THE EFFECTS OF LONG TERM AGRICULTURAL PRODUCTION ON SOIL MICROBIAL DIVERSITY

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ABSTRACT

Soil microorganisms are important facilitators of ecosystem functions including: crop and pesticide residue decomposition, carbon sequestration, Nitrogen (N) fixation and nutrient cycling. Therefore, the abundance and diversity of soil microorganisms may act as a buffer against stress and change, ensuring that soils remain productive. The Rotation ABC Long term soil experiment (LTSE) at Lethbridge, Alberta provides an opportunity to use modern molecular tools to study microbial dynamics in response to over a century of agricultural land management. My goals were to assess cumulative effects of N fertilizer and different cropping intensities (CI) on soil microbial community abundance and diversity, both in soils collected in 2012 and in the soils archived over 100 years of wheat production. Soil microbial community abundance and diversity of soils collected in 2012 were influenced by N fertilization and CI. Phospholipid fatty acid (PLFA) analysis of the 2012 soils revealed a positive relationship between N fertilizer application and CI on the total active biomass. Quantitative PCR analysis showed that N functional gene abundances were affected by significant interactions between N fertilizer and CI, and the abundances of denitrifier genes (*nosZ* and *nirK*) were also strongly influenced by higher levels of CI in the 2012 soils. A short term soil storage experiment indicated that storage time and fertilizer treatment (45 kg N ha^{-1}, 20 kg P ha^{-1} vs. unfertilized) influenced the amount of DNA extracted. In addition, the results suggested that N and P fertilized soils had greater bacterial diversity than unfertilized soils. Through the use of 16S rRNA gene profiling with these soils the results suggested that bacterial diversity and richness was lowest in the oldest samples, implying a shift over decades of agricultural production. Soils collected after the introduction of N fertilizer displayed an increase in N function gene abundances, indicating an increase in N cycling potential. Overtime, the changes in agricultural land management led to increased plant

and soil productivity, resulting in a positive relationship between years under agricultural land management and soil bacterial diversity and abundance.

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LIST OF ABBREVIATIONS

A45/AN	Rotation A with 45 kg N per ha^{-1}
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
AOA	ammonia oxidizing archaea
AOB	ammonia oxidizing bacteria
C45/CN	Rotation C with 45 kg N per ha^{-1}
C_1	Rotation C first year of WWF
C_2	Rotation C second year of WWF
C_3	Rotation C third year of WWF
CI	cropping intensity
DNA	deoxyribonucleic acid
GHG	greenhouse gas
Gr+	Gram positive
Gr-	Gram negative
ISA	Indicator Species Analysis
IV	Indicator value
LTSE	long term soil experiment
MAP	mean annual precipitation
MAT	mean annual temperature
MDS	multidimensional scaling
N	Nitrogen
nirK	nitrite reductase
nirS	cytochrome cd1 dependent nitrite reductase
nosZ	nitrous oxide reductase
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PLFA	phospholipid fatty acid
QPCR	quantitative polymerase chain reaction
rRNA	ribosomal ribonucleic acid
RT	room temperature
16S	16S ribosomal RNA
S_{obs}	single observed species
WWF	wheat wheat fallow rotation sequence for Rotation C

1.0 General Introduction

1.1. Introduction

Nitrogen (N) composes 78% of earth's atmosphere and is an essential molecular component of amino and nucleic acids which are the building blocks of terrestrial life. In agricultural soils, N is significant because it is a limiting factor to plant growth and is also the only essential plant nutrient that is not released from the weathering of soil minerals (Schulten and Schnitzer, 1997). The addition of N is well known to increase crop productivity and in turn increase plant residues. Soil microbial communities contribute to decompose proteins, peptides and amino acids from plant and animal residues (Sowden et al., 1977), and mediate the biogeochemical processes regulating C and N cycles, therefore influencing plant productivity and global climate (Griffiths and Philippot, 2013). The biodiversity of soil microbial communities provides resilience to changes in agronomic and environmental conditions and prevents loss of important soil functions (Rousk et al., 2009).

Crop rotation also has an influence on soil microbes, in part because of the influence of plant residue inputs (Phillips et al., 2015). Through microbial decomposition, both above and below ground plant residues become C energy substrates that shape microbial community structure and diversity (Freschet et al., 2013). Soils with reduced cropping intensity (CI) or fallow undergo changes in the abundance and structure of their soil microbial communities because of the lack of plant inputs during in this non-cropped time period (Acosta-Martínez et al., 2007). Traditionally the use of fallow in crop rotation is used to retain moisture and soil N (O'Dea et al., 2015), however these benefits do not outweigh the drawbacks with respect to plant inputs. The reduction of plant inputs during fallow reduces the amount of C substrates available for microbial growth and drives change in the soil microbial community structure over time

(Paterson et al., 2011). Carbon from growing plants has a different composition than older soil C, and therefore promotes the growth of different microbial groups (Kemmitt et al., 2008). In addition, variability in litter inputs between different crop rotations is known to alter biological processes (O'Dea et al., 2015). Different crops have different C:N ratios and the C:N ratio of everything on and in the soil can affect crop residue decomposition and nutrient cycling (USDA, 2011). The land use history of a soil impacts a soil microbial community's ability to degrade other residues (Griffiths and Philippot, 2013). In contrast to fallow, decreased tillage and fertilizer usage are practices associated with increased microbial biomass, which in turn improves soil quality and fertility (Mbuthia et al., 2015).

Soils may take decades after a change in land management to resume a steady state (Janzen, 1995). The Rotation ABC Long term soil experiment (LTSE) at Lethbridge, Alberta provides an opportunity to study microbial dynamics in response to 100 years of wheat crop production and 45 years of N fertilizer implementation. Although many studies have characterized soil properties of Rotation ABC (Janzen, 1995; Smith et al., 2012), none have used modern biochemical and molecular biology tools to investigate the microbial response to long term land management. Use of DNA and PLFA based methods provide culture independent means to study soil microbial diversity. While culture based methods are valuable, they may not be as representative as culture independent approaches owing to the fact that only 1% of bacteria can be grown in culture (Kirk et al., 2004).

The overall goal of this project was to characterize the active and total soil bacteria community of Rotations A and C, with the aim learning more about the role of microbial abundance and diversity in key biogeochemical processes affecting the productivity and sustainability of these agroecosystem soils. The step wise changes in soil microbial diversity and

community structure over decades of changing agronomic practices were studied through the nucleic acids extracted from soil archives. The cumulative changes to soil microbial communities evident after 100 years of wheat crop production were determined using fresh soils collected in 2012. These goals were accomplished by optimizing DNA extraction from archived soils, performing 16S rRNA gene targeted amplicon sequencing, quantitative PCR (qPCR) to estimated genetic potential for nitrification and denitrification and PLFA extractions to determine differences in broad level community structure of living biomass in soils with different treatment histories.

1.2. Organization of the Dissertation

The research presented in this dissertation is organized in manuscript format. Following this introduction and the literature review presented in Chapter 2, three studies are presented in Chapters 3 and 4. These research chapters are organized around the LTSE evolution, with Chapter 3 investigating the whole cumulative change over 100 years of wheat production, and Chapter 4 examining stepwise shifts in the soil microbial community over time in response to management changes using archived soils. The goal of the research presented in Chapter 3 was to compare the microbial biomass of soil microbial communities under continuous wheat monocropping to the 2nd year in a 3 year wheat-wheat-fallow (W<u>W</u>F) cycle, as measured with PLFA biomarkers. In Chapter 3 the hypotheses were 1) that soil microbial communities under continuous cropping (100% CI) would have larger biomass than those with W<u>W</u>F management (67% CI) management, and 2) that soil bacteria and archaeal community structures and N functional groups will become more abundant and less diverse over time due to the influence of N fertilizer. Chapter 4 furthers the work of the previous chapter with a similar objective of determining the relationship between soil bacterial and archaeal communities and N functional

group abundance with increased N fertilizer treatments and continuous wheat cropping over decades. In Chapter 4, the hypotheses were 1) soil bacterial populations would lose genetic diversity over decades of wheat monocropping and 2) the DNA yield and quality would decrease over time in air dried soils stored at RT.

2.0 LITERATURE SURVEY

2.1 Importance of biodiversity for agroecosystem sustainability

In agriculture, biodiversity contributes to the productive capacity and resilience of an agroecosystem (Kennedy and Smith, 1995; Giller et al., 1997; Torsvik and Øvreås, 2002). It is important to increase the working knowledge of the relationships between agricultural management practices, biodiversity and ecosystem services in order to develop more sustainable farming practices to feed an ever increasing global population (Wood et al., 2015). Biodiversity allows soil functions to occur even during periods of stress or changes to the ecosystem because multiple organisms are able to complete the same ecosystem tasks, and with different environmental tolerances (Bhatia, 2008; Rousk et al., 2009). Biodiversity is influenced by quantity and quality of amendments, human activities, climate, plant host and soil types (Wu et al., 2008). In addition to soil microbes, the biodiversity in an agroecosystem is comprised of the different types and cultivars of crops in rotation, the types and breeds of animals raised, the weeds and the wildlife present (Jackson et al., 2007; Frison et al., 2011).

Soil microorganisms are part of the biodiversity of an agroecosystem and perform a range of ecosystem services including decomposition of crop and pesticide residues, carbon sequestration, and N fixation, therefore regenerating minerals that limit plant productivity (Venter et al., 2016). Furthermore, bacteria in agricultural soils are potential agents of bioremediation, biocontrol, and plant growth promotion (Fox, 2003; Lupwayi et al., 1998; Top, 2003). The reduction of soil microbial diversity as a result of a land management change is a potential loss of these functional traits and biological products. In addition, losses of biodiversity impact the sustainability of an agroecosystem because of the potential for increased reliance on fertilizer inputs to increase crop yields (Giller, 1997; Wood et al., 2015; Trivedi et al., 2016). Thi

increased reliance on inputs results in the intensification of agriculture which in turn increases costs to both the producer and the environment (Tilman et al., 2001).

2.2 LTSEs and soil archives

A long-term soil experiment (LTSE) becomes a historical record of microbial community adaptation in an evolving agroecosystem. A time frame of greater than ten years is more suitable for observing microbial adaptation in soils under agricultural crop production (Janzen, 1995). When observations are made over a shorter term, functional redundancy can make up for changes to a soil microbial community caused by changes in soil environmental conditions (Rousk et al., 2009). In studies that compared contrasting treatments in similar soils, the full impacts on soil microbial community structure were best observed over a longer time frame (Clark and Hirsch, 2008). Due in part to the technology available at the start of many historical experiments, soil samples associated with LTSEs were stored air dried at room temperature (Clark and Hirsch, 2008).

Many of the global LTSEs maintain collections of archived soil samples including TAGA in the Netherlands, Broadbalk Wheat Experiment in the U. K., Sanborn Field in the U.S. and Breton Plots and Rotation ABC in Canada. The maintenance of soil archives allows samples previously collected to be re-investigated, and help answer questions about long term effects of land management changes on soil microbial communities (Dolfing et al., 2004; Cary and Fierer , 2014). Archived soils also provide the baseline for the assessment of events such as disease, pollution and climate change. The TAGA long term study in the Netherlands has been used for public health purposes to monitor the levels of antibiotic resistance genes from 1940 to 2008 (Knapp et al., 2009). Similarly, the Broadbalk Wheat Experiment is a LTSE in Rothamsted, UK that was founded in 1843 and used to study impacts of N application types and rates on the soil

microbial diversity (Clark and Hirsch, 2008). Sanborn field was initiated in 1888 to study the impact of crop rotation and manure on grain crop production (Rasmussen et al., 1998). The Breton Classical Plots were established in 1930, with the original intent to find a system of farming suitable for a wooded soil belt (Izaurralde et al., 1996). Recent publications describing such historical experiments have demonstrated that by using DNA based methods, the effects of agricultural practices on total bacterial diversity can be studied in archived soils (Clark and Hirsch, 2008; Dolfing et al., 2004; Knapp et al., 2009). Specifically, the DNA obtained from archived soils can be used to characterize molecular targets and N functional groups (Clark and Hirsch, 2008). Rotation ABC at Lethbridge Research Center was established in 1911 to assess the effects of crop rotation on soil quality. Like most LTSEs, Rotation ABC has evolved over time to reflect technological advances in crop production (Janzen, 1995; Smith et al., 2012) and has applied N and P fertilizer treatments since 1967 and 1972 respectively.

2.3 Effect of crop production on soil microbial abundance and diversity

Crop production is a component of agriculture that involves the growth of plants for food and fiber. Crop rotation is the agricultural management practice of growing a sequence of plant species on the same land (Yates, 1954). In monoculture cropping, the same plant species is grown repeatedly on the same land (Power, 1990). It was observed that above ground diversity influences below ground diversity (Giller, 1997) and this relationship was demonstrated in many recent studies where increased above ground diversity, such as crop rotations with alternating crop types, were reported to have increased soil microbial diversity, bacterial biomass (Acosta-Martinez et al., 2008; Venter et al., 2016) and crop yield (Di Falco , 2012) over a low diversity system such as monoculture cropping. Acosta-Martinez et al. (2008) showed that a rotation of cotton-wheat had a significantly greater bacterial biomass and was 37% more diverse than a

cotton monoculture in the same soil type. Shifting from monoculture to a crop rotation of alternating crop types results in varied plant residues because of differences in root lengths, root exudates, plant litter and N and P mineralization (Kennedy, 1999; Soon and Arshad, 2002; Wardle et al., 2004; Costa et al., 2006) which can impact the soil microbial community. For example, *Brassica sp.* and C3 grasses such as *Triticum sp*. in a rotation were reported to promote disease-suppressive bacteria (Mazzola et al., 2004; Hollister et al., 2013). In contrast, legumes, in addition to the significant effects on their N fixing symbiont partners *Rhizobium sp.*, are associated with reduction in disease suppressive bacteria (Latz et al., 2012; Latz et al., 2015).

Cropping intensity (CI) describes the amount of fallow in a cropping sequence, for example wheat-fallow is 50% CI, wheat-wheat-fallow 67% and 100% occurs when there is no fallow time and a crop is harvested each growing season. Cropping intensity is a driver of microbial biomass, with higher microbial biomass reported at 100% cropping intensity (CI), as compared to 50% CI (Acosta Martinez et al., 2007). Lower CI decreased soil microbial abundance and diversity because of a lack of C inputs during this non cropped time period (Zelles et al., 1992; Lupwayi et al., 1998). Traditionally, the purpose of fallow was to conserve soil moisture; however the practice was also correlated with losses in C from soils (Janzen, 1995; Acosta Martinez et al., 2007). It is likely that resource availability has more impact on soil microbial community structure than the type of fallow used (chemical or tillage) (Helgason et al., 2010).

2.4 Effect of N fertilizers on microbial communities

2.4.1 Nitrifiers

Nitrogen is a macronutrient that is pivotal to global biogeochemical cycles. As a macronutrient, N is a growth limiting factor in crop production, and therefore the biodiversity of

soil organisms involved in the N cycle are important topics of study (Top, 2003). Soil N pools are comprised of inorganic and organic forms. A classic view point is that plants compete with soil microbes for inorganic N (NH_4^+, NO_3^-), and plants do not directly take up much organic N (Date, 1972). Immobilization is the action of microbial uptake of inorganic N, and therefore makes N inaccessible to plants. The inorganic N is resulting from microbial decomposition (mineralization) of organic N containing compounds such as amino acids, amino sugars, peptides, proteins, and chitin (Harmsen and van Schreven, 1955). More recently, N cycling is viewed as driven by microbial and mycorrhizal extracellular enzyme depolymerisation of N containing polymers (Schimel and Bennet, 2004). In low N environments, plants and microbes compete for available N (Hodge et al., 2000). Nitrification can occur in high N environments with enough excess NH_4^+ to support both plant and microbial populations (Schimel and Bennet, 2004).

The first step in the nitrification process is ammonia oxidation, catalyzed by ammonia monooxygenase (*amoA*) part of a diverse group of copper containing membrane associated monoxygenase enzymes (Hatzenpichler, 2012). Ammonia oxidation is influenced by oxygen levels, and the availability of ammonium (Wang et al., 2009). The *amoA* enzyme is produced by both ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) though they are phylogenetically distant with different cell size and structure (Prosser and Nicol, 2008; Schauss et al., 2009). Ammonia monooxygenase (AMO) converts NH_4^+ to hydroxylamine which is then converted by hydroxylamine oxidase to nitrite. Ammonia oxidation is considered the rate limiting step in this two-step conversion of NH_4^+ to NO_2^- because ammonia oxidizers lack ecological redundancy (Top, 2003). Many studies have determined that AOA are more abundant while AOB are more active, because of higher rates of *amo*A per cell that are not correlated with

increased activity (Schauss et al., 2009; Nicol et al., 2008; Tourna et al., 2008). For example, a study by Di et al. (2009) found that though AOA were abundant, an increase in ammonia substrate did not increase their activity. The higher numbers of AOA may suggest that they are not associated with nitrification (Di et al., 2009) or possibly that AOA provide resilience under differing conditions (Schauss et al., 2009).

Soil environmental conditions of temperature, pH and soil management practices influence ammonia oxidation (Braker and Conrad, 2010; Wang et al., 2009). The pH has an impact because of speciation which changes the chemical form and availability of substrates. Prosser and Nicol (2008) found that increases in pH decreased AOA abundance, while lower pH decreased AOB abundance. Differences in soil environments create habitat niches, as demonstrated by AOA which are adapted to low nutrient conditions and AOB which have adapted to be competitive in high nutrient conditions (Schleper and Nicol, 2010).

The second step in nitrification is nitrite oxidation. Nitrite oxidizing bacteria produce Nitrite oxidoreductase to convert nitrite (NO_2^-) to nitrate (NO_3^-). Nitrite oxidizing bacteria are more functionally redundant than ammonia oxidizers and their activity is linked to ammonia oxidizers as a source of substrate (Ward and Bouskill, 2011). Nitrification has been linked to direct emissions of N_2O (Bateman and Baggs, 2005). In some nitrite oxidizers the reaction of NO_2^- to NO_3^- is reversible, causing denitrification and a loss of plant available N.

2.4.2 Denitrifiers

Denitrification is a part of the N cycle that allows for microbial respiration in low oxygen conditions (Clark et al., 2012) however this function has a cost, resulting in losses of N (Wallenstein, et al., 2006). There are four steps during bacterial denitrification: nitrate reduction,

nitrite reduction, nitric oxide reduction and nitrous oxide reduction (Philippot et al., 2007). Abundance and diversity of the genes for nitrite reductase (*nirK, nirS*) and nitrous oxide reductase (*nosZ*) can be used to estimate denitrification potential of a soil (Hallin et al., 2009; Philippot et al., 2007; Enwall et al., 2010). The reduction of nitrous oxide results in the production of N_2 gas, instead of GHG N_2O, a potent GHG. Many identified denitrifier genomes do not have genes coding for *nosZ* (Throback et al., 2004) which has implications on GHG emissions. Most denitrifiers do not produce all the required enzymes to complete the reaction making it a community process (Zumft, 1997).

The abundance and diversity of a denitrifier community allows functional redundancy which provides tolerance to environmental conditions and stress (Phillipot et al., 2007). Wallenstein et al. (2006) describes denitrification rates as controlled by distal and proximal soil conditions. Distal soil conditions that affect microorganisms over the long term are: climate, land management, soil type and texture. Proximal soil conditions have immediate impact on microorganisms and include: C and NO_3^- substrate concentration, O_2, soil H_2O and temperature. The response of a denitrifier community to N inputs is dependent on how the N substrate impacts the proximal and distal controls. Carbon substrates influence denitrification activity primarily because denitrification is carried out by heterotrophs. Unlike autotrophs that synthesize their own food, heterotrophs depend on external C sources for energy. The addition of C substrate has been observed to prevent NO_3^- leaching by stimulating denitrification, when NO_3^- is not limited (Greenan et al., 2006). Soil microbial communities respond to different C substrates differently, as observed by Giles et al. (2017) where it was reported that there was a significant difference in N_2O emissions from an agricultural soil treated with 3 different C substrates. Carbon substrates can be used by denitrifiers for growth or respiration, depending on conditions. Soil bacterial

populations that use the substrate more efficiently are likely to increase in abundance (Giles et al., 2017). In a study by Morley and Baggs (2010), a significant interaction between C substrate and O_2 concentration was reported. Plant derived C is known to increase both soil respiration and denitrification rates, and as soil respiration increases, O_2 is consumed (Morley and Baggs, 2010).

3.0 Impact of 100 Years of Land Management Practices on Soil Microbial Community Structure and Nitrogen Functional Gene Abundance in a Long Term Soil Experiment

3.1 Preface

Soil microbes are drivers of agroecosystem functions that perform biogeochemical cycling of nutrients relevant to crop production. The biodiversity of soil microbes acts to buffer against potentially detrimental changes in environmental conditions, consequently permitting ecosystem services to be maintained. In order to better conserve soil microbial diversity, it is important to gain understanding of the impact that intensive agriculture practices such as nitrogen (N) fertilizer application and continuous cropping have on soil microbial community structure. Long term soil experiments (LTSEs) provide us with unique sites to observe the cumulative changes in biodiversity as influenced by decades of soil management. This study characterized the soil microbial communities in an LTSE in which N fertilizer (45 kg ha^{-1}) had been applied for 45 years on a >100 year old continuous wheat and wheat-wheat-fallow (WWF) rotations. This site allows an opportunity to observe a system in a steady state, allowing study of a soil microbial community structure formed in response to long term agricultural management practices. The first objective of my study was the evaluation of soil microbial biomass under continuous wheat monocropping in comparison to the WWF rotation, both with N fertilizer application and without it. The second objective was to estimate soil bacterial and archaeal communities and N functional group abundance changes under long term N fertilizer treatments and continuous wheat cropping management.

3.2 Abstract

In Canada, N fertilizer and crop rotation are common practices used to increase crop yields, reduce crop pests, preserve soil moisture and also promote soil fertility. The objective of my

study was to determine if the long term use of N fertilizer (45 kg ha^{-1} ammonium nitrate) and different cropping intensities (CI) impact soil microbial community structure and abundance. This study focused on the subplots check (N_0P_0) and N ($N_{45}P_0$) of Rotation ABC, a LTSE in Lethbridge, Alberta. Soil samples were collected post-harvest along four transects, in September 2012. Soil microbial community abundance and structure was altered in response to N fertilizer and CI. Phospholipid fatty acid (PLFA) data indicated a statistically significant interaction of N fertilizer application and CI on the total active microbial biomass. The 100% CI soils with added N had levels of total biomass that were two times greater than the 67% CI soils with added N and three times greater than both the 100% and 67% CI soils without N. Quantitative PCR analysis determined ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) N functional gene abundances were affected by significant interactions between N fertilizer and CI,. Abundances of denitrifier functional genes *nirK and nirS* were significantly impacted under by the effects of cropping intensity and N fertility management, whereas *nosZ* was not significantly influenced. The broad level changes observed in this study indicated that variations in soil N amendment rate and cropping intensity did alter soil microbial community structure. Continuous cropping and N fertilizer use resulted in increased potential for N cycling, including genes for both the production and consumption of N_2O, an important greenhouse gas (GHG).

3.3 Introduction

The early 20th century marked the beginning of mechanized agriculture on the Canadian prairies, and the establishment of Rotation ABC experiment in Lethbridge, Alberta. For decades afterwards, farmers commonly relied on in the inherent fertility of soils to sustain their crops. Over time it became apparent that nutrient additions in the forms of crop residue return and N fertilizer were necessary to maintain or improve crop yield levels and soil fertility. The

application of inorganic N fertilizer has been a land management practice since the 1960s, necessary to bolster crop growth and maintain soil productive capacity. However, the application of N fertilizers alone does not ensure soil productivity. Microbial transformations are important in the biochemical cycling of N and it is well established that microbial biodiversity facilitates important soil functions under changing environmental conditions (Fox, 2003).

