Effects of nitrogen stabilizers and crop rotation on mineral nitrogen, N cycle genes and microbial community structure and abundance in podzolic soil in boreal climate

By

Irfan Mushtaq

A thesis submitted to the School of Graduate Studies

In partial fulfillment of the requirements for the degree of

Master of Science

Boreal Ecosystems and Agricultural Sciences

School of Science and the Environment

Grenfell Campus

Memorial University of Newfoundland and Labrador

August 2022

St. John’s, Newfoundland and Labrador

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Approved by:

Dean of the Graduate School

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Abstract

Nitrogen (N) is an essential macronutrient required to enhance crop growth and yield. Excessive N application can result in N losses through nitrate leaching and denitrification which can be greater than 50% of applied N. Nitrogen losses can be minimized by application of synthetic N stabilizers and crop rotation that can inhibit volatilization, nitrification and denitrification processes for a few weeks and affect the N mineralization. Field experiment was conducted to investigate the effect of N stabilizers and crop rotation on soil pH, soil mineral N, genes involved in nitrification and denitrification process and the microbial community structure. The experiment was conducted in a randomized complete block design (RCBD) with split plot arrangement and replicated four times with plot size 3 m × 4 m. Experimental treatments were crop rotations (silage corn-silage corn, silage corn-wheat, silage corn-faba bean) and five nitrogen stabilizers and fertilizer (control, urea, agrotain, entrench, and super-U). N stabilizers significantly reduced the gene abundance involved in nitrification (*amo*A AOB, *amo*A AOA, *nxr*), and denitrification (*nir*K and *nos*Z) compared to plots treated with urea. N stabilizers had no significant effect on soil NH_4^+ and NO_3^- as compared to urea. The results further revealed that N sources had no significant effects on fungal and bacterial community structure and abundance. However, crop rotation significantly affected both fungal and bacterial community composition and fungal Shannon diversity in boreal climate. Overall, cereal-legume rotation significantly affected the soil microbiome during nitrification and denitrification processes in boreal climate. Further, long term studies are required to determine the effects of N sources and crop rotation on microbiome diversity and abundance in boreal climate.

General summary

The use of nitrogenous fertilizers is increasing day by day due to their role in improving crop growth, yield and quality. Increased N fertilizer application result in more N losses. This may result in increased cost of production, adverse environmental effects due to water contamination and production of nitrous oxide (N_2O) a potent greenhouse gas with 298 times global warming potential. However, N stabilizers application could reduce N volatilization, leaching and denitrification losses, improve nitrogen use efficiency and enhance crop yield. In present study, N stabilizers increased soil NH_4^+ level, decreased soil NO_3^- and nitrogen gene abundance indicating a disruption of the N mineralization process. N stabilizers improve N availability while crop rotation significantly increased soil microbial communities and abundance. This study suggests that N stabilizers have the potential to increase soil NH_4^+ and decreased soil NO_3^- compared to urea application. However, Crop rotation also significantly affected bacterial community structure and abundances due to root exudation.

Acknowledgments

First and foremost, I want to thank Almighty Allah, who has continuously blessed humanity inexpressibly and given us this chance to discover our diverse talents and expertise in the practical field. My supervisor, Dr. Mumtaz A. Cheema, whose competence and knowledge boosted my understanding, given me with substantial professional and personal mentoring. He taught me a great deal about how to be smart in both scientific research and life in general, is someone I want to express my sincere thanks to. I am also appreciative of his moral support during several trying times and situations. He has taught me more than I could ever credit him with here as a mentor and instructor. He has exemplified for me what a good scientist ought to be. I want to express my gratitude to my co-supervisor Dr. Svetlana Yurgel as well as the members of the committee Dr. Lakshman Galagedara and Dr. Raymond Thomas for their assistance and direction throughout the project.

Without the funding assistance of the Research Office and School of Graduate Studies Grenfell Campus at Memorial University of Newfoundland, this work would not have been possible. Clean Tech project research support is much appreciated. For their assistance in the lab and field, I am also grateful to Dr. Muhammad Nadeem, Dr. Yeukai Katanda, Nathieli S., Tao Yuan, Jiaxu Wu, Bilal Javed, Muhammad Mashaallah Farhain, and Jayamini.

Last but not least, I want to express my gratitude to my parents, siblings, friends and other family members for their kindness, love, and unwavering support as I finished my degree program.

Irfan Mushtaq

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List of Abbreviations

% - Percentage
µl - Microliter
ºC - Degree centigrade
NH_4^+ - Ammonium
AOA - Ammonia Oxidizing Archaea
AOB - Ammonia Oxidizing Bacteria
Amo - Ammonia monooxygenase
cmol/kg - centimoles per kilogram
d.f - Degree of freedom
DCD - Dicyandiamide
DNA - Deoxyribonucleic acid
EC - Electrical Conductivity
G - Gram
H^+ - Hydrogen ion
h – Hour
ITS – Intergenic Transcribed Spacer
ha – Hectare
Kg - Kilogrm
Kg/ha – Kilogram per hectare
KCl – Potassium Chloride
L – Liter
mg/ha – Milligram per hectare
MRF – Mean Relative Frequency
mg/L – Milligram per litre

µl – Microliter

m^2 – Square meter

N – Nitrogen

NO_3^- - Nitrate

nxr – Nitrite oxidoreductase

nir – Nitrite reductase

NI – Nitrification inhibitor

NBPT - N-(n-butyl) thiophosphoric triamide

NO_2^- - Nitrite

NH^3 – Ammonia

NMDS – Non-metric multi-dimensional scaling

NUE – Nitrogen use efficiency

NL – Newfoundland and Labrador

nM – Nanomole

ng – Nanogram

ng/µl – Nanogram per microlitre

pH – Power of hydrogen ion

p – Probability or level of significance

rpm – Revolutions per minute

rRNA – Ribosomal ribonucleic acid

rDNA – Ribosomal deoxyribonucleic acid

SMN – Soil mineral nitrogen

s – Second

t – Ton

UI – Urease inhibitor

Chapter 1

1. General introduction

1.1 Introduction

Newfoundland and Labrador (NL) faces challenges of insufficient locally produced food and feed to support the Province population. The Provincial Government and the Newfoundland and Labrador Federation of Agriculture (NLFA) have jointly developed an Agriculture Sector Work Plan to increase food production from 10 to 20 % of NL by 2022 (NL 2018). NL's weather is highly influenced by cool ocean currents resulting in extremely low crop growth temperature which is not suitable to meet the required heating units for crop production in the Province (Resources 2004). Additionally, the land base is dominated by conifer species with shallow acidic stony soils that exhibit low fertility, further inhibiting agricultural development in the province (Resources 2018).

The soil physiochemical properties, quality and health can be improved to support superior crop growth and yield by adopting beneficial management practices (BMPs) such as crop residue/organic matter incorporation, manure, compost, charcoal or waste material (wood ash and wood sludge) application (Alkharabsheh et al. 2021; Azu et al. 2019; Imran et al. 2021). Manure and synthetic nitrogen (N) fertilizer application increase crop growth as well as yield indicated by Iqbal et al. (2020), However, N losses can be more than 50% of applied N as ammonia due to volatilization, nitrate (NO_3^-) leaching, immobilization, or via gaseous losses which eventually increase the global warming potential and contamination of natural water sources (Prasad 2013). To enhance global food security and nitrogen use efficiency (NUE), it is important to introduce innovative products or technologies (N stabilizers, slow release fertilizers), BMPs {(conservation tillage, no-till, crop rotation and cover crops/intercropping, 4R- (right time/right rate/right place/right source)} to reduce these N losses in different cropping systems (Ghosh et al. 2015; Reetz 2016). N fertilizer stabilizers such as Agrotan, eNtrench and SuperU contain either urease inhibitors (UIs), nitrification inhibitor (NIs) or both, and are known to reduce N losses by delaying urea hydrolysis and nitrification processes via suppressing the active soil microbial activities or metabolism (Martins et al. 2017; Meng et al. 2021; Zaman et al. 2008). Recent findings have demonstrated success with N stabilizers in significantly reducing N losses via inhibition of the nitrification and denitrification processes (Wu et al. 2017; Xi et al. 2017). UIs such as N-(n-butyl) thiophosphoric triamide (NBPT) can delay urea hydrolysis by 7 – 14 days resulting in a 35% reduction in ammonia losses (Zaman et al. 2008). NIs are chemical compounds [(nitrapyrin (2-chloro-6-(trichloromethyl) pyridine; NP, Dicyandiamide (DCD)] that slow down bacterial oxidation of ammonium ion (NH_4^+) by inhibiting the ammonia oxidizer activity, which result in slow conversion of NH_4^+ to nitrite (NO_2^-) and then to NO_3^- during the nitrification process (Wu et al. 2017).

Crop rotation has numerous benefits, including increased cycling of nutrients, soil health and as well as soil physical characteristics like bulk density, porosity and soil texture (Davis et al. 2012; Gaudin et al. 2015; Munkholm et al. 2013). Conversion of organic N to mineral N (N mineralization) can be affected by crop rotation, through changing soil temperature, moisture, and soil pH (Riyo 2018). Crop rotation is considered as a management strategy for decreasing fertilizer N input and reducing the danger of excess N leaching during wet conditions (Riyo 2018). Including legume crops in crop rotation can add a significant amount of N for subsequent crops (Joshi 2017). Hence, crop rotation could be used an innovative strategy not only to improve crop yield, soil quality, and health, as well as limit soil nitrogen losses (McKee 2021). Furthermore, net emission of greenhouse gasses (GHGs) from agricultural activities can potentially be decreased by crop rotation discussed by Xiao et al. (2022), and possibly increase C sequestration or soil organic carbon (SOC) (Ramesh et al. 2019). For example, Drury et al. (2008) have shown that including legume crop in crop rotation lowered nitrous oxide (N_2O) emission 5 fold compared to continuous silage corn cultivation. Another study explained that

SOC can be increased by including legumes in a diverse rotation with high residue cropping sequence such as silage corn (Ortega et al. 2002). Residue quantity, quality, and recalcitrance levels associated with crop rotations can affect microbial biomass, diversity, and frequency (White and Rice 2009).

