

**Long-Term Impacts of Tillage, Cover Crops, and Nitrogen Rates on
Microbial Community Dynamics and Soil Quality Parameters under
Continuous Cotton Production in West Tennessee**

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DEDICATION

In loving memory of Dr. Carl Jones, for believing in me and making the connections that have made this dissertation possible. I will always be grateful for his kindness and support.

To my Dad, the late Hezekiah Karanja Mbutia, for believing in education and always pushing us to aim for the best, your memory lives on.

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ABSTRACT

Microbial communities play a central role in nutrient cycling and soil quality in agro-ecosystems. This research focused on a comparative analysis of the microbial community structure and activity of soils on long-term (31 years) continuous cotton- *Gossypium hirsutum* L., production in West Tennessee under conservation agricultural (CA) and conventional tillage practices that included: Nitrogen (N) fertilizer rates (N-rates) (0, 34, 67 and 101 kg N per ha); Cover crops (Hairy vetch-*Vicia villosa* and winter wheat- *Triticum aestivum*, and a No Cover control); and Tillage (Till and No-till). It was expected that microbial diversity, activity and soil quality would be greater under CA practices relative to conventional tillage.

The microbial community structure profiled using Fatty Acid Methyl Ester extractions (FAME) revealed FAME indicators for Gram positive bacteria, actinomycetes and mycorrhiza fungi to be significantly greater ($p < 0.05$) in the No-till treatments relative to Till. In contrast, the saprophytic fungi indicators were significantly greater ($p < 0.05$) in the Till treatments resulting in significantly greater fungi to bacteria FAME ratio under Till than No-till. N-rate had a significant effect on the relative abundance of the mycorrhiza biomarker which decreased with increasing N-rate. Results from high-throughput 16S rRNA gene sequencing analysis revealed microbial diversity in soils under 101 N-rates to be significantly ($p < 0.05$) less diverse than the 34 and 67 N-rates. However, tillage and cover crop did not significantly influence bacterial diversity.

Soil quality properties revealed significantly greater ($p < 0.05$) total carbon and N in the combination of No-till treatments having cover crops, with the No-till treatments also having

significantly greater extractable nutrients (phosphorous, potassium, and calcium), and enzymatic activity (beta-glucosidase, beta-glucosaminidase, and phosphodiesterase) indicating an improvement in soil quality and fertility.

This study reveals that CA practices involving No-till and cover crops promote conditions that support an increase in the abundance and activity of soil microbial communities, in turn leading to greater soil nutrient cycling capacity and soil quality. This long-term assessment was able to provide an overview of the benefits of C sequestration with these CA practices for low biomass crops like cotton under a monoculture production.

TABLE OF CONTENTS

INTRODUCTION	1
Statement of purpose.....	3
Literature Review.....	4
The role of microbial communities in agro-ecosystems	6
Characterization of microbial community structure: approaches, challenges and opportunities	10
Concepts of microbial structure and diversity measures	18
Influence of agricultural management practices on microbial structure and functions	20
References.....	25
CHAPTER I TILLAGE, COVER CROPS, AND NITROGEN FERTILIZATION EFFECT SOIL QUALITY, MICROBIAL COMMUNITY STRUCTURE AND FUNCTION IN A LONG-TERM (31 YRS) CONTINUOUS COTTON SYSTEM.....	34
Abstract.....	35
Introduction.....	37
Materials and Methods.....	41

Study Site and Soil Sampling	41
Characterization of Soil Chemical and Physical Properties	42
Characterization of soil biological properties	43
Calculation of the soil management assessment framework (SMAF) quality indices	45
Data Analysis	48
RESULTS	48
Soil chemical and physical properties.....	48
Soil microbial community biomass, structure and activity.....	54
Soil Quality Index	61
DISCUSSION	65
Soil physicochemical properties after 31 years of different tillage options, cover crops and varying N-rates.....	65
Status of Microbial Community Structure and Enzymatic Activity after 31 years of tillage, cover crops, and varying N-rates	72
Soil quality index and conclusions	79
References.....	82

Appendix.....	90
CHAPTER II NITROGEN FERTILIZER, COVER CROPS AND TILLAGE EFFECTS ON SOIL BACTERIA TAXONOMIC COMPOSITION IN A LONG-TERM (31 YEARS) CONTINUOUS COTTON STUDY IN WEST TENNESSEE	91
Abstract.....	92
Introduction.....	94
Materials and Methods.....	98
Study Site and Soil Sampling	98
DNA Extraction, PCR Amplification and Sequencing.....	99
Bioinformatics and statistics	100
RESULTS	103
Sequence coverage.....	103
Alpha and Beta diversity measures.....	105
Bacterial taxonomic composition	108
Relationship between soil properties and bacterial taxonomic abundance.....	115
DISCUSSION.....	120

CONCLUSIONS.....	133
References.....	136
CHAPTER III SOILS AND CIVILIZATIONS: USING A GENERAL EDUCATION COURSE TO TEACH AGRICULTURAL RELEVANCE.....	
Abstract.....	146
Introduction.....	147
Materials and Methods.....	151
Results and Discussion	152
Summary.....	158
References.....	159
CONCLUSIONS.....	161
VITA.....	166

LIST OF TABLES

Table 1 Effect of nitrogen fertilizer rates (N-rate) on selected soil chemical and physical properties.....	52
Table 2: Soil physicochemical properties-tillage and cover crop effect.....	53
Table 3: Soil biological properties (microbial biomass, moisture, respiration, and enzyme activities) as affected by cover crop, tillage and N-rates	57
Table 4: Microbial community composition according to FAME profiles as affected by tillage, cover crop and N-rates	58
Table 5: PCA species coordinate loading scores of identified FAME microbial groups	60
Table 6: Tillage and cover crops effect on soil quality indicators based on the scores as determined by soil management assessment framework (SMAF)	63
Table 7: Effect of nitrogen fertilizer rates (N-rate) on selected soil chemical and physical properties.....	64
Table 8: Alpha diversity index measures for richness (PD-whole tree and Chao) and diversity (Shannon and Simpson) across N-rate, cover crop and tillage treatments	106
Table 9: The relative abundance of phyla that differed significantly across treatments (N-rate, cover crops and tillage).....	112

Table 10: Significant soil properties identified by variable selection as influential factors driving the relative abundance of the abundant phyla.....	117
Table 11: Student responses at the end of 2012 Fall Semester using a Likert scale based are questions/statements asked with answers on a scale of importance	154
Table 12: Student responses at the end of 2012 Fall Semester using a Likert scale based are questions/statements asked with answers on a scale of importance	155
Table 13: Comparison of the mean responses to survey questions at the start and end of 2013 Spring Semester to questions based on the scale of importance shown in table 1	156
Table 14: Comparison of the mean responses to survey statements at the start and end of 2013 Spring Semester to statements based on the scale of agreement shown in Table 2.....	157

LIST OF FIGURES

Figure 1: Tillage by cover crop effect on soil total carbon (C) (upper) and nitrogen (N) (lower).	51
Figure 2: Microbial community structure according to FAMES as influenced by tillage and nitrogen (left) and cover crop (right).	59
Figure 3: Rarefaction curves showing sequence coverage for all treatment factors.....	104
Figure 4: Non-metric dimensional scaling representing the community structure differences across all samples.....	107
Figure 5: Relative abundances of the dominant bacterial phyla (top) and order (bottom).....	111
Figure 6: Phyla observed to significantly differ at each treatment factor.....	113
Figure 7: Significantly different bacteria at the order lineage as influenced by each treatment factor:	114
Figure 8: Heat map of Pearson correlation coefficients showing the relationship between all soil properties and the top abundant phyla as represented by the relative sequence abundance of each taxon.	118
Figure 9: Variable selection profiles showing the relationship between variables selected as predictors of the abundance of individual bacteria phyla.	119

INTRODUCTION

Ongoing climate change, depletion of natural resources and food security are the main factors driving the need for sustainable agricultural management production systems. Conservation agricultural (CA) principles are some of the main approaches being spearheaded as a means of mitigating the negative environmental impacts attributed to conventional agricultural practices while maintaining sustainable crop productivity. This dissertation presents research conducted to address some of the knowledge gaps that exist in understanding how different CA practices would influence microbial community dynamics related to their role in facilitating nutrient transformation processes, soil physiochemical properties (soil quality), as well as plant growth promotion factors.

This dissertation is divided into three main sections that include: 1) an introduction encompassing a statement of purpose, and a literature review of the current knowledge base concerning microbial community dynamics under agricultural ecosystems; 2) three main chapters in manuscript form, with the first two based on the research on microbial community profiling plus one published manuscript based on a survey from my role as a teaching assistant and a final section that includes the overall conclusion of the research findings and recommendations.

Chapter 1 focused on the microbial community structure and the interplay between their activity and probable role in influencing the soil quality status after 31 years of tillage, cover crops and nitrogen fertilization rates. The chapter focused on how conservation agriculture practices and fertilizer usage affect the soil quality and soil microbial community structure. Soil quality as

used in this paper is understood to be the state of the soil as it results from different management properties determined as an index which integrates soil physical, chemical and biological properties together (Andrews, 2004). Microbial community structure was determined through Fatty acid methyl esters (FAME) analysis which provided insight on the structural composition of the microbial biomass based on major bacterial and fungal biomarkers. Microbial activity was assessed based on basal microbial respiration and select soil enzymes.

Chapter 2 focused on the characterization of the bacterial taxonomic species composition under these varying practices and how their diversity may influence ecological soil functions and activity. The objectives of this paper were to identify: impacts of different management practices on specific bacterial species; overall microbial diversity and structure; and their interrelations to soil edaphic properties and probable ecological functions. To gain an in-depth understanding of the importance of different microbial species in agro-ecological functioning, it is necessary to identify the specific taxonomic species that shift under different management practices. While the use of FAME analysis revealed that there were indeed shifts in microbial community structure, the method is limited in that it cannot differentiate the specific taxonomic species that are impacted by these management practices. In the second chapter, a genomic sequencing approach that is able to capture taxonomic species composition based on the conserved phylogenetic marker, ribosomal ribonucleic acid (rRNA) gene. To understand different controlling factors for these different bacterial species, specific bacterial groups were regressed and correlated with selected soil properties.

Chapter 3 is a manuscript entitled, “Soils and Civilizations: Using a General Education Course to Teach Agricultural Relevance,” published in a special September 2013 issue of the North

American Colleges and Teachers of Agriculture (NACTA) Journal featuring 24 peer-reviewed manuscripts dealing with the theme of “Globalization: Implications for teaching and learning in postsecondary agricultural education.” This is a paper that I co-authored as part of my involvement as a graduate teaching assistant in a general education course unit “Soils and Civilizations” geared towards increasing students enrollment to agricultural disciplines taught at the university of Tennessee. This study measured changes in student perception of population growth, food security and civilization stability and the relationship these concepts have with agricultural production and environmental sustainability. While it may not be directly related to soil microbial dynamics and conservation agriculture, the paper highlights the need of creating awareness of these important issues to upcoming scholars and sensitizing them to the fact that they have a role to play towards promoting food security and environmental sustainability. The study showed that such a course can have an impact in student perception of agriculture and soil science. This course can be an important tool in raising awareness about the role of soils and agriculture in food security and environmental sustainability to increase enrollment in agricultural disciplines.

Statement of purpose

Soil microorganisms play an integral role in the functioning, productivity and sustainability of agro-ecosystems. Agricultural management practices will impact the structure, composition and diversity of microbial communities that will in turn have an effect on the ecosystem’s productivity. Understanding the microbial dynamics in terms of composition, structure as well as their inter-relations to soil functions is therefore necessary in establishing and integrating management practices that promote sustainable agro-ecosystem functioning.

While the general contribution and functions of microorganism within agricultural systems are known, there is still a knowledge gap on the specific roles of the different microbial groups as well as how these are influenced by the interaction of different management practices. A better understanding of the contribution of microorganisms and impact of management effects would require the characterization of the microbial community shifts under different management practices more so on the long-term basis. Long-term experiments provide a comparative basis of being able to link probable cause and effects of ecosystem shifts.

The project presented here was therefore focused on three main goals. The first goal was to characterize the microbial community structure, composition and activity under contrasting agricultural management practices; the second goal was to characterize the soil physicochemical properties that are linked to soil quality; and finally, to determine the inter-relations of observed shifts in microbial community to probable functions. The project was based on long-term continuous cotton research plots in Jackson, Tennessee established in 1981 that were focused on assessing the benefits of CA management practices.

The specific objectives and hypotheses as well as the methodological approach taken are given in details in subsequent chapters.

Literature Review

Ongoing climate change, depletion of natural resources and food security are the main factors driving the need for sustainable agricultural management production systems. Conventional agricultural practices employ several principles mainly aimed at maximizing production yields but at the expense of natural resources and the environment (Hobbs et al., 2008; Powlson et al.,

2011). These practices include clearing land, deep tillage that incorporates crop residues into the soil often using heavy machinery that compact the soil, and heavy reliance on additional inputs like mineral fertilizers and chemical pesticides. Continuous tilling disrupts the soil structure, incorporates crop residues into the soil leaving the soil surface bare and prone to erosion. The incorporation of surface residue material into soil through tilling also makes it more accessible to soil microbes and also increases greater oxygen diffusion into the soil resulting in greater rates of organic matter decomposition. The increased rates of decomposition result in greater emissions of carbon dioxide (CO₂) that further contributes to the greenhouse effect (Six et al., 2006; Roldan et al., 2007; Hobbs et al., 2008; Powlson et al., 2011, Halvorson et al., 2002). Exhaustion of soil organic matter as well as a decrease in natural biota leads to a reliance on mineral fertilizers and chemical inputs for nutrients, pest and disease management. This reliance on chemical inputs can contribute to the buildup of soil toxicity and environmental pollution (Govaerts et al., 2009). It is in consideration of the above factors that policy makers are calling for the development of sustainable farming practices. The focus of conservation agricultural management principles is to establish management practices that integrate the efficient use of natural resources and external inputs with an aim of improving crop productivity while conserving the environment and maintaining soil quality (Hobbs et al., 2008; Powlson et al., 2011). Conservation agricultural (CA) management practices that include reduced tillage, cover cropping and crop rotation are some of the practices being endorsed by policy makers to mitigate soil erosion, minimize the emission of greenhouse gasses, as well as increase soil quality and crop productivity (Hobbs et al., 2008).

The replenishment of soil organic matter (SOM) and the gradual increase in the relative abundance, diversity and activity of soil microbial communities are some of the factors attributed

to improved soil quality under CA (Tikhonovich and Provorov, 2011; Mohammadi et al., 2011). Reduction in soil disturbance, maintenance of residual material, use of cover crops, and crop rotation have been recognized as factors that lead towards changes in microbial abundance, diversity and activity (Hobbs et al., 2008). These changes call for a strategy to evaluate management practices that can be used depending on the different cropping system, soil type, climatic conditions, as well as potential pests and diseases.

It is clear that microbes play an important function in agricultural production, and that different management practices have an influence on microbial structure and functions, as will be discussed in the next sections. The questions that arise are which microorganisms and microbial activities are amplified or moderated under different management practices, how do the members of the microbial community symbiotically or competitively interact with each other and how this in turn influences sustainable crop production and ecosystem functions. The goal of CA management practices would be to achieve a long-run equilibrium of a microbial community structure that would facilitate factors such as increased nutrient capacity, soil structural buildup and aeration, as well as plant disease suppression (Hobbs et al., 2008).

The role of microbial communities in agro-ecosystems

Soils form one of the most complex ecosystems teeming with a vast range of microbes with the identity and functions of a majority of these still being unknown (Torsvik and Øvreås, 2002; Fitter et al., 2005; Little et al., 2008). It has been estimated that one gram of soil may contain up to 10 billion microorganisms (Torsvik and Ovreas, 2002). These include a wide range of species of bacteria, fungi, algae and protozoa. The microbial community plays a critical role in the maintenance of soil quality and agro-ecosystem functioning. Soil quality has been defined as

“the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, maintain the quality of air and water environments, and promote plant, animal, and human health” (Doran and Zeiss, 2000).

One of the key roles of soil microbial communities is their integral role in regulating soil biogeochemical cycling processes, through decomposition of soil organic matter (Powlson et al., 2011; Tikhonovich and Provorov, 2011). For example, microbes contribute to the carbon cycle in several ways; firstly, soil microbes are involved in the decomposition of organic matter releasing CO₂ to the atmosphere, and secondly they also act as a carbon sink contributing to the pool of SOC. The balance between these processes and stability of the microbial derived organic matter will determine carbon sequestration, a key goal in sustainability. The key driver of achieving this is the efficiency of biomass incorporation into fungal and bacterial biomass that is referred to as carbon use efficiency (CUE) or microbial growth efficiency (MGE) (Six et al., 2006; Jastrow et al., 2006). The CUE/MGE determines the balance between microbial cell biomass production (growth) and the rate of microbial respiration and excretion (metabolism). The stability of the microbially-derived organic matter (MOM) will then depend on the nature of the MOM and also on the degree of its protection by soil aggregates.

Different groups of soil microbes will differ in their MGE, the composition of cell wall structures and MOM, and in the enzymes produced to break down SOM. The major groups of microbes in the soil are bacteria, archaea and fungi which make up approximately >90% of soil microbial biomass (Six et al., 2006; Jastrow et al., 2006). The cell walls of fungi are mainly composed of melanin and chitin which are complex molecules that are more resistant to degradation. On the other hand, bacterial cell walls are mainly composed of phospholipids which are more readily

degradable (Bailey et al., 2002). Due to this fact the contribution of fungi to the microbial biomass pool is typically larger compared to that of bacteria. Fungi also have a higher C:N ratio of about 10, while that of bacteria is around 4 (Six et al., 2006). The nature of the extracellular enzymes that bacteria and fungi produce also differ: Fungi mostly produce enzymes that can attack lignitic material promoting condensation reactions, while bacteria produce enzymes that would favor the breakdown of nonlignitic material. This difference in the breakdown of lignitic vs nonlignitic material is important because the degradation of lignitic material will lead to the buildup of monomers that are the constituents of recalcitrant humic material of SOM. It has also been hypothesized that fungi have a more efficient MGE compared to bacteria, which means that the amount of new biomass C produced per unit substrate of C metabolized by fungi is greater compared to that of bacteria (Six et al., 2006; Jastrow et al., 2006). Given the above stated factors, it is clear that an increase in microbial biomass would contribute to the pool of SOC but how long it is retained in soil would be dependent on the microbial community composition, its overall MGE, the quality of available substrate as well as the degree of protection of the substrate.

Another role of soil microbes involves their contribution to soil aggregate formation and soil structure stabilization. Soil microbes have been shown to promote the process of soil aggregate formation and stabilization through different mechanisms that include the mixing and formation of channels within the soil matrix, production of extra-cellular and polymeric substances that coagulate soil particles, degradation and alteration of soil organic matter, and the attachment of their cells to soil particles (Powlson et al., 2011). In particular, fungi are said to facilitate macroaggregate formation and stabilization as hyphae and mycelium channels through soil

(Rillig, 2004; Borie et al., 2008) , while bacteria are said to contribute to microaggregate stabilization (Caesar-TonThat et al., 2007; 2010).

Microbes play an important role in plant health through control of diseases, and in adaptation to physiological stresses like drought. Studies have shown that different pests can either increase, decrease or remain constant after the onset of conservation practices (Garbeva et al., 2004). It is believed that the incorporation of crop rotations and cover crops would have an effect of reducing pest and disease incidences by increasing microbial diversity and in turn increasing competitive advantage of beneficial organisms versus the pathogenic (Patzek, 2008; Govaerts et al., 2009). Microbial diversity has also been considered a key factor in the development of soil suppressiveness, i.e., the ability of soil to naturally suppress soil borne-diseases (Garbeva et al., 2004).

A recent study undertaken to characterize the soil fungal community structure along a disease severity gradient of soil borne pathogen affecting field peas demonstrates that microbial communities differ between soils with diseased plants and healthy plants (Xu et al., 2012).

Garbeva et al. (2004), in their review on microbial diversity and soil suppressiveness concluded that understanding shifts in microbial diversity would be necessary towards development of agricultural management practices that would maximize microbial communities that promote building up of soil suppressiveness.

Understanding the microbial dynamics in terms of composition, structure as well as their inter-relations to soil functions is therefore necessary in establishing and integrating management practices that promote sustainable agro-ecosystem functioning.

Characterization of microbial community structure: approaches, challenges and opportunities

Understanding the dynamics of soil microbial communities and their interacting factors can be a daunting task. First, many of the microorganisms thought to enhance soil quality are difficult to culture or cannot be cultured. It is also difficult to devise experimental designs capable of simulating exact field conditions thus complicating the analysis of interacting environmental effects. In addition, most of the methods are limited in their capabilities to determine microbial composition and linking this to soil functioning (Nannipieri and Ascher, 2003; Six et al., 2006) as well as the fact that most methods that can be used are rigorous and time consuming (Ghazanfar et al., 2010).

Because soil microbial biomass (SMB) is the living component of SOM, assessing the changes in the SMB is used as an early indicator of improvements in soil quality as it responds more quickly to changing soil conditions (Brookes, 2001). In general, it is accepted that an increase in SMB would be beneficial to the functioning of a given ecosystem. It is based on this premise that the evaluation of SMB can be used as a comparative measure of improvements in soil quality between different management practices. However, questions have been raised on the meaning and interpretation of the values of SMB (Carter et al., 2011; Gonzalez-Quiñones et al., 2011). The challenge lies in the fact that there are no benchmark values of SMB that reflect the normal functioning of a given soil ecosystem (Gonzales-Quinones et al., 2011). SMB is also prone to temporal variability due to its sensitivity to seasonal environmental factors, soil types, and soil sampling and handling which further confounds its interpretations (Carter et al., 2011; Gonzales-Quinones et al., 2011). A clear understanding of the desirable range of SMB for maintaining

normal soil ecological functions would enable effective monitoring and evaluation of soil quality as influenced by management practices. Scoring of SMB by factoring/constraining it to inherent soil characteristics and site climatic factors has been proposed as one approach to determining critical attainable SMB values within a given soil ecosystem (Gonzales-Quinones et al., 2011).

Characterization of the microbial community structure, composition and diversity provides an added avenue of further understanding the role of microbes in influencing key soil ecological functions. The methods for studying microbial community diversity and structure can be categorized into classical, biochemical, and molecular techniques that can be either culture-based or culture-independent (Kirk et al., 2004; Little et al., 2008). The classical approach is the plate count technique that relies on culturing of bacteria or fungi on agar media followed by identification and quantification of specific taxonomic or functional groups. This technique is, however, limited by the fact that a majority of microbes are uncultivable with the estimate being only 1% of microbes in soil can be cultured. The method is also biased towards fast growing microbial groups and is therefore not suitable in studies geared towards investigating microbial community diversity and structure especially in the environment like soil

The biochemical techniques that include the sole carbon utilization patterns, and fatty acid methyl ester analysis (FAME), and various molecular techniques are the common methods of choice used in the characterization of microbial community structure. The basis for selection, the advantages and disadvantages of specific methods are described in reviews by Kirk et al. (2004) and Little et al. (2008). For this dissertation FAME and 16S rRNA gene sequencing techniques were the methods used.

FAME is a biochemical approach that relies on the extraction and characterization of signature cell wall phospholipid linked fatty acids that are associated with different microbial groups (Zelles, 1999a). This method is founded on the basis that different microbial groups have some unique fatty acid characteristics. For example, the classification of bacterial groups is mainly based on saturated, branched, monounsaturated and cyclopropane fatty acid carbon chains, while fungi are classified mainly based on presence of linoleic acids (Frostegard and Baath, 1996; Zelles, 1999a; b). Based on this classification several fatty acid biomarkers have been identified that are associated with bacterial groups of gram negative/positive bacteria, and actinomycetes and fungi (Zelles, 1999a).

The advantages of the FAME analysis are that: 1) it is culture independent and thus not biased to the culturable microorganisms; 2) it is based on essential living cell membranes thus it can then be used as an indicator for viable microbial biomass; and 3) can be used to calculate the ratio of the relative abundance between fungal and bacterial biomass (Kirk et al., 2004 and Nannipieri and Ascher, 2003). The drawback to this method is that FAME signatures cannot separate individual taxa and different taxa may have overlapping FA biomarkers. Also FAME is limited in its application for determining diversity indices; and therefore caution should be taken in interpretation and discussion of the overall implication of the FAME patterns and consistency in signatures used comparing results under different ecological conditions (Frostegård et al., 2011).

It would be important to note that different techniques are available that utilize the FAME approach. These include the standard FAME that first fractionates/separates out different lipids and methylation is then carried out on the basis of the different phospholipids. The other technique relies on direct methylation of whole cell fatty acid without separating out the

glycolipids and neutral lipids, a technique that has been commercialized referred to as microbial identification systems (MIDI) and a modification of the MIDI technique commonly referred to as EL-FAME (Zelle, 1999a). The whole cell fatty acid approaches are criticized for including the storage fatty acids which may be more sensitive to growth condition.

Recent advancement in molecular techniques in the last two decades has revolutionized soil microbiology by providing culture-independent methods that have better resolution in the taxonomic identification of species composition diversity and functional potential of microorganisms within a given ecosystem. The advent of the next generation sequencing (NGS) platforms has boosted the field of soil microbiology by availing more affordable and faster means of large scale analysis of genetic information from soil microbial communities (Ghazanfar et al., 2010; Doolittle and Zhaxybayeva, 2010; Wooley et al., 2010; Simon and Daniel, 2011). These mainly involve the extraction and sequencing of nucleic acids from environmental samples directly (metagenomics) or based on specific phylogenetic markers (microbiomics) (Wooley et al., 2010; von Mering et al., 2007; Simon and Daniel, 2011).

Several environmental sequencing studies demonstrate the impact this approach has in answering a wide range of ecological diversity and functionality questions in different scientific fields. In a comparative analysis of the microbial communities based on metagenomics from contrasting environments, Tringe et al. (2005) demonstrated that different environments exhibited a wide range of species complexity. The environments they characterized ranged from agricultural soils to three deep sea whale carcasses. Not surprisingly, the agricultural soil had a greater species complexity compared to those of the whale carcasses. The applications of environmental

sequencing are clearly wide with the potential of having several testable hypotheses from one metagenome/microbiome dataset (Rodriguez-Brito et al., 2006).

Nevertheless, the application of environmental sequencing is also faced with some challenges. One challenge involves the isolation and extraction of high-quality DNA that encompasses all the microorganisms found within an environmental sample (Simon and Daniel, 2011). This is due to the fact that many microbial cells may be difficult to lyse using the most common DNA extraction protocols. Extraction of representative DNA is even more challenging for complex soil environments due to the interaction of the microorganisms with the physiochemical properties of soil (Lombard et al., 2011). This challenge has been addressed by the development of protocols that allow the isolation of high quality DNA from different environments and thus selection of an appropriate extraction protocol is crucial to obtaining optimum DNA yield (Simon and Daniel, 2011; Lombard et al., 2011). Another challenge involves obtaining a sample that is representative of the particular environment and one that can be utilized in comparative analysis studies. Sequencing based on phylogenetic markers is also stated to have biases mainly due to the PCR amplification steps involved and thus direct sequencing is stated to be ideal in giving the global view of the species composition within a given environment. Concerns regarding soil environmental sequencing are extensively addressed in a review by Lombard et al. (2011).

Perhaps one of the more challenging aspects of next generation sequencing relates to data handling and analysis - bioinformatics. The analysis of environmental sequencing is not only faced with the challenge of handling large data sets and short sequence reads but is further hampered by the fact that the sequences originate from a wide range of organisms (Lombard et

al., 2011; Tringe et al., 2005). This raises difficulties in the analyzing and interpreting the large data output generated. The depth of sequencing, referred to as coverage, varies from one sequencing platform to the other and usually depends on the read length, i.e. the platforms that give longer reads will usually give less depth and vice versa. The different next generation sequencing platforms generate base pair (bp) sequence read lengths ranging from as short as 35bp to 400bp (Morozova and Marra, 2008; Glenn, 2011). The platform currently recommended for deeper sequencing is the Illumina Mi/HiSeq which can generate millions of sequence reads lengths ranging from 35-300bp. The technique used for sequencing, i.e. metagenome or amplicon sequencing, the length of sequences, and depth of coverage are all factors to consider when deciding on bioinformatics program software. This has led to the release of various metagenome/microbiome analysis software platforms that perform processes that include sequence quality control, classification and comparative analysis based on different programming languages and mathematical algorithms. Several open source software applications that are commonly used include QIIME (Caporaso et al., 2010), Mothur (Schloss et al., 2009), and MG-RAST (Meyer et al., 2008).

A measure of potential microbial activities is valuable in gaining a more comprehensive understanding of the contribution of microbial communities towards agro-ecological functions like nutrient cycling. There are several approaches used for estimating microbial activities that take advantage of the need of microorganisms to utilize substrates for growth and reproduction. The sole carbon source utilization pattern technique also referred to as community level physiological profiling (CLPP) is a method that is used to differentiate between microbial communities based on potential functional diversity. It relies on grouping bacteria and fungi communities based on their ability to utilize different carbon sources. The advantage of this

method is the availability of commercially prepared plates that are set to analyze for different carbon sources making it highly reproducible, relatively inexpensive and allows for the analysis of many samples at the same time (Little et al., 2008). The drawback to this technique is that like the plate count method it only represents culturable fractions of the community and favors' fast growing organisms.