The biochemical cycling of N involves N fixation from the atmosphere via symbiotic and free living microbes. The second stage of the N cycle is termed assimilation or immobilization, which is the process of N incorporation from inorganic to organic forms (Booth et al., 2005). The pool of inorganic N in soils can be derived from microbial decomposition (mineralization) of organic compounds such as plant or animals residues or from anthropogenic sources such as N fertilizers (urea, ammonium nitrate). Plants incorporate inorganic N as NH_4^+ or NO_3^- and can also utilize organic N once it has undergone microbial mediated decomposition from polymers to monomers (Schimel and Bennett, 2004)

Nitrification can occur once there is an excess NH_4 available to support both plant and soil microbial populations (Schimel and Bennett, 2004). The nitrification process transforms NH_4^+ to nitrite and nitrate, and begins with ammonia oxidation catalyzed by ammonia monooxygenase (*amoA*) and the process is dominantly influenced by oxygen levels, and ammonium availability (Wang et al., 2009). Ammonia monooxygenase converts ammonia to hydroxylamine in a rate limiting step because ammonia oxidizers lack ecological redundancy(Topp, 2003). The *amoA* enzyme is produced by both ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) (Schauss et al., 2009; Zhang et al., 2012), which are phylogenetically distant and have different cell size and structure (Prosser and Nicol, 2008). Soil environmental conditions of

temperature and pH, as well as soil management practices, all impact ammonia oxidation because these parameters control general microbial growth (Wang et al., 2009; Braker and Conrad, 2011).

Denitrification is a part of the N cycle resulting in a loss of N with rates primarily controlled by nitrate, oxygen, pH and carbon (Wallenstein et al., 2006). There are four steps during bacterial denitrification: nitrate reduction, nitrite reduction, nitric oxide reduction and nitrous oxide reduction (Philippot et al., 2007). Abundance and diversity of the genes for nitrite reductase (*nirK*, *nirS*) (Coyne et al., 1989) and nitrous oxide reductase (*nosZ*) can be used to estimate denitrification potential of a soil (Philippot et al., 2007; Hallin et al., 2009). Many denitrifier genomes identified do not have genes coding for *nosZ* (Throback et al., 2004; Clark et al., 2012), which has implications on GHG emissions because the reduction of nitrous oxide results in the production of N_2 gas, instead of a GHG.

Most denitrifiers do not produce all the required enzymes to complete the reaction, suggesting denitrification is a community scale process (Zumft, 1997). Denitrifier communities are abundant and diverse with high functional redundancy that allows tolerance of environmental conditions and stress (Philippot et al., 2007). The response of denitrifier communities to N inputs is dependent on how the N increase impacts the proximal and distal controls on soil conditions (Wallenstein et al., 2006). Proximal controls include substrate concentration, O_2, soil H_2O and temperature have immediate impact on microorganisms. Distal controls affect microorganisms over the long term and include climate, land management, soil type and texture.

Soils with reduced cropping intensity (CI) or fallow undergo changes in the abundance and structure of their soil microbial communities because of the lack of plant residue inputs during this non-cropped time period (Acosta-Martínez et al., 2007). Examples of cropping intensity (CI)

include a wheat-fallow rotation (50% CI), and wheat-wheat-fallow (67% CI). A cropping intensity of 100% occurs when a crop is produced each growing season without any years in fallow. In Acosta-Martinez et al. (2007), biomass was found to be greater in soils under 100% CI, as compared to 50% CI. The reduction of plant residue inputs during fallow (non-cropped time period) reduces the C substrates available for microbial growth and gradually shifts the soil microbial community structure over time to those microorganisms that tolerate low energy conditions (Paterson et al., 2011). Fresh organic C from growing plants has a different composition than older soil organic C, and therefore promotes the growth of different microbial groups (Kemmitt et al., 2008).

It takes decades after a change in land management for a soil to resume a steady state (Janzen, 1995). The Rotation ABC LTSE at Lethbridge, Alberta provides an opportunity to study microbial dynamics in response to 100 years of wheat rotation management and 45 years of N fertilizer implementation. Although many studies have characterized soil properties of Rotation ABC (Smith et al., 2012) none have used modern biochemical and molecular biology tools to investigate the microbial response to long term land management. The two hypotheses for this work are:ammonia oxidizer and denitrifier communities will become more abundant over time due to N fertilizer use and cropping intensity will cause changes in soil microbial community composition. The objective was to characterize total populations of ammonia oxidizers and denitrifiers using NFGs. This work aims to grow the understanding of the function of microbial abundance and diversity in key biogeochemical processes affecting the productivity and sustainability of an agroecosystem.

3.4 Materials and methods

3.4.1 Soil sampling methodology

Soils were collected from Rotation ABC, a historic Agriculture and Agri-Food Canada long term soil experiment (LTSE) established in 1911 at Lethbridge, Alberta to assess the effects of crop rotation on soil quality (Janzen, 1996). The site is located on an Orthic Dark Brown Chernozem has a mean annual precipitation (MAP) of 386.0 mm and mean annual temperature (MAT) of 5.7°C. Rotation ABC was divided into plots under different crop rotation management (Fig. 3.1) with rotations A and C being the focus of this study. Rotation A had been continuously sown with wheat (W) for over 100 years (Table A.1, Cultivars of wheat grown on Rotation ABC, Appendix A) and Rotation C had been wheat-wheat-fallow rotation. Rotation C is comprised of 3 plots, C1, C2 and C3 to allow all phases of the rotation to exist in all years. For this study, the second year wheat phase was selected for sampling (C1 in 2012) to de-emphasize the short term effects of the fallow phase on microbial abundance.

Land management of Rotation ABC evolved over time to reflect technological advances in crop production (Janzen, 1995; Clark and Hirsch, 2008). The advent of combine harvesters resulted in crop residues remaining in the field after 1943 and the green revolution influenced the addition of N (as ammonium nitrate) and P treatments from 1967 and 1972 respectively. Fertilizer was applied to plots cropped to wheat, however not to fallow plots, as follows: N (34-0-0) broadcast prior to seeding at a rate of 45 kg N ha^{-1} and P (0-45-0) applied with seed at a rate of 22.5 kg P_2O_5 $ha-^{1}$.

This study focused on the subplots check (N_0P_0) and N ($N_{45}P_0$). Samples were collected post-harvest in September 2012. Each subplot was sampled along four transects that function as pseudoreplicates to compensate for an experimental design that pre-dates the application of

modern statistical methods with replication to field studies. Prior to soil sampling, any large masses of crop residue present were removed. Soil cores were collected with truck-mounted, hydraulic driven soil coring equipment (Giddings Machine Company, Fort Collins, CO) along transects 1 to 4 (Fig. 3.1) with four cores 3.75 cm in diameter and 0 to 10 cm depth for each replicate point. Cores were bulked for each replicate and stored on ice in portable coolers during transport then processed within 48 h. Soil cores were homogenized by sieving to 2.0 mm. Soils that were subsampled for nucleic acid isolation were stored at -80.0 °C while subsamples for PLFA were first lyophilized then stored at -20.0°C. Soils subsampled for general soil property analyses were stored at 4.0°C.

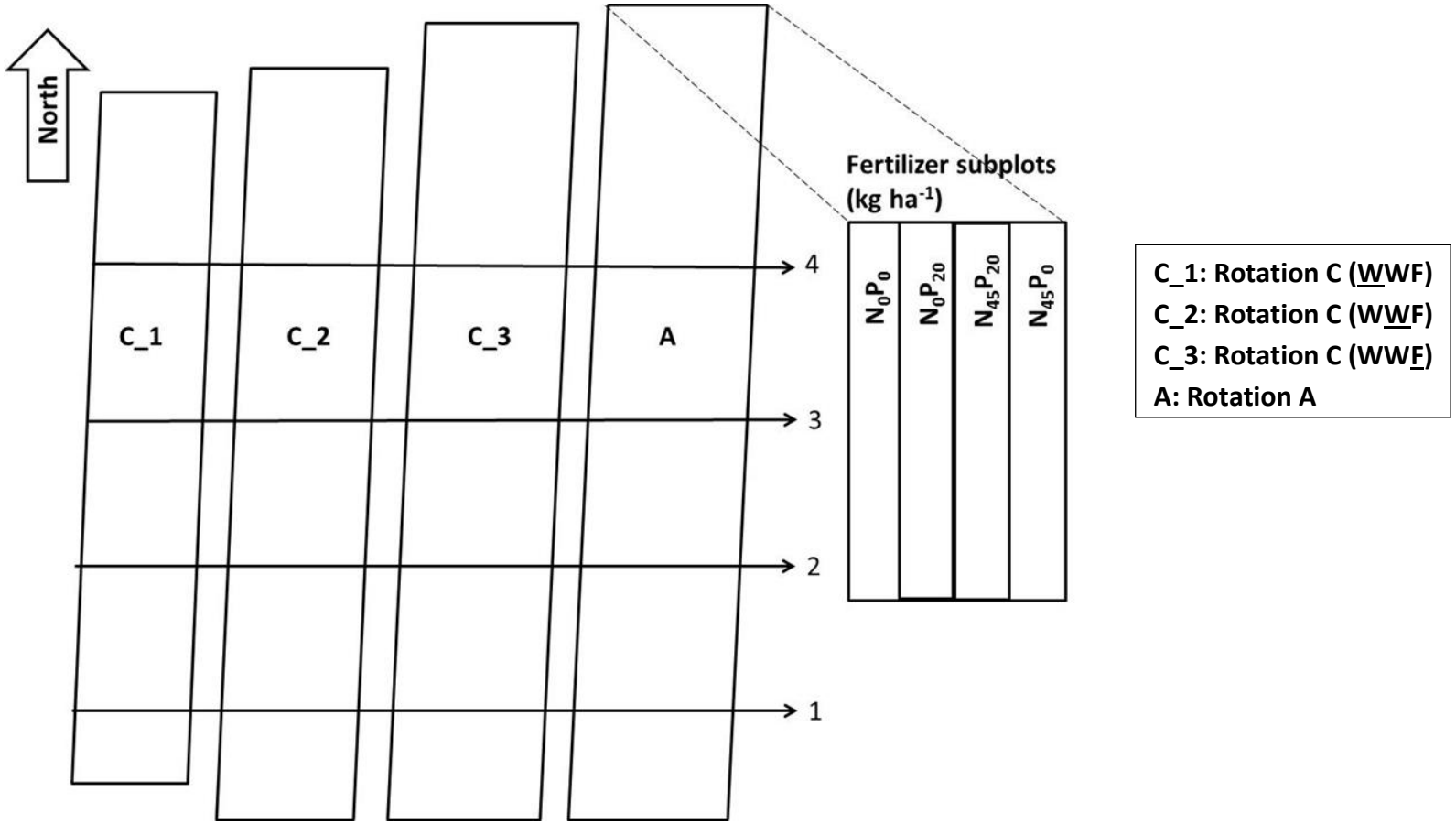

Fig. 3.1 Rotation A (A) and C (all phases of WWF rotation, C_1, C_2, C_3) soil sampling locations, modified from Ellert, 2003. Sampling transects are denoted as 1 to 4. Each Rotation consists of 4 fertilizer subplots, with 0 kg ha^{-1} of N and P (N_0P_0), 0 N and 20 kg P ha^{-1}(N_0P_{20}), 45 kg N and 20 kg P ha^{-1} ($N_{45}P_{20}$) and 45 kg N and 0 kg P ha^{-1} ($N_{45}P_0$).

3.4.2 Characterization of soil properties

The general soil characteristics such as soil pH and inorganic nutrients N, P, Potassium (K) and Sulfate (S) were determined by ALS Environmental Lab (Saskatoon, Saskatchewan). The pH was measured with a 1:2 soil: water solution; S and available Nitrate via a $CaCl_2$ extraction (Alberta Agriculture, 1988 p. 19, 28); Potassium and P with the modified Kelowna method (Qian and Schoenau, 1994) was $CaCl_2$ extracted (Table B.1, Appendix B).

3.4.3 Phospholipid fatty acid (PLFA) analysis

Phospholipid fatty acid analysis was used to characterize changes in microbial abundance and community structure in the N_0P_0 (kg ha^{-1}) and $N_{45}P_0$ (kg ha^{-1}) of Rotation A and C (W<u>W</u>F). The modified PLFA extraction of White (1979), adapted from Bligh and Dyer (1959) was used to perform the extraction, as described in Helgason et al. (2010). All solvents used were HPLC grade, and deionized water was used for all analyzes. The N_2 for sample evaporation was of ultra-high purity (Praxair Canada Inc., Mississauga, ON). All glassware was prepared in a 4% (v/v) Extran 300 soap bath for 2h then scrubbed and tripled rinsed with dH_2O. To remove any remaining lipids, glassware was soaked in 10% HCl (4 h for glass, 2 h for Teflon lined vial caps). Vials and caps were again tripled rinsed with dH_2O. Dry glassware was then heated to 400°C for 4h in a muffle furnace (Thermo Fisher Scientific Inc., Waltham, MA). The extraction of fatty acids was from 4.0 g of freeze dried, ground whole soil in 50 mL glass vials with 15.0 mL of 2:1:0.8 (v/v) of MeOH:$CHCl_3$:P solution. Samples were protected from light and shaken horizontally for 2 hours then centrifuged for 10 min at 1000 rpm (25°C). Next, the supernatant was transferred to another 50mL screw cap glass tubes and 5.0 mL P buffer and 4.0 mL $CHCl_3$ were added prior to mixing the solution. The lower non-polar phase was transferred to an 8.0 mL tube and concentrated with N_2 at 30°C with 2 sample washes with 1.0 mL methanol. Samples

were resuspended in 1.0 mL chloroform prior to lipid separation step. Neutral, glycol and phospholipids were sequentially eluted from a solid phase extraction column (0.50 g Si; Varian Inc. Mississauga, ON) with 5.0 mL chloroform, 10.0 mL acetone and 5.0 mL methanol, respectively. The samples were then evaporated with N_2 and stored at -20°C. Samples then underwent base catalyzed methylation in an excess of methanol which converted the phospholipids to methyl esters. Methyl esters were then analyzed with a Hewlett Packard 5890 Series II gas chromatograph (Hewlett Packard, Palo Alto, CA) fitted with an Agilent Ultra 2 cross-linked 5%-Phenyl-methylpolysiloxane column; 23.85 m x 0.2 mm ID x 0.33 µm film thickness (Thomas Scientific, Swedesboro, NJ). The injector temperature was set at 250°C, and the flame ionization detector at 300°C. The temperature program began with an oven temperature of 170°C, ramping to 260°C at 5°C $minute^{-1}$ and then to 310°C at 40°C $minute^{-1}$ with a total run time of 20.75 minutes. Sample peaks were identified with MIDI software using the TSBA 40 library (version 4.1) and MIS Calibration Standard Mix 1200-A (MIDI Inc., Newark, DE). Microbial biomass was calculated as per Hedrick (2005), based on the addition of an internal standard, methyl nonadecanoate (Sigma-Aldrich, Oakville, ON). Total bacterial biomass was determined with lipid biomarkers i14:0, i15:0, a15:0, i16:0, i17:0, a17:0 (Hedrick *et al.*, 2005) and 16:1ω7t, 16:1ω9c, 16:1ω7c, 18:1ω7c, 18:1ω9c, cy17:0, and cy19:0 (Macdonald et al., 2004). The Gram positive (Gr +) community was identified based on the detection of i14:0, i15:0, a15:0, i16:0, i17:0, a17:0 (Hedrick et al., 2005), and Gram negative (Gr -) with 16:1ω7t, 16:1ω9c, 16:1ω7c, 18:1ω7c, 18:1ω9c, cy17:0, and cy19:0 (Macdonald et al., 2004). Arbuscular mycorrhizal fungi (AMF) were measured with 16:1ω5c (Olsson, 1999). The ratios of cy17:0/16:1ω7c represent the Stress 1 biomarker, and cy19:0/18:1ω7c the Stress 2 biomarker as described in Grogan and Cronan, (1997).

3.4.4 Nitrifier and denitrifier gene abundances

DNA extraction. DNA was extracted from four technical replicate samples of 0.25 g of fresh soil with Mo Bio Power Soil kits according to the manufacturer's instructions (Mo Bio Laboratories, Carlsbad, CA). DNA extracts were pooled and then the quantity of DNA was determined spectrophotometrically using a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA).

Construction of quantitative PCR standard curves. A set of 6 plasmids containing gene fragments of interest were used as standards for absolute quantification (Table 3.1). DNA was extracted from source materials and insert DNA amplified using oligonucleotide primers and reaction conditions as found in literature references. PCR products were agarose gel purified with a Qiagen QIAquick gel extraction kit (Qiagen Inc., Toronto, Ontario) then quantified with NanoDrop 2000, as described above. Gene fragments of interest were ligated into plasmid vectors using Invitrogen TOPO TA Cloning Kit (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions. Recombinant *Escherichia coli* DH5αT1R strains carrying successful ligations were inoculated into 3.0 mL LB broth (16 h, 37.0°C, 150 rpm) with 50.0 μg/mL selective antibiotic, kanamycin or ampicillin, depending on plasmid. Plasmid DNA was extracted from 2.0 mL of culture with a Qiagen QIAprep Spin Miniprep kit following manufacturer's instructions. Plasmids were quantified and then linearized using the HindIII restriction enzyme (Thermo Fisher Scientific Inc.). Following digestion, reactions were pooled, purified from the agarose gel and quantified as described above. The linearized plasmid with target DNA insert was then utilized to create dilution series and used to derive standard curves.

Quantitative PCR assays. Quantitative real-time PCR (qPCR) was performed in triplicate using an ABI Step One Plus, and Platinum SYBR Green qPCR SuperMix, (Life Technologies,

Burlington, ON) to detect the abundances of 16S rRNA, bacterial and archaeal ammonia monooxygenase (*amoA*), nitrous oxide reductase (*nosZ*), cytochrome cd1 type nitrite reductase (*nirS*) and copper nitrite reductase (*nirK*) gene copies g^{-1} soil (Table 3.1). Bovine serum albumin (Life Technologies, Burlington, ON) was included in the assays at a rate of 0.2 μg μL^{-1} to reduce potential PCR inhibition caused by residual humic acids in the DNA extractions. The passive reference dye 5-carboxy-X-rhodamine (ROX) was added to the reactions to normalize for differences in fluorescent reported signal between wells at a concentration of 50 nM. Melt curve analysis was performed after each assay to test for the presence of primer dimer formation and assess the specificity of the reaction.

Table 3.1 Quantitative PCR gene targets, primers and reaction parameters.

Gene	Source	Primer Name	Primer conc.(µM)	PCR vol.(µL)	DNA † vol.(µL)	PCR conditions	References
16S	*Pseudomonas stutzei*	EUB338/518	0.03	20.0	4.0	95°C 120s ,40 of (95°C 60s, 53°C 30s, 72°C 60s, 80°C 60s), 1 of (95°C 15s, 60°C 60s,+0.3°C per min to 95°C 15s)	Fierer *et al.*, 2005
Bacterial *amoA*	*Nitrosomonas europa*	amoA1F/2R	0.60	25.0	4.0	95°C 120s, 46 of (95°C 45s, 55°C 45s, 72°C 45s, 80°C 60s),1 of (95°C 15s, 60°C 30s,+0.3°C per min to 95°C 15s)	Hallin *et al.*, 2009; Rotthauwe *et al.*, 1997;Stephen *et al.*, 1999
Archaeal *amoA*	Fosmid 54d9	crenamo23F/ 616R	0.13	20.0	4.0	95°C 120s, 45 of (95°C 60s, 55°C 60s, 72°C 60s, 80°C 60s),1 of (95°C 15s, 60°C 60s, +0.5°C per min to 95°C 15s)	Shauss *et al.*, 2009; Tourna *et al.*, 2008
nosZ	*Pseudomonas stutzei*	nosZ 2F/2R	0.60	25.0	3.0	95°C 120s, 6 of (95°C 15s, 65°C 30s [-1°C with each cycle], 72°C 30s, 80°C 15s) ,40 of (95°C 15s, 60°C 30s, 72°C 30s, 80°C 15s),and 1 of (95°C 15s, 60°C 15s, +1.0°C per min to 95°C 15s)	Clark *et al.*, 2012; Henry *et al.*, 2006

nirS	*Pseudomonas stutzei*	cd3aF/ R3cd-R	1.00	25.0	5.0	95°C 120s, 6 of (95°C 15s, 65°C 30s [-1°C with each cycle], 72°C 30s, 80°C 15s) ,40 of (95°C 15s, 60°C 30s, 72°C 30s, 80°C 15s),and 1 of (95°C 15s, 60°C 15s, +0.5°C per min to 95°C 15s)	Clark *et al.*, 2012; Thröback *et al.*, 2004
nirK	*Sinorhizobium meliloti*	nirKH1F, nirK1R/R3Cu	0.60, 0.80	25.0	3.0	95°C 120s, 6 of (95°C 15s, 63°C 30s [-1°C with each cycle], 72°C 30s, 80°C 30s) ,40 of (95°C 15s, 58°C 30s, 72°C 30s, 80°C 30s),and 1 of (95°C 15s, 60°C 15s, +0.3°C per min to 95°C 15s)	Dandie *et al.*, 2011

†qPCR reaction sample template 10ng μL^{-1}

3.4.5 Data analysis

PLFA. The absolute concentration of PLFA was calculated from GC peak data using Equation 3.1, adapted from Hedrick (2005).

$$\text{PLFA nmol g}^{-1}\text{ soil} = \frac{PA_{PLFA} \bullet [IS] \bullet (IS_{vol}) \bullet 1000 \text{ nmol } \mu\text{mol}^{-1}}{Soil_{dw} \bullet PA_{IS} \cdot MW_{PLFA}}$$

Where PA_{PLFA} is the peak area of the PLFA, [IS] is the concentration of methyl nonadecanoate (19:0), (IS_{vol}) is the volume of 19:0 (μL), $Soil_{dw}$ is the weight of soil extracted (g dry weight), PA_{IS} is the peak area of 19:0, MW_{PLFA} is the molecular weight of the target PLFA (μg mol^{-1}).

Microbial abundance data were analyzed using ANOVA in SPSS v.20 and community profiles were subjected to nonmetric multidimensional scaling (nMDS) ordination v.5.0 (Kruskal, 1978).

Quantitative PCR assays. Gene copy numbers were standardized by the mass of DNA and g dry soil analyzed for each sample, then $\log_{10}$ transformed. Statistics were performed using a one-way ANOVA in SPSS v.20 to determine the significance of cropping intensity and N fertilizer additions on gene abundances.

3.5 Results

3.5.1 Impact of land management practices on soil microbial communities

Phospholipid fatty acid data indicated total biomass in the soils increased with N fertilizer application at 45 kg ha^{-1} and CI at 100%. Both treatments had a significant interaction effect together on total microbial biomass (p<0.02, Table 3.2). When different treatment levels were compared, 100% CI soils with N fertilizer had 2-3 fold greater total microbial biomass than the other soils tested. The bacterial biomass (nmol PLFA g^{-1} soil) was significantly increased in response to both N fertilizer and CI; however, fungal biomass was only significantly increased by N (Table 3.2). The fertilized soils (45 kg N ha^{-1}) soils had higher biomass than the unfertilized

soils (0 kg N ha^{-1}) soils. The abundance of both Gr- and Gr+ bacteria populations increased significantly in response to N fertilizer (Table 3.2). The abundance of AMF was not significantly influenced by N or CI (Table 3.2) possibly due to the lack of P amendments in the soils studied (N_0P_0, $N_{45}P_0$). The abundance of AMF was greatest in the continuous wheat $N_{45}P_0$ soil.

Nonmetric multidimensional scaling (MDS) ordination analysis of PLFA profiles (expressed as log transformed mol %) grouped each soil PLFA profile according to similarity, with a final stress of 7.67 (Fig 3.2). The soil microbial community structure of Rotation A $N_{45}P_0$ and C N_0P_0 were distinctive, whereas A N_0P_0 and C $N_{45}P_0$ were more similar. Cropping intensity and N fertilizer application (Table B.1) had a significant (p<0.01) effect on pH with the pH decreasing in response to increased cropping intensity (CI) and $N_{45}P_0$ (kg ha^{-1}). There was a significant interaction of CI and N fertilizer on NO_3 availability (p<0.01, Table B.1). The results from average wheat yield from 1912 to 1967 for continuous wheat (A) and W-W-F(C) under N0 (kg ha^{-1}) and N45 (kg ha^{-1}) treatment suggest that yield increased with N fertilizer additions (Table C.1).