The functional genes of coded enzymes are the functional markers of microbes which play significant role in metabolic activities. Crop rotation had been proven to have a significant effect on different gene abundance involved in N cycle (Linton 2020). Previous studies focused on either the effect of long-term crop rotation or N stabilizers on gene abundance involved in nitrification and denitrification (Fu et al. 2020; Maul et al. 2019; Munroe et al. 2020). However, it is still unclear about the effects of N stabilizers and short-term crop rotation on N mineralization, genes associated with nitrification and denitrification processes and microbial community structure in podzolic soils in boreal climate or ecosystem. We hypothesized that short term crop rotations and N stabilizers can inhibit the activity of microbes involved in nitrification and denitrification resulting in increased N uptake by plants in podzols in boreal climate. The specific objectives of this study were: 1) to investigate the effects of N sources and short-term crop rotation on soil pH, mineral nitrogen, and relative abundance of genes involved in nitrification and denitrification processes, 2) to determine the effects of N sources and crop rotation on soil microbial composition and abundance during nitrification and denitrification processes.

1.2. Review of literature

1.2.1. Functions of nitrogen in crop growth, development, and biomass yield

N was named “nitrogene” by a French scientist named J. C. Chaptal in 1790 and it is an element number 14 in the periodic table and thereafter, in 1872, G. K. Rutherford, a Scottish chemist discovered the importance of nitrogen for plant growth (Fageria et al. 2003). An essential macronutrient like N is required in large quantity to enhance plant growth, development, and biomass/yield (Kumar et al. 2021). It plays a vital role in plant photosynthesis, cellular respiration, metabolism (amino acids and proteins), and heredity (nucleotides and nucleic acids) (Leghari et al. 2016). It is a necessary component of life and is found in a variety of biomolecules, such as nucleic acids, proteins, and constituents of compounds like chlorophyll and alkaloids (Fageria and Baligar 2005). It enhances the leaf area index, photosynthetic rate, light interception, biomass yield, food quality as well as net assimilation rate of plants (Barraclough et al. 2010; Ullah et al. 2010). Protein, of which N is a crucial component, is involved in all major processes in plants, including sucrose synthesis and usage, a crucial source of energy for plant growth and cell maintenance (Zinati et al. 2001). For substantial outcomes, crops with high yield potential require higher N application (Kiani et al. 2016). Enhanced N fertilization increased wheat growth and yield (Ali et al. 2011). In silage silage corn, N application rate of 200 kg ha^{-1} increased silage corn yield and improves quality compared to an application rate of 100 kg N ha^{-1} (Safdarian et al. 2014). In faba bean, grain and dry matter yields were significantly enhanced with increasing N fertilization up to 150 kg ha^{-1} (Daur et al. 2008).

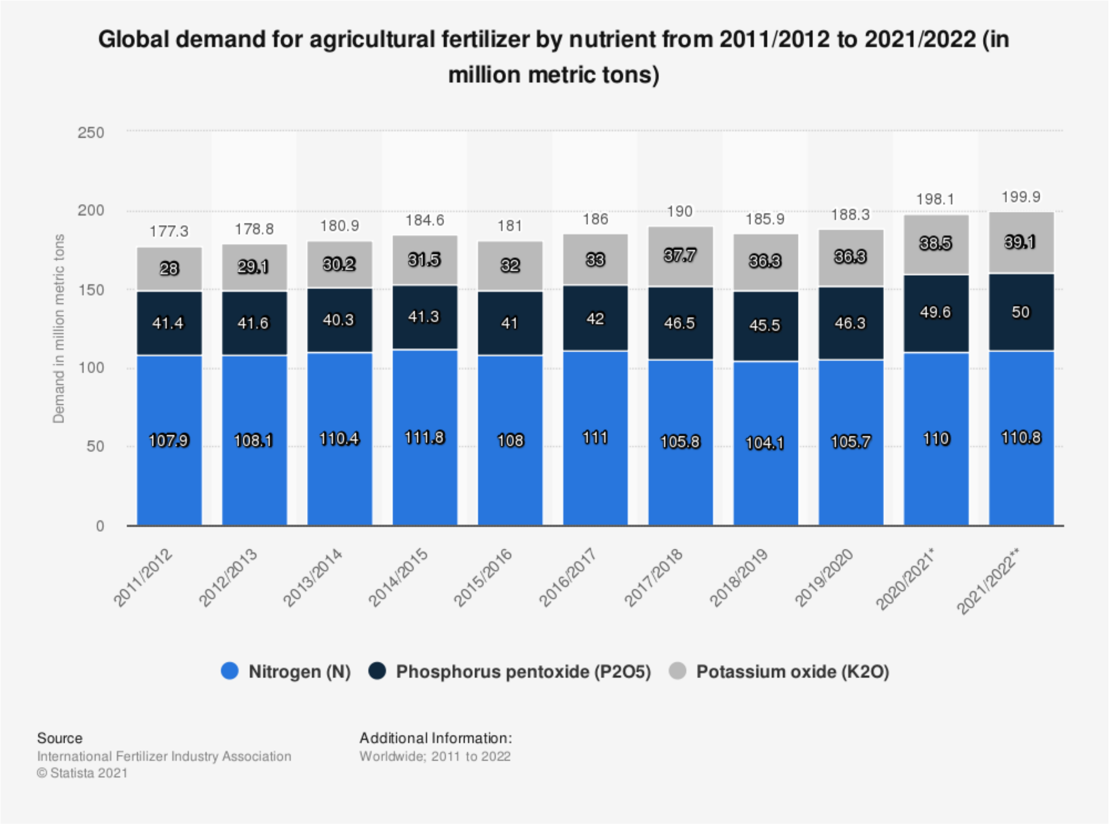
The older leaves are the first to show signs of N deficiency since N is a highly mobile nutrient in plants. Severe N deficiency results in stunted plant growth, yellowish leaves, reduced number of tillers in cereals, reduced number of pods in legumes, and reduced yield in both cereals and legumes (Fageria and Baligar 2005). In the early growth stages, leaves become yellowish-green and pale, in later growth stages they become more yellow according to the deficiency level (Fageria and Baligar 2005). In case of severe N deficit, the leaf area index and leaf area duration decrease, which lowers light interception, radiation use efficiency, and photosynthetic rate (Barbieri et al. 2000; Fageria and Baligar 2003; Sinclair and Horie 1989; Uhart and Andrade 1995) and low protein in grains (Fageria and Baligar 2005). Therefore, there is a need to review

N fertilizers production, consumption, management practices and technologies to reduce N losses and enhance NUE in different cropping systems.

1.2.2. Nitrogen fertilizers scenario across the globe

The world population is increasing continuously and expected to reach 9.9 billion in 2050 from current 7.9 billion (Kaneda et al. 2018). If the population growth rate continues with this pace, then food production needs to grow exponentially to meet the global food insecurity challenges. For example, FAO (2009) reported that by 2050, the demand for cereals for human food and animal feed is projected to increase to 3 billion tons from the current level of close to 2.1 billion tons. The total global food demand between 2010 and 2050 is expected to increase by 35% - 56% (van Dijk et al. 2021). To meet this target of food production from the limited and decreasing arable land resource (Malhi et al. 2001), N fertilizers is projected to exceed 186 million Mg yr^{-1} by 2050 (del Pilar Muschietti-Piana et al. 2018). Therefore, the use of innovative technologies/products, tools and BMPs that could enhance NUE, crop yield and reduce N losses are considered key factors to ensure adequate global food security (Erisman et al. 2008; Matson et al. 1997).

The success story of "Green Revolution" was based on higher N application to wheat cultivars and semi-dwarf rice that boosted grain yield and food security; however huge damage to the environment occurred during the process (Hungate et al. 2003; Matson et al. 1999; Tilman et al. 2001). Industrial and corporate agriculture requires abundant supply of inputs particularly synthetic fertilizers application. Additionally, monocropping system depends on heavy use of synthetic fertilizers and pesticides which result in ground water contamination and human health problems (Schlesinger 2009; Subbarao et al. 2006). For example, fast and uncontrolled nitrification due to excessive application of N fertilization results in the loss of approximately 70% N in different cropping systems (Peterjohn and Schlesinger 1990; Raun and Johnson 1999; Vitousek and Howarth 1991).


Global demand for agricultural fertilizer by nutrient from 2011/2012 to 2021/2022 (in million metric tons)

Source
International Fertilizer Industry Association
© Statista 2021

Additional Information:
Worldwide; 2011 to 2022

Figure 1.1: Global demand for agriculture fertilizer by NPK in million metric tons from 2012 to 2022 (Fernández 2022).

The worldwide annual N fertilizer application is about 150000 Tg and the estimated economic N loss is about US$ 81 billion per year (Galloway et al. 2008). Urea is the most often utilized type of N fertilizer in agricultural or crop production, representing more than half of global N use (Khan et al. 2013; Modolo et al. 2015). It is expected that urea fertilizer demand could increase exponentially due to high N (46%), low cost compared to other N sources, high water soluble, ease of handling, and storage safety and to meet the global food production (FAO 2019). To enhance NUE and reduce N losses, it is imperative to review biogeochemical processes.