Respiration, a vital process of all living organisms provides a basis of estimating the potential of microbial communities to breakdown/oxidize organic materials and release nutrients i.e. mineralization. Soil microbial respiration is determined by measuring the amount of carbon dioxide released by a given mass of soil per unit of time after a given period of incubation (Pell et al., 2006). The amount of CO₂ evolved can be measured by several methods that include: the trapping of CO₂ in a sodium hydroxide solution and back-titrating it with hydrochloric acid; the use of infra-red gas analyzers; and the use of gas chromatography (Pell et al., 2006). Soil microbial respiration can be employed to determine the functional potential of soil microbial communities based on two main approaches. One approach involves the estimation of respiration from a given field sample without addition of any substrate and is referred to as the basal respiration rate (Pell et al., 2006). Comparative analysis of the basal respiration rate can then be used as an estimate of the quantity and quality of substrate between soil samples. The second approach involves the measurement of soil respiration in the presence of an added substrate such as glucose referred to as the substrate-induced respiration. The substrate induced respiration method is used to provide an estimate of microbial biomass (Anderson and Domsch, 1978) and can also be modified to provide a measure of the contributions of bacterial and fungal populations to soil metabolism by inhibiting the activity of either one of the microbial groups

(fungi or bacteria) and then measuring substrate induced respiration using specific substrates that are utilized by the active microbial group (Anderson and Domsch, 1973).

Microorganisms produce a wide range of enzymes that govern the breakdown and assimilation of substrates a process that is central to biogeochemical nutrient cycling. Thus a measure of enzyme activities in soil is used as a means of gauging the probable functional potential of soils in cycling and retention of certain nutrients like C, N, P and sulfur (Dick, 2011). The most common enzyme assays that have been developed involve the assessment of extracellular hydrolytic or oxidative enzymes. The hydrolytic enzymes are substrate specific and catalyze reactions that cleave specific bonds that link different monomers (Dick, 2011). An example of these includes the glycosidases such as β -glucosidase that catalyzes the hydrolysis of β -D-glucopyranosides in the degradation of cellulose. The oxidative enzymes on the other hand act on broader classes of substrates that have similar bonds. An example of these includes peroxidases that are involved in the breakdown of lignin (Dick, 2011).

Standardized enzyme assays mainly involve the determination of changes in the concentration of the reaction substrate or product under buffered conditions mainly using artificial substrates that are linked to a chromophore or fluorogenic component that can be detected by spectrophotometry. Examples of these include the p-nitrophenyl (PNP) and 4-methylumbelliferone (MUF)-linked substrates that are used for the assays of hydrolytic enzymes. Based on these substrates, several protocols have been developed targeting different enzyme activities with more recent developments involving the use of micro-plate techniques that enable the analysis of several enzymes and many samples within a shorter period of time (Nannipieri et al., 2012; Deng et al., 2011; Popova and Deng, 2010).

Concepts of microbial structure and diversity measures

Diversity, structure and function are some of the descriptors used in characterization of microbial communities. Structural properties mainly aim to describe the microbial community in terms of members who are within a particular community while functional properties, on the other hand, aim to describe how the microbial community behaves in performing various processes (Little et al., 2008). Diversity is a term used to describe the size, distribution and variability within and among communities in terms of structure and function (Torsvik and Ovreas, 2002).

In characterization of structural diversity, various components are given consideration. These include the members who are within a particular community (species composition), their numbers (richness), and the distribution of individuals among species (evenness) (Torsvik and Ovreas, 2002; Nannipieri and Ascher, 2003; Little et al., 2008). Measures of bio-diversity within a given community referred to as α -diversity, and among the communities referred to as β -diversity can be calculated based on different diversity metrics (Whittaker, 1960; Whittaker, 1972, Ovreas, 2000; Nannipieri and Ascher, 2003).

It is believed that microbial diversity is an attribute that can be used to estimate how well a given ecosystem will perform (Nannipieri and Ascher, 2003) and maintain its function and structure which is termed as its robustness/stability (Little et al., 2008). The robustness of an ecosystems/community refers to its ability to resist change in structure or functioning after a significant perturbation (Nannipieri and Ascher, 2003; Little et al., 2008). Robustness can be looked at in three different ways, temporal stability-how well the community maintains its structure over time; resistance – ability to resist change after a perturbation; and resilience – the

ability to return to its native state after significant perturbations/disturbances. It is believed that microbial diversity is directly correlated to ecological stability.

On the other hand, there is still an ongoing debate on whether an increase in community diversity necessarily leads to an increase in functionality and robustness on the basis of functional redundancy (Nannipieri and Ascher, 2003; Little et al., 2008). Functional redundancy has been defined as the ability of one microbial taxon to carry out a process at the same rate as another under the same environmental conditions (Allison and Martiny, 2008). The concept of functional redundancy addresses a challenge to the diversity theory above because it implies that the loss of diversity/or loss of certain species in a given ecosystem would not necessarily alter the ecosystem function and stability as other species would easily replace its function. The main reason why this would be an important concept to soil microbial ecologists lies in the fact that it would have direct implications on the response of an ecosystem functioning to shifts in microbial composition that may arise due to stress and disturbances. In a review on this topic, Nannipieri and Ascher (2003) hypothesized that “a minimum number of species are essential in ecosystem functioning under steady conditions but a large number of species maybe essential for maintaining stable processes in changing environments.”

The question that arises then on the basis of functional redundancy is whether there are certain ecological functions that are more prone to be altered or maintained as a result of shifts in microbial composition. The proposition is that broad processes that can be carried out by different microbial groups like respiration, mineralization and decomposition of organic matter, would be more prone to functional redundancy than functions that are carried out by more

specialized microorganisms like nitrogen fixation, nitrification, denitrification, methanogenesis, sulphur reduction, and pathogenicity among others.

Influence of agricultural management practices on microbial structure and functions

The microbial community structure and function, is influenced by the interaction of various factors such as the soil physical and chemical properties, climate, crop type, and cultural practices like tillage, crop rotations, cover crops as well as fertilizer and pesticide application (Six et al., 2006; Govaerts et al., 2009). Soil physical and biochemical changes associated with CA practices have been attributed as factors that would alter the soil microbial ecology (Doran, 1980a; b, 1987; Fraser et al., 1988; Young and Ritz, 2000; Doran and Zeiss, 2000; Drijber et al., 2000). Reduced tillage practices have been associated with greater soil water content and bulk density that promotes greater abundance of anaerobic microbial species (Linn and Doran, 1984). The disturbance of the soil physical framework through tillage has been shown to disrupt fungal hyphae networks, and it's therefore expected that soils under reduced tillage would promote the proliferation of fungi (Beare et al., 1992; Young and Ritz, 2000). It is expected that crop residue left on the soil surface would promote the dominance of saprophytic fungi that are able to breakdown more resistant carbon substrates (Beare et al., 1992). On the other hand, mixing of surface residue material with soil through tillage not only makes it more accessible to soil microbes, but has been postulated to typically favor the dominance of aerobic bacteria with a greater capacity to breakdown labile substrates (Linn and Doran, 1984; Beare et al., 1992; Spedding et al., 2004; Simmons and Coleman, 2008).

Surface residue and cover crops not only serve as physical protection from soil erosion but also act as a source of additional organic C to soil and substrates to microbes. The additional C input

from cover crops has mostly been shown to correlate with an increase in microbial biomass (Wardle, 1992). The quantity and quality of additional substrates plays an important role in influencing the microbial community structure and activity (Drijber et al., 2000; Bailey et al., 2002; Bending et al., 2002). The plant residue stoichiometry in terms of its C: N: P ratios will have an influence on microbial biomass and activity (Bell et al., 2014). The C:N ratios in plant residue will drive the dynamics of mineralization vs immobilization, while the lignin/cellulose content will drive the decomposition rate as well as the dominance of bacteria vs fungi. The cover crop used for crop rotation would therefore have an influence on microbial dynamics which has been observed in several studies (Acosta-Martínez et al., 2003, 2010a; Acosta-Martínez, 2004; Wortman et al., 2013). For example, mycorrhizal colonization levels in cotton production have been shown to be greater in crop rotations that included wheat or corn than continuous cotton (Wright et al., 2008; Acosta-Martínez et al., 2010a), supporting the theory that plant species diversity may correlate with microbial diversity.

The inclusion of cover crops with different substrate quality, either high C residue crops and/or leguminous N fixing cover crops on the other hand usually necessitates changing strategies in the application of N based fertilizers (Reiter et al., 2008). This warrants increasing N-rates when using high C residue crops that would counteract possible immobilization. In contrast, for low C residue crops N-rate would be decreased to compensate for N mineralization. The manipulation of N-rate application introduces another influential factor on microbial community dynamics. N additions has been shown to have variable effects on microbial biomass and activity (Wardle, 1992; Treseder, 2008). N can be beneficial by promoting plant growth and thus increasing the quantity of residue that can be returned to soil (Alvarez, 2005). The added residue then acts as an additional source of C substrate to soil microbes that may promote their proliferation and

diversity. On the other hand, N can change the osmotic potential and soil chemistry creating conditions that can be toxic to soil microorganisms. For example, high levels of N can lead to acidic conditions, which in turn limit availability of magnesium and calcium, and increase aluminum solubility, which can be toxic to microbes (Treseder, 2008).

Several studies have shown that tillage, soil type, crop species, and residue management can alter the diversity, structure and distribution of soil microbial community, microbial activity, as well as soil quality parameters (Lupwayi et al., 1998; Feng et al., 2003; Spedding et al., 2004; Roldán et al., 2007; Reganold et al., 2010; Reeve et al., 2010).

Reeve et al. (2010) investigated the effects of soil type and farm management on various microbial activities that included microbial respiration, enzyme assays and ecological functional genes. By correlating microbial activities to gene functions, their study showed that management had an influence on functional activity and diversity of the microbial community, with soils that were managed organically having a greater microbial diversity relative to conventionally managed soils. Based on their results, management method was indicated to have a more significant effect on microbial activity compared to the soil type. Spedding et al. (2004) compared different tillage techniques i.e. minimum tillage, conventional tillage and No-till with and without crop residue. Their results showed that residue had a greater impact on microbial dynamics compared to the different tillage systems, with the plots that included retained residue having greater microbial biomass C and N and was greater by 61 and 96 %, respectively.

Lupwayi et al. (1998) and Feng et al. (2003) both did studies looking at the microbial community structure and diversity under conventional tillage and no till systems based on substrate utilisation patterns. Lupwayi et al. (1998) investigated the microbial community

structure and diversity under wheat in no till and conventional tillage with or without crop rotation. Their results showed that the diversity and distribution of bacteria species was significantly reduced in plots under tillage. On the other hand, the diversity was significantly greater in fields under crop rotation of wheat with clover in comparison to fields under continuous wheat. Feng et al. (2003) investigated the microbial dynamics under conventional till and No-till continuous cotton system at different time of the season and at different depths. They analysed the soil organic C and N, the microbial biomass and did a microbial community profiling based on phospholipid ester-linked fatty acid (FAME). Their results showed an improvement of soil quality indicators in the No-till system based on significantly greater levels of soil organic C, and N, and microbial biomass in the surface layers compared to the conventional till system. Tillage also influenced the relative abundance of soil microbes which was greater in the No-till systems particularly during the fallow period and prior to cotton establishment.

Research shows that tillage and management practices do significantly influence the dynamics of the microbial community significantly. However, due to the limitation in most methods used in studying microbial dynamics, very few studies have characterized the microbial species composition under these different management practices. The recent advancements in molecular techniques in the last two decades provides several approaches that can now be applied to characterize the microbial community structure and carry out a comparative analysis of microbial communities under different management practices. These include the sequencing of environmental DNA samples based on conserved marker genes like the 16S ribosomal RNA.

Most studies that have used the sequencing approach have characterized microbial taxonomic composition across contrasting land uses for example between pasture, forest soils, grassland, and cropland (Lauber et al., 2008, 2009; Acosta-Martínez et al., 2010b; Shange et al., 2012). Few studies have been done to characterize the microbial community profile within one land use comparing soils under different conservation and tillage management practices based on this approach. One of the studies was done by Ceja-Navarro et al. (2010), where they characterized the bacterial communities under contrasting tillage practices. Their study was able to elucidate the species composition within the contrasting environments and demonstrated how they differed from each other. In their study, they looked at the effect of surface crop residue management under No-till and conventional tillage (CT) system by using phylogenetic markers and multivariate analyses of sequences done based on 16S rRNA bacterial amplicons (Ceja-Navarro et al., 2009, 2010). They demonstrated that retention of surface crop residue led to the increase of several groups of beneficial bacteria, such as Pseudomonadales, which have several species involved in reduction of soil borne diseases. The treatments without surface crop residue had relatively lower abundance of the same bacterial groups. They further illustrated that soils under No-till and crop residue retention on the soil surface had the highest level of species diversity compared to the tilled soils. Some of the bacterial groups that were greater in treatments under No-till with crop residue retention included species such as *Pseudomonas*, *Xanthomonas*, *Rhizobium*, and *Rhodospirillales*. Residue retention also reduced the relative abundance of species within the *Acidobacteria* and *Actinomycetes*. Their research demonstrates that rRNA profiling can be used to illustrate how different management strategies can influence various bacterial species and also how these groups interact based on the management practices.

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CHAPTER I

TILLAGE, COVER CROPS, AND NITROGEN FERTILIZATION EFFECT

SOIL QUALITY, MICROBIAL COMMUNITY STRUCTURE AND

FUNCTION IN A LONG-TERM (31YRS) CONTINUOUS COTTON

SYSTEM

Abstract

Conservation agricultural (CA) practices impact the structure and activity of microbial communities that in turn influence nutrient transformations processes, soil structural properties, and plant growth and health factors. This study aimed to characterize the soil microbial community structure and activity as well as soil physicochemical properties as influenced by long-term (31 yrs.) CA practices that included: Nitrogen fertilizer rates (N-rate) including the rates 0, 34, 67 and 101 kg N ha⁻¹; Cover crops (Hairy vetch- *Vicia villosa* and winter wheat- *Triticum aestivum*, and a No Cover control); and Tillage (Till and No-till) on a continuous cotton (*Gossypium hirsutum*) production located at Jackson, West Tennessee.

The microbial community structure determined using Fatty Acid Methyl Ester (FAME) revealed FAME biomarkers for Gram + bacteria, actinomycetes and the mycorrhiza fungi to be significantly greater ($p < 0.05$) in the No-till treatments compared to the Till. In contrast, the saprophytic fungi biomarkers were significantly greater ($p < 0.05$) in the Till treatments. The overall relative abundance of fungi to bacteria (F:B) FAME biomarker ratio was surprisingly greater in Till than No-till treatments. N had a significant effect on the relative abundance of the mycorrhiza fungi biomarker which decreased with increasing N-rate and was also significantly ($p < 0.05$) less under the vetch cover crop.

Soil quality properties revealed significantly greater ($p < 0.05$) total C and N in the combination of No-till treatments having cover crops, with the No-till treatments also having significantly greater extractable nutrients (P, K, and Ca), and enzymatic activity indicating an improvement in soil quality and fertility.

Total C and N increased with increasing N-rates being significantly greater ($p < 0.05$) at the high N-rate (101N) but in turn resulted in a significant decrease of the extractable nutrients (P, K, Ca), and soil pH. Nevertheless, it was interesting to note that treatments under the Hairy vetch cover crop did not show a response to N-rates having similar levels of total C and N at all N-rates.

These results show that CA management practices involving No-till in combination with cover crops would be the most beneficial management practices for enhancing soil quality while maintaining sustainable yield production especially for low biomass monoculture crop production systems.

Introduction

Conservation agricultural (CA) management practices that include reduced tillage, cover cropping and crop rotation are practices being endorsed by policy makers to mitigate soil erosion, minimize the emission of greenhouse gasses, as well as increase crop productivity and soil quality (Hobbs et al., 2008). The replenishment of soil organic matter (SOM) and an increase in the abundance and activity of soil microbial communities are some of the factors attributed to improved soil quality under CA as they play a central role in governing key soil functions and properties (Mohammadi et al., 2011; Tikhonovich and Provorov, 2011). Changes in SOM and microbial abundance influence soil nutrient cycling and retention, soil structural build up, soil aeration, water holding capacity, and root proliferation (Al-Kaisi, Yin, & Licht, 2005; Doran, 1980a; b, 1987; Fraser et al., 1988; Young and Ritz, 2000; Doran and Zeiss, 2000; Drijber et al., 2000). Consequently, changes occurring in the soil physical and biochemical properties of soil due to these management practices are not only mediated by, as much as they are also mediators of the microbial community and their functions.

Due to the interrelations of soil physical and biochemical soil properties with microbial communities in influencing soil functions, the measurement of different soil properties therefore provides a more comprehensive assessment of soil quality. These include properties like soil C and N, soil bulk density, soil pH, electrical conductivity, and extractable crop nutrients like N, P and K to mention but a few (Arshad and Martin 2002; Arias et al., 2005).

The scoring of soil quality indicators based on site specific-factors and their correlation to specific identifiable ecosystem services has been proposed as an accepted approach of

monitoring and assessment of changes in soil quality (Doran and Parkin 1994; Karlen and Stott, 1994; Andrews et al., 2004; Sojka et al., 2003; Zobeck et al., 2008). The soil management assessment framework (SMAF) tool is one of the recommended soil quality indexing approach for evaluation of soil quality (Zobeck et al., 2008). The SMAF is based on modelling of dynamic soil quality indicators by integrating site specific soil inherent properties, climatic factors, and requirements of crops under production to obtain unit less values ranging from 0-1 representing increment of attaining associated soil potential functions (Andrews et al., 2004). SMAF currently avails scoring curves for calculating soil indices for thirteen soil properties like soil organic matter, extractable soil nutrients, total C, soil pH, microbial respiration quotient, microbial biomass carbon among others and offers the option of selecting the most suitable depending on the purpose of study (http://soilquality.org/tools/smaf_intro.html).

The indicators are selected on the basis of how they contribute to both crop productivity and environmental quality. For example, organic matter helps to define soil fertility, retention and cycling of nutrients, soil pH determines nutrient availability and the mobility while extractable nutrients demonstrate the capacity to support plant growth (Arshad and Martin, 2002).

Interpretation of soil quality indicators could be based either on each indicator or their integration into what is known as the soil quality index (Doran and Perkin, 1994; Arshad and Martin, 2002). While it is easy to interpret each individual indicator depending on their set critical limits, it is challenging to interpret the integrated quality index as there are no baseline set and conclusions are made on a higher is better basis (Sojka et al., 2003). Nevertheless the information on the individual indices would still be valuable in informing (determining) some of the limitations of the soils under study. This information can be incorporated as a provision for

offering management recommendations on ways of maximizing the potential of the measured soil quality properties as well as informing policies.

The effects of management practices on many of these soil quality indicators are dependent on time (West and Post, 2002; Alvarez, 2005; Al-Kaisi et al., 2005;). For example, a change or increase in soil C and N upon shifting to CA practices (No-till, cover cropping and N-fertilization) becomes apparent after 10 years, while a time period of less than 10 years may result in varying and conflicting results (Havlin et al., 1990). Based on a meta-analysis by Alvarez (2005), C sequestration upon shifting to reduced tillage practices starts reaching a steady state between 25-30 yrs.

Although studies have shown that No-till, cover crops and N-fertilization result in changes on soil microbial structure, and soil physical and biochemical properties (Linn and Doran, 1984; Beare et al., 1992; Spedding et al., 2004; Simmons and Coleman, 2008; Acosta-Martinez et al., 2011), there is still paucity of information on how these practices interact together over long-term (> 30 years) on soil microbial structure, activity and overall soil quality for cotton cropping systems (Harman et al., 1989; Halvorson et al., 2002). This information is crucial as cotton produces low biomass return compared to other crops and the integration of CA practices could help to compensate for this limitation. Not all CA practices are always possible, for example, the use of cover crops has been difficult in certain regions where cotton is significantly produced (e.g. Southern High Plains) that are faced with water limitations (Harman et al., 1989; Acosta-Martínez et al., 2011).

The research presented here aimed to determine the changes in soil microbial community structure and activity after the long-term implementation of CA practices and their interrelation

to soil properties. This is based on a long-term No-till research project under continuous cotton production in West Tennessee. The research plots were established in 1981 in a randomized block split-split plot experimental design consisting of N-rates as the main plot, tillage as the split plot and cover crop as the split-split plot.

The specific objectives of this study were to: 1) Evaluate the microbial community structure and activity as affected by the different CA management practices according to the individual response indicators including microbial biomass C and N, microbial community composition (via fatty acid methyl ester (FAME) analysis) and microbial activity (microbial respiration and measurement of select enzyme activities of C, N and P cycling) 2) Evaluate the long-term effects of the different CA practices on selected soil physicochemical properties that included total C and N, extractable chemical nutrients (P, Ca, K and Mg), soil pH, and bulk density; 3) Evaluate how the microbial structure and activity may have impacted the overall changes in the soil physicochemical properties and use the SMAF tool to score the different soil properties into quality indices representing their different soil functions

The hypotheses for this study were that they would be: 1) No-till, cover crops (vetch and wheat) and higher nitrogen rates would result in increased abundance of both bacteria and fungi biomarkers and microbial activity relative to Till, No cover crop and low N-rates.; 2) No-till, cover crops (vetch and wheat) and higher nitrogen rates would result in increased total C and N and extractable soil nutrients relative to Till, no cover crop and low nitrogen rates; 3) Soil quality, as measured by the SMAF soil quality index (Andrews et al., 2004) will improve/aggrade under No-till, cover crops (vetch and wheat).and high rate of N-rate treatments relative to Till, No cover crop and low N-rate treatments.

Materials and Methods

Study Site and Soil Sampling

This study was conducted on long-term conservation agriculture research plots under continuous cotton at West Tennessee Research and Education Center (WTREC), Jackson. The soil at the site are classified as Lexington silt loam (fine-silty, mixed, thermic, Ultic Hapludalf), well-drained with a 0 to 2 percent slope. The soils are derived from marine deposits overlaid by loess deposits. The mean annual rain fall of the region is 1375 mm. The research plots under this study were established in 1981 and focused on the assessment of different tillage systems (No-till and conventional Till), cover crops: No Cover, winter wheat (*Triticum aestivum*. L), hairy vetch (*Vicia villosa*), and different nitrogen (N) fertilizer rates (0, 34, 67, 101 kg N ha⁻¹) on continuous cotton (*Gossypium hirsutum* L.) production.

The treatment and experimental design were set up in a randomized complete block design (RCBD) with a split-split plot. The whole plot was N fertilization rates with 4 levels (0 Kg N ha⁻¹, 34 kg N ha⁻¹, 67 kg N ha⁻¹ and 101 Kg N ha⁻¹), the split plot was cover crops with three levels (Hairy Vetch, Winter wheat, and No Cover crop) and the split-split plot was tillage systems with two levels (No-till and Till). Each treatment factor had four replications with the experimental units of 12 m by 8 m in size having 8 rows of cotton. Tillage is usually performed two times before planting by a standard disc harrow followed by smoothing and breaking up of clods by a harrow. It is important to note that since the plots were established in 1981, liming has only been applied once in 1995 and was targeted to plots having a soil pH < 6.0 (Cochran et al., 2007).

Field operations on the sampled plots were carried out in May 2013. The plots that are under conventional tillage were disked on the 16th May. On the 17th of May all plots including those under No-till were planted with cotton variety Phytogen 375 WRE. Fertilizer was applied on the 18th of May, both phosphorus (P) and potassium (K) fertilizers were applied at uniform rate across all the treatments. P was applied as triple superphosphate at $101 \text{ kg P}_2\text{O}_5 \text{ ha}^{-1}$ and K was applied as murrate of potash at $134 \text{ kg K}_2\text{O ha}^{-1}$. Nitrogen fertilizer was applied as NH_4NO_3 four days after planting (21st May) in accordance to the N-rate treatment levels of 0 kg N ha^{-1} ; 34 kg N ha^{-1} , 67 kg N ha^{-1} and 101 kg N ha^{-1} .

Soils were sampled on June 12, 2013 after the cotton plants had established. Soil was sampled at a 0-7.5 cm depth using a 2.5 cm diameter soil probe. Sampling was done randomly within the plot approximately 10 -15 cm away from the crop with approximately 20-25 subsamples. The subsamples from each plot were mixed together into one composite sample. Sampling for bulk density was done in October 2013 in order to minimize any confounding effects of tilling from the current season.

Characterization of Soil Chemical and Physical Properties

Soil sampling for the analysis of total C (TC) and N (TN), and soil elemental composition was done at the beginning of the cotton growing season in June of 2013. Subsamples of soils were sent to the University of Tennessee, Soil, Plant, and Pest Center laboratories in Nashville for analysis. TC and TN were measured using a Thermo Flash EA 1112 NC combustion analyzer after samples had been dried and sieved through a 30 um sieve. Soil elemental analysis was done for extractable phosphorous (P), potassium (K), calcium (Ca), and magnesium (Mg) based on a Mehlich 1 extraction and measured using a Perkin-Elmer 5300 & 7300 DV Inductively Coupled

Plasma (ICP) unit. Soil pH was measured using a 1:1 soil/water suspension and the buffer capacity subsequently determined by adding 10 ml Moore-Sikora buffer (Sikora and Moore, 2008).

Soil bulk density was calculated based on the dry soil mass of intact soil cores of a known volume (7.5 cm long and 7.5 cm diameter metal cylinder) after drying at 105°C for 24 h based on the soil core method (Hartge et al., 1986). The bulk density values were then used to calculate the soil C and N to a volume/area basis (Kg ha^{-1}).

Characterization of soil biological properties

Microbial biomass C (MBC) and N (MBN) were determined using the chloroform fumigation direct extraction (CFDE) method on 10 g oven-dry equivalent samples (Horwarth and Paul, 1994). Samples were fumigated in the dark for 48 h after which C and N of fumigated and non-fumigated samples were extracted using 0.5 M K_2SO_4 . Total dissolved organic C (DOC) and total extractable N were measured on a Shimadzu TOC/TN analyzer. Values for non-fumigated samples were then subtracted from fumigated samples and a K_{ec}/K_{en} of 0.35 for C and N respectively applied (Voroney et al. 1991).

Microbial respiration was determined based on soil incubation in 500 ml mason jars and sampling the headspace for CO_2 . 50 g moist soil (field condition) was placed into the jar and sealed tightly with a cap fitted with a septum suitable for gas sampling. Jars were then sampled with a needle attached to a 1 ml syringe and measured with an infrared gas analyzer, LI-COR, 820 (LI-COR Biosciences, NE, USA). The samples were then incubated at 25°C and resampled every other day for a period of two weeks. CO_2 was calculated in comparison to CO_2 standards

of known concentration that were measured at each of the CO₂ samplings to generate a standard curve.

Soil potential biogeochemical cycling was evaluated according to the activities of select enzyme activity, i.e. phosphodiesterase, β-glucosidase and β-glucosaminidase using air-dried soil (0.5 g < 5 mm) with their appropriate substrate (p-nitrophenyl derivate) and incubated (37°C) at their optimal pH following the assay conditions described in Tabatabai (1994) or Parham and Deng (2000). The enzyme activities determined according to the release from p-nitrophenol as the reaction product were expressed in mg of p-nitrophenol released kg⁻¹ soil h⁻¹. All enzyme activities were assayed in duplicate with one control, to which substrate was added after incubation and subtracted from the sample value.

Soil microbial community structure was determined using Ester-linked Fatty Acid Methyl Ester (EL-FAME) analysis method (Schutter and Dick, 2000). Saponification and methylation of ester-linked fatty acids (FA) was done by incubation of 3 g of moist equivalent soil in 15 mL of 0.2 M KOH in methanol at 37°C for 1 h with vortexing done every 10 min; neutralisation of the extracts was then done by adding 3 ml of 1.0 M acetic acid and the FA were then partitioned into an organic phase using 10 ml of hexane followed by centrifugation at 480 x g for 15 m; the organic layer was transferred to a clean glass test tube and the hexane was evaporated under a stream of nitrogen gas; the FA were then re-dissolved in 0.5 mL of 1:1 hexane:methyl-tert butyl ether containing methyl nonadecanoate (19:0) as an internal standard, transferred to 2 ml GC glass vials and FA concentrations measured using an Agilent 6890 N gas chromatograph with a 25 m × 0.32 mm × 0.25 μm (5 % phenyl)-methylpolysiloxane Agilent HP-5 fused silica capillary column (Agilent, Santa Clara, CA) and flame ionization detector (Hewlett Packard, Palo Alto,

CA) with ultra-high purity hydrogen as the carrier gas. Absolute amounts of FA (nmol g^{-1} soil) were calculated according to Zelles (1996) using the 19:0 internal standard which was then used to calculate molar percent (mol %). Twenty-six fatty acids (FA) that were consistently present in all samples were used for the data analysis with fourteen of these identified as FA representing different bacteria and fungi biomarkers as determined in previous studies (Frostegard and Baath, 1996; Zelles, 1997, 1999; Feng et al., 2003; Mathew et al., 2012). FA having the length of 14 carbon chain and higher were used to calculate total FAME (nmol g^{-1} soil), which is usually used as an estimate of microbial biomass. The bacteria biomarkers identified included five Gram positive (Gram+) bacteria (i15:0, a15:0, i16:0, i17:0, a17:0); three Gram negative (Gram-) bacteria (cy17:0, cy19:0, 16:1w7c) and three actinomycetes (10Me16:0, 10Me17:0, 10Me18:0). The bacterial sum was calculated based on the summation of the Gram +, Gram- and actinomycetes biomarkers. Fungal indicators included two saprophytic biomarkers (18:2 ω 6c, 18:3 ω 6c) and an arbuscular mycorrhizal fungi (AMF) associated biomarker (16:1 ω 5c). The fungal sum was calculated based on the summation of the 18:2 ω 6c and 18:3 ω 6c. The AMF biomarker was not included in the fungi summation as it is not considered saprophytic fungi but as a symbiotic fungi and thus unique in its soil function. The fungal/bacterial ratio was calculated by dividing the fungal sum by the bacterial sum.