Table 3.2 Quantities of Total Bacterial and Fungal biomass, Gram positive (Gr+), Gram negative (Gr-) bacteria, arbuscular mycorrhizal fungi (AMF) and Stress 1 and Stress 2 biomarkers as indicated from PLFA characterization of 2012 soil samples (n=16). Standard errors indicated in parenthesis, and means followed by different letters indicate a significant difference ($p<0.05$).

Rotation	N Fertilizer kg ha^{-1}	Total biomass	Bacterial biomass	Fungal biomass	Gr+	Gr-	AMF	Stress 1	Stress 2
		nmol PLFA g^{-1} soil							
A	0	12.9(1.1)b	6.6(1.9)b	0.5(0.2)b	1.67(0.2)b	4.3(0.3)b	0.8(0.1)a	0.4(0.0)a	0.3(0.0)a
A	45	36.1(6.5)a	16.7(1.9)a	2.1(0.6)a	3.9(1.3)a	12.0(3.0)a	3.8(2.3)a	0.4(0.1)a	0.3(0.0)a
C	0	12.5(1.3)d	6.2(0.5)d	0.4(0.1)b	1.6(0.1)b	4.0(0.2)b	0.7(0.1)a	0.4(0.0)a	0.2(0.1)a
C	45	17.2(2.5)c	8.5(0.8)c	1.1(0.6)a	3.3(0.8)a	5.6(0.9)a	1.0(0.1)a	0.4(0.0)a	0.3(0.0)a
					ANOVA p-values				
	CI	NS	*	NS	NS	NS	NS	NS	NS
	N	*	**	*	*	**	NS	NS	NS
	CIxN	*	NS	NS	NS	NS	NS	NS	NS

*,**,*** Significant at $p \leq 0.05, 0.01, 0.001$

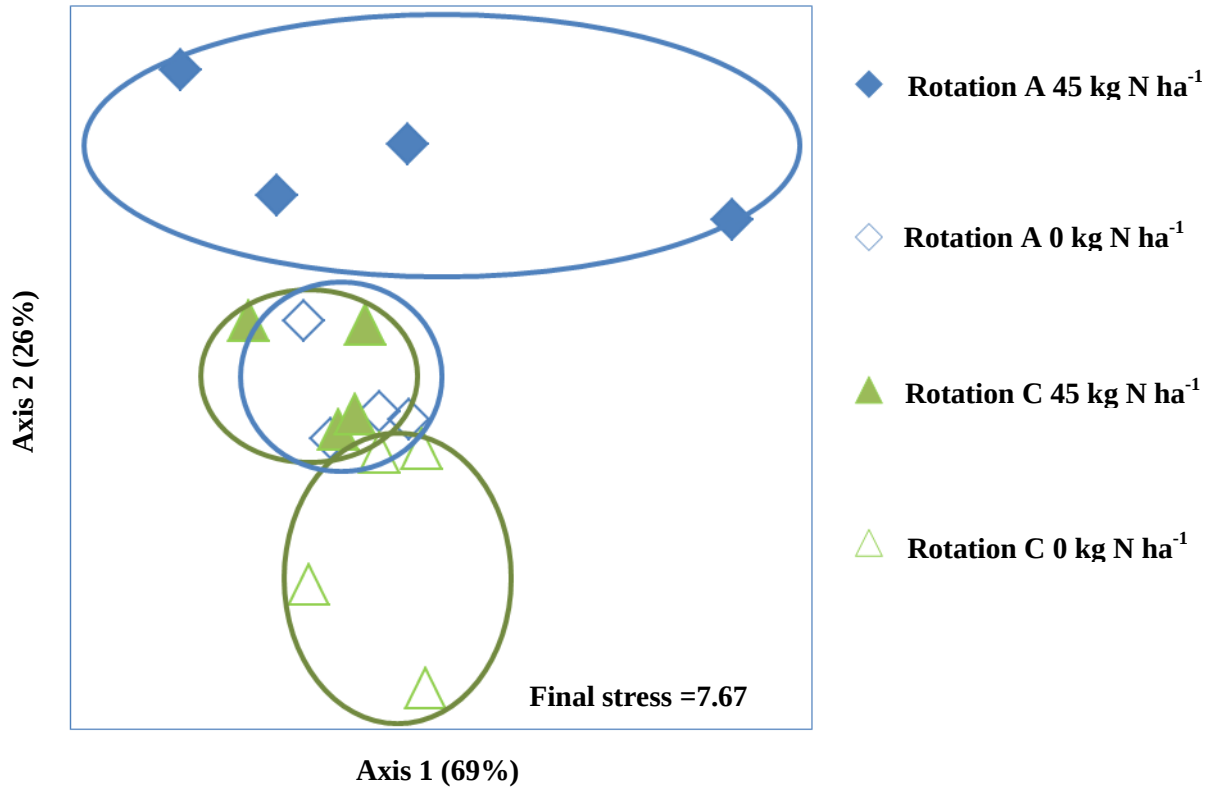

Fig 3.2 Nonmetric multidimensional scaling (MDS) analysis (Final stress=7.67) of N fertilizer and cropping intensity effects on PLFA profiles (log transformed mol% PLFA abundances) from Rotation A (continuous wheat) and C (WWF) from 2012 soils. Circles indicate PLFA profiles of similar composition: Rotation A plus 45 kg N ha^{-1} (dark blue), Rotation A at 0 kg N ha^{-1} (light blue), Rotation C plus 45 kg N ha^{-1}(dark green) and Rotation C at 0 kg N ha^{-1} (light green).

3.5.2 The effect of land management practices on N functional gene abundances

Real time quantitative PCR was used to determine the abundance of both ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) N functional genes. The gene copies per g^{-1} of dry soil of both AOA and AOB were affected by significant interactions between N fertilizer (0 and 45 kg ha^{-1} ammonium nitrate) and CI. Ammonia oxidizing bacteria abundance ranged from 7.1 to 8.0 log gene copies g^{-1} dry soil and AOA abundance was measured at 7.6 to 8.1 log gene copies g^{-1} dry soil (Fig. 3.3). Real time PCR was used to determine the abundance of three targeted denitrifier functional marker genes *nosZ*, *nirK* and *nirS*. Cropping intensity and N fertilizer treatment had a significant ($p<0.05$) effect on *nirK* abundance, with 7.35 to 7.90 log gene copies g^{-1} soil. The cytochrome cd_1 dependent Nir gene *nirS* was observed to be significantly influenced by cropping intensity and N fertilizer treatment ($p<0.05$), with 5.40 to 5.90 log gene copies g^{-1} soil (Fig. 3.4). The abundance of *nosZ* ranged from 9.41 to 9.96 log gene copies g^{-1} soil, and was not significantly influenced by CI or N fertilizer treatment (Fig. 3.4).

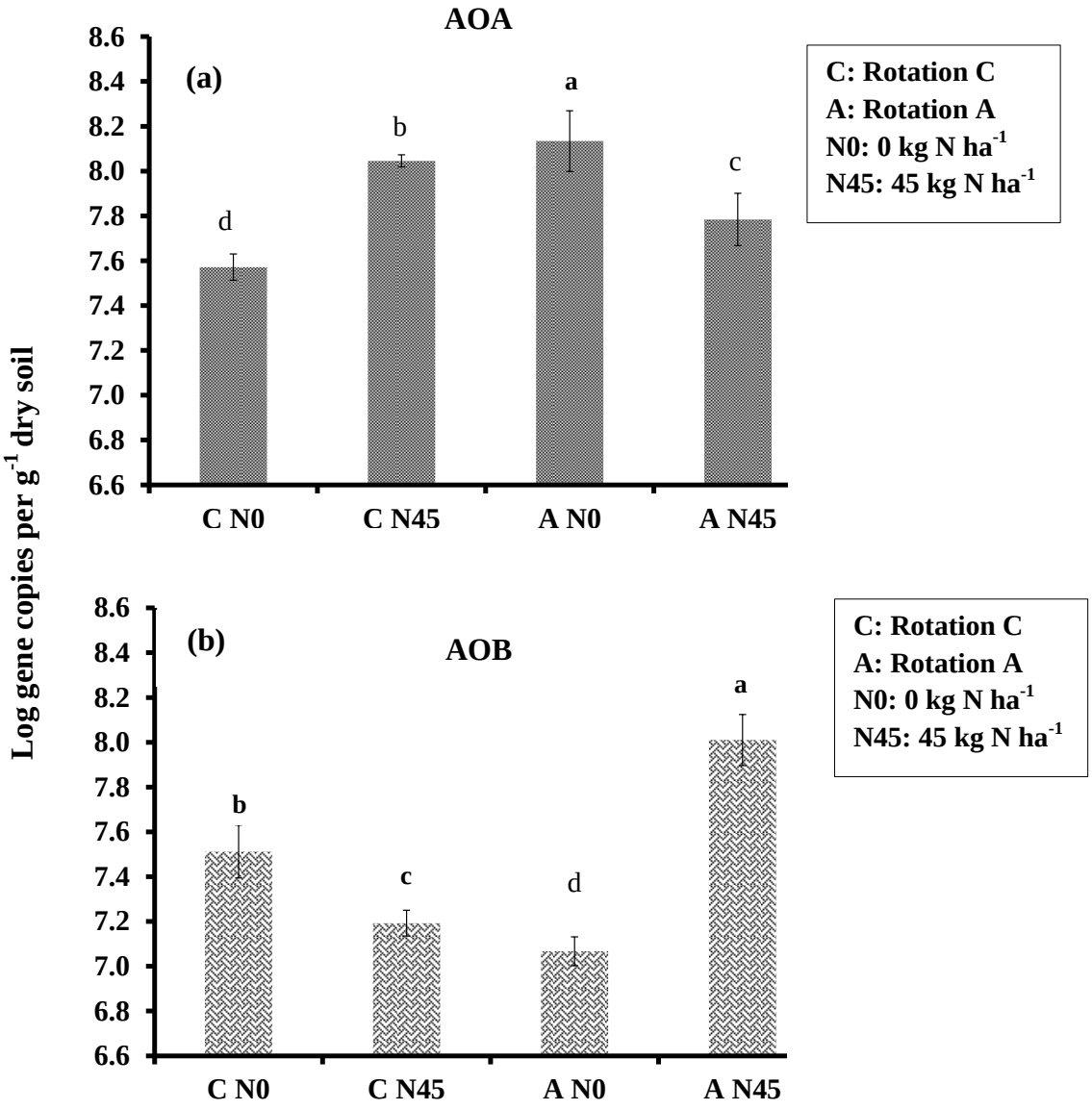

Fig. 3.3 Ammonia oxidizing archaea (a) and ammonia oxidizing bacteria (b) abundance as affected by cropping intensity and N fertilizer levels (kg N ha^{-1}); bars denote standard error. Means followed by different letters indicate a significant difference ($p<0.05$).

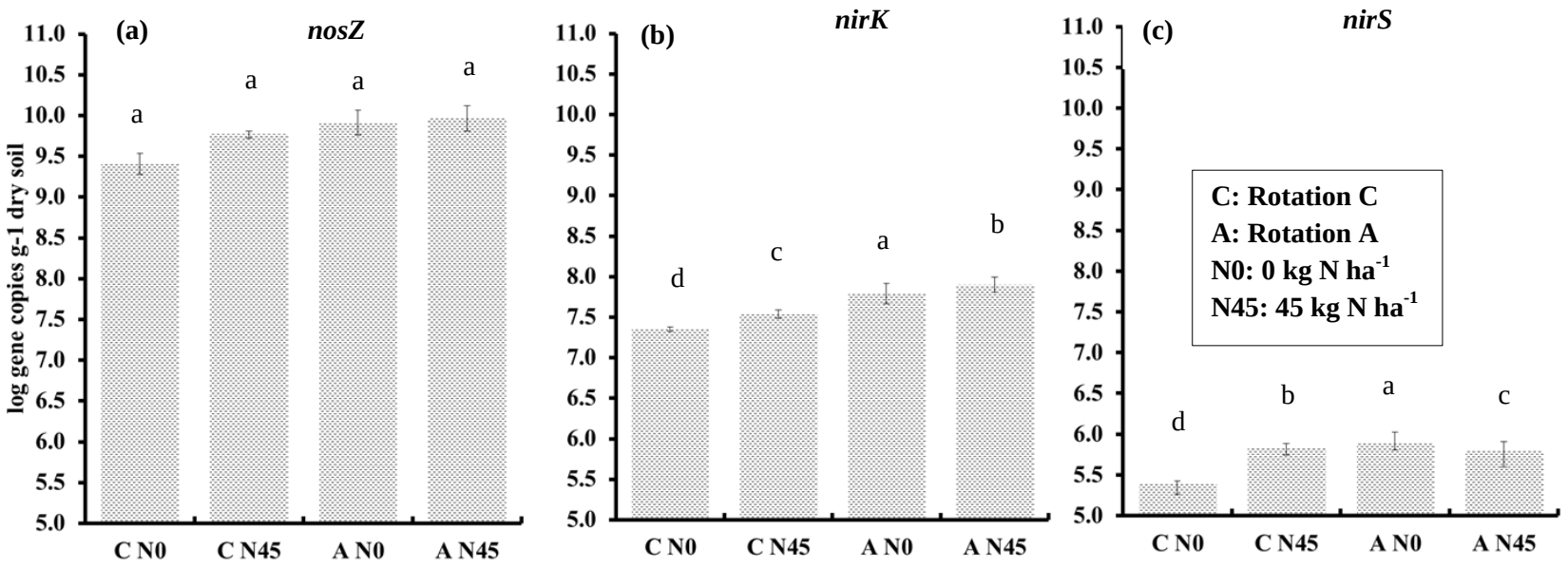

Fig. 3.4 Denitrifier abundance as estimated with log gene copies *nos*Z (a), *nirK* (b) and *nirS* (c) g^{-1} soil; bars denote standard error. Means followed by different letters indicate a significant difference ($p<0.05$).

3.6 Discussion

3.6.1 Impact of land management practices on soil microbial communities

The abundance and diversity of the soil microbial community was altered in response to different land management practices, specifically to N fertilizer and CI rates. Through the comparison of different treatment levels, it was observed that 100% CI soils with 45 kg N fertilizer ha^{-1} had 2-3 fold greater total biomass than the other soils studied. The presence of a consecutive crop f for 100 years and N fertilizer for 45 years in Rotation A soils increased plant productivity and also the supply of C substrates available to soil microbes, and as a result these organisms were estimated as more abundant than those in the Rotation C soils. It has been reported that concentration of organic matter in Rotation A was higher than in Rotation C (Janzen, 1995) and that N fertilizer tends to increase plant productivity in both rotations (Janzen, 1987). In addition, average grain yield data from Rotation A and C (1912-2011) indicated that N fertilizer tended to increase yields (Fig.C.1, Appendix C). Similar results were found by Zhang et al. (2012), where PLFA biomarkers were used to detect that both N fertilizer and straw residues were factors in total biomass and bacteria increases of a long term rice-wheat rotation. Zhang and coworkers found significantly more biomass in the N-P-K plus straw residue soils than in the N-P-K without straw residue or unfertilized check soils. The stimulating effect of C inputs on total biomass was also observed in a study by Shi et al. (2015) which linked an increase in corn stalk application rates to increased abundance of total biomass, as well as broad bacterial and fungal groups. Bardgett et al. (1999) found positive correlation between increased plant productivity and increased total PLFA, while Börjesson et al. (2012) demonstrated a link between additions of N and increases in total biomass in soils that were not C starved.

The abundance of bacteria significantly increased in response to both N fertilizer and CI and fungal biomass was significantly increased by N (Table 3.3). In low N systems such as the N_0P_0 soils, plants compete with microbes for organic N, which limits the microbial growth (Schimel and Bennett, 2004). The optimal C:N ratio for microbial growth is 25:1, and so with wheat residue at 80:1, an N deficit occurs that curtails microbial growth without the addition of more N. Consequently, N fertilizer is a factor in the greater biomass of 100% CI over 67%CI, evident because the $N_{45}P_0$ soils have higher abundance than the N_0P_0 soils. In contrast, many studies have found that the addition of N decreased bacterial abundance (Langer and Klimanek, 2006; Rousk et al., 2011; Shi et al., 2015) or had no impact (Verdenelli et al., 2013; Zhao et al., 2014b). Increased fungal abundance has been linked to N fertilizer both by Zhao et al. (2014b) and Ngosong et al. (2010), while Börjesson et al. (2012) did not observe any differences between soils with and without N amendments. Börjesson et al. (2012) didn't specify if the soils were ground prior to extraction, as grinding increases the amount of fungal PLFA extracted (Allison et al., 2005).

The abundance of both Gram negative (Gr-) and Gram positive (Gr+) bacteria populations increased significantly in response to N fertilizer, with Gr- in greater abundance overall than Gr+ (Table 3.3). The link of N to abundance suggests that N is the more limiting growth factor than plant residue derived C for these groups of bacteria, either directly or indirectly. Directly, the addition of fertilizer reduces N limitations, allowing microbial growth to occur, and indirectly the addition of N increases plant productivity and crop residue inputs resulting in greater C inputs. In a 10 year experiment, Verdenelli et al. (2013) evaluated the effects of six different fertilizer treatments on soil microbes. Their results indicated that both Gr- and Gr+ bacteria were

stimulated with the addition of balanced fertilization with NPS and micronutrients (Verdenelli et al., 2013).

Although the unfertilized check plots are lower in nutrients than the other soils studied, none of the soils differed significantly for stress markers measured (Table 3.3). Stress markers are a measure of environmental pressures which can cause increased fluidity of cell membranes, and in order to compensate for these detrimental changes to molecule transport, changes in phospholipid composition occur (Kaur et al., 2005). Neither the N fertilization treatment nor the fallow frequency led to detectable differences in stress markers, so the results suggest that N and C are not the most limiting factor for these soil bacterial populations. Both stress marker ratios were low in comparison to values reported in a tillage study by Helgason et al. (2010), where ratios of 0.3 to 1.2 were observed in typical Canadian prairie agricultural soils. Another potential explanation for the low stress markers is that soil fertility was partially augmented by atmospheric deposition of N. Anthropogenic sources of N are found in the environment, including emission from fertilizers, manures, fossil fuel and biomass combustion. The N_2O remains in the atmosphere, but he remainder are deposited (Bobbink et al., 2010).

The abundance of AMF was greatest in the continuous wheat N fertilized soil, suggesting that plant residue C inputs and nitrate have some effect on their growth. A recent study utilized a qPCR assay to measure AMF abundance with symbiosis (SYM) gene targets (Nouri et al., 2014) and observed that NO_3^- enhanced the colonization of AMF species *Rhizophagus irregularis* on *Petunia hybrida* at lower concentrations and inhibited AMF at higher concentrations.

Whole community analysis with PLFA profiles was used to determine soil microbial communities that are similar or different (Zelles et al., 1999). The soil microbial community

structure of Rotation A $N_{45}P_0$ and C N_0P_0 were distinctive, while A N_0P_0 and C $N_{45}P_0$ were more similar. The significant differences in growth conditions as determined by soil nutrient analysis (Table B.1, Appendix B) may be driving these distinct soil microbial communities suggested by the nonmetric MDS analysis (Fig. 3.2). Cropping intensity and N fertilizer application (Table B.1, Appendix B) had a significant ($p<0.01$) effect on pH, with the pH decreasing in response to increased cropping intensity (CI) and $N_{45}P_0$ (kg ha^{-1}). There was a significant interaction of CI and N fertilizer on NO_3^- availability ($p<0.01$, Table B.1, Appendix B). These results indicate that important parameters of microbial growth (pH and N availability) were influenced by the agricultural management practices of N fertilization and 100% CI and provide evidence of divergence from a shared origin.

Soil microbial communities perform key biological functions in agroecosystems including nutrient cycling and N fixation. Therefore it is worthwhile to study the impact of N fertilizer and cropping intensity on soil microbial community structure. Phospholipids are essential components of living cells, and therefore the measurement of total PLFA from soils are useful indicators of viable microbial biomass in environmental samples (Zelles, 1999; Frostegård et al., 2011). The use of mineral fertilizers is known to induce changes in microbial biomass (Verdenelli et al., 2013) and PLFA is a culture independent means to assess broad level changes coinciding with land use history (Schloter et al., 2003). This work with PLFA provides a broad overview of the soil microbial response to the cumulative effects of 100 years of continuous wheat cropping and 45 years of N fertilizer.

3.6.2 The effect of land management practices on N functional gene abundances

The copy numbers of AOB have been adjusted as in Norton et al. (2002) to reflect that there are two to three ammonia monooxygenase gene copies per cell. In terms of AOA:AOB,

unfertilized 100% CI soils were 13:1, fertilized 67% CI soils were 7:1, while unfertilized 67% CI and fertilized 100% CI were lower, with 1:1 and 1:2 respectively. The results indicate that although continuous wheat cropping significantly increased bacterial abundance over the 67% CI soils (Fig. B.1, Appendix B); the distributions of AOB and AOA do not follow the same trend.

Until 10 years ago, the only known ammonia oxidizers were bacteria (Prosser and Nicol, 2008) and it is only more recently that archaea have been identified with the ammonia monooxygenase gene (*amoA*) (Jia and Conrad, 2009). Very few groups of organisms have the ability to oxidize ammonia to nitrate. Rates of oxidization likely differ in AOA and AOB based on abundance and activity rates of amoA per cell. The phylogenetic and physiological differences between AOA and AOB (Shauss et al., 2009) might provide a basis for the differential growth rates in response to the different growth conditions created by long term N and C gradients in these soils. The presence of both types of ammonia oxidizers is an example of functional redundancy with two phylogenetically separate groups performing the same step in the cycling of N.

In my study, the soils differ in C and N substrate inputs. Many other studies have documented responses of AOA and AOB to changes in soil conditions. In Chu et al. (2007), long term mineral fertilizer (NPK) was reported to shift AOB DGGE patterns, implying genetic adaptations in response to changes in growth conditions. In my work the abundances of AOB vary in response to a significant interaction of CI and N. In a long term fertilizer experiment with a wheat and maize rotation, He et al. (2007) had similar results, where AOB abundance increased following additions of N fertilizer paired with organic matter versus N fertilizer alone. The abundance of AOB and AOA in the He et al. (2007) study were 6.0 to 8.0 and 6.6 to 8.0 log gene copies respectively, which is similar to the ranges observed in the 2012 fall Rotation A and C

soils. In Leininger et al. (2006) the addition of N stimulated both AOA and AOB growth in N fertilized soils; however other studies have observed an AOA preference for low ammonia concentrations (Di et al., 2009; Hatzenpichler, 2012).

