Microbial Communities in Agricultural Soil – Diversity, Abundance and Activity Impacted by Fertilization, Cropping and Tillage Practices

by

Nicola Frances Linton

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements for the degree of Doctor of Philosophy in Environmental Sciences

Guelph, Ontario, Canada

© Nicola Frances Linton, January, 2020
ABSTRACT

MICROBIAL COMMUNITIES IN AGRICULTURAL SOIL – DIVERSITY, ABUNDANCE AND ACTIVITY IMPACTED BY FERTILIZATION, CROPPING AND TILLAGE PRACTICES

Nicola Frances Linton
Univeristy of Guelph, 2019

Advisors:
Dr. Kari Dunfield

Soil is a complex ecosystem that supports diverse communities of microorganisms carrying out multiple biogeochemical cycles, which contribute to its health and functioning. Agricultural management practices such as nutrient inputs, tillage and crop rotations impact physiochemical and biological soil qualities, and the provision of multiple ecosystem services. The objectives of this thesis are to understand how management impacts soil bacterial functional groups involved in nitrogen cycling, as well as total bacterial and fungal diversity. We found that delayed release urea fertilizer increased nitrogen cycling gene abundance and decreased nitrous oxide emissions in a soil microcosm study. We further explored links between nitrogen cycling microbial communities and nitrous oxide emissions in a long-term field study with simple and diverse crop rotations under no till. Long-term no tillage and diversification of corn-soybean rotations with winter wheat and red clover cover increased total, nitrifying, and denitrifying (nirK and nosZ2) bacterial functional groups during the second-year corn
phase. Nitrous oxide emissions occurred after urea ammonium nitrate fertilization and were higher in the diverse rotation, highlighting that agricultural management practices can improve soil health but also lead to alteration of nutrient cycling pathways as a tradeoff. The effects of long-term tillage and crop rotational diversity on total bacterial and fungal diversity were also explored through use of high throughput sequencing of soil sampled during the second-year corn phase. Tillage decreased bacterial and fungal diversity but increased community evenness. Alfalfa alone or in rotation with corn increased fungal diversity metrics but had less of an impact on bacterial diversity. Bacterial and fungal community composition was shaped by both tillage and crop rotational diversity. Tillage and crop rotational history were associated with bacterial and fungal taxonomic groups that were relatively more abundant in soils under each long-term management strategy. This thesis shows how a multitude of bacterial functional groups, as well as total bacterial and fungal communities can be shaped by agricultural management practices and influence the processes that provide many soil ecosystem benefits.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Kari Dunfield, for her inspiration, support and guidance throughout my PhD studies as I entered the wonderful world of soil microbiology. I feel very fortunate to have worked under your expertise, in an environment where I could learn so much, and which has been an incredible journey both personally and professionally. I am very thankful to have you as my PhD supervisor. I would also like to thank Dr. Shu Chen and Dr. Claudia Wagner-Riddle for your mentorship, advice and support while acting as members of my advisory committee. I am most fortunate to have had such a strong group of mentors.

I also would like to thank and extend my gratitude to members of the Dunfield and Wagner-Riddle labs for their support in the lab, field, and with data analysis. I am incredibly grateful to have learned from your own research, experience and many discussions during my graduate studies. My time in SES has always felt very supported by a wonderful team of fellow students and colleagues who were always willing to share their time and help. I am also very thankful to the faculty and staff in SES who helped me learn many facets of soil science and for helping me with all aspects of my graduate work.

And of course, many thanks to my friends and family who provided their unwavering support as I embarked on one of the most challenging and exciting journeys
in my research and academic career. Thank you for your patience and enthusiasm as I shared my many puzzles and discoveries about soil microbiology.

I would like to acknowledge and thank the funding sources of my research project and program, including the Ontario Graduate Scholarship (OGS), the Natural Sciences and Engineering Research Council of Canada (NSERC), the University of Guelph and the Food from Thought program, and the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA).
TABLE OF CONTENTS

Abstract ........................................................................................................................................ ii

Acknowledgements .................................................................................................................... iv

Table of Contents .......................................................................................................................... vi

List of Tables .................................................................................................................................. xiii

List of Figures ................................................................................................................................. xiv

List of Abbreviations ...................................................................................................................... xvii

List of Appendices .......................................................................................................................... xx

1 Introduction .................................................................................................................................... 1

1.1 Soil Ecosystems and Agriculture ............................................................................................. 1

1.2 The Soil Environment .............................................................................................................. 2

1.3 Biogeochemical Cycling ......................................................................................................... 3

1.4 Biogeochemical Cycling of Nitrogen ....................................................................................... 4
1.5 Agricultural Management Effects on Soil Health and Microbial Communities .11

1.6 Research Objectives and Thesis Format.................................................................15

2 Nitrogen Cycling Soil Bacteria Respond Differently to Urea and SuperU Amendment ........................................................................................................17

2.1 Abstract ................................................................................................................18

2.2 Introduction ............................................................................................................19

2.3 Materials and Methods..........................................................................................24

2.3.1 Soil Sampling and Microcosm Experimental Setup ...........................................24

2.3.2 Soil Sampling for Microbial Analysis .................................................................25

2.3.3 Soil Nucleic Acid Extraction and RNA Reverse Transcription to cDNA ....25

2.3.4 Quantification of Total Bacteria and Nitrogen Cycling Bacterial Genes and Transcripts .............................................................................................................26

2.3.5 Soil Inorganic Nitrogen .......................................................................................28

2.3.6 Nitrous Oxide Measurements ............................................................................29

2.3.7 Statistical Analysis .............................................................................................29
2.4 Results ........................................................................................................................................30

2.4.1 Soil Ammonium and Nitrate ........................................................................................................30

2.4.2 Soil Nitrous Oxide Emissions ......................................................................................................30

2.4.3 Nitrogen Cycling and Total Bacterial Community Abundance and Activity
in Urea and SuperU Treated Soil ........................................................................................................33

2.4.3.1 Total bacterial community ........................................................................................................33

2.4.3.2 Bacterial Communities Involved in Dissimilatory Nitrate Reduction to
Ammonium .........................................................................................................................................33

2.4.3.3 Urease bacterial community .....................................................................................................34

2.4.3.4 Nitrifying bacterial community .................................................................................................34

2.4.3.5 Denitrifying bacterial community – nitrite reducers .................................................................35

2.4.3.6 Nitrous oxide reducers ..............................................................................................................36

2.5 Discussion .....................................................................................................................................41

2.6 Conclusion .....................................................................................................................................48
3 Wheat and Cover Crop alter Nitrogen Cycling Microbial Communities and a Nitrous Oxide Emission Event in a Corn-Soybean Cropping System........................................49

3.1 Abstract........................................................................................................................................50

3.2 Introduction ......................................................................................................................................51

3.3 Materials and Methods ..................................................................................................................56

3.3.1 Site Description and Experimental Design .................................................................56

3.3.2 Soil Sampling for Microbial Analysis .................................................................................57

3.3.3 Soil Nucleic Acid Extraction and RNA Reverse Transcription to cDNA....58

3.3.4 Quantification of Total Bacteria and Nitrogen Cycling Bacterial Genes and Transcripts..................................................................................................................58

3.3.5 Nitrous Oxide Measurements ...............................................................................60

3.3.6 Soil Inorganic Nitrogen and Moisture Measurement......................................................61

3.3.7 Soil Collection and Processing for Bacterial Diversity Analysis.........................62

3.3.8 Statistical Analysis .................................................................................................................63

3.4 Results ........................................................................................................................................64
3.4.1 Site characterization and N₂O Emission Measurements .....................64
  3.4.1.1 Air Temperature, Precipitation and Water Filled Pore Space ..........64
  3.4.1.2 Soil Ammonium and Nitrate ........................................64
  3.4.1.3 Soil Nitrous Oxide Emissions ........................................65
  3.4.1.4 Bacterial Diversity ..........................................................67
3.4.2 Bacterial Community Abundance and Activity in Simple and Diverse Corn Rotations .................................................................68
  3.4.2.1 Total Bacterial Community .................................................68
  3.4.2.2 Nitrifying Bacterial Community ...........................................68
  3.4.2.3 Denitrifying Bacterial Community – Nitrite Reducers .................70
  3.4.2.4 Complete Denitrification – Nitrous Oxide Reducers ....................71
3.4.3 Bacterial Abundance and Activity – Links to Soil Nitrogen ...............74
3.5 Discussion ....................................................................................77
  3.5.1 Nitrifying Bacteria in Simple and Diverse Crop Rotations ..............79
  3.5.2 Denitrifying Bacteria from Simple and Diverse Crop Rotations .........82
4 Three and a Half Decades of Tillage and Rotation alters Bacterial and Fungal Communities in a Corn Based Cropping System

4.1 Abstract

4.2 Introduction

4.3 Materials and Methods

4.3.1 Site Description and Experimental Design

4.3.2 Soil Sampling for Microbial Analysis

4.3.3 Soil Nucleic Acid Extraction and Preparation for High Throughput Sequencing

4.3.4 Bioinformatics Pipeline and Sequence Analysis

4.3.5 Diversity Analysis and Statistical Tests

4.4 Results

4.4.1 Bacterial and Fungal Sequence Analysis

4.4.2 Bacterial and Fungal Alpha Diversity
4.4.3 Bacterial and Fungal Community Composition........................................99

4.4.4 Bacterial and Fungal Taxonomic Groups Shaped by Long-Term Tillage ..... 105

4.4.5 Bacterial and Fungal Taxonomic Groups Shaped by Long Term Crop Diversity ................................................................................................................. 109

4.4.5.1 Tilled Corn Rotations .............................................................................. 109

4.4.5.2 No Till Corn Rotations ........................................................................... 111

4.5 Discussion..................................................................................................... 114

4.5.1 Bacterial and Fungal Communities are Impacted by Tillage .............. 114

4.5.2 Bacterial and Fungal Communities are Impacted by Crop Rotation ...... 119

4.6 Conclusion .................................................................................................... 124

5 General Discussion and Conclusion ............................................................... 126

References ........................................................................................................ 131

Appendices....................................................................................................... 151
**LIST OF TABLES**

Table 2.1 Quantitative PCR Primers, Conditions and PCR Efficiencies ...........................27

Table 2.2 Results of ANOVA for total and nitrogen cycling bacterial genes in urea and SuperU treated soils. ................................................................................................................37

Table 2.3. Results of ANOVA for total and nitrogen cycling bacterial transcripts in urea and SuperU treated soils. ...........................................................................................................39

Table 3.1 Alpha diversity metrics and tests of significance for bacterial diversity in soil from no till CCSS and CCSWrc (n=4). ..............................................................................................................67

Table 3.2 Results of ANOVA of soil total and nitrogen cycling bacterial gene and transcript abundance in simple (CCSS) and diverse (CCSWrc) long term crop rotations. ..........................................................................................................................72

Table 4.1 P-values for test of significance differences in bacterial and fungal alpha diversity metrics from long-term crop rotational diversity and tillage. .................................................98

Table 4.2 PERMANOVA of the bacterial 16S rRNA Bray Curtis dissimilarity matrix from long-term corn rotations with tillage and no till management. ............................................102

Table 4.3 PERMANOVA of the fungal ITS Bray Curtis dissimilarity matrix from long-term corn rotations with tillage and no tillage management. .........................................................105
LIST OF FIGURES

Figure 1.1 Nitrification pathway ........................................................................................................5

Figure 1.2 Denitrification pathway ....................................................................................................6

Figure 1.3 Nitrifier denitrification .....................................................................................................8

Figure 1.4 Dissimilatory nitrate reduction to ammonium .................................................................8

Figure 2.1 Soil NH\textsubscript{4}\textsuperscript{+}, NO\textsubscript{3}\textsuperscript{-}, daily N\textsubscript{2}O emissions, and cumulative N\textsubscript{2}O in a 35-day microcosm incubation study with urea and SuperU. .................................................................32

Figure 2.2 Gene abundance of total and nitrogen cycling bacterial groups in soil microcosms treated with urea and SuperU. .................................................................................................39

Figure 2.3 Transcript abundance of total and nitrogen cycling bacterial groups in soil microcosms treated with urea and SuperU. .................................................................................................41

Figure 3.1 Weather, inorganic N, WFPS and N\textsubscript{2}O emissions in long term no till CCSS and CCSWrc rotations .................................................................................................................................66

Figure 3.2 Gene and transcript abundance of total and nitrogen cycling bacterial groups in soil from simple (CCSS) and diverse (CCSWrc) long term crop rotations under no till management. ........................................................................................................74
Figure 3.3 Redundancy analysis of soil nitrogen cycling gene abundance, NH$_4^+$, NO$_3^-$, N$_2$O emissions and WFPS from CCSS and CCSWrc. ................................................................. 75

Figure 4.1 Principle coordinate analysis of the Bray Curtis dissimilarity index for bacterial 16S rRNA amplicon sequences. ................................................................. 101

Figure 4.2 Principle coordinate analysis of the Bray Curtis dissimilarity index for fungal ITS amplicon sequences. ................................................................. 104

Figure 4.3 Cladogram showing bacterial taxa with higher relative abundance in till (green) and no till (red) corn rotations according to LEfSe. ........................................... 107

Figure 4.4 Cladogram showing the fungal taxa from division to genus with higher relative abundance in till (green) and no till (red) corn rotations according to LEfSe... 108

Figure 4.5 Cladogram for bacterial taxa with higher relative abundance in long-term corn rotations under tilled management according to LEfSe. ........................................ 110

Figure 4.6 Cladogram showing the fungal taxa with higher relative abundance in long-term corn rotations under tillage management according to LEfSe. ....................... 111

Figure 4.7 Cladogram showing significant bacterial taxa with higher relative abundance in long-term corn rotations under no till management according to LEfSe.......... 113
Figure 4.8 Cladogram showing the fungal taxa from division to genus with higher relative abundance in long-term corn rotations under no till management according to LEfSe.
LIST OF ABBREVIATIONS

AAAA: continuous alfalfa
AMF: arbuscular mycorrhizal fungi
AMO: ammonia monooxygenase
amoA: ammonia monooxygenase gene
ANOVA: analysis of variance
ASV: analysis sequence variant
BMP: best management practice
C: carbon
CCAA: corn-corn-alfalfa-alfalfa rotation
CCCC: continuous corn
CCSS: corn-corn-soybean-soybean rotation
CCSWrc: corn-corn-soybean-winter wheat with red clover cover
CCWS: corn-corn-soybean-winter wheat rotation
CO₂: carbon dioxide
DCD: dicyandiamide
DNRA: dissimilatory nitrate reduction to ammonium
Faith-PD: Faith’s phylogenetic diversity
GHG: greenhouse gas
HAO: hydroxylamine oxidoreductase
HTS: high throughput sequencing
K: potassium

LEfSe: linear discriminant analysis of effect size

N: nitrogen

N₂: dinitrogen gas

N₂O: nitrous oxide

NAP/NAR: nitrate reductase

NBPT: N-(n-butyl)-thiophosphoric triamide

NH₃: ammonia

NH₄: ammonium

NIR: nitrite reductase

nirS/nirK: nitrite reductase genes

NO: nitric oxide

NO₂⁻: nitrite

NO₃⁻: nitrate

NOR: nitric oxide reductase

NOS: nitrous oxide reductase

nosZ1/2: nitrous oxide reductase genes

nrfA: nitrite reductase gene used in DNRA

NXR: nitrite oxidoreductase

O₂: oxygen

OTU: operational taxonomic unit

P: phosphorus
PCoA: principle coordinate analysis
PERMANOVA: permutational analysis of variance
PLFA: phospholipid fatty acid
qPCR: quantitative PCR
RDA: redundancy analysis
SOC: soil organic carbon
SOM: soil organic matter
UAN: urea ammonium nitrate
ureC: urease gene
WFPS: water filled pore space
LIST OF APPENDICES

Appendix 5.1 Quantitative PCR Primers, Conditions and Efficiency. .......................... 151

Appendix 5.2 PCA of bacterial sequence variants in four replicate plots of long-term no till CCSS and CCSWrc. ........................................................................................................ 152

Appendix 5.3 Spearman rank correlation of total and nitrogen cycling gene abundance with mean daily N₂O emissions, inorganic nitrogen and water filled pore space. .......... 153

Appendix 5.4 Spearman rank correlation of total and nitrogen cycling transcript abundance, ratio of nir to nosZ, mean daily N₂O emissions, inorganic nitrogen and water filled pore space. .................................................................................. 154

Appendix 5.5 Bacterial richness and diversity indices in till and no till (corn rotations only) .................................................................................................................................. 154

Appendix 5.6 Bacterial richness and diversity indices in 6 long-term crop rotations. 155

Appendix 5.7 Bacterial richness and diversity indices in 5 long-term corn rotations under tillage. .................................................................................................................................. 156

Appendix 5.8 Bacterial richness and diversity indices in 5 long-term crop rotations under no till. .................................................................................................................................. 157
Appendix 5.9 Fungal richness and diversity indices in till and no till (corn rotations only).
........................................................................................................................................157

Appendix 5.10 Fungal richness and diversity indices in 6 long-term crop rotations.....158

Appendix 5.11 Fungal richness and diversity indices in 5 long-term corn rotations under tillage. ........................................................................................................................................................................159

Appendix 5.12 Fungal richness and diversity indices in 5 long-term corn rotations under no till. ........................................................................................................................................................................160

Appendix 5.13 Pairwise comparisons of bacterial community composition between long-term crop rotations. ........................................................................................................................................................................161

Appendix 5.14 Pairwise comparisons of fungal community composition between long-term crop rotations. ........................................................................................................................................................................162
1 Introduction

1.1 Soil Ecosystems and Agriculture

Soil is a complex ecosystem which supports a diverse and interconnected network of micro- and macro organisms. In an agricultural context, soil functions as a substrate for crop growth, providing essential water and nutrients. Management practices and nutrient inputs used to maximize crop yields will impact the physiochemical and biological qualities of soils, affecting the health of the soil ecosystem. There is a need to maintain and even improve soil health and quality, to ensure sustainable crop production in light of a growing world population and in the face of a changing climate (De Vries et al., 2012; Tilman, 1999). The concept of soil quality was introduced as “the capacity of soil to function” (Karlen et al., 1997). Soil quality and soil health are sometime used interchangeably and in agriculture, a healthy soil not only supports plant growth but provides other ecosystem services such as reduction in greenhouse gas (GHG) emissions, improvement of water quality, retention of soil organic matter (SOM), and efficient nutrient cycling (Kibblewhite et al., 2008; Mann et al., 2019). Soil microbial communities, encompassing bacteria, fungi and archaea, are drivers of plant residue decomposition, SOM formation, carbon (C) and nitrogen (N) cycling, stabilization of aggregates, and improvement of soil structure, all of which contribute to healthy soil functioning (Lehman et al., 2015; Saccà et al., 2017). There is a recognized link
between anthropogenic soil management, microbial biodiversity and functioning, soil health, and human health (Mann et al., 2019; Maron et al., 2018; Roger-Estrade et al., 2010; Thiele-Bruhn et al., 2012; Wall et al., 2015). The biodiversity of soil and the multitude of functions that communities carry out are recognized as a component of soil resistance and resilience to perturbations (Griffiths and Philippot, 2013). It is important to understand how agricultural management affects soil physicochemical properties but also soil biodiversity and functions, to contribute to our understanding of the relationship between them and the impact on provision of beneficial soil ecosystem services.

1.2 The Soil Environment

Soil health tests such as the Cornell or Ontario Soil Health Assessments incorporate physical, chemical and biological measures to assess the current status and impact of past management practices (Bünemann et al., 2018; Congreves et al., 2015). Physical properties include good structure and aggregate stability, water holding capacity, and bulk density, which require management to ensure optimal health of the soil system. The chemical properties of soil, such as pH, buffer capacity, nutrients such as N, phosphorus (P) and potassium (K), SOM, and soil organic C (SOC) are all influenced to a degree by the soil physical environment, with optimal pH ranges necessary for particular crops, and ensuring vital macro and micronutrients are available and accessible. The biological component of soil includes the microbial
communities residing there, which carry out numerous important functions. Due to the interconnected nature of soils, alterations to soil chemical and physical properties from management practices, will influence the biological component, and together they will interact to shape the soil ecosystem. For example, increases in SOM change available nutrients and increase moisture, which in turn alter the environment within which soil microbial communities are living (Turmel et al., 2015). The use of manure, chemical fertilizer, and crop residue addition or removal causes sudden changes in nutrients and can shift biogeochemical cycling in agricultural systems, increasing the turnover of nutrients such as N or C.

1.3 Biogeochemical Cycling

Soil microbial communities are the drivers of biogeochemical cycling of nutrients in soils. The C, N, P, and K in plant inputs, crop residues, inorganic fertilizer, and manure, are metabolized by various soil bacterial, fungal and archaeal groups. The majority of soil microbial communities are involved in decomposition of available materials resulting in the formation of SOM (Schimel and Schaeffer, 2012; Smith et al., 2015). Broad taxonomic groups are involved in decomposition and organic matter formation. For example, a wide phylogenetic range of fungi and bacteria decompose organic material and contribute to organic matter turnover, however fungi are better able to use lignin, while bacteria are better at utilizing higher quality, more labile materials (Fabian et al.,
Soil microbial communities can produce a variety of SOM compounds with both simple and complex chemistry (Bonanomi et al., 2017; Kallenbach et al., 2016). Higher ratios of fungi to bacteria have been linked to greater C use efficiency and accumulation of SOC (Malik et al., 2016). In comparison to the broad phylogenetic groups involved in C turnover, a relatively narrower range of soil microbial communities are involved in N cycling, with only a subset of fungi, archaea and particular bacterial genera involved in the various pathways (Hayatsu et al., 2008). Due to the use of N fertilizer in intensively managed agricultural systems it is important to understand how N is cycled by microbial groups within the soil environment, as the production and release of certain N compounds such as nitrous oxide (N\textsubscript{2}O), an important GHG, ammonia (NH\textsubscript{3}) volatilization, and nitrate (NO\textsubscript{3}\textsuperscript{-}) production has important environmental consequences.

### 1.4 Biogeochemical Cycling of Nitrogen

Understanding the N cycle is a key step in determining which pathways are playing a role in soil N transformations, and the potential routes of N loss. The N cycle is a set of interconnected pathways where N is transformed from one form to another through the actions of microorganisms, as well as abiotic chemical decomposition of intermediates (Butterbach-Bahl et al., 2013; Hayatsu et al., 2008). Soil N and the role microorganisms play in its biogeochemical cycling has been under investigation since
the 1800s, when the importance of N for plant growth was first recognized (Galloway et al., 2013). Initially two main pathways were identified, nitrification and denitrification, but our understanding of the pathways involved in N transformations has expanded to comprise 6 interlinked processes (Firestone and Davidson, 1989; Kuypers et al., 2018; Stein and Klotz, 2016). In addition to nitrification and denitrification there are pathways that provide N assimilation, ammonification, anaerobic ammonium oxidation, and N fixation (Kuypers et al., 2018; Stein and Klotz, 2016). Decades of research has focused on factors controlling N transformations and in particular N₂O production and consumption in soils (Abbasi and Adams, 2000; Baggs, 2011; Butterbach-Bahl et al., 2013; Firestone and Davidson, 1989; Thompson et al., 2016).

The nitrification pathway involves ammonia/ammonium (NH₄⁺) oxidation to NO₃⁻ in a stepwise set of enzymatic reactions that produce two intermediates, hydroxylamine and nitrite (NO₂⁻) (Butterbach-Bahl et al., 2013; Stein and Klotz, 2016) (Figure 1.1).

\[
\text{NH}_4^+ \xrightarrow{} \text{NH}_2\text{OH} \xrightarrow{} \text{NO}_2^- \xrightarrow{} \text{NO}_3^-
\]

**Figure 1.1 Nitrification pathway**

Ammonium is a positively charged cation that can be produced through hydrolysis of organic compounds such as urea or supplied from inorganic fertilizer. Ammonium, when present in ammoniacal form, can lead to NH₃ volatilization, unless
conditions are suitable for the process of nitrification. The first and rate limiting step of nitrification involves oxidation of NH$_4^+$ to hydroxylamine (Bothe et al., 2000). Hydroxylamine can be further oxidized to NO$_2^-$, with nitric oxide (NO) now known to be produced as an additional intermediate, but can also undergo chemical decomposition leading to N$_2$O formation (Butterbach-Bahl et al., 2013; Kuypers et al., 2018). The final step in nitrification, NO$_2^-$ oxidation to NO$_3^-$ is essential in preventing NO$_2^-$ accumulation. The steps in nitrification are dependent on substrate availability and sufficient oxygen (O$_2$) for the enzymatic reactions to be carried out (Sahrawat, 2008).