Calculation of the soil management assessment framework (SMAF) quality indices

Soil quality indices were calculated based on the soil management assessment framework (SMAF) as described in Andrews et al. (2004). Seven of the 13 indices with scoring algorithms that are currently available under the SMAF quality scoring algorithms were used for this study. These include, Organic C estimated from the total C, soil pH, bulk density, soil extractable P,

and K, microbial biomass (MBC) and β -glucosidase activity. The selection of the SMAF indices is based on their role in certain soil functions that can be used as measures for attaining specific management goals. MBC, soil pH, P, K and β -glucosidase activity are indices selected for their role in nutrient cycling. Organic C and bulk density are selected for their role in soil-water relations, aggregate stability as well as filtering and buffering. All the selected indices are measures used for the assessment of crop productivity and ecosystem functioning (Andrews et al., 2004).

The soil quality indices were calculated based on modelled nonlinear scoring curves developed for each indicator. The scoring curves are developed based on algorithms and logical statements that factor in the relationship of normalized scores of the empirical values of the indicator to controlling factors that limit or enhance its performance for the representative soil function. Based on the scoring algorithm used, each indicator is then transformed into unitless scores ranging from 0-1 with a score of 1 representing the highest potential of the indicator for its associated function within the given system (Andrews et al., 2004). The factors taken into consideration for determining the scoring curves include the inherent soil properties (for example, the expected organic matter content for a given soil type), climatic factors, and cropping history. The relationship between the indicator and controlling factors determines the fitting of the model to known predictor modelling curves that determine the interpretation of each indicator. These include the upper asymptotic sigmoid curve that assumes that more-is-better, lower asymptote curve that factors less-to be better, and the gaussian function with a mid-point optimum. The scores obtained from each indicator are then integrated into a soil quality index by dividing their sum by the total number of indicators used and multiplying that by 100. An Excel sheet containing the modeled algorithms for each SMAF indicator is available from the

developers of the SMAF index and was used for the calculation in this study

(http://soilquality.org/tools/smaf_intro.html).

The factor class groupings used for this study based on the provisions of the SMAF modelling for inherent soil properties, climatic factors and cropping consideration were as follows: An organic matter class that factors in the expected range of organic matter for soils within the taxonomic sub-order classification of udalfs; textural classification for silt loam soils; climate classification based on the average annual precipitation (1200 mm) and average annual cropping days (180 to 220 frost free days) ; spring seasonal class based on the sampling time (June 2013); regional class for a humid temperate climate; crop classification of crops with similar growth requirements in terms of soil pH, electrical conductivity, and P range; weathering class based on the soil order classification (Alfisol); a slope class for slopes ranging between 0-2 %; and the P extracting method (Mehlich 1). These factors were used as necessary for the fitting of the scoring curves for each individual soil indicator. For example, for the fitting of organic C, the factors put into consideration include the taxonomic sub-order, textural class, weathering class, climate class and regional class.

The interpretation of MBC, organic C, and extractable K, are based on the more-is-better asymptote curve. Bulk density is based on the less-is-better function on the assumption that high bulk density would have inhibitory effect for root growth and porosity. P is based on the mid-optimum curve criteria based on the balance between its availability for the seasonal crop need and minimizing risk of surface water contamination through run off.

Data Analysis

Data were analysed by a mixed Model Analysis of Variance (ANOVA) and means separated using Fisher's protected LSD using SAS (SAS Institute, Cary, NJ, 2012). Given that N-rate resulted in greater differences in soil physicochemical properties that made it challenging to separate the effect of other overriding factors like pH, the analysis was split based on each N-rate in order to determine the effect of tillage and cover crop treatments. The effect of N-rates was determined on the full RBD-split split plot model. The analysis for microbial properties were run on the full RBD-split split model since N-rate did not seem to play a great influential role on the significant differences. Exploratory principal component analysis (PCA) performed on a correlation matrix using the Vegan package (ver. 2.0-2) in R (Oksanen et al., 2011) was used to distinguish treatment separation of the microbial community structure. Variable selection by forward selection of the PCA loading factors was run to determine the factors contributing to the variance explained by each component.

RESULTS

Soil chemical and physical properties

Total C and N

Tillage practice (No-till and till) and cover crops (vetch, wheat or No Cover) had a significant ($p < 0.05$) effect on the soil C and N with the effect varying based on nitrogen application rate (N-rate) (Figure 1). There was an interaction between tillage and cover crops with treatments having

cover crop (vetch or wheat cover) having significantly higher soil C and N compared to No Cover under the lower N-rates (0, 34 and 67 N). At the higher N-rate (101 N), there were no significant differences in soil C and N across tillage or cover crop treatments (Figure 1). It is interesting to note that in treatments with vetch cover crop, increasing N-rate did not make a difference in the levels of soil C and N. Soil C and N in treatments under wheat and No Cover showed a response to N-rate increment with the greatest level at the 101 N-rate.

N-rate had a significant effect ($p < 0.05$) on soil C and N with the greatest soil C and N levels recorded at the highest N-rate (101 N kg ha⁻¹) (Table 1).

Soil extractable nutrients, pH, and bulk density

Among the extractable soil nutrients that were analyzed, tillage and cover crop had a significant effect ($p < 0.05$) on P, Ca, and K but this varied dependent on N-rate (Table 2). For instance, P and Ca were greater in No-till compared to till within the 0 N, 34 N and 101 N-rates. K was significantly greater in No-till compared to till but only within the 0N-rate but was greater in till compared to No-till at the 67 N-rate.

In terms of cover crop, P and K were significantly greater under wheat and No Cover compared to the vetch which had the lowest levels but only at the lower N-rates (0 N and 34 N).

Significant differences ($p < 0.05$) in Ca only occurred at the 0 N-rate with vetch having significantly greater levels than wheat (Table 2).

N-rate also had a significant effect ($p < 0.05$) on P, K, and Ca which showed a decreasing trend with increasing N-rate (Table 1). P levels were significantly greater at 0 N-rate and decreased with the input of N-fertilizer but were not significantly different between 34, 67 and 101 N-rate.

K significantly decreased with N- rate in the order of 0 N < 34 N < 67 N = 101 N. Ca also decreased with N-rate in the following order 0 N < 34 N = 67 N < 101 N. Mg levels were not significantly affected by tillage, cover crop or N-rate. It is important to note that the levels of P and K reported are greater than expected which may be explained by the fact that sampling was done only a few weeks after fertilization and the soil test may have captured the residual fertilizer effect.

Among the treatment factors, tillage, cover crop and N-rate, only N-rate had a significant effect on soil pH. Soil pH decreased with increasing N-rate with significantly less pH ($p < 0.05$) recorded at 67 N-rate and least at 101 N-rate (Table 2).

Bulk density (BD) differed significantly between the two tillage with No-till treatments having significantly higher BD but only within the lower N-rates (0 N, 34 N and 67 N) (Table 2).

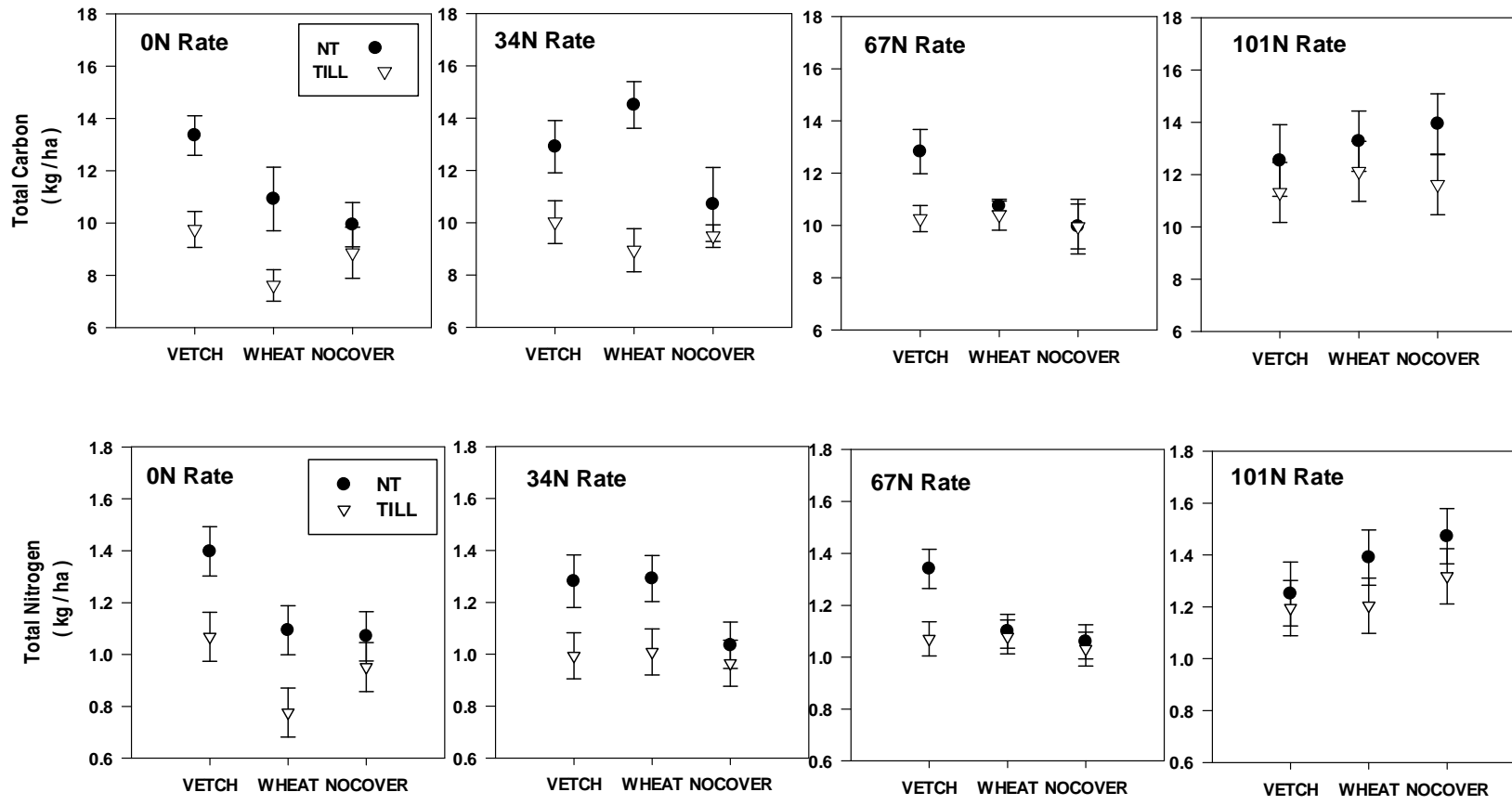


Figure 1: Tillage by cover crop effect on soil total carbon (C) (upper) and nitrogen (N) (lower).

Each point represents means (n=4) at each cover crop within each N-rate level. Overlapping standard error bars are not significantly different (LSD protected, $p \leq 0.05$)

Table 1 Effect of nitrogen fertilizer rates (N-rate) on selected soil chemical and physical properties

N-rate	TC ---- kg /ha-----	TN	P -----	K mg/kg soil	Ca -----	Mg	pH	BD g/cm ³
0 N	10.08 (0.85)b	1.06 (0.08)b	112.52 (7.52)a	247.67 (13.84)a	988.44 (33.78)a	75.25 (8.64)a	5.80 (0.15)a	1.21 (0.03)a
34 N	11.12 (0.90)b	1.10 (0.08)b	91.98 (6.64)b	223.98 (9.99)b	909.20 (28.66)b	75.33 (5.96)a	5.63 (0.12)a	1.18 (0.03)b
67 N	10.61 (0.67)b	1.10 (0.06)b	84.20 (7.84)b	200.46 (14.7)c	860.63 (40.01)b	76.33 (5.47)a	5.39 (0.14)b	1.17 (0.03)b
101 N	12.47 (1.17)a	1.31 (0.11)a	85.02 (8.38)b	207.43 (14.00)c	760.91 (61.34)c	70.54 (10.53)a	5.01 (0.0.13)c	1.18 (0.04)b

Treatment effect means (standard errors in brackets) n=4, followed by the same lower case letter across N-rate (0 N, 34 N, 67 N and 101 N Kg ha⁻¹) are not significantly different (LSD protected, $p \leq 0.05$). P (phosphorous), K (potassium), Ca (calcium) and Mg (magnesium) are Mehlich 1 extractable nutrients.

Table 2: Soil physicochemical properties-tillage and cover crop effect

N-rate	Treatments	P	K	Ca	Mg	pH	BD
0 N	Cover						
	Vetch	94.93 (9.49)b	217.50 (18.35)b	1046.25 (42.81)a	82.13 (7.33)a	5.80 (0.16)a	1.19 (0.01)a
	Wheat	122.31 (8.13)a	253.06 (8.06)a	922.38 (34.25)b	68.06 (11.39)a	5.94 (0.002)a	1.23 (0.012)a
	No Cover	120.31 (4.95)a	253.06 (15.10)a	996.69 (24.30)ab	75.56 (7.18)a	5.64 (0.31)a	1.22 (0.21)a
	Tillage						
	No-till	129.21 (7.18)a	265.21 (11.80)a	1056.13 (40.71)a	79.29 (9.62)a	5.80 (0.13)a	1.27 (0.02)a
	Till	95.83 (7.87)b	230.13(15.87)b	920.75 (26.86)b	71.21 (7.66)a	5.79 (0.16)a	1.16 (0.04)b
34 N	Cover						
	Vetch	79.31 (6.52)b	203.50 (14.95)b	919.62 (25.80)a	79.94 (4.86)a	5.59 (0.09)a	1.17 (0.03)a
	Wheat	98.93 (4.53)a	231.56 (8.18)a	918.06 (23.40)a	72.56 (4.27)a	5.60 (0.12)a	1.18 (0.03)a
	No Cover	99.61 (8.88)a	238.84 (12.07)a	891.57 (36.79)a	73.62 (8.74)a	5.71 (0.13)a	1.21 (0.23)a
	Tillage						
	No-till	101.36 (9.86)a	227.36 (12.07)a	967.82 (40.31)a	78.95 (6.96)a	5.52 (0.19)b	1.23 (0.03)a
	Till	83.38 (3.43)b	220.88 (11.40) a	855.46 (17.02)b	72.00 (4.95)a	5.74 (0.13)a	1.14 (0.03)b
67 N	Cover						
	Vetch	66.84 (8.39)b	179.61 (13.87)a	799.06 (36.92)a	74.7 (4.98)a	5.38 (0.32)a	1.16 (0.03)a
	Wheat	88.19 (6.81)a	206.56 (14.83)a	904.31 (57.9)a	78.13 (4.16)a	5.42 (0.11)a	1.19 (0.02)a
	No Cover	94.81 (8.33)a	210.31 (15.40)a	867.75 (24.80)a	77.06 (7.28)a	5.36 (0.13)a	1.19 (0.04)a
	Tillage						
	No-till	86.59 (7.68)a	186.50 (11.74)b	850.68 (42.73)a	75.23 (6.39)a	5.31 (0.17)a	1.23 (0.02)a
	Till	82.00 (8.00)a	213.25 (17.67)a	869.75 (45.28)a	77.33 (4.56)a	5.47 (0.14)a	1.12 (0.03)b
101 N	Cover						
	Vetch	73.24 (7.60)b	199.82 (20.93)a	679.71 (83.07)a	66.50 (12.63)a	4.84 (0.211)a	1.15 (0.2)a
	Wheat	88.56 (9.33)ab	208.31 (10.46)a	781.19 (55.06)a	73.94 (10.99)a	5.08 (0.13)a	1.19 (0.04)a
	No Cover	92.25 (7.45)a	212.56 (10.61)a	830.50 (45.88)a	72.75 (7.96)a	5.11 (0.11)a	1.18 (0.04)a
	Tillage						
	No-till	93.95 (7.60)a	201.32 (14.61)a	811.09 (81.10)a	72.23 (13.14)a	5.00 (0.15)a	1.19 (0.03)a
	Till	76.83 (9.15)b	213.04 (13.39)a	714.92 (45.58)b	69.00 (7.91)a	5.02 (0.078)a	1.16 (0.04)a

Tillage and cover crop means (standard errors in brackets) n=4, followed by the same lower case letter within each nitrogen rate (0N, 34N, 67N and 101N Kg/ha) and N-rate means followed by the same lower case letter are not significantly different (LSD protected, $p \leq 0.05$). P (phosphorous), K (potassium), Ca (calcium) and Mg (magnesium) are Mehlich 1 extractable nutrients.

Soil microbial community biomass, structure and activity

Treatment effects on microbial biomass were only significantly ($p < 0.05$) different for the MBN due to cover crop, which was greater under vetch cover (Table 3). Calculation of total FAMES (all FA's having > 14 C), which also represents microbial community biomass, was only significantly greater under vetch cover but also showed an increasing trend with N-rate (Table 4). FAMES indicators for Gram + bacteria (sum of FA's: *i15:0*, *a15:0*, *i16:0*, *i17:0*, *a17:0*), actinomycetes (sum of FA's: *10Me16:0*, *10Me17:0*, *10Me18:0*), and the mycorrhiza fungi (FA: *16:1 ω 5c*) were significantly ($p < 0.05$) greater in the No-till treatments. In contrast, the saprophytic fungi (sum of FA: *18:2 ω 6c* and *18:3 ω 6c*) and FA *18:1 ω 9c* were significantly greater in the till treatments. It is interesting to note that vetch cover resulted in a significantly ($p < 0.05$) greater relative abundance of the Gram + bacteria and a corresponding lower abundance in the Gram - bacteria (sum of FA: *cy17:0*, *cy19:0*, *16:1 ω 7c*) and mycorrhiza fungi biomarker. The mycorrhiza fungi biomarker also significantly decreased ($p < 0.05$) with the increase in N-rate (Table 4). Although there were significant differences in the abundance of the major bacterial groups due to tillage and cover crop, the overall abundance of total bacteria (sum of Gram + bacteria, Gram - bacteria and actinomycetes) did not reveal significant differences ($p > 0.05$) between treatments. Nevertheless, there was a significant difference ($p < 0.05$) in the ratio of fungi: bacteria FA biomarkers due to tillage, which was significantly higher in the till treatments relative to the No-till.

A PCA analysis of twenty-six FA's that were consistently present in all the samples was able to differentiate microbial community structure based on tillage, cover crop and N-rate (Figure 2).

Included in the PCA plots are the known bacterial and fungal biomarkers including the FA biomarker 18:1 ω 9c which has been identified as a fungi biomarker (Feng et al., 2003; and Acosta-Martinez; Simmons and Coleman, 2008) by several researchers and a gram - bacteria (Fierer et al., 2003) by others. The first principal component (PC 1) (which explained 23.4 % of variability) mainly differentiated the No-till and vetch cover treatments from the Till, wheat and No Cover treatments. On the other hand, PC2 (which explained 18.5 % of variability), mainly differentiated the high N-rate 101 N from the lower N-rates 0 N and 34 N. A variable selection to determine the FA's that significantly ($p > 0.05$) contributed to the variation ($R^2=0.99$) of each PC (Table 5) revealed significantly greater relative abundance of two G+ bacteria (*i*17:0, *i*16:0, *a*15:0, *a*17:0), two G- bacteria (*cy*19:0 ω 8c, 16.1 ω 7c), and actinomycetes (10Me16:0, 10Me17:0, 10Me18:0) to be more associated with the No-till, vetch treatments. In contrast the saprophytic fungi biomarker 18:2 ω 6c and the 18:1 ω 9c associated more with the till, No Cover and wheat treatments. The high N-rate (101 N) treatment did not show a significant association with any of the biomarkers, while the low N-rates associated with a greater relative abundance of the mycorrhiza fungi (16.1 ω 5c).

The potential soil metabolic capacity as determined by microbial respiration and selected enzyme activities also differed based on tillage, cover crop and N-rate (Table 3). The enzyme activities mainly showed significant differences ($p < 0.05$) due to tillage practice with the No-till treatments having significantly greater activities of β -glucosidase, β -glucosaminidase, and phosphodiesterase. Enzyme rates were mostly greater in the vetch treatments compared to wheat and No Cover, but this was only significantly greater for the β -glucosaminidase activity. There

was an increasing trend in β -glucosaminidase activity with N-rate and a decreasing trend in the activity of phosphodiesterase. Microbial respiration on the other hand was mainly influenced by an interaction of tillage and cover with the vetch, No-till treatments having the greatest rate of microbial respiration.

Table 3: Soil biological properties (microbial biomass, moisture, respiration, and enzyme activities) as affected by cover crop, tillage and N-rates

Treatment	Microbial biomass (community size)			Soil Metabolic Capacity				
	MBC	MBN (mg/kg)	MBC:N	Moisture	Respiration ($\mu\text{mol g}^{-1}$ dry soil)	Enzyme activities of biogeochemical cycling		
						β -GD	β -GAD (mg PNP g^{-1} soil h^{-1})	PPD
Cover								
Vetch	400.79(105.88)a	99.31(22.16)a	4.19(0.89)a	23.69(0.46)a	6.24(1.04)a	79.98(7.43)a	37.31(4.59)a	46.80(8.56)a
Wheat	351.74(100.41)a	78.82(15.80)b	4.63(1.25)a	21.44(0.46)b	4.42(1.12)b	70.95(7.02)a	31.44(3.41)b	43.27(6.41)a
No Cover	336.42(101.74)a	78.03(15.22)b	4.20(0.95)a	21.08(0.45)b	4.45(0.103)b	72.11(11.14)a	32.26(4.33)b	45.53(8.12)a
Tillage								
No-till	357.51(103.46)a	82.06(26.47)a	4.40(1.18)a	23.24(0.42)a	5.24(1.02)a	84.78(9.90)a	38.35(5.42)a	49.55(8.25)a
Till	368.46(89.03)a	88.72(17.80)a	4.28(0.87)a	20.90(0.42)b	4.83(1.08.41)a	63.92(7.15)b	28.99(2.79)b	40.84(7.14)b
N-rate								
0N	340.64(81.01)a	81.89(14.87)a	4.09(0.71)a	20.61(0.77)a	4.42(1.23)a	74.16(6.59)a	29.52(4.51)a	48.71(8.89)a
34N	295.44(72.79)a	68.33(10.01)a	4.32(0.94)a	21.58(0.77)a	3.79(0.96)a	79.06(11.72)a	30.88(3.83)a	47.97(8.54)a
67N	397.09(119.85)a	84.82(15.08)a	4.54(1.10)a	22.95(0.77)a	6.30(1.02)a	74.44(7.40)a	36.37(4.56)a	43.95(6.38)a
101N	418.77(146.16)a	106.52(30.95)a	4.42(1.35)a	23.14(0.77)a	5.64(0.98)a	69.75(8.41)a	37.92(3.54)a	40.16(6.98)a
ANOVA TABLE (Significance level (p=0.05))								
N-rate	0.8446	0.2058	0.9896	0.1160	0.1166	0.4923	0.1026	0.552
Cover	0.2001	0.029	0.9073	<0.0001	0.0002	0.0695	0.007	0.5696
N x C	0.8946	0.1857	0.2594	0.1671	0.3739	0.1966	0.9015	0.7631
Tillage	0.2947	0.2642	0.7751	<0.0001	0.3017	<0.0001	<0.0001	0.0021
N x T	0.5759	0.3163	0.7841	0.6296	0.6097	0.2953	0.5929	0.9918
C x T	0.5956	0.6202	0.9249	0.0054	0.0055	0.1101	0.1862	0.1661
N x C x T	0.6626	0.3212	0.6956	0.8665	0.3208	0.2487	0.5066	0.3085

Means (n=4) with standard errors in brackets for each treatment factor: Cover crop- Hairy vetch, Winter wheat, and No Cover; Tillage-No-till and Till and; Nitrogen fertilization rate (N-rate)-0, 34, 67 and 101 N kg/ha followed by the same lower case letter are not significantly different (LSD protected, $p \leq 0.05$). Microbial biomass carbon and nitrogen (MBC and MBN); Moisture content; Basal microbial respiration and Enzyme activities (β -glucosidase (β -GD), β -glucosaminidase (β -GAD), and phosphodiesterase (PPD))

Table 4: Microbial community composition according to FAME profiles as affected by tillage, cover crop and N-rates

TRT	G+ Bact	G- Bact	Actino	Bacteria	Fungi	AMF	18:1w9c	F:B	Total Fame
Cover									
Vetch	13.27(0.22)a	9.60(0.16)b	5.73(0.12)a	28.60(0.37)a	8.66(0.24)a	3.09(0.14)b	13.15(0.34)a	0.30(0.01)a	240.01(9.22)a
Wheat	12.59(0.22)b	10.25(0.16)a	5.55(0.12)a	28.41(0.37)a	8.60(0.24)a	3.91(0.14)a	13.85(0.34)a	0.30(0.01)a	196.01(9.22)b
No Cover	12.47(0.23)b	9.89(0.17)ab	5.57(0.12)a	27.94(0.38)a	8.62(0.24)a	3.93(0.15)a	14.28(0.35)a	0.31(0.01)a	203.83(9.38)b
Tillage									
No-till	12.71(0.30)a	9.87(0.15)a	5.78(0.10)a	28.70(0.31)a	8.07(0.20)b	3.94(0.12)a	13.01(0.27)b	0.28(0.01)b	211.79(7.96)a
Till	12.15(0.30)b	9.95(0.15)a	5.46(0.10)b	27.93(0.30)a	9.19(0.20)a	3.36(0.11)b	14.50(0.27)a	0.33(0.01)a	214.77(7.87)a
N-rate									
0N	12.41(0.26)a	9.81(0.22)a	5.60(0.15)a	27.85(0.41)a	8.62(0.28)a	4.92(0.16)a	13.69(0.39)a	0.31(0.01)a	193.87(10.39)b
34N	12.68(0.27)a	9.91(0.22)a	5.57(0.16)a	28.16(0.43)a	8.91(0.28)a	3.83(0.17)b	14.20(0.40)a	0.32(0.01)a	205.60(10.65)ab
67N	13.14(0.26)a	10.10(0.22)a	5.68(0.15)a	28.93(0.42)a	8.11(0.27)a	3.32(0.17)c	13.25(0.39)a	0.28(0.01)a	228.36(10.39)a
101N	12.88(0.26)a	9.83(0.22)a	5.62(0.15)a	28.31(0.42)a	8.87(0.27)a	2.50(0.17)d	13.90(0.39)a	0.31(0.01)a	225.30(10.39)a

ANOVA TABLE (LSD protected, $p \leq 0.05$)

N-rate (N)	0.2523	0.7432	0.9610	0.2901	0.1371	<.0001	0.3979	0.1896	0.0406
Cover (C)	0.0327	0.0038	0.4183	0.4086	0.9842	0.0002	0.0744	0.8700	0.0008
N x C	0.9464	0.7822	0.1422	0.9814	0.7880	0.6018	0.9741	0.9170	0.5821
Tillage (T)	0.0495	0.5871	0.0149	0.0628	<.0001	0.0008	0.0002	0.0005	0.7588
N x T	0.5436	0.2301	0.0155	0.0638	0.3214	0.2664	0.0259	0.1241	0.3962
C x T	0.8100	0.2192	0.4513	0.3664	0.7260	0.1715	0.5852	0.9177	0.7206
N x C x T	0.2017	0.8118	0.8304	0.5924	0.7757	0.4304	0.5572	0.7621	0.0775

Treatment (Trt) means of each cover crop, tillage method and nitrogen rate (n=4) with standard errors in brackets for each fatty acid methyl ester (FAME) microbial group followed by the same lower case letter are not significantly different (LSD protected, $p \leq 0.05$). G+ and G- = Gram positive and negative bacteria respectively; Actino = Actinomycetes; AMF=Arbuscular mycorrhiza fungi; F: B=Fungi: Bacteria ratio; Bacteria= Total bacterial abundance (sum of G+, G-, and Actinomycetes); Fungi=Total fungi abundance (sum of 18:2w6c and 18:3w6c); Total Fame=Sum of all FAME biomarkers over 14 Carbon chain.

