Microbial biomass C		Basal respiration		FDA hydrolysis		Soil moisture	
	CK ( $\mu\text{g C g}^{-1}$ soil)	FI	CK ( $\mu\text{g CO}_2\text{-Cg}^{-1}$ soil)	FI	CK ( $\mu\text{g fluroscein g}^{-1}$ hr $^{-1}$ )	FI	CK	FI
Nov 2006	139.4	153.2	59.5	45.7	40.1	22.5	0.05	0.04
May 2007	141.6	122.6	38.4	43.4	26.1	20.9	0.03	0.04
Sep 2007	177.1	150.3	81.1	34.9	40.5	18.5	0.06	0.05
LSD <sub>(0.05)</sub>	47.6	50.7	36.2	16.2	21.7	12.4	0.01	0.01

CK= Control and FI= Fertigation

**Table 2.4 Correlation analysis of microbial biomass C, microbial activity, and moisture content for different treatments**

	Microbial biomass C				Basal respiration				FDA hydrolysis				Soil moisture			
	CK	FERT	IRR	FI	CK	FERT	IRR	FI	CK	FERT	IRR	FI	CK	FERT	IRR	FI
<b>Microbial biomass C</b>	1.00	1.00	1.00	1.00	0.44†	0.41†	0.53†	0.61†	0.40†	0.11	0.31†	0.25†	0.05	0.01	0.06	0.21*
<b>Basal respiration</b>	0.44†	0.41†	0.53†	0.61†	1.00	1.00	1.00	1.00	0.36†	0.34**	0.48†	0.39†	0.07	0.02	0.02	0.05
<b>FDA hydrolysis</b>	0.40†	0.11	0.31†	0.25†	0.36†	0.34**	0.48†	0.39†	1.00	1.00	1.00	1.00	0.05	0.01	-0.12	-0.01
<b>Soil moisture</b>	0.05	0.01	0.06	0.21*	0.07	0.02	0.02	0.05	0.05	0.01	-0.12	-0.01	1.00	1.00	1.00	1.00

†Significant at  $P \leq 0.01$ , \*\*Significant at  $P \leq 0.05$ , and \*Significant at  $P \leq 0.1$

CK= Control, FERT= Fertilization, IRR= Irrigation, FI= Fertigation

**Table 2.5 PLFA having scores  $\geq |\pm 0.42|$  for principal component loadings**

<b>Fatty acid</b>	<b>Score</b>	<b>Specificity as a biomarker*</b>
PC 1		
18:0	-0.81	Bacteria
16:0	-0.42	Nonspecific
PC 3		
cy19:0	-0.60	Anaerobic bacteria
18:1 $\omega$ 7	0.51	Aerobic bacteria

\*Source: Findlay (2004), and Paul and Clark (1996)

**Table 2.6 PLFA having scores  $\geq |\pm 0.42|$  for canonical loadings**

<b>Fatty acid</b>	<b>Score</b>	<b>Specificity as a biomarker*</b>
Can 1		
18:0	0.92	Bacteria
10Me16:0	-0.88	Bacteria
18:3 $\omega$ 6	0.83	Fungi
20:1 $\omega$ 9c	0.83	Fungi
16:0	0.70	Nonspecific
i15:0	0.66	Bacteria
Can 2		
i15:0	0.61	Bacteria
18:3 $\omega$ 6	0.42	Fungi

\*Source: Findlay (2004), and Paul and Clark (1996)

**Table 2.7 PLFA biomarkers and PLFA ratios in nmol g<sup>-1</sup>**

Month	Sampling position	Bacteria		Fungi		Fungi/bacteria ratio		AMF (16:1ω5)		Cy19:0/precursor ratio		Total PLFA	
		CK	FI	CK	FI	CK	FI	CK	FI	CK	FI	CK	FI
Nov 2006	North	26.3	22.9	1.9	1.0	0.07	0.02	1.6	1.5	1.2	0.8	44.1	36.7
	Center	24.2	33.8	2.4	4.7	0.09	0.13	1.1	1.0	1.1	2.2	41.3	58.8
	South	23.1	20.8	2.5	1.4	0.11	0.06	1.3	1.1	0.8	1.1	39.3	34.1
May 2007	North	26.9	23.5	1.4	1.0	0.05	0.04	1.1	1.0	1.6	0.9	42.7	38.3
	Center	32.7	24.3	2.3	1.8	0.07	0.08	1.1	0.9	1.7	0.9	54.4	41.8
	South	29.6	26.9	2.3	3.7	0.09	0.11	1.3	0.8	1.1	1.2	49.3	46.9
Sep 2007	North	20.2	8.0	1.0	0.3	0.05	0.04	0.9	0.5	1.5	0.6	30.4	12.4
	Center	13.7	13.5	0.6	1.7	0.05	0.10	0.6	0.5	1.3	0.9	21.5	23.6
	South	15.6	8.4	1.1	0.4	0.08	0.05	0.6	0.4	1.3	0.6	25.3	11.8
LSD <sub>(0.05)</sub>		13.1	11.9	1.2	2.4	0.04	0.05	0.5	0.6	0.9	0.7	20.6	21.5

**Table 2.8 Correlation between PLFA biomarkers and soil properties**

Soil properties	PLFA biomarkers and ratio				Total PLFA
	Bacteria	Fungi	AMF	Cy 19:0/precursor ratio	
Microbial biomass C	0.31	NS*	0.26	0.31	0.27
Basal respiration	NS*	NS*	NS*	0.32	NS*
FDA hydrolysis	0.46	0.31	0.30	0.53	0.42
Soil moisture content	-0.24	-0.18	NS*	NS*	-0.25
Soil organic carbon	NS*	0.29	NS*	NS*	NS*

\* Not significant at  $P \geq 0.07$

**Table 2.9 Analysis of similarity (ANOSIM) R statistic for bacterial and fungal ARISA**

	<b>B-ARISA</b>		<b>F-ARISA</b>	
	<b>R statistic</b>	<b>P value</b>	<b>R statistic</b>	<b>P value</b>
Sampling time	0.32	0.01	-	NS*
Treatments	-	NS*	0.20	0.02
Sampling positions	-	NS*	-	NS*

\*Not significant at  $P > 0.05$

## Figure Legends

Fig. 2.1 Daily average soil temperature and daily rainfall at the study site from March 2006 to October 2007. Sampling months for microbial community analyses are marked with asterisks.

Fig. 2.2 Basal respiration and FDA hydrolysis for different treatments and sampling positions.

Fig. 2.3 Microbial biomass C, basal respiration, FDA hydrolysis and soil moisture content for control and fertigated treatments in the loblolly pine plantation over sampling time.

Fig. 2.4 Principal component analysis of PLFA profiles for control (CK) and fertigation treatments from three sampling positions: North (N), Center (C), and South (S).

Fig. 2.5 Canonical discriminant analysis of phospholipid fatty acids for control (CK) and fertigation (FI) treatments from three sampling positions: North (N), Center (C), and South (S).

Fig. 2.6 Principal component analysis of ARISA profiles (a) bacteria and (b) fungi for control (CK) and fertigation (FI) treatments from three sampling positions: North (N), Center (C), and South (S).

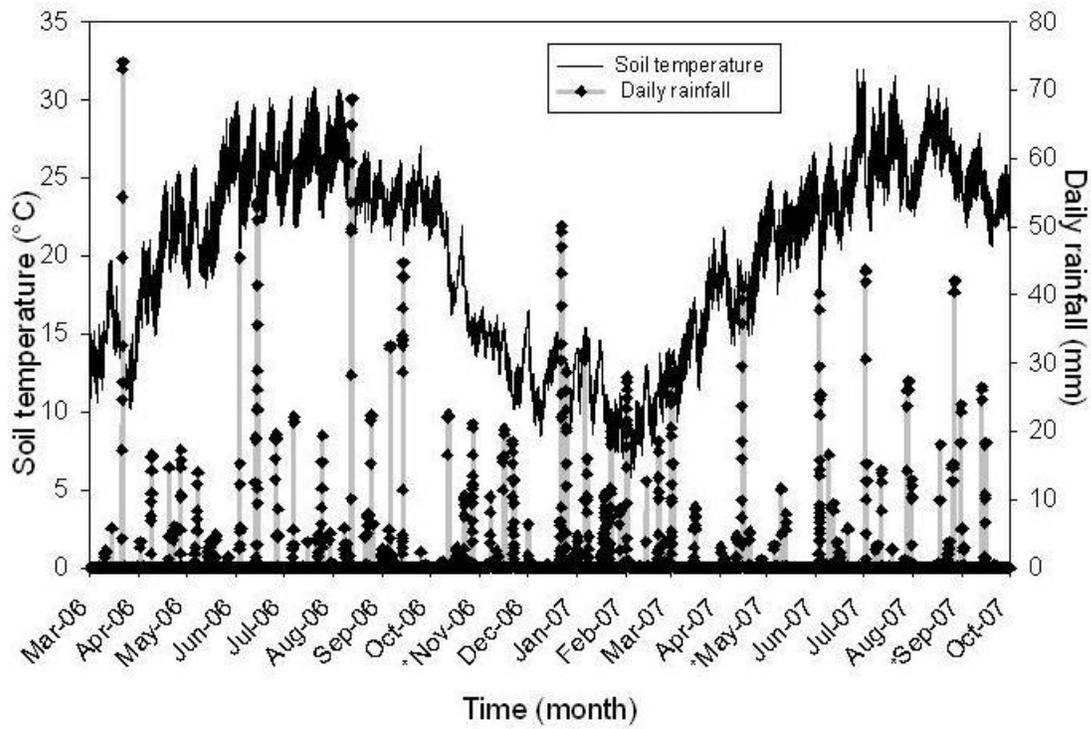


Fig. 2.1 Daily average soil temperature and daily rainfall at the study site from March 2006 to October 2007. Sampling months for microbial community analyses are marked with asterisks.

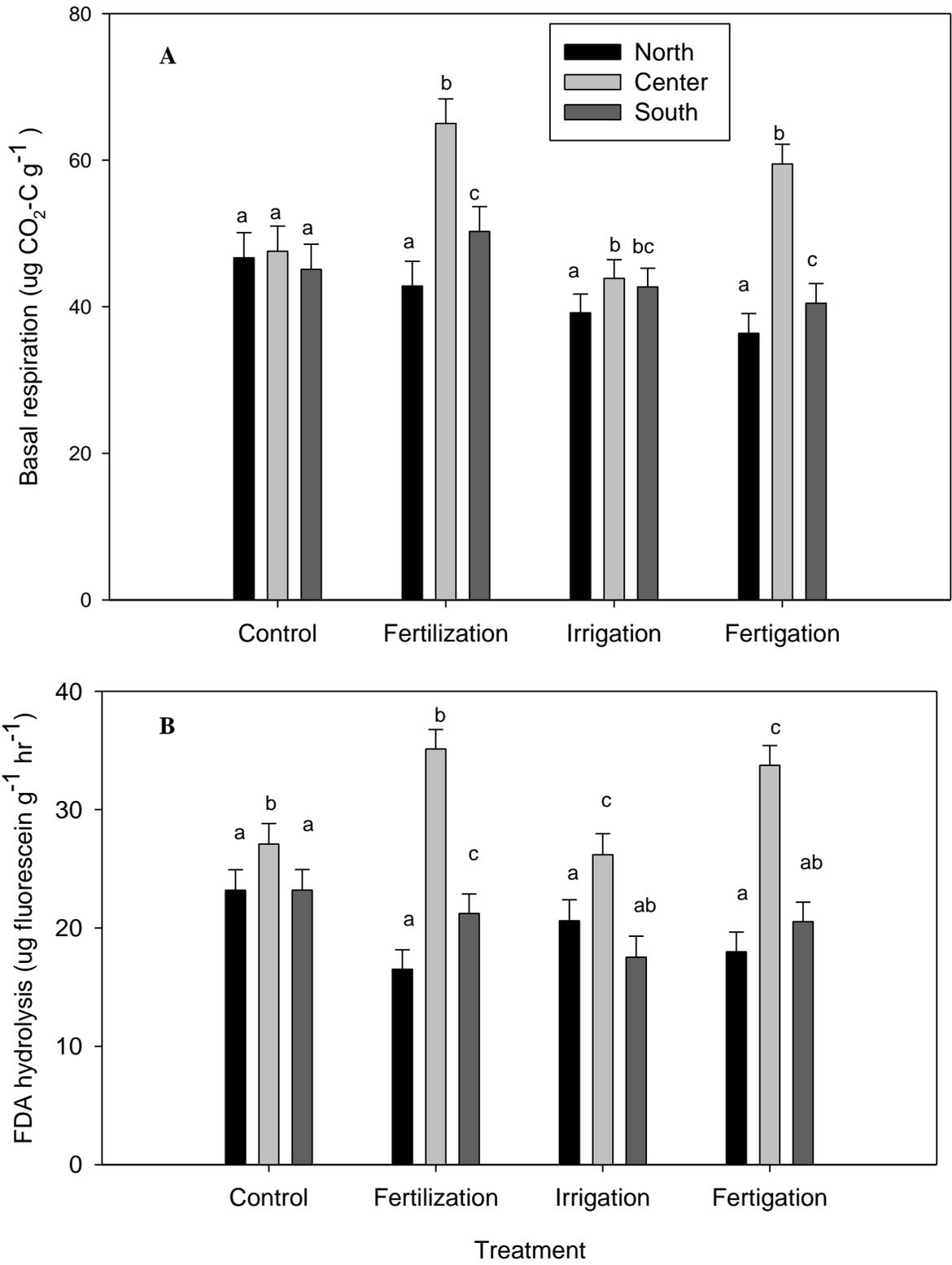


Fig. 2.2 Basal respiration (A) and FDA hydrolysis (B) for different treatments and sampling positions

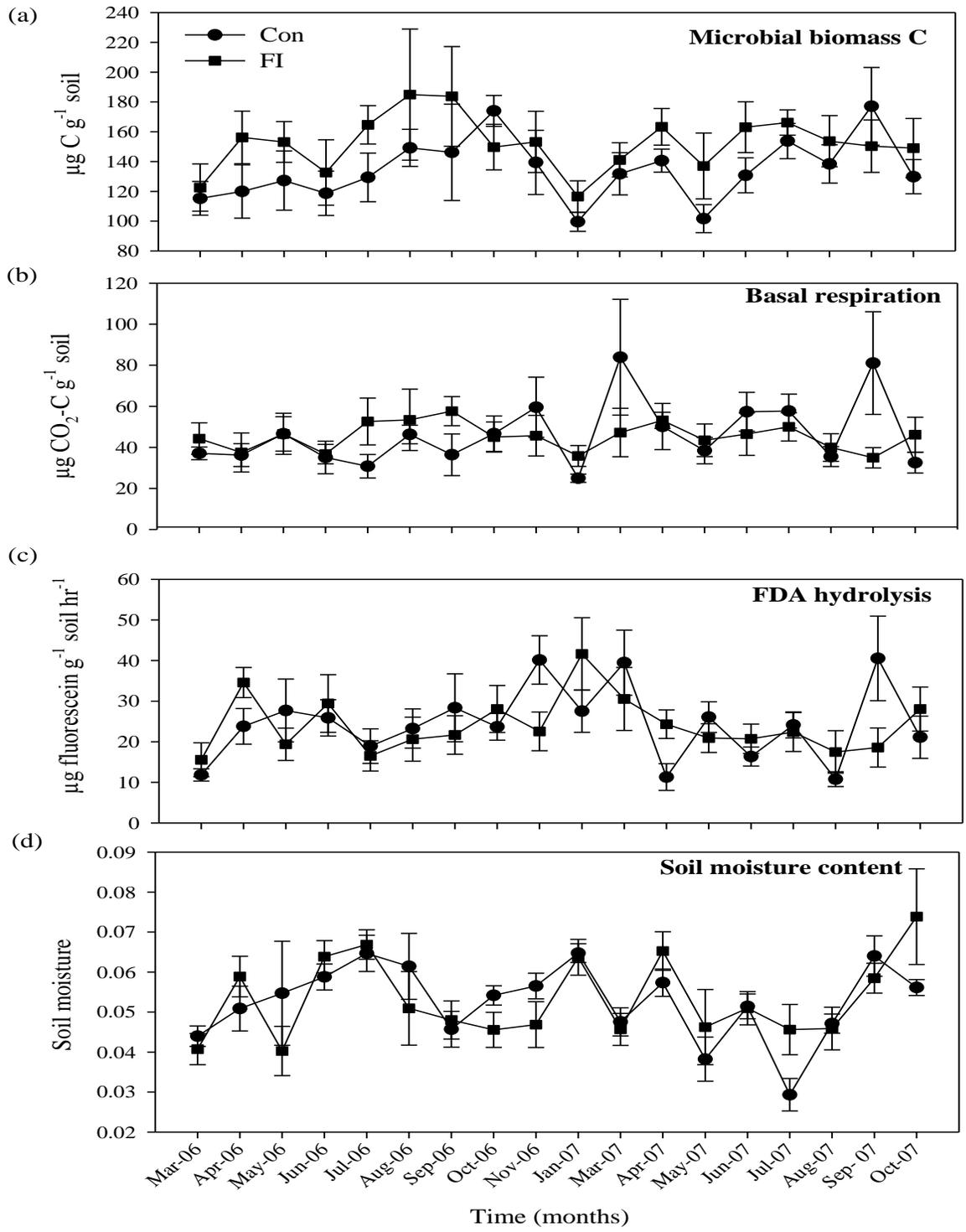


Fig. 2.3 Microbial biomass C, basal respiration, FDA hydrolysis and soil moisture content for control and fertilized treatments in the loblolly pine plantation over sampling time.