Quantification of denitrifier gene abundance provides some insight into the influence of decades of N fertilizer and a century of variations in CI on these important soil organisms. It is important to study the potential changes to the denitrifying bacteria community in agricultural systems because these processes may result in the both loss of N from plant production, and the release of the greenhouse gas (GHG) N_2O. Nitrite reductase genes (*nirK*, *nirS*) are the most commonly used molecular markers for denitrifier studies (Wallenstein, 2006) because nitrite reduction is the first step in the reaction that results in a loss of N as a gaseous product. Abundance and diversity of the genes for nitrite reductase (*nirK*, *nirS*) (Coyne et al., 1989) and nitrous oxide reductase (*nosZ*) can be used to estimate denitrification potential of a soil (Hallin et al., 2009; Philippot et al., 2009; Enwall et al., 2010). The *nirK* gene was detected in greater abundance than *nirS,* implying that bacteria with *nirK* are more responsive to the growth conditions influenced by N and CI treatments. Cropping intensity also has a significant (p<0.001) effect on *nirK* abundance, which was found to increase in response to CI, so was greater in the continuous wheat soils than the WWF soil. The cytochrome cd_1 dependent Nir gene *nirS* was observed to have similar abundance in all plots (Fig. 3.4) and remained unaffected by CI or N fertilizer treatment. The nitrous oxide reductase gene *nos*Z expresses the enzyme responsible for the conversion of N_2O to N_2 and is not found in all denitrifying bacteria. If a soil has lower *nos*Z abundance, it may have environmentally damaging consequences because without it, denitrification results in N_2O emissions, instead of merely losing N as dinitrogen gas to the atmosphere (Clark *et al.*, 2012; Throback *et al.*, 2004). In my study I observed that

continuous cropping resulted in greater abundance of *nos*Z, and so may be more environmentally beneficial than the use of fallow. The abundance of *nos*Z was significantly influenced (p<0.02) by CI with 100% CI soils having significantly greater abundance than 67% CI soils. In Hallin et al. (2009), an increase in *nirK* and *nosZ* gene abundances was reported with 100% CI (maize) when compared to 0% CI (bare fallow). My results suggest that fertilizer application contributed to a slight change in pH (Table B.1), and Hallin et al. (2009) also found that the type of fertilizer used influences denitrifier abundances as a result of pH changes. In Dandie et al. (2011), the denitrifier communities were studied in both riparian and agricultural soils (maize). Nitrous oxide reductase abundance was observed to be greater in the riparian soils, so therefore the more influential factor was soil moisture rather than the amount of N. Dandie et al. (2011) had similar findings because *nirK* abundance was greater than *nirS*, suggesting the *nirK* microbial community responded more positively to N fertilized maize production than those harbouring the *nirS* gene. In Clark et al. (2012), the abundance of denitrifier communities was assessed in a LTSE experiment composed of five different treatments: an unfertilized plot, two levels of N (144 kg ha^{-1}, 288 kg ha^{-1}), a farm yard manure plot and woodland plot. With the exception of the woodland plot, all soils were continuous wheat cropped. Their results were similar to Rotation A and C, with the abundances of *nosZ* and *nirK* responding to changes in N rate and were greater than *nirS*.

3.7 Conclusions

Ecosystem services help to make agricultural crop production viable (Giller et al., 1997; Topp, 2003) and the size and composition of a soil microbial community determines the range ecosystem services. The LTSE Rotations A and C (Lethbridge, AB) were studied to gain insight in to the impact of 100 years of agricultural management on nitrogen functional genes in

soil microbial communities. These soils differed in terms of C returns and N input, and because of the tremendous buffering potential of soils, it often takes decades for soils to adjust to changes in treatments. Decades of N fertilizer application combined with over a century of continuous wheat production resulted in increased total and bacterial biomass, as compared to unfertilized WWF. The biomass of fungal, Gr – and Gr+ bacterial groups were significantly increased by N fertilizer. Overall, the continuous presence of a crop every growing season combined with N fertilization in Rotation A soils increased the supply of C substrates (from crop residues) available to soil microbes, and as a result these organisms grew more abundantly than those in the WWF soils. Characterization of ammonia oxidizer and denitrifier populations studied suggested these N cycling microorganisms responded to differences in C and N inputs resulting from decades of differential soil amendments. In my study, the ratio of archaeal ammonia oxidizers was much higher than bacterial ammonia oxidizers in unfertilized soils. In contrast, the AOA and AOB populations were more equivalent in the fertilized soils indicating a tolerance for different soil environments. Denitrifier abundance was positively influenced by CI, with significantly greater *nosZ* and *nirK* populations in the 100% CI soils. These results indicate a potential for losses of N_2 gas, given suitable anoxic soil conditions. The changes observed in this study indicated that variation in soil amendments and crop production methodology did alter soil microbial community structure.

4.0 Characterization of Bacterial Diversity and N Functional Gene Abundance based on DNA Extracted from Archived Soils

4.1 Preface

In Chapter 3, the cumulative response to decades of nitrogen (N) fertilizer amendments and a century of varied cropping intensity (CI) on soil microbial community structure and N functional gene abundance were discussed. The study of the relationship between N fertilizer and soil microbes was important because both are influential components of sustainable crop production. In the following chapter, my work investigates the soil archives that accompany the Rotation ABC. The following study helps to answer the questions: do soil bacterial and archaeal communities and N functional groups become more abundant over time with N fertilizer treatments; do soil bacterial populations lose genetic diversity during 100 years of wheat monocropping; and, does DNA yield and quality decrease over time in air dried archived soils? Since it has not been determined if viable DNA can be extracted and characterized in order to better understand the long term effects of CI and N on soil microbial communities at this location, my work fills a knowledge gap.

4.2 Abstract

The Rotation ABC LTSE at Lethbridge, Alberta was established in 1910 to study the effect of fallow and fertilizer in a spring wheat rotation. The associated soil archives provide a long-term record of change in many common agricultural practices. The storage conditions of archived soil collections (air dried and at room temperature) present challenges for the preservation of microbial diversity and for the extraction of DNA representative of microbial populations. In a short term study, fresh soils were air dried, ground and then stored at room temperature with DNA extracted at 8 time points (T_0 to T_8) over 30 months. Both storage time and $N_{45}P_{20}$ fertilizer treatment influenced the amount of DNA extracted. The diversity of the soil

microbial community did not change from T_0 to T_8, with Inverse of Simpson scores higher in $N_{45}P_{20}$ fertilized soils than in unfertilized check. Rotation ABC soil samples archived in 1910, 1940, 1967, 1993 and 2011 were characterized using 16S rRNA gene profiling. An increased relative abundance of several bacterial groups was observed in the soil samples including: Proteobacteria, Acidobacteria, Planctomycetes, Armatimonadetes, and Nitrospira. The diversity of soil bacteria was estimated with S_{obs} and increased between soil samples from 1910 and 2011. Quantitative PCR (qPCR) results suggested 100% CI and N fertilizer treatments increased nitrogen functional gene (NFG) abundances more than 67% CI and check soils did, likely due to greater C and N substrate availability. My results indicated the abundance of ammonia oxidizing archaea was more influenced by CI than N fertilizer, while ammonia oxidizing bacteria were more stimulated by N fertilizer additions than CI. Denitrifiers with *nos*Z responded positively to both 100% CI and N fertilizer, while populations with *nir*K and *nir*S were more stimulated by 100% CI than N fertilizer.

4.3 Introduction

Many research institutions exist globally that maintain collections of archived soils obtained through short and long term field experiments (Dolfing and Feng, 2015). Soil archives function as references for assessing change to soil and environmental quality. As new techniques become standard, soils collected before environmental disturbances are baselines that allow the assessment of effects to soil microbial communities to be more certain (Cary and Fierer, 2014). DNA based microbial studies of soil archives are advantageous compared to culture based investigations because bacterial DNA survives the archiving process much better than culturable bacteria (Clark and Hirsch 2008), and also because the unculturability of the majority of soil microbes leads to biased results (Martin-Laurent et al., 2001). Although culture based studies of

archived soils have shown that spore forming bacteria are better adapted to survive the dry storage conditions, the DNA of the non-surviving organisms can also persist for long periods of time, protected by clay particles and soil organic matter (Clark and Hirsch 2008; DeNobili et al., 2006).

In recent studies of soil archives, DNA based methods were utilized to uncover detailed information on the long term effects of agricultural practices on soil microbial communities, monitor the levels of antibiotic resistance genes over time, and changes to soil fertility (Clark and Hirsch, 2008; Feng et al., 2015; Hirsch et al, 2012; Knapp et al., 2009). By using the soil microbial communities in archived soils as a baseline, alterations to soil microbial community diversity and abundance can be observed. These observations are important to determine if certain land use practices are leading to decreased soil bacterial diversity. Soil microbes are important to the biogeochemical cycling of nutrients, and when greater diversity is present, the ecosystem is better able to withstand environmental stress (Giller et al., 1997; Topp, 2003). This increased microbial diversity permits functional redundancy which means that multiple organisms are able to perform the same ecosystem services under different conditions.

The Rotation ABC LTSE is a Canadian prairie resource with a soil archive collection and an actively monitored field experiment. The soil samples collected at various intervals over the last century were initially utilized to assess the effects of crop rotation on soil quality (Smith et al., 2012). Through the use of modern molecular biology tools, these same soil archives provided the basis for examining change in soil microbial community abundance and diversity over 100 years of agricultural land management. In common with most historic field experiments, Rotation ABC has evolved over time to reflect technological advances in crop production (Clark and Hirsch, 2008; Janzen, 1995) and has applied N and P treatment since 1967 and 1972

respectively. To date, none of the studies conducted in the Lethbridge long-term plots utilized modern molecular tools to examine microbial nucleic acids. My work will begin to fill this knowledge gap with two studies. The first study examines the viability of DNA in air dried soils, with the objective of determining the effects of long-term storage of soil samples on microbial DNA abundance and quality. The second study was a survey of the bacterial DNA extracted from archived soils with the objective to assess changes in both the bacterial diversity and abundance of key N functional genes.

4.4 Materials and methods

4.4.1 Effect of soil storage conditions on soil bacterial community structure

4.4.1.1 Soil sampling, grinding and storage

The standard procedure for archiving soils for Rotation ABC involves air drying and grinding soils followed by storage in sealed glass vials at room temperature (RT). In order to demonstrate putative changes in soil bacterial communities during the archiving process, fresh soils were processed in the same manner as the long-term soil archive, stored for 30 months and the microbial community analysed periodically with DNA profiling. These fresh soil samples were collected prior to seeding in April 2012 from the N_0P_0 and $N_{45}P_{20}$ treatments of both Rotations A and C to examine soils with and without fertilizer inputs. Soil cores were collected with truck-mounted, hydraulic driven soil coring equipment (Giddings Machine Company, Fort Collins, CO) along transects 1 to 4 with four 10 cm cores (3.75 cm diam.) for each replicate (Fig. 3.1). Soils were stored on ice in portable coolers during transport and processed within 48 h. Soil cores were homogenized prior to sieving to 4.0 mm and subsampling. Soils were then air dried for 7 days at RT, ground, subsampled and stored at RT to mimic the conditions used to preserve soils for archival storage.

4.4.1.2 Soil DNA extraction and storage time course

DNA was extracted from soils using a modified method for nucleic acid extraction (Clark and Hirsch, 2008; Knapp et al., 2010) using four technical replicates of 0.2 g each (n=4). Prior to extraction, a rehydration step was performed in the Mo Bio Powersoil Bead tubes (Mo Bio Laboratories, Carlsbad, CA), modified from Clark & Hirsch, 2008. The archived soils were rehydrated to 8.5% water with nuclease free PCR grade water (Life Technologies Inc., Burlington, ON), then incubated for 20 minutes at 4˚C (Clark and Hirsch, 2008). The remainder of the DNA isolation protocol followed the Mo Bio Power Soil kit instructions (Mo Bio Laboratories, Carlsbad, CA). A DNA elution pooling method was performed to increase DNA concentration, in which the four technical replicates were concentrated into 100 µL eluent per soil eluted (Fierer et al., 2012). Specifically, the first replicate was eluted in 100 µL TE buffer. The eluent was then used to elute a second replicate column, followed by elution of a third and fourth replicate in the same manner. DNA was quantified using a Qubit 2.0 Fluorometer (Life Technologies Inc., Burlington, ON) following the manufacturer's instructions for dsDNA high sensitivity (HS) reagents (Table 3.1). To establish a soil storage time course, DNA was extracted from the N_0P_0 and $N_{45}P_{20}$ soils at 8 sampling points from the instigation of the experiment. The DNA extractions for time 1 to 7 were at 3 month intervals and from time 8 were performed 12 months later for the total storage duration of 30 months.

4.4.1.3 Illumina MiSeq next generation sequencing of 16S rRNA v4 region

Following extraction and quantification, DNA was sent to Genome Quebec at McGill University for characterization using an Illumina MiSeq platform (Montreal, QC, Canada). PCR was performed for each genomic DNA samples to amplify the V4 region of bacterial rRNA gene DNA using oligonucleotides 515F (GTGCCAGCMGCCGCGGTAA) and 806R

(GGACTACHVGGGTWTCTAAT) from Caporaso et al.(2012). In the following step, PCR amplicons were purified with Ampure XP beads (Beckman Coulter, Canada) and ligated to index adapters, pooled in equal concentrations, denatured and sequenced according to Illumina guidelines (Illumina, San Diego CA, USA). Bioinformatics were performed with Mothur v.1.35.1 and with the Schloss MiSeq protocol (accessed August 25, 2015). The quality control parameters were to remove sequences > 310 bp, along with ambiguous base calls > 0 bp and homopolymers runs > 8 bp. The operational taxonomic units (OTUs) were defined at 97% similarity and classified with the Silva and RDP databases, with specifications to omit non-bacterial taxonomy. A sequencing depth of 19,836 sequences was selected prior to further analysis. Rarefaction curves and diversity measures of single observed species (SOBs), and Inverse Simpson were calculated using Mothur v. 1.35.1. Operational taxonomic units were subjected to nonmetric multidimensional scaling (MDS) ordination (McCune and Grace, 2002) in PCOrd v.6.0., using the Slow and Thorough automated method with a distance metric of Sorensen (Bray-Curtis).

4.4.2 Bacterial Diversity and N Functional Gene Abundance of DNA Extracted from Archived Soils

4.4.2.1 Soil sampling for the soil archive and management history of the Rotation ABC experiment

Soils were collected from Rotation ABC, an Agriculture and Agri-Food Canada long term soil experiment (LTSE) initiated in 1911. The site is located on Orthic dark brown Chernozem soils, with a MAP 386.0 mm and a MAT 5.7°C. The standard procedure for archiving soil samples was to take two 67 mm diameter cores from 0.0 to 30.0 cm with 5 replicate sites per treatment, cleaning the core with a wire brush between samples. To homogenize the soils, stones and plant residues were removed prior to sieving to 2mm particle size. Following sieving, soils

were air dried for a minimum of 72 hours at ambient temperatures (18-28°C), in unsterile field buildings, with precautions taken to minimize air born contaminants. Soils samples were then ground and stored in glass vials at room temperature. With the exception of soils sampled in 2011, subsamples from each 5 replicates of the top 0 to 7.5 cm layer were combined and used to characterize microbial communities. In 2011 the field soils were sampled 0 to 15cm so this subsample was used in place of a 0 to 7.5 cm subsample.

In this LTSE, the soils were not sampled and archived every year, but rather periodically. Soil archived in 1910, 1940, 1967, 1993 and 2011 were chosen for this preliminary study because relatively large amounts of soil were collected in those years, however only one composite sample was available from each soil. The 1910 soils were collected when relatively diverse native prairie was plowed for wheat crop production. The 1940 soils represent 30 years of unfertilized wheat production. Beginning in 1910, the harvesting practice was to export crop residues from the fields, to a central threshing location. A change in harvesting method to combine harvesters occurred in 1943, and resulted in crop residues remaining in the field. In 1967 an N fertilizer treatment was overlaid on the LTSE: 45.0 kg nitrogen (N) ha^{-1} as ammonium nitrate (34.5-0-0), dividing each phase into a check (N_0) or N fertilized (N_{45}) subplot. This practice continued as N broadcast prior to seeding to all phases until 1985, at which time it was adapted to include only the cropped phases. In 1972, a second fertilizer treatment was imposed on the sub plots: triple superphosphate (0-46-0) fertilizer applied with the wheat seed, 45 kg ha^{-1}, until 2010 when it was reduced to 22.5 kg ha^{-1} (Smith et al., 2012). This addition of P resulted in two new sub plots of P only (N_0P_{20}) and also with N ($N_{45}P_{20}$) per rotation.

4.4.2.2 DNA extraction

DNA was extracted from the air dried soils as in section 4.4.1.2.

4.4.2.3 Illumina MiSeq DNA sequencing of 16S rRNA v4 region

DNA samples were sequenced and analyzed as in section 4.4.1.3 using the Schloss MiSeq protocol (accessed May 25, 2015). A sequencing depth of 5,702 sequences was selected prior to further analysis.

4.4.1.4 Nitrifier and Denitrifier gene abundances

The construction of quantitative PCR standard curves was detailed in section 3.4.3. Quantitative real-time PCR (qPCR) was performed in triplicate using an ABI Step One Plus, and Platinum SYBR Green qPCR SuperMix, (Life Technologies, Burlington, ON) to detect the abundances of 16S rRNA, bacterial and archaeal ammonia monooxygenase (*amoA*), nitrous oxide reductase (*nosZ*), cytochrome cd1 type nitrite reductase (*nirS*) and copper nitrite reductase (*nirK*) gene copies. As in the previous chapter, optimized conditions for each target, including reaction mix components and genomic DNA concentrations used are listed in Table 3.1. Bovine serum albumin (Life Technologies, Burlington, ON) was included in the assays at a rate of 0.2 $\mu g\ \mu L^{-1}$ to reduce potential PCR inhibition caused by residual humic acids in the DNA extractions. The passive reference dye 5-carboxy-X-rhodamine (ROX) was added to the reactions to normalize for differences in fluorescent reported signal between wells at a concentration of 50 nM. Melt curve analysis was performed after each assay to test for the presence of primer dimer formation and assess the specificity of the reaction. Gene copy numbers were standardized by the dry weight (g^{-1}) of soil analyzed for each sample, then $\log_{10}$ transformed. Statistics were performed using a one-way ANOVA in SPSS v.20 to determine the significance of cropping intensity and N fertilizer additions on gene abundances.

4.5 Results

4.5.1 Soil bacterial community stability in soils under simulated archival conditions

The amount of DNA extracted was affected by both storage time and fertility treatment. There was a significant interaction of time in storage and fertility treatment (p<.0001). The highest amount of DNA was extracted at 18 months in $N_{45}P_{20}$ soils and the lowest at 3 months from N_0P_0 soils (Table 4.2).

Table 4.2 DNA concentration (μg g^{-1}soil) of air dried soils from Rotation A. Standard error indicated in parenthesis.

Time (months)	**DNA conc. (μg g^{-1}soil)**	
	N_0P_0 (kg ha^{-1})	$N_{45}P_{20}$(kg ha^{-1})
0	0.38(0.01)	5.76(0.88)
3	0.85(0.07)	1.32(0.18)
6	1.00(0.12)	1.54(0.30)
9	1.45(0.13)	2.64(0.37)
12	2.04(0.28)	2.70(0.31)
15	2.15(0.48)	4.30(0.13)
18	4.94(0.25)	8.97(0.51)
30	3.07(0.18)	3.42(0.15)
	ANOVA p-value	
	Time	**0.0001******
	Fertility	**0.0001******
	Time x Fertility	**0.0001******

To eliminate biases due to uneven sequencing depths between sample libraries, high throughput sequencing of 16S rRNA genes were analysed at the same value of 19,836 sequences per sample. There were bacteria from 16 phyla identified (Fig. 4.2). Changes in the relative abundance of 10 phyla (Actinobacteria, Proteobacteria, Bacteriodetes, Acidobacteria, Planctomycetes, Firmicutes, Chloroflexi, Verrucomicrobia, Gemmatimonadetes, and

Armatimonadetes) accounted for most of the change in community structure amongst the samples.

Storage time (to 30 months) did not significantly change the relative abundance of Actinobacteria, Proteobacteria, Bacteriodetes, Acidobacteria, Planctomycetes, Verrucomicrobia, Armatimonadetes or unclassified bacteria. A significant increase (p<0.0001) in the relative abundance of Firmicutes (Fig. 4.1) occurred as time in storage increased, in both fertilized and unfertilized soils. The $N_{45}P_{20}$ fertility treatment had significantly increased the relative abundance of Actinobacteria (p<0.0001), Proteobacteria (p=0.04), Bacteriodetes (p<0.0001), Acidobacteria (p<0.0001), Planctomycetes (p<0.0001), Verrucomicrobia (p<0.0001), Armatimonadetes (p=0.0014) and unclassified bacteria (p=0.0167). A significant interaction between storage time and fertility treatment occurred in Chloroflexi (p=0.0245) and Gemmatimonadetes (p=0.0125).

The diversity of the soil bacterial community during storage time was determined with the inverse of Simpson index (1/D) and it was relatively stable during the study (Fig. 4.2). The bacterial diversity increase in the unfertilized soils (1713 to 1877 OTUs), in contrast, a decrease was shown in the fertilized soils (2234 to 2161 OTUs). The total number of species observed in a sample (S_{obs}) results did not indicate loss of bacterial richness during storage time because almost double the S_{obs} value was observed in fertilized when compared to unfertilized soils, 116.64 to 112.77 vs. 57.65 to 60.62, respectively. The nonmetric MDS analysis of OTUs did not show a relationship between storage time and OTU profiles (Fig. 4.3). Rather, field replicate was a strong influence shaping bacterial communities. This result was further supported by Indicator Species Analysis (ISA) which found more OTUs to be significant indicators of fertilizer treatment (IV 60-100%, p<0.005) rather than storage time (IV 38-53%, p<0.05).

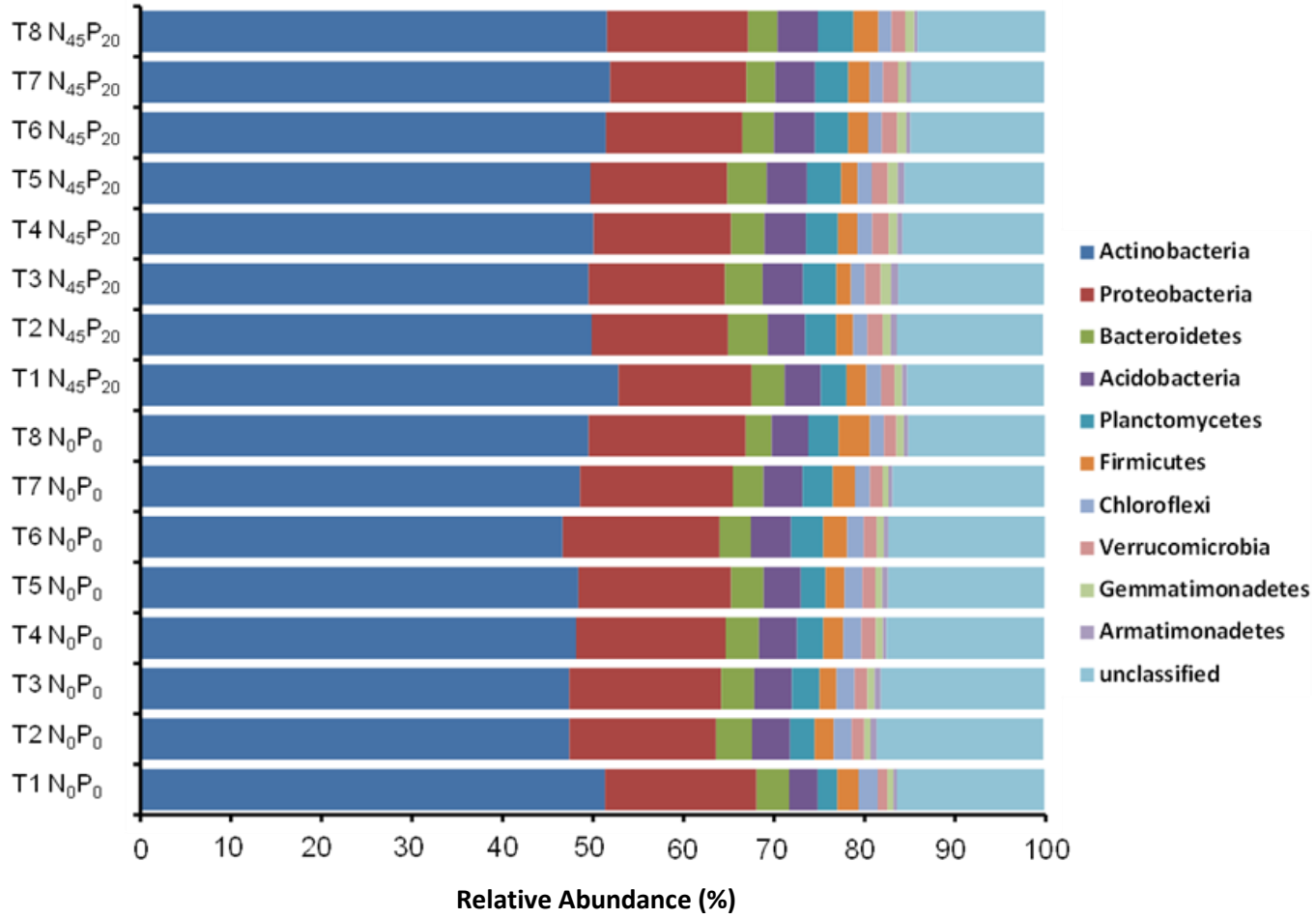

Fig. 4.1 Relative distribution of bacterial phyla (%) from DNA profiles of fertilized ($N_{45}P_{20}$ kg ha^{-1}) and unfertilized (N_0P_0 kg ha^{-1}) and Rotation A soils stored for 30 months.