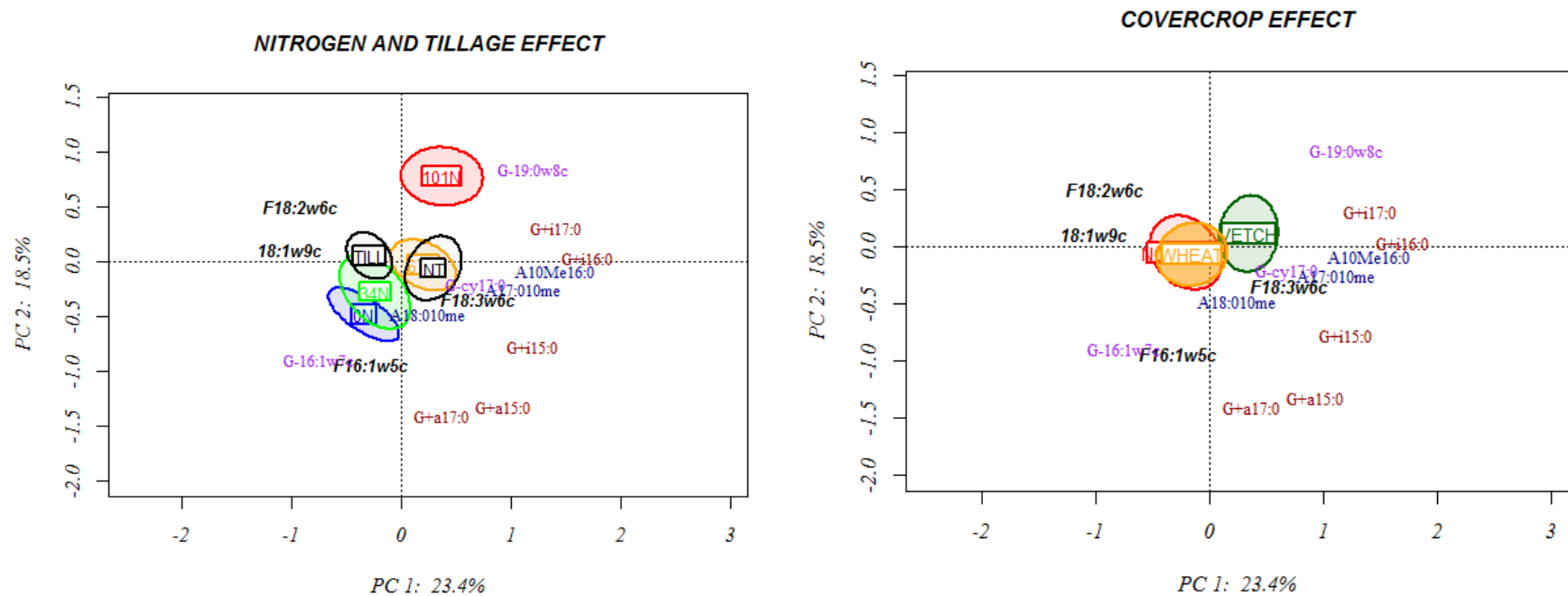


Figure 2: Microbial community structure according to FAMES as influenced by tillage and nitrogen (left) and cover crop (right).

The PCA shows each FAME with the actual group represented. For example, bacteria biomarkers identified included five Gram positive (Gram+) bacteria (i15:0, a15:0, i16:0, i17:0, a17:0); three Gram negative (Gram-) bacteria (cy17:0, cy19:0, 16:1w7c) and three actinomycetes (10Me16:0, 10Me17:0, 10Me18:0). Fungal markers included two saprophytic biomarkers (18:2w6c, 18:3w6c) and an arbuscular mycorrhizal fungi (AMF) associated biomarker (16:1w5c). The fungal sum was calculated based on the summation of the 18:2w6c and 18:3w6c.

Table 5: PCA species coordinate loading scores of identified FAME microbial groups

FA's Biomarkers	PC1	PC2
G+ i15.0	0.865***	-0.55***
G+ a15.0	0.668***	-0.945***
G+ i17.0	1.012***	0.221**
G+ a17.0	0.267*	-1.004***
G+ i16.0	1.215***	0.026
G- cy17.0	0.423***	-0.178**
G- cy19.0w8c	0.753***	0.707***
G-16.1w7c	-0.467***	-0.746***
A 10Me.16.0	0.938***	-0.078*
A 10me17.0	0.743***	-0.25***
A 10me18.0	0.163***	-0.479***
F 16.1w5c	-0.265***	-0.931***
F 18.2w6c	-0.918***	0.51***
F 18.3w6c	0.164***	-0.048
18.1w9c	-1.004***	0.48***

Loading factors of FA (Fatty acid biomarkers) within

the first two PCA components for Gram + bacteria

(i15:0, a15:0, i16:0, i17:0, a17:0); Gram – bacteria

(cy17:0, cy19:0, 16:1w7c); Actinomycetes

(10Me16:0, 10Me17:0, 10Me18:0); and Fungi

(AMF-16:1ω5c, and 18:2ω6c and 18:3ω6c)

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared: 0.9943, Adjusted R-squared: 0.9924

F-statistic: 504.9 on 25 and 72 DF, p-value: < 2.2e-16

Soil Quality Index

Among the soil quality parameters assessed (Table 6), the extractable nutrients P, K, pH, MBC, and bulk density had the highest quality scores ranging from 0.85 to 1.00 while TOC and β -glucosidase (BG) had scores below 0.50 resulting in an overall soil quality index (SQI) ranging between 61-71%.

Tillage had a significant effect ($p < 0.05$) on the quality scores for TOC, K, BG, pH and BD mainly only at the lower N-rates (Table 6). At the 0 N-rate TOC, K and BG, had significantly greater quality scores under No-till compared to till, while BD and P had greater quality scores under till compared to No-till. At 30 N-rate the quality score for TOC was greater under No-till compared to till while pH, P, and BD were greater under till compared to No-till. At 67 and 101 N-rates tillage did not have an influence on any of the quality scores, besides the quality score for BG that was higher under No-till compared to till. The overall quality score did not differ by tillage at any of the N-rates (Table 6).

Cover crop had significant effects on the quality scores for K, BG and on the SQI differing based on N-rate. K quality score was significantly greater in wheat and No Cover compared to vetch which had the lowest score but only at the 0 and 34 N-rates. BG quality score was significantly greater in vetch compared to wheat and No Cover but only at the 0 N-rate. The SQI score only differed at the 0 N-rate where vetch had the highest quality score compared to wheat and No Cover.

N-rate (Table 7) had a significant effect ($p < 0.05$) on the quality scores for TOC and P which showed an increasing trend with N-rate increase having the greatest scores at the 101 N. On the

other hand, the scores for K and pH decreased having the least scores at the 101 N-rate. All the other scores (MBC, BD, and BG) did not differ with N-rate. N-rate did not have any significant effect on the SQI.

Table 6: Tillage and cover crops effect on soil quality indicators based on the scores as determined by soil management assessment framework (SMAF)

N-rate	Treatment	TOC	PTOC	K	pH	BD	β-BG	MBC	SQI
Scores									
0 N	Cover								
	Vetch	0.41 (0.04)a	0.96 (0.03)a	1.01 (0.01)b	0.99 (0.01)a	0.95 (0.02)a	0.30 (0.04)a	0.94(0.04)a	70.84 (1.37) a
	Wheat	0.25 (0.05)b	0.85 (0.03)a	1.04 (0.00)a	1.00 (0.00)a	0.91 (0.02)a	0.14 (0.032)b	0.91(0.04)a	63.24 (1.37) b
	No Cover	0.27 (0.05)b	0.92 (0.04)a	1.04 (0.02)a	0.97 (0.03)a	0.94 (0.03)a	0.20 (0.05)b	0.93(0.04)a	64.55 (1.37) b
	Tillage								
	No-till	0.37 (0.07)a	0.84 (0.03)a	1.04 (0.00)a	1.00 (0.01)a	0.90 (0.02)b	0.27 (0.05)a	0.92(0.03)a	61.14 (1.70) a
34 N	Cover								
	Vetch	0.43 (0.05)a	0.99 (0.01)a	1.00 (0.02)b	0.98 (0.01)a	1.00 (0.01)a	0.25 (0.05)a	0.80(0.08)a	70.08 (1.41) a
	Wheat	0.42 (0.05)a	0.97 (0.01)a	1.03 (0.01)a	0.98 (0.01)a	0.94 (0.01)a	0.21 (0.05)a	0.94(0.07)a	70.48 (1.53) a
	No Cover	0.31 (0.06)a	0.96 (0.01)a	1.03 (0.01)a	1.00 (0.01)a	0.94 (0.03)a	0.37 (0.13)a	0.79(0.07)a	68.51 (1.53) a
	Tillage								
	No-till	0.47 (0.07)a	0.95 (0.01)a	1.02 (0.01)a	0.98 (0.00)b	0.92 (0.03)b	0.34 (0.07)a	0.79(0.06)a	0.47 (0.07)a
67 N	Cover Crop								
	Vetch	0.44 (0.04)a	1.00 (0.05)a	0.97 (0.02)a	0.96 (0.02)a	0.96 (0.01)a	0.21 (0.04)a	0.94(0.07)a	71.44 (1.65) a
	Wheat	0.35 (0.03)ab	1.00 (0.05)a	1.00 (0.01)a	0.97 (0.02)a	0.97 (0.01)a	0.23 (0.05)a	0.85(0.07)a	68.69 (1.56) a
	No Cover	0.31 (0.05)b	0.99 (0.05)a	1.00 (0.02)a	0.96 (0.01)a	0.96 (0.01)a	0.19 (0.03)a	0.90(0.08)a	66.73 (1.56) a
	Tillage								
	No-till	0.34 (0.07)a	0.99 (0.01)a	0.98 (0.01)a	0.96 (0.02)a	0.95 (0.02)a	0.25 (0.06)a	0.85(0.07)a	68.32 (1.45) a
101 N	Cover Crop								
	Vetch	0.47 (0.14)a	1.00 (0.04)a	0.99 (0.02)a	0.90 (0.03)a	0.99 (0.01)a	0.21 (0.07)a	0.99(0.03)a	70.19 (1.68) a
	Wheat	0.49 (0.07)a	1.00 (0.04)a	1.01 (0.01)a	0.93 (0.01)a	0.96 (0.02)a	0.17 (0.03)a	0.97(0.03)a	70.54 (1.68) a
	No Cover	0.51 (0.05)a	0.99 (0.04)a	1.01 (0.01)a	0.93 (0.01)a	0.96 (0.02)a	0.19 (0.07)a	0.93(0.03)a	70.19 (1.53) a
	Tillage								
	No-till	0.54 (0.09)a	0.99 (0.00)a	1.00 (0.02)a	0.92 (0.02)a	0.96 (0.02)a	0.24 (0.07)a	0.95(0.03)a	70.55 (1.41) a
	Till	0.45 (0.08)a	1.00 (0.00)a	1.01 (0.01)a	0.93 (0.01)a	0.98 (0.01)a	0.14 (0.02)a	0.97(0.03)a	70.07 (1.25) a

Tillage and cover crop soil quality means (standard errors in brackets) followed by the same lower case letter within each nitrogen rate (0 N, 34 N, 67 N and 101 N Kg/ha) are not significantly different (LSD protected, $p \leq 0.05$). TOC- total organic carbon; PTOC-phosphorous based on TOC levels; K-potassium; BD-soil bulk density; β-BG- β-glucosidase; MBC- microbial biomass C; SQI- soil quality index (an integration of all the individual quality score).

Table 7: Effect of nitrogen fertilizer rates (N-rate) on selected soil chemical and physical properties

N Rate	TOC	PTOC	K	pH	BD	BG	MBC	SQI
	Scores							
0 N	0.31 (0.05)b	0.91 (0.01)b	1.03 (0.01)a	0.99 (0.01)a	0.93 (0.02)a	0.21 (0.03)b	0.93 (0.04)a	66.21 (1.02)a
34 N	0.39 (0.05)b	0.97 (0.01)a	1.02 (0.01)a	0.98 (0.01)a	0.95 (0.02)a	0.27 (0.07)a	0.84 (0.04)a	69.71 (1.05)a
67 N	0.36 (0.04)a	1.00 (0.01)a	0.99 (0.01)b	0.96 (0.01)b	0.97 (0.01)a	0.21 (0.04)b	0.90 (0.04)a	68.98 (1.03)a
101 N	0.49 (0.09)a	1.00 (0.01)a	1.00 (0.01)b	0.92 (0.01)c	0.97 (0.01)a	0.19 (0.05)b	0.96 (0.04)a	60.24 (1.05)a

Nitrogen rate (N-rate) means (standard errors in brackets) n=4, followed by the same lower case letter across N-rate (0 N, 34 N, 67 N and 101 N Kg ha⁻¹) are not significantly different (LSD protected, $p \leq 0.05$). P (phosphorous), K (potassium), Ca (calcium) and Mg (Magnesium) are Mehlich 1 extractable nutrients.

DISCUSSION

Soil physicochemical properties after 31 years of different tillage options, cover crops and varying N-rates

The effect of tillage and cover crops on soil C and N

After 31 years of No-till/Conservation agriculture (CA) practices, our results demonstrate the value of cover crops in increasing soil organic C and N in reduced tillage practices for low residue producing crops like cotton under a monoculture production. Greater levels of soil C and N were recorded in treatments under No-till than till (approximately 19% and 10% greater for TC and TN respectively) in treatments having cover crops (vetch and/or wheat) particularly within the lower N-rates. Greater levels of soil C and N under No-till than Till has been reported in several studies (Halvorson et al., 2002; Wright and Hons, 2004; Al-Kaisi et al., 2005). Al-Kaisi et al. (2005) found No-till to have as high as 15 to 21 % greater soil organic C than soils that were chisel ploughed in their study of seven years under corn-soybean rotation on a Mollisol in Iowa. Though it is expected that reducing tillage would decrease the rate of organic carbon decomposition by occluding it from soil microbes, the degree of to which this has on soil C build-up has been shown to vary based on climate, soil type, amount of crop residue returned to soil, crop species, and duration under reduced tillage (West and Post, 2002; Al-Kaisi et al., 2005).

This long-term assessment is able to provide an overview of the benefits of C sequestration with these CA practices for cotton production under monoculture. A study by Acosta-Martínez et al. (2011a) observed no changes in soil C under continuous cotton production after 5 years of no-

tillage on sandy soils in the semiarid region of the Texas high plains. They only detected an increase in soil C when the cover crops were introduced into the system under a crop rotation of forage sorghum and a winter cover crop that did not involve cotton. Similarly, Halvorson et al. (2002) found that after 12 years of reduced tillage practices on a silt loam soil in North Dakota observed no significant increases in soil C in No-till treatments under spring wheat-fallow production. In contrast they recorded significant increases in soil C in No-till treatments under a cropping rotation sequence of annual spring wheat, winter wheat and sunflower. Halvorson et al. (2002) attributed the soil C increases under the crop rotation sequence to significantly greater residue return recorded from the crop rotations compared to less residue levels from the spring wheat-fallow system. Wrights and Hons (2004) after 20 years of No-till on a silty clay loam in Central Texas also found the greatest soil organic C and N in No-till under a sorghum-wheat-soybean rotation in comparison to No-till under a continuous sorghum production.

Altogether these results and those of the studies mentioned above signify the importance of cover crop as a source of C inputs through crop residues returned to soil to be a key driver towards facilitating soil C and N build up, especially under the production of low biomass crops like cotton.

The Effect of Different Cover Crop Species on Soil C and N

This study further demonstrates that the effect of cover crops on soil C and N is species dependent. It is interesting that after 31 y, soil C and N as well as cotton yield (see Appendix 1, page 79) in treatments under vetch cover crop did not differ based on N-rate. The contribution of vetch cover crop on both soil C and N was the same for all the N-rates (0 N, 34 N, 67 N, 101 N). However, treatments under wheat and No Cover showed a positive response to increase of N-rate

having the greatest levels at the highest N-rate (101 kg N ha⁻¹). These differences in cover crop effect on soil C and N storage as well as yield is reflective of their differences in nutrient acquisition ability and their C:N ratio. Nutrient acquisition capacity would lead to differences in crop biomass production. Vetch, being an N-fixing crop, would be able to produce substantial crop biomass regardless of N fertilizer usage. Wheat on the other hand, is more dependent on availability of N in the soil for increasing its crop biomass production and that explains the positive response it had to N-fertilization. At the high N-rates, the crop biomass production of both vetch and wheat would depend on the applied N-fertilizer since with high availability of N in soil, the N fixing activity of leguminous crops is inhibited. This could be the probable reason why the soil C and N as well as yield at the high N-rates did not differ based on cover crop or tillage. Jagadamma et al. (2007) reported a difference of 2.1 Mg ha⁻¹ of above ground biomass yield between corn-soybean and continuous corn cropping system at 0 N Kg ha⁻¹ fertilizer rate with corn-soybean having the higher yield. However, at high N fertilizer rate (280 kg N ha⁻¹), the difference was reversed, with continuous corn having a higher yield (0.94 Mg ha⁻¹). The higher above ground biomass yield of continuous corn at higher N-rate could be due to the fact that corn is a higher biomass crop than soybean.

The C:N ratio of the crop residue determines mineralisation and immobilisation rates. The lower C:N ratio of the vetch residue would lead to faster mineralisation rates than that of residue from wheat and No Cover. The higher mineralisation rates would result in faster accumulation of decomposed organic matter, while the higher C:N ratio of wheat might mean more accumulation of non-decomposed organic matter on the soil surface. Nevertheless, higher N-rates would provide soil microbes with the needed N to facilitate higher mineralization rates of the high C:N crops like wheat. Indeed, preliminary results on a study focusing on differences in mineralization

and nitrification under these plots show that the potential mineralisation rate at the highest N-rate (101 N kg ha⁻¹) for both vetch and wheat did not differ (data yet to be published).

From these results it is apparent that after a continuous period of growing a legume cover crop like vetch, crop productivity and soil C can be maintained without the need for additional N-fertilizer. The lack of difference in soil C and N at 0 N-rate under vetch cover crop and at 101 N-rates under either wheat or vetch cover crop shows that higher levels of soil C could be achieved without a need of increased fertilizer rates which is believed to have a positive effect on soil C sequestration.

The Effect of Increasing N-rates on Soil C and N

An additional factor to this study was on the influence of increasing N-rate interaction with tillage practice and cover crops on the levels of soil C and N. The greatest soil C and N levels were recorded at the highest N-rate (101 kg N ha⁻¹).

Increasing N-rates has been reported to have a positive effect on soil C storage, more so where crop residue are returned to soil, although a lack of effect has also been shown in some studies (Alvarez, 2005). For example, on a long-term (26 years) study under continuous corn and corn-soybean rotation cropping system on a Mollisol in Illinois, Jagadamma et al. (2007) found the above ground residue yield to have a positive correlation with N-rates. Their study showed the above ground residue yield to increase with increasing N-rate, from 0 N up to 150 kg N ha⁻¹ beyond which no further increase in yield were observed. Furthermore, Jagadamma et al. (2007) found that 64 % of soil organic C build up (kg ha⁻¹ y⁻¹) could be explained by the increased N-rates. Similarly, Halvorson et al. (1999) in an 11-year study at Akron, Ohio, under a spring-

barley-cotton rotation observed increasing N-rate to explain almost 90 % of the soil organic carbon. This positive response of soil C storage to N-rate could mainly be attributed to an increase in the net crop production and quantity of residues returned to soil. Where there has been a lack of response to N-fertilization it has been attributed to differences in soil texture, N-fertilizer effect on the quality of residue returned to soil and the interaction of climatic factors (Alvarez, 2005).

In the same plots, cotton yield (Appendix 1) records also show a similar response as that observed with soil C and N levels. Generally, treatments under No-till had greater cotton yields but this varied based on cover crop and N-rate. The greatest yields were recorded at the high N-rate (101N kg ha⁻¹) regardless of tillage or cover crop treatment. Nevertheless, cotton yield for the No-till treatments under vetch were constantly highest at each of the N-rate. Indeed, this parallel increase of yield and soil C and N levels depicts that organic C levels in soils is a function of crop yield. These trends are indicative of N being a limiting factor towards crop growth in these soils and further supports our hypothesis that added residue either from cover crop or enhanced crop growth by N-fertilization plays a significant role in promoting C and N storage in these soils. The significance of residue additions towards the build-up of C and N in these soils may also be driven by the fact that the soils in this region are generally characterised by low organic matter content with the main crop in this study, cotton, being a low biomass crop and would therefore have minimal contribution to residue additions besides when heavily fertilized (Causarano et al., 2006).

Soil extractable nutrients, soil pH and bulk density

Similar to what was observed on soil C and N under the tillage treatments, the soil nutrient status based on extractable P, K, and Ca was also greater under the No-till treatments than till, but Mg did not differ between tillage treatments. The higher P, K, and Ca concentration under No-till could be the effect of biological cycling that result from the crop residues mainly attributed to the concurrent increase of soil organic matter under No-till. Greater levels of organic matter not only serve as a source of nutrients as it is mineralised, but it also act as a chelating agent of competing Al species, and release organic acids that mediate the solubilisation of P, making it more available. Furthermore, under No-till, P sorption to soil colloids is reduced due to the fact that tilling results in the ions within the soil having more contact with soil particles thus increasing the surface reactivity of the particles that would result in greater P sorption under Till as opposed to No-till therefore leading to greater exchangeable P in No-till than in till.

P, K and Ca, had a negative relationship with N-rate with higher N-rates resulting in significantly lower concentrations. Jagadamma et al. (2008) observed greater exchangeable K at 0 N-rate and the least concentration under a higher N-rate (280 kg N ha⁻¹). The negative correlation of soil nutrients with increasing N-rate observed in our study was not surprising as the soil pH also decreased. Given that the fertilizer applied in our plots was in the form of ammonium nitrate, the long-term application of this fertilizer type would result with acid production through NH₄⁺ oxidation by nitrifying bacteria. The increase in soil acidity due to N fertilizer application will result with increased levels of exchangeable aluminium (Al) in soil solution. The increased level of Al in solution reacts with P forming the precipitate reducing P concentration in soil. Another effect of higher Al concentration in soil solution is that, Al will exchange with basic cations (Ca,

K and Mg) on the soil exchange site. This results in increased concentration of these base cations in solution where they would be susceptible to being lost through leaching. Blevins et al. (1977) and Schroda et al. (2011) observed lower percent base saturation under increased soil acidity. Barak et al. (1997) also observed a concurrent decline in both base saturation and cation exchange capacity (CEC) with increased soil acidity. The decline in CEC results in reduced soil adsorption capacity hence lower concentrations of exchangeable cations on the soil exchange sites. The effect of this would be greater concentration of the exchangeable cations in the soil solution, the fate of which may be leaching from the soil (Barak et al., 1997). Blevins et al. (1977) saw a decline of about 30% of CEC in an alfisol in Lexington, Kentucky at fertilizer application rates of 336 kg N ha⁻¹ and 21% CEC reduction at the application rate of 168 kg N ha⁻¹ over a period of five years without soil liming.

It is also probable that the difference in extractable P, K and Ca between higher N-rates and lower N-rates could be related to the observed yield differences. It may be assumed that the greater yields observed at the high N-rate would have in turn led to greater uptake of other nutrients like P, K and Ca as well, therefore reducing their availability at the high N-rate.

Under the high N-rate (101 N) the greater cotton yield could have resulted with more extraction of P, K and Ca but under 0 N-rate, lack of N might have affected the amount of these nutrients that might have been taken up by the plants. Though the higher N-rate resulted with less concentration of P and K, the vetch cover crop at 0N had the same effect. The fixation of N by vetch at this lower N-rate might have promoted more plant up take of these nutrients whereas the lack of N under wheat and No Cover led to the opposite. However Ca was higher under vetch at

0N than under both wheat and No Cover but it was the same across other N-rates (34, 67 and 101 N). Mg was not affected by either of the treatment factors.

Contrary to what is expected with the reduced tillage systems, till had a lower soil bulk density than No-till. No-till had a bulk density of 1.23 g cm^{-3} while till 1.15 g cm^{-3} . However, this may be a difference that exists only within the upper soil surface as sampling was only restricted to a 7.5 cm depth. This difference could be due to the fact that under till, the upper soil surface is periodically loosened through tilling while under No-till there is more aggregation of soil particles at the soil surface without any loosening.

Bulk density at the 0 N-rate was also greater compared to treatments having N-fertilizer applied. This could be due to the fact that with N-fertilizer application, crop growth increases resulting in higher root biomass at the upper soil surface which would result in increased porosity from the root channels. Although cover crop did not have a significant influence on bulk density, we observed lower bulk density values for treatments under vetch compared to wheat or No-cover. Also, at the highest N-rate, bulk density did not differ between the two tillage treatments which may be explained by the increased level of crop biomass in both systems.

Status of Microbial Community Structure and Enzymatic Activity after 31 years of tillage, cover crops, and varying N-rates

The soil microbial biomass (SMB) based on both chloroform fumigation extraction and FAME analysis revealed subtle differences based on cover crop and N-rate but did not reveal differences between tillage treatments. Treatments under hairy vetch had significantly greater microbial biomass N and total FAME compared to wheat or No Cover, but there were no significant

differences in microbial biomass C between the cover crops. Total FAME revealed differences due to N-rate which was significantly higher at the 67 and 101 N-rates. This suggests the possible response of microbial community to increases in both rooting activity and additional substrate as a result of increased N availability. This is in agreement with what we observed in the response of total C and N (as well as yield) as discussed in the previous section. The quantity and quality of additional substrates based on higher N fertility status has also been shown to significantly increase the levels of microbial biomass in several other studies (Fraser et al., 1988; Drijber et al., 2000; Bailey et al., 2002; Bending et al., 2002).

Although SMB is expected to be greater under reduced tillage systems, significantly greater levels were not observed in this study. Nevertheless, significant differences in the abundance of specific microbial groups between No-till and till treatments based on FAME analysis were observed. No-till treatments were characterised by a greater relative abundance of fatty acid (FA) biomarkers associated with Gram+ bacteria, actinomycetes and mycorrhiza fungi. These results are in agreement with those reported by several other studies (Drijber et al., 2000; Feng et al., 2003; Helgason et al., 2009). Tillage, in comparison to reduced tillage systems, has been shown to typically favor the dominance of aerobic bacteria with a greater capacity to breakdown labile substrates (Linn and Doran, 1984; Beare et al., 1992; Spedding et al., 2004; Simmons and Coleman, 2008; Acosta-Martinez et al., 2011). Nevertheless, over time the accumulation of substrate quantity on the surface soils of reduced tillage systems usually results in greater soil porosity at the upper surface and may result in proliferation of aerobic bacteria (Mathew et al., 2012). Helgason et al. (2009), in a study comparing microbial communities under No-till and till demonstrated an increase in the abundance of both bacteria and fungi biomarkers under No-till. The interaction of increased substrates and other factors like substrate quality and environmental

conditions created by conservation tillage practices may provide for the selection of specific bacterial groups. Greater abundance of Gram+ bacteria and Actinomycetes FA biomarkers has been related to greater recalcitrant aromatic C content as well as anaerobic soil conditions (Feng et al., 2003). Conditions under No-till are known to promote increased infiltration, and a higher water holding capacity that leads to cooler and wetter conditions that have been shown to favor the abundance of anaerobic bacteria species (Linn and Doran, 1984). The 10Me16:0, a FA biomarker for actinomycetes has also been associated with sulfate-reducing bacteria that indicate anaerobic conditions (Feng et al., 2003). The gravimetric soil moisture content (Table 4) for No-till in this study was significantly greater compared to the till treatments which may have led to the greater abundance of the 10Me16:0 as observed in our results.

The similarity in microbial community composition of soils under vetch with that of the No-till treatments further indicates the possibility that substrate quality might have been an influential factor in driving the differences in bacterial abundance. The relative abundance of the Gram+ bacteria was observed to be greater in treatments under vetch compared to wheat and the No Cover treatment. The yields under vetch as well as soil C and N were greater indicating increased substrate quantity. Vetch having a lower C:N ratio would also have more labile residue than that of wheat or cotton, resulting in higher decomposition rates that would lead to the greater accumulation of decomposed organic matter. This may imply an increase in the availability of substrates for microbial proliferation. However, we do not rule out the possibility of the interaction of other factors that have been shown to play an important role in driving microbial community structure such as soil water potential, redox potential, bulk density, soil pH among others.

The relative abundance of the mycorrhiza fungi FA biomarker under No-till was also higher compared to till which is consistent with what most studies have reported (Drijber et al., 2000; Acosta-Martinez et al., 2007; Wang et al., 2012). Tillage is known to decrease the abundance of mycorrhiza due to the disruption of their hyphal network (Drijber et al., 2000). It is well known that mycorrhiza play an important role in nutrient acquisition by increasing root surface through extraradical hyphae (Smith and Read, 1997). Mycorrhizae have also been shown to play an important role in macro-aggregate formation and stabilization through production of glycoproteins (Rillig and Mummey, 2006; Six et al., 2006). The macro-aggregate formation and stabilization by mycorrhiza fungi has also been attributed to protect soil organic C (Rillig and Mummey, 2006; Six et al., 2006). The greater abundance of FA biomarkers associated with mycorrhizal fungi, Gram + bacteria and actinomycetes under No-till may therefore explain the higher soil C due to their associated roles in C sequestration. The greater abundance of mycorrhiza biomarkers may also have contributed to greater yield values obtained under No-till due to their associated role in nutrient acquisition.