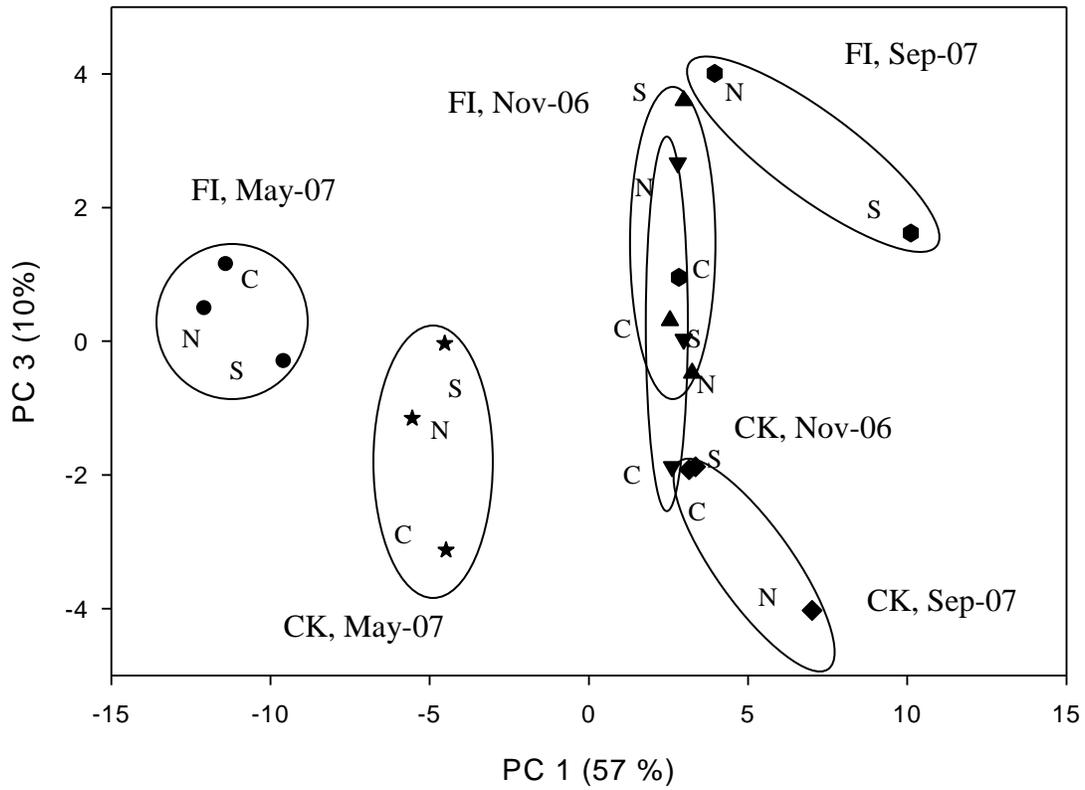


Fig. 2.4 Principal component analysis of phospholipid fatty acids for control (CK) and fertigation (FI) treatments from three sampling positions: North (N), Center (C), and South (S).

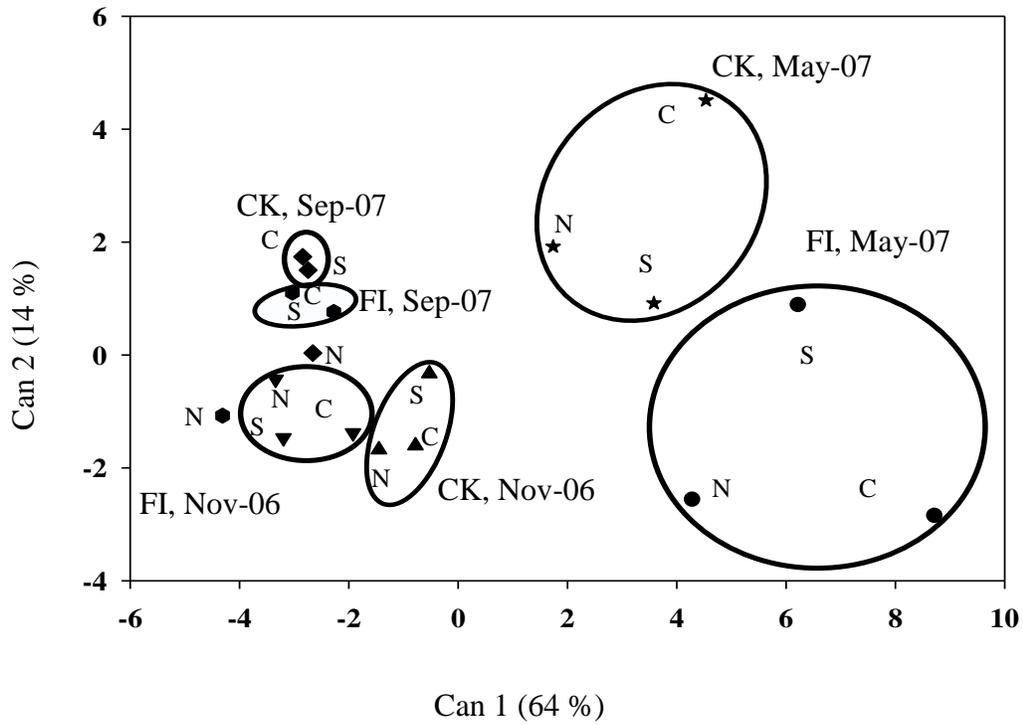


Fig. 2.5 Canonical discriminant analysis of phospholipid fatty acids for control (CK) and fertigation (FI) treatments from three sampling positions: North (N), Center (C), and South (S).

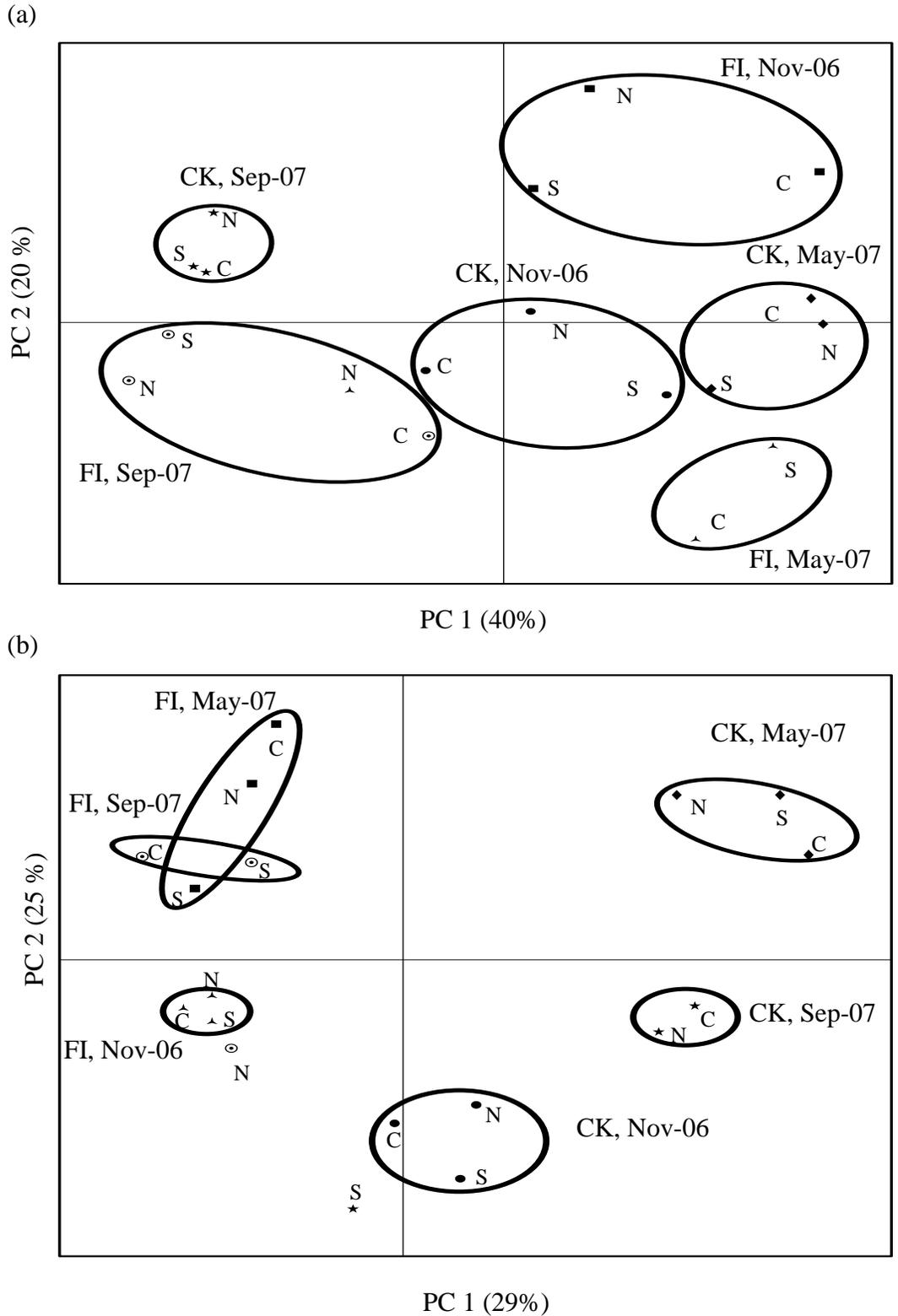


Fig. 2.6 Principal component analysis of ARISA profiles (a) bacteria and (b) fungi for control (CK) and fertigation (FI) treatments from three sampling positions: North (N), Center (C), and South (S).

### **Chapter III. Influence of Nitrogen Sources and Soil pH on Soil Microbial Communities in a Long-Term Crop Rotation System**

#### **Abstract**

Agricultural management practices such nitrogen fertilization and lime application can influence soil physical and chemical characteristics and may cause changes in soil microbial community structures. Long-term field experiments can serve as a useful tool in studying the effects of agricultural management practices on soil physical, chemical and biological properties. The objective of this study was to examine effects of nitrogen sources and soil pH on soil microbial communities in a long-term crop rotation system. The field experiment, Cullars Rotation, consisting of a three-year rotation of cotton, corn, wheat, soybean and clover, was established in 1911 on a Marvyn sandy loam soil. Soil samples were collected in June and October 2008 and February 2009 at two depths (0-5 and 5-15 cm). Soil pH values for no input and no lime treatments were significantly lower than other treatments. Analysis of variance for microbial biomass carbon and basal respiration showed that nitrogen sources, soil depth, and the interaction effects were significant ( $P=0.001$ ), and total PLFA (phospholipid fatty acid) was significant for nitrogen sources and soil depth ( $P=0.05$ ). Analysis of variance for microbial biomass C, basal respiration and total PLFA showed significant sampling time and soil depth effects ( $P=0.05$ ). Multivariate analyses of phospholipid fatty acids and automated ribosomal intergenic spacer analyses profiles showed that changes in soil microbial communities were associated with nitrogen sources and soil pH. The fungal biomarker (18:2 $\omega$ 6,9) was lower in the

surface soil for no input treatment than inorganic fertilizer treatments. Bacterial biomarkers, the fungal biomarker (18:2 $\omega$ 6,9) and the arbuscular mycorrhizal biomarker (16:1 $\omega$ 5) were positively correlated to soil organic carbon content. PLFA and ARISA profiles showed no input, no lime, and no nitrogen with no legume treatments. These results indicate that changes in soil microbial communities were associated with soil pH and nitrogen sources (inorganic fertilizer vs legumes).

## **Introduction**

Agricultural management practices like fertilization, lime application and use of cover crops are important in maintaining sustainability of agroecosystem. Inorganic fertilizers and leguminous cover crops are used to enhance crop productivity by increasing nitrogen availability to plants. Nitrogen inputs to soil also influence changes in soil microbial community structure. Studying the changes in the size, composition and activity of soil microbial communities due to different types of nitrogen inputs help determine fertilizer management practices that can improve nutrient cycling and carbon sequestration.

Soil microbial communities in agricultural soil are influenced by many factors. The different physical, chemical and biological factors that can influence microbial community in soil are moisture (Buckley and Schmidt, 2001a), tillage (Buckley and Schmidt, 2001b; Feng et al., 2003), soil pH (Lauber et al., 2009), soil carbon availability (Ramirez et al., 2010), fertilization (Hallin et al., 2009) and crop rotation (Ngosong et al., 2010). The application of fertilizers and crop rotation can also cause shifts in the soil PLFA profile due to changes in soil microbial community structure (Bohme et al., 2005).

Long-term experimental sites are field experiments with permanent plots that are sampled periodically to quantify changes in soil physical, chemical and biological variables (Hofmockel

et al., 2007). Significant changes in soil quality are slow and it takes a significant time period to achieve a steady state condition after a change in management practice (Kennedy, 1999). These long-term, continuous experiments, which have documented changes and results thoroughly and continuously managed treatments, can be invaluable for studying changes in soil physical, chemical and biological parameters. Rousk et al. (2010b) investigated the influence of soil pH on the soil microbial community composition of two major microbial groups, bacteria and fungi in the Hoosfield Acid strip at Rothamstead, UK, using quantitative PCR. Soil pH between 4 and 8 positively influenced the relative abundance and diversity of the bacterial community composition because of the narrower pH range required for optimal bacterial growth. The fungal community was less strongly influenced by soil pH because fungi have a wider pH range for optimal growth. Rousk et al. (2010a) also studied the influence of soil pH on PLFA profiles using soil samples collected from same experimental site with a pH range of 4.5 to 8.3. The soil microbial community as indicated by PLFA composition was strongly influenced by soil pH, but total PLFA were not significantly affected by pH.

Li et al. (2008) studied the effect of long-term applications of N and P fertilizers and organic manure on soil microbial biomass C and bacterial communities in corn fields. The soil microbial biomass was significantly higher in manured as well as N and P fertilized treatments when compared with other treatments. This study showed that addition of organic and inorganic fertilizers, crop growth stage and soil chemical conditions influenced soil microbial characteristics. Sun et al (2004) studied bacterial community composition using PCR-DGGE targeting 16s rRNA gene in a long-term continuous wheat experiment using organic fertilizer (cattle manure) and inorganic fertilizers. Manure application significantly increased soil bacterial diversity compared with inorganic fertilizer-treated soil.

Application of nitrogenous fertilizers must depend on the quantitative requirements of the crop for maximizing crop productivity and minimizing nitrogen loss (Schloter et al., 2003). A study on long-term fertilizer application by Marschner et al. (2003) found that addition of organic and inorganic fertilizers for 31 years caused changes in microbial community composition. The study found soil organic carbon and C/N ratio were influencing bacterial and fungal communities.

There is a need for better understanding of how bacterial and fungal communities respond to long-term application of inorganic nitrogen fertilizer, crop rotation with a winter legume cover and different soil pH conditions. In this study, the response of soil microbial communities to nitrogen sources and soil pH was investigated in a century-old crop rotation system. The overall goal was to determine the influence of long-term management practices on soil microbial community structure. The specific objectives were to examine the effects of nitrogen sources and soil pH on soil microbial biomass C and soil microbial communities in the Cullars Rotation (circa 1911). Since soil microbial communities are extremely complex, a biphasic approach was used in the study. The broad scale structure of both prokaryotic and eukaryotic microorganisms was studied using PLFA analysis; fine resolution structures of bacterial and fungal communities were determined using ARISA. Since phospholipids are rapidly degraded by enzymes in the soil upon cell death, they can provide information on viable microbial communities.