The denitrification pathway is also a stepwise process, where NO$_3^-$ is respired to NO$_2^-$ and N gases NO, N$_2$O, and dinitrogen gas (N$_2$) (Butterbach-Bahl et al., 2013; Hayatsu et al., 2008; Stein and Klotz, 2016) (Figure 1.2). Nitrate in soil can be produced by nitrification reactions, as well as from application of fertilizers containing NO$_3^-$.

$$\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$$

Figure 1.2 Denitrification pathway

Denitrification is used by microbes capable of carrying out one or more of the steps when adapting to declining O$_2$ conditions, in order to maintain cellular respiration (Giles et al., 2012; Khalil et al., 2004). Denitrification is considered to be primarily carried out under anaerobic conditions, and is dependent on availability of N oxides,
sufficiently low O\textsubscript{2}, soil moisture, C, and pH (Baggs, 2011; Bakken et al., 2012; Butterbach-Bahl et al., 2013; Firestone and Davidson, 1989; Giles et al., 2012; Zumft, 1997). When the stepwise reduction of NO\textsubscript{3}\textsuperscript{-} to N\textsubscript{2} is incomplete, N\textsubscript{2}O can be produced as an end product (Braker and Conrad, 2011; Butterbach-Bahl et al., 2013; Khalil et al., 2004; Zumft, 1997). Low O\textsubscript{2} and higher C availability have been found to promote complete denitrification, as O\textsubscript{2} can inhibit the N\textsubscript{2}O reductase enzyme (Morley and Baggs, 2010). Low pH has been shown to inhibit N\textsubscript{2}O reductase assembly, thereby producing higher N\textsubscript{2}O:N\textsubscript{2} ratios (Bakken et al., 2012).

Denitrification can produce N\textsubscript{2}O as an end product when conditions do not allow for further metabolism to N\textsubscript{2}, but N\textsubscript{2}O can also be a product of nitrification under certain soil N, moisture, and O\textsubscript{2} conditions (Baggs, 2011; Hink et al., 2016; Kool et al., 2009; Zhu et al., 2013). Nitrous oxide can be produced through nitrifier denitrification (Kool et al., 2011; Wrage-Mönnig et al., 2018)(Figure 1.3), coupled nitrification-denitrification, and dissimilatory nitrate reduction to ammonia (DNRA, Figure 1.4) (Butterbach-Bahl et al., 2013; Giles et al., 2012; Mohan et al., 2004; Smith and Zimmerman, 1981).

Dissimilatory nitrate reduction to ammonium occurs when both C availability and O\textsubscript{2} are optimal, as they determine the metabolic benefits of DNRA over denitrification (Morley and Baggs, 2010). Multiple studies have shown that when urea or NH\textsubscript{4}\textsuperscript{+} is supplied and soils are kept under aerobic conditions, with O\textsubscript{2} above 0.5% and soil moisture at 50-55% water filled pore space (WFPS), that bacterial ammonia oxidation and nitrifier
denitrification contribute significantly to N$_2$O release, which was not measured when ammonium oxidation was inhibited (Hink et al., 2016; Liu et al., 2016; Uchida et al., 2013; Zhu et al., 2013).

\[
\begin{align*}
\text{NH}_4^+ & \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \\
\text{NO}_3^- & \rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+ \\
\end{align*}
\]

**Figure 1.3 Nitrifier denitrification**

**Figure 1.4 Dissimilatory nitrate reduction to ammonium**

The steps of the N cycle are mostly carried out by microbially driven enzymatic processes. Nitrification can be performed by a variety of soil microorganisms, including bacteria, archaea and some fungi (Hayatsu et al., 2008; Prosser and Nicol, 2012; Taylor et al., 2016). The first step, ammonia oxidation, is thought to be performed primarily by bacteria and archaea using the enzyme ammonia monooxygenase (AMO), whereas further oxidation of hydroxylamine from hydroxylamine oxidoreductase (HAO) is currently only known to be carried out by bacteria (Kuypers et al., 2018). Nitrite
oxidation is catalyzed by nitrite oxidoreductase (NXR), present in a wider variety of bacteria than those with AMO (Kuypers et al., 2018; Schreiber et al., 2012; Stein and Klotz, 2016). Previously, organisms were thought to carry out either ammonia oxidation or nitrite oxidation, but recently complete nitrification was shown for Candidatus Nitrospira inopinata (Daims et al., 2015; Koch et al., 2019). Denitrification can also be carried out by soil bacteria, archaea and some fungi (Hayatsu et al., 2008; Kuypers et al., 2018; Stein and Klotz, 2016). In denitrification the reduction of NO$_3^-$ to NO$_2^-$ and NO is carried out by groups capable of producing the enzymes nitrate reductase (NAP/NAR) and nitrite reductase (NIR) respectively (Kuypers et al., 2018). Reduction of the N gases NO and N$_2$O requires the enzymatic activity of nitric oxide and nitrous oxide reductases (NOR and NOS) (Kuypers et al., 2018). It is important to remember that soil is heterogenous in nature with pockets and niches of varying nutrient concentration and availability, moisture, and O$_2$. Experiments have shown that nitrification and denitrification can be carried out and produce N$_2$O simultaneously (Abbasi and Adams, 2000; Khalil et al., 2004), so prevalent conditions may not limit various groups of microorganisms to one process or the other and measured N products will be the culmination of multiple enzymatic and chemical processes.

Soil N cycling microbial groups are the mechanism of N$_2$O production and understanding conditions and management practices that impact their activities can assist in designing systems that provide beneficial soil ecosystem services, while
minimizing N\textsubscript{2}O production. Important groups of bacteria and archaea that are involved in ammonium oxidation are Betaproteobacteria, Gammaproteobacteria, and Thaumarchaeota, while nitrite oxidation can be performed by Alpha-, Beta-, and Gammaproteobacteria, as well as Chloroflexi, Nitrospinae and Nitrospirae (Kuypers et al., 2018; Stein and Klotz, 2016). Denitrification on the other hand can be carried out by a much wider range of microorganisms, and like nitrification some have the cellular machinery to carry out the complete pathway, while others are restricted to certain steps (Giles et al., 2012). The ability to denitrify, in part or fully, is not restricted to any particular set of bacterial groups but can be performed across phylogenetic groups, and it has also been demonstrated in soil fungi, cultured archaea and some protists (Giles et al., 2012; Hayatsu et al., 2008; Stein and Klotz, 2016).

Soil bacteria and specific functional groups involved in N cycling can be investigated using molecular techniques. By targeting the gene for each of the enzymes involved we can use quantitative measures of gene abundance as proxies for community size. As soil microorganisms may be present in soils but not necessarily active, measuring the RNA copies of each gene gives us a greater indication of whether that step in the pathway is being used. Transcript copy numbers, as they are an intermediate step in protein expression, provide a proxy for measuring the potential activity of soil functional groups. Targeting both DNA and RNA allows researchers to
answer questions regarding the community response to perturbations and any changes in activity.

1.5 Agricultural Management Effects on Soil Health and Microbial Communities

Agricultural practices involve various management strategies to ensure optimal yields, as well as ensuring soils can support continued crop production over the long term. The use of inorganic or organic fertilizers, pesticide application, tillage, as well as rotation of crops are the main options for agroecosystem management. Their direct or indirect effect, as well as interactions between them will shape the soil physicochemical and biological qualities.

Manure or inorganic fertilizer application to soil can supply both simple, readily accessible compounds to microbial communities, as well as more complex substrates. Increasing SOM through inputs of manure, fertilizer, and crop residues not only changes soil chemistry but can also improve soil physical parameters such as soil structure, water retention, and cation exchange capacity (Manns et al., 2016; Munkholm et al., 2013; Smith et al., 2015; Van Eerd et al., 2014). Changes to the soil environment will indirectly influence soil microbial communities, who can also be shaped by the direct effects of nutrient additions. Inorganic fertilizer use provides an immediate influx of accessible substrates for microbial communities in the form of urea, NH$_4^+$, NO$_3^-$, or
combinations thereof, often with other elements such as P and K. Nitrogen is an important crop nutrient but its metabolism by soil N-cycling microbial groups can reduce the amount of N accessible to crops and also lead to GHG emissions such as N₂O. As the soil physical, chemical and biological characteristics will determine which N cycle processes can be preferentially carried out, and which N products are produced, soil management will influence N cycling (Butterbach-Bahl et al., 2013; Firestone and Davidson, 1989; Giles et al., 2012; Morley and Baggs, 2010; Sahrawat, 2008). One way of managing N availability is through the use of delayed release N fertilizers. They can be urea, NH₄⁺, or NO₃⁻ based with additional features such as protective coatings making them temporarily inaccessible to soil microorganisms, or with compounds designed to prevent or limit microbial enzymatic processes (Schwab and Murdock, 2010).

Tillage can be used to change soil structure, bulk density, porosity, incorporate residues left after harvest, and break up weeds (Cates et al., 2016; Pires et al., 2017; Van Eerd et al., 2014). Tillage causes changes in soil moisture, and O₂, from its impact on the structure and porosity of the soil physical environment, which influences water infiltration and movement, as well as gas and nutrient diffusion (Karlen et al., 1997; Pires et al., 2017). Tillage also physically breaks up crop residues and increases the depth to which they are incorporated in soil, making different substrates available to a wider variety of soil microorganisms, which can lead to increased microbial biomass in
the short-term (Roger-Estrade et al., 2010). Tillage can also impact soil microorganisms directly, by physically disrupting their communities, a feature that is useful in order to break up fungal networks that are plant pathogens, but this will also affect plant beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) (Roger-Estrade et al., 2010; Wilpiszeski et al., 2019). Numerous studies are using fingerprinting and molecular tools to investigate how soil microbial communities are shaped from anthropogenic activities, including tillage and crop production (Banerjee et al., 2019; Shange et al., 2012; Stagnari et al., 2014; Wang et al., 2017; Yin et al., 2010). Earlier studies have found mixed results regarding tillage impacts on microbial communities. Tillage has been shown to have a negative impact on fungal but a positive impact on bacterial diversity (Lienhard et al., 2014). However another study found that bacterial and archaeal diversity in soils was lower after 15 years of tillage compared to no tilled soils (Schmidt et al., 2018).

The type and diversity of crops grown either in monoculture or as part of a rotation strategy will supply a wider variety of plant inputs and residue types to the soil environment. In Ontario the major crops grown (area grown, in hectares of land) have been soybean, followed by grain corn, but more diverse rotations including wheat, oats, barley, or perennials and legume cover crops are promoted for their ability to improve grain yields, SOC, water and nutrient use efficiencies, as well as soil aggregation (Cates et al., 2016; Gaudin et al., 2013; Jarecki et al., 2018; Maiga et al., 2019; Renwick et al.,
Crop rotations are used in order to break plant pathogen disease cycles but provision of different inputs to soil from more varied crop exudates and residues of different composition has other beneficial impacts. Both soybean and corn are examples of crops planted in spring and harvested in fall, leaving the soil fallow throughout the non-growing season. Increasing crop diversification through use of cover crops in corn-soybean systems can prevent soil from lying fallow and increase the amount and diversity of crop residue inputs. Cover crops can improve soil through reduced loss of topsoil and nutrients from erosion and runoff, improved water infiltration and storage capacity, as well as increased soil organic matter and nitrogen through N-fixation for legume cover crops (Gaudin et al., 2013).

The link between increased crop diversity and belowground diversity is not clear. A meta-analysis of crop diversity effects on soil microbial diversity found that of 27 studies, 14 did not show any relationship, 9 showed a positive effect, and 2 a negative effect (Venter et al., 2016). Soil microbial communities are incredibly diverse, and little is known about their diversity, functions and redundancy in this environment. An increasing number of studies are utilizing molecular tools available to shed light into the impacts of agricultural management on microbial functioning and diversity, to better understand and promote beneficial changes to soil and maintain or improve the ecosystem services they provide.
1.6 Research Objectives and Thesis Format

The overall aim of the research presented in this thesis was to investigate:

- The response of N cycling bacterial groups to urea fertilizer with and without urease and nitrification inhibitors (Chapter 2)
- The response of N cycling bacterial groups to urea ammonium nitrate (UAN) fertilization after long-term crop rotational diversification (Chapter 3)
- Bacterial and fungal diversity and shifts in community composition from long-term crop diversity and tillage practices (Chapter 4)

In the second chapter quantitative PCR (qPCR) was used to investigate urea metabolizing, nitrifying and denitrifying bacterial groups in a 35-day soil microcosm study with two treatments, urea and urea with urease and nitrification inhibitors. Measurements of bacterial abundance and activity were linked to N₂O emissions, and soil NH₄⁺ and NO₃⁻. In chapter 3 qPCR measurement of nitrifying and denitrifying bacterial groups in a long-term field trial with simple and diverse corn rotations under no till management was performed by repeated sampling during the growing season of the second-year corn phase after UAN application. The abundance and activity of N cycling groups was linked to plot N₂O emissions, inorganic N and soil moisture. Finally, in chapter 4 high throughput sequencing (HTS) of the bacterial V4 region and fungal ITS1 region was utilized to investigate shifts in the total bacterial and fungal diversity and
community composition from long term tillage and crop rotations with corn, soybean, winter wheat (with and without red clover), and alfalfa.
2 nitrogen cycling soil bacteria respond differently to urea and superu amendment

*n Nicola F. Linton¹, Amanda Anderson, Katelyn Congreves², Claudia Wagner-Riddle¹, Kari Dunfield¹

¹School of Environmental Sciences, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada

²Department of Plant Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, S7N5A8, Canada

*corresponding author: lintonn@uoguelph.ca

Author Contributions:

N. Linton was the primary author for this study, having conducted soil sampling and lab work for microbial analyses, as well as data and manuscript preparation. Authors A. Anderson and K. Congreves are credited for study design and microcosm establishment, as well as provision of inorganic nitrogen and nitrous oxide data. Author C. Wagner-Riddle is credited with help in data interpretation and manuscript editing. Author K. Dunfield is credited with results interpretation and manuscript editing.
2.1 Abstract

Inefficient N fertilizer use leads to N losses from agroecosystems, resulting in economic losses and potential environmental harm from N cycling products such as N\textsubscript{2}O. Urease and nitrification inhibitors (N-(n-butyl)-thiophosphoric triamide and dicyandiamide) added to urea-based fertilizers can temporarily inhibit microbial urease and ammonia monooxygenase enzyme activity. Urea hydrolysis and nitrification inhibiting fertilizers aim to mitigate N loss and reduce N\textsubscript{2}O emissions by delaying urea conversion to NO\textsubscript{3}\textsuperscript{-}. However, there is conflicting evidence regarding urease and nitrification inhibitor reduction of N\textsubscript{2}O emissions and the impact of inhibition on key bacterial N cycling groups utilizing N cycling pathways for growth and survival. There are several bacterial genes that code for enzymes involved in N cycling pathways, and they can be used as markers of bacterial communities and their activity. These include urease (ureC) and ammonia monooxygenase (amoA), nitrite reductase (nirS/nirK in denitrification and nrfA in DNRA) and nitrous oxide reductase (nosZ\textsubscript{1} and nosZ\textsubscript{2}, typical and atypical). The objective of this research was to compare short-term N\textsubscript{2}O emissions from soil receiving SuperU or urea in an incubation study, and link differences to size and activity of N cycling bacterial communities. Soil samples collected the 0-15 cm top layer of a field in Elora, Ontario, were homogenized and 30g placed in microcosms maintained at 60% WFPS. SuperU and urea were applied at 120kg N/ha on day 0, mixed into soil and N\textsubscript{2}O emissions, soil nitrate and ammonium concentrations were
measured 7 times over 35 days. For microbial analyses, soil was sampled in triplicate, flash frozen in liquid N, and DNA and RNA extracted for qPCR quantification of the total bacterial community and nitrogen cycling functional groups using primers targeting 16S rRNA, ureC, amoA, nirS/nirK, nosZ1, nosZ2 and nrfA genes. Results indicate that SuperU reduced N\textsubscript{2}O emissions by 37\%, compared to urea treatment and soil NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{−} concentrations were lower early in the study. The SuperU treated soil had increased N cycling gene abundances for groups involved in DNRA, nitrification, and denitrification pathways, indicating communities were not negatively affected by urease and nitrification inhibitors. In both treatments we captured temporal changes in gene abundance as well as increased activity of N cycling pathways in response to the accumulation of N pathway substrates. This research provided information on a panel of N cycling bacterial groups and how they were impacted by urease and nitrification inhibitors under controlled conditions.

### 2.2 Introduction

Application of inorganic N fertilizer is a commonly used practice to optimize economic returns in crop production, including in Southern Ontario (OMAFRA, Agronomy Guide for Field Crops). However, N applied to the soil in agroecosystems can be lost to the environment, which reduces N use efficiency and can also cause environmental harm (Liu et al., 2013; Schwab and Murdock, 2010; Woodley et al.,
Pathways of N loss include NH$_3$ volatilization, NO$_3^-$ leaching, runoff and erosion, and N$_2$O and N$_2$ emissions to the atmosphere (Cameron et al., 2013). There is global concern regarding the potential negative consequences for ecosystems from release of N compounds to the environment (Coskun et al., 2017; Galloway et al., 2013; Liu et al., 2010; Ollivier et al., 2011). Nitrous oxide emissions are of particular interest due to its global warming potential 265 times that of carbon dioxide (CO$_2$) and its long lifespan in the atmosphere (~100 years, EPA online). Nitrous oxide emissions from agricultural activities in Canada accounts for 71% of national N$_2$O emissions, of which N fertilizer application contributed 23% (Environment and Climate Change Canada, 2019).

Decreasing loss of N from soils can be achieved through timing of application, by application of N during periods of increased plant requirement and uptake, but also through use of N fertilizer products with urease and nitrification inhibitors (Drury et al., 2012; Woodley et al., 2018). Urease inhibitors reduce NH$_3$ volatilization, and delaying nitrification using urease and nitrification inhibitors slows down conversion to NO$_3^-$ and reduce potential NO$_3^-$ leaching, as well as N$_2$O emissions (Cameron et al., 2013). Urea hydrolysis can be inhibited by compounds such as N-(n-butyl)-thiophosphoric triamide (NBPT) and phenylphosphorodiamidate, while the first step of nitrification, ammonia oxidation, can be inhibited by dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate, through temporary and reversible inhibition of the urease and AMO
enzymes (Cameron et al., 2013; Cantarella et al., 2018; Schwab and Murdock, 2010; Silva et al., 2017).

The use of enhanced efficiency N fertilizers for reduction of N loss in soils has been investigated in a number of studies. Urea treated with NBPT reduced NH$_3$ volatilization in field soils (Liu et al., 2019; San Francisco et al., 2011). In a microcosm study it was shown that NBPT reduced the rate of urea hydrolysis as well as nitrification, but increased N$_2$O emissions, in alkaline but not acidic soils (Fan et al., 2018). Other urease inhibitors such as phosphoric acid triamide, used alone or in conjunction with nitrification inhibitors reduced the amount of N lost as N$_2$O by 78 to 94% (Khalil et al., 2009). The reduction in loss of N as N$_2$O was observed in soils from a pot as well as field experiment (Khalil et al., 2009). Inhibition of nitrification using DCD can reduce nitrate leaching, N$_2$O emissions and gross nitrification rates, and temporal changes in NH$_4^+$ and NO$_3^-$ levels in both laboratory and field studies (Cameron et al., 2014; Chen et al., 2014; Harty et al., 2017, 2016; Lan et al., 2013; Yang et al., 2017). Altering the transformation of urea and ammonium through use of NBPT and DCD can shift the N products in soil, although this action is not consistent. Urine application to pasture soil incubated with DCD caused a slower conversion of NH$_4^+$ to NO$_3^-$, and reduced NO$_3^-$ accumulation compared to urine alone (O’Callaghan et al., 2010). Similar results were found in another study by Chen et al. (2014) where NH$_4^+$ increased sharply after urea application and the steady decline over time was slowed in treatments with DCD (Chen
et al., 2014). The use of DCD as a nitrification inhibitor increased soil inorganic N and shifted it to more \( \text{NH}_4^+ \), as well as increasing crop yields and plant N uptake (Liu et al., 2013). However in another study, when urea was used with NBPT in grassland there was no measurable effect on \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) or \( \text{N}_2\text{O} \) emissions at both low and high WFPS (Menéndez et al., 2009). The ability of combined urease and nitrification inhibitors to reduce \( \text{N}_2\text{O} \) emissions and alter N product concentrations varies due to multiple other soil factors that also influence enzymatic reaction rates, which can be controlled more in a laboratory incubation study.

Urease and nitrification inhibitors act on the microbial enzymes that catalyze urea hydrolysis and nitrification reactions. Nitrogen cycling pathways are used by soil microbial communities for growth and respiration. Inhibiting these processes could alter the activity and potential of the microbial group carrying out the process, leading to changes in community response. Studies have tested both urease and nitrification inhibitor effects on soil microbial communities, with the majority of research on nitrification inhibitors. Urease inhibition has been found to increase numbers of ureolytic organisms, but also to decrease their abundance as well as the abundance of nitrifiers (Fan et al., 2018; Li et al., 2019). Nitrification inhibitors have been shown to reduce nitrifier abundance in numerous studies, although the efficacy of DCD was not consistent, with some studies also reporting no effect (Chen et al., 2014; Dai et al., 2013; Di and Cameron, 2011; O’Callaghan et al., 2010; Yang et al., 2017). Some
studies also measured denitrifying communities, with studies reporting a reduction or no effect on abundance of nirS, nirK, nosZ1 and nosZ2 (Di et al., 2014; Kou et al., 2015).

The majority of studies have looked at a subset of either nitrification or denitrification genes, with fewer also measuring transcript abundance as a way to track changes in microbial activity over time. Here we used a panel of 7 N cycling gene targets to measure changes in community abundance as well as activity. This study used soil microcosms treated with urea as well as SuperU in order to investigate N cycling bacterial groups involved in urea hydrolysis, nitrification, as well as denitrification and DNRA in controlled conditions using both NBPT and DCD in combination. The objectives of this study were to investigate the efficacy of combined urease and nitrification inhibitors in reduction of N₂O emissions and delay in urea hydrolysis and nitrification, assess the impact of inhibition on 7 groups of N cycling soil bacteria, and link shifts in N to measures of gene and transcript abundance. We hypothesized that the Super U treated soils, compared to the urea treated soils, would have 1) lower N₂O emissions, 2) reduced NH₄⁺ and NO₃⁻ accumulation early in the study, 3) decreased ureC and amoA gene and transcript abundance in response to pathway inhibition, and 4) reduced denitrifier and DNRA gene and transcript abundances due to reduction in NO₃⁻ and N₂O which are substrates for these pathways.
2.3 Materials and Methods

2.3.1 Soil Sampling and Microcosm Experimental Setup

Soil was collected from the Elora, Ontario Research Station (43°39'N 80°25'W) in June 2016. The site used for soil collection was adjacent to a long-term field trial and was covered with natural vegetation consisting of grass. Soils from this site are classified as a silty clay loam (Grey Brown Luvisol) with 27 % sand, 57 % silt and 17 % clay and pH ranging from 7.2 to 7.5 (Congreves et al., 2015; Munroe et al., 2016). Soil from the top 15 cm was collected, transported back to the lab and allowed to dry. Soil was sieved to 2 mm and 30 g of soil placed into 50 ml conical tubes. Soil was wetted using deionized water to 30 % WFPS for initial pre incubation before treatments applied and set up at a field equivalent bulk density of 1.14 g cm$^{-3}$. Microcosms were pre incubated for 2 weeks at room temperature after initial wetting to allow microbial populations to stabilize and remove effects of initial wetting event. Fertilizer treatments consisted of urea and SuperU applied at a field equivalent rate of 120 kg-N ha$^{-1}$ and the WFPS brought to 60%. Four replicates of each treatment were established for N$_2$O emission measurements, which were done repeatedly over the course of the 35-day incubation. For inorganic N and microbial analysis, four replicates of each treatment were also established and as these measurements required destructive sampling, sufficient replicates of each treatment were established for all planned sampling time
points. After treatments were applied, microcosms were incubated in a completely random design at room temperature (20-21 °C) in the dark for a period of 35 days. During incubation microcosms were covered with parafilm and periodic weighing of the tubes was conducted to ensure WFPS remained constant.