1.2.3. Nitrogen cycling processes

The biogeochemical N cycle is the planetary-scale cycling of N among ecosystems which is made up of a series of redox processes that inter-convert the different nitrogenous chemicals found in nature (Schaechter 2009). These processes include N fixation, uptake, mineralization, immobilization nitrification, and denitrification. Nitrogen fixation is carried out by bacteria like *Azotobacter* and *Bradyrhizobium* that can fix atmospheric N in the soil and make it available for plant uptake (Gaudin et al. 2013; Isobe and Ohte 2014). Plants can uptake N in different forms including NH_4^+ and nitrate NO_3^- (Hachiya and Sakakibara 2017). Soil NH_4^+ is a less dominant form of mineral N with a low concentration compared to NO_3^- (Li et al. 2013). N immobilization result in conversion of inorganic N to organic form making it unavailable to plants (Li et al. 2021). However, N mineralization process involved the decomposition of organic N to NH_4^+ and NO_3^- and then make it available for plants uptake (Guntiñas et al. 2012). Enzymes including protease, urease, and transaminase directly correlated with microbial biomass N which derives a large portion of the mineralizable N (55%–89%) (Ajwa et al. 1999). Moreover, the mineralization rate can be influenced by other factors such as precipitation and total soil N, through changing soil microbial biomass (Li et al. 2019).

1.2.4. Nitrification and denitrification

Nitrification is a biologically mediated process in which $NH_4^+$is transformed to NO_3^- via NO_2^- by *Nitrosomonas* and *Nitrobacter* (Yao et al. 2016). Ammonia oxidizers convert NH_4^+ to NO_2^-, while NO_2^- oxidizing bacteria convert NO_2^- to NO_3^- (Stein 2015). According to a previous study by Farquharson (2016), the nitrification process is not fully effective, and a significant quantity of N is lost from the soil as N_2O gas. The nitrification process in the soil is an essential mechanism for N conversion in ecosystems, producing NO_3^- that impacts net primary production, whereas N_2O, a consequence of denitrification process, is a significant GHG (Li et al. 2020). Denitrification is largely an anaerobic process in which microorganisms use nitric oxide (NO) and N_2O as intermediates to convert NO_3^- and NO_2^- to dinitrogen (N_2) (Zhao et al. 2020). Denitrification is a vital process that regulates N loss from soil ecosystems (Kou et al.

2020).

1.2.4.1. Microbes and genes involved in nitrification and denitrification processes

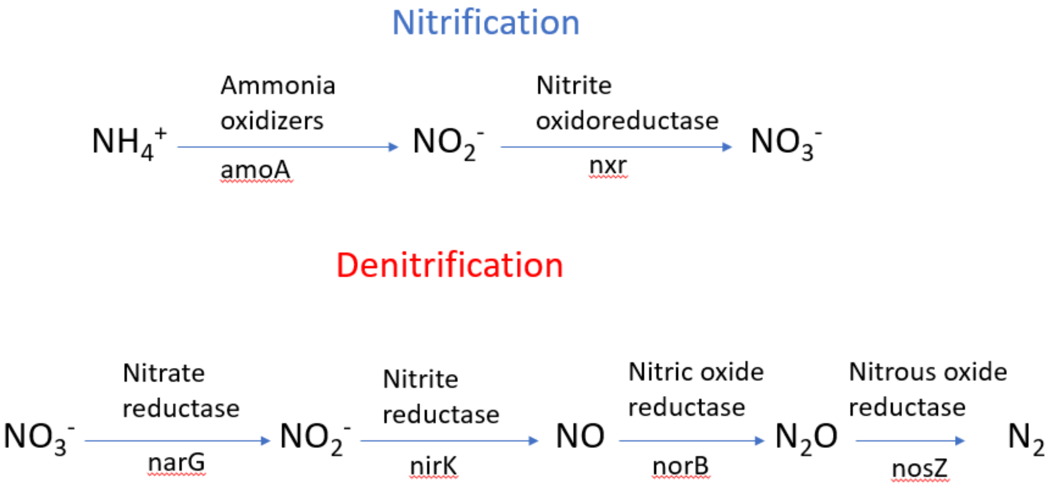


Figure 2.2: Figure explaining the role of microbes and genes in nitrification and denitrification processes in soil system. In nitrification process, NH_4^+ is converted to NO_3^- via NO_2^-, while in denitrification process, there are multiple steps where enzymes and genes play role in transforming NO_3^- to different forms of nitrogen gases.

Nitrification is a microbial-driven N transformation process that occurs in a variety of soil environments (Zhao et al. 2020). NO_2^- is an essential intermediary in several N transformation processes, including nitrification and denitrification (Van Cleemput and Samater 1995). Microbes including ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) are largely responsible for nitrification (Canfield et al. 2010). Nitrification is a two-stage process, with ammonia oxidation being the first and rate-limiting phase (Huang et al. 2020). Ammonia monooxygenase, a major enzyme encoded by ammonia monooxygenase subunit A, catalyzes ammonia oxidation (*amo*A) to NO_2^- via NH_2OH. Coexisting microbial communities such as AOA, AOB reported by Prosser et al. (2020), and nitrite-oxidizing bacteria (NOB/NXR), which oxidize NO_2^- to NO_3^- in soil ecosystems, are thought to be primarily responsible for these processes (Huang et al. 2020). *Nitrospira* is a genus of bacteria which can convert NH_3 to NO_3^- in a single organism called a comammox, which may be found in a variety of habitats (Huang et al. 2020).

The denitrification process is carried out by many microorganisms including *Thiobacillus denitrificans*, and *Lactobacillus* (Tazeen 2022). NO_3^- is reduced to NO_2^- by the enzyme nitrate reductase, NO_2^- to NO by nitrite reductase, NO to N_2O by nitric oxide reductase while nitrous oxide reductase is responsible for conversion of N_2O to N_2 gas during the denitrification process (Thies et al. 2019). *nar*G and *nap*A are the genes that code for nitrate reduction, whereas *nir*K and *nir*S are the genes that code for nitrite reductase, and *nos*Z gene that code for nitrous oxide reductase (Philippot et al. 2011). Apart from many biotic and abiotic variables that can affect denitrification in soil, these genes operate as functional indicators for evaluating denitrifier populations (Azevedo et al. 2015; Kou et al. 2020). In soils, nitrification and denitrification are critical processes and are major contributors of atmospheric N_2O emissions and may cause about 70% ozone-depleting N_2O emissions (Li et al. 2017).

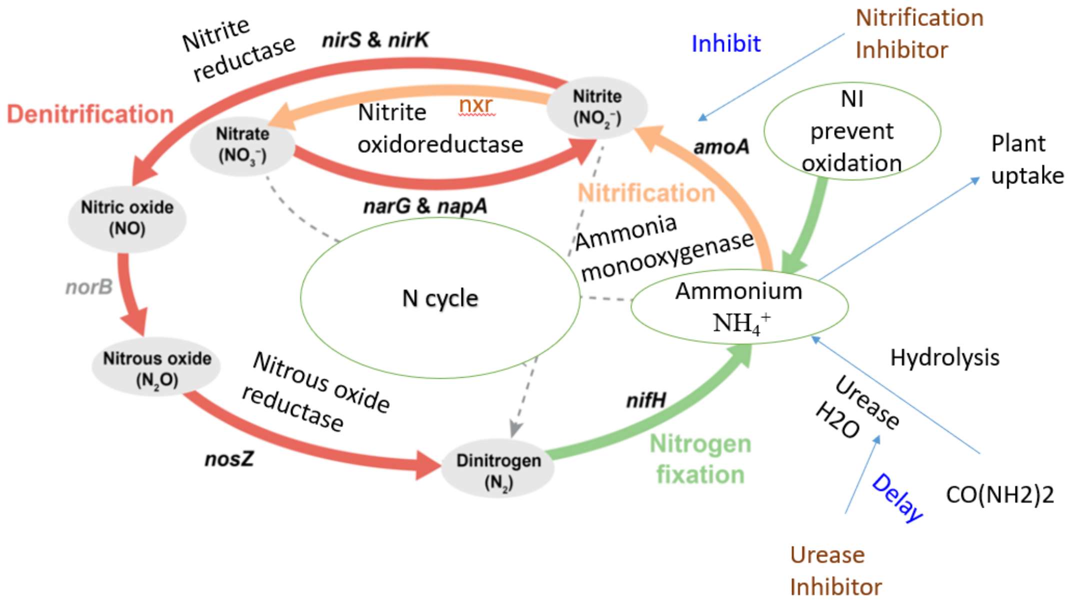


Figure 1.2: Role of genes in N cycle (Griffith 2016). However, author of this thesis has modified this flow chart/diagram by adding urease inhibitors and nitrification inhibitors and genes responsible for nitrification and denitrification in soil systems. For example, urease inhibitor delay hydrolyses process at step one, then nitrification inhibitors inhibit nitrification process due the presence of *amo*A AOB, *amo*A AOA, *nir*S, *nir*K, *nxr* and *nos*Z.