## **Materials and Methods**

### ***Study Site***

The Cullars Rotation in Auburn, AL, established in 1911, is the oldest soil fertility crop rotation study with replicated treatments in the USA (Mitchell et al., 2011). The experimental

field consists of 14 treatments (6 m wide x 30 m long) with a 1 m alley between adjacent plots and 6 m between blocks. Since the establishment of the experiment in 1911, three treatments (A, B and C) were added in 1914 to study the effect of winter legumes on crop rotation. The soil at the site is Marvyn sandy loam (fine-loamy siliceous, thermic Typic Kanhapludults) consisting of 68% sand, 16% silt, and 16% clay. The experiment was arranged in ordered block design replicated three times with one replicate for each of the three crops in the 3-year rotation of cotton, corn, wheat and soybeans. Crops were planted and cultivated using conventional tillage prior to 1997. Since then, all crops have been planted using in-row subsoiling and conservation tillage with no irrigation. The six treatments used in this study were winter legume without N fertilizer, no N fertilizer and no winter legume, no input, NPK fertilizer without winter legume, NPK fertilizer with winter legume, and no lime (Table 3.1). Detailed descriptions of the field experiment can be found in Mitchell et al. (2005) and Mitchell et al. (2011).

#### ***Soil sampling and chemical analysis***

Soil samples were collected from six random positions in each plot using a soil auger with plastic liner 15 cm in length and 4.5 cm in diameter. The collected soil cores were divided into two depths (0-5 and 5-15 cm), mixed, and passed through a 4-mm sieve after thorough mixing. Soil samples were collected in June and October 2008 and February 2009. Crops and crop residues present in the field at the time of sampling are shown in Table 3.1. Homogenized sub-samples were taken for determination of moisture content, soil microbial biomass C, basal respiration, and extraction of lipids and DNA. Sub-samples taken from initial and final samples were air-dried for total carbon and nitrogen analysis using a TruSpec CN analyzer (Leco Corp., St. Joseph, MI). Soil pH was determined using 1:1 soil/water and 1:2 soil/CaCl<sub>2</sub> suspensions. Field-moist soil samples were stored at 4°C for no more than 2 weeks before lipid extraction.

Soil samples for DNA extraction were stored at -20°C. Chemical analyses were performed in duplicate.

### ***Soil microbial biomass C***

Soil microbial biomass C was determined using the chloroform fumigation-incubation method (Horwath and Paul, 1994). Soil samples (25 g dry weight equivalent of moist soil) were adjusted to 50% of their water holding capacities were placed in 150 ml beakers, pre-incubated for 5 days, fumigated with chloroform for 24 hours, and then incubated in closed Mason jars for additional 10 days at 23 ± 1 °C. Carbon dioxide produced during the incubation period was trapped in 1 M NaOH solution (5 ml) and quantified by titration using 0.25 M HCl.

### ***Soil microbial activity***

Basal respiration was measured by static incubation-titrimetric determination (Alef and Nannipieri, 1995). As described above, soil samples (25 g dry weight equivalent of moist soil) adjusted to 50% of their water holding capacities were placed in 150 ml beakers, preincubated for five days, and then incubated for additional 10 days in closed Mason jars at 23 ± 1°C. Carbon dioxide produced during the 10-day period was determined following the above-described procedure.

### ***Soil microbial community analyses***

#### ***Phospholipid fatty acid analysis (PLFA)***

PLFA analysis was performed as described by Feng et al. (2003). It involved extraction of total lipids from soil, fractionation of total lipids, derivatization of fatty acids to form FAMES, and analysis of FAMES. Briefly, duplicate moist field soil samples (8 g dry weight) were used for extracting total lipids using a single-phase citrate buffer-chloroform-methanol solution (1:2:0.8 v/v/v, pH 4). The phospholipids were separated from neutral lipids and glycolipids using

silicic acid column chromatography. The phospholipids were then subjected to a mild alkaline methanolysis, and resulting FAMES were extracted using hexane and dried under nitrogen gas. The FAMES containing 19:0 methyl ester as an internal standard were analyzed using a Hewlett Packard 5890 gas chromatograph with a 25-m HP Ultra 2 capillary column and a flame ionization detector. FAME peaks were identified using the MIDI peak identification software (MIDI, Inc., Newark, DE) and quantified based on the internal standard added. The nomenclature for fatty acids used here was described by Feng et al. (2003).

*Automated ribosomal intergenic spacer analysis (ARISA)*

ARISA involves total community DNA extraction from soil, polymerase chain reaction (PCR) amplification using fluorescence-tagged oligonucleotide primers targeting the intergenic transcribed spacer region, automated electrophoresis, laser detection of fluorescent DNA fragments, and analysis of ARISA peak banding patterns. Total soil DNA was extracted from 10 g moist soil samples using a PowerMax™ Soil DNA Kit (MoBio Labs Inc., Carlsbad, CA) following the manufacturer's instructions. The extracted DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and stored at -80°C until use. Both bacterial and fungal ARISA were performed to determine soil microbial community structure.

The bacterial primers used in the PCR reactions were ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale et al., 2004). The reaction mixture contained 12.5 µL of 2X GoTaq colorless master mix (Promega, Madison, WI), 25 µg of bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO), 0.2 µM of ITSF primer, 0.2 µM of ITSF primer labeled with IRD800 fluorochrome (LI-COR, Lincoln, Nebraska), 0.4 µM of ITSReub primer, 5 µL of template DNA (~20 ng) and nuclease

free water to make the final volume to 25  $\mu$ L. Amplification was performed on a Biometra T-Gradient thermo cycler (Whatmann, Goettingen, Germany) using the following cycling parameters: 3 min at 94°C, 30 cycles of 60 s at 94°C, 30 s at 55°C and 60 s at 72°C, and a final 5 min at 72°C (Ranjard et al., 2000).

The fungal automated intergenic spacer analyses were performed using ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3') (Nicolardot et al., 2007; Ranjard et al., 2006). The reaction mixture (25  $\mu$ L) consisted of 12.5  $\mu$ L of 2X GoTaq colorless master mix, 25  $\mu$ g of bovine serum albumin, 0.3  $\mu$ M of ITS1F primer, 0.1  $\mu$ M of ITS1F primer labeled with IRD800 fluorochrome, 0.4  $\mu$ M of 3126T primer, and 5  $\mu$ L of template DNA (~20 ng). The thermocycling conditions were as follows: 4 min at 95°C, 35 cycles of 60 s at 95°C, 30 s at 53°C and 60 s at 72°C, and a final 7 min at 72°C (Kennedy et al., 2005a; Kennedy et al., 2005b).

A total of 5  $\mu$ L amplified PCR products (2.5  $\mu$ L from each replicate) was mixed with 2.5  $\mu$ L of stop buffer (LI-COR Blue Stop Solution), denatured at 95°C for 2 min, and then placed on ice. The denatured PCR products (0.8-1  $\mu$ L) were loaded on 6% polyacrylamide gel along with 0.8  $\mu$ L of the IRD800 50-700 bp sizing standard (LI-COR). ARISA fragments were resolved under denaturing conditions for 9 hours at 1,500 V using the LI-COR 4300 sequencer. The laser scanned banding pattern image from the LI-COR sequencer was converted to 8-bit TIFF using Kodak 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY).

### *Data analysis*

Microbial biomass carbon and soil microbial activity data were analyzed with SAS software (Version 9.1.3) using PROC MIXED and a correlation procedure. The mole percent distribution of PLFAs was analyzed using PROC STEPDISC (stepwise discriminatory analysis)

and PROC CANDISC (canonical discriminant analysis). Analysis of PLFA profiles was performed using a set of 57 fatty acids that were present in most of the samples. The stepwise discriminatory analysis selected 24 fatty acids for June and October 2008 and 17 for February 2009), respectively, for the canonical discriminant analysis. Bacterial biomass was calculated using the sum of 15 bacterial markers, i.e., 14:0, 15:0, a15:0, i15:0, i16:0, 16:1 $\omega$ 5, 16:1 $\omega$ 7, 16:1 $\omega$ 9, 17:0, a17:0, i17:0, 18:0, 18:1 $\omega$ 7, cy17:0 and cy19:0 (Frostegard and Baath, 1996; Spedding et al., 2004). Fungal biomass was assessed using 18:2 $\omega$ 6,9 (Kaiser et al., 2010). The concentration of biomarkers cy19:0 to precursor ratio was used to study changes due to soil pH conditions (Rousk et al., 2010a). The concentration of biomarker 16:1 $\omega$ 5 was used to assess the presence of arbuscular mycorrhizal fungi (Joergensen and Wichern, 2008; Ngosong et al., 2010). The fungal to bacterial PLFA ratio was calculated using 18:2 $\omega$ 6,9/sum of bacterial markers (Boyle et al., 2008; Spedding et al., 2004). The concentration of biomarker 10Me16:0 was used to assess the presence of actinobacteria (Findlay, 2004). The PLFA biomarkers were analyzed using PROC MIXED and correlation procedures.

ARISA banding pattern images were processed using the software BIONUMERICS ver. 5.0 (Applied Maths, Belgium). Each image was normalized using the 50-700 bp sizing standard as the external reference standard, which allowed for comparison of multiple gels. Levels of similarity between DNA fingerprints were compared using a densitometric curve-based method with a cosine coefficient after the conversion, normalization, and background subtraction with mathematical algorithms of banding patterns. Dendrograms were developed using cluster analysis performed with a cosine similarity coefficient and an unweighted pair-group method using average linkages (UPGMA). The position tolerance was set at an optimization of 0.5%,

and band comparison was made using a position tolerance of 1%. PCA was used to determine the distribution of fingerprint patterns according to treatments and soil depths.

An Analysis of Similarity (ANOSIM) was used to test whether observed separation among treatment groups based on ARISA profile was significant (Clarke, 1993; Edenborn et al., 2011; Kent et al., 2007). This procedure uses the Bray-Curtis similarity matrix to calculate the test statistic R (Clarke, 1993). ANOSIM is analogous to a one-way ANOVA based on multi-group data and generates an R value that ranges from -1 to +1 with values greater than 0 indicating greater dissimilarity between treatment groups than among samples and P value indicating the level of significance. Generally, R lies between 0 and +1; values smaller than zero have been considered unlikely because they would indicate dissimilarity among replicates within treatments rather than between treatments (Chapman and Underwood, 1999). The magnitude of R indicates degree of separation between treatments, with score of +1 indicating complete separation and 0 indicating no separation between treatments.

## **Results**

### ***Crop yields***

Table 3.2 shows crop yields for the 2007-2009 crop rotation cycles. The NPK +no legume and NPK + legume treatments had highest yields for cotton and soybean from 2008-2009 while no nitrogen+ legume treatment had highest yield for cotton in 2007. The NPK +no legume and NPK + legume treatments had highest yields for corn and wheat while no nitrogen+ no legume had highest yield for corn in 2008. Crop yields were lowest or had no yield recorded for no input and no lime treatments for all the crops from 2007 to 2009. Cotton, corn, soybean and wheat recorded lower yields in the no lime treatment compared to other treatments. Cotton and soybean were more sensitive to acidic soil condition in no lime treatment. Crimson clover

produced more dry matter in no N+ legume and NPK+ legume treatments compared to other treatments. The long-term yield trends from 1997 show periods of yield increases and dramatic decreases. Year to year variability was high because Cullars Rotation is a non-irrigated field experiment. The short-term drought and other adverse weather related factors during growing season caused dramatic reduction in the yield.

### ***Soil chemical and biochemical properties***

Chemical properties of the surface soil differed among treatments (Table 3.3). Soil organic carbon and pH were significantly lower in the no input treatment. Soil pH for the no lime treatments was the lowest among all treatments. The soil organic carbon contents were highest in the no nitrogen + legume and NPK + legume treatments and the lowest in the no input treatment. There was a significant depth effect with surface soil having higher soil organic carbon and pH than sub-surface soil. When averaged across sampling time, microbial biomass C and basal respiration were significantly lower in no input and no lime treatments (Table 3.3). Total PLFA was the lowest in the no input treatment. Microbial biomass C, basal respiration, and total PLFA in the surface soil were at least two times more than those in the subsurface soil. Table 3.4 shows the results of these three biochemical parameters by sampling time average across treatments. For the surface soil, microbial biomass C, basal respiration, and total PLFA were highest in February and lowest in June. There were no significant differences at the 5 to 15 cm depth.

### ***Soil microbial community analysis by PLFA analysis***

In this study, canonical discriminant analysis of PLFA showed that the first two canonical components explained 68%, 78% and 69% of the total sample variation for June 2008, October 2008, and February 2009, respectively (Fig. 3.1). For the June 2008 sampling period, the no nitrogen with legume and NPK fertilizer both with legume and without legume treatments

clustered together for the surface soil, while no lime, no input and no nitrogen with no legume treatments were separated from the cluster. The no input treatment clustered along with the treatments of no nitrogen with legume, and NPK fertilizer both with legume and without legume treatments in the subsurface soil. The no lime and no nitrogen with no legume treatments were separated from the cluster. The influential fatty acids for the first canonical component were bacterial biomarkers (10Me16:0, and 15:0) and a nonspecific fatty acid (20:1 $\omega$ 9c) (Table 3.5). The second canonical component was influenced mostly by two bacterial biomarkers (18:1 $\omega$ 7c and cy19:0).

For the October 2008 sampling period data points for no nitrogen with legume, and NPK with legume and without legume treatments clustered together for both surface and subsurface soil. The no lime, no input and no nitrogen with no legume treatments were separated from the cluster at both surface and subsurface soils; the data point for surface soil from the no input treatment was mixed with data points for subsurface soil. The influential fatty acids for the first canonical component (Table 3.5) were the bacterial biomarker (15:0) and two nonspecific fatty acids (16:0 and 19:1 $\omega$ 11c). The second canonical component was influenced by the bacterial biomarker (i17:0) and a nonspecific fatty acid (16:1 2OH).

In February 2009, no nitrogen with legume, no input, NPK with legume and without legume treatments clustered together for both surface and subsurface soils. The influential fatty acids for the first canonical component were actinobacterial biomarker (10Me16:0), the fungal biomarker (18:2 $\omega$ 6, 9c) and a nonspecific fatty acid (17:1 $\omega$ 8c). The second canonical component was influenced mostly by bacterial biomarker (16:1 $\omega$ 7c) and nonspecific fatty acid (16:0) (Table 3.5).

Overall, the canonical discriminant analyses of PLFA profiles showed that soil microbial communities in no lime and no N + no legume treatments were different from those in other treatments. The no input treatment was separated from other treatments in June and October 2008. The PLFA profiles also showed significant differences in microbial communities between the surface and subsurface soils and over time.

Table 3.6 shows concentrations of PLFA biomarkers averaged across sampling periods. Bacterial biomarkers were lowest in the no input treatment. There was also a significant depth effect for bacterial biomarkers, with significantly higher concentrations in the surface soil. The fungal biomarker (18:2 $\omega$ 6,9) was the lowest in the no input treatment at the surface. The fungal biomarker (18:2 $\omega$ 6,9) showed a depth effect with higher concentration in surface soil for all other treatments. The arbuscular mycorrhizal biomarker (16:1 $\omega$ 5) was the lowest at both soil depths for the no input treatment. The fatty acid cy19:0 to precursor ratio concentration was the highest for the no input treatment at both soil depths. The actinobacterial biomarker was lowest in the no lime treatment in surface and subsurface soil compared to other treatments.

#### ***Soil microbial community analysis by ARISA***

The DNA concentrations in of the Cullars Rotation soil ranged from 1.6 to 9.8  $\mu\text{g g}^{-1}$  dry soil. The number of bacterial ARISA bands ranged from 16 to 116 for June 2008, from 22 to 142 for October 2008 and from 24 to 147 in February 2009. The number of fungal ARISA bands ranged from 16 to 86 for June 2008, from 39 to 113 for October 2008 and from 28 to 118 in February 2009. The first two components of bacterial and fungal ARISA profiles for June 2008 explained 64 and 65% of the total variation (Fig. 3.2). The bacterial ARISA profile formed separate clusters for the no input, NPK with legume, NPK without legume, and no lime treatments. The fungal ARISA profile formed separate clusters for no lime and no input

treatments. The NPK with legume treatment formed a cluster within NPK without legume cluster for fungal ARISA. Analysis of similarity (ANOSIM) of the bacterial ARISA profile for June 2008 found differences between different treatments to be significant (global  $R=0.11$ ,  $P\leq 0.05$ ) (Table 3.7). There was significant separation between surface (0-5 cm) and subsurface (5-15 cm) depth (global  $R=0.10$ ,  $P\leq 0.05$ ) but no significant separation among treatments for the fungal ARISA profile.

The first two components of bacterial and fungal ARISA profile for October 2008 explained 66 and 61% of the total variation (Fig. 3.3). The bacterial and fungal ARISA profiles formed separate clusters for the no input, NPK with legume, NPK without legume, and no lime treatments. The fungal ARISA profile formed separate clusters for no lime, no input, no nitrogen with no legume and NPK with no legume treatments. The bacterial ARISA profile for October 2008 between different treatments were found to be significant with a global  $R$  value close to 0 showing no difference between treatments (global  $R=0.04$ ,  $P\leq 0.05$ ) (Table 3.7). There was no significant separation for fungal ARISA based on ANOSIM global  $R$  value among treatments and soil depths.