The abundance of mycorrhizae fungi FA biomarkers in this study was dramatically impacted by N fertilization and showed a significant negative relation to increasing N-rates. Increased amounts of readily available forms of key nutrients to plants especially P have been shown to decrease mycorrhiza colonization (Azcon-Aguilar and Bago, 1994; Smith and Read, 2008; Wang et al., 2009). This is mainly attributed to the fact that plants would not need to invest the extra energy cost of maintaining the symbiosis as they can easily contract the nutrients directly. Mycorrhizal response to N enrichment is mediated by ambient soil fertility. Nitrogen enrichment often dramatically increases aboveground productivity and as plants become enriched with mineral nutrients, they tend to allocate more photosynthate to shoots and leaves and less to roots

and mycorrhiza fungi. N enrichment therefore reduces the value of mycorrhiza for nutrient uptake. In this study, the influence of N on mycorrhiza FA biomarker was also observed in treatments under the vetch cover crop which had significantly lower abundance of mycorrhiza that may be attributed to the higher N availability through N-fixation by vetch. The lower mycorrhiza abundance under vetch may also indicate the possibility of competition between rhizobium nodulation and mycorrhiza colonization.

The ratio of fungi to bacteria FA biomarkers (F:B ratio) under conservation tillage soils is generally expected to be greater comparatively to tilled soils. Indeed, several studies have reported greater F:B ratio under reduced tillage systems (Frey et al., 1999; Helgason et al., 2009; Stahl and Parkin, 1999). Besides minimal disruption of their hyphal networks, the abundance of fungi has been hypothesized to be greater under reduced tillage mainly because: they have a higher microbial growth efficiency; their cell walls are more resistant to degradation than bacteria; and they are able to utilize more recalcitrant residue (Six and Jastrow, 2002; Jastrow et al., 2006; Waring et al., 2013). These properties of fungi have therefore been linked with greater soil C sequestration under reduced tillage systems (Six and Jastrow, 2002). Nevertheless, the generalization that the abundance of all fungi species would be greater under conservation tillage systems as has been postulated is questionable. Several studies have reported the lack of fungal dominance in reduced tillage systems (Feng et al., 2003; Helgason et al., 2009; Mathew et al., 2012).

In this study, the fatty acid biomarkers associated with saprophytic fungi (18:2w6 and 18:3w6c) were significantly higher under till resulting in a higher F:B ratio compared to No-till. Correspondingly, Calderon et al. (2001) did not see a decrease of similar fungal biomarkers

(18:2w6 and 18:3w6c) after tillage in an experiment setup to investigate the short term effects of rotor-tilling a previously fallow soil. Nonetheless, they observed an effect of tillage on a eubacteria biomarker (18:1w7t) implying a stronger effect of tillage on this bacteria than to the fungi which is contrary to the common notion that fungi are more sensitive to tillage. The relative abundance of FA 18:1w9c that has also been used as a fungal biomarker by several researchers (Feng et al., 2003; and Acosta-Martinez; Simmons and Coleman, 2008) was also greater under Till in our soils. While the results reported here are from a onetime sampling point, preliminary data collected the prior year showed similar trends (data not shown). A study based on similar soils in Jackson West TN comparing different tillage regimes under a continuous soybean production system reported greater population of nematode parasitic fungi in tilled treatments compared to No-till treatments (Bernard et al., 1997). The results of this study and those of other studies reported above indicate that other factors may have a stronger influence on the response of these fungal bio-markers other than direct tillage events.

Fungi have been shown to be adaptive to extreme environmental stress conditions such as soil moisture conditions, osmotic stress, temperature, and soil pH among other factors (Stromberger et al., 2007). Simmons and Coleman (2008) demonstrated an increase in fungi FAs over bacteria FAs due to higher air temperatures and lower precipitation regardless of management practice indicating that these environmental factors had a stronger influence on fungi than management practices. It is therefore possible that the higher fungi abundance under Till in our study may be due to the response of some environmental stress factors that may have been accentuated by continuous tillage. The year prior to our sampling, 2012 was reported to be a drought year in most of Tennessee and thus might have triggered the increase in fungi under Till that carried over to the following year.

To obtain a better picture of the long-term effects of management practices on microbial communities we assessed their metabolic capacity based on basal respiration and some key enzymes (β -glucosidase, β -glucosaminidase, and phosphodiesterase) that play a role in C, N, and P cycling. The results revealed significant differences on all potential enzyme activities due to tillage. However, it was surprising that the basal microbial respiration between tillage at the time of this sampling point was not significantly different between the two tillage practices.

No-till treatment resulted in significantly greater enzyme activities than till which is in agreement with what has been reported in other studies (Acosta-Martinez 2008; Deng and Tabatai, 1996; 1997). The differences in enzyme activities between tillage practices may be reflective of the differences in microbial community composition or functional potential based on the interaction of substrates accumulated over the years and environmental conditions created by the management practices. Given a similarity in the patterns observed on soil C, N and extractable P levels with those of enzyme activities these results indicate that No-till practices induce microbial communities and conditions that favor C, N and P cycling compared to those under Till.

It has been documented that the nature of the extracellular enzymes that bacteria and fungi produce differ. Fungi produce enzymes able to degrade more recalcitrant organic materials like lignin, while bacteria are more efficient in degrading more labile substrates. It is therefore expected that a higher soil C content would be correlated with a higher F: B ratio and higher enzyme activities. In this study, the F:B ratio was lower under No-till (Table 4) but we observed higher levels of gram+ bacteria and actinomycetes which have also been associated with more recalcitrant aromatic C (Feng et al., 2003). We also observed higher levels of mycorrhiza which

have been attributed to the protection of soil C through promoting soil aggregation. Based on these observations, we may deduce that No-till creates conducive conditions that increase the functional potential of microbial communities in C cycling despite the lack of fungal dominance and also promotes protection of organic C from further degradation resulting in C sequestration.

The effect cover crop treatments on metabolic function were only evident for β -glucosaminidase and basal microbial respiration which were significantly greater under the vetch cover crop compared with wheat and No Cover. This implies that vetch cover promotes greater microbial activity compared to No Cover and wheat.

Soil quality index and conclusions

The overall quality index (Table 6) was above average (61-71%) of the maximum score across all treatment factors which reflects that our soils are within the range of acceptable crop production and ecosystem functioning. We are using the term acceptable cautiously since there are no defined critical values for the soil quality index which is mainly based on higher being better (Andrews et al., 2004).

The use of a soil quality index was intended to define the different response of the individual parameters to tillage, cover crop and N-rates. According to the SMAF quality scoring technique, scores range from 0.0-1.0 with values closer to 1 indicating the highest potential of the indicator for its associated soil function (Andrews et al., 2004). Based on our results (Tables 6 and 7), we can therefore deduce that the soils under our study are not limited by extractable soil P and K, soil pH, bulk density and microbial biomass C which had scores ranging from 0.85-1.00

regardless of tillage, cover crop or N-rate. The main limiting factors seem to be total organic C (TOC) and β -glucosidase which had scores below 0.50.

These results imply that management practices for these soils need to be geared towards increasing total organic C and microbial activity as indicated by less β -glucosidase. Besides the fact that the TOC score was low in our soils we nevertheless were able to see significant differences based on tillage and cover crop at lower N-rates (0 N and 34 N). No-till and vetch cover had significantly greater levels (Table 6) indicating the potential for No-till in combination with cover crop to improve the soil organic matter in these soils. The quality scores for TOC were greater at 67 N and 101 N reflecting the importance of N fertilizer in the functioning of the soil. We see the potential of being able to improve the TOC without the application of higher N-rates by continued use of vetch cover under No-till systems.

It was interesting to note that the microbial biomass C level attained in these soils was not a limiting factor to its functional potential towards nutrient cycling according to the SMAF scores. Nevertheless, the low β -glucosidase score indicated that the potential of certain microbial activity were still limiting. The β -glucosidase score was significantly greater under No-till compared to till (Table 6) indicating that No-till practices possibly results in conducive conditions that promote potential for its activity. This may also be a factor of the differences in the relative abundance of microbial groups under No-till as discussed in the previous sections. The β -glucosidase score was also greater in vetch compared to wheat and No Cover but only under the 0 N-rate.

Based on the above discussion, it is apparent that No-till combined with vetch would be the recommended management option for optimising on the functional potential of microbial

biomass and increasing TOC in these soils. Nevertheless, other management practices may need to be incorporated in order to further maximize the potential of attaining greater TOC under these soils. This may involve the incorporation of higher residue crops such as maize as a rotation with cotton.

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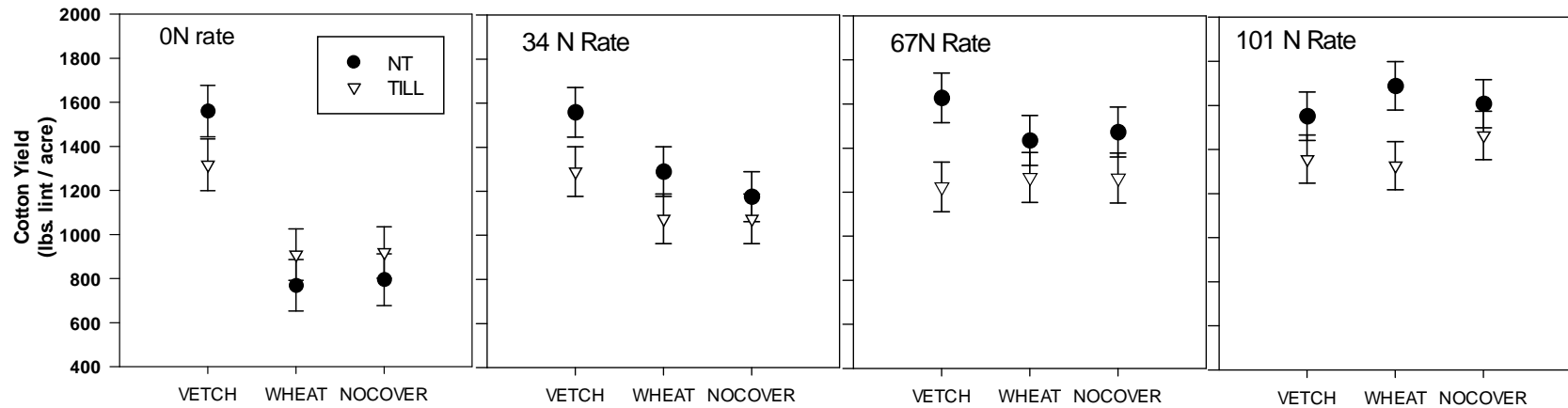
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Appendix



Appendix 1: Cotton lint yield for 2013 under the different N-rates (0, 34, 67 and 101 N kg /ha); Cover crop (Hairy vetch, winter wheat, and No Cover) and tillage (Till and No-till (NT)). Each point represents means (n=4) at each cover crop within each N-rate level. Overlapping standard error bars are not significantly different (LSD protected, $p \leq 0.05$)

Data provided by Dr. Don Tyler.

CHAPTER II

NITROGEN FERTILIZER, COVER CROPS AND TILLAGE EFFECTS ON SOIL BACTERIA TAXONOMIC COMPOSITION IN A LONG-TERM (31 YEARS) CONTINUOUS COTTON STUDY IN WEST TENNESSEE

Abstract

Soil bacterial communities are central to the functioning of agro-ecosystems. However, there is a lack of information on their characterization as they relate to specific soil functions and how the community structure is affected by different agronomic practices. This study aimed to characterize bacterial community structure shifts under different agricultural practices consisting of: nitrogen (N) fertilization rates (N-rate) (0, 34, 67 and 101 kg N ha⁻¹); cover cropping with (hairy vetch (*Vicia villosa*), winter wheat (*Triticum aestivum*), and a No Cover); and tillage (No-till and till). High throughput Illumina MiSeq 16S rRNA gene sequencing was used to generate bacterial community sequences.

Bacterial diversity differed significantly ($p < 0.05$) across N-rate with the 101 N-rates generally being less diverse than the 34 and 67 N-rates. Cover crops and tillage did not have significant effect on microbial diversity.

N-rates, cover crop and tillage had significant ($p < 0.05$) effects in the relative abundance of bacterial taxa at the phyla, class and order levels. Orders belonging to Acidobacteria and Nitrospirae generally decreased with increase in N-rate while those belonging to Planctomycetes increased with increasing N-rate. Other groups that differed across N-rates belonged to the phyla Proteobacteria and Chloroflexi. An important phylum that differed across both cover crop and tillage treatments was the Actinobacteria. No-till resulted in a significantly greater relative abundance of Actinobacteria compared to till. The use of cover crops also resulted in a significantly greater relative abundance of Actinobacteria which increased in the order of Wheat

> Vetch > No Cover. Other groups that differed across cover crop and tillage included Bacteroidetes Verrucomicrobia, and Armatimonadetes.

This study reveals that agricultural management practices involving reduced tillage and cover cropping lead to significant shifts in bacterial species composition that may significantly alter nutrient cycling capacity under these production systems.

Introduction

Soil microbial communities play an integral role in agro-ecosystems services that include mediating key nutrient transformation process, improvement of soil structural properties, stimulation of plant growth, and in the control of plant diseases and pests. The structural composition and diversity of microbial communities and their activities are critical in the productivity and sustainability of agro-ecosystems. Microbial diversity is often low in agro-ecosystems due to the dynamic nature of disturbances associated with agricultural management practices. Conservation agricultural (CA) management practices that include reduced tillage methods, maintenance of adequate soil cover by use of crop residue and/or cover crops, and crop rotation are practices that are also associated with an increase in the relative abundance, diversity, and activity of microbial species.

Soil physical and biochemical changes associated with these practices have been attributed as factors that alter the soil microbial ecology (Doran, 1980a; b, 1987; Fraser et al., 1988; Young and Ritz, 2000; Doran and Zeiss, 2000; Drijber et al., 2000). For example, shifting to minimum tillage practices has been associated with greater soil water content and greater soil bulk density resulting in a greater abundance of anaerobic microbial species (Linn and Doran, 1984). The accumulation of suitable C substrate on the soil surface of conservation tillage systems is another factor attributed to the proliferation of microbial communities (Helgason et al., 2009; Mathew et al., 2012). Helgason et al. (2009) compared soil microbial structure under both tilled and reduced tillage practices using Fatty Acid Methyl Ester profiling, demonstrating an increase in the abundance of both bacterial and fungal biomarkers under reduced tillage practices.

The inclusion of cover crops with different intrinsic substrate qualities, either belonging to the high C residue grass species and/or leguminous nitrogen (N) fixing cover crop species, often necessitates changing strategies in the application rates of N-based fertilizers (Reiter et al., 2008). High C residue crops usually require additional N to counteract immobilization, while N-fertilization rate (N-rate) would be decreased for low C:N residue crops to compensate for N mineralization. The manipulation of N-rate introduces another factor influencing microbial community dynamics with variable effects on microbial biomass and activity (Wardle, 1992; Treseder, 2008). Supplementary N can be beneficial by promoting plant growth and thus increasing the quantity of residue that can be returned to soil (Alvarez, 2005). On the other hand, high N-rates will change soil chemistry creating potentially toxic conditions to soil microorganisms. For example, high levels of N-fertilization can lead to acidic conditions, which can hinder the activity of microorganisms, limit the availability of certain nutrients to microorganisms and also inhibit enzyme production and activity (Treseder, 2008; Ramirez et al., 2010, 2012).

Implementing CA practices has been shown to have a significant effect on soil microbial structure that lead to an increase in species diversity as reported under previous studies (Lupwayi et al., 1998; Feng et al., 2003; Spedding et al., 2004). However, knowledge regarding the long-term impact and interactions of CA management practices on bacterial species composition and diversity, and the significance on associated ecological functions is still limited and not fully substantiated (Fierer et al., 2007). It has been stated that changes in microbial diversity may be relatively constrained under different management practices within agricultural landscapes (Bowles et al., 2014). There are also questions on whether a shift in microbial community composition and diversity necessarily leads to changes in soil ecological functions due to species

functional redundancy. In a review on this topic, Nannipieri and Ascher (2003) hypothesized that a few number of species would be sufficient to maintain steady state ecosystems, while a large number of species may be vital under dynamic environmental conditions such as those found in agricultural environments.

To gain insight on the ecological significance of microbial community shifts under long-term CA management practices, it is necessary to characterize the shifts in specific microbial taxonomic species and determine how those shifts may relate to observed changes in soil quality properties attributed to CA practices. The identification of specific microbial species that shift after implementation of CA management practices would be beneficial in determining the importance of these species on soil agro-ecological functioning and identifying management practices that enhance microbial communities towards a sustainable agro-system production.

This dissertation research focused on conducting a comparative analysis of soil bacterial species composition under long-term CA management practices compared to conventional tillage on continuous cotton, *Gossypium hirsutum* L. production in West Tennessee. The research plots were established in 1981 in a randomized block split-split plot experimental design consisting of four N-rates (0, 34, 67 and 101 N kg/ha) as the main plot, contrasting tillage practices (till and No-till) as the split plot and different cover crops species (Hairy vetch (*Vicia villosa*), Winter wheat (*Triticum aestivum*) and No Cover as the split-split plot.

Next Generation DNA sequencing was used to characterize soil bacterial communities based on the universal 16S rRNA phylogenetic marker. The specific objectives of this research were to:

1. Characterize the bacterial species composition and diversity under differing tillage options, cover crop species and N-rates and;
2. Determine the relationship of the increase or decrease of specific bacterial taxa with changes in soil physicochemical properties observed under the CA practices and their possible significance on ecological functions.

The hypotheses based on these objectives were that the following significant shifts in bacterial species composition would be observed:

1. An overall increase in bacterial species diversity in No-till and cover crop treatments compared to till and No Cover treatments;
2. A decrease in bacterial species diversity as N-rates increased with low diversity under the higher level of N-fertilization 101 N compared to the lower N-rates (0, 34 and 67 N) and;
3. Greater shifts in bacterial groups with specialized functions than bacterial groups with broad functions.
4. Greater relative abundance of bacterial groups involved with carbon cycling with No-till and cover crop treatments.

Materials and Methods

Study Site and Soil Sampling

The research site is located at the West Tennessee Experiment Station (WTES), Jackson, Tennessee. The soils at the site are classified as a Lexington silt loam (fine-silty, mixed, thermic, Ultic Hapludalf), well-drained soil with a 0-2% slope. The plots were established in 1981 under continuous cotton- *Gossypium hirsutum* L., production in a randomized block, split-split plot design consisting of four N-rate treatment levels (0, 34, 67, and 101kg/ha) as the main plots; the main plots were subdivided into sub-plots of four cover crop treatments hairy vetch (*Vicia villosa*), winter wheat (*Triticum aestivum*) and a No Cover; each of the sub-plots were then further subdivided into two with one portion tilled (till) and the other portion left untilled (No-till). All treatment sets have four replications. Tillage is usually performed two times before planting by a standard disc harrow followed by smoothing and breaking up of clods. It is important to note that since the plots were established in 1981, liming has only been applied once in 1995 and was targeted to plots having a soil pH < 6.0 (Cochran et al., 2007).

Sampling was performed at the beginning of the cotton growing season of 2013 in June after cotton planting. Two main cover crop species representing a grass species (winter wheat) and a leguminous cover crop species (hairy vetch) were selected for this study, under all four nitrogen fertilization rate (0, 34, 67, and 101 kg N ha⁻¹) from both till and No-till plots. The soil was sampled from the top 0-7.5 cm with a minimum of 20 randomly selected points next to the rows of cotton plants in each plot. Care was taken not to contaminate soils from different plots by cleaning the soil sampling probes between plots with 70% ethanol. Sub-samples from each plot

were homogenized and portioned into three sampling bags with the portion meant for DNA extraction immediately flash frozen using liquid nitrogen. Samples were stored in a cooler containing dry ice for transportation back to the lab after which the soils were stored at - 80°C until needed.

DNA Extraction, PCR Amplification and Sequencing

Total soil DNA was extracted from each soil samples from each plot using the Power Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) as directed by the manufacturer's instructions, with slight modifications. Specifically, soil samples (0.35 g) were pre-heated in a hot water bath at 65°C for 20 min to optimize the homogenization step. The remaining steps were performed as directed by the manufacturer. DNA extracted from each replicate plot sample were quantified using the PicoGreen® (Ahn et al., 1996) dsDNA quantitation assay and stored at 20°C.

In preparation for DNA sequencing, samples from replicate plots of each treatment were pooled together based on equal concentration resulting in a total of 24 pooled samples. PCR amplification and subsequent sequencing was completed using tag encoded rRNA primers (F515/R806) targeting the V4 region of the 16S rRNA as developed for paired end sequencing on the Illumina Miseq platforms (Caporaso et al., 2012). The sequencing was completed at The Center for Environmental Biotechnology (CEB), at the University of Tennessee, Knoxville. Following sequence Demultiplexing, all individual Fastq files for each sample were uploaded onto MG-RAST (Meyer et al., 2008) for quality control and annotation under the project number 6978 with individual sample identification numbers starting from 4544835.3 to 4544858.3 and are available for public access.

Bioinformatics and statistics

Sequence quality filtering and bioinformatics was performed using the QIIME v1.4.0 (Caporaso et al., 2010) pipeline following the guidelines provided for multiplexing and de-multiplexing of paired end illumina sequence data. A total of 5.1 million reads were obtained from the illumina MiSeq sequence run having a phred quality score > 30 . The quality filtering and de-multiplexing step in QIIME resulted in sequence reduction to 4.9 million reads with a mean sequence length of 253 base pairs (bp) joined paired-end reads. Clustering of reads was accomplished using the QIIME open-reference picking process, whereby sequences were first clustered against a reference database, and reads which failed to match a reference were subsequently clustered de novo. Clustering of sequences into Operational Taxonomic Units (OTUs) was achieved based on the UCLUST algorithm method with 97% sequence-identity cut-off and sequences were aligned to the bacterial Greengenes reference database (DeSantis, et al., 2006) for taxonomic assignment (RDP-Classifer confidence cut-off = 0.6). Further quality filtering steps within the open reference picking process included removal of chimera's (Chimera Slayer), singletons, non-bacterial lineages and low abundant OTUs comprising less than 0.005 % of the total sequences. Sub-sampling for data analysis was set at the smallest library size.

Microbial diversity measures within each community (α -diversity) and between the communities (β -diversity) was determined using both taxonomic based (species lineages and/or OTUs) and phylogenetic based (incorporates phylogenetic relatedness) approaches (Lozupone and Knight, 2008). For the richness estimation, the Faith's Phylogenetic Diversity (PD-whole tree) that adds up all the branch lengths of a phylogenetic tree was used as the divergence diversity metrics (Faith and Baker, 2006). PD-whole tree measures the fraction of diversity contained in one

community (as measured by the total of all branch lengths in the community) relative to the total amount of diversity contained in all communities (total of all branch lengths from all communities). The Chao richness estimator (Chao, 1984), a taxonomic based diversity index was also used as a comparison to the PD-whole tree. The Simpson (1949) and Shannon (1948) diversity indices were used to determine the quantitative α -diversity. Statistical significance of the diversity measures between treatments was analyzed by parametric t-test based on Monte Carlo permutations.

β -diversity was assessed by computing un-weighted (richness) and weighted (quantitative-richness and evenness) UniFrac distance metrics which are both phylogenetic divergence based methods. The Sorenson (1948) (qualitative-richness) and Bray-Curtis (1957) (quantitative-richness and evenness) were used as β –diversity distance metrics based on taxonomic species composition. Analysis of the strength and statistical significance of the β –diversity measures between each treatment factors was carried out using ADONIS and ANOSIM nonparametric statistical tests that partition a distance matrix among sources of variation in order to describe the strength (R-square) and significance (p-value > 0.05) that a categorical or continuous variable has in determining variation of distances. Significance was determined based on 1000 permutations. Visualization of the β –diversity distance matrix was then done by dimensionality reduction methods that include PCoA (Principle Coordinates Analysis) and/or NMDS (non-Metric dimensional scaling).

To determine differences in taxonomic species composition at different lineages data-mining and statistical analysis was carried out using STAMP (Parks et al., 2014) and Calypso version 3

(<http://bioinfo.qimr.edu.au/calypso/>) software packages that are used for the exploration and visualization of microbial community profiles.

To determine the relationship between soil edaphic factors (soil physicochemical and biological properties) and observed differences in taxonomic species composition, variable selection analysis was performed following the step-wise variable selection procedure using the JMP statistical program. The best model was identified based a combination of a low AIC (Akaiki information criteria) and a mallow's C_p value lower than the number of variables within the model. Predictor variables identified were then validated through multiple linear regression analysis with any variable having a p-value greater than 0.05 being dropped only leaving the variables that helped to explain the variability within the model. The data on soil properties used in the variable selection are based on soil chemical and biological analyses done within the same soil samples in a study focused on soil quality and microbial structure (Mbuthia et al., 2014- chapter 2 of dissertation). The soil properties used in model prediction included: total soil carbon (C) and nitrogen (N), C:N ratio, microbial biomass C (MBC) and N (MBN), microbial biomass C:N ratio (MBC: N), soil enzyme activities (phosphodiesterase (PPD), β -glucosidase (GD) and β -glucosaminidase (GAD)), basal microbial respiration, extractable soil nutrients (Phosphorous (P), Potassium (K) Calcium (Ca), and Magnesium (Mg)), soil pH, moisture content, and bulk density.

RESULTS

Sequence coverage

After the initial quality filtering and de-multiplexing step in QIIME, clustering of reads and removal of chimera's (Chimera Slayer) and singletons reduced the sequence reads from 4.9 to 4.5 million with a total of 87,843 OTUs. A final quality filtering step involving removal of non-bacterial lineages and low abundant OTUs comprising less than 0.005 % of the total sequences resulted in a final total of 3.5 million sequences, with a total of 3069 OTUs. To determine sequencing depth coverage, rarefaction of all the sequences was performed using the QIIME rarefaction script. This revealed that the sequencing depth achieved maximum coverage for all samples even for the sample with the lowest sequence means (Figure 3). Based on the rarefaction curves, normalization for sampling for further statistical analysis was set to 62,400 sequences per sample based on the least sample size obtained.

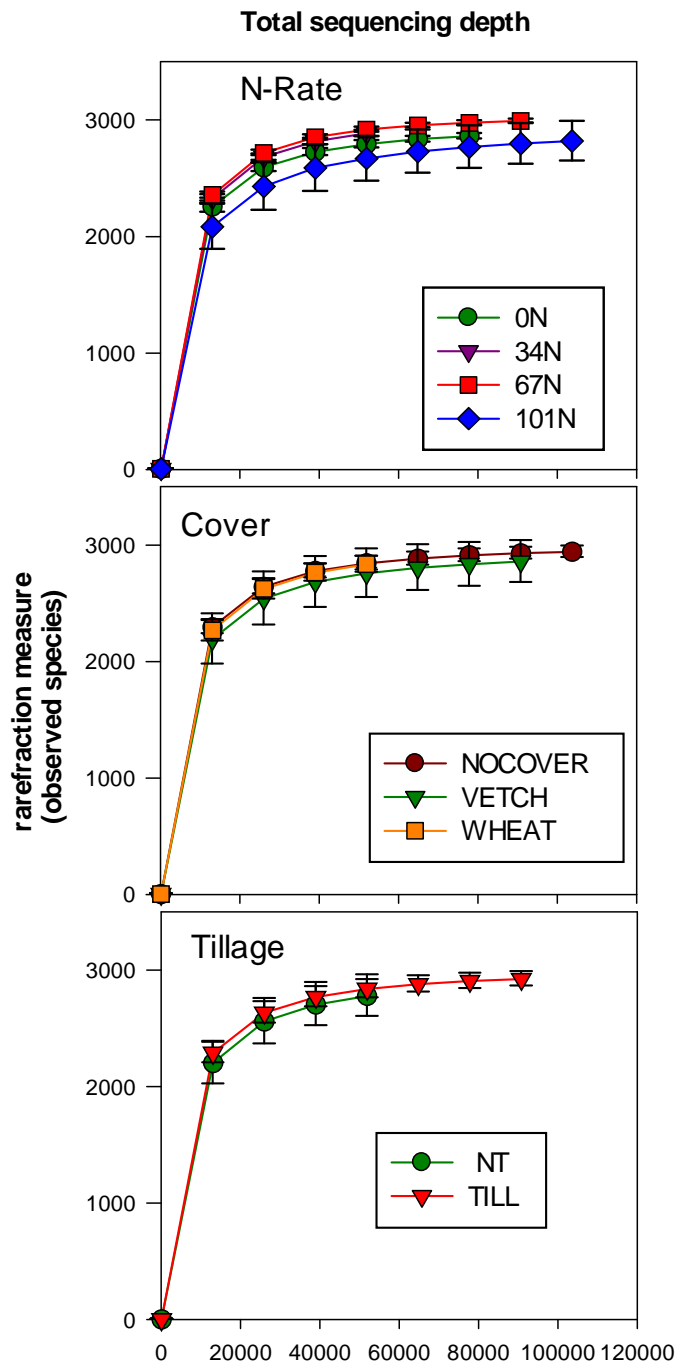


Figure 3: Rarefaction curves showing sequence coverage for all treatment factors
Rarefaction curve shows maximum coverage having been attained at approximately 20,000 sequences/sample.

Alpha and Beta diversity measures

Among all the treatment factors, both richness and evenness differed significantly across N-rate ($p < 0.001$) with 101 N-rate being significantly less diverse than the 34 and 67 N-rate. However there were no differences in richness or evenness associated with cover crop and tillage treatments (Table 8). The species richness based on both taxonomic classification (Chao index) and divergence measure (PD-whole tree) revealed similar trends in N-rate. Treatments under the 34 and 67 N-rate had a microbial community with a significantly greater species richness compared to the microbial community under the 0 and 101 N-rate. Factoring in species abundance, the α -diversity indices (Shannon and Simpson), revealed a less even distribution of microbial species in treatments under the highest N-rate (101 N). The Shannon index also revealed a lower evenness in treatments under 0 N-rate compared to treatments under 34 and 67 N-rate but was still significantly greater than that under the 101 N-rate.