Microbes are responsible for the existence of all living forms on the planet (Davies 1999). Various microorganism communities exist in various environments across the globe (Akkermans et al. 1994). These communities govern all stages of biogeochemical cycles as producers, consumers, and decomposers (Bertrand et al. 2015). Krumins et al. (2009) reported that microbes play an important role in soil nutrient cycling, and hence are responsible for N buildup and losses in the ecosystems. Heterotrophic nitrifying microorganisms (derive energy from organic compounds), nitrifying archaea, denitrifying fungi, aerobic denitrifying bacteria, and anammox bacteria are some of the players involved in N cycling (Hayatsu et al. 2008). N fixation is the first step in the microbial N transformation process, which is followed by microbial ammonification, N absorption, nitrification, and lastly dissimilatory nitrate reduction (Canfield et al. 2005). Highly specialized bacteria with the nitrogenase enzyme break the triple bond in atmospheric N and mix it with hydrogen to produce NH_4^+ during the fixation process (Fowler et al. 2015).

Microbial enzymatic activities are often employed as soil quality signs, and they play a significant role in mineralization and other processes in the ecosystem (Dindar et al. 2015). Understanding the reaction of microbial enzymes under such settings is essential for forecasting ecosystem function in the future, especially in the context of anthropogenic environmental change (Alster et al. 2013). In the subject of microbial ecology, understanding biogeochemical processes and associated ecosystem functions might be aided by identifying functional microbial communities and their spatial pattern characterization (Wessén et al. 2011). Soil fertility and local climate conditions are also driving factors in N cycling microbial communities (Ochoa-Hueso et al. 2016).

1.2.5. Factors affecting nitrification, denitrification, and immobilization process

Different environmental variables such as soil temperature, soil moisture, soil texture, and aeration can impact nitrifying microbial populations (Yang et al. 2019). The soil pH affects nutrient availability (substrate content), ammonia-oxidizer activity and nitrification process (Saarenheimo et al. (2015). Meinhardt et al. (2018) observed that nitrification occurs more readily in high pH soils and AOB contributes more to ammonia oxidation than AOA, even with higher population.

Soil temperature also affects nitrification. The optimum temperature for nitrification is nearly 35 °C, while low and high temperature reduced nitrification rate (Myers 1975). Under high soil moisture conditions, water occupies soil pore spaces that reduce the soil aeration as well nitrification rate. It was observed that lower moisture content in the soil has a less adverse effect on nitrification than that higher moisture content (Craswell and Martin 1974). Nitrification rate is affected by the soil texture like clay soil has a higher nitrification rate than sandy soils (Sahrawat 2008). Similarly, highest nitrification rate was observed in silty clay followed by loam and smallest was in sandy loam (Aulakh et al. 1996). Nitrogen fertilizers application can boost autotrophic nitrification while preventing ammonium immobilization in the soil (Stone et al. 2015). However, under substantial N application, AOA or AOB dominates nitrification process is still under debate (Yurtseven et al. 2018).

Denitrification is influenced by soil moisture, tillage, soil texture, temperature, and nitrate concentration (Kou et al. 2020). Soil moisture triggers denitrification rate, denitrification rate is minimal when the moisture content in the soil is less than 60% of the maximum water-holding capacity (Nishio et al. 1988). Tillage can affect the denitrification process, no-till increase denitrifier communities activity and hence denitrification (Wang et al. 2019b). Denitrification rate increases with increase in temperature, twice denitrification rate was observed at 25 ° C compared to 8 ° C (Herrman et al. 2008). The texture of the soil might affect the denitrification process. For example, sandy soil has a higher affinity for N leachability due to bigger pore spaces in the form of NO_3^- fertilizers. While clayey and loamy soils have a lower affinity for NO_3^- leaching as compared to sandy soil due to low porosity and tightly packed together. Under slightly high pH, silty-clay soils exhibited enhanced denitrification reported by Chamindu Deepagoda et al. (2020), while at high soil pH up to 9, denitrification is inhibited in sandy loam soil (Obia et al. 2015).

Denitrification processes and N transformation can be influenced by types of fertilizers utilized (Zhao et al. 2020). The addition of N-based fertilizers increased the activity of soil microbial populations that are involved in nitrification and denitrification processes and play an important part in N transformations (Zhao et al. 2020).

Soil N immobilization is a process in which soil inorganic N is converted to organic form (Jansson and Persson 1982). N immobilization helps in reducing the leaching and gaseous losses by retaining N in organic form in the soil system (Li et al. 2021). Soil N immobilization rate (NIR) is influenced by soil moisture, temperature, pH, quantity, and quality of soil organic matter, NH_4^+, and NO_3^- contents via changing the soil microbial biomass. An increase in temperature from 10°C to 15°C result in increased soil NIR in most soils (Lang et al. 2010). Soil moisture is also an important factor determining NIR, when moisture content increase from 30% to 60% then NIR increase several times (Sun et al. 2019). Wang et al. (2019a) reported that increase in soil pH decrease the NIR, while high carbon-to-nitrogen ratio (C/N) enhance NO_3^- immobilization (Cheng et al. 2017). In boreal forests, the addition of NH_4^+ and NO_3^- substrate for nitrification and denitrification increased NIR (Lavoie et al. 2011). Furthermore, biotic N immobilization is also affected by the activity of microbes which depend on soil microbial biomass (Johnson et al. 2000).

1.2.6. Nitrogen losses in different cropping systems

N losses can occur in most terrestrial ecosystems via greenhouse gas emissions, inert forms, or NO_3^-leaching (Fang et al. 2015). When UR application done to the surfaces of both low and high pH soils, the loss of N by NH_3 volatilization is a major problem since it is easily hydrolyzed by enzymes like urease, raising the pH of the soil near the fertilizer granules (Chien et al. 2009). NH_3 and soil N_2O emissions are also affected by the application source of inorganic N fertilizer, application rate and soil temperature (Snyder 2008; Snyder et al. 2009).

However, there have been claims that cereals can only uptake 40% of applied N fertilizer including maize, rice, barley, wheat, and sorghum due to poor management practices (Giambalvo et al. 2018). The average NUE is approximately 30-50% in cereals due to higher N losses (Haroon et al. 2019). As UR is hydrolyzed to generate NH_3, up to 40% NH_3 was volatilized (Drury et al. 2017; Rochette et al. 2009; Salazar et al. 2012). Bouwman et al. (2002) also reported higher NH_3 volatilization in warm climates. For example, many field studies conducted in warmer climates (25-28°C) reported 20-30% NH_3 volatilization losses (Cantarella et al. 2003; Cantarella et al. 2008), but in tropical weather these losses can be near to 60% (Sparovek and Jong Van Lier 1997). Moreover, NH_3 losses may negatively affect the ecology and the quality of atmosphere. Atmospheric quality can be impacted by NH_3 losses may have a negative ecological impact (Zaman and Blennerhassett 2010; Zaman et al. 2008; Zaman et al. 2009).

Low NUE leads to increase in cost of crop production, as well as associated environmental problems. N fertilizers is easily transformed into NO_3^- and lost through processes such as leaching (Bowles et al. 2018). Ground water pollution is caused by an excess of NO_3^- generated by microorganisms during nitrification. Globally, the estimated NO_3^- leaching losses from the agriculture system are 19% of applied N reported by Lin et al. (2001) and is estimated to reach 61.5 Tg N year^{-1} by 2050. In the maize-wheat cropping system, NO_3^- leaching losses were 22% and 15%, when N was applied @ 240 and 180 kg ha^{-1}, respectively (Zhou and Butterbach-Bahl 2014). The major route of NO_3^- removal from boreal ecosystems is microbial gaseous N generation via denitrification (Fang et al. 2015). N is also lost via immobilization when inorganic N is transformed to organic form and remains unavailable for plant uptake (Jansson and Persson 1982). Gross N immobilization losses in the terrestrial ecosystem are more than 1400 Tg N per annum (Kuypers et al. 2018). Surface runoff leads to N lost in-stream and lakes, it was estimated that average runoff losses in wheat soybean, wheat-cotton, wheat-silage corn and wheat-fallow were 61, 108, 111, 146 mm, respectively (Jiao et al. 2012).

However, It was observed that 2% N was lost as N_2O in the denitrification process in maize cropping system (Grace et al. 2011). Bouwman et al. (2005) reported that N can be lost to the atmosphere up to 27% as a result of denitrification. As expected, increasing synthetic N fertilizer rate generally increases N_2O emissions (Bouwman et al. 2002; Dusenbury et al. 2008; Halvorson et al. 2014; Hoben et al. 2011; Rochette et al. 2013; Van Groenigen et al. 2010). N losses are critical in terms of human health, economics, and the environment. Therefore, it is mandatory to use modern approaches/technologies to reduce N losses not only to improve NUE but also to improve environmental conditions.

1.2.7. Management practices and technologies to reduce N losses

There are several BMPs used to reduce N losses, such as conservation tillage, no-till, crop rotations, cover crops, and 4Rs (right time of application, right rate, right source, right place) (Ghosh et al. 2015; Reetz 2016). The right rate involves supplying the required quantity of nutrients to the crop based on soil test reports (Ghosh et al. 2015). The right source involves formulations that can improve NUE and reduce environmental concerns by considering the crop needs and the soil properties (Johnston and Bruulsema 2014; Reetz 2016). For instance, slow-release fertilizers (polymer and sulfur coated fertilizers), are designed to release nutrients in synchrony with plants requirement (Ghosh et al. 2015; Trenkel 2010). However, most of the slow-release fertilizers are expensive and by restricting nutrient availability may limit the growth of the fast-growing crops (Ghosh et al. 2015). The right place refers to nutrient placement which depends on crop type and soil conditions, e.g., banding, side or top dressing or broadcasting (Ghosh et al. 2015; Grant et al. 1996). Right time involves matching nutrient supply with the crop requirement (Johnston and Bruulsema 2014). Therefore, it is essential to use new approaches/technologies to control N losses. One of the promising approach currently being practiced to minimize N losses and improve crop N uptake is the use of N fertilizers coated with UIs or NIs termed as N stabilizers which delay the UR hydrolysis and nitrification process through blocking the microbial activities (Akiyama et al. 2010; Cameron et al. 2013). UIs and NIs seem to be more efficient as they simplify and reduce the frequency of fertilizer applications, which allows flexibility in the timing , saving time, fuel, and labor (Grant et al. 2005).