The first two components of bacterial and fungal ARISA profile for February 2009 explained 69 and 61% of the total variation (Fig. 3.4). The bacterial and fungal ARISA profiles formed separate clusters for the no input, NPK with legume, NPK without legume, no nitrogen with legume, no nitrogen without legume, and no lime treatments. The NPK with legume treatment and NPK without legume formed overlapping clusters for both bacterial and fungal ARISA clusters for no N with and without legume treatments also overlapped. Treatment effects for ARISA profiles were more pronounced in February 2009. The bacterial ARISA profile for February 2009 between treatments were significantly different (global  $R=0.42$ ,  $P\leq 0.05$ ) while

there was no significant separation by soil depth (global  $R=0.04$ ,  $P\leq 0.05$ ) (Table 3.7). The fungal ARISA profile for February 2009 showed significant differences by treatment (global  $R=0.36$ ,  $P\leq 0.05$ ) and by soil depth (global  $R=0.12$ ,  $P\leq 0.05$ ). Bacterial and fungal ARISA profiles showed that data points for no lime and no input treatments formed their own individual clusters for all three sampling times. Bacterial ARISA also revealed the impact of legumes on microbial community structure for treatments with NPK application except for February 2009.

#### ***Interactions between soil chemical and biochemical variables with PLFA biomarkers***

Correlation analyses were performed to determine interactions between soil physicochemical and biochemical variables. Total PLFA were positively correlated with soil moisture content ( $r=0.42$ ), microbial biomass C ( $r=0.66$ ), basal respiration ( $r=0.69$ ), and soil organic carbon ( $r=0.70$ ) (Table 3.8). Bacterial biomarkers were positively correlated with soil moisture content ( $r=0.40$ ), microbial biomass C ( $r=0.65$ ), basal respiration ( $r=0.66$ ), and soil organic carbon ( $r=0.67$ ). The fungal biomarker (18:2 $\omega$ 6,9) was positively correlated with soil moisture content ( $r=0.46$ ), microbial biomass C ( $r=0.68$ ), basal respiration ( $r=0.73$ ), and soil organic carbon ( $r=0.63$ ). The arbuscular mycorrhizal fungal biomarker (16:1 $\omega$ 5) was positively correlated with soil moisture content ( $r=0.38$ ), microbial biomass C ( $r=0.73$ ), basal respiration ( $r=0.75$ ), soil organic carbon ( $r=0.70$ ) and soil pH ( $r=0.30$ ). The cy19:0 to precursor ratio was negatively correlated with soil moisture content ( $r=-0.24$ ), microbial biomass C ( $r=-0.43$ ), basal respiration ( $r=-0.40$ ), soil organic carbon ( $r=-0.20$ ) and soil pH ( $r=-0.73$ ). The actinobacterial biomarker was positively correlated with soil moisture content ( $r=0.27$ ), microbial biomass C ( $r=0.47$ ), basal respiration ( $r=0.50$ ), soil organic carbon ( $r=0.29$ ) and soil pH ( $r=0.42$ ). The soil pH was weakly correlated with total PLFA ( $r=0.21$ ), bacterial biomarkers ( $r=0.26$ ) and fungal biomarker (18:2 $\omega$ 6,9) ( $r=0.20$ ).

## Discussion

Long-term application of nitrogenous fertilizers and use of winter legumes as cover crops results in improvement of soil quality especially in the southeastern USA with low fertility soils. Cullars Rotation, the oldest continuous soil fertility experiment to study long-term effect of fertilization showed dramatic changes in crop yields. The no input and no lime treatments had significantly lower soil carbon and soil pH than other treatments. The accumulation of crop residues, especially in treatments containing winter legumes, results in enrichment of soil organic matter in the surface layer and subsequently increases abundance and activity of microorganisms. Hiltbold et al. (1985), conducting a study in same experimental field, found that omitting winter legumes and N fertilizers resulted in decreased soil organic matter content. Soil in the no lime treatment had a  $\text{pH}_{\text{water}}$  of 4.6 and organic carbon content of 6.1 mg/g of soil. The relatively high soil organic carbon content in the no lime treatment observed in our study and Hiltbold et al. (1985) suggest that organic matter decomposition is slow at low pH conditions.

Microbial biomass C and total PLFA was significantly lower in June 2008 than in February 2009. This can be due to unfavorable or stress conditions affecting soil microbial community due to low soil moisture and high temperatures during summer months. Schloter et al. (2003) quantified microbial biomass using PLFA found that soil water content and temperature influence soil microbial community structure. Microbial biomass and basal respiration were significantly lower in no input treatment compared to other treatments. This result is consistent with another study conducted in the same experimental field found significant differences in microbial biomass C between unfertilized and fully fertilized treatments (Insam et al., 1991). The same study found no lime and no input treatments had a soil  $\text{pH}_{\text{salt}}$  of 4.2 and 4.3, respectively.

The basal respiration for the NPK + legume treatment was significantly higher than other treatments. This increase in basal respiration is consistent with another study conducted using both organic and chemical fertilizers in a long-term experiment (Hu and Qi, 2011). They showed that an increase in basal respiration was resulted from higher organic carbon levels due to continuous application of organic fertilizers. In another study involving long-term application of integrated organic (manures) and inorganic (chemical fertilizers) amendments, results indicate an increasing accumulation of soil organic matter, which in turn had a significant effect on soil microbial biomass (Chakraborty et al., 2011).

Phospholipid biomarkers can be used to classify microbial groups and to evaluate their physiological conditions (Zelles, 1999). Concentrations of the fatty acid cy19:0 to precursor ratio increased with change in soil pH, indicating environmental stress on the soil bacterial community (Rousk et al., 2010a). The no lime treatment showed an increase in concentration of fatty acid cy19:0 to precursor ratio compared to other treatments. This agrees with other studies showing an increase in the stress indicator ratio due to environmental stresses such as anaerobic conditions or other unfavorable conditions for microorganisms. The arbuscular mycorrhizal fungi (16:1 $\omega$ 5) was detected in higher concentrations in all treatments in surface soil compared to subsurface soil. This agrees with a previous study conducted in agricultural soil cropped to wheat (Ngosong et al., 2010). The fungal biomarker (18:2 $\omega$ 6,9) concentration was higher in no nitrogen with legume, no nitrogen without legume, NPK with legume, and NPK without legume treatments compared to no input treatment. Soil organic carbon was positively correlated with bacterial ( $r=0.67$ ) and fungal biomarkers ( $r = 0.63$ ). This result agrees with the findings from a study conducted in agricultural soil cropped to corn (Allison et al., 2007).

Polyphasic approaches are often used to study soil microbial communities. PLFA analysis has been shown to be the best approach to discern a treatment effect on soil microbial communities and is able to differentiate treatments that are not resolved by PCR-based methods in some cases (Ramsey et al., 2006). In this study, both PLFA analysis and ARISA revealed the shift in soil microbial communities associated with nitrogen sources and soil pH. The observed changes in soil microbial communities can be due to favorable physical and chemical soil conditions associated with nitrogen sources and soil pH. The PLFA analysis showed a more pronounced depth effect than ARISA. This suggests that in addition to bacteria and fungi, other microorganism (e. g., protozoa and nematodes) may be influencing soil microbial community structure. The PLFA analysis also separated no N + no legume and no lime treatment from other treatments for both surface and subsurface soils.

Changes in microbial community composition were determined using ARISA, an automated DNA fingerprinting method targeting the intergenic spacer regions of bacteria and fungi. This method is highly reproducible and effective in detecting changes in soil microbial community structures. Bacterial and fungal ARISA were previously used in studies conducted in agricultural and forest soil (Prevost-Boure et al., 2011; Ranjard et al., 2001). This is the first time that ARISA was used to determine the influence of nitrogen sources and soil pH on soil microbial communities in a century old long-term continuous fertility experiment. Although the procedure can provide information on genetic community structure of soil bacteria and fungi, the intergenic spacer regions targeted by ARISA cannot be used to identify dominant organisms. In a long-term agro-ecosystem study, the impact of organic and inorganic fertilizers on soil bacterial community was determined using PCR-DGGE (Sun et al., 2004). The inorganic fertilizer treated soils had similar bacterial community structure and lime treatment did not influenced bacterial

community structure. The maximum number of ARISA bands detected in this study was 147 which represent number of operational taxonomic units (OTU). This molecular method could detect only a portion of bacterial diversity present in soil because the number of species of bacteria per gram of soil varies between  $10^3$  and  $10^7$  (Gans et al., 2005; Roesch et al., 2007). The treatment effect for ARISA profiles was more pronounced during February 2009 and it may be due to low plant activity due to winter weather conditions.

The soil microbial community analyses using PLFA and ARISA showed a shift in soil bacterial communities for the no input and no lime treatments. This observation confirms the finding that soil organic carbon can influence the bacterial community structure (Demoling et al., 2007). Soil organic carbon was positively correlated with total PLFA, bacterial, fungal, arbuscular mycorrhizal fungal and cy19:0 biomarkers. Lauber et al. (2008) used quantitative PCR to study microbial community structure and found that fungal populations were influenced by soil nutrient status, especially soil C/N ratio.

## **Conclusions**

In this study, PLFA and ARISA profiles showed the influence of no input, no lime, and no nitrogen with no legume treatments. The fungal biomarker (18:2 $\omega$ 6,9) concentration was lower in the surface soil for the no input treatment than inorganic fertilizer treatments. The arbuscular mycorrhizal biomarker (16:1 $\omega$ 5) concentration was lower in the surface soil and subsurface soil for the no input treatment than other treatments. Bacterial biomarkers, the fungal biomarker (18:2 $\omega$ 6,9) and arbuscular mycorrhizal biomarker (16:1 $\omega$ 5) were positively correlated with soil organic carbon content. These results indicate that changes in soil microbial community structure were influenced by changes in soil properties due to management practices, i.e., lime application and nitrogen source (inorganic fertilizer vs legumes).

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**Table 3.1. Crops present in the field at each sampling time**

Plot no:	Fertility treatment		Crop		
	Description	Input	West	Middle	East
<b>June 2008</b>					
A	No N + legume	Leg, P, K, lime	Cotton in soybean residues	Wheat residues	Corn in crimson clover residues
B	No N + no legume	P, K, lime	Cotton in soybean residues	Wheat residues	Corn in crimson clover residues
C	No input	None	Cotton in soybean residues	Wheat residues	Corn in crimson clover residues
1	NPK + no legume	N, P, K, lime	Cotton in soybean residues	Wheat residues	Corn in crimson clover residues
3	NPK + legume	Leg, N,P, K, lime	Cotton in soybean residues	Wheat residues	Corn in crimson clover residues
8	No lime	Leg, N,P, K	Cotton in soybean residues	Wheat residues	Corn in crimson clover residues
<b>October 2008</b>					
A	No N + legume	Leg, P, K, lime	Cotton residues	Soybean	Corn residues
B	No N + no legume	P, K, lime	Cotton residues	Soybean	Corn residues
C	No input	None	Cotton residues	Soybean	Corn residues
1	NPK + no legume	N, P, K, lime	Cotton residues	Soybean	Corn residues
3	NPK + legume	Leg, N,P, K, lime	Cotton residues	Soybean	Corn residues
8	No lime	Leg, N,P, K	Cotton residues	Soybean	Corn residues
<b>February 2009</b>					
A	No N + legume	Leg, P, K, lime	Crimson Clover	Soybean residues	Wheat in corn residues
B	No N + no legume	P, K, lime	Crimson Clover	Soybean residues	Wheat in corn residues
C	No inputs	None	Crimson Clover	Soybean residues	Wheat in corn residues
1	NPK + no legume	N, P, K, lime	Crimson Clover	Soybean residues	Wheat in corn residues
3	NPK + legume	Leg, N,P, K, lime	Crimson Clover	Soybean residues	Wheat in corn residues
8	No lime	Leg, N,P, K	Crimson Clover	Soybean residues	Wheat in corn residues

**Table 3.2 Crop yields in various treatments from 2007 to 2009**

<b>Plot</b>	<b>Fertilizer input</b>	<b>Cotton lint lb/A</b>	<b>Corn grain bu/A</b>	<b>Soybean grain bu/A</b>	<b>Wheat grain bu/A</b>	<b>Clover dry matter lb/A</b>
<b>2007</b>						
A	No N +legume	915	46.1	27.9	21.5	*
B	No N + no legume	740	33	22.1	11.7	NA
C	No input	0	0	0	0	0
1	NPK + no legume	490	79.1	20	44.7	NA
3	NPK + legume	540	55.8	21.8	46.1	*
8	No lime	0	31.5	0	14.3	*
<b>2008</b>						
A	No N +legume	767	35.8	43.1	19.1	1320
B	No N + no legume	906	42.3	41.3	18.3	NA
C	No input	0	0	0	0	0
1	NPK + no legume	984	39.8	40.9	38.7	NA
3	NPK + legume	932	39.8	41.8	45.6	2090
8	No lime	427	13.4	15.5	15.4	0
<b>2009</b>						
A	No N +legume	1051	64.7	50.8	34.5	1404
B	No N + no legume	977	31.1	55.4	28.8	NA
C	No input	0	0	0	0	0
1	NPK + no legume	1233	126.5	62.2	57.6	NA
3	NPK + legume	1150	132.4	62.2	57.4	2656
8	No lime	298	29.1	7.8	11.3	0

\*No dry matter data collected due to weak and erratic stand.

**Table 3.3 Selected soil chemical and biochemical properties averaged over the sampling period**

Treatment	Soil organic carbon mg g <sup>-1</sup> soil*		Soil pH <sub>water</sub> 1:1 Soil/water*		Soil pH <sub>salt</sub> 1:2 Soil/CaCl <sub>2</sub> *		Microbial biomass C, µg C g <sup>-1</sup> soil**		Basal respiration µg CO <sub>2</sub> -C g <sup>-1</sup> soil**		Total PLFA nmol g <sup>-1</sup> soil*	
	0-5 cm	5-15 cm	0-5 cm	5-15 cm	0-5 cm	5-15 cm	0-5 cm	5-15 cm	0-5 cm	5-15 cm	0-5 cm	5-15 cm
No N + legume	9.6	5.1	6.1	5.4	5.7	4.6	307.3	106.2	78.7	20.4	62.4	21.5
No N + no legume	8.3	4.1	6.2	5.7	5.8	4.9	270.2	78.5	75.4	13.1	55.6	17.3
No input	4.8	4.0	4.8	4.4	4.0	3.7	126.3	38.0	32.1	12.2	26.1	7.1
NPK + no legume	7.7	4.3	5.8	5.7	5.4	4.8	248.3	83.1	76.6	21.3	50.6	18.5
NPK + legume	9.6	5.2	6.0	5.6	5.2	4.6	299.5	96.0	113.6	22.5	58.6	21.5
No lime	9.2	5.6	4.2	3.9	3.5	3.4	154.7	78.9	53.0	14.6	63.0	25.7
LSD <sub>(0.05)</sub>	1.4	0.8	0.7	0.6	0.7	0.5	58.3	25.2	29.4	12.6	21.9	9.1

\*\*Significant at P≤0.001, \*Significant at P≤0.05.