Diversity across treatments (β -diversity) revealed differences in taxonomic species (Sorenson-dice, 1948) that differed significantly based on N-rate ($R^2 = 0.41$, $p < 0.05$), and tillage ($R^2 = 0.09$, $p < 0.05$) with no differences in species richness between cover crops ($R^2 = -0.05$, $p > 0.05$). β -diversity based on phylogenetic divergence (unweighted UniFrac distances) revealed significant differences only with N-rate ($R^2 = 0.43$, $p < 0.05$) with no significant differences in richness due to tillage ($R^2 = 0.08$, $p > 0.05$) and cover crops ($R^2 = 0.05$, $p > 0.05$). β -diversity factoring in relative abundance in taxonomic species (Bray-Curtis, 1957), revealed N-rate to be the main driving factor of species evenness ($R^2 = 0.60$, $p < 0.05$). Tillage treatment also had a significant effect but explaining less variation on species evenness ($R^2 = 0.18$, $p < 0.05$) while cover crop had no significant effect ($R^2 = 0.04$, $p > 0.05$). Species evenness based on phylogenetic divergence

(Weighted UniFrac distances) revealed significant differences based only on N-rate ($R^2=0.5106$, $p < 0.05$) with no significant differences based on tillage ($R^2=0.0979$, $p > 0.05$) or cover crop ($R^2=0.0236$, $p > 0.05$).

Table 8: Alpha diversity index measures for richness (PD-whole tree and Chao) and diversity (Shannon and Simpson) across N-rate, cover crop and tillage treatments

	PD-whole tree	Chao	Shannon	Simpson
N-Rate				
0N	112.82(0.80)b	2918.40(23.06)b	10.31(0.05)b	0.9983(9.06E-05)a
34N	116.11(0.84)a	2990.86(15.86)a	10.34(0.05)ab	0.9983(1.24E-04)a
67N	117.07(0.67)a	3018.22(14.08)a	10.40(0.03)a	0.9984(1.17E-04)a
101N	110.41(5.96)ab	2858.76(156.2)ab	10.05(0.27)b	0.9978(4.60E-04)b
Cover Crop				
Vetch	112.38(6.11)a	2902.16(155)a	10.20(0.30)a	0.9981(5.12E-04)a
Wheat	114.90(1.78)a	2968.00(35)a	10.30(0.13)a	0.9983(2.39E-04)a
No Cover	114.76(1.20)a	2962.57(47)a	10.32(0.05)a	0.9982(6.21E-05)a
Tillage				
No-till	113.12(5.33)a	2923.17(135)a	10.22(0.25)a	0.9981(4.5E-4)a
Till	114.77(2.14)a	2962.02(51)a	10.32(0.10)a	0.9983(1.74E-4)a

Alpha-diversity means (standard errors in brackets) represent the calculated alpha diversity indices within each treatment factor: Nitrogen fertilization rates (N-rate: 0, 34, 67 and 101 N kg/ha, n=6); Cover crops-Hairy vetch, Winter wheat and No Cover (n=8); and tillage- No-till, and Till (n=12). Means followed by the same letter group across each treatment factor are not significantly different ($p < 0.05$)

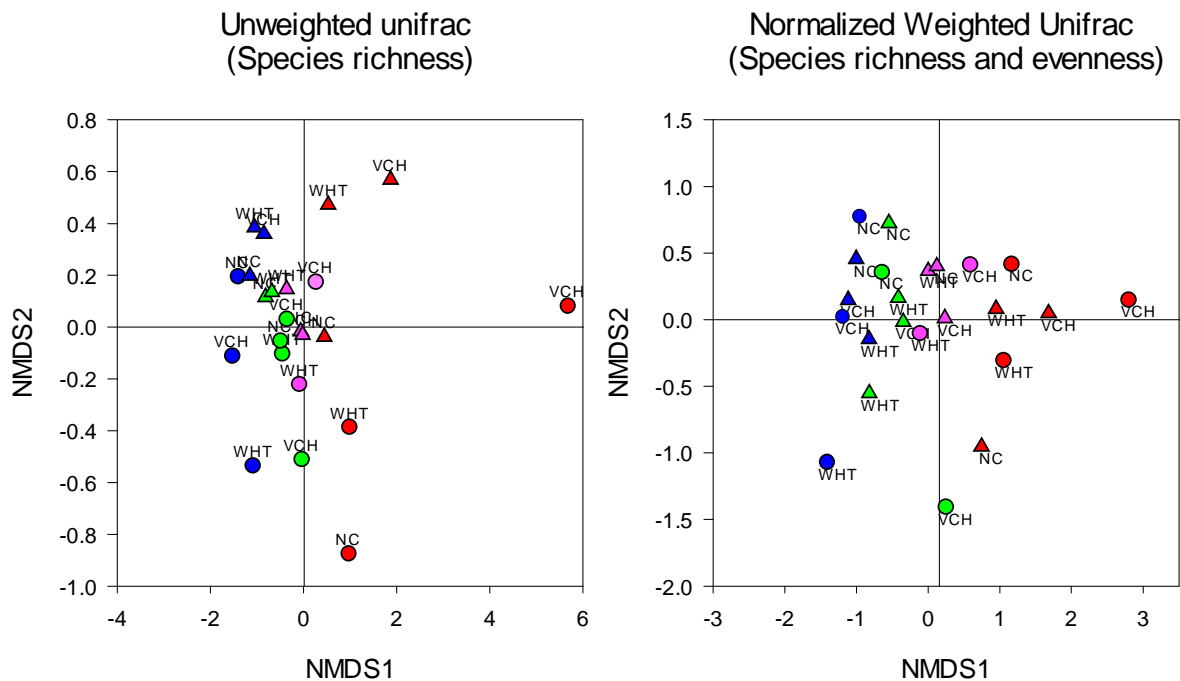


Figure 4: Non-metric dimensional scaling representing the community structure differences across all samples.

N-rate treatments represented by color; Red= 101N-rate, Pink= 67N-rate, Green=34N-rate, and Blue= 0N-rate; symbol shapes represents the tillage treatment; Circles= NoTill treatments, Triangles= Till treatments; Symbol names designate the cover crop treatments: VCH=Hairy Vetch, WHT=Winter Wheat and NC=NoCover crop. NMDS stress unweighted normalized=0.057 and weighted normalized =0.098

Bacterial taxonomic composition

Of the classifiable sequences, 25 phyla were identified across the sample set. Taxonomic level differences revealed a microbial community that was more similar at the high taxonomic rank (phyla), but revealing diverse and more species specific trends at the lower ranks (class-order) (Figure 5). The dominant phyla were Proteobacteria, Acidobacteria, Actinobacteria, Planctomycetes, and Chloroflexi with the percent relative abundance across treatments ranging approximately between 27-30%, 17-21%, 13-19%, 10-17%, and 7-13% respectively. Among the treatments, N-rate had a greater number of phyla with significant differences ($p < 0.05$) in the relative abundance of 6 out of the 25 phyla identified with cover crop and tillage each having significant effect on only having two and three significant phyla respectively (Table 9). It is interesting to note that the distribution of the top 10 most abundant phyla differed between treatments under the lower N-rates (0, 34, 67 N) and those under the highest N-rate (101 N). Acidobacteria was the second most abundant phylum under the 0, 34 and 67 N-rate, but at the 101 N- rate Planctomycetes was the second most abundant. Concomitantly, the phyla Nitrospirae and Bacteroidetes did not feature among the top 10 abundant phyla under the 101N and were replaced by candidate bacterial phyla designated as WPS-2 and AD3 (Figure 5).

Among the top 10 most abundant phyla, N-rate had a significant influence on the relative abundance of Acidobacteria, Planctomycetes, Nitrospirae, and WPS-2 (Figure 6). The relative abundance of Acidobacteria and Nitrospirae decreased as N-rate increased being significantly lower at the 101 N-rates, while the relative abundance of Planctomycetes, WP-2 and AD3 increased as N-rate increased being significantly greater at the 101 N-rate. The phyla that differed among the cover crop treatments were Actinobacteria, and Bacteroidetes. The relative

abundance of Actinobacteria was significantly greater under the wheat cover compared to the vetch and No Cover while Bacteroidetes was significantly greater under No Cover compared to vetch or wheat (Fig 6). Between the tillage treatments, the phyla that significantly differed were Verrucomicrobia, Armatimonadetes, AD3, and Chlorobi. Verrucomicrobia and AD3 were significantly greater under No-till compared to till, while the abundance of Armatimonadetes and Chlorobi were greater under till compared to No-till (Figure 6).

To further delve into the differences in bacterial community composition, relative abundance was assessed at the class and order level. This revealed more differences in bacterial groups that included orders from phyla that had not been identified as being significantly different at the phylum level (Figure 7). These included groups from Gemmatimonadetes, Chloroflexi, and Proteobacteria. It was interesting to note that groups from the same phyla would exhibit contrasting trends with the increase in N-rate. These contrasting trends may be the reason why differences at the phyla level were not detected for some of the phylum.

Specific groups that significantly differed across N-rate (Figure 7) included: Four orders from the Acidobacteria phylum that included Chloracidobacteria (3-10 %), Acidobacteria-6 (5-10 %), Acidobacteria-iii1-8 (1-2 %), were decreasing with N-rate and Acidobacteriia (1-4 %), and Solibacteres (1-2 %) increasing with N-rate. It was interesting to note that the dominant Acidobacteria groups had a negative correlation with N-rate. The phyla Chloroflexi had two classes that significantly differed across N-rate at the class lineage which were, Ktedonobacteria (1-4%) that showed an increasing trend with N-rate increase and a class designated as Chloroflexi (1-2%) that had a decreasing trend with increasing N-rate increase. From the Proteobacteria phylum, Deltaproteobacteria (2-5%) and Gammaproteobacteria (1-4%) differed

significantly at the class lineage exhibiting a decreasing and increasing trend respectively with N-rate increase. At the order level, Rhodospirillales from Alphaproteobacteria and Xanthomonadales from Gammaproteobacteria also differed significantly across N-rate both having an increasing trend with N-rate; from the phylum Planctomycetes, two important groups were identified as being significantly different across N-rate. These included the class Phycisphaerae (3-5 %) and Planctomycetia (6-12 %) both of which exhibited an increasing trend with N-rate increase. The strain Ellin5290, the only classified member of Gemmatimonadetes significantly increased with N-rate. Nitrospirae does not have any specific lower lineages but continued to show significant differences across N-rates even at the order level.

Significant differences due to the cover crop were only exhibited in one or two groups of each significant phylum (Figure 7). From the phyla Actinobacteria, the order Actinomycetales (4-6 %) which had the highest abundance under wheat, followed by the vetch cover, with No Cover having the least abundance; and from the phylum Proteobacteria, the order Rhizobiales (4-5%) a nitrogen-fixing bacteria, differed between cover crops. Interestingly, Rhizobiales exhibited the greatest abundance at the No Cover treatment with vetch and wheat being significantly less but not differing from each other.

Significantly different orders as driven by tillage treatments (Figure 7) included: Gaiellales (3-5%) from the phylum Actinobacteria and Chthoniobacterales (4-5%) from the phylum Verrucomicrobia having a greater relative abundance under No-till compared to till. Sphingobacteriales (2-3%) from the phylum Bacteroidetes, Burkholderiales (2-3%) and Myxococcales (2-2.5%) from the phylum Proteobacteria exhibiting a greater abundance under till compared to No-till.

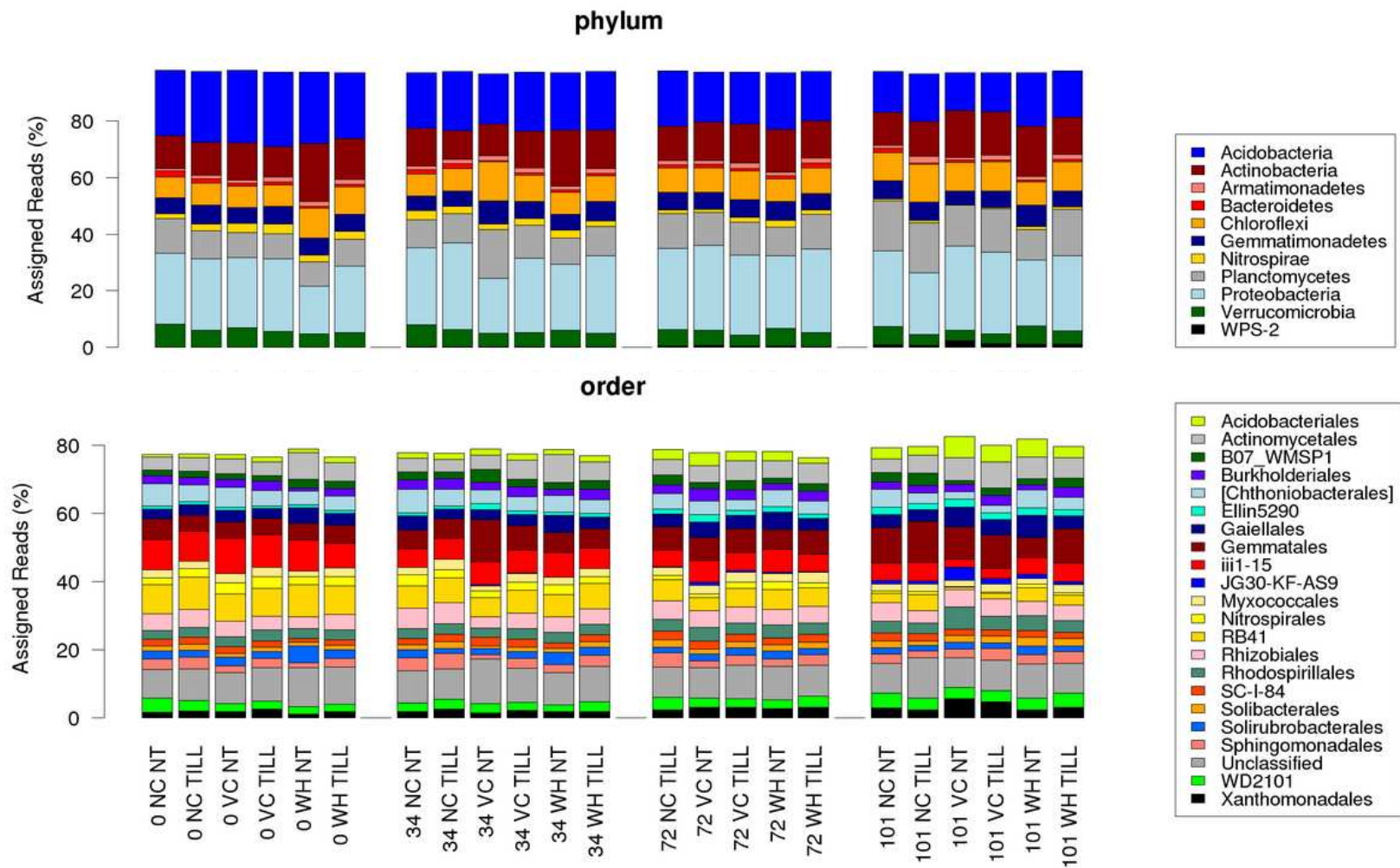


Figure 5: Relative abundances of the dominant bacterial phyla (top) and order (bottom)

Under tillage (NoTill (NT) and Till) and cover crops (Hairy vetch (VC), winter wheat (WH) and NoCover (NC)) across all nitrogen fertilization rate (0, 34, 67 and 101N-rates)

Table 9: The relative abundance of phyla that differed significantly across treatments (N-rate, cover crops and tillage)

Taxon	0N	34N	67N	101N	p-values (corrected)
N-Rate					
Acidobacteria	24.81(1.29)a	20.04(1.15)ab	18.77(0.97)b	15.70(1.85)c	3.35e-08
Planctomycetes	9.73(1.30)b	11.46(2.59)b	11.69(0.83)b	15.41(2.49)a	1.83e-03
Nitrospirae	2.73(0.56)a	2.44(0.45)ab	1.60(0.45)b	0.71(0.30)c	3.80e-06
WPS-2	0.02(0.01)b	0.17(0.05)b	0.39(0.12)b	1.18(0.61)a	5.82e-05
AD3	0.25(0.13)c	0.44(0.26)b	0.54(0.22)ab	0.85(0.18)a	0.002
WS3	0.51(0.15)a	0.48(0.09)a	0.34(0.09)b	0.14(0.10)c	1.47e-04
COVER CROP					
	Vetch	Wheat	No Cover		p-values (corrected)
Actinobacteria	13.45(1.75)b	15.93(2.76)a	11.86(0.85)b		5.00e-03
Bacteroidetes	0.86(0.22)b	0.92(0.31)b	1.52(0.01)a		5.38e-03
TILLAGE					
	No-till	TILL			p-values(corrected)
Verrucomicrobia	6.02(1.28)a	4.97(0.80)b			0.038
Actinobacteria	14.95(3.00)a	12.79(1.36)b			0.04
Armatimonadetes	1.28(0.28)b	1.73(0.24)a			0.0008

Mean relative abundance (standard errors in brackets) of bacterial phyla that differed across the Nitrogen fertilization rates (N-rate: 0, 34, 67 and 101 N kg/ha, n=6); Cover crops-Hairy vetch, Winter wheat and No Cover (n=8); and tillage- No-till, and Till (n=12). Means followed by the same letter group across each treatment factor level are not significantly different ($p < 0.05$) P-value: adjusted to minimize for multiple comparison errors.

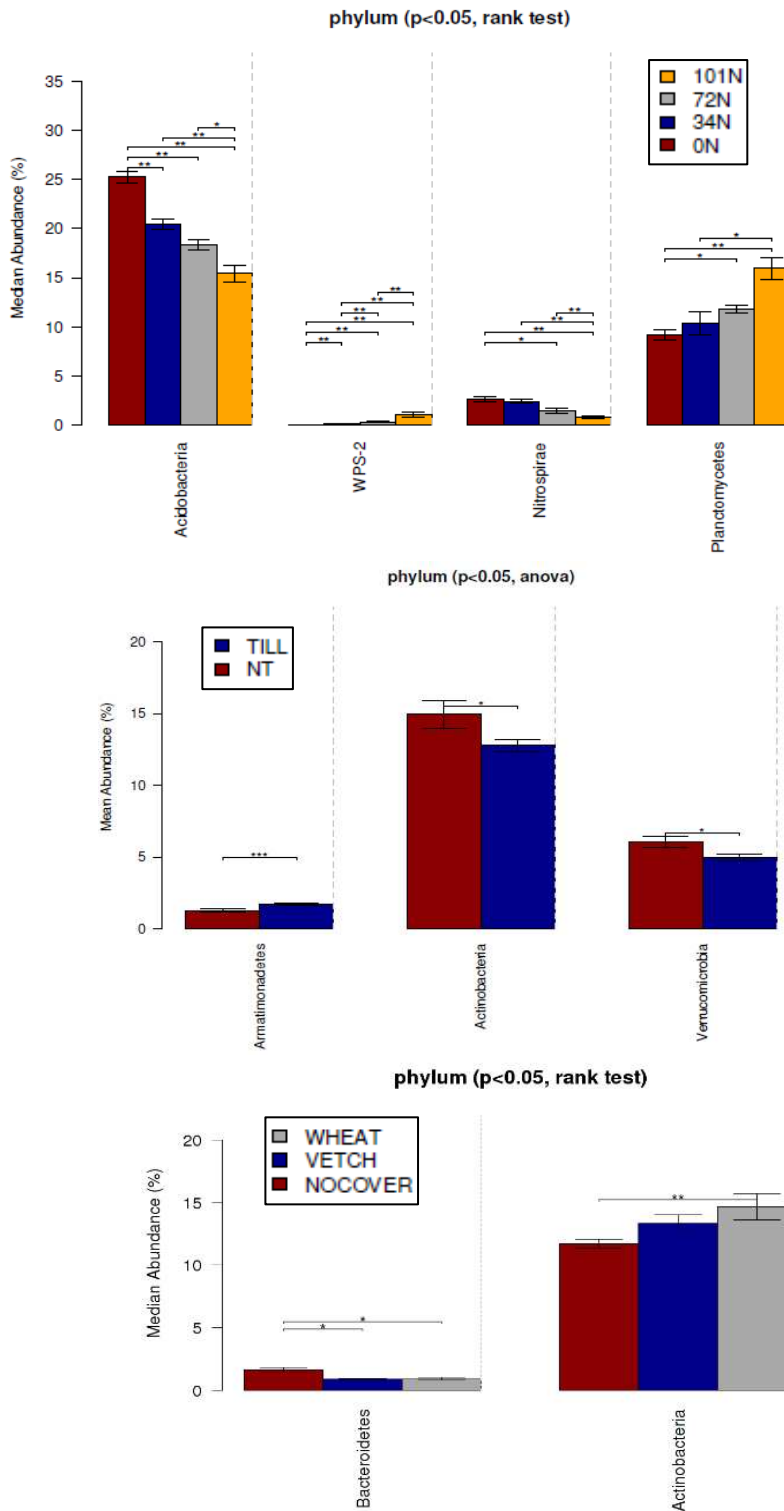


Figure 6: Phyla observed to significantly differ at each treatment factor
N-rate 0, 34, 67 and 101 N kg/ha (Top); tillage No-till (NT) and Till, (middle); and cover crop, Hairy vetch, Winter wheat and No Cover (bottom).

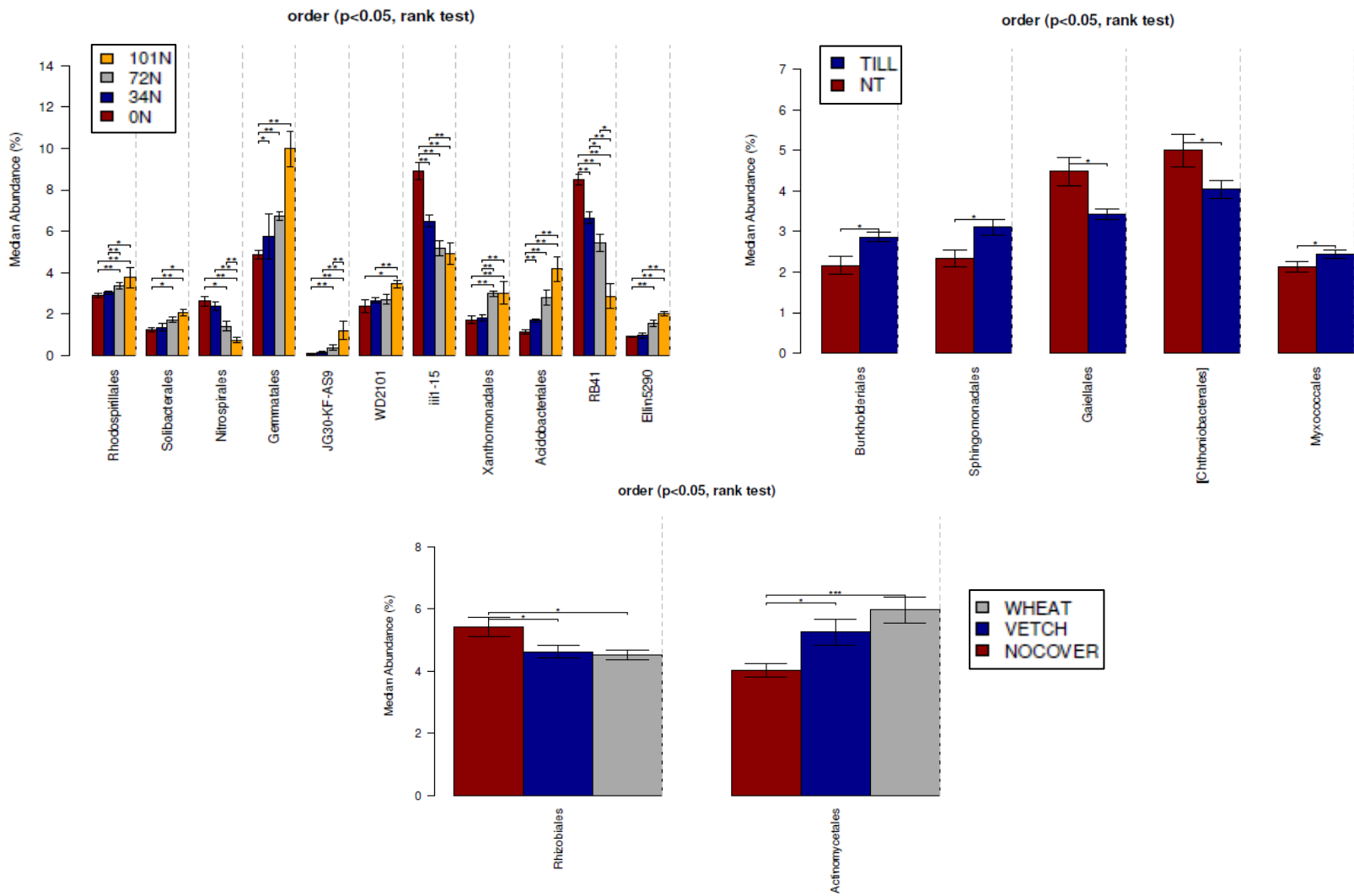


Figure 7: Significantly different bacteria at the order lineage as influenced by each treatment factor: N-rate-0, 34, 67 and 101 N kg/ha (top left), tillage- till and NoTill (top right) and cover crop- winter wheat, hairy vetch and No Cover (bottom)

Relationship between soil properties and bacterial taxonomic abundance

Variable selection analysis was used to determine the possible relation of soil properties factors on microbial species composition. This was run against the phyla identified to be significant among the treatment factors. Table 10 shows the properties identified for each phylum based on the best fit model including the adjusted R^2 and p-values, and Figure 9 shows the relationship between phyla and selected variables. Among the soil properties, pH had a significant relationship with four of the phyla exhibiting a significant positive correlation with Acidobacteria, and Armatimonadetes; and a negative correlation with Nitrospirae and Planctomycetes. β -glucosidase (GD) exhibited a significant correlation with three of the phyla exhibiting a positive correlation with Nitrospirae, and Bacteroidetes; and a negative correlation with Armatimonadetes. Bulk density also had a significant correlation with three of the phyla showing a positive relationship with Actinobacteria, Acidobacteria and Verrucomicrobia. Soil moisture content was another factor exhibiting significant relationship with three phyla that included a positive correlation with Chloroflexi and negative correlation with Bacteroidetes and Proteobacteria. Total C:N ratio had significant relationship with the abundance of two phyla that included a positive correlation with Actinobacteria, and a negative correlation with Acidobacteria. Other related factors included: microbial biomass C:N ratio exhibiting a negative correlation with Planctomycetes and Chloroflexi; Phosphodiesterase (PPD) that had a negative correlation with both Chlorobi and Chloroflexi; Mg exhibiting a positive correlation with Chlorobi and negative correlation with Actinobacteria; phosphorous (P) having a negative correlation with Proteobacteria and Armatimonadetes; calcium (Ca) exhibiting a positive correlation with Verrucomicrobia ; and finally total carbon (TC) having a negative correlation with Chlorobi.

Pearson correlation between all the soil properties and the top abundant phyla classified the soil properties into two main groups that either positively or negatively correlated with a group of specific bacterial phyla (Fig 8). One group of soil properties consisted of: total C and N, microbial biomass C (MBC) and N (MBN), β -glucosaminidase (GAD), and basal microbial respiration; with the second group mainly consisting of the extractable soil nutrients P, K Ca, and Mg, soil pH, bulk density, phosphodiesterase (PPD), β -glucosidase (GD) and C:N ratio.