N stabilizers play a significant role in enhancing NUE by minimizing the N losses through suppressing the activity of microbes and delaying NH_3 oxidation. UIs delay the UR hydrolysis process up to 7 -14 days, for example, N-(*n*-butyl) thiophosphoric triamide (NBPT), a UI, blocks three of the urease enzyme's active sites by creating a tridentate bond with two nickel atoms in the center by the carbamate bridge connecting with one oxygen atom and two metals. This decreases the chances that UR will reach the nickel atoms, inhibits hydrolysis, and improves the N supply cycle (Cantarella et al. 2018). By limiting the rate of substrate availability, NBPT slows the UR hydrolysis process and is anticipated to influence NH_3 oxidation and subsequent denitrification (Ding et al. 2011).

While NI suppresses the activity of microbes and slows down the process of nitrification as well as denitrification, leading to more uptake of NH_4^+ instead of being lost. Increasing the N uptake by plants, increases crop yield, and ultimately improvement in NUE. For instance, Dicyandiamide (DCD), and nitrapyrin (2-chloro-6-(trichloromethyl) pyridine; NP), are common NI (Modolo et al. 2018). DCD is cheap, less volatile, easily soluble in water, widely used, and can effectively utilized with N fertilizer (Trenkel 2010). Ammonia monooxygenase (AMO), a metallo enzyme involved in the initial step of nitrification, is readily bound by DCD, which reduces NO_3^- leaching (Menneer et al. 2008; Vogeler et al. 2007). It slows down the NH_3 oxidation process and subsequent NO_3^- production, leaching, and denitrification in the form of N_2O emission (Dennis et al. 2012; Di et al. 2007; Zaman et al. 2009). However, efficacy of DCD can be affected by many factors including soil moisture and temperature like other NIs (Subbarao et al. 2006). .

Similar to DCD, NP plays a significant role in reducing N leaching losses. In general, most of NIs act as biostatic (inhibit the growth of an organism) but not biocidal (destroy the growth of an organism) (Byrne et al. 2020). However, the direct effects of NIs on microbes often require high doses except for NH_3 oxidizers. For example to completely inhibit the activity of AOA and AOB (e.g., *N. europaea*, Ca. *N. frankandus*, Ca. *N. sinensis*) just <30 μM of NP required while >100 Mm of NP was required to suppress the bacteria such as nitrite-oxidizing (e.g., *Nitrobacter* sp.) activity (Papadopoulou et al. 2020).

Several studies have shown that UIs and NIs often reduce NH_3 and N_2O emissions in different cropping systems (Drury et al. 2012; Halvorson and Del Grosso 2012; Halvorson et al. 2010; Halvorson et al. 2011; Halvorson et al. 2014; Hyatt et al. 2010; Soares et al. 2012; Soon et al. 2011; Venterea et al. 2010). DCD application not only reduced N_2O emission by 76% but also greatly reduced NO_3^- in runoff and leaching in subtropical climates (Meng et al. 2021). Whereas, NBPT reduced UR hydrolysis by 35% in silage corn (Martins et al. 2017).

Urea treated with combine application of NIs and UIs has been proven to have less N losses in the form of NH_3 volatilization, NO_3 leaching, and N_2O emissions reported by Zaman et al. (2008), which may improve NUE and crop yields (Nastri et al. 2000; Zaman et al. 2008; Zaman et al. 2009). Zaman and Blennerhassett (2010) found that for maximum GHG reduction, additional applications of 5, 7, and 10 kg ha^{-1} DCD result in the reduction of N_2O by 25, 47, and 47%, respectively. It was also investigated that the application of NP, DCD, and DMPP had a reduction potential of N_2O emission from 30-50% (Ruser and Schulz 2015). Gilsanz et al. (2016) reported that highest inhibitory effect of NIs in reducing N_2O emissions was observed in grassland and followed by cropland, upland, and paddy cropping system.

1.2.9. Effect of N stabilizers on relative abundance of genes involved in nitrification and denitrification

N stabilizers like NIs and UIs have been shown to impede the nitrification process and are commonly utilized as nutrient sources in agricultural soils (Wu et al. 2017; Xi et al. 2017). By inhibiting the NH_3 monooxygenase enzyme by copper chelation, NIs prevent NH_3 oxidizer activity. It has been observed that NIs don't just target AOB or AOA, the two most common NH_3 oxidizers (Huang et al. 2012). AOA communities predominate in different types of soil reported by Clark et al. (2020), and their abundance is positively linked with NO_3^-, NH_4^+, and NO_2^- concentrations (Clark et al. 2020). In general, Kawakami *et al.* (2012) reported that DCD application inhibited AOB rather than AOA, although NP inhibited AOA reported by Ruser and Schulz (2015), and DCD severely hampered AOA growth in very low pH soils (Huang et al.

2012). It also has been observed that DCD has non-significant impacts on microbial communities (O'Callaghan et al. 2010; Wakelin et al. 2013).

Fu et al. (2020) reported that DCD significantly reduced *amo*A gene copies in sandy loam soil in wheat crop. NP application significantly reduced *nir*K gene copies in rice cropping system (Meng et al. 2020). However, Fisk et al. (2015) explained that NP had no significant effect on *amo*A gene abundances. The DCD and NBPT application lowered 40-56% abundance of *amo*A, *nir*K and *nos*Z as compared to urea ammonium nitrate application (Tosi et al. 2020).

Further, some NH_3 oxidizers have also been reported to possess the urease enzyme and use UR directly as an energy source. This would then imply that NBPT could influence these ureolytic NH_3 oxidizers directly by inhibiting their intracellular urease. However, the molecular mechanisms involving UR hydrolysis and its delay by NBPT or its effect on NH_3 oxidizers is not well understood. Few researchers have examined how NBPT affects NH_3 oxidizers (Fan et al. 2018; Shi et al. 2017; Xi et al. 2017), it delays the UR hydrolysis process, therefore there is a need for further investigation under limited substrate availability, effects on NH_3 oxidizers, nitrification, and denitrification genes abundance. There is no clear understanding about combined application of UI+NI coated UR influences the microbial community structure and genes involved in nitrification and denitrification in different crop rotations in podzolic soils in boreal climate that need further investigation. Moreover, the effects of such combinations on soil N cycling microbes involved in hydrolysis and NH_3 oxidation have received little attention, and more studies are required (Dong et al. 2018). Previous studies have investigated the effects of NIs on NH_3 oxidizers with emphasis on AOA and AOB microbial communities (Chen et al. 2015; Shi et al. 2016). Recently, (*comammox nitrospira*) a single organism is carried out complete NH_3 oxidation (Daims et al. 2015; Van Kessel et al. 2015). There is still a need to understand the contribution of these microbes to nitrification and denitrification, and how they respond to the application of NIs like NP, DCD, and UIs like NBPT. Some production systems such as pasture production receive N input throughout the whole year (Abalos et al. 2014). However, concerns of long-term repeated effects of N application on soil health have been raised (Hartmann et al. 2015). These concerns include changes in soil chemical properties like soil pH. Such changes in soil chemical properties could influence soil microbial community (Geisseler and Scow 2014; Zhou et al. 2015). Many studies have focused on the effects of repeated application of N fertilizer on soil microbial communities (Chen et al. 2016; Hartmann et al. 2015; Wang et al. 2019c). However, the application of UIs and NIs and their effects on N cycling microbes involved in nitrification, denitrification together have rarely been studied and needs further investigation.

1.2.10. Effects of crop rotation (silage silage corn, wheat, and faba bean) on N losses, microbes, and genes involved in nitrification and denitrification processes.

Crop rotation has numerous benefits, including improved weed control and disease management increased cycling of nutrients, soil tilth, and soil physical characteristics (Davis et al. 2012; Gaudin et al. 2015; Munkholm et al. 2013). Crop-soybean rotation reduced soybean cyst nematode prevalence (*Heterodera glycines*) (Chen et al. 2001; Howard et al. 1998). Thus, crop rotation may decrease disease and insect populations as well as weed populations by disrupting the life cycles. The transformation of organic N to mineral N can also be affected by crop rotation through changing soil pH, temperature, soil moisture, and plant residues (Kladivko 2001). Research has demonstrated that compared to their respective monoculture sequences, silage corn-soybean rotations increased silage corn yield (Meese et al. 1991; Porter et al. 1997).

A long-term crop rotation study on soil N availability significantly increased the mineralized net N in soil as compared to continuous monocropping (Gaudin et al. 2013). As a result, crop rotation can be utilized as a management strategy to raise the soil's nitrogen pool while lowering the amount of fertilizer N input and reducing the chance of excessive N leaching during moist weather conditions. A later crop may receive a considerably more N if legumes are included in the crop rotation. Thus, minimizing N losses through legume-cereal crop rotation has improved crop yield and soil health (Gogoi et al. 2018).

Crop rotation with legume can decrease 5-7 times GHG emission by other crops like cereals (Stagnari et al. 2017). For instance, silage corn as a monoculture crop requires high fertilizer inputs, resulting in low fertilizer use efficiency due to higher N_2O emissions and NO_3^- leaching. Long-term rotation and till & No-till (NT) trials in Ontario has shown greater yield stability when silage corn and legume rotations were integrated with winter wheat under-seeded with red clover or alfalfa (Gaudin et al. 2013). Previously it was observed that crop rotation with legume crop lowered N_2O emission by 5.1 times as compared to continuous silage corn (Drury et al. 2008).