2.3.2 Soil Sampling for Microbial Analysis

Microcosms were destructively sampled for microbial analysis on days 1, 3, 7, 10, and 35 (n=3). From each microcosm ~2 g was collected using nuclease free materials and immediately frozen in liquid N. Samples were stored on dry ice immediately after freezing and placed in a freezer at -80°C until RNA/DNA co-extraction was performed. A sub-sample (~5 g) of soil was also taken to determine gravimetric moisture content used to calculate gene and transcript copies per gram dry soil.

2.3.3 Soil Nucleic Acid Extraction and RNA Reverse Transcription to cDNA

Soil was stored at -80°C until processing. DNA and RNA were co extracted using the MoBio RNA Powersoil Extraction Kit with DNA co extraction kit and quantified using NanoDrop (ThermoFisher) prior to storage at -20°C (DNA) or -80°C (RNA). DNA removal from RNA was performed using RQ1 DNase (Promega) and confirmed using gel electrophoresis and qPCR, prior to reverse transcription to cDNA. The Applied Biosystems® High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corp.)
was used to reverse transcribe RNA to cDNA. Reactions with sample RNA were performed in triplicate, after which they were pooled and stored at -20°C. Appropriate no enzyme and no template controls were included. Nucleic acid extracts were tested for presence of inhibitors using qPCR detection of an M13 plasmid with spiked at 10^5 copies μl^-1 into reaction mixes.

2.3.4 Quantification of Total Bacteria and Nitrogen Cycling Bacterial Genes and Transcripts

Total bacterial communities and N cycling bacterial communities were quantified using qPCR by targeting 16S rRNA, ureC, nrfA, amoA, nirS/K, nosZ1 and nosZ2 genes and transcripts, using primer pairs 338F/518R (16S rRNA gene)(Fierer et al., 2005), nrfAF2aw/R1 (Welsh et al., 2014), ureC-F/R (Collier et al., 1999), amoA1F/2R (amoA)(Rotthauwe et al., 1997), cd3af/r3cd (nirS)(Throbäck et al., 2004), F1aCu/R3Cu (nirK)(Hallin and Lindgren, 1999), nosZ2F/2R (nosZ1)(Henry et al., 2006), and nosZ1F/R (nosZ2)(Harter et al., 2016). All qPCR was performed using a Bio-Rad CFX detection system (Bio-Rad Laboratories Inc.) using SsoFast EvaGreen Supermix (for 16S rRNA, ureC, nrfA, amoA, nirS/K, nosZ2) or iQ SYBR Green Supermix (nosZ1), with appropriate plasmid standard curves and diluted DNA/cDNA to reduce impact of PCR inhibition. Primer sequence, qPCR conditions, and qPCR efficiency for each target are shown in Table 2.1.
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer Sequence</th>
<th>Denaturation</th>
<th>Annealing Elongation</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA gene</td>
<td><strong>338f</strong> ACTCCTACGGGAGGCAGCAG</td>
<td>98°C – 5s</td>
<td>110.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>518r</strong> ATTACCGCGGCTGCTGG</td>
<td>55°C – 5s*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>ureC-F</strong> AAGSTSCACGAGGACTGGGG</td>
<td>95°C – 5s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>ureC-R</strong> AGGTGTTGGCASACCATSAGCAT</td>
<td>58°C – 15s</td>
<td>96.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>amoA 1F</strong> GGGGTCTTACTGGTGTT</td>
<td>98°C – 10s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>2R</strong> CCCCTCKGSAAGCCTTCTTC</td>
<td>55°C – 10s</td>
<td>104.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>nirS Cd3af</strong> GTSAACGTSAAGGARACSGG</td>
<td>98°C – 10s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>R3Cd</strong> GASTTCCGRTSGTCTTGA</td>
<td>57°C – 10s</td>
<td>105.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>nirK F1aCu</strong> ATCATGGTSCTGCCGCG</td>
<td>98°C – 10s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>R3Cu</strong> GCCTCGATCAGRTTGTGGTT</td>
<td>56°C – 10s</td>
<td>98.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>nosZ1 2F</strong> CGCRACGGCAASAAGGTSMSGTT</td>
<td>95°C – 10s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>2R</strong> CAKRTGCARKSCTGGCAGAA</td>
<td>60°C – 30s</td>
<td>99.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>nosZ-II-F</strong> CTNGGNCCNYTKCAYAC</td>
<td>95°C – 30s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>nosZ-II-R</strong> GCNGARCARAANTCBGTRC</td>
<td>58°C – 60s*</td>
<td>96.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>nrfA nrfAF2aw</strong> CARTGYCAYGTBGARTA</td>
<td>95°C – 10s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>nrfAR1</strong> TWWGGCATRTGRCARTC</td>
<td>53°C – 30s*</td>
<td>77.4</td>
<td></td>
</tr>
</tbody>
</table>
Primers were used at a final concentration of 0.3 µM for 338f/518r and amoA1F/2R, 0.4 µM for ureC-F/R, nrfAF2aw/R1, cd3af/r3cd and F1aCu/R3Cu, and 2 µM for nosZ2F/2R and nosZ2F/R. Standard curves for relative qPCR were generated from plasmid DNA containing the target gene (10^8-10^1 copies per reaction). Bacterial 16S rRNA, ureC, nrfA, amoA, nirS, nirK, nosZ1 and nosZ2 genes were cloned from Clostridium thermocellum spp. (16S rRNA gene), Nitrosomonas europaea spp. (amoA), Alcaligenes faecalis ATCC 8750 (nirK), Pseudomonas auruginosa PAO1 (ureC, nirS and nosZ1), Escherichia coli 25922 (nrfA), and Gemmatimonas aurantica 27-T DSM 14586 (nosZ2). PCR products were cloned in One Shot DH5α-T1R, Top10 Escherichia coli competent cells using a TOPO TA Cloning kit (Life Technologies Corp.). Cells were grown and plasmid extraction performed using Qiaprep Spin Miniprep Kit (Qiagen). Plasmids were sequenced to confirm target identity. All qPCR assays included no template controls and were optimized to reaction efficiencies of 77-110 % with R values of 0.995-1.000.

2.3.5 Soil Inorganic Nitrogen

The concentrations of soil NH₄⁺ and NO₃⁻ were measured by destructively sampling soil microcosms on days 0, 1, 3, 7, 10, 21 and 35. The soil extraction was performed according to Maynard and Kalra (1993), and the extracts were analyzed for
NH₄⁺ and NO₃⁻ concentrations using a continuous flow autoanalyzer (Seal AutoAnalyzer 3 - AA3, Seal Analytical).

2.3.6 Nitrous Oxide Measurements

Microcosms for gas measurements were set up with 4 replicates per treatment and measurements of N₂O emissions taken on day 0, 2, 4, 7, 13, 16, 21, 27, and 35. Microcosms used for gas analysis were placed in glass jars with a septum lid and each jar was allowed to equilibrate with the atmosphere prior to sealing in between each sample collection date. Air samples were taken from vials using a 10ml syringe with 25-gauge needle and collected until study completion. After sampling, the air in each microcosm was refreshed and lid was sealed until next sampling date. Air samples were sent for analysis by gas chromatography at Agricultural and Agri-Food Canada in Harrow, ON, Canada (Drury et al., 2012).

2.3.7 Statistical Analysis

Gene and transcript abundance data was normalized by copy number per gram of dry soil. Bacterial gene and transcript abundance, as well as soil inorganic N and N₂O emissions were analyzed using analysis of variance (ANOVA) in SAS version 9.4. A factorial GLIMMIX model was used with logarithmic transformation of the data, to test hypotheses of differences in treatments, over time as well as their interaction. Significant differences were determined by $p$-value <0.05.
2.4 Results

2.4.1 Soil Ammonium and Nitrate

Urea and SuperU application caused increases in soil $\text{NH}_4^+$ and $\text{NO}_3^-$, which varied over time in the microcosms. GLIMMIX analysis using both treatment and day as fixed factors showed a significant interaction between treatment and day ($p<0.0001$), for both $\text{NH}_4^+$ and $\text{NO}_3^-$. Interaction means were spliced by day to compare differences in treatment on each day. Soil $\text{NH}_4^+$ was significantly higher in the urea treatment on day 0, after treatments were applied (Figure 2.1A). In both treatments there was an increase in $\text{NH}_4^+$, which peaked on day 1, after which it declined. On day 3 $\text{NH}_4^+$ was significantly higher in the SuperU treatment. A steady decline in $\text{NH}_4^+$ was measured to levels below 11 mg N kg$^{-1}$ soil which occurred on day 7. On day 21 there was a significantly higher amount of $\text{NH}_4^+$ in the SuperU treatment, but levels in both treatments were below 3.5 mg N kg$^{-1}$ soil at this point. Soil $\text{NO}_3^-$ increased after urea and SuperU were added and on day 3 was significantly higher in the urea treated soil (Figure 2.1B). In both treatments soil $\text{NO}_3^-$ increased significantly from day 0 to 7, after which it plateaued.

2.4.2 Soil Nitrous Oxide Emissions

In both treatments $\text{N}_2\text{O}$ emissions started to increase immediately after urea and SuperU were applied to soil. Emissions increased significantly in both treatments from
day 2 and peaked on day 7 before declining on day 13 (Figure 2.1C) (p<0.001). There were higher emissions from the urea treated soil during this time, although no significant differences in daily N\textsubscript{2}O emissions was detected on any day of the study (p=0.19). After day 13, there was a continuous decline in N\textsubscript{2}O emissions from the SuperU treatments until the end of the study. Emissions increased slightly in the urea treatment on day 16 before declining at the end of the study period. Soil treated with SuperU had cumulative N\textsubscript{2}O emissions that were 37 % lower (p=0.23) over 35 days of incubation (Figure 2.1D).
Figure 2.1 Soil NH₄⁺, NO₃⁻, daily N₂O emissions, and cumulative N₂O in a 35-day microcosm incubation study with urea and SuperU. Average values and standard error of soil NH₄⁺ (A), NO₃⁻ (B), daily N₂O emissions (C), and cumulative N₂O (D) for urea and SuperU are shown over the study period (n=4). Significant differences in treatment means for NH₄⁺ and NO₃⁻ on each day (at p<0.05) are indicated with an asterisk.
2.4.3 Nitrogen Cycling and Total Bacterial Community Abundance and Activity in Urea and SuperU Treated Soil

2.4.3.1 Total bacterial community

The total number of bacteria measured by 16S rRNA gene abundance was 5.83x10^9 and 6.78x10^9 copies per gram dry soil, in the urea and SuperU treatments respectively. Gene abundance did not differ between treatments but there was a significant effect of day (p=0.045, Table 2.2). The 16S rRNA gene abundance in both treatments increased over the course of the incubation (Figure 2.2A). Total bacterial community activity, measured by transcript copies of 16S rRNA gene per gram of dry soil, was 3.73x10^{10} and 4.73x10^{10} for urea and SuperU respectively. The 16S rRNA transcript abundance did not differ significantly between the two fertilizer treatments or over time (Table 2.3). Transcript abundance in both treatments was highest in the first few days of the study and decreased over time (Figure 2.3A), but temporal changes were not found to be significant.

2.4.3.2 Bacterial Communities Involved in Dissimilatory Nitrate Reduction to Ammonium

The gene abundance of nrfA was 8.20x10^7 and 9.78x10^7 gene copies per gram dry soil in urea and SuperU respectively. Analysis of variance showed that nrfA gene abundance was significantly higher in the SuperU treatment (Table 2.2). At p≤0.1 there...
was a significant interaction of treatment and day of study. Treatment means were compared on each day of the study and it was found that nrfA abundance was significantly higher in SuperU on day 1 and day 35 (Figure 2.2B). There were no significant differences in nrfA gene copies over time. No nfrA transcripts were detected for samples in the study.

2.4.3.3 Urease bacterial community

The ureC gene abundance was 2.21x10^8 and 2.43x10^8 gene copies per gram dry soil in the urea and SuperU treated soils respectively (Figure 2.2C). Gene copies did not differ between treatments and they did not change significantly over time according to ANOVA (Table 2.2). Transcripts of ureC were targeted but were not detected in either treatment over the course of the study.

2.4.3.4 Nitrifying bacterial community

The abundance of the nitrifying bacterial community was measured using gene copies of amoA and was larger in the SuperU treated soils (6.18x10^6 copies per gram dry soil) compared to urea (4.09x10^6 copies per gram dry soil). Comparison of amoA gene abundance between treatments using ANOVA indicated that at p<0.1 there was a significant difference between urea and SuperU (p=0.052, Table 2.2). Gene abundance of amoA was consistently higher in SuperU treated soils but large variability on certain days may have prevented detection of statistically significant differences between
treatments at p≤0.05. In both treatments we measured increasing gene copies of *amoA* from day 1 to day 7, after which gene abundance remained elevated until the end of the study (Figure 2.2D). Transcript abundance of *amoA* was highly variable and was not significantly different between treatments (Table 2.3). We detected transcript copies in both treatments on day 1, 3, 7 and 10, which started high and declined in both treatments over time (Figure 2.3B). Transcript copies were detected during the main period of N₂O emissions at the beginning of the study.

### 2.4.3.5 Denitrifying bacterial community – nitrite reducers

Both groups of bacteria capable of NO₂⁻ reduction were present in soils from our study, as measured by presence of the *nirS* and *nirK* genes. The abundance of *nirK* gene copies was greater than that of *nirS*. Both nitrite reduction groups were greater in the SuperU treated soils. Gene abundance of *nirS* was 4.04×10⁷ and 4.75×10⁷ gene copies per gram dry soil in urea and SuperU respectively. For *nirK*, gene abundance was 1.45×10⁸ and 1.65×10⁸ copies per gram dry soil for urea and SuperU respectively. Only *nirS* gene abundance was significantly different between the urea and SuperU treated soils (Table 2.2). We compared treatment means on each day of the study as well and found that on day 1 and 10 there were significantly higher *nirS* gene copies in the SuperU treated soils (Figure 2.2E). For *nirK* we also observed higher gene abundance on days 1 and 10 in SuperU but these differences were not significant.
(Figure 2.2F). There was a significant effect of day for *nirS* but not *nirK* gene abundance (Table 2.2). For *nirS* there was a significant increase in gene abundance between day 1 and day 7 in urea treated soil, and the same increase was observed in the SuperU treated soil from day 3 to day 10, but this was not significant. The same increase between day 3 and 7 was found for *nirK* in both urea and SuperU but was not significant. Increases in *nirS* and *nirK* gene abundance were during the time when we measured large increases in soil NO$_3^-$ (Figure 2.1B). No *nirS* transcripts were detected throughout the study and for *nirK* we were able to quantify transcript copies from day 1 to 7 for urea and day 1 and 3 only for SuperU. Transcript copies were not detected for the remainder of the study, despite a second emission event between day 7 and 21 in the urea treatments. High variability in transcript abundance prevented detection of treatment differences.

### 2.4.3.6 Nitrous oxide reducers

Gene abundance for both groups involved in nitrous oxide reduction showed that *nosZ2* was twice as abundant as *nosZ1* (Figure 2.2G and H). For *nosZ1*, gene abundance in urea and SuperU was 2.11x10$^8$ and 2.77x10$^8$ copies per gram dry soil respectively. There were significantly higher gene copies of *nosZ1* in the SuperU treatments (Table 2.2). For *nosZ2* there were 4.58x10$^8$ and 4.95x10$^8$ gene copies in urea and SuperU respectively but no significant difference between treatments (Table 2.2). Gene abundance for both *nosZ1* and *nosZ2* did not vary significantly over time.
Transcript copies for nosZ1 were only detected on days 1 to 7 for the urea treatments, and on day 1 and 3 for SuperU (Figure 2.3D). This mirrored the days on which we detected nirK transcripts in both treatments. There was high variability in nosZ1 and nosZ2 transcript copies and significant differences between treatments were not detected. For nosZ2, transcript copies were detected in both treatments on all days of the study but there was a significant drop in copy numbers from days 1-10 and day 35 of the study (Figure 2.3E).

Table 2.2 Results of ANOVA for total and nitrogen cycling bacterial genes in urea and SuperU treated soils. P-values for test of significance between treatment, day, as well as their interaction, with values p<0.05 shown in bold (n=3).

<table>
<thead>
<tr>
<th>Gene</th>
<th>16S rRNA</th>
<th>nrfA</th>
<th>ureC</th>
<th>amoA</th>
<th>nirS</th>
<th>nirK</th>
<th>nosZ1</th>
<th>nosZ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.06</td>
<td><strong>0.01</strong></td>
<td>0.19</td>
<td>0.05</td>
<td><strong>0.02</strong></td>
<td>0.12</td>
<td><strong>0.03</strong></td>
<td>0.24</td>
</tr>
<tr>
<td>Day</td>
<td><strong>0.04</strong></td>
<td>0.41</td>
<td>0.34</td>
<td>&lt;0.001</td>
<td><strong>0.04</strong></td>
<td>0.24</td>
<td>0.76</td>
<td>0.51</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.65</td>
<td>0.08</td>
<td>0.88</td>
<td>0.74</td>
<td>0.27</td>
<td>0.51</td>
<td>0.12</td>
<td>0.53</td>
</tr>
</tbody>
</table>
Figure 2.2 Gene abundance of total and nitrogen cycling bacterial groups in soil microcosms treated with urea and SuperU. Abundances were quantified using qPCR by targeting genes for 16S rRNA (panel A), dissimilatory nitrate reduction (nrfA, panel B), urease (ureC, panel C), ammonia monooxygenase (amoA, panel D), nitrite reductases (nirS and nirK, panels E and F), and nitrous oxide reductases (nosZ1 and nosZ2, panels G and H). Panels A – H show mean gene abundance (copies per gram of dry soil) for urea and SuperU for each day sampled (n=3). Significant differences between treatment means on particular days are marked with an asterisk (p<0.05).

Table 2.3. Results of ANOVA for total and nitrogen cycling bacterial transcripts in urea and SuperU treated soils. P-values for test of significance between treatment, day, as well as their interaction, with values p<0.05 shown in bold (n=3). N.D indicates no transcripts were detected for target.

<table>
<thead>
<tr>
<th></th>
<th>16S rRNA</th>
<th>nrfA</th>
<th>ureC</th>
<th>amoA</th>
<th>nirS</th>
<th>nirK</th>
<th>nosZ1</th>
<th>nosZ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.11</td>
<td>N.D</td>
<td>N.D</td>
<td>0.39</td>
<td>N.D</td>
<td>0.96</td>
<td>0.65</td>
<td>0.11</td>
</tr>
<tr>
<td>Day</td>
<td>0.13</td>
<td>N.D</td>
<td>N.D</td>
<td>0.50</td>
<td>N.D</td>
<td>0.62</td>
<td>0.23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.56</td>
<td>N.D</td>
<td>N.D</td>
<td>0.70</td>
<td>N.D</td>
<td>0.99</td>
<td>0.91</td>
<td>0.44</td>
</tr>
</tbody>
</table>
No ureC transcripts were detected.

No nirA transcripts were detected.

No nirS transcripts were detected.

---

40
Figure 2.3 Transcript abundance of total and nitrogen cycling bacterial groups in soil microcosms treated with urea and SuperU. Abundances were quantified using qPCR by targeting genes for 16S rRNA (panel A), ammonia monoxygenase (amoA, panel B), nitrite reductase (nirK, panel C), and nitrous oxide reductases (nosZ1 and nosZ2, panels D and E). Quantification of nrfA, ureC, and nirS was not possible as transcript copies were below the assay detection limit. Panels A – E show mean gene abundance (copies per gram of dry soil) for urea and SuperU for each day sampled (n=3). There were no significant differences between treatment means on particular days (p<0.05).

2.5 Discussion

Soil bacterial N cycling communities responded differently, peak N₂O emissions were lower with SuperU, and inorganic N dynamics differed in soils with urea and SuperU addition. On the first day of the study (day 0), after urea and SuperU was added to soils the level of NH₄⁺ increased in both treatments but was higher in the urea treatment. This indicates that urea was metabolized to NH₄⁺ more rapidly than in the SuperU treatment, where it remained lower. On day 3 the opposite treatment effect on NH₄⁺ concentration was observed. The amount of NH₄⁺ had dropped in both treatments but to a greater degree in the urea soils receiving urea. The accumulation of NO₃⁻, which started out the same in both treatments at the beginning of the study, occurred more rapidly and was higher in the urea treatment on day 3. Together this data indicates that the inhibitors present in the SuperU treatment delayed metabolism of urea to NH₄⁺ and further to NO₃⁻ in the first few days of the study. Nitrous oxide emissions were also measured and increased in both treatments after fertilizer addition, but N₂O emissions were lower in the SuperU treated soils during the first week. Declining N₂O emissions were measured after the first 7 days.
Reduced amounts of NH$_4^+$ after urea+NBPT application was also measured by Fan et al. (2018), although the reduced NH$_4^+$ compared to the urea only treatment persisted for a longer time in their study (25 days) compared to ours, possibly due to differences in soil pH and moisture. In our study NH$_4^+$ peaked and did not differ between treatments by day 1 after which it started to decline in both. Reduction in mineralization of organic N to NH$_4^+$ was measured for both NBPT alone or in combination with DCD (Harty et al., 2017). Delayed ureolysis and NH$_4^+$ accumulation, as well as downstream oxidation of NH$_4^+$ to NO$_3^-$ was seen in studies due to use of DCD alone or with NBPT, with urea or urine as an N source (Cameron et al., 2014; Chen et al., 2014; Lan et al., 2013; Yang et al., 2017).

Of the studies examining NH$_4^+$ and NO$_3^-$ dynamics from urea and urea+NBPT/DCD some found that N$_2$O emissions were reduced (Cameron et al., 2014; Dai et al., 2013; Harty et al., 2016; Lan et al., 2013), while another resulted in higher N$_2$O emissions (Fan et al., 2018). Differences in study results and efficacy of inhibitors may be partly due to the soil physiochemical conditions. In the study by Fan et al. (2018) they found that NBPT increased N$_2$O emissions only in the alkaline soils, with no difference between urea and urea+NBPT in acidic soils. However the soils in our study are alkaline and another study showing reduced N$_2$O emissions used soils with pH of <7 (Harty et al., 2016). The ability of NBPT to inhibit urease in soils is also dependent on soil moisture and O$_2$, as aerobic conditions are required for NBPT to be converted to the
active compound that directly inhibits urease (Cantarella et al., 2018). In our study the WFPS was at 60%, and in Fan et al. (2018) it was at 50% water holding capacity, both of which are not fully saturated conditions. It was also noted in a review of urease inhibitor action that although there is no direct effect of nitrification inhibitors on urease inhibitors, prolonging NH$_4^+$ by preventing it’s oxidation to NO$_3^-$ could lead to increased NH$_3$ loss or have no effect, depending on soil properties (Cantarella et al., 2018). Clearly a combination of the soil physical and chemical environment can lead to differences in how N transformations occur and the resulting N products.

Changes in bacterial community abundance and their activity in response to urea and urea+NBPT addition were measured in order to examine the various groups involved in N transformations in soil. We measured the gene and transcript abundance of a number of N cycling bacterial groups over time, to see which pathways were responding to the changes in N that we measured. We found that there was no significant effect of SuperU on ureC abundance. This result differs from two other studies, one of which found a significant decrease in ureC in NBPT treated soils (Fan et al., 2018), and another that measured increased ureC abundance in NBPT treated soils, (Li et al., 2019), both compared to urea alone. Gene abundance of ureC was positively correlated with urea hydrolysis and pH (Fisher et al., 2017). The rapid metabolism of urea to NH$_4^+$ was noted in Cantarella et al. (2018) and was observed in our study. By day 1, 24 hours after treatments were applied, there was no difference in NH$_4^+$.
measured. Sampling for microbial gene abundance did not occur immediately after treatments were applied (day 0) so it is possible that differences in ureC may have been captured if we had sampled the day 0 samples. Rapid increases in ureC gene abundance was seen in Fan et al. (2018) however the peak of NH$_4^+$ was lower than in our study, at ~25 mg kg$^{-1}$ and ~12 mg kg$^{-1}$ soil for the urea and urea+NBPT alkaline soil respectively. In our study NH$_4^+$ on day 1 peaked at 59 mg kg$^{-1}$ soil, potentially from greater urea hydrolysis in both treatments, which prevented detection of any differences. In our study we were not able to measure ureC transcripts in either treatment, and other studies discussed here did not detect them either.