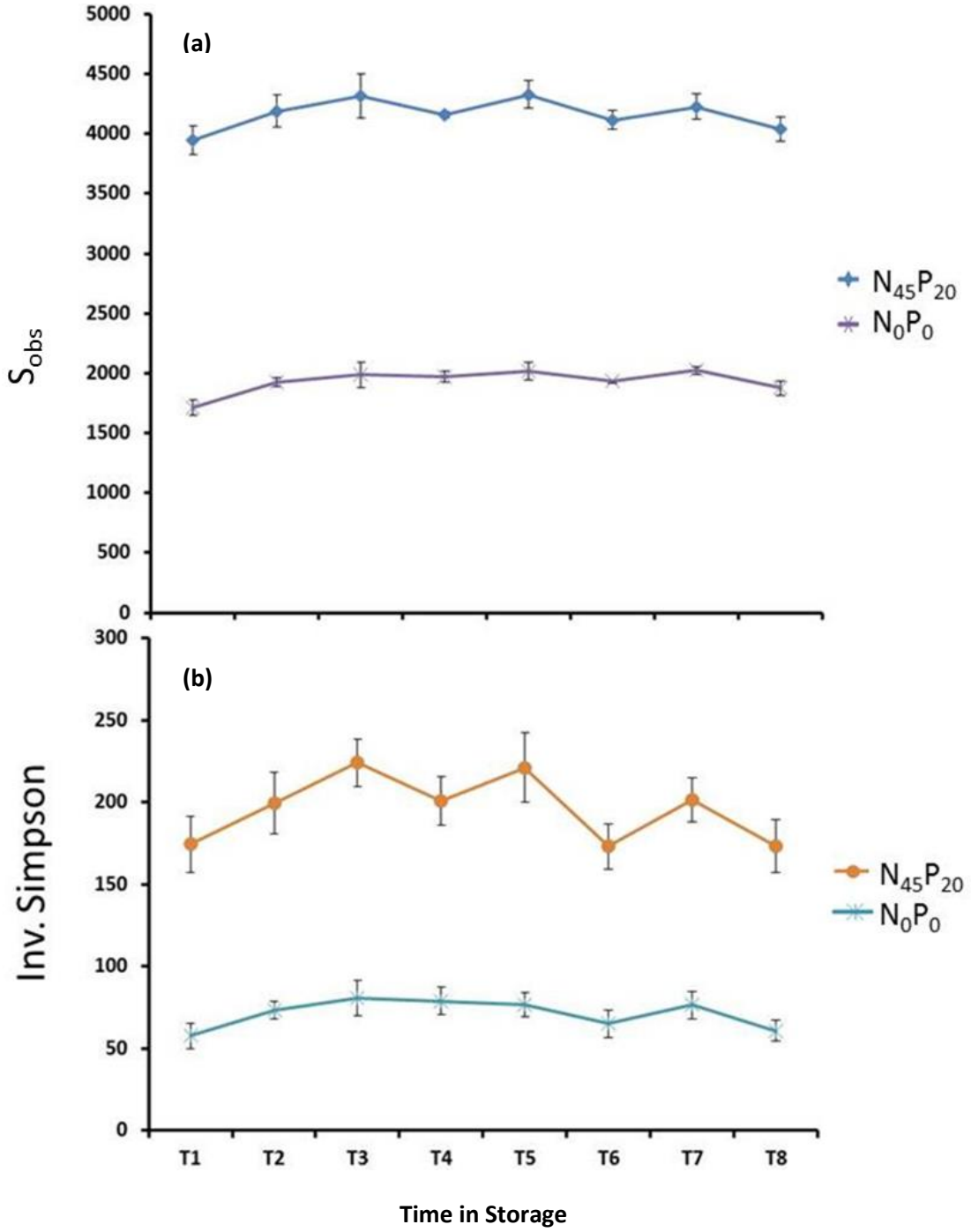

Fig. 4.2 Bacterial diversity indices in fertilized soils ($N_{45}P_{20}$ kg ha^{-1}) and unfertilized soils (N_0P_0 kg ha^{-1}) over 30 months of storage under archival conditions, estimated with (a) S_{obs} and (b) Inverse of Simpson (1/D). Error bars denote standard error.

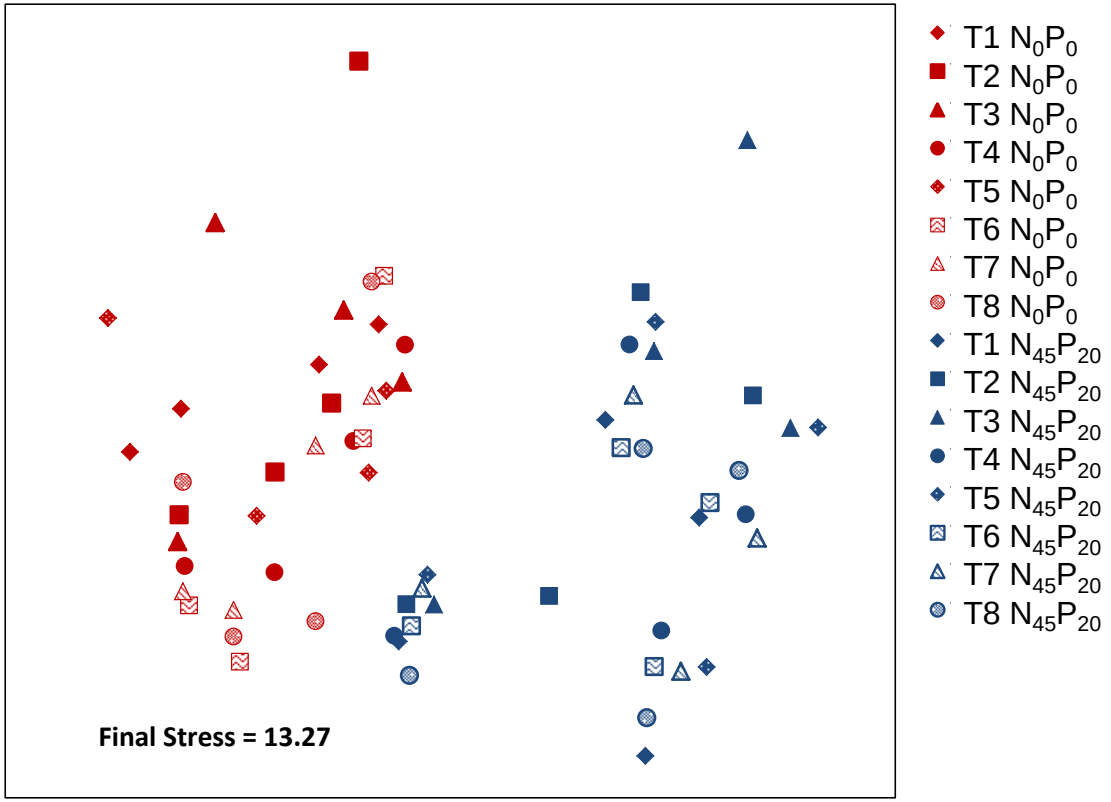

Fig. 4.3 Nonmetric multidimensional scaling (MDS) ordination (stress= 13.27) analysis of OTU profiles of unfertilized (N_0P_0, denoted in red) and fertilized ($N_{45}P_{20}$, denoted in blue) Rotation A soil stored for 30 months.

4.5.2 Variations in phylogenetic groups over 100 years of soil archives

This study assessed soil samples (non-replicated) from five time points in this archived collection spanning over 100 years. The soil samples included native prairie soil from 1910 as well as fertilized split-plot treatments in 1993 and 2011. These soils were characterized for the amount of genomic DNA recovered, 16S rRNA gene phylogenetic distribution and N functional gene abundances. The results showed that at each time point, the amount of DNA recovered did not appear to be influenced by fertility treatment or cropping intensity (CI), (Table D.1, Appendix D). The analysis of high throughput sequencing of 16S rRNA genes was performed on a sampling depth of 3293 sequences per sample. A total of 18 bacterial phyla were detected and of these, 12 phyla accounted for most of the community structure detected within the DNA extracted from archived soils (Fig. 4.4). Sample collection year was associated with changes to relative abundance in bacterial populations more so than N fertilizer treatment status or CI. The relative abundance of Proteobacteria, Acidobacteria, Planctomycetes, Armatimonadetes and Nitrospira increased over time in the DNA extracted from soils collected 1910-2011. The relative abundance of Gemmatimonadetes and Bacteriodetes were more abundant in the DNA extracted from soils collected in 1993 than other time points. The relative abundance of Firmicutes decreased in the DNA extracted from soils collected 1910-2011. The relative abundance of Actinobacteria was observed to decrease in the DNA extracted from soils collected 1910-1993, then increase in 2011. The relative abundance of Crenarchaeota and unclassified bacteria did not appear to change in the DNA extracted from soils collected 1910-2011.

The diversity of soil bacteria detected in DNA extracted from soils collected in 1910-2011 was estimated with S_{obs} and the inverse of Simpson index (Fig. 4.5). Sampling year appeared to be the most influential factor on bacterial diversity, more so than N fertility or CI. Bacterial

diversity increased in DNA extracted from both Rotation A and C soils collected 1910-2011. The results of S_{obs} calculations indicated bacterial richness was similar for both CI levels and increased in a nearly linear manner over the time span studied (Fig. 4.5).

Nonmetric multidimensional scaling (MDS) was completed on the OTU profiles of Rotation A and C soils collected 1910-2011 to observe relationships resulting from time, CI and fertilizer treatments (Fig 4.6). The data points grouped together most strongly by sampling year, followed by CI and fertility treatment. These results suggest greater similarity within a sampling year. The community structures of the OTU profiles from 1910, 1940 and 1967 are more closely grouped than those of 1993 and 2011. Differences in community structures could be attributed to changes influenced by the sample year and CI level.

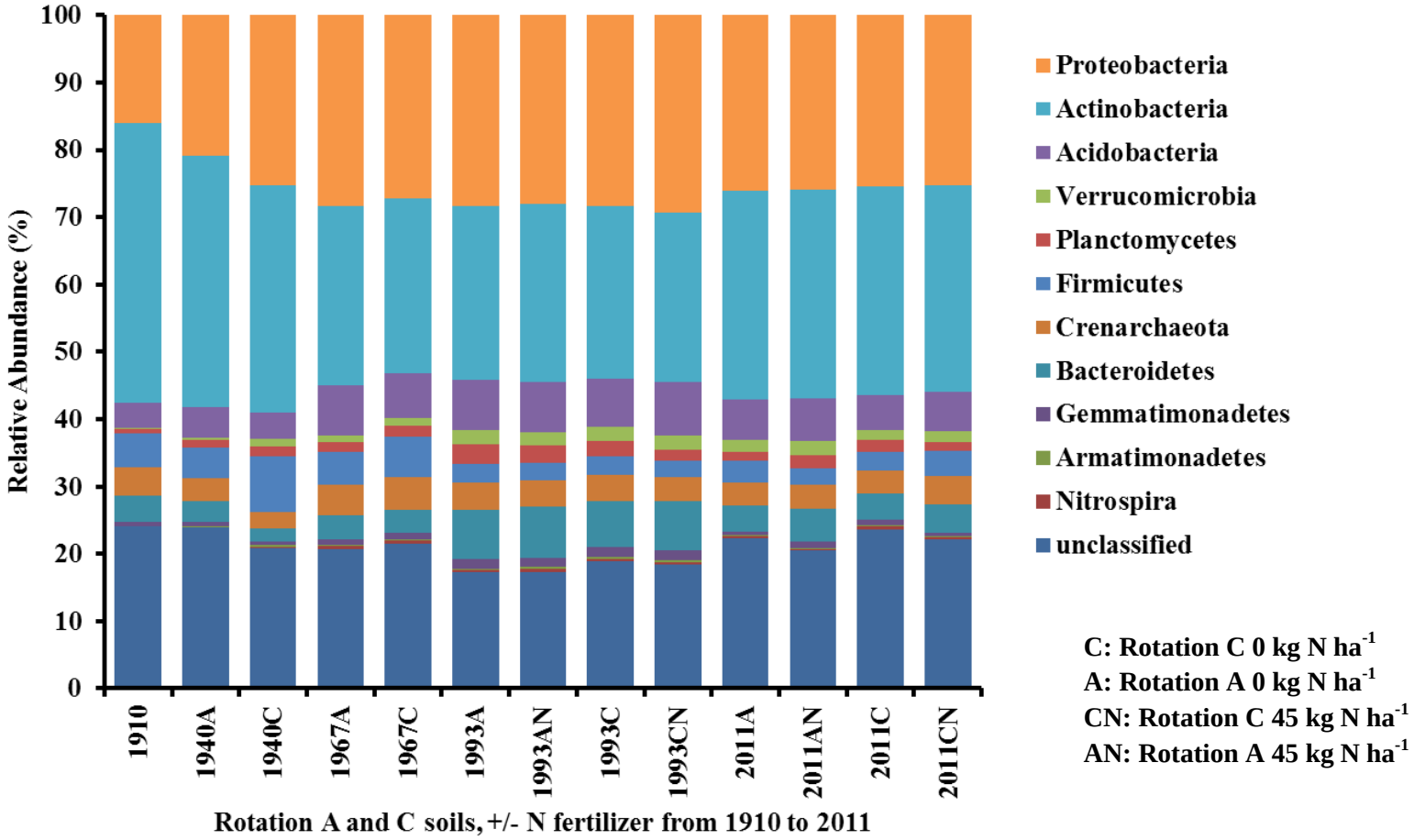

Fig. 4.4 Relative abundance (%) of bacterial phyla in Rotation A and C (0 and 45 kg N ha^{-1}) soils archived in 1910, 1940, 1967, 1993 and 2011.

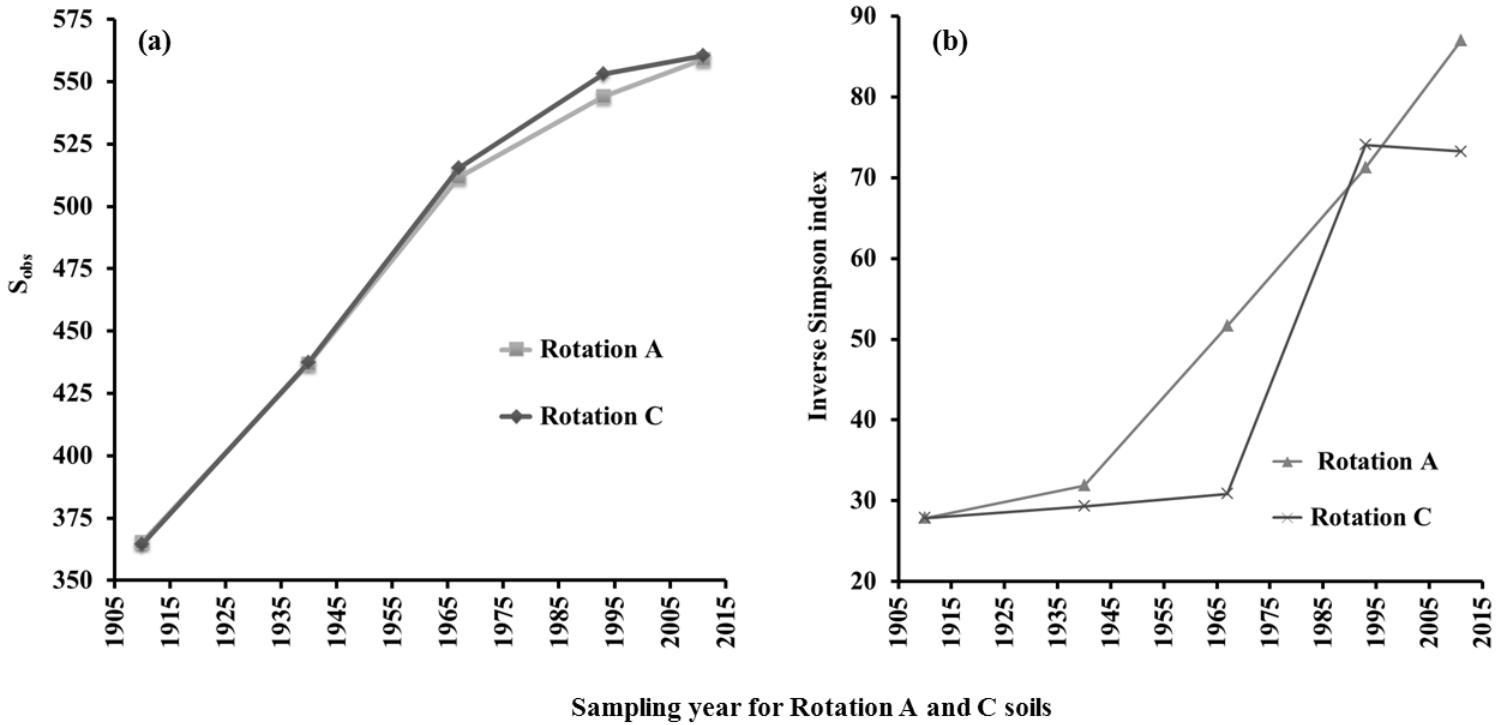

Fig. 4.5 Estimations of bacterial diversity over time as estimated with (a) S_{obs} and (b) Inverse of Simpson diversity indices fertilizer treatments combined for each year.

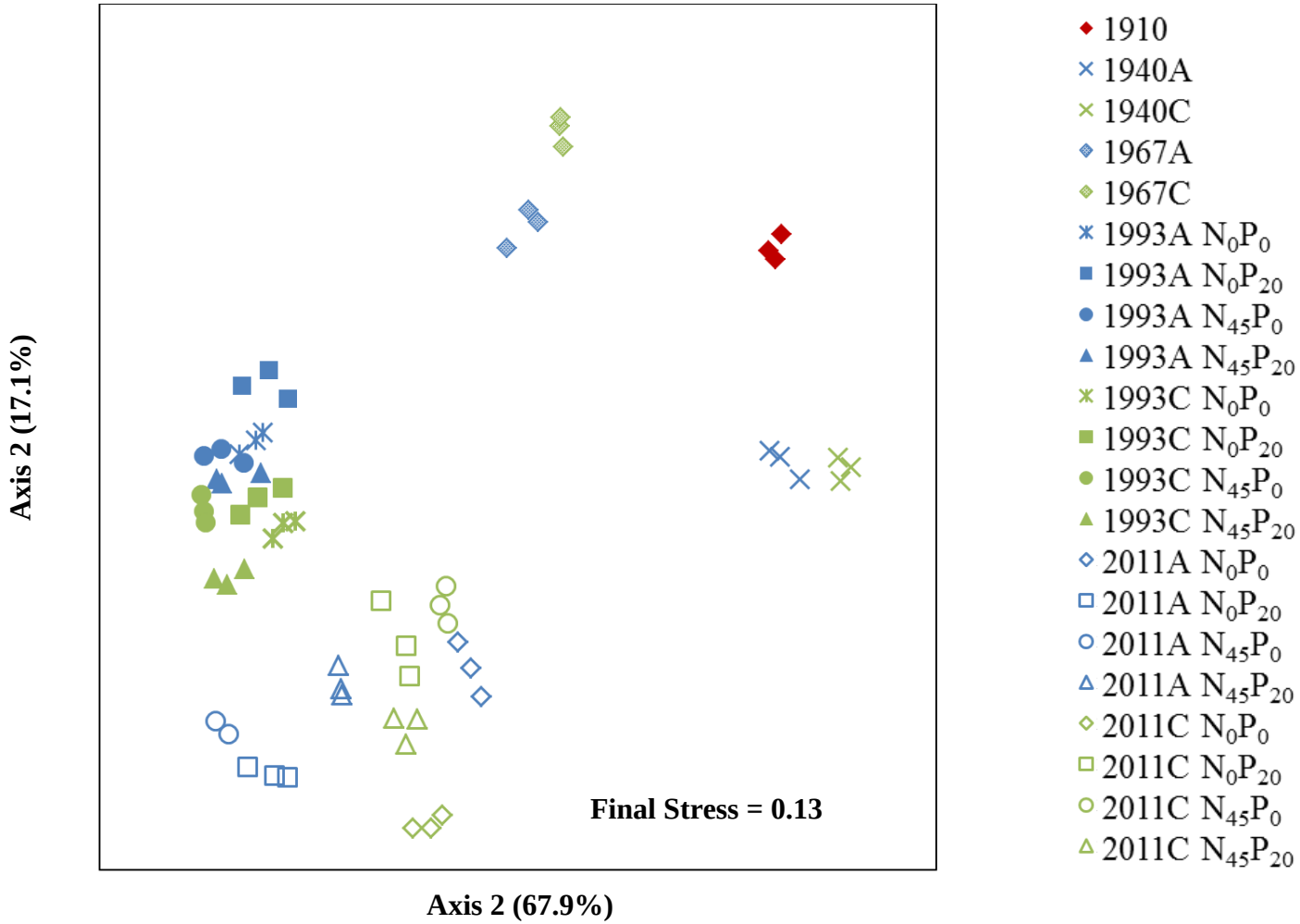

Fig. 4.6 Nonmetric multidimensional scaling ordination of community structures of OTU profiles resulting from 16S rRNA gene profiles of Rotation A (blue) and C (green) soils archived in 1910 (red), 1940, 1967, 1993 and 2011. Final stress 0.13.

4.5.3 N functional gene abundances over 100 years of soil archives

The N functional genes including ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacterial (AOB), nitrous oxide reductase (*nosZ*), copper nitrite reductase (*nirK*) and cytochrome cd1 type nitrite reductase (*nirS*) were estimated using DNA extracted from the archived soils. The qPCR efficiencies ranged from 80.2 to 99.5 %, with R^2 values of .98 to .99. The abundance of AOA increased 2.5 fold (7.5 to 7.9 log gene copies g^{-1}) in DNA extracted from the archived soils of 1910-1967 with the exception of DNA extracted from the 1940 Rotation C soils which in contrast decreased 2.3 fold (7.5 to 7.1 log gene copies g^{-1}) (Fig. 4.8). A further 2.5 fold (7.9 to 8.3 log gene copies g^{-1}) increase in AOA abundance was observed in the DNA extracted from the 1967-1993 soils. The abundance of AOB detected in DNA extracted 1940 soils was 10 times greater than those of 1910, and was uninfluenced by CI levels. In contrast, the AOB abundances in the DNA extracted from the archived soils of 1967 and 1993 demonstrated a difference between N levels, with increased AOB detected in 1993 soils with N fertilizer applied.