Table 10: Significant soil properties identified by variable selection as influential factors driving the relative abundance of the abundant phyla

Bacteria Phyla	Soil property	F:Ratio	Prob > F	Adjusted R-square	model p-value
Acidobacteria	pH	70.377	<.0001	0.78	<0.0001
	C:N	5.3438	0.0322		
	Bulk density	7.7204	0.012		
Nitrospirae	pH	54.6988	<.0001	0.75	<0.0001
	B-Glucosidase	6.7278	0.0174		
Chlorobi	TC	43.8392	<.0001	0.68	<0.0001
	Mg	13.3053	0.0017		
	PPD	5.9562	0.0246		
Planctomycetes	pH	24.6254	<.0001	0.55	0.0001
	MBC:N	6.5972	0.0183		
Actinobacteria	C:N	8.9288	0.0079	0.48	0.002
	Mg	7.63	0.0128		
	Bulk density	5.0735	0.037		
	MBC	4.4624	0.0489		
Verrucomicrobia	Calcium	7.1166	0.0152	0.48	0.0014
	Bulk density	5.2516	0.0335		
Armatimonadetes	B-Glucosidase	10.0862	0.005	0.44	0.003
	Phosphorous	5.8417	0.0259		
	pH	4.8861	0.0395		
Chloroflexi	PPD	12.1061	0.0029	0.40	0.001
	MBC:N	7.3562	0.0148		
	MoistureC	5.1392	0.0367		
Proteobacteria	Phosphorous	14.1996	0.0014	0.39	0.04
	GAD	4.6548	0.0447		
	MoistureC	6.6448	0.019		
Bacteroidetes	MoistureC	8.502	0.0089	0.30	0.02
	B-Glucosidase	6.652	0.0184		
	BasalResp	2.9546	0.1019		

CN: total soil carbon (C) and nitrogen (N) ratio; MBC: microbial biomass C; soil enzyme activities (GD: β -glucosidase, PPD: Phosphodiesterase and GAD: β -glucosaminidase); BasalResp: basal microbial respiration: extractable soil nutrients (P: phosphorous, Ca: calcium, Mg: magnesium and K: potassium); pH: soil pH; MoistureC: moisture content; and BD: bulk density. Values in red indicate negative correlation and green a positive correlation

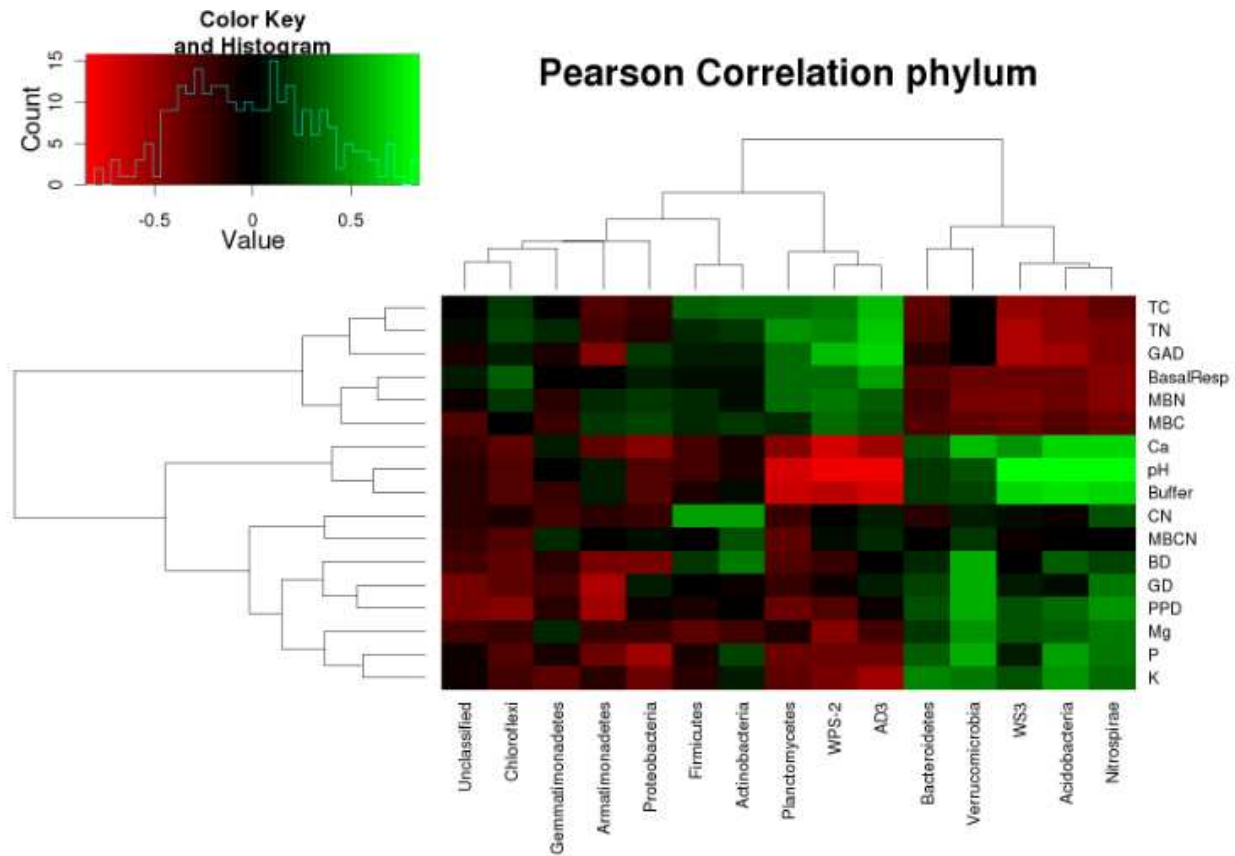


Figure 8: Heat map of Pearson correlation coefficients showing the relationship between all soil properties and the top abundant phyla as represented by the relative sequence abundance of each taxon. CN: total soil carbon (C) and nitrogen (N) ratio; MBC: microbial biomass C; soil enzyme activities (GD: β -glucosidase, PPD: Phosphodiesterase, and GAD: β -glucosaminidase); BasalResp: basal microbial respiration; extractable soil nutrients ((P: phosphorous, Ca: calcium, Mg: magnesium and K: potassium); pH: soil pH; MoistureC: moisture content; and BD: bulk density. Values in red indicate negative correlation and green a positive correlation

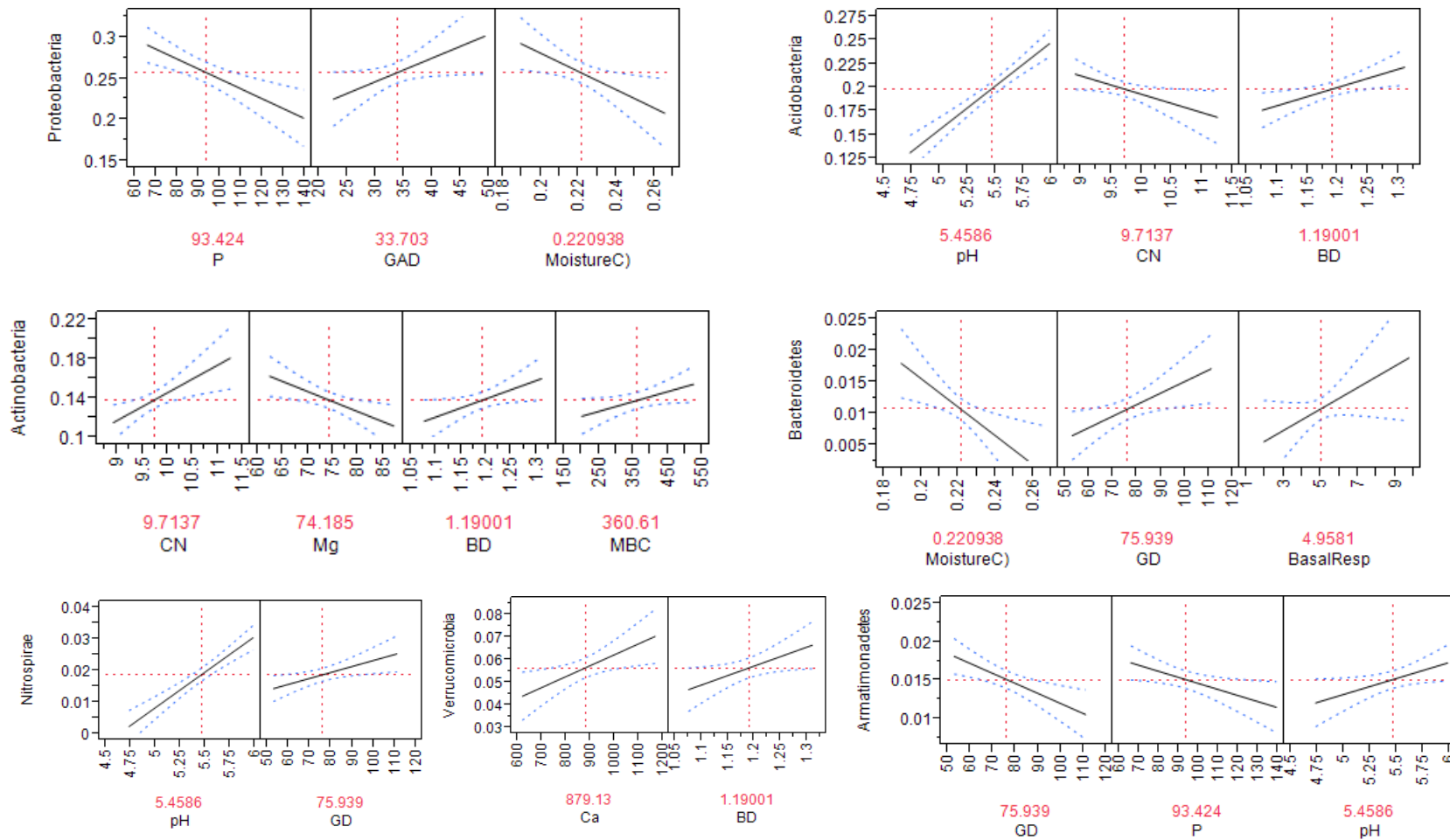


Figure 9: Variable selection profiles showing the relationship between variables selected as predictors of the abundance of individual bacteria phyla.

The Y-axis represents the relative sequence abundance of each taxon. CN: total soil carbon (C) and nitrogen (N) ratio; MBC: microbial biomass C; soil enzyme activities (GD: β -glucosidase and GAD: β -glucosaminidase); BasalResp: basal microbial respiration; extractable soil nutrients (Phosphorous (P), Calcium (Ca), and Magnesium (Mg)); soil pH; MoistureC: moisture content; and BD: bulk density. Values in red indicate the mean values of observed variable factors across treatments.

DISCUSSION

Microbial diversity measures in this study revealed bacterial species richness and evenness to be strongly driven by N-rates. Bacterial diversity exhibited a positive response to increasing N-rate with the highest diversity being observed at the 72 kg/ha N-rate but then showing a declining trend at the higher N-rate (101 N kg/ha). Increasing N-rates has been demonstrated to significantly alter soil microbial abundance (Treseder, 2008; Ramirez et al., 2010, 2012; Chen et al., 2014). This has been postulated to several controlling factors that include: an increase in net primary production promoting changes in substrate quantity and quality available to soil microbes; altering the soil pH, the overall soil chemistry and osmotic potential of soil solution that may result in toxic effects on soil microbes; and by inhibiting certain microbial enzyme activities that may in turn limit microbial population growth (Treseder, 2008; Chen et al., 2014).

In this study, the observed effect of N-rate on microbial diversity may be attributed to a combination of several of the above stated factors. A significant increase in crop yield, and total C and N content with increasing N-rate were recorded in the research plots under this study (Mbutia L.W., dissertation-chapter 2) pointing to an increased availability of substrate quantity that could be utilized by soil microbes resulting in increased microbial abundance. The declining trend in diversity at the highest N-rate could be attributed to the significant decrease in soil pH and levels of extractable phosphorous (P), calcium (Ca), and potassium (K) that were also reported at the high N-rate (101 N-rate). The increase in acidic conditions will in turn increase aluminum solubility promoting conditions that are toxic to soil microbes that in combination with the decline in availability of soil P, K and Ca would limit microbial growth (Treseder, 2008).

Contrary to what we had hypothesized, we did not observe any significant differences in bacterial species diversity due to cover crops and tillage treatments. Nevertheless, an NMDS of the microbial community structure revealed greater community dissimilarity between the cover crop and tillage treatments at the highest N-rate (101 N). In particular, the vetch No-till treatment under the 101 N-rates was a clear outlier as observed in the NMDS's plots. The high N-rate combined with vetch and No-till may create distinct environmental conditions that exclude some bacteria and promote specific bacteria adapted to the extreme conditions. The higher substrate quality resulting from an interaction effect of both inorganic N and additional organic N from vetch as well No-till combined with a lower pH may explain this result.

An analysis of the abundance distribution of bacterial taxonomic composition in the soils under this study revealed the dominant taxa to belong to the phyla: Proteobacteria (27-30%), Acidobacteria (17-21%), Actinobacteria (13-19%), Planctomycetes (10-17%), and Chloroflexi (7-13%). This is in agreement with the dominant taxa that has been reported across a majority of soils although the order of dominance and relative abundance of each taxa varies dependent on land use (Janssen, 2006; Youssef and Elshahed, 2009). Most studies have looked at the bacterial taxonomic composition across contrasting land uses for example between pasture, forest soils, grassland, and cropland (Lauber et al., 2008, 2009; Acosta-Martínez et al., 2010; Shange et al., 2012). The study presented here is unique in that it represents an assessment of the bacterial distribution within one land use, i.e. a cropping system, with the variation being a long-term history of differing management practices. An assessment of the bacterial composition as influenced by the management practices at the different taxonomic lineages revealed significant differences that are of value to agro-ecosystem functioning.

As observed with the diversity measures, N-rate had the most pronounced effect on the significant differences in the relative abundance of bacterial taxonomic groups. A noteworthy case in point is the trend observed in the shift of the Acidobacteria groups as influenced by increasing N-rate and by association decreasing soil pH. It is commonly expected that Acidobacteria would elicit a negative correlation with soil pH (increasing acidity) as their name implies. Deviating from this expectation, the relative abundance of the dominant Acidobacteria sub-groups in our study elicited a significant positive correlation with soil pH with their abundance being significantly less at the lowest N-rate. Acidobacteria are ubiquitous bacteria in soils known to thrive in a wide range of habitats adapting to a wide range of temperatures, salinity, organic matter content, and soil pH (Rawat et al., 2012). Therefore, it would not be surprising that some Acidobacteria sub-groups would thrive under higher soil pH. Several other studies have reported the differential response of Acidobacteria to soil pH but with the dominance of the acid tolerant sub-groups being reported in most soils (Jones et al., 2009; Lauber et al., 2009; Rousk et al., 2010). The sub-groups that show a positive correlation with pH were mainly observed in soils having a pH > 6 (Barns et al., 1999). The dominance of Acidobacteria groups that increase with an increase in soil pH in these soils is therefore striking and indicates their possible importance in maintaining their associated ecological functions under this system. While the ecological significance of Acidobacteria in soil is yet to be fully established, recent research based on comparative genomics have shown three sub-groups to have the functional potential in carbon and nitrogen cycling as well as adaptation to stress and starvation (Ward et al., 2009; Rawat et al., 2012). Given the probable ecological functions of Acidobacteria in C and N cycling, their adaptation to increasing soil pH would be an important

adaptation for agro-ecosystems, since liming of soil to reduce acidity is a common practice for most agricultural crop production systems.

Other bacterial taxa that differed significantly ($p < 0.05$) due to N-rates included the Nitrospirae, Planctomycetes, and a candidate bacteria phylum designated as WPS-2 (Fig 6). Members of the Nitrospirae and Planctomycetes have been identified as key players in the N cycle, or /and organic matter decomposition (C-cycling). Nitrospirae are nitrite oxidizing bacteria that play a role in nitrogen transformations by driving one of the key steps in nitrification by oxidizing nitrite (NO_2^-) to nitrate (NO_3^-) (Kowalchuk and Stephen, 2001; Arp et al., 2002). Nitrospirae are chemolithoautotrophic nitrifiers that utilize inorganic carbon (like HCO_3^- and CO_2) as a source of energy using NO_2^- as an electron acceptor. In our study, Nitrospirae decreased as the N-rate increased from a mean of 2.73% at the 0 N-rate down to 0.71% at the 101 N-rate (58% reduction). Nitrospirae also had a strong positive correlation with pH indicating that an increase in acidic conditions might be a limiting factor to their growth. The relative abundance of nitrifying bacteria has been shown to be negatively impacted by high levels of ammonium and/or low pH which seem to result in toxic conditions for the nitrifiers (Belser, 1979). Given the fact that the source of N-fertilizer in our study was ammonium nitrate, the high ammonium level and combined decrease in pH observed may explain the decrease of Nitrospirae with increasing N-rate. Nitrospirae also had a positive correlation with β -glucosidase enzyme activity. This may suggest that Nitrospirae are dependent on the C released as CO_2 from β -glucosidase activity.

Planctomycetes are a unique group of bacteria that share some characteristics with eukaryotic cells in that they have intra-cytoplasmic membranes that compartmentalize the cell. This characteristic gives them the ability to undertake endocytosis, i.e. the ability to take up

macromolecules such as protein into their cells via their membranes. Most members of this phylum are slow growing aerobic or facultative chemoheterotrophs specialized in carbohydrate metabolism with one divergent species that is a chemolithoautotrophs. An outstanding member of this phylum is the chemolithoautotrophic anaerobic ammonium oxidizing bacteria (annamox) that are able to oxidize ammonium under anaerobic conditions. Other members of this phylum include the class Planctomycetacia said to play a role in the initial breakdown of complex organic matter into simpler compounds and probable aggregate formation (Fuchsman et al., 2012). The dominant groups in the soils under this study belonged to the class Planctomycetacia and Phycisphaerae which exhibited a positive relationship with N-rate increasing from 7-12 % and 2-4 % respectively from 0 -101 N-rate. This indicates their preference to higher fertilized environments and a possible adaptation to acidic conditions as was recorded at the high N-rate (101 N). Members of the Planctomycetes have been shown to adopt to extreme environments ranging from hot springs, suboxic and sulfidic conditions, polluted environments and have been utilized for bioremediation (Nogales et al., 2001; Wagner and Horn, 2006; Elshahed et al., 2007; Fuchsman et al., 2012). Including soil pH, variable selection identified microbial biomass C:N ratio as possible drivers/predictors of the abundance of Planctomycetes. The relative abundance of Planctomycetes exhibited a negative correlation with MBC:N ratio and soil pH. MBC:N ratio may be used as an indirect indicator of the substrate quality. A lower MBC:N may indicate a greater C:N ratio substrate quality that would result in reduced assimilation of C and N into the microbial biomass. It may then postulate that the negative correlation of Planctomycetes with MBC:N ratio may be indicative of their affiliation to complex organic matter corroborated by the greater C levels recorded at the high N-rate treatments.

At the lower lineages, N-rate had a significant ($p < 0.05$) influence to bacterial species belonging to the Proteobacteria phylum, with increasing N-rate having a negative correlation with Deltaproteobacteria and a positive correlation with Gammaproteobacteria and Alphaproteobacteria. In particular, Rhodospirillales (order) belonging to Alphaproteobacteria and Xanthomonadales (order) from Gammaproteobacteria differed significantly across N-rate showing a positive trend with N-rate increase (Figure 7). Proteobacteria are ubiquitous in soils usually recorded as the most dominant phyla in most soils (Spain et al., 2009). Members of the Proteobacteria constitute a wide range of morphological, physiological and metabolic capacity and have been indicated to play an integral role the global C, N and S (sulfur) cycling. The differential response of the Proteobacteria groups to N-rate is thus indicative of their specificity in adaptation to specific ecological environments and probable functions. For example, species belonging to Xanthomonadales are recognized as important plant pathogenic species (Vauterin et al., 1995; Van Sluys et al., 2002). The significant increase in the relative abundance of Xanthomonadales with increasing N-rate may indicate their affinity to N. This implies that given conducive conditions and susceptible crop species, disease incidences might be greater with increased N levels. On the other, hand it may be indicative of the competitive ability of pathogenic microbial species in being able to survive extreme environmental conditions that may result with high N levels. The phylum Chloroflexi had two groups that significantly differed across N-rate at the class lineage Ktedonobacteria and Chloroflexi (class) showing an increasing and decreasing trend with N-rate respectively. The phylum Chloroflexi is stated to be phylogenetically diverse. Several groups within this phylum have been associated with anoxic environment and rely on sulfur compounds as a source of energy (Costello and Schmidt, 2006). It is therefore not surprising that Chloroflexi had a positive correlation with moisture content

attesting to its affinity to anaerobic conditions. Chloroflexi also had a negative correlation with microbial biomass C:N ratio and phosphodiesterase which may indicate they may be more adapted to nutrient poor environments.

It was interesting to note the differences in the distribution of the top 10 most abundant phyla between the lower N-rate treatments (0, 34, 67 N-rate) to that under the highest N-rate (101 N-rate), (Figure 5). While Acidobacteria was the second most dominant phyla under the 0, 34 and 67 N- rate, its dominance at the 101 N- rates was decreased by the significant increase in the dominance of Planctomycetes. Concurrently, the abundance of Nitrospirae also diminished at the high N-rate with the relative abundance of candidate phyla WPS-2 being more pronounced. These differences in taxonomic composition between the lower N-rates and higher N-rates are indicative of distinct differences in the environmental conditions. The pronounced increase of the candidate phyla WPS-2 may indicate its probable adaptation to high N and low pH (acidic conditions) and probable toxic environment. WPS-2 was isolated from a Polychlorinated Biphenyl-Polluted Soil where a rare Planctomycetes species was also isolated indicating similar adaptation properties to extreme environments (Nogales et al., 2001).

Despite the lack of significant differences in bacterial diversity measures due to cover crop and tillage treatments, we were able to detect significant differences in the abundance of specific bacterial groups. An important phylum that differed across both cover crop and tillage treatments was the Actinobacteria. No-till resulted in a significant increase in the relative abundance of Actinobacteria which was greater under No-till compared to till. The use of cover crops also resulted in a significantly greater relative abundance of Actinobacteria which increased in the order of Wheat>Vetch>No Cover (Figure 6). Bacterial groups belonging to the Actinobacteria

phylum have long been recognized as important key players in the decomposition of soil organic matter with their abundance being greater in habitats with more recalcitrant C (Conn, 1916; Goodfellow and Williams, 1983). Actinobacteria are also unique from other bacteria in that they have a mycelial growth habit more similar to soil fungi that makes it possible to explore the bulk soil in search of water and nutrients (McCarthy and Williams, 1990). Several groups of the Actinomycetes have also been recognized for their ability to produce secondary metabolites that play a role in plant growth promotion and suppression of pathogenic microbes (Goodfellow and Williams, 1983). These properties of the Actinobacteria make them a vital component of the agro-ecosystem and thus an increase in its abundance may be equated to a contributed increase in their ecological function. In our study, soils under No-till as well as under cover crops in general had greater levels of total C and N which may have been contributed to by in part by the probable activity of Actinobacteria. A variable selection to determine probable soil properties that relate to the Actinobacteria abundance identified C:N ratio, microbial biomass C (MBC), Mg, and bulk density as probable driving factors in determining their relative abundance. MBC, C:N ratio and bulk density exhibited a positive correlation with Actinobacteria while Mg showed a negative correlation. The positive correlation of Actinobacteria with both C:N ratio and MBC may signify its role in carbon cycling and nitrogen cycling and might be indicative of their ability to utilize both labile and recalcitrant forms of substrates. The significant positive correlation of Actinobacteria with bulk density may indicate an adaptation to reducing levels of oxygen availability. The role of Mg in influencing Actinobacteria abundance is not clear but Mg is known to play an important role as a co-factor to several enzyme activities and is also an important nutrient.

Other bacterial groups that were influenced by tillage included species from the Verrucomicrobia and Armatimonadetes (Figure 6). Treatments under No-till had a greater abundance of Verrucomicrobia and lower abundance in Armatimonadetes compared to till. Verrucomicrobia are relatively slow growing taxa that have been shown to follow abundance pattern that follow conditions of limited nutrient availability (Ramirez et al., 2012; Fierer et al., 2013). The relative abundance of Verrucomicrobia has also been shown to decline under agricultural soils amended with nutrients (Ramirez et al., 2012; Carbonetto et al., 2014). The low abundance of Verrucomicrobia (5-6 % sequence means) in our system is in agreement with the influence of nutrient amendments but the response of their abundance to tillage practices indicates other underlying influential factors. Functionally, Verrucomicrobia have been associated with carbohydrate metabolism and degradation of more recalcitrant carbon indicating their probable role in C cycling (Fierer et al., 2013). The ecological significance of Armatimonadetes is yet to be established. Nevertheless their response to tillage practices indicates an importance in a given potential ecological function for this agro-ecological system.

The contrasting response of Verrucomicrobia and Armatimonadetes to tillage may imply differing mechanisms of adapting to the differing ecological environments that arise from tillage or the lack of tillage. Tilling is associated with more homogenous environmental conditions which indicates easier accessibility of nutrients to microorganisms and has been indicated to promote copiotrophic bacteria that are able to use readily available nutrient fractions and have high growth rate.

Reduced tillage systems on the other hand are usually characterized with spatial heterogeneity thus having pockets of readily available and non-available nutrients and may thus be able to harbor both copiotrophic and oligotrophic bacteria.

Differences at the order lineages revealed significantly greater relative abundance of Sphingobacteriales, Burkholderiales and Myxococcales which were all significantly greater under till compared to No-till. The order Burkholderiales entails several bacterial strains that are known to be plant pathogenic but a majority are also exploited for biological control of soil pathogens, plant growth promotion and/or bioremediation (Nogales et al., 2001; Coenye and Vandamme, 2003). Myxococcales are micropredators and are known to form spores in response to starvation, and physiochemical stresses (Huntley et al., 2011). We may therefore attribute the greater abundance of Sphingobacteriales, Burkholderiales and Myxococcales under till compared to No-till to their capabilities of being able to: exploit and outcompete other bacterial taxa under limited nutrient resources, prey on other microorganisms and develop survival mechanisms to starvation and stressful conditions.

To explore on the possible biotic and abiotic factors that may drive the shifts in the abundance of bacterial taxa and their association to probable ecological adaptations, regression and correlation analysis was run. The association of the abundance of microbial taxa with biotic and abiotic soil characteristics has been propositioned as an acceptable approach of being able to characterize microbial shifts into ecologically meaningful categories that can help elucidate the ecological roles of different bacterial groups (Fierer et al., 2007). This has mainly seen the differentiation of bacterial taxa within a given ecosystem into copiotrophic and oligotrophic strategic growth

categories (Fierer et al., 2007; Lauber et al., 2008; Ramirez et al., 2010; Shange et al., 2012; Carbonetto et al., 2014).

In this study, soil pH was identified as the best predictor of four of the significant phyla exhibiting a positive correlation with Acidobacteria, and Armatimonadetes; and a negative correlation with Nitrospirae and Planctomycetes. This is in agreement with other studies which have shown pH to be the biggest driver of shifts in the abundance of bacterial taxa (Lauber et al., 2008, 2009; Rousk et al., 2010). Nevertheless, soil pH has been shown to differentially influence different groups of taxa under different studies. For example, Rousk et al. (2010) recorded a significant influence of soil pH on Acidobacteria (negative correlation), and a positive correlation with Nitrospira, and Alphaproteobacteria. Lauber et al. (2009) demonstrated a significant influence of soil pH on Acidobacteria (negative correlation) and a positive correlation with Actinobacteria, and Bacteriodetes. Lauber et al. (2008) showed pH to have a significant influence on Acidobacteria and Proteobacteria having a negative and positive correlation respectively. This differential influence of soil pH indicates that soil pH may be acting as an indirect mediator of other controlling factors that drive microbial abundance.

It would be important to note that in our study, we cannot separate the influence of pH from the effect of N-fertilization. N enrichment has also been shown to have both direct and indirect effects on the abundance of soil bacteria with the indirect effects being closely associated with soil pH (Ramirez et al., 2012).

Soil pH has been shown to affect microbial community in a number of ways which can be direct or indirect. An indirect way could be through the influence of other soil chemical properties such as: nutrient availability; cationic metal solubility for example on boron, and aluminum; and

osmotic potential which are factors that would in turn influence changes in microbial community structure. Directly, soil pH may limit the metabolic functioning of different bacterial taxa based on their physiological tolerance range. From the studies mentioned above and results from our study, Acidobacteria is the only phylum that has been consistently reported as being significantly influenced by soil pH. This implies that the effect of pH is probably more direct on Acidobacteria while its effect on other bacterial taxa may be more indirect dependent on other underlying factors. This implies that the greater effect of soil pH on Acidobacteria is more likely a direct effect and vice versa on a majority of the other bacterial taxa.

Other soil properties (Table 10) that were found to be related include β -glucosidase, bulk density, and soil moisture which had an influence on three bacterial taxa each. β -glucosidase is an enzyme that catalyzes the last step of cellulose degradation. The association with β -glucosidase may therefore indicate the involvement of a given taxa to C- cycling or a need for C as an energy source. Bulk density and moisture would both have an influence on the soil-moisture and soil-aeration relationship and may thus be an indication of the sensitivity of a given taxa to aerobic/anaerobic conditions. Surprisingly, total C only had an association with one phylum while several phyla showed an association with C:N ratio and MBC:N ratio indicating an involvement with C and N cycling and sensitivity to substrate quality. It was interesting that both P and phosphodiesterase had negative correlation with several of the taxa, P having a negative correlation with Proteobacteria and Armatimonadetes with phosphodiesterase having a negative correlation with Chlorobi and Chloroflexi. This may be an indication of these taxa being less competitive in a nutrient rich environment. It was surprising that none of the bacterial taxa associated with β -glucosaminidase activity, an enzyme involved in chitin degradation. The lack

of association between bacteria and β -glucosaminidase has been postulated to indicate that this enzyme is mainly produced by fungal populations (Acosta-Martínez et al., 2010).

A correlation analysis (Figure 8) between all the soil properties and the dominant phyla revealed two main clusters of the bacterial taxa based on association with two sets of soil properties: with one set of soil properties consisting of total C and N, microbial biomass C (MBC) and N (MBN), β -glucosaminidase, and basal microbial respiration; and the second set consisting of the extractable soil nutrients P, K, Ca, and Mg, soil pH, bulk density, phosphodiesterase, β -glucosidase and C:N ratio.

The observed patterns are indicative of the proposed copiotrophic/oligotrophic hypothesis (Fierer et al., 2007). This hypothesis postulates that slow growing oligotrophic (K-strategists) microorganisms would be prevalent in soils with high amounts of recalcitrant organic matter while fast growing copiotrophic (R-strategists) microorganisms would be prevalent in nutrient rich soils having high amounts of labile nutrient fractions (Fierer et al., 2007; Dion and Nautiyal, 2008). Given the fact that agro-ecosystems are mainly characterized by nutrient enriched soil environments, it may be challenging to categorize soils within these systems into these broad based categories. Within this context and based on our observations, we would propose that soil environment characterized by greater levels of the extractable soil nutrients; the phosphorous nutrient acquisition enzyme- phosphodiesterase; and β -glucosidase activities would be characterized as copiotrophic. On the other hand, soil environment with greater levels of total C and N; microbial biomass C and N; and β -Glucosaminidase – an enzyme involved with breakdown of more recalcitrant organic materials to be characterized as oligotrophic.