**Table 3.4 Microbial biomass C, basal respiration and total PLFA for different sampling periods**

Sampling Periods	Microbial biomass C		Basal respiration		Total PLFA	
	$\mu\text{g C g}^{-1} \text{ soil}^*$		$\mu\text{g CO}_2\text{-C g}^{-1} \text{ soil}^*$		$\text{nmol g}^{-1} \text{ soil}^*$	
	0-5 cm	5-15 cm	0-5 cm	5-15 cm	0-5 cm	5-15 cm
Jun 2008	219.2	80.4	51.5	14.8	37.3	12.8
Oct 2008	229.2	78.2	70.3	16.8	52.9	26.5
Feb 2009	254.2	81.8	93.2	20.5	67.8	16.5
LSD <sub>(0.05)</sub>	29.5	19.8	16.6	12.5	10.8	10.8

\*Significant at  $P \leq 0.05$

**Table 3.5 PLFA having scores  $\geq |\pm 0.72|$  on the first two canonical components for all three sampling periods**

<b>Fatty acid</b>	<b>Score</b>	<b>Specificity as a biomarker*</b>
<b>June 2008</b>		
Can 1		
10Me16:0	-0.90	Actinobacteria
20:1 $\omega$ 9c	0.86	Nonspecific
15:0	0.81	Bacteria
Can 2		
18:1 $\omega$ 7c	0.78	Bacteria
cy19:0	-0.72	Bacteria
<b>October 2008</b>		
Can 1		
16:0	0.94	Nonspecific
19:1 $\omega$ 11c	0.90	Nonspecific
15:0	0.84	Bacteria
Can 2		
i17:0	0.78	Bacteria
16:1 2OH	0.72	Nonspecific
<b>February 2009</b>		
Can 1		
10Me16:0	-0.90	Actinobacteria
18:2 $\omega$ 6,9c	0.88	Fungi
17:1 $\omega$ 8c	0.88	Nonspecific
Can 2		
16:1 $\omega$ 7c	-0.81	Bacteria
16:0	0.75	Nonspecific

\*Source: Findlay (2004) and Paul and Clark (1996)

**Table 3.6 PLFA biomarkers in nmol g<sup>-1</sup> of soil for different treatments by sampling depths**

Treatment	Bacteria		Fungi		AMF (16:1 ω5)		Cy19:0/pre ratio		Actinobacteria	
	0-5 cm	5-15 cm	0-5 cm	5-15 cm	0-5 cm	5-15 cm	0-5 cm	5-15 cm	0-5 cm	5-15 cm
No N + legume	30.97	12.84	3.55	0.61	2.11	0.59	0.56	1.00	2.38	1.60
No N + no legume	28.27	10.62	3.20	0.53	2.00	0.58	0.48	0.86	3.00	1.89
No input	13.58	4.47	1.16	0.20	0.78	0.15	0.97	1.84	2.83	1.64
NPK + no legume	25.28	10.91	3.70	0.66	1.84	0.54	0.61	0.99	3.27	1.94
NPK + legume	28.93	12.72	4.02	0.72	2.00	0.61	0.58	1.21	3.15	1.68
No lime	29.67	13.33	3.03	1.13	1.85	0.62	2.14	4.41	1.55	0.65
LSD (0.05)	9.94	5.24	2.41	0.47	0.74	0.26	0.86	0.63	0.94	0.69

**Table 3.7 Analysis of similarity (ANOSIM) R statistic for bacterial and fungal ARISA for different sampling periods**

	Sampling period					
	June 2008		October 2008		February 2009	
	Treatment	Depth	Treatment	Depth	Treatment	Depth
B-ARISA	0.11	NS*	0.04	NS*	0.42	0.04
F-ARISA	NS*	0.10	NS*	NS*	0.36	0.12

\*Not significant at  $P \leq 0.05$

**Table 3.8 Correlation between PLFA biomarkers and soil properties**

Soil properties	PLFA biomarkers					
	Total PLFA	Bacteria	Fungi	16:1 $\omega$ 5	Cy19:0/pre ratio	Actinobacteria
Soil moisture content	0.42	0.40	0.46	0.38	-0.24	0.27
Microbial biomass C	0.66	0.65	0.68	0.73	-0.43	0.47
Basal respiration	0.69	0.66	0.73	0.75	-0.40	0.50
Soil organic carbon	0.70	0.67	0.63	0.70	-0.20	0.29
Soil pH	0.21	0.26	0.20	0.30	-0.73	0.42

Significant correlation at  $P \leq 0.05$

### Figure legends

Figure 3.1 Canonical discriminant analysis of PLFA profiles for all three sampling periods: No N + legume (A), no N + no legume (B), no input (C), NPK + no legume (1), NPK + legume (3) and no lime (8).

Figure 3.2 Principal component analysis of ARISA profiles for June 2008: No N + legume (⊙), no N + no legume (■), no input (⊛), NPK + no legume (●), NPK + legume (★) and no lime (◆).

Figure 3.3 Principal component analysis of ARISA profiles for October 2008: No N + legume (⊙), no N + no legume (■), no input (⊛), NPK + no legume (●), NPK + legume (★) and no lime (◆).

Figure 3.4 Principal component analysis of ARISA profiles for February 2009: No N + legume (⊙), no N + no legume (■), no input (⊛), NPK + no legume (●), NPK + legume (★) and no lime (◆).

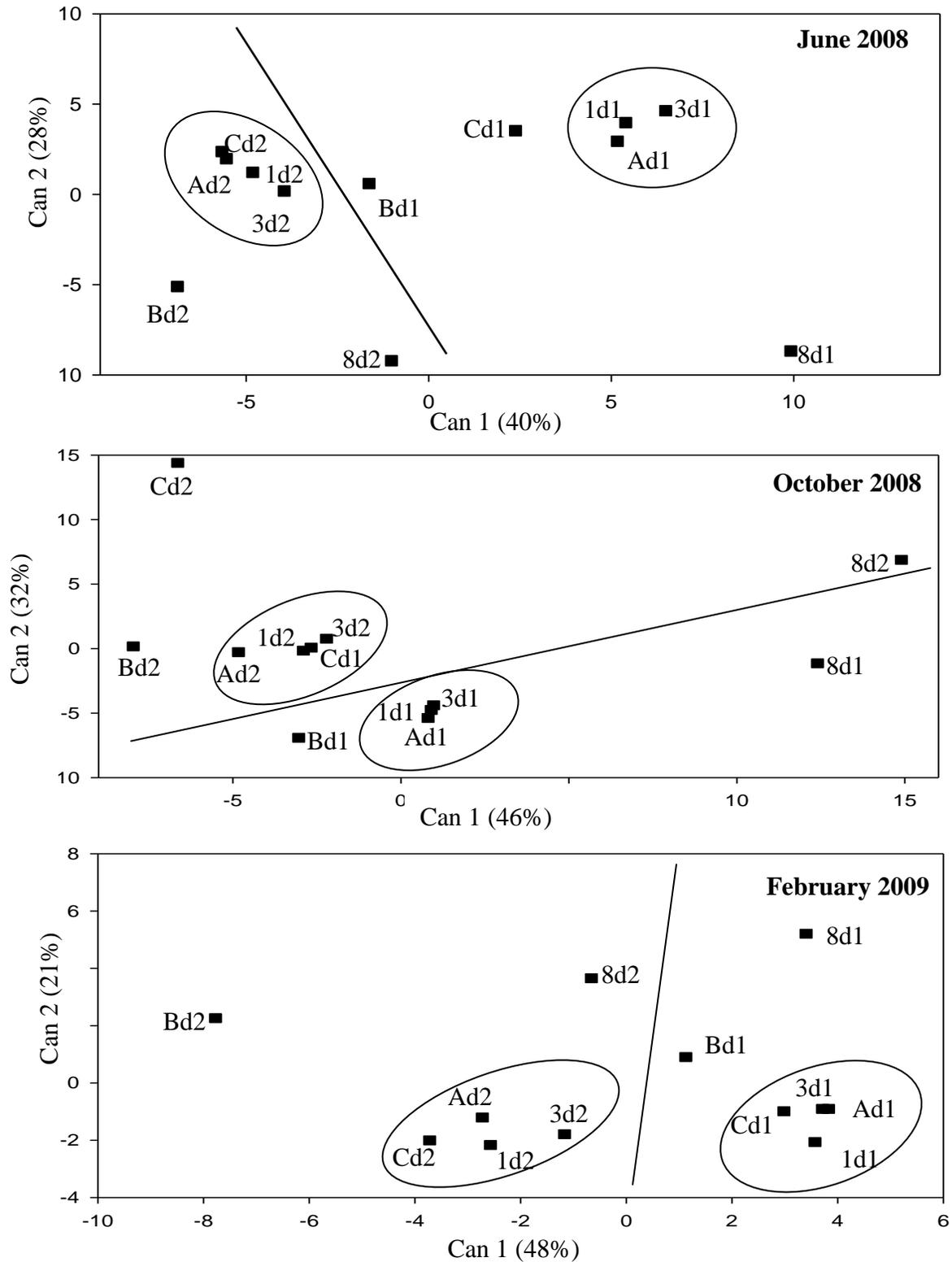


Fig 3.1 Canonical discriminant analysis of PLFA profiles for three sampling periods: No N + legume (A), no N + no legume (B), no input (C), NPK + no legume (1), NPK + legume (3) and no lime (8). The number following letters indicates soil depth (1: 0-5cm; 2: 5-10cm).

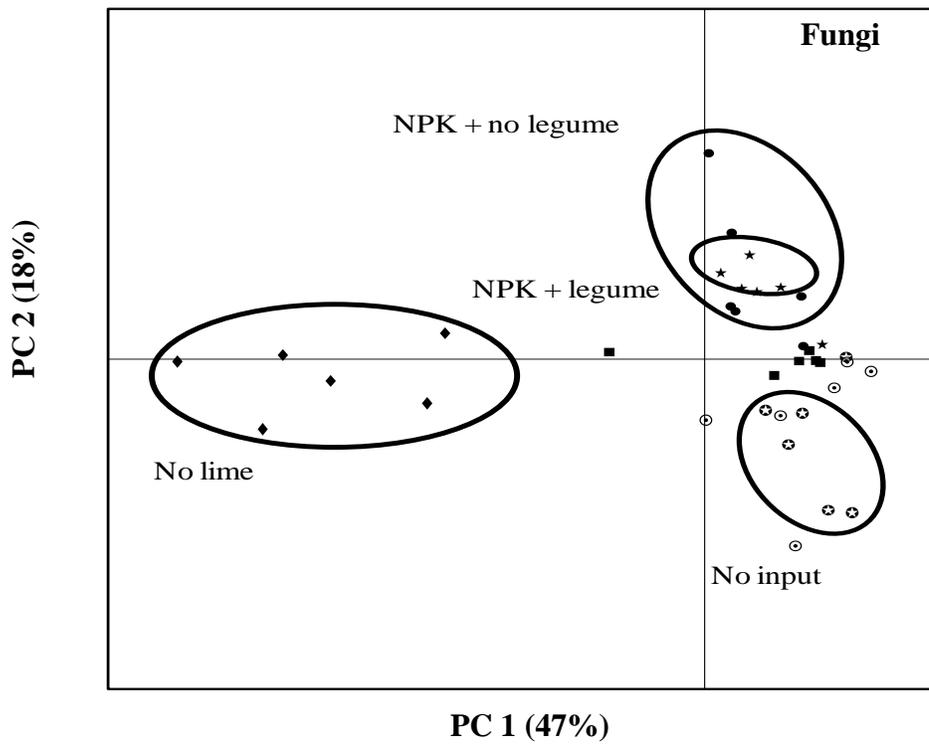
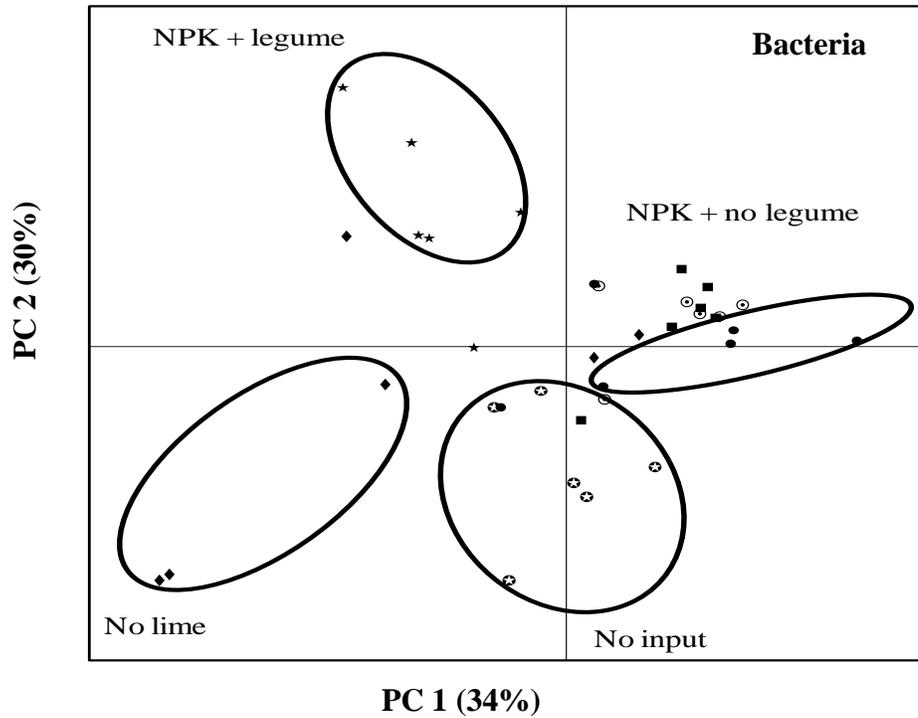


Fig. 3.2 Principal component analysis of ARISA profiles for June 2008: No N + legume (⊙), no N + no legume (■), no input (★), NPK + no legume (●), NPK + legume (★) and no lime (◆)

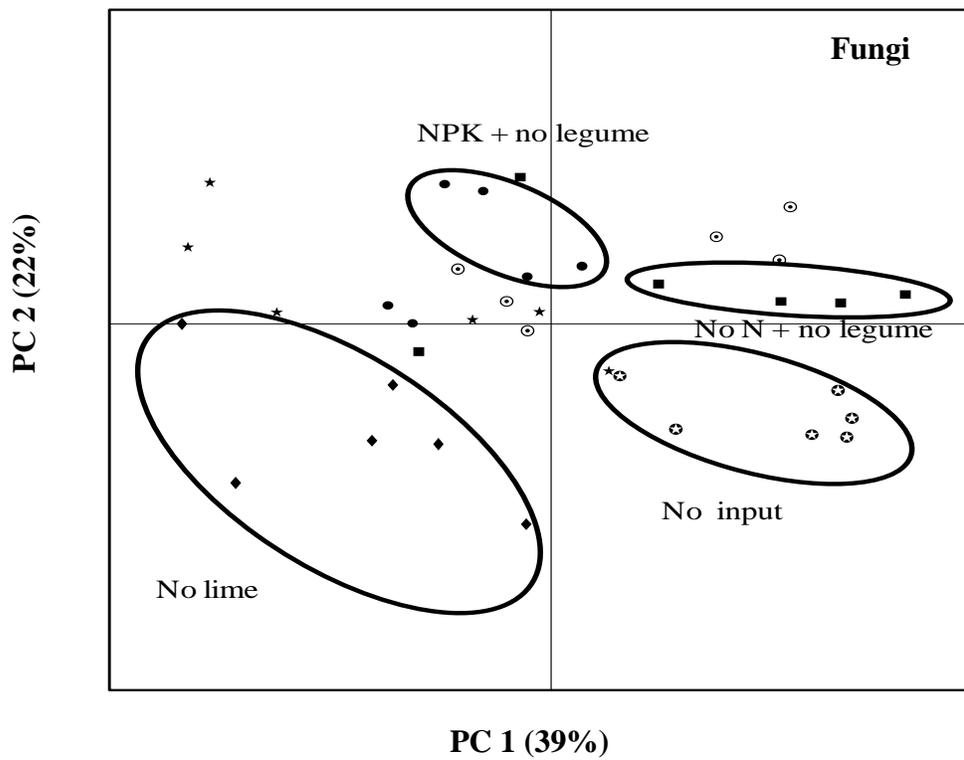
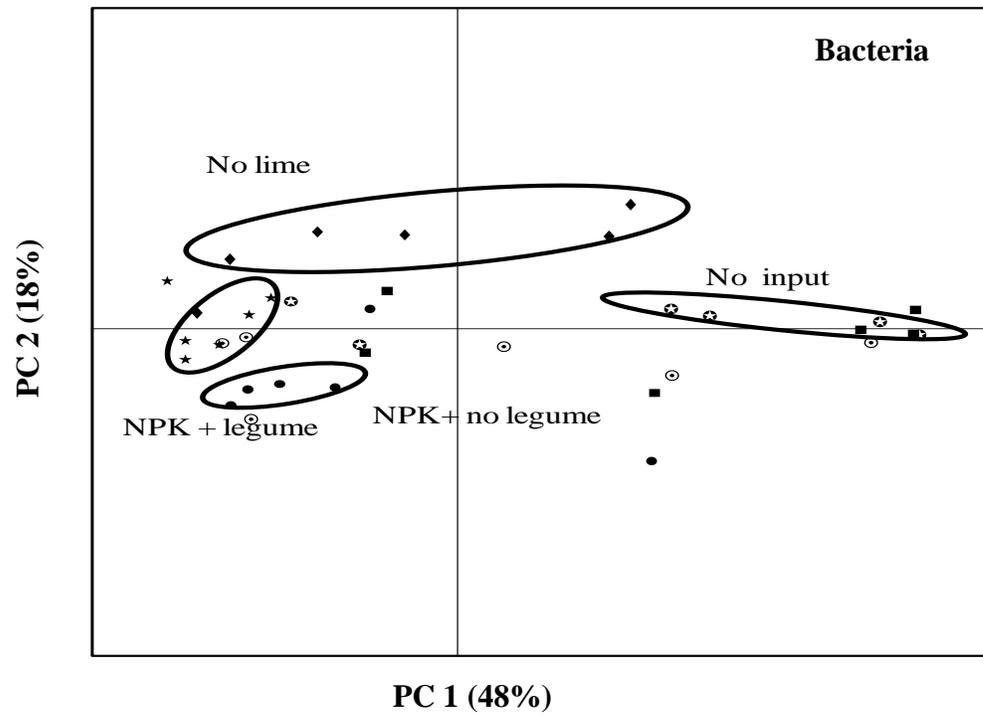