Increased amoA gene abundance over time was observed in both treatments and the overall amoA abundance was greater in soils with SuperU (Figure 2.2A). In the SuperU treatment the gene abundance of amoA rose more rapidly and remained higher towards the end of the study. This was interesting as other studies have found that nitrification inhibitors such as DCD decrease bacterial amoA gene abundance (Chen et al., 2014; Di et al., 2014; Di and Cameron, 2011; Fan et al., 2018; O’Callaghan et al., 2010; Yang et al., 2017). The other studies do show similar temporal dynamics although the time from urea or NH$_4^+$ addition to peak abundance varies, as does the magnitude of the decrease in gene abundance caused by DCD. In general, peak gene abundance occurred within 7-14 days in other studies, although in Di et al. (2014) gene abundance peaked more than 50 days after treatments were applied. Significant differences
between treatments were evident in the first 7-14 days post treatments (Chen et al., 2014; Fan et al., 2018), or did not occur until after that time (O'Callaghan et al., 2010; Yang et al., 2017). The other studies did not measure bacterial amoA transcript abundance. Transcript copies of amoA were measurable in our study and increased after urea or SuperU addition, before declining below detectable limits of qPCR. Interestingly, in this and other studies it was observed that despite conditions suitable for denitrification and NO$_3^-$ reduction to N$_2$O, NO$_3^-$ remained elevated in both treatments.

Nitrate can be used as an alternate electron acceptor in denitrification, but also in a less studied pathway called DNRA. The DNRA pathway was investigated as nitrate remained elevated despite WFPS providing conditions suitable to denitrification. Results showed that bacterial communities with nrfA were present and more abundant in the SuperU treated soils, particularly on days 1 and 35 (Figure 2.2B). Transcript copies of nrfA could not be detected in either treatment. These findings are similar to that of Harty et al. (2017), who measured DNRA and found it to be significantly higher in urea+DCD and urea+DCD+NBPT treated soil, compared to urea alone. Other studies comparing urea and urea+DCD/NBPT have not looked at the gene or transcript abundance of nrfA. In another laboratory incubation experiment, denitrification and DNRA competed for NO$_3^-$ and DNRA was carried out with rates one third that of denitrification (Chen et al., 2015). In their study DNRA was not a significant contributor to N$_2$O emissions; however,
when N₂O emissions decreased together with NO₃⁻ towards the end of the study, DNRA was a more significant proportion of N cycling. In a study where soils were supplied with non-limiting NO₃⁻ (as KNO₃) and either glucose, red clover, or other straw residues, towards the end of a 72 hour incubation soil NH₄⁺ was significantly increasing (Henderson et al., 2010). These results highlight that although groups with nrfA are not always contributing to N₂O emissions they are involved in maintaining and retaining N in soil.

The genes involved in denitrification were also examined and NO₂⁻ reductase genes, nirS and nirK were detected in both treatments. Both nirS and nirK gene abundance was higher in SuperU treated soils and they followed the same trends over time, but greater abundance in SuperU was only significant for nirS, on days 1 and 10 (Table 2.2). As for amoA, nirK transcripts were detected in the first part of the study but were not significantly different between treatments. Transcript copies of nirK were highest in the first few days of the study and declined to below detectable levels by day 10, likely in response to increasing nitrate levels at the beginning of the study. What is interesting however is that although NO₃⁻ gradually increased and plateaued for the remainder of the microcosm experiment, nitrite reducer gene abundance remained stable, but nitrite reductase transcript copies declined. As WFPS was kept constant and in a range suitable for denitrification to proceed we can conclude that microbial communities were utilizing a multitude of pathways as part of N metabolism. Higher nirS
and nirK in the SuperU treated soils was in contrast to a study where DCD was shown to decrease copy numbers of nirK in urine treated soil (Di et al., 2014). Interestingly in their study they found that the dynamics of nirK mirrored that of amoA, which were decreased by the DCD addition, likely due to the fact that communities with amoA can also harbor nirK and utilize it in nitrifier denitrification (Di et al., 2014). In this study there was a significant increase in amoA and nirS, but not nirK, from day 1 to day 10.

The reduction of N₂O to N₂ can be carried out by two groups of bacteria in soils, those with the genes nosZ1 and nosZ2. Gene and transcript abundance of both nosZ1 and nosZ2 were measured and it was found that both groups were more abundant in SuperU treated soils, but this was only significant for nosZ1, on day 35 in particular. The abundance of both nosZ genes did not change significantly over the course of the incubation study but the abundance of transcripts did. For both nosZ1 and nosZ2, transcript abundance was highest during the first 7-10 days of the study, when N₂O emissions were greatest in both urea and SuperU. After this period, transcript abundance decreased in both treatments, with nosZ2 transcripts no longer above the detectable limit on day 10. Transcript copies of nosZ2 continued to decline and were at their lowest level on day 35. Gene abundance differences indicate that the communities in SuperU are larger than in urea treated soils and as a result there may be increased complete denitrification occurring, resulting in the lower amount of N₂O emissions measured. Other studies have examined gene abundances of nosZ1, and nosZ2 to a
lesser extent, and in one study both increased when soil was treated with urine but no effect from DCD was apparent (Di et al., 2014). The study by Di et al. (2014) did not examine either nosZ transcript abundances.

### 2.6 Conclusion

In conclusion, SuperU addition did alter inorganic N dynamics and reduced N$_2$O emissions in soil microcosms with field equivalent rates of N. The bacterial functional groups involved in N cycling were impacted by the addition of NBPT and DCD in SuperU. Soil treated with SuperU had increased bacterial community sizes for a number of agriculturally relevant N cycling processes including DNRA, nitrification and denitrification. This highlights that more than just the groups directly targeted by inhibitors can be impacted due to the interconnected pathways that govern biogeochemical cycling of nutrients such as N in agricultural soils.
3 Wheat and Cover Crop alter Nitrogen Cycling Microbial Communities and a Nitrous Oxide Emission Event in a Corn-Soybean Cropping System

*Nicola F. Linton¹, Pedro Vitor Ferrari Machado¹, Bill Deen², Claudia Wagner-Riddle¹, Kari Dunfield¹

¹School of Environmental Sciences, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada

²Department of Plant Agriculture, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada

*corresponding author: lintonn@uoguelph.ca

N. Linton was the primary researcher and author for this study, having conducted field and lab work for microbial analyses, and manuscript preparation. Author P. Ferrari-Machado is credited for field work, collection and provision of data, methods and results for inorganic nitrogen and nitrous oxide. Author B. Deen is credited for the use of his OMAFRA-funded field plots used for this research project. Author C. Wagner-Riddle is credited for project conception, advice and for funding through the NSERC strategic grant. Author K. Dunfield is credited for her mentorship, support and advice on soil
sampling, molecular analyses, data interpretation and manuscript focus and preparation.

3.1 Abstract

Agriculture accounts for a majority of global soil N\textsubscript{2}O emissions. Changes to crop rotation are known to alter belowground bacterial communities. Several soil bacterial groups contribute to soil N cycling and production of N\textsubscript{2}O emissions from use of N fertilization. The goal of this study was to understand the impacts of 35 years of crop diversification in shaping diversity and the community size and activity of nitrifier and denitrifier bacteria and N\textsubscript{2}O emissions after corn N fertilization. Long-term four-year rotations of corn-corn-soybean-soybean (simple) and corn-corn-soybean-wheat underseeded with red clover cover crop (diverse) were established in 1980 in Elora, Ontario. In 2017, N\textsubscript{2}O emissions were measured 11 times per day using automatic chambers. Soil was collected every 2 days during peak N\textsubscript{2}O emissions and every week otherwise by immediately flash freezing in liquid N in the field. DNA and RNA were co-extracted and 16S rRNA, amo\textit{A}, nir\textit{S}, nir\textit{K}, nosZ\textsubscript{1} and nosZ\textsubscript{2} genes and gene transcripts were quantified to determine community abundance and activity of total bacteria, and N cycling groups involved in ammonia oxidation and denitrification. An N\textsubscript{2}O emission event occurred in both rotations following UAN application but was higher in the diverse rotation. The diverse rotation had persistent higher total and denitrifying
(nirK and nosZ2) bacterial abundance. Bacterial amoA significantly increased in both rotations shortly after UAN addition, but gene detection decreased significantly in the simple rotation and remained elevated in the diverse. Gene transcript levels did not differ significantly by rotation. Transcripts for amoA and nirK were only detected after UAN addition. Transcripts for 16S rRNA and atypical nosZ2 were consistently detected but higher after UAN addition. NirS transcripts were not detected at any point in the study. Total bacterial diversity did not differ between simple and diverse rotations sampled. The data from this study shows that although diversity did not differ, microbial pathways leading to soil N2O emissions are elevated in more diverse crop rotations, indicating that best management practices (BMPs) that improve soil health need to take biological components into account in order to try and minimize GHG production.

3.2 Introduction

Inorganic N fertilizer is commonly used to maximize corn yields in Southern Ontario (Ontario Ministry of Agriculture Food and Rural Affairs, 2014). Nitrogen applied to the soil in agroecosystems can be lost, reducing the crop N use efficiency and in some cases causing environmental harm. Forms of N that can be lost and are a cause of concern include NH4+ (through ammonia volatilization), NO3− (leaching with water) and N2O, a potent GHG, which have negative consequences for ecosystems on a global scale (Coskun et al., 2017; Galloway et al., 2013; Liu et al., 2010; Ollivier et al.,
In particular, N$_2$O emissions from anthropogenic activities is a concern due to its
global warming potential 265 times that of CO$_2$ and its long lifespan in the atmosphere
(~100 years, EPA online). Nitrous oxide emissions from agricultural activities in Canada
accounted for 77% of national N$_2$O emissions, of which N fertilizer application
contributed 23% (Environment and Climate Change Canada, 2019). Limiting potential
N$_2$O emissions and loss of nutrients such as N from biogeochemical cycling and
agronomic practices has been a recognized area in need of research to mitigate
environmental harm (Tilman, 1999; Vitousek et al., 1997).

A number of studies have examined agricultural management practices and their
impacts on soil physicochemical properties and nutrient cycling in agroecosystems, with
a focus on improving soil and reducing N loss (Congreves et al., 2015; Lehman et al.,
2017; Renwick et al., 2018). Such practices include using reduced tillage and/or
increasing crop rotational diversity in corn and soybean production systems. Using
reduced or no tillage can improve soil health scores, organic C and total nitrogen
(Congreves et al., 2015; Van Eerd et al., 2014). In some soils the use of no till can lower
yields due to compaction, delayed soil warming and drying, although the negative
impacts to soil structure and yield reduction can be mitigated when diversifying crop
rotations are used (Lundy et al., 2015; Munkholm et al., 2013). Diversifying crop
rotations by inclusion of wheat and a legume cover crop into corn-soybean rotations can
improve soil structure, increase soil C, improve corn and soybean yields, and decrease
the rate of fertilizer N needed to produce maximum corn yield (Congreves et al., 2015; Gaudin et al., 2015a; Munkholm et al., 2013). Increasing N use efficiency and lowering the required N to achieve maximum yields through crop diversification has the potential to minimize N loss from agroecosystems. These potential benefits of diversification to N cycling could alter GHG emissions, in addition to the other benefits for soil. Meta analyses examining cover crop effects on soil N\textsubscript{2}O emissions found reduced levels in 40\% of the cases, but in 60\% there was increase in emissions during the cash crop season and lower emissions only during the cover crop growth period, when N is incorporated into its biomass before release during decomposition (Basche et al., 2014; Han et al., 2017). This highlights that growing cover crops can improve soil health, increase N and organic C, but at the same time result in increased GHG emissions such as N\textsubscript{2}O. The impact of cover crops and rotational diversity on N\textsubscript{2}O emissions is therefore an important area for study that warrants further attention. Studies linking rotational diversity, inclusion of cover crops, and N\textsubscript{2}O emissions have shown reductions in N\textsubscript{2}O emissions, in addition to other soil health benefits. In one study, diversification of continuous corn by inclusion of oat and alfalfa resulted in 12\% lower N\textsubscript{2}O emissions (Drury et al., 2014). Reduced soil N\textsubscript{2}O emissions were also measured when field peas and winter wheat were included in a no till corn-soybean system (Lehman et al., 2017). The reduction of N\textsubscript{2}O emissions from cover crop inclusion can depend on timing and use of additional N input in the form of inorganic fertilization. For example, Mitchell et al.
(2013), found that inclusion of a winter rye cover crop in a no till corn-soybean system resulted in lowered N₂O emissions when no fertilizer was used but when economically optimal rate of N was applied N₂O emissions increased, possibly due to increased mineralizable C in the diverse rotation soil (Mitchell et al., 2013). The authors point out that more work is needed to understand the effect of crop diversification and cover crop use on overall yearly N₂O emissions.

Soil N transformations and potential routes of loss include abiotic factors such as chemical decomposition of N intermediates that can lead to N₂O formation, NO₃⁻ leaching during periods of high precipitation, but also the microbially mediated pathways of the N cycle (Butterbach-Bahl et al., 2013; Carswell et al., 2017). Two main pathways of the N cycle, nitrification and denitrification, have been the focus of decades of research investigating factors controlling microbial N transformations and N₂O production and consumption in soils (Baggs, 2011; Butterbach-Bahl et al., 2013; Firestone and Davidson, 1989; Thompson et al., 2016). Nitrification involves oxidation of ammonium/ammonia through intermediate N compounds to nitrate and can be performed by a variety of soil microorganisms, including bacteria, archaea and some fungi (Hayatsu et al., 2008; Prosser and Nicol, 2012; Taylor et al., 2016). Denitrification has been considered a main pathway of N₂O production, but N₂O can also be a product of nitrification under certain soil N, moisture, and O₂ conditions (Baggs, 2011; Hink et al., 2016; Kool et al., 2011, 2009; Zhu et al., 2013). Soil moisture can determine O₂ and
substrate availability for soil bacteria, including nitrifiers (Orchard and Cook, 1983; Stark and Firestone, 1995). In a study where urea was added to soils with O₂ concentrations ranging from 0.5 to 21%, N₂O was a byproduct of nitrifier nitrification and nitrifier denitrification, with greater than 50% contribution from these processes (Zhu et al., 2013). When soil moisture conditions are between 50-55% WFPS this leads to conditions that are both aerobic and anaerobic in the heterogeneous soil environment, and significant emissions of N₂O from nitrification have been measured (Liu et al., 2016; Uchida et al., 2013). Denitrification is an anaerobic multistep process where nitrate is reduced to gaseous N₂, with the exception of incomplete denitrification, where N₂O is produced as an end product (Braker and Conrad, 2011). Soil conditions such as O₂ availability, moisture, C and pH control the ability of various denitrifying microbial groups to fully or partially carry out the steps leading to incomplete or complete denitrification, which determines the balance of N₂O to N₂ produced (Baggs, 2011; Firestone and Davidson, 1989; Giles et al., 2012; Morley and Baggs, 2010). The known contributions of both nitrification and denitrification pathways to N₂O formation and reduction highlight the importance of examining multiple pathways of the N cycle to fully understand the complexity of conditions that govern microbially mediated N transformations in soil.

Increased crop diversification can change the soil physical and chemical environment, increasing SOM, water retention and structure, parameters which will impact soil microbial communities and their functions (Gaudin et al., 2013; Maiga et al., 2013).
As crop diversification has been shown to lower GHG emissions we can understand the biotic mechanisms underlying this by measuring bacterial communities involved in N cycling in agricultural soil. Bacteria capable of the initial rate limiting step in nitrification, ammonia oxidation, as well as the groups involved in the denitrification reaction (nitrate reductases and nitrous oxide reductases) were targeted using qPCR. The objective of this study was to examine the long-term crop rotation effect of simple (corn-soybean) vs. diverse (corn-soybean-winter wheat underseeded with red clover) rotations on bacterial diversity and bacterial N cycling groups and their activity after application of N fertilizer to corn and to link this to N$_2$O emissions.

3.3 Materials and Methods

3.3.1 Site Description and Experimental Design

The experiment was established in second year corn plots at the long-term field trial, established in Elora, Ontario (43°39’ N 80°25’ W) in 1980. This long-term research trial consists of a randomized complete block design with four-year crop rotations as the main factor and tillage as a split plot treatment. Soil at this site is classified as a silty loam (Grey Brown Luvisol) with 27% sand, 57 % silt and 17 % clay (Congreves et al., 2015). This experiment was conducted in plots under no till management with simple (corn-corn-soybean-soybean - CCSS) and diverse rotations (corn-corn-soybean-winter
wheat + red clover, CCSWrc) during June to July, when N$_2$O emissions were measured and detailed soil sampling for microbial community analysis was conducted. Two duplicate plots under each crop rotation treatment, both under no till management were used. All four plots sampled during the study were under second year corn in the summer of 2017. Starter fertilizer was applied at corn planting and nitrogen fertilizer was applied as side dress at the corn six leaf stage on June 12, 2017 as UAN injected to a depth of 5-7 cm at 150 kg-N ha$^{-1}$.

### 3.3.2 Soil Sampling for Microbial Analysis

Soil was sampled on June 7 (5 days prior to UAN application), as well as on June 15, 19, 21, 23, 26, 30, July 4 and 18, using a sterile 3 ml syringe to collect the top 5 cm. Six soil samples were collected from each half plot, from across a UAN injection line adjacent to gas measurement chambers to capture spatial variability within the target zone of injected N fertilizer. Soil was homogenized, and ~2 g was immediately subsampled using nuclease free materials and frozen in liquid nitrogen. Samples were stored on dry ice for transport back to the laboratory and kept at -80°C until RNA/DNA co-extraction was performed. A sub-sample (~10 g) of the remaining composite soil was used to determine gravimetric moisture content.
3.3.3 Soil Nucleic Acid Extraction and RNA Reverse Transcription to cDNA

DNA and RNA were co extracted using the MoBio RNA Powersoil Total RNA Extraction Kit according to the manufacturer’s instructions and quantified using NanoDrop (ThermoFisher) prior to storage at -20°C (DNA) or -80°C (RNA). DNA removal from RNA was performed using RQ1 DNase (Promega) and confirmed using gel electrophoresis and qPCR, prior to reverse transcription to cDNA. The Applied Biosystems® High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corp.) was used to reverse transcribe RNA to cDNA. Reactions with sample RNA were performed in triplicate, after which they were pooled and stored at -20°C. Appropriate no enzyme and no template controls were included. Nucleic acid extracts were tested for inhibition using qPCR detection of an M13 plasmid spiked at $10^5$ copies μl$^{-1}$.

3.3.4 Quantification of Total Bacteria and Nitrogen Cycling Bacterial Genes and Transcripts

Total bacterial communities and nitrogen cycling bacterial communities were quantified using qPCR by targeting 16S rRNA, amoA, nirS/K, nosZ1 and nosZ2 genes and transcripts, using primer pairs 338F/518R (16S rRNA gene) (Fierer et al. 2005), amoA1F/2R (amoA) (Rotthauwe, Witzel, and Liesack 1997), cd3af/r3cd (nirS) (Throbäck et al. 2004), F1aCu/R3Cu (nirK) (Hallin and Lindgren 1999), nosZ2F/2R
(nosZ1) (Henry et al. 2006), and nosZII/F/R (nosZ2) (Harter et al. 2016). All qPCR was performed using a Bio-Rad CFX detection system (Bio-Rad Laboratories Inc.) using SsoFast EvaGreen Supermix (for 16S rRNA, ureC, nrfA, amoA, nirS/K, nosZ2) or iQ SYBR Green Supermix (nosZ1), with appropriate plasmid standard curves and diluted DNA/cDNA to reduce impact of PCR inhibition. Primer sequence, qPCR conditions, and qPCR efficiency for each target are shown in the appendix (Appendix 5.1)

Primers were used at a final concentration of 0.3 µM for 338f/518r and amoA1F/2R, 0.4 µM for cd3af/r3cd and F1aCu/R3Cu, and 2 µM for nosZ2F/2R and nosZII/F/R. Standard curves for relative qPCR were generated from plasmid DNA containing the target gene (10^-8 to 10^1 copies per reaction). Bacterial 16S rRNA gene, amoA, nirS, nirK, nosZ1 and nosZ2 were cloned from Clostridium thermocellum spp. (16S rRNA gene), Nitrosomonas europaea spp. (amoA), Alcaligenes faecalis ATCC 8750 (nirK), Pseudomonas auruginosa PAO1 (nirS and nosZ1), and Gemmatimonas aurantica 27-T DSM 14586 (nosZ2). PCR products were cloned in One Shot DH5a-T1R, Top10 Escherichia coli competent cells using a TOPO TA Cloning kit (Life Technologies Corp.). Cells were grown and plasmid extraction performed using Qiaprep Spin Miniprep Kit (Qiagen). Plasmids were sequenced to confirm target identity. All qPCR assays included no template controls and were optimized to reaction efficiencies of 85-105 % with R values of 0.99.
3.3.5 Nitrous Oxide Measurements

Nitrous oxide emissions at the soil atmosphere interface were measured using eight opaque, vented, automatic non-steady-state chambers (LiCor model 8100-104; Li-Cor, Lincoln, NB), interfaced with a Gasmet DX 4015 FTIR analyzer (Brummell et al., 2012). One day before the start of data collection, eight PVC collars (314.2 cm$^2$) were installed into the soil (to reach 3 cm headspace). Collars were removed for UAN fertilizer injection and reinserted on top of the injection lines after UAN application, to capture emissions from soil where fertilizer was added. Measurements were performed continuously, with a multiplexer cycling through each of the 8 chambers in turn, where at each sampling event the chamber closed and the change in N$_2$O concentration was measured over a 20 min period with the on-board Calcmet software (Calcmet™ ver. 12.15, analysis module version 4.42.2), recording N$_2$O concentrations on a 1-minute basis. A 60 s dead band was discarded from the data to account for small disturbances and pressure changes that occur immediately after chamber closure (Davidson et al., 2002). Before the start and after the end of each measurement a 90 s pre and post-purge was performed with atmospheric air to avoid cross contamination between the eight chambers. Supplementary measurements of chamber temperature and pressure were continuously performed by the LiCor + Gasmet system. N$_2$O concentrations increased near-linearly after the dead band period and the slope (dC dt$^{-1}$) was calculated by plotting N$_2$O concentration vs. time and fitting the data to a linear least-
squares regression with aid of the SoilFluxPro software (Li-Cor, Lincoln, NB). N$_2$O emissions were then calculated following the procedures described by de Klein and Harvey (2015).

3.3.6 Soil Inorganic Nitrogen and Moisture Measurement

The concentrations of soil NH$_4^+$ and NO$_3^-$ at 0-15 cm were determined after the sidedress N fertilization of the corn phase of the simple and diverse crop rotation. Six cores were collected to form a composite sample per experimental unit. The cores were taken across the injected N bands, according to Brouder and Mengel (2003). To avoid N losses, samples were placed in a cooler with ice in the field and then stored in a 4°C laboratory cooler and extracted the following day or placed directly in a -20°C freezer for further extraction. The soil extraction was performed according to Maynard and Kalra (1993), and the extracts were analyzed for NH$_4^+$ and NO$_3^-$ concentrations using a continuous flow autoanalyzer (Seal AutoAnalyzer 3 - AA3, Seal Analytical). Bulk density of each plot was measured by taking 4 soil cores using a 4.8 cm x 5 cm (D x H) cylinder. Soil was weighed before and after drying at 105°C for 48 hours volumetric water content calculated using gravimetric water content and bulk density values. Soil water filled pore space was calculated from volumetric water content, with particle density assumed to be 2.65 g cm$^{-3}$. 
3.3.7 Soil Collection and Processing for Bacterial Diversity Analysis

Soil was collected from four replicate plots, per treatment on October 27, 2017 to perform total microbial diversity analysis using HTS. Soil samples were collected from the top 10 cm of each plot using a sterilized soil probe with 2 cm diameter. Twelve soil samples per plot were collected and combined to form a composite sample. Soil composites were homogenized and stored in a cooler until transported back to the lab. Duplicate extractions were performed using the Qiagen DNeasy Powersoil Kit, according to the manufacturer’s instructions, and DNA was quantified using NanoDrop (ThermoFisher) prior to storage at -20°C. DNA from duplicate extractions were pooled by volume and submitted to Genome Quebec for HTS using an Illumina MiSeq 2x250 paired end run. The bacterial V4 hypervariable region of the 16S rRNA gene was amplified using modified primers 515F (5’-GTGYCAGCMGCCGCGGTAA-3’) and 860R (5’-GGACTACNVGGGTWTCTAAT-3’) from the Earth Microbiome Project. Bioinformatics processing of the sequence files was performed using QIIME2 version 2018.6 and above. The demultiplexed sequences were imported into QIIME2 and primer sequences trimmed using the cutadapt plugin. Trimmed sequences were checked for quality at their 3’ end to determine optimal length cutoff before read joining, denoising and chimera removal using DADA2. Resulting amplicon sequence variants (ASVs), which correspond to 99.9% similarity, were classified against the SILVA 16S/18S taxonomic database version 132. Sequences not assigned to kingdom
bacteria were filtered, together with sequences assigned to archaea, mitochondria and chloroplast for all downstream analyses. Diversity estimates were calculated by subsampling without replacement to rarefy sequences/sample. Samples were rarefied to 30,700 ASVs. Alpha diversity estimates were calculated including Observed ASVs, Faith’s Phylogenetic Diversity (Faith-PD) and Evenness. Differences in community composition were tested using PERMANOVA (permutation analysis of variance) of the Bray-Curtis distance matrix. Ordination was performed using PCA in STAMP software.