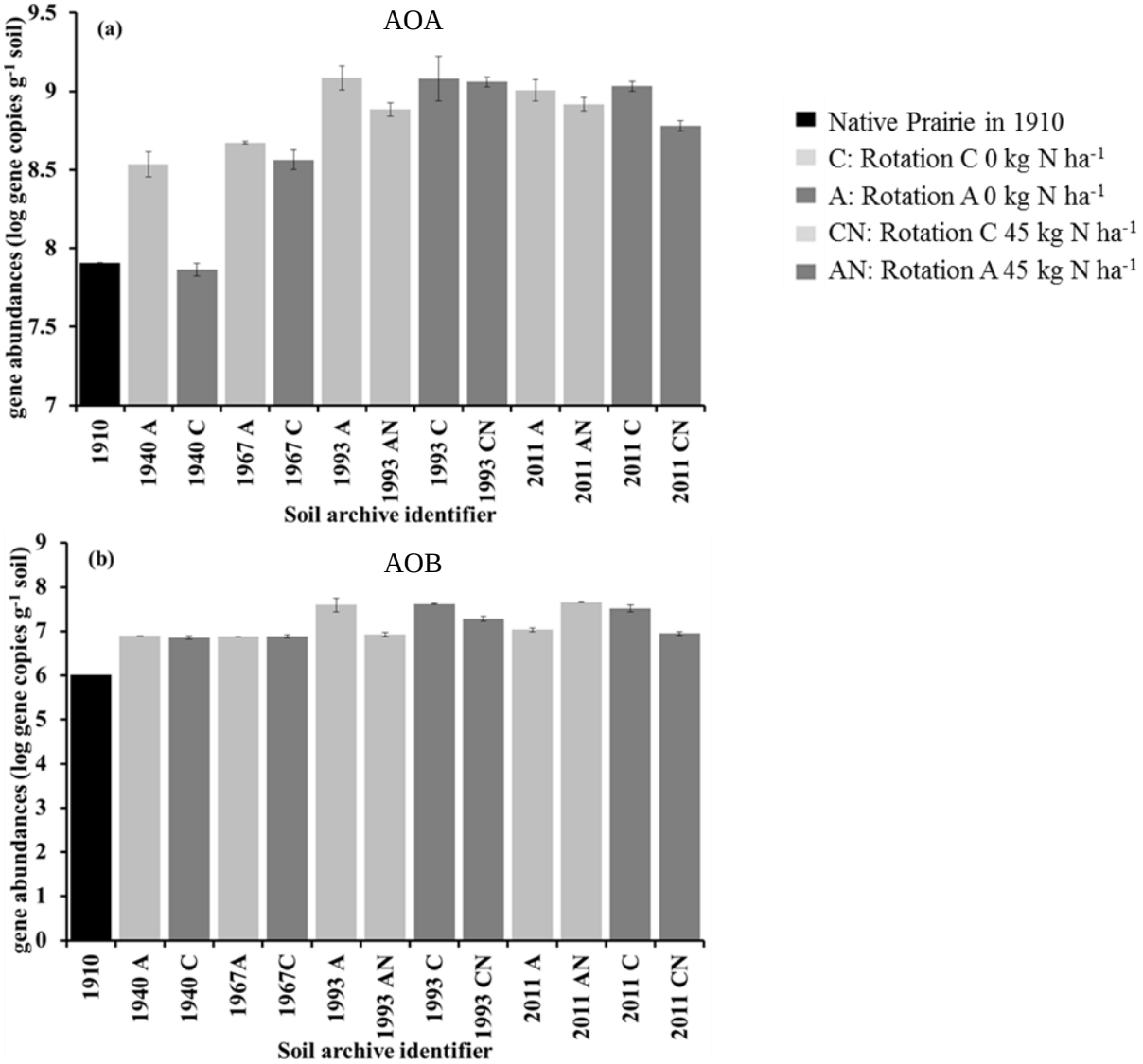

Fig. 4.7 Ammonia oxidizing archaea (AOA) (a) and ammonia oxidizing bacteria (AOB) (b) log gene copies g^{-1} soil; error bars denote standard error in technical replicates.

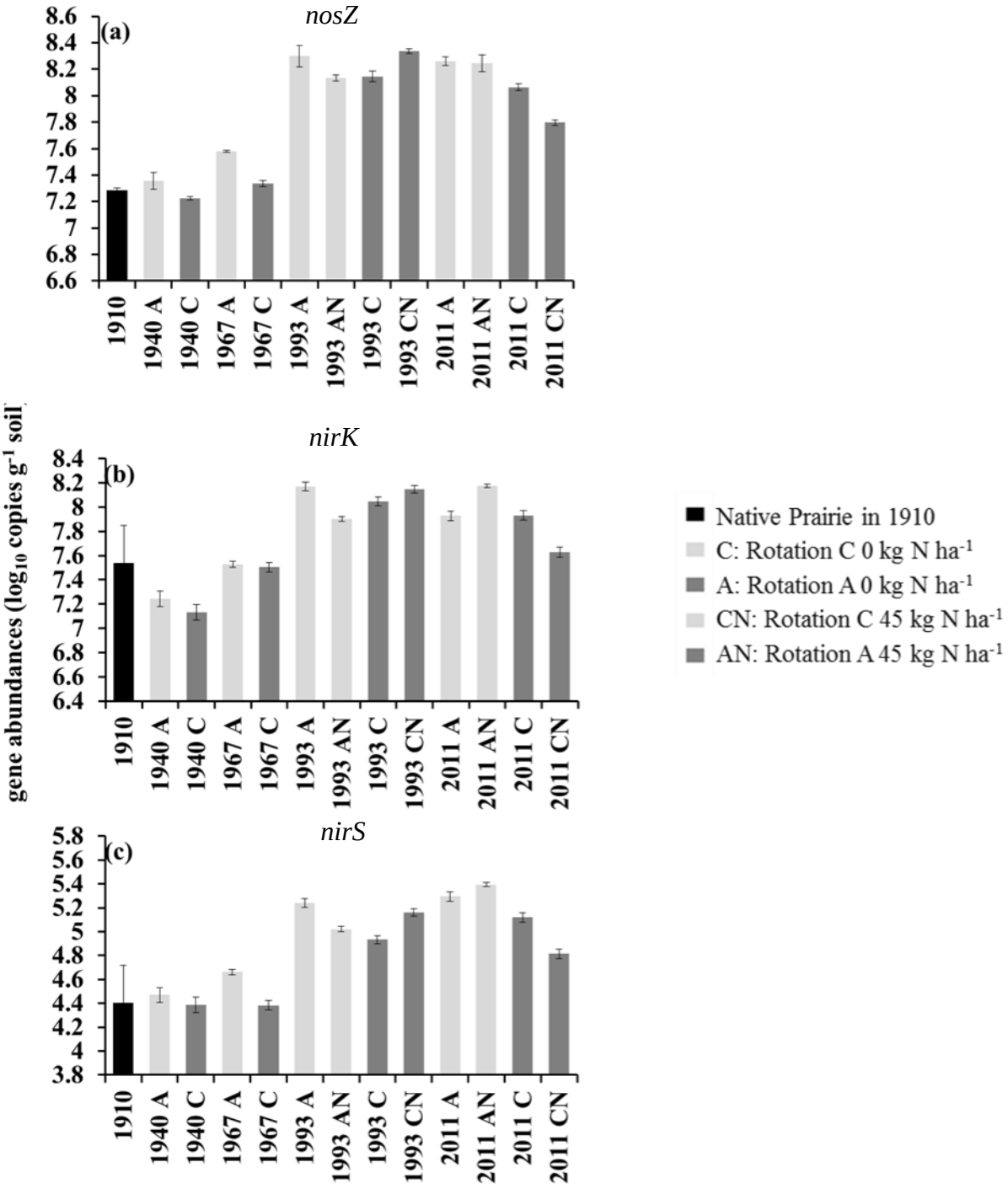

Fig. 4.8 Denitrifier gene abundance of *nosZ* (a), *nirK* (b), and *nirS* (c), g^{-1} soil; error bars denote standard error in technical replicates.

The estimated abundance of the bacterial denitrification genes *nosZ*, *nirK* and *nirS* decreased in DNA from 1910 and 1940 soils and then increased in the 1967, 1993 and 2011 soils (Fig. 4.8). The estimated abundance of *nosZ* gene copies in DNA extracted from 1910 and 1940 soil samples were greater at 100% than at 67% CI (Fig 4.8a). The 1967 and 1993 soils show increased *nosZ* genes abundance for both levels of N and CI. The estimated abundances of *nirK* gene copies in the 1940 soils were lower than in 1910 and 1967 soils (Fig 4.8b). This suggested an influence of crop residue, because crop residue return was adopted as common practice in 1943. The estimated abundance of *nir*K gene copies in 1993 soils was greater than those in 1910 and 1967 soils. The estimated abundance of *nir*K gene copies in the 2011 soils was less abundant than of the 1993 soils. The estimated abundance of *nir*S in 1910 soils was greater than in 1940 soils under both 100% and 67% CI levels (Fig 4.8c). In contrast, the estimated abundance of *nir*S gene copies in the DNA extracted in 1967 from 100% CI soils surpassed both those of 1910 and 1940. The distribution of the estimated abundances of *nirS* gene copies in 1993 and 2011 soils at 100% CI were less influenced by N fertility treatments than soils at 67% CI. The estimated abundances of *nirS* gene copies followed the same trend as observed with *nosZ* and *nirK* profiles, with an increase in estimates as time in storage decreased.

4.6 Discussion

4.6.1 Soil bacterial community stability in soils under simulated archival conditions

This work demonstrated that modern molecular biology approaches can be applied to the investigation of DNA viability and bacterial community structure during the soil archiving process. To better understand the potential influence that soil archive conditions have on bacterial community structure, fresh soils were air dried, then ground and stored at RT. The soils

were subsampled for DNA extractions over 30 months. The N and P fertility treatments and storage time had a combined significant effect on the amount of DNA extracted (Table 4.2). The N and P fertilized soils had significantly greater quantities of DNA extracted than unfertilized soils. In Hirsch et al. (2016), DNA extraction yields were used as a proxy for microbial biomass and they found DNA yield significantly increased with the addition of plant cover and N fertilizer. In Chapter 3, PLFA abundances were measured as indicators of active microbial biomass and the results were soils with N fertilization and 100% CI had significantly greater total PLFA than unfertilized soils. In many agroecosystems, N fertilized soils have been found to have greater bacterial biomass than unfertilized soils (Mbuthia et al., 2015). Fertilized soils have greater plant productivity (Olsson, 1999) which in turn increases the level of soil organic matter. N and P are considered limiting nutrients to plant growth in most ecosystems (Agren et al., 2012) and fertilized soils allow higher plant productivity (Spohn et al., 2016). Increased plant productivity increases C substrates available to soil microbes, resulting from increased root exudates, and above and below ground residues (Geisseler and Scow, 2014).

Soil organic matter content is positively associated with increased soil DNA recovery (Terrat et al., 2012) and is known to protect microbial biomass and DNA from degradation in desiccated soils (Denobili et al., 2006; Clark and Hirsch, 2008). This positive association between SOM and DNA recovery may be in part because DNA is protected from degradation when bound by humic substances, as well as clay minerals, and sand particles (Burlage, 1998; Pietramellara et al., 2008). In addition, the desiccated condition of air dried soil is thought to be beneficial to storage because of inhibited enzymatic and microbial degradation of extracellular DNA (Levy-Booth et al., 2007). However, the archiving process does result in some degradation of DNA (Clark and Hirsch, 2008) and currently frozen storage is recommended for soil samples to allow optimal recovery of microbial DNA (Lauber et al., 2010; Tatangelo et al., 2014).

Another factor to consider is the possibility that DNA extracted from soils also contains extracellular DNA from plant and microbial origin (Pietramellara et al., 2009) as well as DNA resulting from cell lysis during the extraction procedure. The lysis of dead cells is thought to be the main source of extracellular DNA in soil (Levy-Booth et al., 2007) and the potential presence of extracellular DNA implies that the quantity of DNA isolated from a soil sample is not only a measure of living microbial biomass because it contains DNA from multiple origins and time periods.

The bacterial community structure appeared stable over time as 16S rRNA gene profiles did not change significantly during time in storage (Fig. 4.1), with the exception of the Firmicutes. The relative abundance of Firmicutes increased in the soils stored for longer periods of time, in part due to the ability produce endospores allowing resistance to desiccation. The hardiness of endospores was noted in Clark and Hirsch (2008), when it was reported that archived soils had greater relative abundance of Firmicutes than fresh soils (Davidson and Janssens, 2006). However, a recent study indicated that soil organic matter content increased the relative abundance of Firmicutes (Feng et al., 2015). The soil microbial community did not appear to lose diversity over the course of the study suggesting that DNA was protected from degradation over time in storage (Fig. 4.2) which was supported by an Indicator Species Analysis (ISA). This analysis at the OTU level suggested that time in storage had minimal impact on OTU profiles (Fig. 4.3). During storage, there is potential for the initial desiccation of the soil caused cell lysis in many microbial cells. These cell lysis events meant that DNA was released from the cells and this newly extracellular DNA might have been decomposed, or absorbed to clay minerals and humic substances (Nguyen and Chen, 2007; Pietramellara et al., 2008; Morrissey et al., 2015). Although the archiving process inhibited the enzymatic and microbial degradation of extracellular DNA, the DNA degradation rates of nucleic acids of different origin cannot be

assumed equivalent (Clark and Hirsch, 2008; Levy-Booth et al., 2007). Soils fertilized with N and P were estimated to have greater bacterial diversity (Fig 4.2) than the unfertilized soils and these results suggested that soil fertility levels were associated with maintaining the stability of the soil microbial community structure. The increased bacterial diversity in the N and P soils may also be a result of increased soil organic matter (SOM) due to increased plant productivity that in turn meant there were more humic acids available to bind DNA. In other recent soil storage studies, duration and temperature of the storage did not significantly influence bacterial community structure, at least in the short term (Campbell et al., 2009; Lauber et al., 2010; Tatangelo et al., 2014). In contrast, Rubin et al. (2013), storage time and temperature did cause changes in alpha diversity; however, this study found that a soil remained more similar to its original environment than to non-related habitats. My results indicate that air dried storage of soils did not change the relative diversity of soil microbial communities, and therefore historic soils stored in this manner may be successfully studied with molecular biology methods.

4.6.2 Variations in phylogenetic groups and N functional gene abundances over 100 years of soil archives

With the evidence that DNA persisted under 30 months of archival storage conditions, DNA was isolated from select archived soils and characterized with 16S rRNA gene sequencing. Out of the 18 phyla identified in soils collected 1910-2011, Proteobacteria and Actinobacteria were most abundance (Fig. 4.4). Proteobacteria are dominant phyla in terrestrial soils (Hirsch et al., 2016; Tardy et al., 2015; Jangid et al., 2011; Acosta-Martinez et al., 2008). Actinobacteria are known to form endospores (Macagnan et al., 2006) and are drought tolerant (Yandigeri et al., 2012) which is a potential explanation for the high relative abundance of Actinobacteria in archived soils vs. fresh (Ventura et al., 2007; Acosta-Martínez et al., 2010). Other phyla identified included Acidobacteria, Verrucomicrobia, Planctomycetes, Firmicutes, Crenarchaeota,

Bacteriodetes, Gemmatimonadetes, Armatimonadetes and Nitrospira (Fig. 4.4), and these phyla were consistently found in typical agricultural soil DNA profiles (Buckley et al., 2006; Tian et al., 2015). Acidobacteria relative abundance was greater in soils with N fertilizer additions, which may be related to the lower pH of these soils (Wessén et al., 2010) or indicate a faster growth rate (Ramirez et al., 2012). Verrucomicrobia are found in both water and soil and have been reported to increase in relative abundance during succession from cropland to grassland (Fierer et al., 2009; Jangid et al., 2011). Planctomycetes are related to Verrucomicrobia (Hou et al., 2008) and contain a lineage that is capable of anaerobically oxidizing ammonium (Strous et al., 1999). Planctomycete abundance has been correlated with the soil characteristics of pH, C, NO_3 concentration and N content (Buckley et al., 2006). Bacteriodetes are Gr-, spore forming, anaerobic rod shaped bacteria that are widely distributed in soil, water and animals. The relative abundance of Bacteriodetes in the archived soils was similar to fresh soils (Hirsch et al., 2016; Tardy et al., 2015; Jangid et al., 2011; Acosta-Martinez et al., 2008) and was greater in soils with N additions (Fig. 4.5). Bacteriodetes are reported to be copiotrophic, and the increased relative abundance was potentially in response to the greater amount of C inputs resulting from increased plant productivity in the N fertilized soils (Ramirez et al., 2010). Crenarchaeota are Gr- archaea which are morphologically diverse containing both thermophilic and cold dwelling prokaryotes (Goldman and Green, 2015). The relative abundance of Firmicutes increased with "sample age", potentially due to storage degradation effects, as discussed previously.

Bacterial diversity and richness of archived soil was lowest in the samples with the longest storage time, increasing as length of storage time decreased (Fig. 4.5). Contrary to my hypothesis, changes to N fertilizer and CI did not consistently impact diversity, a trend also observed in Zhao et al. (2014). This observation is important to note because losses of microbial

richness and diversity can impact N cycling in soil (Philippot et al., 2013). Although microbial community composition is sensitive to N fertilization (Allison and Martiny, 2008), a recent study reported the influence of N on microbial diversity might only become significant at concentrations higher than used in our study (Fierer et al., 2013; Zhong et al., 2015). As the amount of N fertilizer applied is increased, plant productivity is also increased (Spohn et al., 2016), resulting in more plant residues for microbial decomposition (Geisseler and Scow, 2014) which can shift the bacterial community (Ramirez et al., 2010; Ramirez et al., 2012). Bacterial diversity and richness in the soil archives were calculated based on the downsampled sequence number. The observed number of OTUs (SOBs) is a richness measure which is reliable as an index of richness if coverage is sufficient (Hill et al., 2000) which was confirmed using rarefaction analysis. The alpha (α) diversity was estimated with the Inverse Simpson index (1/D). In a recent study of grassland soil, results demonstrated that decreases in microbial richness and diversity can impact N cycling in soil (Philippot et al 2013). This research studied microcosms based on a diversity gradient and observed that denitrification rates were significantly lower in the diluted biomass soils as compared to the undiluted soils. A study of a wheat-rice rotation with several different N and P treatments observed an increase in overall microbial abundance with addition of N and P fertilizer with crop residue; however there was no significant effect on diversity (Zhao et al., 2014). The influence of N on microbial diversity might only become significant at very high concentrations, as observed in a study of winter wheat with applications of 0 to 360 kg N ha-1 (Zhong et al., 2015). When the amount of N fertilizer is increased by a moderate amount, plant growth and thus C availability can increase, which influences the bacterial community (Arcand et al., 2017; Ramirez et al., 2010). As land use intensity increases through the use of N fertilizers, there are significant effects on the richness and diversity of soil bacterial communities (Tardy et al., 2015).

The estimated abundances of both the archaeal (AOA) and bacterial (AOB) ammonia oxidizer genes were greater in the 1993 and 2011 soils as compared to the 1910, 1940 and 1967 soils (Fig 4.7). The agricultural land management following 1940 changed to harvesting completed in the field, which meant increased crop residue return. Another change was adopted following 1967 with the incorporation of N fertilizer which also stimulated increased crop residue return. In a broad sense, the AOA population detected in the soils were more abundant than the AOB. These findings are consistent with many reports that AOA populations are more abundant than AOB in many environments (Leininger et al., 2006; Nicol et al., 2008; Munroe et al., 2016). In addition, the AOA and AOB seem to respond differently to changes in N availability. The AOA populations appear more abundant in low N soils (i.e. unfertilized soils), suggesting an adaptation to low nutrient conditions (Schleper and Nicol, 2010). In contrast, AOB were not as abundant in low N as in soils where N amendments were added (with the exception of Rotation C soils in 2011). Current research has reported that AOA and AOB adapt to different habitats, with AOA demonstrating a preference for NH_3 originating from mineralized organic material and AOB one for NH_3 derived from NH_4^+ or urea fertilizer (Hatzenpichler et al., 2012; Munroe et al., 2016). However there have also been studies that found that the addition of N increased both AOA and AOB populations (Glaser et al., 2010; Xue et al., 2016) or had no impact (Wessén et al., 2010).

Long term studies such as Rotation ABC allow a system to reach a steady state (Janzen, 1995) while shorter term studies may only capture the initial changes caused by a treatment. Another factor influencing AOA and AOB abundance is CI. The 100% CI in Rotation A meant more competition for soil nutrients than in Rotation C because the fallow year in Rotation C allows for mineralization of soil organic matter, resulting in nutrient release (Cruz et al., 2008).

Crop residues have been reported to enhance AOB population size as compared to N additions alone (He et al., 2007; Xue et al., 2016*)*. This relationship between increased C inputs from crop residues and N may explain the similarity in AOB abundances at both CI levels, despite the fact Rotation C receives less fertilizer than Rotation A because ammonium nitrate is only applied in non-fallow years when a crop is sown.

Both N amendments and crop residues are known to affect nitrogen functional gene abundances (Hallin et al., 2009; Wallenstein et al., 2006). Gene abundances are part of a complex system of N transformations through an ecosystem, and conditions that suppress or enhance these abundances are important (Ning et al., 2015). In this study, the relative abundances of denitrification genes differed between unfertilized native prairie and agricultural soils, regardless of N amendment status. The total populations of denitrifiers followed a pattern of *nosZ* with similar abundances to *nirK*, and greater than *nirS*, which has also been observed in recent studies of agricultural soils (Dandie et al., 2011; Henry et al., 2006). Although larger populations do not necessarily mean greater denitrification activity, a correlation between population size and function has been observed (Braker and Conrad, 2011; Hallin et al., 2009). The detection of *nosZ* in each soil is important because the gene product is responsible for the conversion of N_2O to N_2, and the gene is not found in all denitrifiers (Braker and Conrad, 2011; Clark et al., 2012; Throback et al., 2004).

The Nir genes are commonly used for denitrifier studies (Wallenstein et al., 2006) and studying both populations helps to capture the range of diversity of denitrifiers (Butterbach-Bahl et al., 2016). The larger population of *nirK* to *nirS* implies a niche differentiation to environmental conditions, also reported in recent work (Enwall et al., 2010; Jones and Hallin, 2010; Su et al., 2010). The sum of Nir genes as compared to *nosZ* gene abundance (as estimated

with primers nosZ2F/2R) has been used a proxy for total denitrification potential (Philippot et al., 2007; Shrewsbury et al., 2016). In these results, the denitrification potential does not appear to have increased over a century of wheat cropping with or without nitrogen fertilizer additions. It is possible that the rates of N used in this study were sufficiently low as to avoid suppression of NFG abundances, which has been reported at higher levels of N (Philipot 2009 et al., 2015; Wallenstein et al., 2004).

4.7 Conclusions

This study utilized modern molecular techniques to investigate soil microbial communities preserved in soil archives. These archives spanned a century of wheat crop production. It was unknown if DNA could be extracted, and would be of sufficient quality for the evaluation of 16S rRNA genes profiles and N functional gene abundances. The findings suggest that molecular biology studies can be conducted on DNA extracted from archived soils which have been air dried and stored at room temperature for years and even decades. The short term soil storage study validated the use of the original Rotation ABC archived soils by demonstrating the persistence and stability of DNA profiles under the similar soil storage conditions. These archived samples were from soils that originated from native prairie and shifts in soil microbial community were more likely due to fertilizer treatment and CI, than time in storage. The exploration of the soil archives also indicated the presence of nitrifiers AOA and AOB, and of denitrifiers containing *nosZ*, *nirK* and *nirS* genes in all time points. The ratio of Nir genes to *nosZ* gene abundance used a proxy for total denitrification potential does not appear to have increased over a century of wheat cropping with or without nitrogen fertilizer additions. This study has indicated that soil archives are valuable resources because they provide

opportunities to investigate soil bacterial communities of the past, providing a baseline for future studies.

5.0 Summary and Conclusions

Soil microbial communities mediate the biogeochemical processes regulating C and N cycles, and influence plant productivity (Griffiths and Philippot, 2013). N is a limiting factor to plant growth because plants do not synthesize N, and the amount of N effects the C:N, an important control on the availability of nutrients released through microbial turnover of plant residues (Sowden et al., 1977). Microbial decomposition transforms above and below ground plant residues into C energy substrates that shape microbial community structure and diversity (Freschet et al., 2013). Crop rotation shapes soil microbes because of the influence of plant residue inputs on soil microbial community structure (Phillips et al., 2015). Soil microbial communities in soils with reduced CI or fallow undergo changes in their abundance and structure in part from a reduction of plant inputs during in this non-cropped time period (Acosta-Martínez et al., 2007).

LTSEs are a valuable resource for learning more about soil microbial communities because soils may take decades after a change in land management to resume a “steady state" (Janzen, 1995). In short term studies, functional redundancy can make up for alterations in a soil microbial community structure resulting from changes in soil environmental conditions (Rousk et al., 2009). The soil microbial communities in archived soils serve as a baseline, permitting disturbances to soil microbial community diversity and abundance to be observed (Dolfing et al., 2015). These observations help determine if certain land use practices are sustainable or if they are creating conditions that decrease soil bacterial diversity. The Rotation ABC LTSE provided

an opportunity to study soil microbial dynamics during 100 years of WWF management and 45 years of N fertilizer implementation. Many studies have characterized soil properties of Rotation ABC (Janzen, 1995; Smith et al., 2012); however none have employed modern biochemical and molecular biology tools to investigate the soil microbial response to this long term land management.