Using more complex crop rotation is an important management strategy to improve soil health and/or SOC is to increase planting/crop diversity, particularly temporal variation. It has been proposed that greater crop diversification will result in greater environmental stability (Naeem 2002; Tilman et al. 2006). Cereal-legume rotation is important for maintaining agroecosystem diversity that not only increase yield discussed by Gaudin et al. (2013), but also influence bacterial communities (DeBruyn et al. 2011). Greater temporal crop diversification, especially with legumes, along with lower C/N residues increase C retention in soils (Drinkwater et al. 1998). Consequently, rotating legume with silage corn rotation (a high residue cropping sequence) may increase greater SOC or residue addition (Ortega et al. 2002). Crop rotation can affect soil microbial biomass, diversity, and frequency through quantity and quality of residues (White and Rice 2009). Crop rotation significantly decreased AOB abundance in diverse crop rotation as compared to simpler continuous silage corn rotation but did not affect AOA abundance, when silage corn-soybean rotated with wheat-red clover increased the *nir*K and *nos*Z abundances (Linton 2020). Munroe et al. (2020) reported that simple silage corn-silage corn rotation significantly reduced AOB gene copies as compared to diverse crop rotation (silage corn-soybean-wheat). It was observed that AOA abundance was higher in wheat-faba bean intercropping as compared to monocropping (Xu et al. 2018). Another study reported that crop rotation and years of rotation can affect *nir*K gene copy number, while the cropping system determines the *nos*Z gene abundance (Maul et al. 2019). Many studies have reported the long-term effect of different crop rotations on genes and microbial communities involved in the N cycle in tropical climates. However, the effect of short-term crop rotation on relative gene abundance associated with nitrification, microbial diversity, and structural composition in podzolic soils in boreal climate has not been previously investigated.

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1.4. Co-authorship statement

Manuscripts based on the chapter 2, entitled “Effects of nitrogen stabilizers and crop rotation on relative gene abundance involved in nitrification and denitrification processes in podzolic soil under boreal climate” and chapter 3 “Effect of crop rotation and nitrogen fertilizer stabilizers on soil microbial diversity and structure in podzolic soils of boreal ecosystem” will be submitted to frontiers in microbiology and Scientific Reports (Mushtaq, I., Yurgel, S., Nadeem, M., Katanda, Y., Galagedara, L., Thomas, R., Ellsworth, S., Cheema, M. 2022). The primary author will be Irfan Mushtaq, the author of the thesis, and the corresponding author will be Dr. Cheema (supervisor). Dr. Svetlana Yurgel (co-supervisor) and Dr. Raymond Thomas and Dr. Lakshman Galagedara (committee members) will be second, fifth and sixth authors, respectively. Sabrina Ellsworth, research collaborator, Department of Fisheries, Forestry and Agriculture will be the eighth author. Dr. Cheema wrote the research grants, developed the plan of this field experiment, and assisted in writing the results and discussion for the work in Chapters 2 and 3. The data was collected, analyzed, and the manuscript was written by Mr. Irfan. Dr. Yeukai, Dr. Nadeem, and Sabrina Ellsworth helped with experimental setup, field samplings, sample preparation, and lab analysis. Dr. Cheema edited and reviewed the manuscript and supervised overall experimental activities.

Chapter 2

2. Effects of nitrogen stabilizers and crop rotation on soil pH, N dynamics and relative abundance of genes involved in nitrification and denitrification processes in podzolic soil in boreal climate

2.1. **Abstract**

Nitrogen (N) is an essential macronutrient required to increase crop growth, development and yield. Increase N application result in N losses which can vary between 50-70% in different cropping systems. Crop rotation and N stabilizers may affect the N mineralization and consequently limit N losses. This study was designed to evaluate the effect of crop rotation and N stabilizers on soil pH, soil mineral N, and genes involved during nitrification and denitrification processes. The experiment was conducted in small plots (3 m × 4 m) and laid out in a randomized complete block design (RCBD) with split plot arrangement with four replications. The experimental treatments were three crop rotations [(Silage corn-Silage corn (c-c), Silage corn-Wheat (c-w), Silage corn-Faba bean (c-fb)] and five nitrogen sources (Control, Urea, Agrotain, Entrench, and Super-U). Results showed that both N sources and crop rotation had no significant effect on soil EC. However, soil pH was significantly affected by N sources and non-significantly affected by crop rotation. Furthermore, c-fb crop rotation significantly reduced soil NH_4^+ and NO_3^- and relative abundance of all genes involved in N cycle as compared to c-c rotation. However, N stabilizers had no significant effect on soil NH_4^+ and NO_3^- as compared to urea. N stabilizers significantly suppressed the relative abundance of genes involved in nitrification (*amo*A AOB, *amo*A AOA, *nxr*), and denitrification (*nir*K and *nos*Z) compared to urea. According to the results of this study, we may conclude that N stabilizers can impede the normal nitrification and denitrification processes and can improve N uptake by plants.

2.2. Introduction

Nitrogen is the most limiting macronutrient for crop growth, development and yield and its efficient use determines the profitability and environmental sustainability of cropping systems (Kumar et al. 2021; Shahzad et al. 2019). Increased N application enhance crop growth, photosynthesis and biological yield potential (Nasim et al. 2016). To achieve global food security, application of synthetic N fertilizers or manure application is inevitable (Chen et al. 2008; Khan et al. 2014). However, synthetic N fertilizers such as urea (UR) is prone to volatilization, nitrate (NO_3^-) leaching, and gaseous losses (N_2O and NO), which contaminate water bodies negatively impacting the global warming budget (Sgouridis and Ullah 2014). N fertilizers application is projected to exceed 186 Tg N yr^{-1} by 2050 reported by del Pilar Muschietti-Piana et al. (2018) that can further increase the N losses in different cropping systems.

There are several BMPs or approaches such as conservation tillage, no-till, crop rotation, cover crops, 4Rs (right place, right source, right rate, right time) as well as technologies that includes slow release fertilizers and N stabilizers that can be used to reduce N volatilization, greenhouse gas (GHG) emission and NO_3^- leaching losses (Ghosh et al. 2015; Reetz 2016). Nitrogen fertilizers stabilizers are urea coated with urease inhibitors (UIs) or nitrification inhibitors (NIs) or both and used as source of N fertilizers in agricultural production system to reduce NO_3^- leaching and gaseous losses (Wu et al. 2017; Xi et al. 2017). NIs on the other hand are chemical compounds that slow down bacterial oxidation of NH_4^+ to NO_3^- by inhibiting ammonia monooxygenase activity. UIs are chemical compounds that block the activity of the enzyme urease and reduce NH_3 losses by delaying urea hydrolysis from 7 to 14 days explained by Zaman et al. (2008). Ammonia monooxygenase is a major enzyme encoded by subunit A, which catalyzes ammonia oxidation (*amo*A) via copper chelation resulting in slowed nitrification and denitrification processes. In denitrification process, NO_2^- reduced to NO by nitrite reductase (*nir*K and *nir*S), and nitrous oxide reductase (*nos*Z) is responsible for conversion of N_2O to N_2 gas.

Crop rotation is important to preserve soil fertility and boost microbial biomass and activity (Munkholm et al. 2013; Munroe et al. 2020). Legume crop rotation also contribute N to the soil system and the subsequent crop's need for N (Gaudin et al., 2013). A study conducted by Linton (2020) observed an increase in denitrifying genes (*nir*K and *nos*Z) in silage corn-soybean rotation with wheat-red clover. Maul et al. (2019) reported that crop rotation and time of year affect *nir*K gene copy number, while the same study reported that cropping systems (conventional and organic) determine the *nos*Z gene abundance. Furthermore, rotation of silage corn and oat with alfalfa (legume) decreased the *amo*A gene abundance (Drury et al. 2021). Many studies have reported the long-term effects of crop rotation and N stabilizers on different genes involved in the N cycle as a function of growing climates (tropical, dry) (Castellano-Hinojosa et al. 2020; Fu et al. 2020; Meng et al. 2020). However, very little is known of the effects of short-term crop rotation and N stabilizers on the relative gene abundance of involved in the nitrification and denitrification processes occurring in podzolic soils under agriculture production in boreal climate. Thus, we hypothesized that N stabilizers could confer the following: 1) effectively increase the soil NH_4^+ concomitant with decreased soil NO_3^- concentration; 2) significantly decreased the relative gene abundance of involved in nitrification and denitrification. The specific objectives of this study were:

I. To investigate the effect of short-term crop rotation and N stabilizers on soil pH, EC, and mineral N.

II. To evaluate the effect of short-term crop rotation and N stabilizers on relative gene abundance involved in nitrification and denitrification processes occurring in podzolic soil in boreal climate.

2.3. Materials and Methods

2.3.1. Experimental site and treatments

The field experiment was conducted at Pynn's Brook Research Station (49.087° N, 57.541° W), Pasadena, NL during 2019 and 2020 growing seasons. Soil texture was analyzed to be loamy sand with 78.1% sand, 18.1% silt, and 3.7% clay. Liming was done following the recommendation of lab analyses report. During the 2019 growing season, silage corn was grown as a primary crop whereas, wheat and faba bean were added in rotation in the 2020 growing season.