Fig. 3.3 Principal component analysis of ARISA profiles for October 2008: No N + legume (⊙), no N + no legume (■), no input (⊛), NPK + no legume (●), NPK + legume (★) and no lime(◆)

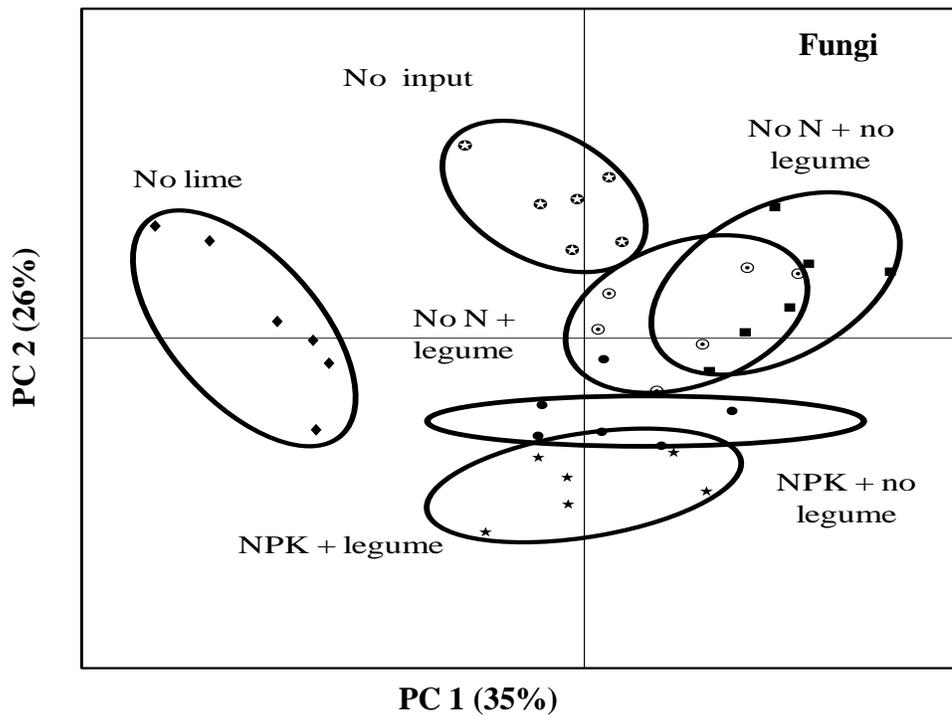
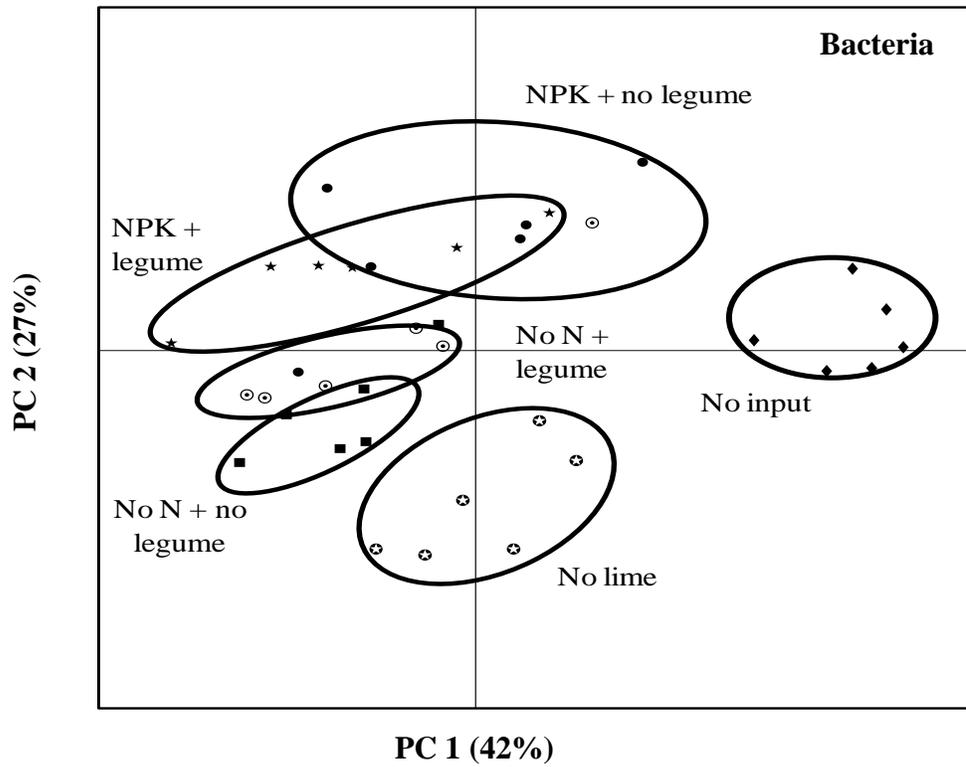


Fig. 3.4 Principal component analysis of ARISA profiles for February 2009: No N + legume (⊙), no N + no legume (■), no input (⊛), NPK + no legume (●), NPK + legume (★) and no lime (◆).

## **Chapter IV. Impact of No-tillage and Conventional Tillage Systems on Soil Microbial Communities\***

### **Abstract**

Soil management practices influence soil physical and chemical characteristics and bring about changes in the soil microbial community structure and function. In this study, the effects of long-term conventional and no-tillage practices on microbial community structure, enzyme activities, and selected physicochemical properties were determined in a continuous corn system on a Decatur silt loam soil. The long-term no-tillage treatment resulted in higher soil carbon and nitrogen contents, viable microbial biomass, and phosphatase activities at the 0-5 cm depth than the conventional tillage treatment. Soil microbial community structure assessed using PLFA analysis and ARISA varied by tillage practice and soil depth. The abundance of PLFAs indicative of fungi, bacteria, arbuscular mycorrhizal fungi, and actinobacteria was consistently higher in the no-till surface soil. Results of principal components analysis based on soil physicochemical and enzyme variables were in agreement with those based on PLFA and ARISA profiles. Soil organic carbon was positively correlated with most of the PLFA biomarkers. These results indicate that tillage practice and soil depth were two important factors affecting soil microbial community structure and activity, and conservation tillage practices improve both physicochemical and microbiological properties of soil.

\*This chapter has been published in the journal *Applied and Environmental Soil Science* (2012). The authors are Reji P. Mathew, Yucheng Feng, Leonard Githinji, Ramble Ankumah and Kipling S. Balkcom. The bulk density and soil phosphatase analyses were done by Leonard Githinji.

## **Introduction**

Tillage systems influence physical, chemical, and biological properties of soil and have a major impact on soil productivity and sustainability. Conventional tillage practices may adversely affect long-term soil productivity due to erosion and loss of organic matter in soils. Sustainable soil management can be practiced through conservation tillage (including no-tillage), high crop residue return, and crop rotation (Hobbs et al., 2008). Studies conducted under a wide range of climatic conditions, soil types, and crop rotation systems showed that soils under no-tillage and reduced tillage have significantly higher soil organic matter contents compared with conventionally tilled soils (Alvarez, 2005). Conservation tillage techniques are widely used in the southeastern United States.

Conservation tillage is defined as a tillage system in which at least 30% of crop residues are left in the field and is an important conservation practice to reduce soil erosion (Uri, 1999). The advantages of conservation tillage practices over conventional tillage include (1) reducing cultivation cost; (2) allowing crop residues to act as an insulator and reducing soil temperature fluctuation; (3) building up soil organic matter; and (4) conserving soil moisture (Schwab et al., 2002; West and Wilfred, 2002).

Different tillage practices cause changes in soil physical properties, such as bulk density (Wander et al., 1998), water holding capacity (Trojan and Linden, 1998), pore size distribution (Azooz et al., 1996) and aggregation (Chan and Mead, 1988). Stratification of soil organic matter and differences in nutrient distribution have also been observed in long-term conservation tillage systems (Kandeler et al., 1999; Staley, 1999). Thus, altered soil physical and chemical conditions under conservation tillage create significantly different habitats for microorganisms and result in shifts of the soil microbial community structure (Feng et al., 2003; Helgason et al., 2009;

Kandeler et al., 1999; Staley, 1999). Conventional tillage can lead to soil microbial communities dominated by aerobic microorganisms, while conservation tillage practices increase microbial population and activity (Staley, 1999) as well as microbial biomass (Balota et al., 2003; Kandeler et al., 1999).

Several studies have examined the effects of tillage practices on soil microbial communities in different cropping systems. In a long-term continuous cotton system, the tillage treatment effect varied by soil depth and over time; the impact of treatments was more pronounced during the fallow period and early in the growing season (Feng et al., 2003). Although fungal dominance is commonly assumed in no-till soils, the relative abundance of fungi over bacteria is not consistently greater in the Northern Great Plain soils under long-term no-till practices compared with intensive tillage (Helgason et al., 2009). Ibekwe et al. (2002) used biochemical (i.e., PLFA) and nucleic-acid based approaches to study the effect of tillage on soil microbial communities in four eastern Washington State soils. PLFA and denaturing gradient gel electrophoresis (DGGE) analyses showed a common pattern of clustering from the four soils, and revealed that soil microbial communities respond more to soil management than annual precipitation.

Various culture-independent methods are available for characterizing soil microbial communities; these methods vary in their sensitivity for detecting microbial community changes. Polyphasic approaches are often used to study soil microbial communities due to the extraordinary magnitude of community size and diversity. PLFAs are a major constituent of cell membranes and have been used to identify individual species of bacteria and fungi. Since they are degraded rapidly upon cell death, PLFAs can be used to characterize living microbial biomass. PLFA analysis also provides insight into the broad scale structure of both bacteria and

eukaryotic microorganisms (Frostegard et al., 1993). The automated ribosomal analysis (ARISA) is a nucleic acid based method, which has a finer resolution for bacterial and fungal communities. This method involves polymerase chain reaction (PCR) amplification of the intergenic region between the small and large subunit ribosomal RNA genes (Fisher and Triplett, 1999). Since the intergenic region exhibits considerable heterogeneity in both length and nucleotide sequence, ARISA has been used to provide rapid estimation of microbial diversity and community composition.

Soil enzymes play key biochemical functions in the decomposition of organic matter in the soil (Burns, 1983; Sinsabaugh et al., 1991). They are process level indicators that reflect past soil biological activity as influenced by soil management. Phosphatases are a broad group of enzymes that are capable of catalyzing hydrolysis of esters and anhydrides of phosphoric acid, and have been reported to be good indicators of soil fertility (Dick et al., 1994; Dick and Tabatabai, 1992). Phosphatases play key roles in phosphorus cycling, including degradation of phospholipids. In this study, the effects of conventional and no-tillage practices on soil microbial communities were investigated in a continuous corn production system by determining microbial community structure using PLFA analysis and ARISA, as well as microbial activities as indicated by soil phosphatases. The central hypothesis was that adoption of no-tillage practice over a long term would cause shifts in soil microbial community structure relative to conventional tillage practices.

## **Materials and Methods**

### ***Study site and soil sampling***

The study site was located at the Tennessee Valley Research and Extension Center in Belle Mina, Alabama, USA. The soil type was a Decatur silt loam (Fine, kaolinitic, thermic

Rhodic Paleudults). The field experiment was arranged in a randomized complete block factorial design of four replications with tillage being the main factor. The no-tillage plots were established in 1990, and conventionally tilled plots in 1994 from previously established no-till plots. Conventional tillage involved disking and chisel plowing in the fall followed by disking and field cultivating in the spring. Cotton was planted at the study site until 2003; corn was planted beginning the following year. Winter rye was seeded in the fall in no-tillage plots and terminated before spring planting with glyphosate application. A detailed description on the history of the field experiment can be found in Schwab et al. (2002). Soil sampling was performed in April of 2008 prior to planting to minimize the effect of plant growth on microbial communities in order to observe the tillage treatment effect. Soil cores (40 to 45 cores) were collected using tube samplers (2.5-cm in diameter) from randomly selected locations in each plot. Soil cores were separated into two depths (0-5 and 5-15 cm) in the field, composited by depth, and thoroughly mixed. Field-moist samples were transported to the laboratory on ice and then passed through a 4-mm sieve within 24 hours. Three additional intact soil cores were collected from each plot for bulk density determination at two depths.

#### *Characterization of soil physical and chemical properties*

Sub-samples from each of the 16 composite samples were taken for gravimetric moisture content determination and chemical analysis after air drying. Total carbon and nitrogen were analyzed using a TruSpec CN analyzer (Leco Corp., St. Joseph, MI). Since there is no appreciable carbonate carbon in this inherently acid soil, the total carbon content is equivalent to the soil organic carbon content. Soil pH was measured using 1:1 soil/water and 1:2 soil/0.01 M CaCl<sub>2</sub> suspensions. Bulk density was determined by measuring the moisture loss from intact soil cores of a known volume after drying at 105°C for 24 hours.

### ***Soil phosphatase activities***

Air-dried soil samples passed through a 2-mm sieve were used to analyze phosphomonoesterases (acid and alkaline phosphatases) and phosphodiesterase activities as described by Tabatabai (1994). The methods are based on colorimetric determination of *p*-nitrophenol released by phosphatase activity when soil is incubated with buffered substrates at each enzyme's optimal pH (Tabatabai, 1994). Acid and alkaline phosphatase assays were performed in a modified universal buffer containing 10 mM *p*-nitrophenyl phosphate at pH 6.5 and pH 11, respectively. Phosphodiesterase assay was performed at pH 8 with 10 mM *p*-nitrophenyl phosphate serving as the substrate. All analyses were done in triplicate.

### ***Soil microbial community analyses***

The homogenized sub-samples were taken for extraction of lipids and DNA. Field moist soil samples were stored at 4°C for no more than two weeks before lipid extraction and at -20°C until soil DNA extraction.

### ***Phospholipid fatty acid (PLFA) analysis***

Phospholipid fatty acid analysis was performed as described by Feng et al. (2003). It involved extraction of total lipids from soil, fractionation of total lipids, derivatization of fatty acids to form FAMES, and GC analysis of FAMES. Briefly, duplicate field moist soil samples (8 g dry weight) from each of the 16 composite samples were used for extracting total lipids using a single-phase citrate buffer-chloroform-methanol solution (1:2:0.8 v/v/v, pH 4). The phospholipids were separated from neutral lipids and glycolipids using silicic acid column chromatography. The phospholipids were then subjected to a mild alkaline methanolysis, and resulting FAMES were extracted using hexane and dried under nitrogen gas. The FAMES containing 19:0 methyl ester as an internal standard were analyzed using a Hewlett Packard 5890

gas chromatograph with a 25-m HP Ultra 2 capillary column and a flame ionization detector. FAME peaks were identified using the MIDI peak identification software (MIDI, Inc., Newark, DE) and quantified based on the internal standard added. The nomenclature for fatty acids used here was described by Feng et al. (2003).

*Automated ribosomal intergenic spacer analysis (ARISA)*

ARISA involved total community DNA extraction from soil, PCR amplification using fluorescence-tagged oligonucleotide primers targeting intergenic transcribed spacer region, automated electrophoresis, laser detection of fluorescent DNA fragments, and analysis of banding patterns. Total soil DNA was extracted from 8 g of moist soil using a PowerMax™ Soil DNA Kit (MoBio Labs Inc., Carlsbad, CA) following the manufacturer's instructions. The extracted DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and stored at -80°C until use. Both bacterial and fungal ARISA were performed to determine soil microbial community structure.

The bacterial primers used in the PCR reactions were ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale et al., 2004). The reaction mixture contained 12.5 µL of 2X GoTaq colorless master mix (Promega, Madison, WI), 25 µg of bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO), 0.2 µM of ITSF primer, 0.2 µM of ITSF primer labeled with IRD800 fluorochrome (LI-COR, Lincoln, Nebraska), 0.4 µM of ITSReub primer, 5 µL of template DNA (~20 ng) and nuclease-free water to make the final volume to 25 µL. Amplification was performed on a Biometra T-Gradient thermo cycler (Whatmann, Goettingen, Germany) using the following cycling parameters: 3 min at 94°C, 30 cycles of 60 s at 94°C, 30 s at 55°C and 60 s at 72°C, and a final 5 min at 72°C (Ranjard et al., 2000).