3.3.8 Statistical Analysis

Gene and transcript abundance data was normalized by copy number per gram of dry soil. Bacterial gene and transcript abundance, as well as soil inorganic nitrogen concentration and N$_2$O emissions were analyzed using ANOVA in SAS version 9.4 using a GLIMMIX model with logarithmic transformation of the data, to test hypotheses of differences in treatments, over time as well as their interaction with a repeated measures design. For bacterial diversity analysis significant difference in means between crop rotations for the alpha diversity metrics was tested using Kruskal Wallis test and differences in community composition (beta diversity) was tested using PERMANOVA using Bray-Curtis dissimilarity. Significant differences were determined by $p$-value $<$0.05. Redundancy analysis was performed to relate variation in N cycling gene abundances (response variables) and soil NH$_4^+$, NO$_3^-$, N$_2$O emissions, and WFPS
(explanatory variables) using CANOCO software (Biometris, Wageningen, The Netherlands).

3.4 Results

3.4.1 Site characterization and N\textsubscript{2}O Emission Measurements

3.4.1.1 Air Temperature, Precipitation and Water Filled Pore Space

Mean air temperature gradually climbed from 11.3°C on June 1, 2017 to a monthly average of 17.4°C and 18.7°C during June and July respectively (Figure 3.1A). Total daily precipitation (Figure 3.1A) was less than 6mm in days prior to fertilizer application in June but after UAN addition, during the period from June 12 to June 23, daily precipitation ranged from 0 to 33.7 mm. Water filled pore space declined early in June but increased in line with precipitation amounts (Figure 3.1B). Rainfall amounts were the same for all plots but WFPS increased >60 %, the level at which denitrification is thought to dominate, 2 days earlier in the CCSWrC plots and remained higher until July 4.

3.4.1.2 Soil Ammonium and Nitrate

Soil ammonium and nitrate did not differ significantly between CCSS and CCSWrC rotations during the period of study (p=0.59 and 0.48 for NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}⁻);
however, levels of inorganic nitrogen did vary significantly over time (p<0.001). Before UAN application both NO$_3^-$ and NH$_4^+$ were low and increased significantly up to and including June 16, after which concentrations gradually declined. Concentration of NO$_3^-$ remained above baseline at the end of the experiment but NH$_4^+$ concentration was back to baseline levels on July 12 (Figure 3.1B).

### 3.4.1.3 Soil Nitrous Oxide Emissions

In the period from June 5$^{th}$ to UAN addition, average soil N$_2$O emissions were 4.2 and 6.2 g N$_2$O-N ha$^{-1}$ day$^{-1}$ for CCSS and CCSWrc respectively. The application of UAN fertilizer was followed by two N$_2$O emission events, the first lasting for $\sim$12 days with a peak on June 23, and the second from June 29 to July 4, with a peak on June 30. Daily N$_2$O emissions started to increase above baseline levels in the CCSWrc rotation immediately after UAN fertilization, whereas in CCSS there was a lag of $\sim$3 days before rapid increases in N$_2$O emissions started. After UAN injection soil N$_2$O emissions increased significantly and remained elevated from June 13 to July 18 (Figure 3.1C). Cumulative N$_2$O emissions from June 5 to July 31 were 36.7 % higher in the diverse rotation (4596 g and 3346 g N$_2$O-N ha$^{-1}$ for CCSWrc and CCSS respectively). Cumulative N$_2$O emissions during the first emission event were significantly higher in the diverse rotation according to a t-test (p=0.04). In a repeated measures ANOVA, significantly higher daily N$_2$O emissions from CCSWrc plots were measured on June 5, 14, 15, 16 and 17 (p<0.05).
Figure 3.1 Weather, inorganic N, WFPS and N\textsubscript{2}O emissions in long term no till CCSS and CCSWrc rotations. Air temperature and precipitation (A.) displaying average, minimum and maximum air temperature (solid line and dot-lines, respectively) and total precipitation (bars). Soil inorganic N and moisture graph (B.) showing WFPS, soil ammonium (NH\textsubscript{4}\textsuperscript{+}) and nitrate (NO\textsubscript{3}\textsuperscript{-}) concentration. Shaded foreground on the WFPS plot highlights periods above 60%, and the dashed lines on the inorganic N plots represent the standard error of the measurement. Nitrous oxide emissions graph (C.) for the CCSWrc and CCSS rotations, with significant differences in daily N\textsubscript{2}O emissions marked with an asterisk. Shaded foreground on N\textsubscript{2}O emission plots represents the standard error of the measurement. NF represents the N fertilization date. Arrows along the x axis indicate soil sampling dates for microbial analyses.
3.4.1.4 Bacterial Diversity

Bacterial diversity was measured in four replicate plots of each rotation under no till, for the purpose of evaluating the total bacterial diversity from long term crop rotations. Bacterial alpha diversity metrics, including observed ASVs, Faith-PD and community evenness were not significantly different between CCSS and CCSWrc (Table 3.1). The bacterial community composition also did not differ based on PERMANOVA test of significant differences between groups (pseudo-F=1.07, p=0.32, Appendix 5.2).

Table 3.1 Alpha diversity metrics and tests of significance for bacterial diversity in soil from no till CCSS and CCSWrc (n=4).

<table>
<thead>
<tr>
<th></th>
<th>Observed ASVs</th>
<th>Faith’s Phylogenetic Diversity</th>
<th>Pielou Evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCSS (Mean±SD)</td>
<td>1422±152.8</td>
<td>78.8±5.29</td>
<td>0.938±0.003</td>
</tr>
<tr>
<td>CCSWrc (Mean±SD)</td>
<td>1420±119.1</td>
<td>78.2±3.78</td>
<td>0.937±0.004</td>
</tr>
</tbody>
</table>

Kruskal Wallis Test of Significant Difference in Means between Rotations

<table>
<thead>
<tr>
<th></th>
<th>H</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

p-value 0.39
3.4.2 Bacterial Community Abundance and Activity in Simple and Diverse Corn Rotations

3.4.2.1 Total Bacterial Community

The total number of bacteria, measured by 16S rRNA gene abundance, was on average 1.4 times greater (ranging from 1.1 to 1.6 times) in the diverse rotation – CCSWrc than total bacterial abundance in the simple rotation – CCSS (p=0.02, Table 3.2). Total bacterial community activity, measured by transcript copies of 16S rRNA gene per gram of dry soil, was not significantly different between the two crop rotations (Table 3.2). Both gene and transcript abundance showed temporal changes (p=0.0003 and 0.01 for gene and transcript copy number respectively). Total bacterial abundance and activity increased following UAN application and remained elevated throughout the duration of the study (Figure 3.2A and G).

3.4.2.2 Nitrifying Bacterial Community

The nitrifying bacterial community, measured by amoA, showed a significant interaction between crop rotation and day of year so the main effect of rotation on each day of the study is reported. The amoA bacterial community was significantly greater in CCSWrc on June 21, 26, 30, July 4 and 18 (Figure 3.2B). During the period of June 21 to July 18 amoA abundance was on average 2.6 times greater in soil from CCSWrc (range was 1.6-4.1 times greater). The abundance of amoA had the largest increase
over time amongst the nitrogen cycling bacterial communities measured in this study. The size of the amoA community, in both CCSS and CCSWrc, was lowest in soils prior to UAN application. On June 15\textsuperscript{th}, 3 days after UAN application and the start of increasing N\textsubscript{2}O emissions, there was a significant 2-fold increase in amoA gene abundance in both rotations. By June 19\textsuperscript{th} (7 days after UAN addition and during large N\textsubscript{2}O emission period) there was an additional 9- and 7-fold increase in amoA in CCSWrc and CCSS respectively. After the peak in gene abundance on June 19\textsuperscript{th} the community size significantly decreased in CCSS on June 21, accompanied by a short term drop in N\textsubscript{2}O emissions. After June 21 amoA abundance in CCSS did not change for the remainder of the study but was still significantly higher than before UAN addition. In the CCSWrc samples amoA gene abundance also dropped on June 21, before increasing and peaking on June 26, after which it varied for the remainder of the study, but these changes were non-significant in comparison to gene abundance on June 21.

Measurement of amoA transcript abundance was below the limit of qPCR quantification prior to UAN addition (Figure 3.2H). On the first sampling date after UAN application (June 15) amoA transcripts were detected only in CCSWrc, at which point N\textsubscript{2}O emissions were already increasing in this rotation. Transcript copies increased in soils from both rotations from June 19\textsuperscript{th} onward, during the first and main N\textsubscript{2}O emission period, and were highly variable until detection fell below the detection limit in both rotations on June 30\textsuperscript{th} and beyond.
3.4.2.3 Denitrifying Bacterial Community – Nitrite Reducers

Bacterial communities involved in nitrite reduction and downstream N₂O production were targeted by measuring gene and transcript abundance of nitrite reducers, with gene copies per gram of dry soil of nirS on average 50 times lower than nirK (range was 42-69 times lower, Figure 3.2C and D). Gene abundance of nirS was not found to be significantly different in soils under long term CCSS and CCSWrc (Table 3.2). Overall, nirK gene abundance was greater in CCSWrc (p=0.035, Table 3.2) but means comparisons on individual sampling days were only greater in CCSWrc on June 7th, prior to UAN addition (Figure 3.2D). Day of year had a significant impact for both nirS and nirK. After UAN was added the nirS gene abundance decreased 1.4 fold and nirK gene abundance decreased by 1.8-fold between June 7 and 15. After June 15th nirK started to increase over time until June 30, during the period that encompasses both N₂O emission events, after which there were no significant changes in gene abundance. For nirS the population size continued to decrease until June 21, just before the main N₂O emission peak (a 1.6- and 1.95-fold decrease for CCSS and CCSWrc respectively), before gene abundance started to increase again in both rotations. No nirS transcripts were detected in soils from this study (Table 3.2). No nirK transcripts were detected in soils before UAN addition (Figure 3.2I). After UAN fertilization nirK transcripts were detected in both long-term rotations but did not differ significantly between them. Transcript copies increased and peak expression was measured on
June 19, after which they declined and varied over the course of the study before increasing again on the final two sampling dates in July.

3.4.2.4 Complete Denitrification – Nitrous Oxide Reducers

Gene copies of nosZ1 were not significantly different between CCSS and CCSWrc (Table 3.2) and no significant differences were observed on particular dates. Overall the gene abundance of nosZ2 was higher in CCSWrc (Table 3.2), with an average of 1.3 times greater gene copies in CCSWrc, but there were no significant rotation differences on individual dates of the study. Temporal patterns differed slightly for the two clades, with nosZ1 gene copies increasing from June 7 to June 19, during the first N₂O emission event, while for nosZ2 there was a decrease in gene copies that lasted until June 21, just before the peak emission (Figure 3.2E and F). After the peak N₂O emission event on June 23, gene copies of nosZ1 fluctuated but did not change significantly while nosZ2 abundance increased. Transcript copies for both nosZ1 and nosZ2 were detected before and after UAN addition, indicating consistent expression of these genes (Figure 3.2J and K). Transcript copies of nosZ1 did not differ significantly by crop rotation or over time (Table 3.2). Transcript copies of nosZ2 differed significantly by day but not by crop rotation (Table 3.2) and increased during the first main N₂O emission event, from June 15 to June 19th, after which they remained stable. Transcript copies of both nosZ1 and nosZ2 started to decline by the last sampling date on July 18th.
Table 3.2 Results of ANOVA of soil total and nitrogen cycling bacterial gene and transcript abundance in simple (CCSS) and diverse (CCSWrc) long term crop rotations. P-values for test of significance between treatment, day, as well as their interaction are shown, with values p<0.05 in bold (n=4). N.A indicates transcripts were detected but not enough for ANOVA to be performed. N.D indicates no transcripts were detected for target.

<table>
<thead>
<tr>
<th>16S rRNA</th>
<th>amoA</th>
<th>nirS</th>
<th>nirK</th>
<th>nosZ1</th>
<th>nosZ2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANOVA for Gene Abundance</strong></td>
<td><strong>Rotation</strong></td>
<td>0.02</td>
<td>&lt;0.001</td>
<td>0.10</td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td><strong>Date</strong></td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td>0.03</td>
<td><strong>0.01</strong></td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td><strong>Interaction</strong></td>
<td>0.94</td>
<td><strong>0.03</strong></td>
<td>0.95</td>
<td>0.90</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>ANOVA for Transcript Abundance</strong></td>
<td><strong>Rotation</strong></td>
<td>0.38</td>
<td>N.A</td>
<td>N.D</td>
<td>N.A</td>
</tr>
<tr>
<td><strong>Date</strong></td>
<td><strong>0.01</strong></td>
<td>N.A</td>
<td>N.D</td>
<td>N.A</td>
<td>0.45</td>
</tr>
<tr>
<td><strong>Interaction</strong></td>
<td>0.99</td>
<td>N.A</td>
<td>N.D</td>
<td>N.A</td>
<td>0.85</td>
</tr>
</tbody>
</table>
Figure 3.2 Gene and transcript abundance of total and nitrogen cycling bacterial groups in soil from simple (CCSS) and diverse (CCSWrc) long term crop rotations under no till management. Abundances were quantified using qPCR by targeting genes and transcripts for 16S rRNA (panel A/G), ammonia monooxygenase (amoA, panel B/H), nitrite reductases (nirS and nirK, panels C and D/I), and nitrous oxide reductases (nosZ1 and nosZ2, panels E/J and F/K). Panels show mean abundance (copies per gram of dry soil) for CCSS (dotted line) and CCSWrc (solid line) for each day sampled (n=4), with the period of N₂O emission post fertilization highlighted in grey. Significant differences between rotation means on particular days marked with an asterisk (p<0.05).

3.4.3 Bacterial Abundance and Activity – Links to Soil Nitrogen

Redundancy analysis (RDA) was performed to understand the relationships between soil inorganic N, N₂O emissions and WFPS and N cycling gene abundances. The dataset was split by rotation and RDA for CCSS and CCSWrc were performed and shown separately (Figure 3.3, upper and lower panel). For the CCSS data, the first two axes explained 43.8% of the variation and a permutation test of all axes was significant (pseudo-F=4.5, p=0.002). For CCSWrc the first two axes explained 63.6% of the variation and a permutation test of all axes was also significant (pseudo-F=9.6, p=0.002). In both the CCSS and CCSWrc data we see strong relationships between NH₄⁺, NO₃⁻, N₂O emissions and amoA gene abundance, oriented towards the left side of RDA axis 1. There is also a strong relationship between nirS, nirK and nosZ2 gene abundances, for both rotations, and these are oriented towards the right of RDA axis 1.
Figure 3.3 Redundancy analysis of soil nitrogen cycling gene abundance, NH$_4^+$, NO$_3^-$, N$_2$O emissions and WFPS from CCSS and CCSWrc. Nitrogen cycling gene abundances were used as response variables and soil inorganic N (NH$_4^+$ and NO$_3^-$), N$_2$O emissions and WFPS as explanatory variables.
Spearman rank correlations support the RDA analysis. Gene abundance of *amoA* was significant and positively correlated with daily N$_2$O emissions, NH$_4^+$, NO$_3^-$, and WFPS (Appendix 5.3). No other gene abundance was significantly correlated with daily N$_2$O emissions. The gene abundances of *nirS* and *nirK* were negatively correlated with NH$_4^+$ but not with NO$_3^-$, and only *nirK* gene abundance was positively correlated with WFPS. The ratio of *nosZ2:nosZ1* was negatively correlated with N$_2$O emissions. Nitrous oxide reducer gene abundances both had a significant correlation with NH$_4^+$, with *nosZ1* being positive and that of *nosZ2* being negative. Only the *nosZ1* gene abundance had a significant, and positive, correlation with NO$_3^-$. The gene abundance of both groups was not correlated with WFPS. In our study certain functional group gene abundances were positively correlated with each other. For example, *amoA* was positively correlated with *nosZ1*. The nitrite and nitrous oxide reducers were all positively correlated with each other (Appendix 5.3). Daily N$_2$O emissions were also positively correlated with NH$_4^+$, NO$_3^-$, and WFPS.

Daily N$_2$O emissions were also positively correlated with transcript abundance of *amoA* (Appendix 5.4). No other N-cycling transcripts were correlated with N$_2$O emissions. Transcript abundance of all N-cycling groups in this study were positively correlated with NH$_4^+$, but not with NO$_3^-$. WFPS was positively correlated with *amoA* transcript abundance but negatively with *nirK* transcript abundance. The activity of
amoA, nirK, nosZ1 and nosZ2, as measured by transcript abundance, were positively correlated with each other.

3.5 Discussion

Soil bacterial N cycling communities responded differently and peak N2O emissions were higher after inorganic N application in soils managed under a long-term diverse crop rotation. Before fertilizer addition NH4+ and NO3− were low in both CCSS and CCSWrc soils sampled in this study and N2O emissions were at a baseline level, as shown in Figure 3.1. Emissions were significantly higher in CCSWrc rotations from June 14 to 17 and were consistently higher afterwards. The dynamics of increased N2O emissions from CCSWrc in this study mirrors the findings from another study that observed when N fertilizer application was not reduced in diverse crops, subsequent N2O emissions were higher (Mitchell et al., 2013). The authors noted that this could be due to the fact that crop diversification can lead to increased soil organic C, with the increased mineralizable C able to support microbial denitrification and N2O production in their study. Previous research in soil from our study site measured higher organic matter, active C and significantly higher potentially mineralizable C in diverse rotations including CCSWrc (Congreves et al., 2015), implying that higher soil C could be supporting increased microbial activity and N2O production during N metabolism. Our data supports this as there were no significant differences in overall bacterial diversity,
but we measured greater gene abundance for total bacterial and particular groups involved in N metabolism (amoA, nirK and nosZ2). In another short-term study of legumes and a non-legume control, application of N as urea exacerbated field N$_2$O emissions compared to control and legume-residue fertilizer, and were higher when no till was used under all fertilization regimes (Bayer et al., 2015).

As UAN supplied urea, NH$_4^+$ and NO$_3^-$, microbial transformations of N fertilizer in the nitrification and denitrification pathways could both occur and contribute to the N$_2$O emissions measured. The RDA analysis and correlations indicate close positive relationships between daily N$_2$O emissions measured, inorganic N, amoA gene abundance and WFPS. This implies that nitrification played a role in N$_2$O emissions from both rotations during the study period. During the 2017 growing season there were a number of rain events and WFPS during the emission events was between 41 and 75%, which is in the transition zone (53-78%) considered to allow N$_2$O production by both nitrification and denitrification (Wrage-Mönnig et al., 2018). In this study the WFPS was consistently higher in CCSWrc. Higher soil moisture in complex rotations highlights one of the many benefits of long-term crop diversification as it has been shown to have a protective effect on crop yields when climate conditions are hot and dry. Gaudin et al (2015) found an increase of 7% and 22% for corn and soybean yields respectively were achieved in long term crop diversification and reduced tillage despite hot and dry conditions. Microsite moisture and O$_2$ conditions, together with soil N can affect the
abundance and activity of the various N cycling bacterial groups, leading to differences in the magnitude and rate of N transformations between rotations.

### 3.5.1 Nitrifying Bacteria in Simple and Diverse Crop Rotations

Correlation of N cycling genes and transcripts with N$_2$O emissions showed that only $amoA$ gene and transcript abundance was significantly and positively correlated to N$_2$O emissions. Positive correlations between $amoA$ gene abundance and N$_2$O emissions were also shown in Liu et al. (2016), who examined four soil types and found that the relative contribution of nitrification to N$_2$O production was 71% for cereal cropped soil at 50% WFPS after fertilization. A study by Martins et al. (2015) found that in forest soil under irrigation and fertilization regimes, WFPS, inorganic N, nitrifying bacterial abundance ($amoA$) but to a lesser extent nosZ, contributed most to models explaining variability in N$_2$O emissions. So particularly when conditions are suitable for both nitrification and denitrification, the nitrifier population can have a profound impact on transformation of N and production of N$_2$O after fertilization.

Nitrifier community abundance in soils from both simple and diverse rotations was low before the addition of inorganic N fertilizer, with no difference between rotations on June 7. The significant increase in $amoA$ community one week after UAN addition was also found in other studies measuring $amoA$ bacterial abundance after ammonium addition, in microcosms as well as in a field study (Glaser et al., 2010; Liu et al., 2016).
In this study the nitrifier gene abundance in soils remained higher after UAN addition, and means comparisons on individual days showed greater amoA gene abundance in the diverse rotation. In another study from this field site, higher amoA gene abundance was found in monoculture corn compared to corn-soybean-winter wheat with red clover (Munroe et al., 2016). In the study by Munroe et al. (2016) both till and no till were examined and although gene abundance was lower in the more diverse rotation, transcript copies were higher. Higher gene abundance that persists in both soils, but markedly in the complex rotation, could be due to the long-term increased crop diversity and organic matter inputs. In a study examining the effect of organic matter removal in forest harvesting it was found that soil organic C and total nitrogen was positively correlated with bacterial amoA gene abundance (Mushinski et al., 2017), indicating that when organic inputs are reduced there is a corresponding decrease in the nitrifier population abundance. In another study measuring the long-term impacts of organic residue quality on ammonia oxidizer populations it was also found that soils that had received higher quality residue had greater bacterial amoA gene abundance, compared to corn and control plots (Muema et al., 2015). The diverse plots in this study also had higher amoA gene abundance, and previous work has shown that these plots have higher SOM (Congreves et al., 2015), highlighting that more diverse residue inputs can directly or indirectly shape nitrifier communities. The dynamics of amoA communities are dependent on timing of substrate availability as well as soil conditions. When
exogenous N was added to the soil, the community responded by increasing in both rotations. The persistence of higher amoA in the diverse rotation can be explained by more varied inputs from long-term crop diversification and how those have shaped the soil environment, supporting nitrification for a longer amount of time. The interaction between soil organic matter and exogenous N on soil N cycling bacterial communities has been observed in other studies. In agricultural soils receiving N fertilizer, particularly if organic material is also added, AOB community structure was changed and community abundance and potential nitrification activity increased relative to no fertilizer controls (Ouyang et al., 2016; Tao et al., 2017). Likewise, in a study by Mushinski et al. (2017), nitrification potential when exogenous NH$_4^+$ was added was significantly lower in soils where residue was removed.

We also measured the activity of nitrifying bacterial communities using their transcript copy numbers, which were below the detection limit prior to UAN addition. Transcript copies rose to detectable levels first in soil from the diverse rotation on June 15, followed by detection in soil from the simple rotation on June 19. The activity of nitrifiers in both rotations dropped below detection after June 26, which corresponds to when N$_2$O emissions decreased after the main event. Other studies examining the contribution of nitrification processes on N$_2$O emissions have found that in many cases they are a major contributor. For example, in a microcosm study using alkaline soils, nitrification and nitrifier denitrification were the dominant N$_2$O production processes in a
short-term microcosm experiment (Shi et al., 2017). Huang et al. (2014) also found that
the nitrification pathway contributed significantly to N$_2$O production when high NH$_4^+$ was
applied to soil and slowing this pathway by inhibiting nitrification could reduce N$_2$O
emissions (Huang et al., 2014). The large amount of variability in transcript copy data
prevented detection of differences in nitrifier activity between soils from simple and
diverse rotations, but we have captured the temporal changes and turning on/off of a
proxy measure for nitrification activity following UAN application. The significant
increase in amoA population size corresponded to the period of main N$_2$O emissions
during this time and earlier transcription of genes involved in ammonia oxidation in soils
from the diverse rotation also occurred when large N$_2$O emissions increased right after
UAN application.