The experimental treatments were five N fertilizer sources: 1) control (C), 2) Urea - UR, 3) Agrotain-AG (urea coated with NBPT), 4) eNtrench-EN (urea coated with nitrapyrin), 5) SuperU™-SU (urea coated with DCD and NBPT) and three crop rotations: 1) silage corn-silage corn, 2) silage corn-wheat, 3) silage corn-faba bean. The experiment was laid out in a RCBD with a split-plot arrangement and replicated four times. Each experimental plot size was 3 m × 4 m. Silage silage corn, wheat, and faba bean were fertilized with either UR or N stabilizers at the rate of 115, 100, and 25 kg ha^{-1}, respectively. All N stabilizers, UR, phosphorus, and potassium were applied as a basal dose at seeding time. Silage silage corn was seeded on June 14, 2019, and June 12, 2020, using a SAMCO drill planter (SAMCO 2200 Agricultural Manufacturing, Limerick, Ireland) with seed rate 9 seeds/m^2. Wheat and faba beans were planted on June 12, 2020, using a seed rate of 450 seeds/m^2, and 45 seeds/m^2), respectively. Faba bean, wheat and silage corn was harvested on September 17, October 1st and November 5, respectively.

2.3.2. Soil sampling and analyses

Three soil samples from each experimental treatment in all 4 replications were collected after 15 days of N fertilizer or N stabilizer application. Soil samples were collected from 0-20 cm soil depth using a soil auger (JMC Backsaver N-2 Handle, USA). To avoid contamination during soil sampling among treatments, the soil auger was washed with distilled water and 70% ethanol and then cleaned with a paper towel. All soil samples were kept in an ice cooler and transported immediately to Boreal Ecosystem Research Facility (BERF), Grenfell Campus Memorial University where they were sieved with <2 mm mesh. Thereafter, three soil subsamples each of 5-g were transferred to 15 mL falcon tubes and were stored at -80 ˚C to analyze abundance of genes using quantitative polymerase chain reaction (qPCR). Another 100 g soil samples were stored in a cooler at 4 ˚C to determine soil pH, EC, moisture content, and soil mineral N (SMN: NO_3^- and NH_4^+). For SMN analysis, 5 g soil was extracted in 50 mL of 2 M KCl, shook for 1 h at 180 rpm, incubated for 45 min at room temperature, and then filtered through Whatman's filter paper and the extract stored at -20 ˚C. Soil NO_3^- and NH_4^+ were analyzed using LACHAT flow injection analyzer (Quick Chem QC8500 Series 2, USA). Soil EC and pH were measured in a 1:2 soil:water suspension shaking for 1 h at 180 rpm, incubated for 45 min, then measured with EC/pH meter (Blue lab meter, New Zealand). For determining soil moisture content, 15 g samples were kept for 24 h in an oven at 105 ˚C or until weight remains constant. The dry weight was then recorded and moisture content % calculated as follow:

$$\text{Moisture content \%} = \text{soil weight before oven dry} - \text{soil weight after oven dry/soil weight before oven dry} * 100$$

2.3.3. DNA isolation

Total genomic DNA was isolated from 0.25 g of soil using the DNase PowerSoil™ DNA Isolation Kits (Qiagen, Netherlands), according to the manufacturer's instructions. DNA quality (280/230 and 260/230 ratios) was checked by Nanodrop spectrophotometer (Nanodrop 2000c spectrophotometer, ThermoFisher Scientific, USA) and gel electrophoresis. DNA was further purified by using a DNA cleanup kit (DNA clean & concentrator-10, catalog no. D4011), ZYMO Research (Cedarlane Laboratories, Ltd., Canada) following the manufacturer protocols.

2.3.4. Quantitative polymerase chain reaction (qPCR)

Table 2.1 Primers used for qPCR to assess gene abundance.

Gene	Primer	Sequence	Reference
*amo*A Bacteria	amoA-1F	GGGGTTTCTACTGGTGGT	(Rotthauwe et al. 1997)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC	
*amo*A Archaea	arch-amoAF	STAATGGTCTGGCTTAGACG	(Francis et al. 2005)
	arch-amoAR	GCGGCCATCCATCTGTATGT	
nxr-Nitrospira	nxr-spira-for5	CARTCSAACTTCCGGTAYGG	(Fu et al. 2018)
	nxr-spira-rev6	AGCCACTTGATCATGAAYTC	
*nir*K	FlaCu	ATCATGGTSCTGCCGCG	(Michotey et al. 2000)
	R3Cu	GCCTCGATCAGRTTGTGGTT	
*nos*Z	nosZ-F	CGYTGTTCMTCGACAGCCAG	(Kloos et al. 2001)
	NosZ-1662R	CGSACCTTSTTGCCSTYGCG	
Archaeal 16S rDNA	ARCH1-1369F	CGGTGAATACGTCCCTGC	(Suzuki et al. 2000)
	PROK-1541R	AAGGAGGTGATCCRGCCGCA	
Bacteria 16S rDNA	BACT-1369F	CGGTGAATACGTTCYCGG	(Suzuki et al. 2000)
	PROK-1492R	CGWTACCTTGTTACGACTT	

For qPCR, 3 soil samples collected from each plot were combined to form one biological replicate and the DNA isolated using the methodology listed above from all 15 treatments and all 4 biological replicates. Using a CFX96™ Real-Time System (CFX96™ Optics Module, Bio-Rad, Singapore) qPCR amplifications were carried out in 10 µL volumes consisting of (5 µL of QuantiFast SYBR Green PCR Master Mix (Bio-rad) for DNA), 1.5 µL of each primer (500 nM) forward and reverse, 2 μL of template DNA at 30 ng μL^{-1} and nuclease-free water (Severn Biotech, Kidderminster, UK). At least 30 ng μL^{-1} of the soil-extracted DNA were added to each PCR reaction, higher than 5 ng μL^{-1} minimum recommended to avoid false results (Vestergaard et al. 2017). The thermal cycling protocol for qPCR consisted of initial denaturation at 98˚C for 2 min 30 s followed by 98˚C for 10 s then annealing for 25 s at 60˚C and the plate read with 39 cycles. To evaluate the specificity of each assay, a melt curve analysis (60 to 95˚ C, with incremental readings every 0.5˚C) was performed after each amplification.

2.4. Results

2.4.1. Effect of nitrogen sources and crop rotation on soil pH, EC, and mineral nitrogen

N sources, crop rotation, and their interaction (N × crop rotation) had no significant effects on soil EC. However, N sources had a significant effect ($p < 0.01$) on soil pH, whereas interactive effects of N sources × crop rotation had no significant effects on soil pH. On the other hand, N sources, crop rotation and their interaction had significant effects ($p < 0.001$) on soil NO_3^- and NH_4^+ (Table 2.2).

Table 2.2 Analysis of variance (ANOVA) showing the effects of N sources, crop rotation, and their interaction on soil EC, pH, NH_4^+, and NO_3^-.

Source of variation	**d.f.**	**EC**	**pH**	**NO_3^-**	**NH_4^+**
N sources	4	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$
Crop rotation	2	NS	NS	$p < 0.001$	$p < 0.001$
(N × crop rotation)	8	NS	NS	$p = 0.010$	$p < 0.001$

NS: Non-significant; $p < 0.001$: highly significant

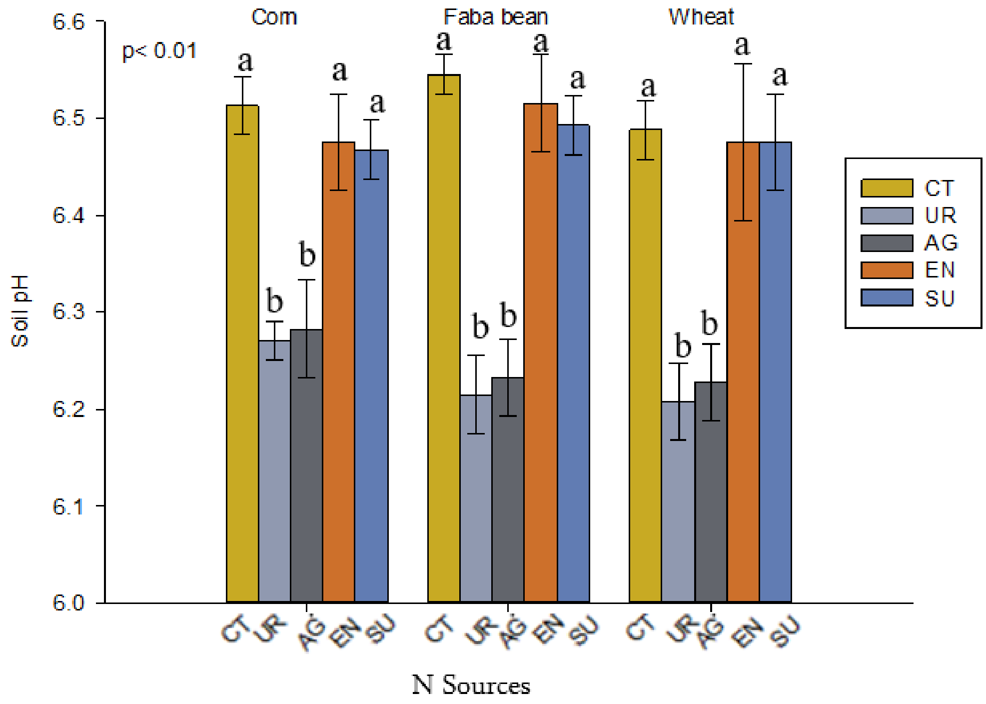

Figure 2.1: Effects of urea and nitrogen stabilizers on soil pH of silage silage corn, faba bean, and wheat cultivated in rotation with silage corn on podzols in boreal climate. The means of four independent replicates are given along standard error bars. Significant differences between treatments are indicated by different letters ($p < 0.05$, Tukey's post hoc test). CT: control, UR: urea, AG: Agrotain (urease inhibitor), EN: eNtrench (nitrification inhibitor), and SU: Super-U (urease inhibitor + nitrification inhibitor).