The fungal automated intergenic spacer analyses were performed using ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3') (Nicolardot et al., 2007; Ranjard et al., 2006). The reaction mixture (25  $\mu$ L) consisted of 12.5  $\mu$ L of 2X GoTaq colorless master mix, 25  $\mu$ g of bovine serum albumin, 0.3  $\mu$ M of ITS1F primer, 0.1  $\mu$ M of ITS1F primer labeled with IRD800 fluorochrome, 0.4  $\mu$ M of 3126T primer, and 5  $\mu$ L of template DNA (~20 ng). The thermocycling conditions were as follows: 4 min at 95°C, 35 cycles of 60 s at 95°C, 30 s at 53°C and 60 s at 72°C, and a final 7 min at 72°C (Kennedy et al., 2005a; Kennedy et al., 2005b).

A total of 5  $\mu$ L amplified PCR products (2.5  $\mu$ L from each replicate) were mixed with 2.5  $\mu$ L of stop buffer (LI-COR Blue Stop Solution), denatured at 95°C for 2 min, and then placed on ice. The denatured PCR products (0.8-1  $\mu$ L) were loaded on 6% polyacrylamide gel along with 0.8  $\mu$ L of the IRD800 50-700 bp sizing standard (LI-COR). ARISA fragments were resolved under denaturing conditions for 9 hours at 1,500 V using the LI-COR 4300 sequencer. Laser scanned banding pattern image from the LI-COR sequencer was converted to 8-bit TIFF using Kodak 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY).

### ***Data analysis***

All microbial parameters were converted to a unit weight of dry soil prior to data analysis. Data for general soil physicochemical and biological properties were analyzed in SAS using PROC MIXED and a multiple comparison procedure as well as PCA. The mole percent distribution of PLFAs was analyzed using principal components analysis (PROC PRINCOMP, SAS ver.9.1.3). Analysis of PLFA profiles was performed using a set of 50 fatty acids that were present in most of the samples. Bacterial biomass was calculated using the sum of 15 bacterial markers, i.e., 14:0, 15:0, a15:0, i15:0, i16:0, 16:1 $\omega$ 5, 16:1 $\omega$ 7, 16:1 $\omega$ 9, 17:0, a17:0, i17:0, 18:0,

18:1 $\omega$ 7, cy17:0 and cy19:0 (Frostegard and Baath, 1996; Spedding et al., 2004). Fungal biomass was assessed using 18:2 $\omega$ 6, 9 (Kaiser et al., 2010), and physiological stress by the ratio of cy19:0/18:1 $\omega$ 7 (Jackson et al., 2003; Moore-Kucera and Dick, 2008). The fungal to bacterial PLFA ratio was calculated using 18:2 $\omega$ 6, 9/sum of bacterial markers (Boyle et al., 2008; Spedding et al., 2004). Gram-negative to Gram-positive bacteria were calculated using (i15:0 + a15:0 + i16:0 + 10Me16:0)/(16:1 $\omega$ 7 + 18:1 $\omega$ 7 + cy19:0). The PLFA biomarkers and ratios were also analyzed using PROC MIXED and a multiple comparison procedure.

ARISA banding pattern images were processed using the software BIONUMERICS Ver. 5.0 (Applied Maths, Belgium). Each image was normalized using the 50-700 bp sizing standard as the external reference standard, which allowed for comparison of multiple gels. Levels of similarity between DNA fingerprints were compared using a densitometric curve-based method with the cosine coefficient after the conversion, normalization, and background subtraction with mathematical algorithms of banding patterns. Dendrograms were developed using cluster analysis performed with the cosine similarity coefficient and unweighted pair-group method using average linkages (UPGMA). The position tolerance was set at an optimization of 0.5%, and band comparison was made using a position tolerance of 1%. PCA was used to determine distribution of fingerprint patterns according to different tillage treatment and soil depth.

## **Results**

### ***Soil physicochemical and biochemical properties***

Physicochemical characteristics of surface soils differed between tillage treatments (Table 4.1). Soil organic C, total N, and C/N ratio were significantly higher in the no-till treatment than the conventional tillage treatment at the 0-5 cm depth, but not at the lower depth. Depth effects were observed only in the no-till treatment. Bulk density for surface soil in both

no-till and conventional-till treatments was lower compared with the subsurface soil although no significant difference was observed between tillage treatments. Soil pH values did not vary by tillage treatment or soil depth.

Total PLFA concentrations, an indicator of viable microbial biomass, ranged from 30 nmol/g of soil for the conventional-till treatment at the 5-15 cm depth to 104 nmol/g of soil for the no-till treatment at the 0-5 cm depth (Table 4.2). The total PLFA concentration in the no-till surface soil was 2.7 times higher than in the conventionally tilled soil. As soil depth increased, total PLFA concentrations decreased in both tillage treatments. Soil phosphatase activities showed a similar trend with no-till soil having significantly higher activities than conventionally tilled soil at the 0- to 5 cm depth (Table 4.2). In the no-till treatment, the enzyme activities were significantly higher at the 0-5 cm than at the 5-15 cm depth except for acid phosphatase. Among three soil phosphatases, acid phosphatase activity was the highest, ranging from 200-367  $\mu\text{g}$  of *p*-nitrophenol  $\text{g}^{-1}\text{hr}^{-1}$ . Alkaline phosphatase activities ranged from 44-321 and phosphodiesterase from 32 to 132  $\mu\text{g}$  of *p*-nitrophenol  $\text{g}^{-1}\text{hr}^{-1}$ .

#### ***Phospholipid fatty acid analysis (PLFA)***

Principal components analysis of PLFA profiles showed that 81% of the total sample variation was explained by the first three principal components (PCs). PC 1 explained 50% of the total variation and separated the soil depth effect. PC 3 explained 7% of the variation and separated the tillage effect (Fig. 4.1). The influential fatty acids for the first principal component (Table 4.3) were an actinobacterial biomarker (10Me16:0), an aerobic bacterial biomarker (16:1 $\omega$ 7), and fungal biomarkers (18:1 $\omega$ 9 and 18:2 $\omega$ 6, 9). The third principal component was influenced mostly by a non-specific fatty acid (i17:1), an anaerobic bacterial biomarker (cy19:0), and an actinobacterial biomarker (10Me16:0) (Table 4.3).

The relative abundance of fungal biomarker (18:2 $\omega$ 6, 9) as indicated by mole percentage did not show tillage treatment effect; however, the concentration of this biomarker was higher in no-till than conventional-till soil at the surface depth (Table 4.4). The sum of bacterial PLFAs showed a similar trend. Similar to the relative abundance of fungal and bacterial PLFAs, the fungal to bacterial PLFA ratios showed depth but not tillage treatment effects. Although arbuscular mycorrhizal (AM) fungi proportions only showed the depth effect, concentrations of the AM fungal biomarker (16:1 $\omega$ 5) showed both tillage and depth effects. The relative abundance of the actinobacterial biomarker (10Me18:0) was similar across tillage treatments and soil depths, whereas its concentrations differed by tillage and depth. Gram-positive to Gram-negative bacterial PLFA ratios (Table 4.4) and the stress indicator ratios (cy19:0/18:1 $\omega$ 7, data not shown) did not show any significant difference for tillage treatment or depth.

#### ***Automated ribosomal intergenic spacer analysis (ARISA)***

Principal components analysis of bacterial ARISA profiles showed that the first and second principal components explained 68% and 23% of the total sample variation, respectively (Fig. 4.2). The first principal component separated the no-tillage from conventional tillage treatment, and the second principal component separated the no-till treatment by soil depth. There was no depth separation for the conventional tillage treatment. Principal components analysis of fungal ARISA profiles showed that the first and second principal components explained 54% and 25% of the total sample variation, respectively (Fig. 4.3). The first principal component separated the tillage effect, while the second principal component separated the surface and sub-surface soil for the no-till treatment.

### ***Interactions between soil physicochemical and biochemical variables***

Correlation and multivariate analyses were performed to determine interactions between soil physicochemical and biochemical variables. Acid and alkaline phosphatases as well as phosphodiesterase activities were positively correlated to soil organic carbon and soil moisture contents (Table 4.5). Soil bulk density was negatively correlated with alkaline phosphatase ( $r = -0.56$ ) and phosphodiesterase ( $r = -0.46$ ) activities, but had no significant correlation with acid phosphatase activities. Total PLFAs were highly correlated with soil organic carbon ( $r = 0.98$ ) and moisture content ( $r = 0.87$ ). The fungal to bacterial PLFA ratios and proportions of the AM fungal biomarker as well as the fungal biomarker were also positively correlated with soil organic carbon (Table 4.5). Bacterial PLFA proportions were negatively correlated to both soil organic carbon and moisture content, but positively correlated to bulk density. The fungal biomarker and the fungal to bacterial PLFA ratio were negatively correlated with soil bulk density. The relative abundance of AM fungal biomarker was positively correlated to soil moisture content.

Multivariate analysis using selected soil physicochemical and enzyme variables (i.e., soil organic carbon, total nitrogen, soil moisture, soil pH, bulk density, acid and alkaline phosphatases, and phosphodiesterase) also revealed tillage and depth effects (Fig. 4.3). PCA showed that the first principal component explained 68% of the total sample variation and the second principal component 17%. Data points for the no-tillage treatment at the surface depth formed a distinct cluster by themselves. Data points for the conventional tillage treatment at both depths clustered together, whereas those for the no-till treatment formed two clusters separated by soil depth. The influential variables for the first principal component were soil organic

carbon, total nitrogen, alkaline phosphatase, phosphodiesterase, and soil moisture, and that for the second principal component was soil pH (Table 4.6).

## **Discussion**

Changes in soil characteristics associated with the adoption of conservation tillage systems generally result in improved soil quality, especially in the southeastern USA where soils are inherently low in fertility and susceptible to aggregate disruption and erosion. In this study, soil under the long-term no-till treatment had higher soil carbon and nitrogen contents, total PLFAs, and phosphatase activities at the 0-5 depth than that under the conventional-till treatment. Tillage treatment effects were less pronounced at the 5-15 cm depth. These observations are in agreement with previous findings reported by, for example, Ceja-Navarro et al. (2010), Drijber et al. (2000), Ekenler and Tabatabai (2003), Feng et al. (2003), Helgason et al. (2009) and Ibekwe et al. (2002). Total PLFAs in the no-till surface soil were much higher than those reported in a previous study during the fallow period (Feng et al., 2003) conducted on the same soil type although organic carbon contents at the two sites were similar. This may be attributed to the difference in the cropping systems: continuous cotton with no winter cover crop in the previous study versus continuous corn with rye as a winter cover crop in this study. Cotton is known to generate lesser residues than corn (Lal, 2004) and the rye cover crop provided additional organic matter input to the soil. Three years of corn/rye cropping system perhaps were not long enough to observe a significant change in soil organic matter; the increase in microbial biomass as indicated by total PLFAs, however, provides another line of evidence that microorganisms are sensitive and early indicators for soil quality evaluation. The findings of tillage treatment and depth effects on phosphatase activities were consistent with the study of Ekenler and Tabatabai (2003). Soil enzymes have been suggested as soil quality indicators owing

to their relationship to soil biology, rapid response to changes in soil management, and ease of measurement (Dick et al., 1996).

In no-till soils, the accumulation of crop residues on the soil surface results in enrichment of soil organic matter in the surface layer and as a consequence increased abundance of microorganisms. This study demonstrated a consistent increase in the abundance of fungi, bacteria, arbuscular mycorrhizal fungi, and actinobacteria in the no-till surface soil. Similar to other reports (e.g., Feng et al. (2003); Helgason et al. (2009); Pankhurst et al. (2002)), this study did not show a fungal dominance in the no-till soil as indicated by the ratio of fungal to bacterial PLFAs. The relative abundance of fungi under no-till practices has been shown to be greater than that under conventional-till practices when fungal biomass was determined by measuring hyphal length (Frey et al., 1999). This discrepancy may be attributed to differences in the methods used. As pointed out by Helgason et al. (2009), microscopic measurements of fungal hyphal length performed by Frey et al. (1999) include both viable and non-viable fungal hyphae. PLFA analysis on the other hand provides a measure of viable microbial biomass. Additional factors to be taken into account include that (1) different groups of microorganisms that share overlapping PLFAs also contribute to the discrepancy and (2) phospholipid concentrations in fungi are lower than in bacteria. Nevertheless, comparison of fungal to bacterial PLFAs ratios between tillage treatments is warranted.

Polyphasic approaches are often used to study soil microbial communities. PLFA analysis has been shown to be the best approach to discern a treatment effect on soil microbial community in its ability to differentiate treatments that are not resolved by a PCR-based methods in some cases (Ramsey et al., 2006). In this study, both PLFA analysis and ARISA clearly demonstrated the shift in soil microbial communities associated with tillage practices. These

findings are consistent with those reported by Drijber et al. (2000), Feng et al. (2003), and Peixoto et al. (2006). The observed changes in soil microbial communities can be attributed to favorable physical and chemical conditions under the no-tillage system for microbial activities. A closer examination of PCA results for PLFA and ARISA profiles (Fig. 4.1 and 4.2) revealed that the depth effect for conventionally tilled soil was more pronounced in PLFA analysis. This suggests that in addition to bacteria and fungi, microfauna (e.g., protozoa and nematodes) may contribute to the discrimination of the subtle difference between soil depths in the relatively well mixed conventionally tilled soil since eukaryotic organisms other than fungi contribute to the soil PLFAs.

ARISA is an automated DNA fingerprinting method targeting the intergenic spacer regions of bacteria and fungi in PCR; it is highly reproducible and effective in detecting changes in soil microbial community structure. Bacterial and fungal ARISA have previously been used in studies conducted on agricultural and forest soils (Prevost-Boure et al., 2011; Ranjard et al., 2001). To our knowledge, this is the first time that ARISA was used to determine the impact of tillage practices on soil microbial communities. Although it provides information on the genetic community structure of soil bacteria and fungi, the intergenic spacer regions targeted by ARISA cannot be used to identify dominant organisms. Little information is available regarding the specific microorganisms affected by different tillage practices. Ceja-Navarro et al. (2010) conducted phylogenetic and multivariate analyses to determine the effects of zero tillage and conventional tillage on soil bacterial communities in a long-term maize-wheat rotation experiment. They found that bacterial communities under zero tillage and crop residue retention have the highest level of diversity and richness. Zero tillage has a positive effect on members of *Rhizobiales*, and crop residue retention increases fluorescent *Pseudomonas spp.* and

*Burkholderiales* group. In a rice-soybean rotation study, the impact of conventional and no-tillage with and without cover crops on soil bacterial community structure was determined using PCR-DGGE without identification of bands through DNA sequencing (Peixoto et al., 2006). Responses of bacterial communities to cultivation, tillage, and soil depth, but not to cover cropping were detected.

Results of principal components analysis based on soil physicochemical and enzyme variables (Fig. 3) were in general agreement with those based on PLFA and ARISA profiles. Soil organic carbon was the most influential factor for PC 1, confirming its critical role in the no-till system. Soil organic carbon was correlated with all biochemical variables except for the relative abundance of bacterial biomarkers. A negative correlation between soil organic carbon- and bacterial PLFAs has also been observed by Zornoza et al. (2009) and Helgason et al. (2009). Lauber et al. (2008) quantified microbial communities by quantitative PCR and also reported a lack of correlation between soil carbon and bacterial population. They showed that soil pH and texture are better predictors of soil bacteria.

## **Conclusions**

In this study, soil under the long-term no-till treatment had higher soil carbon and nitrogen contents, total PLFAs, and phosphatase activities at the 0-5 cm depth than that under the conventional tillage treatment. Differences between tillage treatments at the 5-15 cm depth were negligible with the exception of alkaline phosphatase activities. Soil microbial communities shifted with tillage treatment and soil depth. Tillage practice and soil depth were two important factors affecting soil microbial communities. PLFA analysis and ARISA showed comparable results on treatment effects. PLFA profiles, however, detected differences in microbial communities associated with soil depth in the conventional tillage treatment. This study

demonstrated that tillage systems influence soil microbial communities along with soil physicochemical properties.

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Table 4.1 Selected chemical and physical properties of soils from no-till (NT) and conventional-till (CT) treatments\*

Tillage treatment	Depth (cm)	Organic C (%)	Total N (%)	C/N ratio	Bulk density (Mg m <sup>-3</sup> )	Soil pH (1:2 CaCl <sub>2</sub> )	Soil moisture content
NT	0-5	1.94a	0.13a	14.9a	1.52b	6.1a	0.25a
NT	5-15	0.84b	0.07b	11.7b	1.65a	5.9a	0.18b
CT	0-5	0.92b	0.08b	11.0b	1.53b	6.1a	0.15c
CT	5-15	0.76b	0.07b	10.9b	1.66a	6.2a	0.12d

\*Means (n=4) followed by the same letter in a column are not significantly different (Tukey, P<sub>≥</sub> 0.05).