The overall goal of my work was to characterize the active and total soil bacteria populations within Rotations A (100% CI) and C (67% CI), and discover more about soil microbial abundance and diversity in this agroecosystem. The first objective of my work was to compare the biomass of soil microbial communities under different CI. PLFA results suggested a significant interaction of N fertilizer and CI on the total active microbial biomass. The 100% CI soils with N had levels of total biomass that were twice that of 67% CI soils with N and three times the biomass of the 100% and 67% CI soils without N. Overall, the presence of spring wheat crop every growing season (since 1910) with N fertilizer (since 1967) in 100% CI soils increased the supply of C substrates available to soil microbes, and as a result these organisms grew more abundantly than those in the 67% CI soils. The size and composition of a soil microbial community is important to ecosystem function which is integral to ecosystem services that help to make agricultural crop production viable (Giller et al., 1997; Topp, 2003).

The second objective was to estimate the cumulative result of long term N fertilizer treatments and continuous wheat cropping on soil bacterial and archaeal communities and NFG abundance. Quantitative PCR results suggested that AOA and AOB NFG abundances were driven by significant interactions of N and CI. More specifically, as indicated by ammonia oxidizer and denitrifier groups studied, both were also shaped in response to differences in C and N inputs resulting from decades of differential soil amendments. In this study, the ratio of AOA

was much higher than AOB in unfertilized soils while the populations were more even in the fertilized soils, indicating a tolerance for different soil environments. The abundances of denitrifier functional genes *nosZ* and *nirK* were also significantly increased with 100% CI, while *nirS* was not. These genetic abundances demonstrated functional redundancy in denitrification genes, and also indicated a potential for gaseous N losses given suitable anoxic soil conditions.

The third objective was to assess the viability of DNA in air dried soils, and to characterize the impact that long-term storage of soil samples has on microbial DNA abundance and quality. Using a short term study, fresh soils were air dried, ground and then stored at room temperature with DNA extracted at 8 time points (T_0 to T_8) over 30 months. Results suggested that both storage time and $N_{45}P_{20}$ fertilizer treatment were influential on the amount of DNA extracted. During storage, the initial desiccation of the soil potentially caused cell lysis in many microbial cells which released DNA that might have been decomposed, or absorbed to clay minerals and humic substances (Nguyen and Chen, 2007; Pietramellara et al., 2008; Morrissey et al., 2015) . The results of 16S rRNA gene characterization alluded that the soil microbial community maintained diversity from T_0 to T_8, with diversity scores (Inverse of Simpson and $S_{ob}s$) scores higher in $N_{45}P_{20}$ fertilized soils than in unfertilized soils. The soil microbial community did not appear to lose diversity over the course of the study suggesting that DNA was protected from degradation over time in storage. N and P fertilized soils were estimated to have greater bacterial diversity than the unfertilized soils and these results suggested that soil fertility levels were associated with maintaining the stability of the soil microbial community structure. The increased bacterial diversity in the N and P soils may also be a result of increased soil organic matter (SOM) due to increased plant productivity that in turn meant there were more humic acids available to bind DNA. Analysis at the OTU level did not suggested a strong

relationship between time in storage and OTU profiles. These results suggested that storage duration of air dried soils did not greatly affect the relative diversity of soil microbial communities, therefore is an appropriate approach for investigating the soil archives associated with long term soil experiments.

The fourth objective was to measure changes in soil bacterial diversity and abundance of NFGs in bacterial DNA extracted from archived soils in order to monitor any potential stepwise changes over 100 years of wheat production. Soils archived in 1910, 1940, 1967, 1993 and 2011 were investigated with 16S rRNA gene profiling. The diversity of soil bacteria estimated with S_{obs} was observed to increase between samples from 1910 and 2011, although these values were much lower than in the 2012 soils. The observed increased diversity over time might indicate storage related changes to the community profile, as is suggested by the OTU ordination grouping by year. An alternative explanation could be that as observed in my short term storage study, the level of soil fertility is a driver of SOM, and as such, increases the binding sites for extracellular DNA and protects it from degradation in air dried soils. Over time, the changes in agricultural land management may have increased plant and soil productivity, resulting in a positive linear relationship between time in crop production and bacterial diversity. Quantitative PCR results suggested that a combination of 100% CI and N fertilizer treatments were more influential to NFG than were unfertilized soils at 67% CI. The soil history may have resulted in greater C and N substrate availability in the 100% CI and N fertilizer treated soils from increased plant and microbial productivity. The N fertilizer treatment was a stimulant to microbes by potentially lowering the C:N to be more favourable for decomposition of the increased plant residues made available resulting from increased crop productivity. Estimates of AOA abundance suggested that CI was more influential than N fertilizer, while AOB abundance was

stimulated by N fertilizer additions than CI. Denitrifiers with *nosZ* responded to both 100% CI and N fertilizer, while populations with *nirK* and *nirS* were more stimulated by 100% CI than N fertilizer. The results suggest that molecular biology studies can successfully be conducted on DNA extracted from archived soils which have been air dried and stored at room temperature for years and even decades.

These archived samples were from soils that originated from native prairie and shifts in soil microbial community are more likely due to fertilizer treatment and CI, than time in storage. The exploration of the soil archives also hinted at divergence from native prairie over time, with OTUs profiles grouping by year, and the relative differences in the abundance of nitrifiers AOA and AOB, and of denitrifiers containing *nosZ*, *nirK* and *nirS* genes in all time points. Both N amendments and crop residues are known to affect nitrogen functional gene abundances (Hallin et al., 2009; Wallenstein et al., 2006) and gene abundances are part of a complex system of N transformations through an ecosystem, and conditions that suppress or enhance these abundances are important. This study has indicated that soil archives are valuable resources because they provide opportunities to investigate soil bacterial communities of the past and the findings suggest that molecular biology studies can be conducted on DNA extracted from archived soils which have been air dried and stored at room temperature for years and even decades.

5.1 Future Research Directions

After this initial exploration of the Rotation ABC long term plots, questions remain regarding how imposing new treatments may alter the soil microbial community. Many modern farming operations include a legume in the crop rotation, and the study has to reflected modern practices with the adoption of herbicides, fertilizers and reduced tillage. The new treatments

could include a legume in to the crop rotation and also an increased level of nitrogen to better reflect modern practices. Prior to implementation of any changes, a greenhouse study would offer a controlled environment in which to assess the changes caused by increasing the N treatment to reflect current recommendations for this soil zone, and also with legume residues incorporated prior to a growing cycle of wheat. I propose that including a legume, or legume residue, the C: N would change to favour decomposition after over 100 years of wheat monocropping, which has a high C: N ratio that is known to favour N immobilization. The impacts on the soil microbial community could be observed through use of PLFA, NFG abundances and 16s rRNA profiling. In addition, I would propose that molecular biology tests such as PLFA, NFG abundances and 16s rRNA profiling be conducted at major soil archive sampling events of Rotation ABC, to record and survey the status of the soil microbial community. This data could be included in the archive for future researchers to access, along with the soil archives.

6.0 REFERENCES

Acosta-Martínez, V., Dowd, S., Sun, Y., Allen, V., 2008. Tag-encoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use. Soil Biology and Biochemistry 40, 2762-2770.

Acosta-Martínez, V., Dowd, S.E., Bell, C.W., Lascano, R., Booker, J.D., Zobeck, T.M., Upchurch, D.R., 2010. Microbial Community Composition as Affected by Dryland Cropping Systems and Tillage in a Semiarid Sandy Soil. Diversity 2, 910-931.

Acosta-Martínez, V., Mikha, M.M., Vigil, M.F., 2007. Microbial communities and enzyme activities in soils under alternative crop rotations compared to wheat–fallow for the Central Great Plains. Applied Soil Ecology 37, 41-52.

Agren, G.I., Wetterstedt, J.A., Billberger, M.F., 2012. Nutrient limitation on terrestrial plant growth--modeling the interaction between nitrogen and phosphorus. New Phytolologist 194, 953-960.

Allison, S.D., Martiny, J.B.H., 2008. Resistance, resilience, and redundancy in microbial communities. Proceedings of the National Academy of Sciences 105, 11512-11519.

Allison, V.J., Miller, R.M., Jastrow, J.D., Matamala, R., Zak, D.R., 2005. Changes in soil microbial community structure in a tallgrass prairie chronosequence. Soil Science Society of America Journal 69, 1412-1421.

Arcand, M.M., Levy-Booth, D.J., Helgason, B.L., 2017. Resource Legacies of Organic and Conventional Management Differentiate Soil Microbial Carbon Use. Frontiers in Microbiology 8.

Bardgett, R.D., Mawdsley, J.L., Edwards, S., Hobbs, P.J., Rodwell, J.S., Davies, W.J., 1999. Plant species and nitrogen effects on soil biological properties of temperate upland grasslands. Functional Ecology 13, 650-660.

Bateman, E.J., Baggs, E.M., 2005. Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. Biology and Fertility of Soils 41, 379-388.

Bhatia, C.R., 2008. Role of Microbial Diversity for Soil, Health and Plant Nutrition, In: Nautiyal, C.S., Dion, P. (Eds.), Molecular Mechanisms of Plant and Microbe Coexistence. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 53-74.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology 37, 911-917.

Bobbink, R., Hicks, K., Galloway, J., Spranger, T., Alkemade, R., Ashmore, M., Bustamante, M., Cinderby, S., Davidson, E., Dentener, F., Emmett, B., Erisman, J.W., Fenn, M., Gilliam, F.,

Nordin, A., Pardo, L., De Vries, W., 2010. Global assessment of nitrogen deposition effects on terrestrial plant diversity: A synthesis. Ecological Applications 20, 30-59.

Booth, M.S., Stark, J.M., Rastetter, E., 2005. Controls On Nitrogen Cycling In Terrestrial Ecosystems: A Synthetic Analysis Of Literature Data. Ecological Monographs 75, 139-157.

Börjesson, G., Menichetti, L., Kirchmann, H., Kätterer, T., 2012. Soil microbial community structure affected by 53 years of nitrogen fertilisation and different organic amendments. Biology and Fertility of Soils 48, 245-257.

Braker, G., Conrad, R., 2011. Diversity, structure, and size of N_2O-producing microbial communities in soils-what matters for their functioning? Advances in Applied Microbiology 75, 33-70.

Burlage, R.S., 1998. Techniques in Microbial Ecology. Oxford University Press.

Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S., 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Philosophical transactions of the Royal Society of London. Series B, Biological sciences 368, 20130122.

Campbell, J.H., Clark, J.S., Zak, J.C., 2009. PCR-DGGE comparison of bacterial community structure in fresh and archived soils sampled along a Chihuahuan Desert elevational gradient. Microb Ecol 57, 261-266.

Cary, S.C., Fierer, N., 2014. The importance of sample archiving in microbial ecology. Nature Reviews Microbiology 12, 789-790.

Chu, H., Fujii, T., Morimoto, S., Lin, X., Yagi, K., Hu, J., Zhang, J., 2007. Community structure of ammonia-oxidizing bacteria under long-term application of mineral fertilizer and organic manure in a sandy loam soil. Applied Environmental Microbiology 73, 485-491.

Clark, I.M., Buchkina, N., Jhurreea, D., Goulding, K.W., Hirsch, P.R., 2012. Impacts of nitrogen application rates on the activity and diversity of denitrifying bacteria in the Broadbalk Wheat Experiment. Philosophical Transactions of the Royal Society B: Biological Sciences 367, 1235-1244.

Clark, I.M., Hirsch, P.R., 2008. Survival of bacterial DNA and culturable bacteria in archived soils from the Rothamsted Broadbalk experiment. Soil Biology and Biochemistry 40, 1090-1102.

Costa, R., Götz, M., Mrotzek, N., Lottmann, J., Berg, G., Smalla, K., 2006. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. FEMS Microbiology Ecology 56, 236-249.

Coyne, M.S., Arunakumari, A., Averill, B.A., Tiedje, J.M., 1989. Immunological identification and distribution of dissimilatory heme cd1 and nonheme copper nitrite reductases in denitrifying bacteria. Applied and Environmental Microbiology 55, 2924-2931.

Cruz, A.F., Hamel, C., Hanson, K., Selles, F., Zentner, R.P., 2008. Thirty-seven years of soil nitrogen and phosphorus fertility management shapes the structure and function of the soil microbial community in a Brown Chernozem. Plant and Soil 315, 173-184.

Dandie, C.E., Wertz, S., Leclair, C.L., Goyer, C., Burton, D.L., Patten, C.L., Zebarth, B.J., Trevors, J.T., 2011. Abundance, diversity and functional gene expression of denitrifier communities in adjacent riparian and agricultural zones. FEMS Microbiol Ecol 77, 69-82.

Date, R.A., 1973. Nitrogen, a major limitation in the productivity of natural communities, crops and pastures in the pacific area. Soil Biology and Biochemistry 5, 5-18.

Davidson, E.A., Janssens, I.A., 2006. Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. Nature 440, 165-173.

Denobili, M., Contin, M., Brookes, P., 2006. Microbial biomass dynamics in recently air-dried and rewetted soils compared to others stored air-dry for up to 103 years. Soil Biology and Biochemistry 38, 2871-2881.

Di Falco, S., 2012. On the value of agricultural biodiversity. Annual Review of Resource Economics 4, 207-223.

Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., O'Callaghan, M., Bowatte, S., He, J.Z., 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. Nature Geoscience 2, 621-624.

Dolfing, J., Feng, Y., 2015. The importance of soil archives for microbial ecology. Nature Reviews Microbiology 13, 1.

Dolfing, J., Vos, A., Bloem, J., Ehlert, P.A.I., Naumova, N.B., Kuikman, P.J., 2004. Microbial diversity in archived soils [6]. Science 306, 813.

Enwall, K., Throbäck, I.N., Stenberg, M., Söderström, M., Hallin, S., 2010. Soil resources influence spatial patterns of denitrifying communities at scales compatible with land management. Applied and Environmental Microbiology 76, 2243-2250.

Feng, Y., Chen, R., Hu, J., Zhao, F., Wang, J., Chu, H., Zhang, J., Dolfing, J., Lin, X., 2015. Bacillus asahii comes to the fore in organic manure fertilized alkaline soils. Soil Biology and Biochemistry 81, 186-194.

Fierer, N., Ladau, J., Clemente, J.C., Leff, J.W., Owens, S.M., Pollard, K.S., Knight, R., Gilbert, J.A., McCulley, R.L., 2013. Reconstructing the microbial diversity and function of pre-agricultural tallgrass prairie soils in the United States. Science 342, 621-624.

Fierer, N., Lauber, C.L., Ramirez, K.S., Zaneveld, J., Bradford, M.A., Knight, R., 2012. Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. ISME J 6, 1007-1017.

Fierer, N., Strickland, M.S., Liptzin, D., Bradford, M.A., Cleveland, C.C., 2009. Global patterns in belowground communities. Ecol Lett 12, 1238-1249.

Fox, C.A., 2003. Characterizing soil biota in Canadian agroecosystems: State of knowledge in relation to soil organic matter. Canadian Journal of Soil Science 83, 245-257.

Freschet, G.T., Cornwell, W.K., Wardle, D.A., Elumeeva, T.G., Liu, W., Jackson, B.G., Onipchenko, V.G., Soudzilovskaia, N.A., Tao, J., Cornelissen, J.H.C., Austin, A., 2013. Linking litter decomposition of above- and below-ground organs to plant-soil feedbacks worldwide. Journal of Ecology 101, 943-952.

Frison, E.A., Cherfas, J., Hodgkin, T., 2011. Agricultural biodiversity is essential for a sustainable improvement in food and nutrition security. Sustainability 3, 238-253.

Frostegård, Å., Tunlid, A., Bååth, E., 2011. Use and misuse of PLFA measurements in soils. Soil Biology and Biochemistry 43, 1621-1625.

Geisseler, D., Scow, K.M., 2014. Long-term effects of mineral fertilizers on soil microorganisms – A review. Soil Biology and Biochemistry 75, 54-63.

Giles, M.E., Daniell, T.J., Baggs, E.M., 2017. Compound driven differences in N2 and N2O emission from soil; the role of substrate use efficiency and the microbial community. Soil Biology and Biochemistry 106, 90-98.

Giller, K.E., Beare, M.H., Lavelle, P., Izac, A.M.N., Swift, M.J., 1997. Agricultural intensification, soil biodiversity and agroecosystem function. Applied Soil Ecology 6, 3-16.

Glaser, K., Hackl, E., Inselsbacher, E., Strauss, J., Wanek, W., Zechmeister-Boltenstern, S., Sessitsch, A., 2010. Dynamics of ammonia-oxidizing communities in barley-planted bulk soil and rhizosphere following nitrate and ammonium fertilizer amendment. FEMS Microbiology Ecology 74, 575-591.

Greenan, C.M., Moorman, T.B., Kaspar, T.C., Parkin, T.B., Jaynes, D.B., 2006. Comparing carbon substrates for denitrification of subsurface drainage water. J Environ Qual 35, 824-829.

Griffiths, B.S., Philippot, L., 2013. Insights into the resistance and resilience of the soil microbial community. FEMS Microbiol Rev 37, 112-129.

Grogan, D.W., Cronan Jr, J.E., 1997. Cyclopropane ring formation in membrane lipids of bacteria. Microbiology and Molecular Biology Reviews 61, 429-441.

Hallin, S., Jones, C.M., Schloter, M., Philippot, L., 2009. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. ISME J 3, 597-605.

Harmsen, G.W., van Schreven, D.A., 1955. Mineralization of Organic Nitrogen in Soil, Advances in Agronomy, pp. 299-398.

Hatzenpichler, R., 2012. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Applied Environmental Microbiology 78, 7501-7510.

He, J.Z., Shen, J.P., Zhang, L.M., Zhu, Y.G., Zheng, Y.M., Xu, M.G., Di, H., 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environmental Microbiology 9, 2364-2374.

Hedrick, D.B., Peacock, A., White, D.C., 2005. Interpretation of Fatty Acid Profiles of Soil Microorganisms, Monitoring and Assessing Soil Bioremediation. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 251-259.

Helgason, B.L., Walley, F.L., Germida, J.J., 2010. Long-term no-till management affects microbial biomass but not community composition in Canadian prairie agroecosytems. Soil Biology and Biochemistry 42, 2192-2202.

Henry, S., Bru, D., Stres, B., Hallet, S., Philippot, L., 2006. Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. Applied Environmental Microbiology 72, 5181-5189.

Hill, G.T., Mitkowski, N.A., Aldrich-Wolfe, L., Emele, L.R., Jurkonie, D.D., Ficke, A., Maldonado-Ramirez, S., Lynch, S.T., Nelson, E.B., 2000. Methods for assessing the composition and diversity of soil microbial communities. Applied Soil Ecology 15, 25-36.

Hirsch, P.R., Jhurreea, D., Williams, J.K., Murray, P.J., Scott, T., Misselbrook, T.H., Goulding, K.W.T., Clark, I.M., 2016. Soil resilience and recovery: rapid community responses to management changes. Plant and Soil.

Hodge, A., Robinson, D., Fitter, A., 2000. Are microorganisms more effective than plants at competing for nitrogen? Trends in Plant Science 5, 304-308.

Hollister, E.B., Hu, P., Wang, A.S., Hons, F.M., Gentry, T.J., 2013. Differential impacts of brassicaceous and nonbrassicaceous oilseed meals on soil bacterial and fungal communities. FEMS Microbiology Ecology 83, 632-641.

Izaurralde, R.C., Juma, N.G., Robertson, J.A., McGill, W.B., Thurston, J.T., 1996. The Breton Classical Plots, In: Powlson, D.S., Smith, P., Smith, J.U. (Eds.), Evaluation of Soil Organic Matter Models: Using Existing Long-Term Datasets. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 351-361.

Jackson, L.E., Pascual, U., Hodgkin, T., 2007. Utilizing and conserving agrobiodiversity in agricultural landscapes. Agriculture, Ecosystems and Environment 121, 196-210.

Jangid, K., Williams, M.A., Franzluebbers, A.J., Schmidt, T.M., Coleman, D.C., Whitman, W.B., 2011. Land-use history has a stronger impact on soil microbial community composition than aboveground vegetation and soil properties. Soil Biology and Biochemistry 43, 2184-2193.

Janzen, H., 1987. Effect of fertilizer on soil productivity in long-term spring wheat rotations. Canadian Journal of Soil Science 67, 165-174.

Janzen, H.H., 1995. The role of long-term sites in agroecological research: A case study. Canadian Journal of Soil Science 75, 123-133.

Jia, Z., Conrad, R., 2009. Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environmental Microbiology 11, 1658-1671.

Jones, C.M., Hallin, S., 2010. Ecological and evolutionary factors underlying global and local assembly of denitrifier communities. ISME Journal 4, 633-641.

Kaur, A., Chaudhary, A. , Kaur, A., Choudhary, R., 2005. Phospholipid fatty acid - A bioindicator of environment monitoring and assessment in soil ecosystem. Current Science 89, 1103-1112.

Kemmitt, S.J., Lanyon, C.V., Waite, I.S., Wen, Q., Addiscott, T.M., Bird, N.R.A., O'Donnell, A.G., Brookes, P.C., 2008. Mineralization of native soil organic matter is not regulated by the size, activity or composition of the soil microbial biomass—a new perspective. Soil Biology and Biochemistry 40, 61-73.

Kennedy, A.C., 1999. Bacterial diversity in agroecosystems. Agriculture, Ecosystems and Environment 74, 65-76.

Kennedy, A.C., Smith, K.L., 1995. Soil microbial diversity and the sustainability of agricultural soils. Plant and Soil 170, 75-86.

Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H., Trevors, J.T., 2004. Methods of studying soil microbial diversity. Journal of Microbiological Methods 58, 169-188.

Knapp, C.W., Dolfing, J., Ehlert, P.A.I., Graham, D.W., 2010. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. Environmental Science and Technology 44, 580-587.

Kruskal, J., M. Wish, 1978. Multidimensional Scaling. SAGE Publications, Newbury Park, California.

Langer, U., Klimanek, E.-M., 2006. Soil microbial diversity of four German long-term field experiments. Archives of Agronomy and Soil Science 52, 507-523.

Latz, E., Eisenhauer, N., Rall, B.C., Allan, E., Roscher, C., Scheu, S., Jousset, A., 2012. Plant diversity improves protection against soil-borne pathogens by fostering antagonistic bacterial communities. Journal of Ecology 100, 597-604.

Latz, E., Eisenhauer, N., Scheu, S., Jousset, A., 2015. Plant identity drives the expression of biocontrol factors in a rhizosphere bacterium across a plant diversity gradient. Functional Ecology 29, 1225-1234.

Lauber, C.L., Zhou, N., Gordon, J.I., Knight, R., Fierer, N., 2010. Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS Microbiological Letters 307, 80-86.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806-809.

Levy-Booth, D.J., Campbell, R.G., Gulden, R.H., Hart, M.M., Powell, J.R., Klironomos, J.N., Pauls, K.P., Swanton, C.J., Trevors, J.T., Dunfield, K.E., 2007. Cycling of extracellular DNA in the soil environment. Soil Biology and Biochemistry 39, 2977-2991.

Lupwayi, N.Z., Rice, W.A., Clayton, G.W., 1998. Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. Soil Biology and Biochemistry 30, 1733-1741.

Macagnan, D., Romeiro, R.d.S., de Souza, J.T., Pomella, A.W.V., 2006. Isolation of actinomycetes and endospore-forming bacteria from the cacao pod surface and their antagonistic activity against the witches' broom and black pod pathogens. Phytoparasitica 34, 122-132.

Macdonald, L.M., Paterson, E., Dawson, L.A., McDonald, A.J.S., 2004. Short-term effects of defoliation on the soil microbial community associated with two contrasting Lolium perenne cultivars. Soil Biology and Biochemistry 36, 489-498.

Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J.C., Soulas, G., Catroux, G., 2001. DNA Extraction from Soils: Old Bias for New Microbial Diversity Analysis Methods. Applied and Environmental Microbiology 67, 2354-2359.