3.5.2 Denitrifying Bacteria from Simple and Diverse Crop Rotations

Denitrification both produces and consumes N$_2$O with net emissions depending
on the microbial groups that are involved in the steps of the pathway. This study
measured functional groups upstream of N$_2$O production, nitrite reducers, as well as two
clades that are known to reduce N$_2$O. Denitrifier groups with the gene nitrite reductase
(nirS or nirK) are upstream of N$_2$O production. In this study both nirS and nirK gene
abundance was higher in the diverse rotation soil, but this was only significant for nirK.
The more abundant group involved in nitrite reduction was found to be those with nirK,
as nirS gene abundance was consistently lower than nirK in both rotations. The
The dominance of \textit{nirK} communities in agricultural soil has been found in other studies (Clark et al., 2012; Coyotzi et al., 2016). Once inorganic N was added to soil the abundance of both \textit{nirS} and \textit{nirK} decreased. There was a negative correlation of \textit{nirS} and \textit{nirK} with \textit{NH}_4^+ so it is possible that the increase in \textit{NH}_4^+ concentration suppressed populations of organisms harboring these genes. Gene abundance for either of the nitrite reductases was not correlated with \textit{N}_2\text{O} emissions. However, another field study showed a positive correlation between \textit{nirK} and \textit{N}_2\text{O} emissions (Clark et al., 2012). A study by Jones et al. (2014) found that the ratio of \textit{nirS}:\textit{nirK} was correlated to \textit{N}_2\text{O} emissions from soils but we did not find any correlation between them. An analysis of bacterial genomes found that \textit{nosZ} is more frequently found in organisms with \textit{nirS} (Graf et al., 2014), indicating a greater likelihood that these organisms could perform complete denitrification, and therefore produce \textit{N}_2 as an end product. That same study found that up to 70\% of \textit{nirK}-containing organisms do not possess \textit{nosZ} (for \textit{nirS} this was only 20\%). If \textit{nirK} organisms have less co-occurrence of \textit{nosZ} then \textit{nirK} abundance could be a greater driver of \textit{N}_2\text{O} production. During the first \textit{N}_2\text{O} emission event in this study, \textit{nirS} did not start to increase until after June 21, which was after the main emission period, but for \textit{nirK} the community steadily increased from June 15\textsuperscript{th} onward. As soil moisture increased during this period, there was evidence of the potential for denitrifier contribution to \textit{N}_2\text{O} production. This is backed up by measurement of nitrite reductase gene transcripts. The activity of nitrite reducers was quantified by targeting
both nitrite reductase gene transcripts but we could not detect any for \textit{nirS}. For \textit{nirK}, transcript copies were not detectable prior to UAN addition but they increased in copy number from June 15 to 19th, indicating that the \textit{nirK} community responded to the input of N and increased in activity. Activity did decline right around the peak in N\textsubscript{2}O emission and it was during this time that WFPS also peaked in both soils. It was not until the second N\textsubscript{2}O emission peak that \textit{nirK} transcript copies started to increase again.

Both \textit{nosZ} groups were significantly more abundant in the diverse rotation soil and \textit{nosZ2} was the most abundant of the two types. Higher abundance of \textit{nosZ2} vs. \textit{nosZ1} was also measured in other studies (Graf et al., 2016; Jones et al., 2013; Yoon et al., 2016). Although in another study \textit{nosZ1} was greater in most soils (Jones et al., 2014). Gene abundance for both N\textsubscript{2}O reductases exhibited opposing trends in the early time of the study, during the first N\textsubscript{2}O emission event. There was an increase in \textit{nosZ1} gene abundance but during the same time \textit{nosZ2} declined. The two groups of bacteria with \textit{nosZ} seemed to respond differently to the changing nitrogen and soil moisture conditions. This difference in abundance change was also observed for the second emission event, \textit{nosZ1} remained constant while \textit{nosZ2} abundance increased. Jones et al. (2014) found significant positive correlation with soil N\textsubscript{2}O sink capacity and the ratio of \textit{nosZ2:nosZ1} gene abundance. In this study, in CCSS and CCSWrc, there was a significant but negative correlation between the ratio of \textit{nosZ2:nosZ1} and N\textsubscript{2}O emissions, indicating that as \textit{nosZ2} increased relative to \textit{nosZ1} there was a decrease in
measured N\textsubscript{2}O. Organisms with \textit{nosZ1} are more capable of complete denitrification as they tend to possess both a nitrite reductase and a nitrous oxide reductase gene (Hallin et al., 2017). Organisms with \textit{nosZ2} more often lack nitrite reductases, and are therefore capable of reducing N\textsubscript{2}O produced by other organisms, to N\textsubscript{2} (Graf et al., 2014; Jones et al., 2014). The abundance of \textit{nosZ2} more clearly explained the reduction in N\textsubscript{2}O emissions from various soils in Europe (Jones et al., 2014). In this study, \textit{nosZ2} gene abundance seemed to respond after N\textsubscript{2}O emissions occurred. There was an initial decreasing trend in the first few sampling dates but after the main N\textsubscript{2}O emission peak on June 23 there was a steady increase in \textit{nosZ2} abundance, likely responding to the increased amounts of N\textsubscript{2}O produced by nitrifiers and other denitrifiers. The lack of change in \textit{nosZ1} abundance during this time showed that the two functional groups responded differently. When we measured activity there was minimal \textit{nosZ1} and \textit{nosZ2} expression prior to UAN addition. Transcript copies also responded differently to changing soil N and moisture. The activity of \textit{nosZ1} did not change over time but increased transcription of \textit{nosZ2} was measured during the main emission event, with a second increase in transcript copies that occurred 5 days after the second emission event (small increase in transcripts was also seen for \textit{nosZ1}). There is a larger lag in transcriptional activity for \textit{nosZ2} in the second event, possibly due to the differing soil N and moisture conditions that occurred between the early part of the study and the latter part.
3.6 Conclusion

Long term crop diversification increased soil moisture in no-till corn and soybean rotations, and when exogenous N was applied during the corn phase, there were increased N₂O emissions. There were no differences in total bacterial abundance but functional groups responsible for soil N transformations were more abundant in soil under long-term diverse rotations, leading to increased capacity for nitrification and denitrification to produce N₂O emissions when soil conditions and available N were suitable. Crop diversification provides numerous benefits, such as the promotion and support of microbial populations involved in soil nutrient turnover, and these need to be balanced by also limiting N₂O production. An important consideration and avenue of future research should investigate whether taking the N credits into account and reducing N fertilizer input can provide the same soil benefits, without increased N₂O production from microbially mediated pathways.
4 Three and a Half Decades of Tillage and Rotation alters Bacterial and Fungal Communities in a Corn Based Cropping System

*Nicola F. Linton¹, Bill Deen², Claudia Wagner-Riddle¹, Kari Dunfield¹

¹School of Environmental Sciences, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada

²Department of Plant Agriculture, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada

*corresponding author: lintonn@uoguelph.ca

Author Contributions:

N. Linton was the primary researcher and author for this study, having conducted field and lab work for microbial analyses, and manuscript preparation. Author B. Deen is credited for the use of his OMAFRA-funded field plots for this research. Author C. Wagner-Riddle is credited for project conception, advice and for funding through NSERC strategic grant. Author K. Dunfield is credited for her mentorship, support and advise on soil sampling, molecular analyses, data interpretation and manuscript focus and preparation.
Soil microbial biodiversity is a foundation for provision of diverse ecosystem services but the impact of agricultural management practices on microbial diversity and functioning is not clear. Tillage and crop rotational diversity can change the soil physiochemical environment, leading to potential shifts in communities. A long-term rotation and tillage trial, established in Elora, Ontario in 1980 has found that simple rotations of corn-soybean are consistently associated with reductions in yield, soil health, organic matter, and nutrient use efficiency. The goal of this study was to investigate how long-term rotational diversity shaped soil bacterial and fungal diversity and community composition, under both till and no till management. Four-year rotations ranging from monoculture, 2-crops and 3-crops with legume cover crop included corn, soybean, alfalfa, and winter wheat with/without red clover. Rotations have a tillage split plot treatment with either conventional tillage by moldboard plow or no tillage. Bulk soil samples from the top 10 cm (n=4) were collected in October 2017 during the second-year corn phase. Total DNA was extracted in duplicate and HTS of the bacterial 16S V4 region and fungal ITS1 region was performed, for analysis of microbial community diversity and composition. There was a trend towards reduced bacterial and fungal diversity in soils under tillage, although fungal evenness was higher. Bacterial and fungal community composition was shaped by both tillage and crop rotation. There were bacterial and fungal taxonomic groups that were relatively more abundant in either till or...
no till soil, with certain taxa also associated with long-term rotational history. The shifts in bacterial and fungal communities measured in this study could be linked to the yield stability and increased nutrient use efficiency observed from this long-term trial.

4.2 Introduction

Healthy soils provide a range of benefits to the environment and the numerous ecosystem services they provide impact human health and wellbeing (Kibblewhite et al., 2008; Wall et al., 2015). Agricultural intensification has led to soil degradation and reduced health of those ecosystems, but practices such as reduced tillage, increased rotational diversity and inclusion of legume cover crops has been shown to increase health and resilience (Gaudin et al., 2013). Many key soil ecosystem services are mediated by microbial communities and agricultural management practices can have direct and indirect impacts on the soil environment, impacting communities and their ability to carry out numerous functions (Lehman et al., 2015; van der Heijden and Wagg, 2013).

Previous research on the beneficial impacts of complex rotations has shown that crop rotational diversity, both spatially and temporally, can improve SOM, C sequestration, total N, cation exchange capacity, and aggregate stability (Lehman et al., 2017; Maiga et al., 2019; Prommer et al., 2019; Tiemann et al., 2015). Diverse cropping systems are also associated with greater soil water holding capacity, N use efficiency,
and increased yields and yield stability (Gaudin et al., 2015b; Lehman et al., 2017; Renwick et al., 2018; Williams et al., 2018). Changes to soil physiochemical properties from increased crop diversification will shape this environment, which is inhabited by a very wide range of microorganisms. Diverse soil microbial communities carry out important functions that support healthy soil ecosystem functioning, including nutrient cycling and organic matter breakdown, leading to improved SOM, structure, GHG and C sequestration and water quality (Lehman et al., 2015). The breakdown and processing of soil residues to form SOM is a particularly important attribute of improved soil health, and is mediated by microbial activities (Kallenbach et al., 2016). Soils with more diverse cropping histories have been shown to decompose newly added residue at faster rates, compared to soil from monoculture corn, and this was true for residues ranging from mixtures of two to four species (McDaniel et al., 2016, 2014). The increased capacity of soils with diverse cropping histories to break down residues, accomplished by the soil microbiota, indicates that changes to the soil environment and available niches provided by more complex rotations can impact soil microbial communities, their biogeochemical functioning and resulting changes to SOM formation. In fact, improved microbial C use efficiency has the potential to increase soil C sequestration (Kallenbach et al., 2019). Crop residues offer substrates to microbial communities and an important question is whether increased residue diversity leads to increased microbial community diversity. Crop residues decompose at varying rates, with more rapid initial breakdown for
soybean residues, compared to both corn and wheat, and with different amount and types of culturable bacteria and fungi associated with each (Broder and Wagner, 1988). More recent studies have shown that increased diversity of organic inputs in the form of manure amendments, crop diversity and use of cover crops can increase bacterial and fungal diversity (Hartmann et al., 2015; Schmidt et al., 2019, 2018; Tiemann et al., 2015). Also, the composition of SOM has been shown to shape soil microbial community structure (Thiele-Bruhn et al., 2012). In a meta-analysis of studies examining crop rotational effects on soil microbial diversity it was found that increased rotational diversity was associated with increased microbial richness and diversity in many cases, although some studies did report negative associations or no significant impacts (Venter et al., 2016). The conflicting evidence regarding the link between crop rotational diversity and belowground microbial diversity that is currently available highlights that more studies are needed that investigate agricultural management and soil diversity and functioning.

Another management practice available to improve of soil health is the use of no till. No till or conservation tillage strategies have been shown to improve soil health scores, SOC, and total N (Congreves et al., 2015; Mbuthia et al., 2015; Van Eerd et al., 2014). The use of conservation tillage strategies can increase soil C storage and lessen erosion but can lead to lower quality soil structure and negatively impact yield in some soils such as in South Western Ontario, although these negative effects can be
mitigated by also using increased crop rotational diversity (Lundy et al., 2015; Munkholm et al., 2013). Tillage can impact soil microorganisms directly and indirectly. Direct impacts include breaking up fungal networks, both beneficial and plant pathogens (Roger-Estrade et al., 2010; Wilpiszeski et al., 2019). The indirect influence of tillage on microbial communities is related to the changes tillage can induce to the soil environment, which will impact the physical and chemical components, shaping the niches available to communities for their survival. Tillage has been shown to reduce fungal and bacterial diversity in some cases (De Quadros et al., 2012; Schmidt et al., 2018; Wang et al., 2017), but increase diversity in others (Degrune et al., 2017, 2016). The use of tillage has also been shown to impact the composition of microbial communities (Souza et al., 2015; Sun et al., 2018; Wang et al., 2017).

The interaction of aboveground management on belowground diversity and function is complex. There is an increased interest in examining soil microbial diversity and community composition, as well as functional potential, in systems using different management practices. The presence of links between increased health and resilience of soil under long-term crop diversification and no till poses the question whether there are differences in microbial communities from long-term cropping and tillage practices. Studies from the long-term crop and tillage trial in Elora, Ontario (established 1980) has already shown that diversifying crop rotations resulted in better yield outcomes when weather conditions are wet and cool or hot and dry (Gaudin et al., 2015b). Also, long-
term no tillage has resulted in soils with greater aggregate stability and higher soil health scores, in comparison to rotations with conventional tillage (Congreves et al., 2015). The next step is to investigate how management practices (long-term crop rotational diversity and tillage) have shaped the microbial populations. In this study the legacy effects of 35 years of crop rotational diversity and tillage on soil bacterial and fungal communities was examined. The objectives were to measure and characterize how past crop diversity and tillage have shaped the soil microbial communities by sampling crops of varying complexity in the second-year corn phase. We investigated the extent to which 1) microbial groups in soils under tilled management would differ in diversity and community structure, compared to no till, and 2) long-term crop diversification resulted in greater bacterial and fungal diversity, different community structures, and abundance of particular groups.

4.3 Materials and Methods

4.3.1 Site Description and Experimental Design

For this study, soil samples were taken from a long-term field trial in Elora, Ontario (43°39' N 80°25' W), which was established in 1980 and consists of a randomized complete block design with four-year crop rotations as the main factor and tillage as a split plot treatment. Soil at this site is classified as a silty loam (Grey Brown Luvisol) with 27% sand, 57 % silt and 17 % clay (Congreves et al., 2015). In this study
we were interested in six rotations, continuous corn (CCCC), corn-corn-soybean-soybean (CCSS), corn-corn-soybean-winter wheat (CCSW), corn-corn-soybean-winter wheat + red clover (CCSWrc), corn-corn-alfalfa-alfalfa (CCAA), and continuous alfalfa (AAAA). All rotation except AAAA had a split plot tillage treatment consisting of either fall tillage with moldboard plowing after crop harvest (till), or no tillage (no till). For continuous alfalfa, tillage was performed every 3 years when the crop was terminated and reestablished. Four replicated plots (16.8x 6.1 m) from each crop rotation and tillage treatment were used in this study.

4.3.2 Soil Sampling for Microbial Analysis

Bulk soil samples were collected from each plot on October 27, 2017, during the second-year corn phase before harvest of each long-term rotation with corn, and in alfalfa for the continuous perennial plots. From each plot, 12 bulk soil samples from in between corn rows (for rotations in second-year corn) and between alfalfa plants (for continuous alfalfa) were collected from the top 10 cm using a 2cm soil probe and combined to form a composite sample. Soil composites were homogenized and stored in a cooler until transport back to the lab for microbial analyses. A sub-sample (~10 g) of the composite soil was used to determine gravimetric moisture content.
4.3.3 Soil Nucleic Acid Extraction and Preparation for High Throughput Sequencing

Soil was stored at 4°C until DNA extraction, which was performed in duplicate within 1 week of sampling. DNA was extracted from 0.25g of each soil composite using the Qiagen DNeasy Powersoil Kit according to the manufacturer’s instructions. DNA was quantified and quality checked using NanoDrop (ThermoFisher) prior to storage at -20°C. DNA from duplicate extractions were pooled by volume and submitted to Genome Quebec for HTS using an illumina MiSeq 250 paired end run. For bacteria the V4 hypervariable region of the 16S rRNA gene was amplified using modified primers 515F (5’-GTGYCAGCMGCCGCGGTAA-3’) and 860R (5’-GGACTACNVGGGTWTCTAAT-3’) from the Earth Microbiome Project. For fungi, the ITS1 region was amplified using primers ITS1F (5’-CTTGGTCATTTAGAGGAAGTAA-3’) and ITS2 (5’-GCTGCGTTCTTCTCATCGATGC-3’) (Smith and Peay, 2014).

4.3.4 Bioinformatics Pipeline and Sequence Analysis

Sequences were processed using QIIME2 versions 2018.6 and later (Bolyen et al., 2019). Demultiplexed sequences were imported into QIIME2 and primer sequences trimmed using cutadapt. Trimmed sequences were quality checked at the 3’ end to determine optimal length cutoff before read joining, denoising and chimera removal using the DADA2 plugin. Resulting ASVs, which correspond to 99.9% similarity, were
classified against the SILVA database version 132. Sequences not assigned to kingdom bacteria were filtered by removing sequences assigned to archaea, mitochondria and chloroplast, as well as unassigned sequences, for all downstream analyses. For fungi, unclassified sequences not identified as fungi were filtered. Uneven sequencing depth can affect diversity estimates therefore sample reads after filtering were rarefied to 29,400 for bacteria and 9,000 for fungi.

4.3.5 Diversity Analysis and Statistical Tests

Alpha diversity estimates were calculated in QIIME2 using the core metrics phylogenetic pipeline. Differences in observed ASVs, Faith-PD and Evenness between categorical groups of samples were tested using Kruskal Wallis non-parametric analysis. Samples were grouped by tillage (tilled and no tilled, n=40), crop rotation (corn rotations n=8 and alfalfa n=4) and crop rotation within tillage (n=4) for statistical comparisons. Differences in community composition between samples were calculated using the Bray-Curtis dissimilarity index of rarefied data and significant differences due to long-term tillage, crop rotation as well as crop rotation within tillage were tested using PERMANOVA with 10,000 permutations. Linear discriminant analysis of effect size (LEfSe) (Segata et al., 2011) was used to identify statistically significant bacterial and fungal groups that were relatively more abundant between tillage and crop rotation within tillage categories.
4.4 Results

4.4.1 Bacterial and Fungal Sequence Analysis

A total of 2,719,557 16S sequence reads were generated in total from all 44 samples. Chimeras and other spurious sequences were removed, leaving 1,912,334 sequences that were grouped into 10,641 ASVs. Samples had 44,963 16S rRNA sequences on average with a range of 29,462 to 54,650 per sample. Sequences were rarefied to 29,400 reads per sample by random subsampling without replacement for alpha and beta diversity analyses. For ITS there were 2,017,749 reads total from the sequencing of all 44 samples. After denoising there were 1,088,172 sequences remaining, which were grouped into 2,556 ASVs. Sequences per sample ranged from 9,056 to 33,158. Sequences were rarefied as for 16S rRNA sequences but to 9,000 reads per sample for alpha and beta diversity analyses.

4.4.2 Bacterial and Fungal Alpha Diversity

For bacteria, the main effects of crop rotation, tillage, or crop by tillage combination did not cause any significant differences in alpha diversity metrics (Table 4.1). Fungal alpha diversity metrics were also not significantly different between tillage, crop rotation, and crop by tillage, with the exception of a significant effect of crop by tillage on Faith-PD (Table 4.1). Data for bacterial and fungal diversity and evenness metrics for groups
separated by tillage (Appendix 5.5, Appendix 5.9), crop rotation (Appendix 5.6, Appendix 5.10) and crop by tillage history (Appendix 5.7, Appendix 5.8, Appendix 5.11, Appendix 5.12) are shown in the appendix. There was a trend of decreased bacterial and fungal richness and phylogenetic diversity, compared to soils under no till.

Table 4.1 P-values for test of significance differences in bacterial and fungal alpha diversity metrics from long-term crop rotational diversity and tillage. *Tillage comparisons made by comparing only plots with corn in rotation. **Crop comparisons made for corn plots (till and no till, n=8) and AAAA n=4.

<table>
<thead>
<tr>
<th></th>
<th>Bacteria</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed ASVs</td>
<td>Faith’s Phylogenetic Diversity</td>
</tr>
<tr>
<td>Till vs No Till* (corn only) n=20</td>
<td>0.60</td>
<td>0.24</td>
</tr>
<tr>
<td>Crop** n=8 (AAAA=4)</td>
<td>0.98</td>
<td>0.57</td>
</tr>
<tr>
<td>Crop by Tillage n=4</td>
<td>1.0</td>
<td>0.79</td>
</tr>
<tr>
<td>Till vs No Till* (corn only) n=20</td>
<td>0.32</td>
<td>0.06</td>
</tr>
<tr>
<td>Crop** n=8 (AAAA=4)</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>Crop by Tillage n=4</td>
<td>0.17</td>
<td>0.04</td>
</tr>
</tbody>
</table>

At p<0.1 there are a number of comparisons that are significant for fungal diversity metrics. Tilled corn plots had lower Faith-PD (58.4±5.3) compared to no till.
Fungal evenness was higher in tillage (0.78±0.048) than in no tillage (0.75±0.059). For crop, the highest number of fungal observed ASVs was in AAAA (320±30), followed by CCAA (280±20), monoculture corn (265±32), CCSWrc (260±42), CCSS (259±23), and CCSW (257±25). Faith’s phylogenetic diversity was also greatest in AAAA and CCAA (74.3±7.3, and 63.1±4.8), with Faith-PD values for corn, soybean, and wheat rotations ranging from 58.0±8.2 to 60.1±5.1.

4.4.3 Bacterial and Fungal Community Composition

Principle coordinate analysis (PCoA) of the bacterial 16S rRNA sequences showed that tilled and no till samples separated along the first PCoA axis, which explained 11.5% of the variation (Figure 4.1). Along the second axis, which explained 9.3%, there was a less defined pattern and no distinct clustering of samples based on crop rotational history, in either till or no till, although continuous alfalfa formed a distinct cluster close to the no till samples (Figure 4.1). Analysis of the Bray-Curtis dissimilarity matrix for bacterial communities in the corn plots using PERMANOVA showed that tillage (pseudo-F= 3.83, p=0.0001) and crop rotation (pseudo-F= 1.75, p=0.0001) had a significant effect in shaping the community composition. Within the plots sampled in the corn phase, crop rotational history was also significant when AAAA was excluded from the analysis (pseudo-F=1.45, p=0.0004). Pairwise comparisons of bacterial community composition between long-term rotational history for the corn plots is shown in the
appendix (Appendix 5.13). As tillage had a significant impact on soil bacterial community composition, further evaluation of long-term crop effects was performed on data for tilled and no till plots separately, with AAAA dropped from the analysis. The effect of crop was more apparent in shaping soil bacterial community composition in the no till plots than the tilled plots (Table 4.2). Pairwise comparisons of different long-term crop rotations under tillage showed that bacterial community composition in CCAA differed from CCCC and CCSS but not from the two rotations with CCSW (Table 4.2). Within the no till corn plots, crop rotation had a significant impact on shaping bacterial community composition between CCAA and both CCCC and CCSW (Table 4.2). Additionally, communities in CCCC differed when soybean was added to the rotation, and when CCSS rotations were further diversified from inclusion of winter wheat (Table 4.2).
Figure 4.1 Principle coordinate analysis of the Bray Curtis dissimilarity index for bacterial 16S rRNA amplicon sequences. Each color and symbol combination represent a sample from plots under different long-term crop rotation and tillage management. Continuous corn (CCCC, green), corn-alfalfa (CCAA, red), corn-soybean (CCSS, dark blue), corn-soybean-winter wheat (CCSW, aqua), and corn-soybean-winter wheat with red clover cover crop (CCSWrc, purple) managed with long-term tillage (circles) or no tillage (open squares) are shown with continuous alfalfa (crosses). Open circles used to highlight corn rotations under till (dashed) and no till (solid), as well as AAAA (dark orange).
Table 4.2 PERMANOVA of the bacterial 16S rRNA Bray Curtis dissimilarity matrix from long-term corn rotations with tillage and no till management. Crop main effect within till and no till as well as pairwise differences between crops shown with significant differences in bold (n=4, p<0.05, 10000 permutations).