Founded on the above proposition, our results indicate Bacterioidetes, Nitrospirae, Verrucomicrobia, Acidobacteria and candidate phylum WS3 that fall within a moderate to strong positive correlation with the extractable soil nutrients, phosphodiesterase and β -glucosidase as being copiotrophic. Contrastingly, Planctomycetes, Proteobacteria, Armatimonadetes, Gemmatimonadates, Chloroflexi, Firmicutes, candidate phyla WPS-2 and AD3, would then fall within the oligotrophic category. The phylum Actinobacteria seems to be an intermediary between copio/oligotrophic but more associated with the oligotrophs. However, there are some contradictory results on some of the categorization with those of several studies (Fierer et al., 2007; Ramirez et al., 2012). This may be due to differences in the land use types under which these studies were conducted.

CONCLUSIONS

After 31 years of contrasting agricultural management practices involving N-fertilization, cover cropping and different tillage options, this study reveals shifts in bacterial species diversity and composition that are and/or may be of value to agro-ecological system functions. Though it may have been expected that N-rates would have the greatest impact on microbial community shifts, several of the changes observed are noteworthy. Microbial diversity showed an increasing trend with increasing N-rate, and we observed a declining trend in diversity at the highest N-rate and a resultant significant shift in the species distribution and composition. It was interesting to note that the shifts in microbial composition at the high N-rate (101 N) were characterized by the response of bacteria adapted to environmental stress factors among them bacterial groups belonging to Planctomycetes, the acid-loving Acidobacteria, and candidate phylum WPS-2. This attests that high N levels results in stressful environment that may limit the potential of beneficial

microbial processes. On the other hand, we recorded shifts in bacterial groups known to play probable roles in nutrient cycling (C, N and S). These include groups from alpha, gamma and delta Proteobacteria, Chloroflexi, Nitrospirae and Acidobacteria with specific groups increasing or decreasing relative to increasing N-rate. For instance, Nitrospirae a nitrifying bacterium decreased with increasing N-rate implying that nitrification rates may possibly decrease as higher N-rates are applied. This may in turn imply a greater possibility of environmental pollution through nitrate leaching. This has implications for sustainable agricultural production and augments the need for strategic N-fertilization management guidelines especially within a conservation agriculture context.

Another worthwhile observation was the dominance of the Acidobacteria groups that show a positive correlation with soil pH within this ecosystem which raises questions on their ecological significance that warrants further investigations.

Shifts due to tillage revealed a response of bacteria related to environmental stress factors under till compared to No-till. This implies that No-till promotes conducive conditions that would promote beneficial ecological functions like nutrient cycling. This is indicated by the greater relative abundance of Actinomycetes under No-till that are known to play an important role in organic matter decomposition. The use of cover crops also resulted in a greater abundance of Actinomycetes differences that may have been promoted by changes in substrate quantity and quality leading to a greater need of organic matter decomposition. Surprisingly, we observed greater abundance of Rhizobiales a nitrogen fixing bacteria under No Cover in comparison to vetch and wheat. It is not clear what the ecological implications of this are under a cover crop system.

For many of the shifts observed in bacterial groups within these practices, the specific ecological significance and functionality warrants further studies to ascertain their relevance. We were able to categorize the bacterial groups within this system into possible ecological meaningful categories that provided more information on possible adaptation strategies. The majority of the bacterial taxa within this system seemed to be characterized by the oligotrophs (K-strategists) meaning that they would have relatively lower growth rates, an efficient nutrient uptake system and be able to out-compete the fast growing bacteria under low nutrient conditions given a greater affinity for substrate.

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CHAPTER III

SOILS AND CIVILIZATIONS: USING A GENERAL EDUCATION

COURSE TO TEACH AGRICULTURAL RELEVANCE

A version of this chapter was originally published by the North American Colleges and Teachers of Agriculture (NACTA) Journal in the September 2013 Special Issue:

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This article was a result of research conducted in conjunction with the general education course, "Soils and Civilizations" taught at UT by Professor Eash. Lilian W. Mbutia participated as a teaching assistant in the course, and was a co-author in analyzing and writing up results of data collected to study the course's effects on student attitudes.

Abstract

The enrollment of students to the major scientific disciplines related to agriculture has been on the decline over the past decades. While it is unclear why enrollments change, few would argue that these same disciplines have not been proactive in raising the awareness and importance of environmental disciplines towards sustainable development and the survival and stability of civilizations. Today, most students are unaware of current food production and food security issues and the career opportunities associated with our majors that are hidden inside the “College of Agriculture.” We developed a general education course that addresses relevant food security issues and outlines the sciences contained within agriculture and future opportunities for feeding future generations. The objectives of this paper were to determine how our general education course changes student perception of population, food security and civilization stability and the relationship these concepts have with environmental sustainability. We evaluated student survey responses from two semesters (n=435) of our course. Fifty-two percent of students did not know a major in soil science existed, while 56% responded that they would like to take another course in that discipline. Ninety-nine percent indicated that knowledge of soil science was important in understanding food security, with 43% indicating that their opinion of these issues changed since the beginning of the semester. The food security knowledge and expertise contained within the Agriculture College is seen by students as highly relevant to their future and suggests more forthright marketing through general education courses of our expertise and career opportunities related to these disciplines should be explored further.

Introduction

Climate change, population growth, food security and sustainable intensification are all examples of the buzz words that drive the public discourse shaping our perceptions about the role agriculture and the environment will play in future generations. While roughly 12% of the world's population does not get enough to eat, most health issues in developed countries revolve around obesity and overconsumption. Population growth is occurring in areas with less productive soils that are degraded or rapidly degrading due to unsustainable agricultural practices (Bindraben, et al., 2012). Agriculture can be a source or a sink in regards to greenhouse gases (GHG) and currently produces as much as 13% of GHG emissions (FAO, 2009; FAO, 2011; FAO, WFP and IFAD 2012; Follett, et al.; 2011).

Since 1960 when our population surpassed 3 billion people, more than 4 billion new faces have populated our planet with an increase of nearly 80 million each year. Malthus (1793) warns us about how populations crash when food production does not grow at the same rate as population. By the time our current college graduates arrive at mid-career—in just 20 years—there will be another two billion persons to clothe and feed. This represents a range of problems that will require the best minds to research and solve these pressing issues. Unfortunately, most of the current young generation has a low awareness and inaccurate perceptions with regards to the importance of agriculture (Terry and Lawver, 1995, Gonzalez, 2006). This has mainly been attributed to urbanization and lack of exposure to food production activities. Farm and rural populations have declined, with less than 5% of the U.S. population now living on farms and less than 2% of the labor force working in agriculture (Dimitri, et al., 2005), resulting in less contact by young people with agriculture. Gonzalez (2006) found most high school students either have

misconceptions about agriculture or lack knowledge about agricultural fields of study and employment opportunities.

While the National Academy of Sciences reported significant increases in the number of U.S. college graduates in agricultural and natural resources disciplines from 1987 to 2007, most of the increases were in natural resources conservation, research and animal science fields of study (2009). Several studies have also shown that the enrollment of students to disciplines related to soil and earth sciences has been on the decline since the early 1990's and 2000's (Hartemink, 2008; Collins, 2008). Unfortunately, agricultural scientists and Land Grant Universities have generally adopted a "Field of Dreams" approach to marketing our disciplines whereby we do little to entice students to explore the relevancy of our scientific disciplines to food security and civilization sustainability. In 2010, the Soil Science Society of America conducted a survey to further investigate the trends in soil science education and training (Havlin et al., 2010). One of the concerns that prompted the study was the fact that there was declining academic course offering and enrollment to soil science education programs at land grant universities, a concern also raised by Collins (2008). Havlin et al. (2010) recommended promoting soil science during earlier stages of education and opening general soil science courses up to the wider college student population as part of "general education science credits."

The National Academy of Sciences book, "Transforming Agricultural Education for a Changing World," presented an imperative to change agricultural education (National Academy of Sciences, 2009). The national research priority agenda for 2011-2015 put forth by the American Association for Agricultural Education supports this view (Doerfert, 2011). While many approaches are needed, this paper addresses one ongoing development of a curriculum to

increase knowledge of agriculture and soil science by changing fundamental perceptions about agriculture that would appeal to a broader student population. The “Soils and Civilizations” curriculum presented in this paper blends soil science and agriculture with respect to history and civilization and has success at the University of Tennessee (UT) by increasing the number of degrees pursued within the “College of Agriculture.” This class is populated by a variety of students with undeclared majors to upperclassmen in engineering and nursing.

The course fills a general education requirement at UT and has evolved and grown over the nine years of its offering to over 200 students each semester. Several approaches are used in the course and data is being collected to begin to assess the impact this course has on attitudes about agriculture and soil science. Each semester several students change majors and become students in the College of Agriculture and Natural Resources as a result of taking this course.

The course addresses some of the most important intersections of agriculture and society, including:

1. Distribution of both population and food production and their impact on food security
2. Environmental degradation and its impact on food production
3. Historical analysis of the relationship between civilization success or failure and soil conservation
4. The potential impact of climate change on food production
5. An analysis of climate change as a contemporary example of the “tragedy of the commons” (Hardin, 1968; Ostrom, 1990)

These topics provide a dynamic and cross-disciplinary subject matter that draws students into the material with issues that they can relate to on a personal level. At the outset, few students think there are environmental issues that could impact their livelihood but by semester's end there has been some movement on the educational continuum. That combined with the tragic collapse of civilizations provides a dramatic background for learning about soil science, agriculture, history and geography. For example the disappearance of the Anasazi, Sumerians and Nubians provides a rich backdrop for learning about agricultural practices and the impacts of drought, deforestation and salinization.

The objective of this approach is to:

1. Educate the student populace about agriculture
2. Make knowledge of agriculture more accessible to non-agriculture students by juxtaposing contemporary food security issues with historical collapses
3. Show the importance of agriculture in addressing today's pressing issues, such as food security and climate change
4. Show the relationship between agriculture and natural resource conservation to the rise and fall of civilizations
5. Entice students to learn more about agriculture and soil science with follow-up courses and possible pursuit of a major or career in agriculture and soil science.

Materials and Methods

The course “Soils and Civilizations” was developed nine years ago at the University of Tennessee and has been taught 14 times. The class in spring 2013 had 188 students with 233 registered for Fall 2013. For the past five years enrollment has been capped by the seating capacity of the chosen classroom; in 2013 this course is held in the largest lecture hall on campus. The approach involves presenting interesting historical stories combined with science, problems and solutions and engaging and challenging students.

There is no way to precisely measure the impact of a curriculum on students, as ideas and concepts can be presented and discussed that students may not grasp until later in their academic career. However, this paper is an attempt to quantify more immediate change in perception and attitude. During the 2012 fall semester a survey was conducted at the end of the course to characterize attitudes towards agriculture, climate change and soil science and to determine if the course had an impact on their opinions. The survey response rate was 62% (84 of 135 students). Tables 1 and 2 list the survey questions given to students at the end of the fall 2012 semester and the overall response of the students to the questions based on a Likert scale of importance (Table 1) and scale of agreement to several statements (Table 2). For the spring 2013 semester, surveys were conducted at the beginning and end of the semester to capture the actual change in student perceptions to various topics within the period of the course and to gauge how significant this course is towards enhancing perceptions about the importance of soils and agriculture to development and food security. Questions were modified and student responses are compared between the beginning and end of the semester for scale of importance questions (Table 3) and scale of agreement statements (Table 4).

Results and Discussion

Thirteen percent of respondents in the fall 2012 survey indicated they were freshmen, 34% sophomores, 27% juniors and 26% seniors, with 56% male and 44% female. Based on the responses to the survey in Tables 1 and 2, we are able to make several noteworthy observations. Most of the students signified recognition of the connection between soils, agriculture and food security with 99% of respondents indicating that the class was somewhat or extremely important for understanding why soil is important to food security. Sixty-eight percent indicated it was extremely important for them to understand food security. Seventy-six percent indicated it was extremely important to understand soil resources to avoid environmental catastrophe. Forty percent of survey respondents agreed that their understanding of the topics covered in this course changed since the beginning of this class, while an additional 43% strongly agreed that their understanding of the topics covered in this course changed since the beginning of this class.

Response to the survey also suggests that this course could have an impact on students actually considering a career in soil science. While 52% indicated that soil science was an unknown discipline to them before the course, the survey shows a change in awareness with 56% agreeing or strongly agreeing that they would like to take another class in soil science. Interestingly, 13% agreed that if they had taken the course earlier in their academic career, they might have changed their major to soil science, while an additional 5% strongly agreed they might have changed their major.

The spring semester began with 193 students registered and 181 completed the course. During this session, 175 students took the survey at the beginning of the semester and 176 students

completed the survey at the end. Twenty-nine percent of respondents taking the spring 2013 survey indicated they were freshmen, 21% sophomores, 26% juniors and 23% seniors, with 60% male and 40% female. Forty-six percent indicated they grew up in the suburbs, 16% in the city, 25% in rural areas and 13% on farm. The most significant change in responses by students to survey statements at the end of the course was an increase in the mean from 2.9, where 3 was “No opinion” to 4.1, with 4, being “Agree” in response to the statement, “I have a good understanding of sustainable agriculture.” Another notable change was an increase in the mean from 3.2 to 3.8 in response to the statement, “I think that all students should be required to take a class in agriculture or soil science” and from 3.5 to 4.3 in response to the statement that “The information provided in this course is important for all UT students.” By the end of the course students indicated that the !Kung Bushmen were an example of a sustainable civilization (Figure 1) and our US civilization is similar to most civilizations studied that have disappeared. While the news politicizes climate change issues, students found climate change to be a fact.

Table 11: Student responses at the end of 2012 Fall Semester using a Likert scale based are questions/statements asked with answers on a scale of importance

				Scale of Importance									
				Extremely		Somewhat		No Opinion		Not Very		Not At All	
#	Questions/Statements	Mean	SD	# of 5's	% of 5's	# of 4's	% of 4's	# of 3's	% of 3's	# of 2's	% of 2's	# of 1's	% of 1's
1	The topics covered in this course	4.4	0.59	40	48%	42	50%	1	1%	1	1%	0	0%
2	This class is important for understanding why soil is important to food security	4.7	0.49	60	71%	23	27%	1	1%	0	0%	0	0%
3	It is important to understand intrinsic soil productivity and its link to sustainability	4.5	0.59	44	52%	36	43%	4	5%	0	0%	0	0%
4	How important would it be for you to take a student travel course to further understand food security?	3.4	1.10	14	17%	30	36%	22	26%	14	17%	4	5%
5	How important is it to understand the downfall of the Maya	4.0	0.75	17	20%	51	61%	13	15%	2	2%	1	1%
6	How important is it to understand the downfall of the Greenland Norse?	3.8	0.78	14	17%	49	58%	15	18%	6	7%	0	0%
7	How important is it to understand the role of energy in our lifestyle?	4.8	0.45	66	79%	17	20%	1	1%	0	0%	0	0%
8	How important were the oral readings in lecture?	3.4	1.02	5	6%	45	54%	14	17%	16	19%	4	5%
9	How important is it to you to understand food security?	4.6	0.70	57	68%	20	24%	4	5%	2	2%	0	0%
10	If you were forced to emigrate, how important would it be to evaluate the soils before hand?	4.3	0.82	43	51%	26	31%	13	15%	2	2%	0	0%
11	Understanding soil resources to avoid environmental catastrophe?	4.8	0.46	64	76%	19	23%	1	1%	0	0%	0	0%

Table 12: Student responses at the end of 2012 Fall Semester using a Likert scale based are questions/statements asked with answers on a scale of importance

				Scale of Agreement									
				Strongly Agree		Agree		No Opinion		Disagree		Strongly Disagree	
	Statements	Mean	SD	# of 5's	% of 5's	# of 4's	% of 4's	# of 3's	% of 3's	# of 2's	% of 2's	# of 1's	% of 1's
12	This class has changed my understanding of how we feed ourselves	4.0	0.84	22	26%	45	54%	12	14%	4	5%	1	1%
13	Climate Change is a fact	4.6	0.60	56	67%	23	27%	5	6%	0	0%	0	0%
14	We collectively need to understand the effects of humans on our changing climate	4.6	0.60	57	68%	24	29%	2	2%	1	1%	0	0%
15	The information provided in this course is important for all UT students	4.1	0.96	32	38%	39	46%	7	8%	3	4%	3	4%
16	My understanding of the topics covered in this course has changed since the beginning of this class	4.2	0.85	36	43%	34	40%	11	13%	2	2%	1	1%
17	This class has taught me that understanding population growth is important to understanding our future	4.4	0.72	46	55%	29	35%	8	10%	1	1%	0	0%
18	If I had taken this course earlier in my academic career, I might have changed my major to soil science	2.5	1.09	4	5%	11	13%	24	29%	29	35%	16	19%
19	I would like to take another course in soil science	3.6	1.04	17	20%	30	36%	25	30%	9	11%	3	4%
20	The oral readings in class wasted limited class time	2.5	0.98	2	2%	9	11%	32	38%	25	30%	14	17%
21	If I knew I could make a living as a soil scientist I would become one	2.7	1.14	5	6%	15	18%	27	32%	22	26%	15	18%
22	There is more fiction than fact in this course	1.8	1.01	3	4%	3	4%	8	10%	28	33%	42	50%
23	The Bushmen are an example of a sustainable civilization	3.5	1.19	19	23%	30	36%	14	17%	17	20%	4	5%
24	We—the Americans—are an example of a sustainable civilization	2.0	1.11	1	1%	11	13%	12	14%	23	27%	36	43%

Table 12 continued:

				Scale of Agreement									
				Strongly Agree		Agree		No Opinion		Disagree		Strongly Disagree	
25	Global Warming is a fact and due to human activity	3.7	1.04	20	24%	35	42%	20	24%	5	6%	4	5%
26	Soil science was an unknown discipline to me until I took this course!	3.2	1.45	22	26%	22	26%	6	7%	22	26%	12	14%

Table 13: Comparison of the mean responses to survey questions at the start and end of 2013 Spring Semester to questions based on the scale of importance shown in table 1

#	Questions	Beginning Survey Mean	Ending Survey Mean	Difference
1	How important were the topics covered in this course to you?	4.0	4.2	0.21
2	How important is a course on soils for understanding food security?	4.5	4.8	0.28
3	How important is it to understand intrinsic soil productivity and its link to sustainability?	4.1	4.6	0.42
4	How important would it be for you to take a student travel course to further understand food security?	3.3	3.3	-0.02
5	How important is it to understand the downfall of the Maya?	3.6	3.7	0.10
6	How important is it to understand the downfall of the Greenland Norse?	3.5	3.6	0.11
7	How important is it to understand the role of energy in our lifestyle?	4.6	4.8	0.17
8	How important is it to understand the role of agriculture in climate change?	4.5	4.7	0.17
9	How important is it to you to understand food security?	4.2	4.6	0.42
10	If you were forced to emigrate, how important would it be to evaluate the soils beforehand?	3.8	4.4	0.59
11	Understanding soil resources to avoid environmental catastrophe?	4.5	4.8	0.32

Table 14: Comparison of the mean responses to survey statements at the start and end of 2013 Spring Semester to statements based on the scale of agreement shown in Table 2

#	Statements	Beginning Survey Mean	Ending Survey Mean	Difference
12	I understand how we feed ourselves	3.9	4.2	0.28
13	Climate Change is a fact	4.1	4.8	0.75
14	We collectively need to understand the effects of humans on our changing climate	4.6	4.6	-0.01
15	The information provided in this course is important for all UT students	3.6	4.3	0.78
16	I have good understanding of sustainable agriculture.	3.0	4.1	1.12
17	I think population growth is important to understanding our future.	4.2	4.5	0.35
18	I would like to take another course in soil science	3.3	3.6	0.34
19	I would like to take more agriculture related classes.	3.7	3.8	0.11
20	If I knew I could make a living as a soil scientist I would become one	2.7	2.8	0.06
21	I believe technology can solve all of our problems	2.5	2.6	0.10
22	The Bushmen are an example of a sustainable civilization	3.1	3.9	0.83
23	We—the Americans—are an example of a sustainable civilization	3.0	2.9	-0.09
24	Global Warming is a fact and due to human activity	3.4	3.8	0.32
25	Soil science is an unknown discipline to me	3.4	2.3	-1.10
26	Today more countries have programs on fighting obesity than hunger	3.1	3.4	0.22
27	Climate change is a new phenomenon	2.3	1.8	-0.45
28	Sustainable energy use is an issue that should be addressed	4.2	4.5	0.27
29	Soils have little impact on food security	1.7	1.6	-0.17
30	"Civilizations" are "sustainable"	3.0	2.8	-0.17
31	I think that all students should be required to take a class in agriculture or soil science	3.3	3.9	0.59
32	I think that government has an important role in protecting natural resources	3.8	4.3	0.47

But perhaps more importantly for those of us employed within the Land Grant University System, the survey results suggested that students gained a better understanding of food production and how population growth can cause civilization demise. Student perceptions moved toward the understanding that few of our current civilizations are truly sustainable with sustainable energy use as just one issue that needs to be addressed.

Summary

Based on the responses of this survey, there is a strong indication that this course has an influence/impact on the attitudes of students towards soil, agriculture and their relation to food security and sustainability. Registration for the fall 2013 semester increased 17% to a total of 233 students. Surveys will be used to continue measurements and other methods will be explored to quantify the impact of this course on enrollment to soil science courses.

We think an introductory class is necessary to explain agriculture's role in civilization, subsequent civilization stability and solving global agricultural and food security problems. Quite simply, this course outlines the mission of the Land Grant Universities, a mission that can only be completed if we strive to enlist the best minds to work in agricultural sciences. Our future may depend on our success at marketing our disciplines to future generations and this course is a tool to do so.

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CONCLUSIONS

This study focused on investigating the effects of CA management practices on microbial community shifts and how they interrelate with changes in soil properties and functions that are essential to agro-ecosystem sustainability. While the changes on microbial community structure and activities associated with these practices have been reported in several other studies, this study was unique in that: 1) it was based on long-term experimental plots and therefore provided a basis for relating changes in soil properties that take longer to respond to management practices and how these properties relate to shifts in the microbial community and: 2) the unique experimental set-up, also provided a basis of investigating the long-term results of the interacting effects of the different management practices.

On the basis of literature, the hypotheses of this study were that: 1) an increase in the abundance of both bacteria and fungi would be observed in soils under No-till and cover crop management practices compared to till and treatments without cover crops; 2) the increase in the abundance of these microbial groups (bacteria and fungi) under these management practices (No-till and cover crops) would also result in a greater proportion of fungi over bacteria in comparison to the till and No Cover treatments; 3) the shifts in microbial community would result in greater microbial activity, improved soil properties and in turn lead to greater nutrient cycling and retention capacity under CA practices.

While some of the above mentioned expectations were observed, for example significantly greater abundance of bacterial and mycorrhiza fungi fatty acids (FA's) biomarkers and enzyme activities greater extractable nutrients (P, K, and Ca), total C and N, in No-till treatments

compared to till. This conclusion section highlights some of the noteworthy results and their possible implications.

The importance of including cover crops in a reduced tillage system especially under a continuous mono cropping system of low biomass crops like cotton was apparent with significantly greater total C and N and yield mostly recorded in the combination of No-till with either vetch or wheat cover. Moreover, the unique properties of using leguminous cover crops were also clear. Treatments under vetch cover had similar levels of total C and N as well as yield at all the different fertilization rates. This was most likely a result of greater nutrient cycling capacity in No-till treatments having the vetch cover. This is corroborated by greater microbial biomass N, microbial respiration and β -glucosaminidase activity under the vetch No-till treatments compared to all other treatments. This has important implications for agricultural production systems that are aiming for higher productivity but at the same time focused towards environmental sustainability.

While treatments under the high N-rates had significantly greater total C and N as well as yields, soils under these treatments were on the other hand characterized by significantly less soil extractable nutrients, and low pH. The bacterial species composition in these soils also revealed a response of greater relative abundance of bacterial groups associated with extreme environmental factors like chemical pollution and acidic environments. The use of high N-rates would not only mean an increase in production costs from fertilizer purchase and need for liming, but would also more than likely lead to environmental degradation. In line with the results showing the possibilities of using a leguminous cover crop without N-fertilization, this study ascertains the possibilities of developing strategic management practices involving No-till,

different cover crop species and minimal N-fertilization as optional sustainable agricultural management practices.

Another highlight from this study was on the use of the soil assessment framework (SMAF) quality index tool. This revealed that while the availability of extractable soil nutrients (P and K) were not limiting to production, the limiting factors were mainly total C and N levels as well as the functional potential of the microbial community. It is not surprising that the soil nutrients were not a limiting factor given that agricultural production is characterized by constant inputs of fertilizers to maintain productivity. The soil quality tool further highlighted the need of management practices geared towards increasing the total C and N in these soils and ascertained that No-till and cover crops were some of the potential options, validating the use of CA principals for achieving this. Nevertheless, the overall soil quality index did not show significant differences between the different practices with the score being barely above average of the maximum score expected. Given 31 yrs. under CA management practices, the results of the soil quality index may point to the need of including other optional management practices that would possibly enhance the buildup of soil organic matter in these soils. This may include incorporating crop rotations with high residue crops that have been shown to contribute to greater soil C sequestration.

The results on the fungi to bacteria ratio (F: B ratio) from this study were surprising and contrary to the proposition that fungi will be dominant under reduced tillage systems especially where greater organic C are recorded. While the mycorrhiza relative abundance was greater under No-till, the relative abundance of the saprophytic fungal biomarkers was greater under till leading to a greater F: B under till. This implies that not all fungi species respond in a similar manner to

management practices. The significance of the greater abundance of fungi under till compared to No-till in these soils needs further evaluation using different techniques that would ascertain these findings. One way of determining the importance of fungi and bacteria to carbon mineralization would be to employ methods that specifically inhibit bacteria or fungi and measuring the respiration and biomass production contributed by the non-inhibited group. More mycorrhiza would be expected in the rhizosphere soil and in the top depth within the rooting zone. It is interesting that based on the bulk soil sampling; the treatment effects on mycorrhiza abundance still captured the expected differences in the bulk soil. It would be worthwhile to do an analysis of the rhizosphere soil. This could be a research question to look at the mycorrhiza abundance based on a rhizosphere effect.

The use of high throughput 16S rRNA gene sequencing revealed significant differences in bacterial species community composition that point to differences in the functionality of the microbial community related to soil nutrient cycling as well as adaptation to environmental stresses. For example the greater relative abundance of Actinomycetes under No-till and cover crops corroborates their role in organic matter decomposition. On the other hand till treatments were characterized by a greater abundance of Sphingobacteriales, Burkholderiales and Myxococcales, bacterial groups associated with adaptation to low nutrient conditions and environmental stress factors.

Some differences in the capabilities of the two methods (FAME analysis and 16S sequencing) used to characterize microbial communities as discussed in the literature section were reflected in this research. FAME data showed Actinomycetes FA biomarkers to be greater under No-till compared to till. The 16S data set also showed Actinomycetes to be greater under No-till

compared to till. This indicates some level of agreement between the FAME data and 16S data. Nevertheless, considering the species within Proteobacteria that are known to be gram positive, the results between the FAME and 16S data set does not fully tally. Based on the FAME data set, Gram positive bacteria were greater in abundance under No-till relative to till treatments with the vetch treatments also having a greater abundance of Gram + bacteria relative to wheat and No-cover. On the other hand, the 16S data set showed species belonging to Proteobacteria to be greater under till relative to No-till while one species within the Proteobacteria was greater under vetch which is in agreement with the FAME data. These differences between the FAME and 16S data are reflective of the differences of the methodological approach taken to classify microbial species. While FAME analysis represents a pooling of bacterial species based on a phenotypic characterization while 16S separates out the species based on the genotypic composition. On this basis, the 16S data sets thus gives a provision for an in-depth analysis of individual species that were clustered together based on one biomarker under FAME analysis. Therefore, it would be difficult to do a fair comparison of the two data sets.

While the information revealed by the 16S rRNA gene environmental sequencing are more exploratory than conclusive, the significant trends observed from these analyses would serve as a basis for the development of more specific oriented experiments geared towards gaining a better understanding of the agro-ecological significance of the observed shifts in the relative abundance of specific bacterial groups. This could be achieved by the use of the experimental methods such as soil meta-genomics and transcriptomics which would further elucidate the specific functions of these microbial groups.

VITA

Lilian Wanjiru Mbutia was born and raised in a farming community in Kenya. She got a bachelor's of science degree in Horticulture from Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya. She worked for two years after graduating in an agrochemical company in Kenya after which she decided to pursue further education and got a master's of science degree in International Horticulture from Leibniz Universität Hannover, Germany. She then came to the University of Tennessee where she has been pursuing her PhD in Plants, Soils and Insects with a concentration in Environmental Soil Science. Lilian plans to return back to her country where she hopes to be an agent of change advocating sustainable farming practices in Africa.