Mazzola, M., Funnell, D.L., Raaijmakers, J.M., 2004. Wheat cultivar-specific selection of 2,4-diacetylphloroglucinol-producing fluorescent Pseudomonas species from resident soil populations. Microb Ecol 48, 338-348.

Mbuthia, L.W., Acosta-Martínez, V., DeBruyn, J., Schaeffer, S., Tyler, D., Odoi, E., Mpheshea, M., Walker, F., Eash, N., 2015. Long term tillage, cover crop, and fertilization effects on microbial community structure, activity: Implications for soil quality. Soil Biology and Biochemistry 89, 24-34.

Morley, N., Baggs, E.M., 2010. Carbon and oxygen controls on N2O and N2 production during nitrate reduction. Soil Biology and Biochemistry 42, 1864-1871.

Morrissey, E.M., McHugh, T.A., Preteska, L., Hayer, M., Dijkstra, P., Hungate, B.A., Schwartz, E., 2015. Dynamics of extracellular DNA decomposition and bacterial community composition in soil. Soil Biology and Biochemistry 86, 42-49.

Munroe, J.W., McCormick, I., Deen, W., Dunfield, K.E., 2016. Effects of 30 years of crop rotation and tillage on bacterial and archaeal ammonia oxidizers. J Environ Qual 45, 940-948.

Ngosong, C., Jarosch, M., Raupp, J., Neumann, E., Ruess, L., 2010. The impact of farming practice on soil microorganisms and arbuscular mycorrhizal fungi: Crop type versus long-term mineral and organic fertilization. Applied Soil Ecology 46, 134-142.

Nguyen, T.H., Chen, K.L., 2007. Role of divalent cations in plasmid DNA adsorption to natural organic matter-coated silica surface. Environmental Science and Technology 41, 5370-5375.

Nicol, G.W., Leininger, S., Schleper, C., Prosser, J.I., 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environmental Microbiology 10, 2966-2978.

Norton, J.M., Alzerreca, J.J., Suwa, Y., Klotz, M.G., 2002. Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. Archives of Microbiology 177, 139-149.

Nouri, E., Breuillin-Sessoms, F., Feller, U., Reinhardt, D., 2014. Phosphorus and nitrogen regulate arbuscular mycorrhizal symbiosis in Petunia hybrida. PLoS One 9, e90841.

O'Dea, J.K., Jones, C.A., Zabinski, C.A., Miller, P.R., Keren, I.N., 2015. Legume, cropping intensity, and N-fertilization effects on soil attributes and processes from an eight-year-old semiarid wheat system. Nutrient Cycling in Agroecosystems 102, 179-194.

Olsson, P.A., 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiology Ecology 29, 303-310.

Paterson, E., Sim, A., Osborne, S.M., Murray, P.J., 2011. Long-term exclusion of plant-inputs to soil reduces the functional capacity of microbial communities to mineralise recalcitrant root-derived carbon sources. Soil Biology and Biochemistry 43, 1873-1880.

Philippot, L., Hallin, S., Schloter, M., 2007. Ecology of Denitrifying Prokaryotes in Agricultural Soil. Advances in Agronomy 96, 249-305.

Philippot, L., Spor, A., Henault, C., Bru, D., Bizouard, F., Jones, C.M., Sarr, A., Maron, P.A., 2013. Loss in microbial diversity affects nitrogen cycling in soil. ISME J 7, 1609-1619.

Phillips, L.A., Schefe, C.R., Fridman, M., O'Halloran, N., Armstrong, R.D., Mele, P.M., 2015. Organic nitrogen cycling microbial communities are abundant in a dry Australian agricultural soil. Soil Biology and Biochemistry 86, 201-211.

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M.T., Guerri, G., Nannipieri, P., 2008. Extracellular DNA in soil and sediment: fate and ecological relevance. Biology and Fertility of Soils 45, 219-235.

Power, J.F., 1990. Legumes and crop rotations. Sustainable Agriculture in Temperate Zones, 178-204.

Prosser, J.I., Nicol, G.W., 2008. Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. Environ Microbiol 10, 2931-2941.

Qian, P., Schoenau, J.J., 1994. Assessing nitrogen mineralization from soil organic matter using anion exchange membranes. Fertilizer research 40, 143-148.

Ramirez, K.S., Craine, J.M., Fierer, N., 2012. Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. Global Change Biology 18, 1918-1927.

Ramirez, K.S., Lauber, C.L., Knight, R., Bradford, M.A., Fierer, N., 2010. Consistent effects of nitrogen fertilization on soil bacterial communities in contrasting systems. Ecology 91, 3463-3470.

Rasmussen, P.E., Goulding, K.W.T., Brown, J.R., Grace, P.R., Janzen, H.H., Körschens, M., 1998. Long-Term Agroecosystem Experiments: Assessing Agricultural Sustainability and Global Change. Science 282, 893-896.

Rousk, J., Brookes, P.C., Baath, E., 2009. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Applied and Environmental Microbiology 75, 1589-1596.

Rousk, J., Brookes, P.C., Baath, E., 2011. Fungal and bacterial growth responses to N fertilization and pH in the 150-year 'Park Grass' UK grassland experiment. FEMS Microbiology Ecology 76, 89-99.

Rubin, B.E., Gibbons, S.M., Kennedy, S., Hampton-Marcell, J., Owens, S., Gilbert, J.A., 2013. Investigating the impact of storage conditions on microbial community composition in soil samples. PLoS One 8, e70460.

Schauss, K., Focks, A., Leininger, S., Kotzerke, A., Heuer, H., Thiele-Bruhn, S., Sharma, S., Wilke, B.M., Matthies, M., Smalla, K., Munch, J.C., Amelung, W., Kaupenjohann, M., Schloter, M., Schleper, C., 2009. Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. Environmental Microbiology 11, 446-456.

Schimel, J.P., Bennett, J., 2004. Nitrogen Mineralization: Challenges Of A Changing Paradigm. Ecology 85, 591-602.

Schleper, C., Nicol, G.W., 2010. Ammonia-oxidising archaea - physiology, ecology and evolution, Advances in Microbial Physiology, pp. 1-41.

Schloter, M., Dilly, O., Munch, J.C., 2003. Indicators for evaluating soil quality. Agriculture, Ecosystems & Environment 98, 255-262.

Schulten, H.R., Schnitzer, M., 1997. The chemistry of soil organic nitrogen: A review. Biology and Fertility of Soils 26, 1-15.

Shi, P., Wang, S., Jia, S., Gao, Q., 2015. Effect of 25-year fertilization on soil microbial biomass and community structure in a continuous corn cropping system. Archives of Agronomy and Soil Science, 1-15.

Shrewsbury, L.H., Smith, J.L., Huggins, D.R., Carpenter-Boggs, L., Reardon, C.L., 2016. Denitrifier abundance has a greater influence on denitrification rates at larger landscape scales but is a lesser driver than environmental variables. Soil Biology and Biochemistry 103, 221-231.

Smith, E.G., Ellert, B.H., Janzen, H.H. and Nakonechny, D.J. 2012., 2012. Rotation ABC-Lethbridge, Alberta. Prairie Soils and Crops 5, 147-154.

Soon, Y., Arshad, M., 2002. Comparison of the decomposition and N and P mineralization of canola, pea and wheat residues. Biology and Fertility of Soils 36, 10-17.

Sowden, F.J., Chen, Y., Schnitzer, M., 1977. The nitrogen distribution in soils formed under widely differing climatic conditions. Geochimica et Cosmochimica Acta 41, 1524-1526.

Spohn, M., Pötsch, E.M., Eichorst, S.A., Woebken, D., Wanek, W., Richter, A., 2016. Soil microbial carbon use efficiency and biomass turnover in a long-term fertilization experiment in a temperate grassland. Soil Biology and Biochemistry 97, 168-175.

Su, M., Kleineidam, K., Schloter, M., 2010. Influence of different litter quality on the abundance of genes involved in nitrification and denitrification after freezing and thawing of an arable soil. Biology and Fertility of Soils 46, 537-541.

Tardy, V., Chabbi, A., Charrier, X., de Berranger, C., Reignier, T., Dequiedt, S., Faivre-Primot, C., Terrat, S., Ranjard, L., Maron, P.A., 2015. Land Use History Shifts In Situ Fungal and Bacterial Successions following Wheat Straw Input into the Soil. PLoS One 10, e0130672.

Tatangelo, V., Franzetti, A., Gandolfi, I., Bestetti, G., Ambrosini, R., 2014. Effect of preservation method on the assessment of bacterial community structure in soil and water samples. FEMS Microbioogyl Letters 356, 32-38.

Terrat, S., Christen, R., Dequiedt, S., Lelievre, M., Nowak, V., Regnier, T., Bachar, D., Plassart, P., Wincker, P., Jolivet, C., Bispo, A., Lemanceau, P., Maron, P.A., Mougel, C., Ranjard, L., 2012. Molecular biomass and MetaTaxogenomic assessment of soil microbial communities as influenced by soil DNA extraction procedure. Microb Biotechnol 5, 135-141.

Throback, I.N., Enwall, K., Jarvis, A., Hallin, S., 2004. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol Ecol 49, 401-417.

Tian, W., Wang, L., Li, Y., Zhuang, K., Li, G., Zhang, J., Xiao, X., Xi, Y., 2015. Responses of microbial activity, abundance, and community in wheat soil after three years of heavy fertilization with manure-based compost and inorganic nitrogen. Agriculture, Ecosystems & Environment 213, 219-227.

Tilman, D., Fargione, J., Wolff, B., D'Antonio, C., Dobson, A., Howarth, R., Schindler, D., Schlesinger, W.H., Simberloff, D., Swackhamer, D., 2001. Forecasting Agriculturally Driven Global Environmental Change. Science 292, 281-284.

Topp, E., 2003. Bacteria in agricultural soils: Diversity, role and future perspectives. Canadian Journal of Soil Science 83, 303-309.

Torsvik, V., Øvreås, L., 2002. Microbial diversity and function in soil: from genes to ecosystems. Current Opinion in Microbiology 5, 240-245.

Tourna, M., Freitag, T.E., Nicol, G.W., Prosser, J.I., 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environ Microbiol 10, 1357-1364.

Trivedi, P., Delgado-Baquerizo, M., Anderson, I.C., Singh, B.K., 2016. Response of Soil Properties and Microbial Communities to Agriculture: Implications for Primary Productivity and Soil Health Indicators. Frontiers in Plant Science 7.

Venter, Z.S., Jacobs, K., Hawkins, H.J., 2016. The impact of crop rotation on soil microbial diversity: A meta-analysis. Pedobiologia 59, 215-223.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F., van Sinderen, D., 2007. Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. Microbiol Mol Biol Rev 71, 495-548.

Verdenelli, R.A., Conforto, C.B., Pérez-Brandán, C., Chavarría, D., Rovea, A., Vargas-Gil, S., Meriles, J.M., 2013. Integrated multivariate analysis of selected soil microbial properties and their relationships with mineral fertilization management in a conservation agriculture system. Acta Agriculturae Scandinavica, Section B - Soil & Plant Science 63, 623-632.

Wallenstein, M.D., Myrold, D.D., Firestone, M., Voytek, M., 2006. Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. Ecological Applications 16, 2143-2152.

Wang, Y., Ke, X., Wu, L., Lu, Y., 2009. Community composition of ammonia-oxidizing bacteria and archaea in rice field soil as affected by nitrogen fertilization. Syst Appl Microbiol 32, 27-36.

Ward, B.B., Bouskill, N.J., 2011. The utility of functional gene arrays for assessing community composition, relative abundance, and distribution of ammonia-oxidizing bacteria and archaea, Methods in Enzymology, pp. 373-396.

Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setälä, H., Van Der Putten, W.H., Wall, D.H., 2004. Ecological linkages between aboveground and belowground biota. Science 304, 1629-1633.

Wessén, E., Hallin, S., Philippot, L., 2010. Differential responses of bacterial and archaeal groups at high taxonomical ranks to soil management. Soil Biology and Biochemistry 42, 1759-1765.

White, D.C., Davis, W.M., Nickels, J.S., King, J.D., Bobbie, R.J., 1979. Determination of the sedimentary microbial biomass by extractible lipid phosphate. Oecologia 40, 51-62.

Wood, S.A., Karp, D.S., DeClerck, F., Kremen, C., Naeem, S., Palm, C.A., 2015. Functional traits in agriculture: Agrobiodiversity and ecosystem services. Trends in Ecology and Evolution 30, 531-539.

Wu, T., Chellemi, D.O., Graham, J.H., Martin, K.J., Rosskopf, E.N., 2008. Comparison of soil bacterial communities under diverse agricultural land management and crop production practices. Microb Ecol 55, 293-310.

Xue, C., Zhang, X., Zhu, C., Zhao, J., Zhu, P., Peng, C., Ling, N., Shen, Q., 2016. Quantitative and compositional responses of ammonia-oxidizing archaea and bacteria to long-term field fertilization. Scientific Reports 6.

Yandigeri, M.S., Meena, K.K., Singh, D., Malviya, N., Singh, D.P., Solanki, M.K., Yadav, A.K., Arora, D.K., 2012. Drought-tolerant endophytic actinobacteria promote growth of wheat (Triticum aestivum) under water stress conditions. Plant Growth Regulation 68, 411-420.

Yates, F., 1954. The Analysis of Experiments Containing Different Crop Rotations. Biometrics 10, 324-346.

Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: A review. Biology and Fertility of Soils 29, 111-129.

Zhang, J., Cai, Z., Yang, W., Zhu, T., Yu, Y., Yan, X., Jia, Z., 2012. Long-term field fertilization affects soil nitrogen transformations in a rice-wheat-rotation cropping system. Journal of Plant Nutrition and Soil Science 175, 939-946.

Zhao, J., Ni, T., Li, Y., Xiong, W., Ran, W., Shen, B., Shen, Q., Zhang, R., 2014a. Responses of bacterial communities in arable soils in a rice-wheat cropping system to different fertilizer regimes and sampling times. PLoS One 9, e85301.

Zhao, S., Qiu, S., Cao, C., Zheng, C., Zhou, W., He, P., 2014b. Responses of soil properties, microbial community and crop yields to various rates of nitrogen fertilization in a wheat–maize cropping system in north-central China. Agriculture, Ecosystems & Environment 194, 29-37.

Zhong, Y., Yan, W., Shangguan, Z., 2015. Impact of long-term N additions upon coupling between soil microbial community structure and activity, and nutrient-use efficiencies. Soil Biology and Biochemistry 91, 151-159.

Zumft, W.G., 1997. Cell biology and molecular basis of denitrification? Microbiology and Molecular Biology Reviews 61, 533-616.

Appendix A

Table A.1 Cultivars of Spring Wheat grown on Rotation ABC

Year	Cultivar
1912	Kharkov
1913 - 1916	Red Fife
1914 - 1950	Marquis [1]
1951 - 1954	Rescue
1955 - 1969	Chinook
1970 - 1973	Neepawa
1974 - 1975	Cypress
1976	Canuck
1977 - 1984	Chester
1985 - 1988	Leader
1989 - 1993	Lancer
1994 - 2004	Katepwa
2005 - 2007	AC Abbey
2008 - 2012	AC Lillian

[1] Except for Garnet on Rotation A from 1926 - 1930.

Appendix B

Table B.1 Summary of soil properties of Rotation A and C plots, Fall 2012 soil samples. Results are means of 4 replicates ± standard errors.

	Fertilizer	**pH**	**Nitrate**	**P**	**K**	**S**
	(kg N ha^{-1})		(mg kg^{-1} soil)			
A	N_0P_0	7.52(0.07)	4.2(0.7)	3.3(0.5)	476.3(25.5)	41.8(17.6)
A	$N_{45}P_0$	6.73(0.20)	11.3(1.7)	4.4(0.9)	677.3(80.5)	10.9(1.1)
C	N_0P_0	7.79(0.07)	4.8(0.4)	4.5(0.2)	420.0(15.2)	14.5(0.8)
C	$N_{45}P_0$	7.44(0.24)	4.7(0.4)	4.2(0.4)	501.8(30.8)	8.1(0.4)
ANOVA	CI	*	**	NS	*	NS
	F	**	**	NS	**	NS
	CI x F	NS	**	NS	NS	NS

*,**,*** Significant at $p \leq 0.05, 0.01, 0.001$

Appendix C

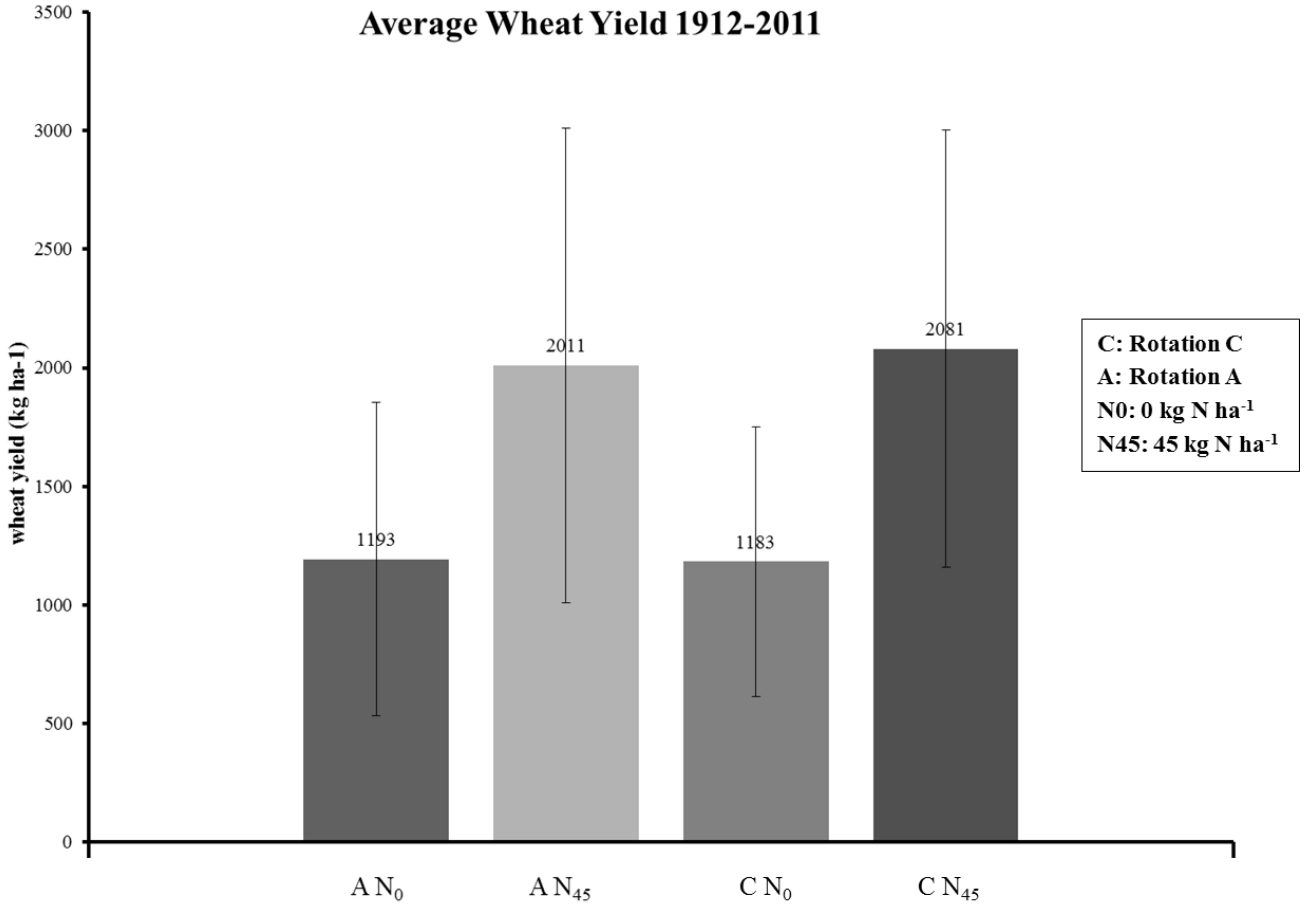

Figure C.1: Average wheat yield from 1912 to 2011 for Rotation A (continuous wheat) and Rotation C (WWF) under 0 kg N ha^{-1} and 45 kg N ha^{-1} treatment. Bars denote +/- 1 standard deviation.

Appendix D

Table D.1 Concentration of DNA (μg g^{-1}soil) extracted from Rotation A and C archived soils under different fertilizer treatments.

Fertilizer	**Code**	**1910**	**1940**	**1967**	**1993**	**2011**
		DNA conc. (μg g^{-1} soil) †				
Native prairie	1910	7.1				
0	A		3.9	8.6	5.8	4.4
45 kg N ha^{-1}	AN				8.9	3.7
45 kg N, 20 kg P ha^{-1}	ANP				3.6	4.5
20 kg P ha^{-1}	AP				6.1	7.7
0	C		3.6	3.7	8.6	4.1
45 kg N ha^{-1}	CN				5.0	2.6
45 kg N, 20 kg P ha^{-1}	CNP				6.0	2.9
20 kg P ha^{-1}	CP				7.8	2.7

†spaces without numerical data reflect time frames prior to fertilizer treatments

Appendix E

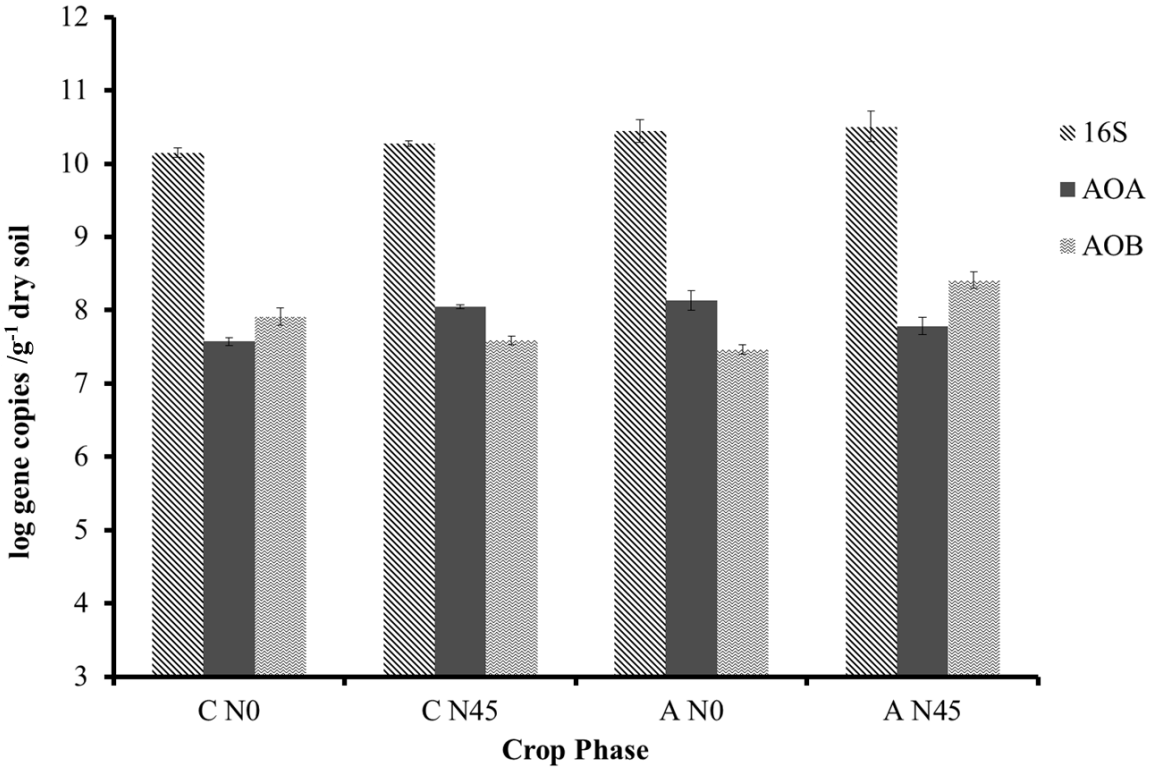

Fig. E.1 Comparison of 16S rRNA abundance to AOA and AOB, as affected by cropping intensity (CI) and N fertilizer levels (kg ha^{-1}); bars denote standard error.