Table 4.2 Total PLFAs and phosphatase<sup>†</sup> activities in no-till (NT) and conventional-till (CT) soils<sup>\*</sup>

Tillage treatment	Depth (cm)	Total PLFAs (nmol g <sup>-1</sup> )	Acid P	Alk P	PDE
			(μg of <i>p</i> -nitrophenol g <sup>-1</sup> hr <sup>-1</sup> )		
NT	0-5	104a	367a	321a	132a
NT	5-15	38b	307ab	44c	36b
CT	0-5	39b	200b	89b	32b
CT	5-15	30c	202b	87b	34b

<sup>†</sup> Acid P, acid phosphatase; Alk P, alkaline phosphatase; PDE, phosphodiesterase.

<sup>\*</sup> Means (n=4) followed by the same letter in a column are not significantly different (Tukey, P ≥ 0.05).

Table 4.3 PLFA having scores  $\geq |\pm 0.23|$  for the first and third principal components

Fatty acid	Score	Specificity as a biomarker*
<b>PC 1</b>		
10Me16:0	-0.65	Actinobacteria
16:1 $\omega$ 7	0.32	Aerobic bacteria
18:1 $\omega$ 9	0.29	Fungi
18:2 $\omega$ 6,9	0.23	Fungi
<b>PC 3</b>		
i17:1	-0.51	Non-specific
cy19:0	-0.34	Anaerobic bacteria
10Me16:0	0.30	Actinobacteria

\* Source: Findlay (Findlay, 2004) and Paul and Clark (Paul and Clark, 1996).

Table 4.4 PLFA biomarkers and ratios<sup>†</sup> in no-till (NT) and conventional-till (CT) soils\*

Tillage treatment	Depth (cm)	Fungi/ bacteria	G+/G- bacteria <sup>‡</sup>	Fungi		Bacteria		AM fungi		Actinobacteria	
				(mol%)	(nmol g <sup>-1</sup> )	(mol%)	(nmol g <sup>-1</sup> )	(mol%)	(nmol g <sup>-1</sup> )	(mol%)	(nmol g <sup>-1</sup> )
NT	0-5	0.08a	1.48a	3.97a	4.47a	53.1a	50.9a	3.89a	4.32a	2.18a	2.09a
NT	5-15	0.03b	1.76a	2.29b	0.76b	56.8b	21.3b	2.93b	1.13bc	2.61a	1.11b
CT	0-5	0.07a	1.54a	3.87a	1.41b	53.0a	20.9b	3.27ab	1.17b	2.41a	0.99bc
CT	5-15	0.04b	1.84a	2.00b	0.68b	57.0b	16.9c	2.83b	0.83c	2.90a	0.77c

<sup>†</sup> G+/G- bacteria: ratio of Gram-positive to Gram-negative bacterial PLFA.

\* Means (n=4) followed by the same letter in a column are not significantly different (Tukey, P<sub>≥</sub> 0.05).

Table 4.5 Correlation coefficients between soil physicochemical and biochemical variables determined in the study

Soil property	Phosphatase activity <sup>†</sup>			PLFA biomarkers and ratios				
	Acid P	Alk P	PDE	Total PLFA	Fungi	Bacteria	Fungi/bacteria	AM fungi
Soil organic carbon	0.72	0.95	0.92	0.98	0.53	-0.65	0.56	0.60
Soil moisture content	0.77	0.84	0.90	0.87	NS	-0.39	NS	0.45
Bulk density	NS*	-0.56	-0.46	-0.53	-0.62	0.49	-0.60	NS

<sup>†</sup> Acid P, acid phosphatase; Alk P, alkaline phosphatase; PDE, phosphodiesterase;

\* NS: No significant correlation ( $P \geq 0.05$ ).

Table 4.6 Soil physicochemical and enzyme variables having scores  $\geq |\pm 0.38|$   
for the first two principal components

Soil properties	Score
<b>PC 1</b>	
Soil organic carbon	0.42
Total nitrogen	0.41
Alkaline phosphatase	0.41
Phosphodiesterase	0.41
Soil moisture	0.38
<b>PC 2</b>	
Soil pH (1:2 CaCl <sub>2</sub> )	0.81

## **Figure Legends**

Figure 4.1 Principal components analysis of PLFA profiles.

Figure 4.2 Principal components analyses of bacterial (A) and fungal (B) ARISA profiles.

Figure 4.3 Principal components analysis using soil physicochemical and enzyme variables.

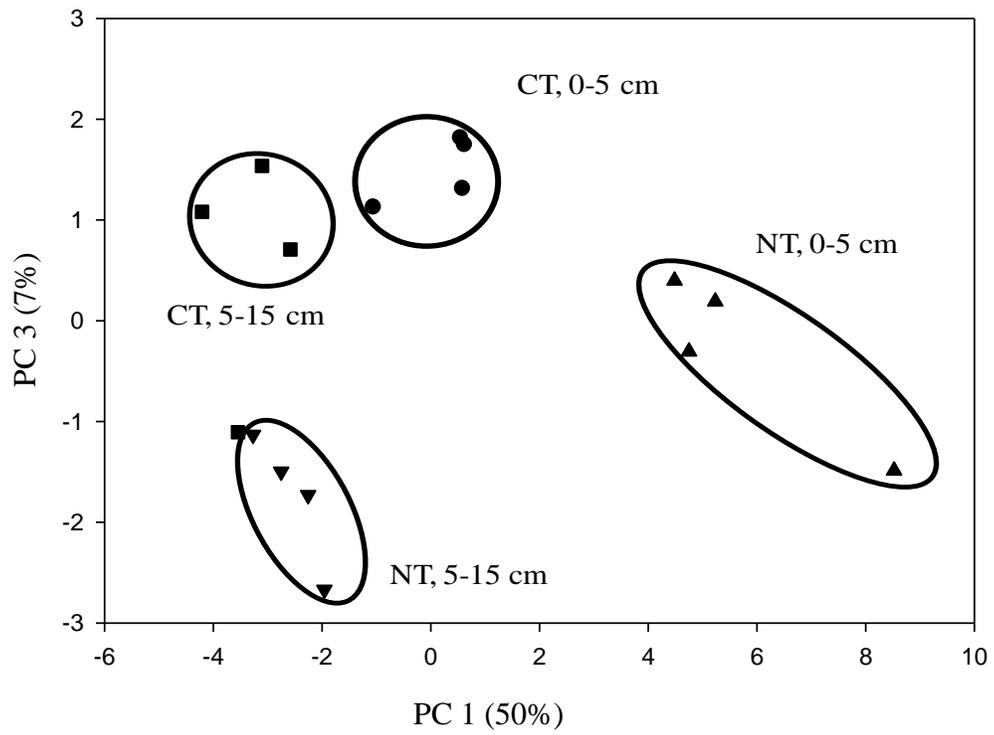


Figure 4.1 Principal components analysis of PLFA profiles.

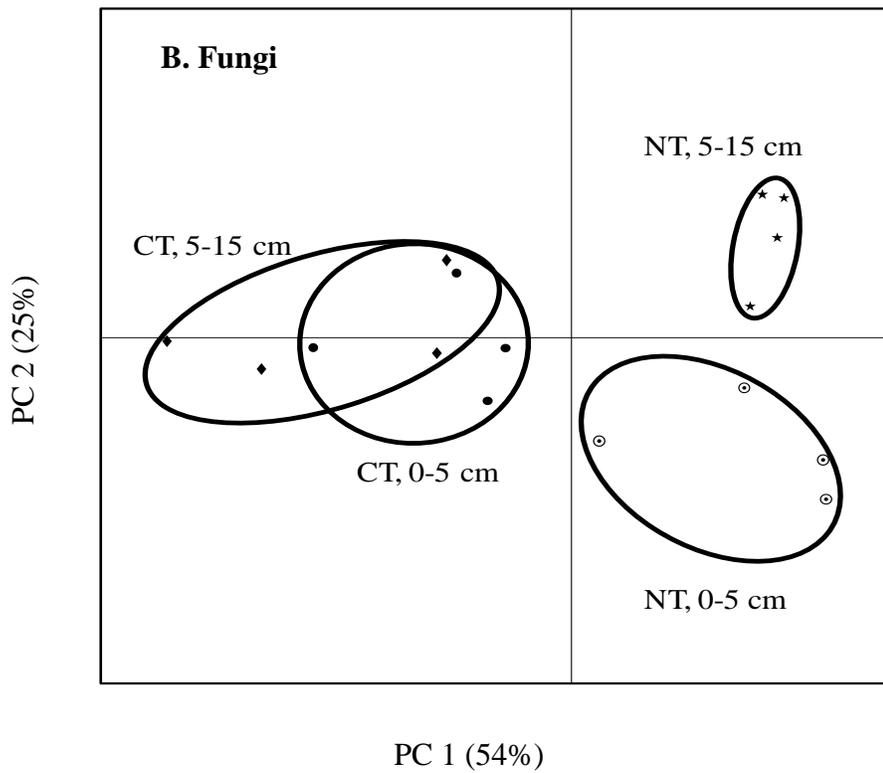
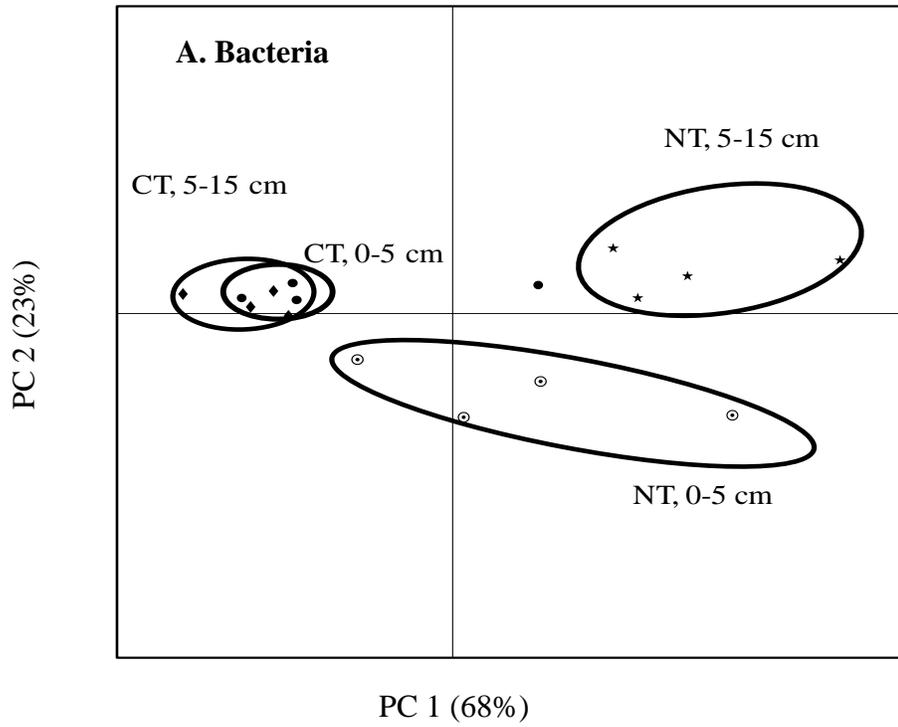


Figure 4.2 Principal components analyses of bacterial (A) and fungal (B) ARISA profiles.

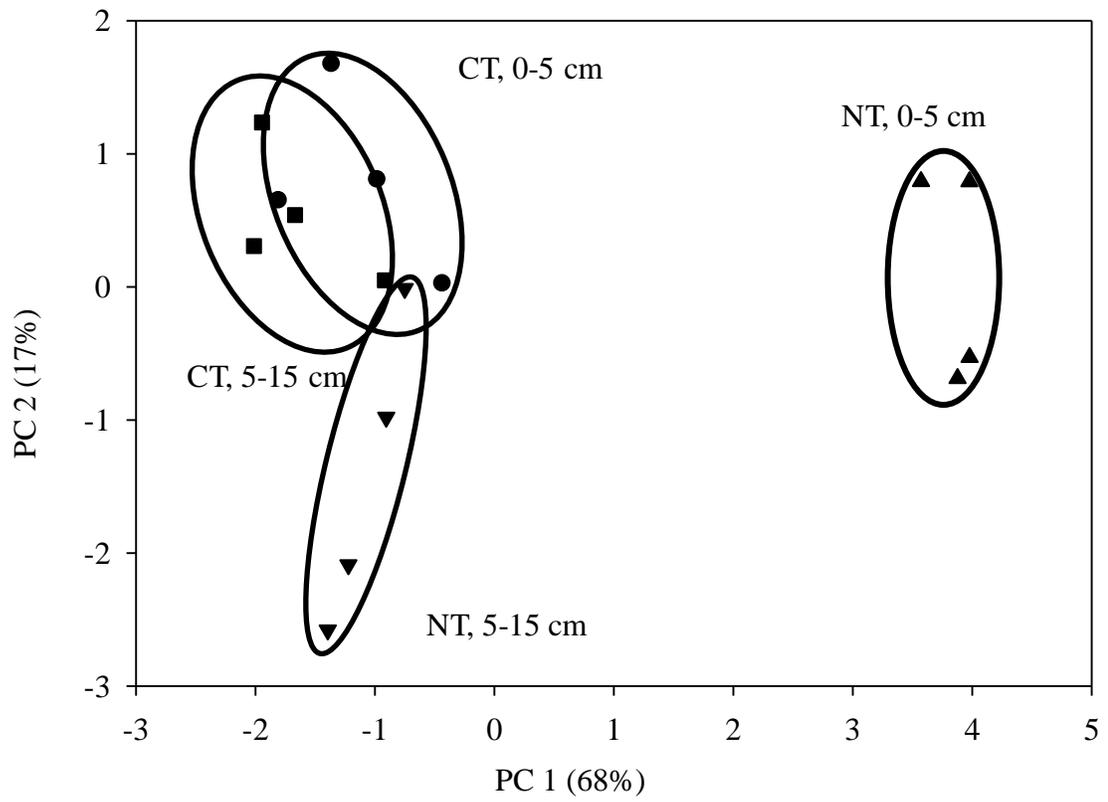


Figure 4.3 Principal components analysis using soil physicochemical and enzyme variables.

## **Chapter V. Conclusions and Future Work**

The effects of management practices such as fertilization, irrigation and tillage on soil microbial community were assessed at one forestry site and two field crop sites. Polyphasic approach using PLFA analysis and ARISA was used to study soil microbial community. The soil microbial communities were influenced by soil organic carbon and soil pH. Fertigation, lime application, nitrogen fertilization, crop rotation with winter legumes and conservation tillage were the other factors influencing changes in soil microbial community.

Chapter II described a study in loblolly pine plantation soil in Aiken, SC. This study showed that soil microbial biomass C and organic carbon were significantly higher for fertilization and fertigation treatments than control. The PLFA and fungal ARISA profiles showed changes in soil microbial community structure due to fertigation and sampling time. Fertilization and irrigation influenced soil microbial community along with soil chemical and biochemical properties. These results indicate that fertigation had a positive effect on soil microorganisms.

Chapter III described Cullars Rotation, a century old long-term fertilizer experiment in Auburn, AL. In this study, microbial biomass C was significantly lower in no input and no lime treatments compared to other treatments. Bacterial biomarkers, fungal biomarker (18:2 $\omega$ 6,9) and arbuscular mycorrhizal biomarker (16:1 $\omega$ 5) were positively correlated with soil organic carbon. PLFA and ARISA profiles showed influence of no input, no lime, and no nitrogen with no legume treatments. Results indicate that changes in soil microbial community structure were associated with soil pH and nitrogen sources (inorganic fertilizers vs. legumes).

Chapter IV described a long-term tillage experiment in Belle Mina, AL. In this study, soil under the long-term no-till treatment had higher soil carbon and nitrogen contents, total PLFAs, and phosphatase activities at the surface soil than that under the conventional tillage treatment. Soil microbial communities shifted with tillage treatment and soil depth, showing that tillage practice and soil depths were two important factors affecting soil microbial communities. PLFA analysis and ARISA showed comparable results for treatment effects. PLFA profiles, however, detected differences in microbial communities associated with soil depth in the conventional tillage treatment. The results from the study indicate that tillage systems influence soil microbial communities along with soil physicochemical properties.

Advanced molecular techniques like next-generation sequencing methods (e.g., pyrosequencing) can provide higher resolution to identify the phylogenetic characteristics of soil microbial community. There are some emerging questions from this study. Does a change in soil microbial community affect key ecological functions like nutrient cycling? Examining microbial community at a phylogenetic scale and linking the microbial community to key ecological functions can help in studying functional diversity of microorganisms in soil. This will help identifying soil and environmental factors that are influencing soil microbial community. Further research is also needed to determine the influence of various agronomic and forestry management practices on soil microbial community composition (i.e., the identity of key organisms) and their dynamics.