<table>
<thead>
<tr>
<th></th>
<th>Tilled p-value</th>
<th>No Tilled p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crop Main Effect</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tilled</td>
<td>1.14 0.07</td>
<td>1.23 0.02</td>
</tr>
<tr>
<td>No Tilled</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pairwise Crop Comparisons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCAA CCCC</td>
<td>1.47 0.027</td>
<td>1.45 0.057</td>
</tr>
<tr>
<td>CCAA CCSS</td>
<td>1.53 0.057</td>
<td>1.21 0.170</td>
</tr>
<tr>
<td>CCAA CCSW</td>
<td>1.00 0.516</td>
<td>1.27 0.084</td>
</tr>
<tr>
<td>CCAA CCSWrc</td>
<td>0.95 0.568</td>
<td>0.85 0.828</td>
</tr>
<tr>
<td>CCCC CCSS</td>
<td>1.20 0.119</td>
<td>1.63 0.031</td>
</tr>
<tr>
<td>CCCC CCSW</td>
<td>1.03 0.312</td>
<td>1.11 0.203</td>
</tr>
<tr>
<td>CCCC CCSWrc</td>
<td>1.11 0.257</td>
<td>1.17 0.136</td>
</tr>
<tr>
<td>CCSS CCCC</td>
<td>1.18 0.168</td>
<td>1.41 0.031</td>
</tr>
<tr>
<td>CCSS CCSW</td>
<td>1.13 0.200</td>
<td>1.10 0.314</td>
</tr>
<tr>
<td>CCSS CCSWrc</td>
<td>0.85 0.858</td>
<td>1.05 0.355</td>
</tr>
<tr>
<td>CCSW CCSWrc</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For fungi, the PCoA of the Bray-Curtis dissimilarity matrix showed effects of both tillage and crop rotation on community composition (Figure 4.2). Samples from tilled and no till plots separated along the first axis, which explained 14.7% of the variation, while the second axis, explaining 12.0% of the variation, showed more separation from long-term crop rotational history. Continuous alfalfa clustered separately and close to those from no till plots. PERMANOVA of the Bray-Curtis dissimilarity matrix for fungal communities in corn plots showed that tillage, independent of crop history had a significant effect on community composition (pseudo-F=6.17, p=0.0001). Crop rotation, independent of tillage also had a significant impact in shaping soil fungal communities.
(pseudo-F=2.95, p=0.0001). Within the plots sampled in the corn phase, crop rotational history was also significant when AAAA was excluded from the analysis (pseudo-F=2.44, p=0.0001). Pairwise comparisons of fungal community composition between long-term rotational history for the corn plots is shown in the appendix (Appendix 5.14). Due to the significant effect of tillage on fungal community composition, further evaluation of long-term crop effects was performed on data for tilled and no till plots separately, with AAAA no longer included in the analysis. Long-term crop rotational history had a significant effect in shaping fungal community composition in both tilled and no till plots (Table 4.3). The effect of crop was more apparent in shaping soil fungal community composition in the no till plots than the tilled plots. For no till plots, fungal communities were significantly different between all long-term crop rotations examined (Table 4.3). For tilled plots, there were pairwise differences between all long-term rotations, with the exception of CCSS and CCSW, and between CCSW and CCSWrc (Table 4.3).
Figure 4.2 Principle coordinate analysis of the Bray Curtis dissimilarity index for fungal ITS amplicon sequences. Each color and symbol combination represent a sample from plots under different long-term crop rotation and tillage management. Continuous corn (CCCC, green), corn-alfalfa (CCAA, red), corn-soybean (CCSS, dark blue), corn-soybean-winter wheat (CCSW, aqua), and corn-soybean-winter wheat with red clover cover crop (CCSWrc, purple) managed with long-term tillage (circles) or no tillage (open squares) are shown with continuous alfalfa (crosses). Open circles used to highlight corn rotations under till (dashed) and no till (solid), as well as AAAA (dark orange).
Table 4.3 PERMANOVA of the fungal ITS Bray Curtis dissimilarity matrix from long-term corn rotations with tillage and no tillage management. Crop main effect within till and no till as well as pairwise differences between crops shown with significant differences in bold (n=4, p<0.05, 10000 permutations).

<table>
<thead>
<tr>
<th></th>
<th>Tilled pseudo-F</th>
<th>p-value</th>
<th>No Tilled pseudo-F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crop Main Effect</td>
<td>1.85</td>
<td>0.0001</td>
<td>2.34</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Pairwise Crop Comparisons

<table>
<thead>
<tr>
<th></th>
<th>CCAA</th>
<th>CCCC</th>
<th>CCA</th>
<th>CCSS</th>
<th>CCSW</th>
<th>CCSWrc</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCAA</td>
<td></td>
<td></td>
<td>3.29</td>
<td>0.029</td>
<td>3.63</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>CCCC</td>
<td>2.01</td>
<td>0.027</td>
<td>2.53</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCA</td>
<td>2.14</td>
<td>0.030</td>
<td>2.79</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCSS</td>
<td>1.57</td>
<td>0.025</td>
<td>2.66</td>
<td>0.031</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCSW</td>
<td>2.21</td>
<td>0.030</td>
<td>1.84</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCSWrc</td>
<td>1.54</td>
<td>0.059</td>
<td>1.81</td>
<td>0.031</td>
<td></td>
</tr>
<tr>
<td>CCCC</td>
<td></td>
<td></td>
<td>2.11</td>
<td>0.029</td>
<td>3.37</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>CCA</td>
<td>1.41</td>
<td>0.114</td>
<td>1.57</td>
<td>0.030</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCSS</td>
<td>1.38</td>
<td>0.029</td>
<td>2.13</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCSW</td>
<td>1.15</td>
<td>0.195</td>
<td>1.68</td>
<td>0.029</td>
<td></td>
</tr>
</tbody>
</table>

4.4.4 Bacterial and Fungal Taxonomic Groups Shaped by Long-Term Tillage

Using LEfSe we identified bacterial and fungal taxonomic groups that are consistently differentially abundant in soil that has been under long-term tillage and no tillage. There were three bacteria phyla more abundant in soil under tillage, Proteobacteria, Bacteroidetes, and Armatimonadetes (Figure 4.3). Soil managed using no till on the other hand showed seven phyla with consistently higher relative abundance. These were Rokubacteria, Latescibacteria, Planctomycetes, Elusimicrobia,
Patescibacteria, Entotheonellaeota and Nitrospirae (Figure 4.3). Within the differentially abundant groups at the phylum level, there were also significantly different groups at lower taxonomic levels as well. For example, Rokubacteria, Nitrospirae, Lathecidibacteria, and Entotheonellaeota were consistently more relatively abundant in no till soil at the phylum through to the genus level. For other phyla there were groups at lower taxonomic levels with higher relative abundance in either tillage regime. An example is within the Proteobacteria, which are greater in tilled soils in this study. Within the Proteobacteria there was also greater relative abundance of gammaproteobacteria under tillage. There were no differences in alpha or delta-proteobacteria (at the class level), but within these two classes some groups were more abundant in tilled soil and others had greater abundance in no till at lower taxonomic levels including order, family and genus levels.
Figure 4.3 Cladogram showing bacterial taxa with higher relative abundance in till (green) and no till (red) corn rotations according to LEfSe. Phyla that differ are labelled and classes annotated with subscripts in the legend.

For fungi, there was a single division more abundant in tilled soil and, whereas four were identified in no tilled soil. In the tilled plots, Ascomycota was significantly greater under tillage, and Mortierellomycota, Olpidiomycte, Chytridiomycota, and unidentified fungi greater under no till (Figure 4.4). Within the Ascomycota, certain classes were relatively more abundant in tilled soil with others were more abundant in no till soil. Leotiomycetes and Pezizomycetes were both greater in till, with
Sordariomycetes instead greater under no till. Within Sordariomycetes, certain orders remained a hallmark of no till, including Sordariales and Coniochaetales, while others were markers of tillage, such as Glomerellales and Chaetosphaeriales (Figure 4.4). The other divisions greater in no till, Mortierellomycota, Olpidiomyctota, and Chytridiomycota, were consistently more abundant at all lower taxonomic levels (Figure 4.4).

Figure 4.4 Cladogram showing the fungal taxa from division to genus with higher relative abundance in till (green) and no till (red) corn rotations according to LEfSe. Divisions that differed are labelled and classes are annotated with subscripts in the legend.
4.4.5 Bacterial and Fungal Taxonomic Groups Shaped by Long Term Crop Diversity

As there were significant differences in bacterial and fungal communities between tilled and no till soil, the data for corn rotations under till and no till was also analyzed separately in order to compare differences in communities due to long-term crop rotation within each tillage regime.

4.4.5.1 Tilled Corn Rotations

Previous crop rotational history was not found to be a significant source of variation in bacterial communities from tilled corn rotations (p=0.07) according to PERMANOVA, but pairwise comparisons of sequences from different rotations indicated some crops differed from each other (Table 4.2). This was explored using LEfSe and various groups were found to be markers of CCAA, CCCC, CCSS, or CCSWrc rotations, dependent on which taxonomic level the comparisons are made at. No bacterial groups at any taxonomic level were found to distinguish soil from CCSS rotations. Two bacterial phyla were found to be relatively more abundant, Entotheonellaeota in CCAA and Dependentiae in CCSWrc (Figure 4.5), and for both phyla the greater abundance was also measured down to the family level. The CCAA rotations were enriched in classes of an unknown Actinobacteria and Acidobacteria subgroup 11, and orders Pseudonocardiales and Nitrosococcales. Soil from CCCC had
greater unknown Elusimicrobia and Patescibacteria, as well as Sphingomonadales (order).

Figure 4.5 Cladogram for bacterial taxa with higher relative abundance in long-term corn rotations under tilled management according to LEfSe. Phyla that differ are labelled and classes and orders annotated with subscripts in the legend.

Fungal communities were found to be significantly different between corn rotations under tillage ($p=0.0001$). Rotations were also found to be significantly different from each other when pairwise comparisons were made, with the exception of CCCC and CCSW, CCSS and CCSW, and CCSW with and without red clover (Table 4.3). Dothideomycetes was found to be a marker of the most diverse rotation, CCSWrc, and Agaricostilbomycetes in CCSS (Figure 4.6). Classes enriched in different crop rotations
were Pleosporales and Myrmecridiales (CCSWrc), Agaricostilbales (CCSS) and Cystofilobasidiales (CCAA).

**Figure 4.6** Cladogram showing the fungal taxa with higher relative abundance in long-term corn rotations under tillage management according to LEfSe. Classes that differ are labelled and orders and families are annotated with subscripts in the legend.

### 4.4.5.2 No Till Corn Rotations

Bacterial and fungal communities were also found to differ between crop rotations in soil from no till plots (p=0.02 and 0.0001 respectively). Pairwise
comparisons of both bacterial and fungal communities under each crop rotation were also found to significantly differ (Table 4.2, Table 4.3). The taxonomic groups that were driving community differences between long-term crop rotations were explored using LEfSe. Unlike in tilled plots, in the no till plots there were bacterial taxa characteristic of each corn rotation we examined. Phylum Armatimonadetes was enriched in CCSW and Fibrobacteres in CCSS. At lower taxonomic levels we saw Acidobacteria subgroup 9 markedly higher in CCAA, Acidobacteriales, Sphingobacteriales and Reyranellales in CCCC, Bacteroidales and Dongiales in CCSWrc, and Fibrobacterales and Azospirillales in CCSS (Figure 4.7).
Figure 4.7 Cladogram showing significant bacterial taxa with higher relative abundance in long-term corn rotations under no till management according to LEfSe. Phyla that differ are labelled and classes and orders annotated with subscripts in the legend.

Two fungal divisions were characteristic of CCSWrc, Olpidiomyco and Mortierellomyco, which were also significantly more abundant in this rotation at all lower taxonomic levels (Figure 4.8). For the other rotations there were certain fungal orders that were found to be markers of long-term crop rotation according to LEfSe. Hypocreales was higher in CCAA, Sodariales in CCSS, Xylariales in CCSW and Agaricales in CCC (Figure 4.8).
4.5 Discussion

4.5.1 Bacterial and Fungal Communities are Impacted by Tillage

The use of long-term tillage resulted in a trend of decreased bacterial and fungal richness and phylogenetic diversity, compared to soils under no till. Bacterial evenness was not affected by tillage; however fungal evenness was higher in the tilled plots. Statistical analysis did not detect significant differences in the alpha diversity metrics at p<0.05, but both fungal phylogenetic diversity and evenness differences were significant.
at p<0.1. Further examination of the data did clearly show that both bacterial and fungal community composition were significantly shaped by tillage practice.

The impacts of tillage on soil microbial diversity in the literature is conflicting, with studies reporting positive, negative and no impacts. There was a clear trend in our data of decreased richness and phylogenetic diversity for bacteria and fungi in soils under tillage, compared to those that were under no till. A negative impact of tillage on bacterial diversity was also found in a number of studies (De Quadros et al., 2012; Schmidt et al., 2018; Wang et al., 2017). For example, in Schmidt et al. (2018) Shannon diversity as well as total bacterial and archaeal numbers were higher in soil with no till. Wang et al. (2017) also found decreased PLFA diversity indices for tilled soils. Studies have also shown positive impacts of tillage on soil microbial diversity. In a study examining 6 years of contrasting tillage regimes on both bacterial and fungal operational taxonomic units (OTUs), richness and Shannon diversity indices were found to be significantly higher in the conventionally tilled system (Degrune et al., 2016). In another study from the same site it was found that bacterial richness was greater and evenness lower in reduced till during the seedling and leaf development stages (Degrune et al., 2017). The authors also found that fungal richness was unaffected by tillage, but evenness was lower at seedling and at the wheat tillering stage. In the studies by Degrune et al. (2016 and 2017) soils were sampled while under wheat. Positive impacts of tillage on the number of bacterial OTUs was also found in another study in a corn-
soybean system, however the significance was less apparent when singleton OTUs were excluded (Smith et al., 2016). For fungi, recent studies found no effect of tillage on overall fungal diversity and evenness metrics (Dong et al., 2017; Schmidt et al., 2019). Due to the complexity of soil systems it is likely that there were other factors that differed in these studies, leading to the differences observed for tillage impacts on microbial diversity. For example, soils sampled in those studies were under different cropping systems suitable to the geographic location and climate, ranging from corn-soybean (Smith et al., 2016), wheat-bean (Degrune et al., 2016), and tomato-cotton (Dong et al., 2017; Schmidt et al., 2019). The studies which found positive impacts of tillage were all sampled in spring and summer, while this study and the other studies which showed a negative impact were sampled during the fall. Clearly there can be both long-term but also short-term influences on microbial diversity during the growing season and over the year. Together these effects may interact to shape the soil and the diversity of microbial communities that can inhabit the niches provided. Timing of tillage and sampling may be another factor influencing whether differences are apparent, and significance detected. Sampling in the fall prior to corn harvest and tillage, as was the case for this study, means that a longer amount of time has elapsed since previous tillage disturbance. Schmidt et al. (2018) noted that redistribution of nutrients from tillage is episodic and favors microorganisms able to capitalize on sudden nutrient availability, and their ability to outcompete others could lower diversity in the short term. On the
other hand, tillage acts to break up established soil structure and can increase turnover of SOM, which could support increased diversity of microorganisms capable of utilizing substrates previously inaccessible. Differences from tillage may become apparent at particular intervals and sampling temporally may be necessary to more fully understand the tillage and microbial diversity relationship.

Bacterial and fungal community structures were clearly shaped by long-term tillage. There were a greater number of bacterial and fungal phyla/divisions in this study that were relatively more abundant in no till. Relatively dominant taxonomic groups were significantly higher in the tilled soils. For bacteria, Proteobacteria and Bacteroidetes were relatively more abundant with tillage, which was also found in two other studies (Degrune et al., 2017; Smith et al., 2016). However this is not always the case and these phyla can be more abundant in no till vs. tilled soils (Dong et al., 2017). Ascomycota, which was the predominant fungal division in soils from this study, was relatively more abundant in tilled soil. Dominance of Ascomycota in agricultural soil using DNA sequencing has often been measured, although others did not detect any significant differences between tillage regimes (Degrune et al., 2016; Dong et al., 2017; Schmidt et al., 2019; Sommermann et al., 2018). In this study it seemed that the soil conditions associated with tillage favored greater abundance of taxonomic groups that are often found to be the dominant taxa in soil ecology studies. Instead, smaller taxonomic groups that encompass fewer groups at lower taxonomic levels were
relatively more abundant in no till soils. Phyla of interest that are more abundant in no tilled soils are the Nitrospirae, which form a monophyletic clade consisting of nitrogen cycling bacteria that are involved in nitrite oxidation as part of the nitrification pathway (Stein and Klotz, 2016). Only Degrune et al. (2017) noted a significant difference in this phylum between tillage treatments and they also found greater abundance in no till soils. As the soil physicochemical environment is disturbed less and more unique niches are available when no tillage is used it is likely that use of no till can support a greater number of less dominant taxonomic groups who can establish themselves more easily. The influence of tillage and how it can provide conditions that favor one functional group or another was demonstrated in a study of soil fungal under contrasting tillage regimes. Symbiotrophs were significantly higher in soils under long-term no till, while saprotrophs were significantly higher in tilled soil (Schmidt et al., 2019). Saprotrophs are responsible for decomposition, organic matter turnover and nutrient cycling. Conditions that provide more varied substrates, which can occur with mechanical disruption of aggregates and physical breakdown of residue and SOM from tillage, will favor these groups. In this study it was found that Mortierella spp. are more abundant in no till and this group is an example of fungi able to degrade chitin and other recalcitrant soil organic materials (Li et al., 2018).

This study clearly shows that despite the time elapsed from prior tillage to soil sampling, long-term tillage management had the capacity to influence soil microbial
diversity, evenness and composition of taxa that are promoted by conditions shaped by each tillage practice.

4.5.2 Bacterial and Fungal Communities are Impacted by Crop Rotation

The use of long-term crop rotational diversity resulted in bacterial and fungal richness and phylogenetic diversity that differed between rotations. For bacteria there was no clear trend of changes in alpha diversity with increasing crop rotational diversity (Appendix 5.6). Fungal diversity was highest in the AAAA and CCAA plots, but relatively even within other corn rotations (Appendix 5.10). Statistical analysis was not able to detect significant differences in alpha diversity metrics, however both bacterial and fungal community composition were shaped by long-term crop rotational history.

It has been suggested that there is increased microbial diversity in soils when more diverse and higher quality inputs are provided (Thiele-Bruhn et al., 2012). There is evidence that when more diverse inputs are provided to soils that soil microbial communities are more diverse. The use of manure, compared to inorganic fertilizer, resulted in higher bacterial and fungal richness, but lower bacterial evenness in a wheat and grass cropping system (Hartmann et al., 2015). The use of cover crops, which provide plant and residue inputs in addition to that of the main crop, has been shown to increase fungal but not bacterial diversity in long-term tomato-cotton rotations (Schmidt et al., 2019, 2018). In this study we sampled rotations with corn in the second-year corn
phase, with the exception of AAAA, which was under alfalfa. The absence of any trends in bacterial alpha diversity could be due to the fact that of the crops in rotation, corn supplies the greatest amount of biomass. Any subtle differences in diversity of bulk soil from long-term cropping history may have been confounded by the effect of (comparatively) short-term inputs of 2 continuous years of corn. For fungi, there was an apparent crop effect on diversity metrics. It was evident that fungal diversity was much greater when soil was sampled under alfalfa, compared to the corn rotations. Interestingly, the fungal diversity in CCAA was lower than in AAAA but higher than in the other corn rotations. This indicates that the alfalfa was able to influence fungal diversity for at least two years, whereas the rotations with two years of soybean did not differ from the other corn rotations. In the study by Schmidt et al. (2018) it was also found that inclusion of cover crops in no till increased the total number of bacteria in the top 5 cm, but for the 5-15 cm depth the same non-significant trend was apparent. In this study the 0-10 cm depth was sampled, which is a common depth for soil microbial studies. It is interesting to consider that perhaps inclusion of increased diversity or certain crops can promote increased bacterial or fungal diversity, but in a depth-dependent manner, particularly or exclusively for no till systems. In the no till soils of this study the residues are left on the surface, and there is less incorporation and mechanical disruption impacting their breakdown. Tillage provides a larger surface area for microbial decomposition of residue and brings material deeper into the soil layer, as
well as impacting soil aggregation, providing more varied niches for microbial groups to inhabit and differentiate. Different sized aggregate fractions have been associated with increased abundance of particular microbial groups when more diverse crop rotations are used (Tiemann et al., 2015). Increased crop diversity was shown to increase the number of total bacterial PLFAs, gram positive bacteria and actinomycetes in micro- and macroaggregates, while non-arbuscular mycorrhizal fungi were greater in the mega-aggregates (Tiemann et al., 2015). The impact of time on residue decomposition and influence on soil microbial diversity was noted in the meta-analysis by Venter, Jacobs, and Hawkins (2016), who found that the magnitude of increased microbial richness was greater in studies of longer duration, although longer term studies had reduced microbial diversity. Over the short term it has been found that bacterial and fungal alpha diversity are significantly influenced by crop growing stage during the growing season (Degrune et al., 2017). Another study also showed that residue microbial communities were found to be gradually replaced by communities likely originating from soil over the course of a single growing season (Kerdraon et al., 2019). It is evident from these studies that soil microbial community diversity can change in response to a short-term change in input, such as from the crop itself during the growing season or after, during residue decomposition, but these effects are perhaps more transient. This study measured long-term changes from 35 years of rotational diversity, but there is also the more short-term effect of two years of corn, which may have made
any differences in microbial diversity difficult to detect at the time that was sampled. Sampling in the spring before initiation of the next crop may have provided another interesting time point to study the long-term impacts of crop rotation with less of an influence of the current crop. Future sampling during the first phase of corn, as well as during other points in the rotations when other crops are present, would provide additional information and understanding regarding both the short- and long-term dynamics of soil microbial diversity in long-term experiments with crop rotational diversity.

It is evident however that long-term crop diversification resulted in changes in the composition of bacterial and fungal communities, particularly for fungi (Figure 4.1, Figure 4.2). For both microbial groups examined there was a clear impact of crop on community structure, as plots from AAAA were different from the corn rotations. For fungi, it was also evident that crop had a greater influence in shaping community structure under no till management. Tillage would cause more frequent homogenization of the soil, while no tillage could allow more niches within aggregates to be established. The results from this study and others have shown that when crop diversity is increased, there are changes to microbial community composition and the abundance of particular groups is shifted. In a study comparing soils managed with corn and only occasional legumes for 20 years, and more diverse cropping that included corn, soybean, winter wheat, red clover, kidney beans and spelt, it was found that there were
significantly different bacterial community compositions between the two (Berthrong et al., 2013).

Within the corn rotations it was evident that as continuous corn was diversified with soybean, and corn-soybean further diversified from inclusion of winter wheat and red clover, that soil microbial communities were shaped differently. Similar differences in diversity of microbial groups, as monoculture corn is diversified with increasingly complex rotations, was shown for actinomycetes and Gram positive bacteria (Tiemann et al., 2015). The study by Tiemann et al. (2015) also ensured sampling was performed while rotations were in the corn phase. Another study examining differences in bacterial community structure between monoculture soybean, corn-soybean and wheat-soybean found that monoculture soybean bacterial communities clustered separately from the soils from soybean with either corn or wheat in rotation, which did not differ from each other (Song et al., 2018). The authors ensured sampling was during the soybean phase and so differences observed were also from the cropping history, not the current crop in rotation. The importance of sampling during the same crop when examining long-term changes from crop diversification is important as there are clear influences of crop on community composition. This was shown in a study examining bacterial community composition between monoculture corn, monoculture soybean, and corn-soybean, where community structure differed between the three but was only significant for corn compared to soybean only (Ashworth et al., 2017). Seasonal effects also shape
community composition, as was shown in a study where the bacterial community composition in bulk soil was found to change during the growing season of a single crop (Sugiyama et al., 2014). This highlights the importance of coordinated sampling while the same crop is in rotation to ensure comparisons of long-term shifts are not confounded by short term crop influences.

In this study bacterial and fungal taxonomic groups were identified that were relatively more abundant and a marker of each long-term crop history, under both tillage regimes. Within each management practice it is evident that there are particular microbial groups that benefit from the soil conditions created and the niches provided. It is worth investigating whether particular functional groups are evenly represented in the simple and more diverse rotations, and whether the community composition of soils provides sufficient diversity to ensure agroecosystems have resilience and resistance to degradation and climate change.

4.6 Conclusion

The legacy of 35 years of long-term no-till and crop rotational diversity altered bacterial and fungal diversity and shifted the composition of microbial communities. Short-term crop effects were also apparent when microbial community diversity and community composition under the corn rotations were compared to those from continuous alfalfa. Tillage also played a significant role in altering both bacterial and
fungal diversity and community composition, having a larger impact on fungi. Crop rotational diversity had a larger impact when soils were under no till management. For both bacterial and fungal groups, when soils were under long-term no till there were greater numbers of taxonomic groups relatively abundant in each of the long-term crop rotations. However, each long-term tillage and crop rotation treatment combination had certain bacterial and fungal groups in higher abundance for that system. This highlights that although microbial groups can be positively or negatively impacted by tillage or the level of diversity of certain crop rotations, particular groups are capable of utilizing niches created from agricultural management practices.
5 General Discussion and Conclusion

Understanding biogeochemical cycling in agricultural soils offers stakeholders with necessary information to guide selection of BMPs for balancing and promoting the various facets of soil health. Previously, the contribution of functional groups to biogeochemical processes such as the N cycle and assessment of microbial diversity, and linking these to agricultural management practice, were challenging to investigate due to the inability to culture most soil microorganisms. In recent years our ability to answer questions regarding the contribution of certain functional groups to N cycling under certain conditions can be achieved through targeting genes from an ever-expanding set of pathways known to be involved. Investigations examining the full soil microbial diversity are also much more accessible with HTS, providing insight into the effects of crop and tillage practices on diversity and community structure.

As efforts to reduce GHG emissions are increasingly being sought as part of agricultural BMPs it is important to understand how the microbial drivers of N cycling are impacted by our manipulation of the soil environment. We investigated this by examining multiple N cycling bacterial communities and their short-term response to urea fertilizer with urea hydrolysis and nitrification inhibitors. We were specifically interested in the groups whose enzymatic activity would be inhibited by the compounds present in the urea with urease and ammonia monooxygenase inhibitors, as well as
groups capable of production and consumption of N$_2$O. Longer term impacts of management on N cycling microbial communities was also investigated through contrasting no till corn rotations with different cropping histories, representing typical rotations found in Ontario (CCSS) as well as those considered part of agricultural BMPs (CCSWrc).

In chapter 2 a panel of N cycling bacterial groups (ureC, nrfA, amoA, nirS, nirK, nosZ1, nosZ2) were measured in response to urea with urease and ammonia monooxygenase inhibitors, and contrasted with urea alone. The urease and nitrification inhibitors in SuperU were successful in delaying N transformations, as well as decreasing N$_2$O emissions from soil microcosms. We found that community abundance of N cycling bacterial groups was higher in the SuperU treated soils, indicating that they responded to the shift in inorganic N dynamics. Through the use of both DNA and RNA targets we were also able to capture activation of gene transcription for the groups involved in urea and downstream N metabolism. Together these findings highlight that the use of urea-based fertilizers with compounds intended to temporarily inhibit biotic N transformations do not have a detrimental impact on soil microbial abundance. They can successfully decrease GHG emissions while still allowing for the activity of the N cycling groups to proceed, although with a shift in metabolism and production of downstream compounds. This allows for use of such products to shift the timing of N
fertilization metabolism to more directly benefit the plant, in order to increase plant N uptake and minimize N loss and environmental harm.

The focus of chapter 3 was to understand how long-term agricultural management shaped bacterial N cycling groups (amoA, nirS, nirK, nosZ1, nosZ2) and altered their response to N fertilization in field conditions. The aim was to link qPCR enumeration of both bacterial N cycling gene and transcripts to daily N₂O emissions. The soils under investigation were under no till management and we contrasted corn-soybean rotations with more diverse rotations including winter wheat underseeded with red clover cover. All measurements were taken while under second year corn, in order to compare the legacy of rotation on soil biogeochemical cycling capacity. The use of increased crop rotational diversity is a BMP promoted with the use of no till in particular as it offers multiple benefit to soil physical and chemical parameters. In our study we found that corn from the diverse rotational history had increased N₂O emissions and increased N cycling gene abundance. This highlights that in order to fully benefit from BMPs we need to carefully select and balance the various ways agricultural management can impact the soil environment. As we learn more about the complexity of the soil environment and its processes, we can potentially harness this information to more carefully manage soils by decreasing N fertilizer application in soils that have great N cycling capacity due to crop history and potential N credit.
The final chapter investigated the legacy of agricultural management practices on bulk soil bacterial and fungal communities, with a focus on total community diversity and community composition using a HTS approach. Bacteria and fungi are major drivers of C cycling, residue decomposition, and organic matter formation and practices aimed at improving these soil health parameters will impact their structure and potential functioning. We have an incomplete picture of how agricultural BMPs such as tillage and the use of crop rotations have shaped microbial communities over long term studies. We found that 35 years of tillage and increasing rotational diversity in corn production systems had a clear impact on the diversity and community composition of groups. In particular it was evident that tillage had a negative impact on diversity and was associated with less groups that were found to be more abundant in those systems. No tillage and the use of diverse crop rotations allowed for more groups to form particular niches, resulting in more heterogenous communities potentially.

Together this thesis expands our understanding how soil microbial communities can be shaped both in the short- and long-term from agricultural management practices. Current BMPs, including the use of N fertilizers with inhibitors, diversified crop rotations, with the use of no tillage, have measurable benefits to soil health and the microbial data from this study supports this. Microbial biogeochemical cycling and diversity are promoted by diverse crop rotations, particularly when no till is used, and this data can be used to highlight how the microbial community contributes to the beneficial
ecosystem services provided by BMPs. Understanding soil microbial functional diversity and assessing the impact agricultural management practices have on potential shifts or losses in the pathways that are carried out is an exciting next step for future research. This thesis has clearly shown that functional groups involved in N cycling are impacted by crop management, and total microbial diversity was shaped by crop rotational diversity and tillage. We now know there are differences in “who” is there, and further work could explore more specifically if certain groups or putative plant pathogens also differ in abundance. Additionally, the differences in community structure measured in this research leads into further questions regarding functions carried out, such as “what are they all doing?".
REFERENCES


Chen, Z., Wang, C., Gschwendtner, S., Willibald, G., Unteregelsbacher, S., Lu, H.,


Gaudin, A.C., Tolhurst, T.N., Ker, A.P., Janovicek, K., Tortora, C., Martin, R.C., Deen,


Harter, J., Guzman-Bustamante, I., Kuehfuss, S., Ruser, R., Well, R., Spott, O., Kappler, A., Behrens, S., 2016. Gas entrapment and microbial N2O reduction reduce N2O emissions from a biochar-amended sandy clay loam soil. Scientific Reports 6, 39574. doi:10.1038/srep39574


managed calcareous Fluvo-aquic soil. Scientific Reports 4, 1–9. doi:10.1038/srep03950


Philosophical Transactions of the Royal Society B: Biological Sciences 363, 685–701. doi:10.1098/rstb.2007.2178


doi:10.1002/ldr.2965

Li, W., Xiao, Q., Hu, C., Liu, B., Sun, R., 2019. A comparison of the efficiency of 
different urease inhibitors and their effects on soil prokaryotic community in a short-
term incubation experiment. Geoderma 354, 113877. 

Lienhard, P., Terrat, S., Prévost-Bouré, N.C., Nowak, V., Régnier, T., Sayphoummie, S., 
Pyrosequencing evidences the impact of cropping on soil bacterial and fungal 
diversity in Laos tropical grassland. Agronomy for Sustainable Development 34, 

nitrous oxide emission, crop yield and nitrogen uptake in a wheat-maize cropping 

Liu, J., You, L., Amini, M., Obersteiner, M., Herrero, M., Zehnder, A.J.B., Yang, H., 
2010. A high-resolution assessment on global nitrogen flows in cropland. 
Proceedings of the National Academy of Sciences 107, 8035–8040. 
doi:10.1073/pnas.0913658107

a primary driver of nitrous oxide production in laboratory microcosms from different 

Volatilization Loss and Corn Nitrogen Nutrition and Productivity with Efficiency 
Enhanced UAN and Urea under No-tillage. Scientific Reports 9, 1–12. 
doi:10.1038/s41598-019-42912-5

Lundy, M.E., Pittelkow, C.M., Linquist, B.A., Liang, X., van Groenigen, K.J., Lee, J., Six, 
doi:10.1016/j.fcr.2015.07.023

Responses of soil organic carbon, aggregate stability, carbon and nitrogen fractions 
doi:10.1071/SR18068

141


Tiemann, L.K., Grandy, A.S., Atkinson, E.E., Marin-Spiotta, E., Mcdaniel, M.D., 2015. Crop rotational diversity enhances belowground communities and functions in an...


148


Molecular Biology Reviews: MMBR 61, 533–616.
## APPENDICES

Appendix 5.1 Quantitative PCR Primers, Conditions and Efficiency. (*combined annealing/elongation step)

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer Sequence</th>
<th>Denaturation</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA gene</td>
<td>338f</td>
<td>ACTCCTACGGGAGGCCAGCAG</td>
<td>98°C – 5s</td>
</tr>
<tr>
<td></td>
<td>518r</td>
<td>ATTACCGCGGTGCTGG</td>
<td>55°C – 5s*</td>
</tr>
<tr>
<td>amoA</td>
<td>1F</td>
<td>GGGGTTTCTACTGGTGGT</td>
<td>98°C – 10s</td>
</tr>
<tr>
<td></td>
<td>2R</td>
<td>CCCCTCKGSAAGCCCTTCTTC</td>
<td>55°C – 10s</td>
</tr>
<tr>
<td>nirS</td>
<td>Cd3af</td>
<td>GTSAACGTAAGARACGG</td>
<td>98°C – 10s</td>
</tr>
<tr>
<td></td>
<td>R3Cd</td>
<td>GASTTCGRTGSGTCTTGA</td>
<td>57°C – 10s</td>
</tr>
<tr>
<td>nirK</td>
<td>F1aCu</td>
<td>ATCATGGTSGCCCGCG</td>
<td>98°C – 10s</td>
</tr>
<tr>
<td></td>
<td>R3Cu</td>
<td>GCCTGATCAGRTTGTGGT</td>
<td>56°C – 10s</td>
</tr>
<tr>
<td>nosZ1</td>
<td>2F</td>
<td>CGCRACGGCAASAGGSGT</td>
<td>95°C – 10s</td>
</tr>
<tr>
<td></td>
<td>2R</td>
<td>CAKRTGCAKSCRTGGCAGAA</td>
<td>60°C – 30s</td>
</tr>
<tr>
<td>nosZ2</td>
<td>nosZ-II-F</td>
<td>CTNGGNNCCNYTKCGAYAC</td>
<td>95°C – 30s</td>
</tr>
<tr>
<td></td>
<td>nosZ-II-R</td>
<td>GCNGARCARAANTCBGTRC</td>
<td>58°C – 60s*</td>
</tr>
</tbody>
</table>
Appendix 5.2 PCA of bacterial sequence variants in four replicate plots of long-term no till CCSS and CCSWrc.
Appendix 5.3 Spearman rank correlation of total and nitrogen cycling gene abundance with mean daily $\text{N}_2\text{O}$ emissions, inorganic nitrogen and water filled pore space. The magnitude of each correlation is shown by the size of circle, with positive correlations in blue and negative in red. Significant correlations are indicated by a grey box ($p<0.05$).
Appendix 5.4 Spearman rank correlation of total and nitrogen cycling transcript abundance, ratio of nir to nosZ, mean daily N\(_2\)O emissions, inorganic nitrogen and water filled pore space. The magnitude of each correlation is shown by the size of circle, with positive correlations in blue and negative in red. Significant correlations are indicated by a grey box (p<0.05).

Appendix 5.5 Bacterial richness and diversity indices in till and no till (corn rotations only). Data rarefied to 29,400 sequences/sample (n=20)

<table>
<thead>
<tr>
<th></th>
<th>Observed ASVs Mean±SD</th>
<th>Faith's Phylogenetic Diversity Mean±SD</th>
<th>Pielou's Evenness Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Till</td>
<td>1432±131</td>
<td>86.5±5.2</td>
<td>0.94±0.006</td>
</tr>
<tr>
<td>No Till</td>
<td>1452±100</td>
<td>88.4±4.4</td>
<td>0.94±0.003</td>
</tr>
</tbody>
</table>
Appendix 5.6 Bacterial richness and diversity indices in 6 long-term crop rotations. Data rarefied to 29,400 sequences/sample (n=4 for AAAA and n=8 for rotations with corn)

<table>
<thead>
<tr>
<th></th>
<th>AAAA</th>
<th>CCAA</th>
<th>CCCC</th>
<th>CCSS</th>
<th>CCSW</th>
<th>CCSWrc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed ASVs</td>
<td>1463±</td>
<td>1439±</td>
<td>1436±</td>
<td>1446±</td>
<td>1471±</td>
<td>1420±</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>37</td>
<td>117</td>
<td>118</td>
<td>158</td>
<td>82</td>
<td>117</td>
</tr>
<tr>
<td>Faith’s Phylogenetic Diversity</td>
<td>86.6±</td>
<td>85.4±</td>
<td>88.6±</td>
<td>87.4±</td>
<td>89.4±</td>
<td>86.4±</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>2.4</td>
<td>5.2</td>
<td>5.3</td>
<td>5.4</td>
<td>3.1</td>
<td>5.0</td>
</tr>
<tr>
<td>Pielou’s Evenness</td>
<td>0.94±</td>
<td>0.94±</td>
<td>0.94±</td>
<td>0.94±</td>
<td>0.93±</td>
<td>0.94±</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>0.002</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.01</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Appendix 5.7 Bacterial richness and diversity indices in 5 long-term corn rotations under tillage. Data rarefied to 29,400 sequences/sample (n=4)

<table>
<thead>
<tr>
<th></th>
<th>CCAA</th>
<th>CCCC</th>
<th>CCSS</th>
<th>CCSW</th>
<th>CCSWrc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Observed ASVs</strong></td>
<td>1420±</td>
<td>1404±</td>
<td>1465±</td>
<td>1455±</td>
<td>1416±</td>
</tr>
<tr>
<td><strong>Mean±SD</strong></td>
<td>123</td>
<td>168</td>
<td>183</td>
<td>102</td>
<td>136</td>
</tr>
<tr>
<td><strong>Faith’s Phylogenetic Diversity</strong></td>
<td>84.4±</td>
<td>86.6±</td>
<td>87.1±</td>
<td>88.6±</td>
<td>86±</td>
</tr>
<tr>
<td><strong>Mean±SD</strong></td>
<td>4.8</td>
<td>6.7</td>
<td>5.7</td>
<td>4.0</td>
<td>6.5</td>
</tr>
<tr>
<td><strong>Pielou’s Evenness</strong></td>
<td>0.94±</td>
<td>0.94±</td>
<td>0.94±</td>
<td>0.93±</td>
<td>0.94±</td>
</tr>
<tr>
<td><strong>Mean±SD</strong></td>
<td>0.004</td>
<td>0.003</td>
<td>0.001</td>
<td>0.01</td>
<td>0.004</td>
</tr>
</tbody>
</table>
### Appendix 5.8 Bacterial richness and diversity indices in 5 long-term crop rotations under no till. Data rarefied to 29,400 sequences/sample (n=4)

<table>
<thead>
<tr>
<th></th>
<th>CCAA</th>
<th>CCC</th>
<th>CCSS</th>
<th>CCSW</th>
<th>CCSWrc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed ASVs Mean±SD</td>
<td>1457±126</td>
<td>1468±41</td>
<td>1426±154</td>
<td>1487±69</td>
<td>1423±116</td>
</tr>
<tr>
<td>Faith’s Phylogenetic Diversity Mean±SD</td>
<td>86.4±6.1</td>
<td>90.6±3.3</td>
<td>87.7±6.1</td>
<td>90.3±2.1</td>
<td>86.8±3.9</td>
</tr>
<tr>
<td>Pielou’s Evenness Mean±SD</td>
<td>0.94±0.003</td>
<td>0.94±0.003</td>
<td>0.94±0.004</td>
<td>0.94±0.001</td>
<td>0.94±0.005</td>
</tr>
</tbody>
</table>

### Appendix 5.9 Fungal richness and diversity indices in till and no till (corn rotations only). Data rarefied to 9,000 sequences/sample (n=20)

<table>
<thead>
<tr>
<th></th>
<th>Observed ASVs Mean±SD</th>
<th>Faith’s Phylogenetic Diversity Mean±SD</th>
<th>Pielou’s Evenness Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Till</td>
<td>261±25</td>
<td>58.4±5.3</td>
<td>0.78±0.048</td>
</tr>
<tr>
<td>No Till</td>
<td>267±33</td>
<td>61.7±6.4</td>
<td>0.75±0.059</td>
</tr>
</tbody>
</table>
### Appendix 5.10 Fungal richness and diversity indices in 6 long-term crop rotations. Data rarefied to 9,000 sequences/sample (n=4 for AAAA and n=8 for rotations with corn)

<table>
<thead>
<tr>
<th></th>
<th>AAAA n=4</th>
<th>CCAA n=8</th>
<th>CCCC n=8</th>
<th>CCSS n=8</th>
<th>CCSW n=8</th>
<th>CCSWrc n=8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Observed ASVs</strong></td>
<td>320±</td>
<td>280±</td>
<td>265±</td>
<td>259±</td>
<td>257±</td>
<td>260±</td>
</tr>
<tr>
<td><strong>Mean±SD</strong></td>
<td>30</td>
<td>20</td>
<td>32</td>
<td>23</td>
<td>25</td>
<td>42</td>
</tr>
<tr>
<td><strong>Faith’s Phylogenetic Diversity</strong></td>
<td>74.3±</td>
<td>63.1±</td>
<td>59.6±</td>
<td>60.1±</td>
<td>59.7±</td>
<td>58.0±</td>
</tr>
<tr>
<td><strong>Mean±SD</strong></td>
<td>7.3</td>
<td>4.8</td>
<td>6.8</td>
<td>5.1</td>
<td>5.0</td>
<td>8.2</td>
</tr>
<tr>
<td><strong>Pielou’s Evenness</strong></td>
<td>0.75±</td>
<td>0.76±</td>
<td>0.75±</td>
<td>0.76±</td>
<td>0.78±</td>
<td>0.77±</td>
</tr>
<tr>
<td><strong>Mean±SD</strong></td>
<td>0.021</td>
<td>0.029</td>
<td>0.076</td>
<td>0.066</td>
<td>0.046</td>
<td>0.058</td>
</tr>
</tbody>
</table>
Appendix 5.11 Fungal richness and diversity indices in 5 long-term corn rotations under tillage. Data rarefied to 9,000 sequences/sample (n=4)

<table>
<thead>
<tr>
<th></th>
<th>CCAA</th>
<th>CCCC</th>
<th>CCSS</th>
<th>CCSW</th>
<th>CCSWrc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Observed ASVs</strong></td>
<td>276±</td>
<td>270±</td>
<td>262±</td>
<td>248±</td>
<td>249±</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>24</td>
<td>28</td>
<td>12</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td><strong>Faith’s Phylogenetic Diversity</strong></td>
<td>60.2±</td>
<td>61.6±</td>
<td>59.8±</td>
<td>56.9±</td>
<td>53.5±</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>4.9</td>
<td>5.4</td>
<td>4.1</td>
<td>4.9</td>
<td>5.5</td>
</tr>
<tr>
<td><strong>Pielou’s Evenness</strong></td>
<td>0.781±</td>
<td>0.763±</td>
<td>0.807±</td>
<td>0.798±</td>
<td>0.750±</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>0.023</td>
<td>0.058</td>
<td>0.022</td>
<td>0.037</td>
<td>0.075</td>
</tr>
</tbody>
</table>
Appendix 5.12 Fungal richness and diversity indices in 5 long-term corn rotations under no till. Data rarefied to 9,000 sequences/sample (n=4)

<table>
<thead>
<tr>
<th></th>
<th>CCAA</th>
<th>CCCC</th>
<th>CCSS</th>
<th>CCSW</th>
<th>CCSWrc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Observed ASVs Mean±SD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>283±</td>
<td>260±</td>
<td>257±</td>
<td>266±</td>
<td>271±</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>38</td>
<td>33</td>
<td>20</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td><strong>Faith’s Phylogenetic Diversity Mean±SD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66.0±</td>
<td>57.5±</td>
<td>60.3±</td>
<td>62.5±</td>
<td>62.4±</td>
<td></td>
</tr>
<tr>
<td>2.9</td>
<td>8.3</td>
<td>6.6</td>
<td>3.6</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td><strong>Pielou’s Evenness Mean±SD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.738±</td>
<td>0.732±</td>
<td>0.716±</td>
<td>0.752±</td>
<td>0.797±</td>
<td></td>
</tr>
<tr>
<td>0.012</td>
<td>0.97</td>
<td>0.065</td>
<td>0.047</td>
<td>0.028</td>
<td></td>
</tr>
</tbody>
</table>
Appendix 5.13 Pairwise comparisons of bacterial community composition between long-term crop rotations. PERMANOVA of the Bray Curtis dissimilarity was performed with 10,000 permutations (n=8)

<table>
<thead>
<tr>
<th>Crop Pairwise Comparisons</th>
<th>pseudo-F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCAA CCCC</td>
<td>1.83</td>
<td><strong>0.012</strong></td>
</tr>
<tr>
<td>CCSS</td>
<td>1.84</td>
<td><strong>0.012</strong></td>
</tr>
<tr>
<td>CCSW</td>
<td>1.36</td>
<td>0.061</td>
</tr>
<tr>
<td>CCSWrc</td>
<td>1.07</td>
<td>0.345</td>
</tr>
<tr>
<td>CCCC CCSS</td>
<td>1.77</td>
<td><strong>0.019</strong></td>
</tr>
<tr>
<td>CCSW</td>
<td>1.19</td>
<td>0.152</td>
</tr>
<tr>
<td>CCSWrc</td>
<td>1.37</td>
<td>0.077</td>
</tr>
<tr>
<td>CCSS CCSW</td>
<td>1.59</td>
<td><strong>0.019</strong></td>
</tr>
<tr>
<td>CCSWrc</td>
<td>1.40</td>
<td>0.077</td>
</tr>
<tr>
<td>CCSW CCSWrc</td>
<td>1.04</td>
<td>0.345</td>
</tr>
</tbody>
</table>
Appendix 5.14 Pairwise comparisons of fungal community composition between long-term crop rotations. PERMANOVA of the Bray Curtis dissimilarity was performed with 10,000 permutations (n=8)

<table>
<thead>
<tr>
<th>Crop Pairwise Comparisons</th>
<th>pseudo-F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCAA CCCC</td>
<td>4.25</td>
<td>0.001</td>
</tr>
<tr>
<td>CCSS</td>
<td>3.03</td>
<td>0.001</td>
</tr>
<tr>
<td>CCSW</td>
<td>3.06</td>
<td>0.001</td>
</tr>
<tr>
<td>CCSWrc</td>
<td>2.07</td>
<td>0.014</td>
</tr>
<tr>
<td>CCCC CCSS</td>
<td>2.56</td>
<td>0.003</td>
</tr>
<tr>
<td>CCSW</td>
<td>2.02</td>
<td>0.014</td>
</tr>
<tr>
<td>CCSWrc</td>
<td>3.07</td>
<td>0.001</td>
</tr>
<tr>
<td>CCSS CCSW</td>
<td>1.69</td>
<td>0.016</td>
</tr>
<tr>
<td>CCSWrc</td>
<td>1.79</td>
<td>0.014</td>
</tr>
<tr>
<td>CCSW CCSWrc</td>
<td>1.27</td>
<td>0.122</td>
</tr>
</tbody>
</table>