N stabilizers and urea had significant ($p < 0.01$) effects on soil pH in all crops (Table 2.2). Urea and AG (N stabilizer) application significantly reduced the soil pH in silage silage corn compared to control. However, EN and SU (N stabilizers) significantly increased soil pH compared to UR but were statistically at par with CT treatment (Figure 2.2). In faba bean, UR and AG application had shown significant reduction in soil pH compared to CT treatment whereas, EN and SU application showed increased soil pH, though statistically at par with CT treatment (Figure 2.2). Similar trend of N stabilizers and UR application in increasing and decreasing soil pH was observed in wheat (Figure 2.2).

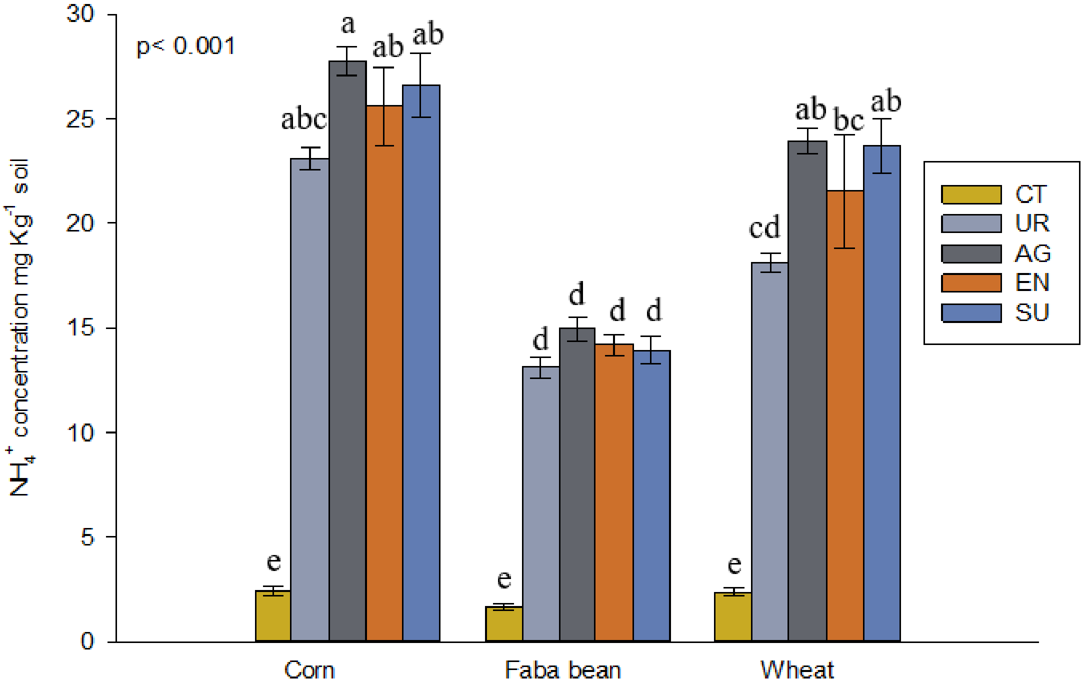

Figure 2.2: Interactive effect of N sources and crop rotations on NH_4^+ concentration mg kg^{-1} soil. The means of four independent replicates are provided together with standard error bars. Significant differences between treatments are indicated by different letters ($p < 0.05$, Tukey's post hoc test). CT: control, UR: urea, AG: Agrotain (urease inhibitor), EN: eNtrench (nitrification inhibitor), and SU: Super-U (urease inhibitor + nitrification inhibitor).

N sources, crop rotation, and their interaction (N × crop rotation) had significant effects ($p < 0.001$) on soil NO_3^- and NH_4^+ (Table 2.2). N stabilizers and UR application both enhanced soil NH_4^+ in all crops compared to CT treatment and these values ranged between 23.10 – 27.75 mg kg^{-1}. Higher

NH_4^+ concentration (27.75 mg kg^{-1}) was observed in silage corn soil with AG application, compared to the lowest (1.65 and 2.42 mg kg^{-1}) in CT treatment in faba bean and silage silage corn soil, respectively. In silage silage corn, higher soil NH_4^+ was observed in AG amended treatment though statistically at par with EN, SU and UR treatments and the lowest soil NH_4^+ was noted in CT treatment. In faba bean, soil NH_4^+ was significantly higher in AG amended treatments compared to the lowest in CT treatment. In wheat, soil NH_4^+ was significantly higher in AG and SU treatments though statistically at par with EN and UR applied treatments and lowest was recorded in CT treatment (Figure 2.3).

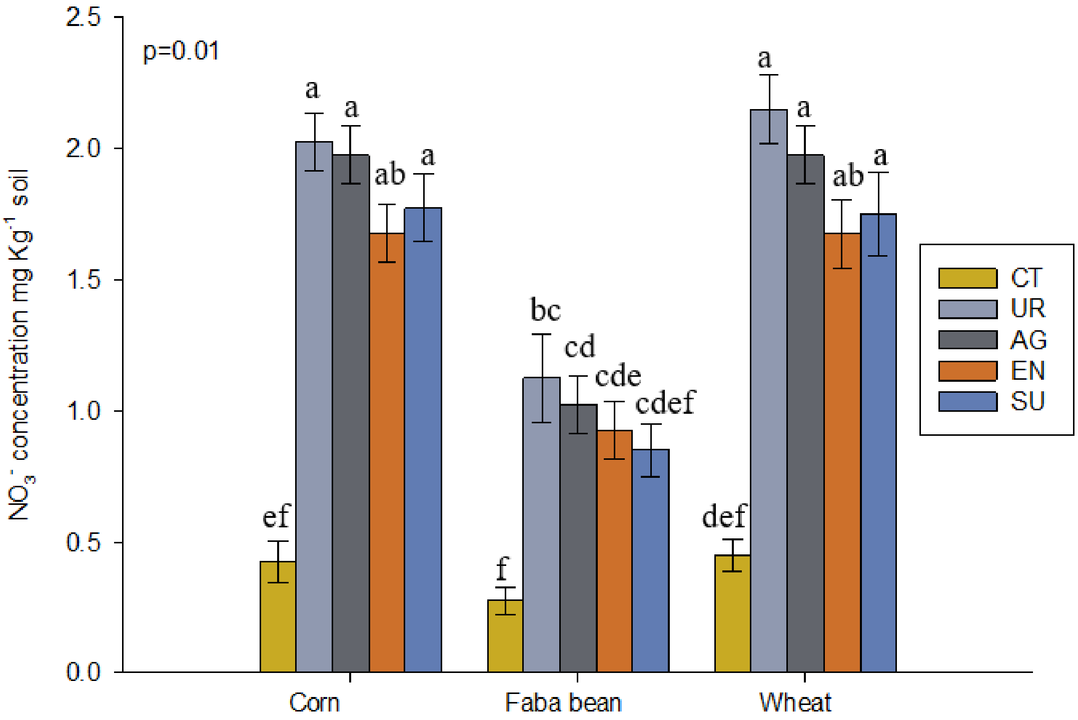

Figure 2.3: Interactive effect of N sources and crop rotations on NO_3^- concentration mg kg^{-1} soil. The means of four independent replicates are provided together with standard error bars. Significant differences between treatments are indicated by different letters ($p < 0.05$, Tukey's post hoc test). CT: control, UR: urea, AG: Agrotain (urease inhibitor), EN: eNtrench (nitrification

inhibitor), and SU: Super-U (urease inhibitor + nitrification inhibitor).

N sources, crop rotation, and their interaction (N × crop rotation) had significant effects ($p = 0.01$) on soil NO_3^- (Table 2.2). Higher soil NO_3^- concentration (2.15 mg kg^{-1}) was observed with UR application in wheat crop, compared to the lowest soil NO_3^- (0.27 mg kg^{-1}) was recorded in CT treatment in faba bean, silage silage corn, and wheat. Higher soil NO_3^- was observed in UR amended treatment though statistically at part with N stabilizers application in all crops and these values vary between 1.67 – 2.15 mg kg^{-1}. Overall, highest soil NO_3^- was observed in wheat, followed by silage corn and lowest was noted in faba bean following either UR or N stabilizers application, respectively (Figure 2.4).

2.4.2. Effect of N sources and crop rotation on relative gene abundance of *amo*A AOB, *amo*A AOA, *nxr-nitrpsipra*, *nir*K and *nos*Z

N stabilizers and urea application had significant ($p<0.001$) effects on the relative abundance of AOB *amo*A gene, AOA *amo*A gene, *nxr-nitrospira*, and *nir*k genes. However, N sources, crop rotation, and their interaction had significant effects on the relative abundance of *nos*Z gene (Table 2.3).

Table 2.3 Analysis of variance showing the effects of N sources, crop rotation, and their interaction on relative abundance of AOB *amo*A, AOA *amo*A, *nxr-nitrospira*, *nir*K, and *nos*Z genes.

Source of variation	**d.f.**	**AOB *amo*A**	**AOA *amo*A**	***nxr-nitrospira***	***nir*K**	***nos*Z**
N sources	4	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
Crops	2	$p = 0.009$	$p = 0.002$	$p < 0.001$	$p =0.015$	$p = 0.008$
N × crop rotation	8	NS	NS	NS	NS	$p = 0.013$

NS: Non-significant; $p < 0.001$; highly significant

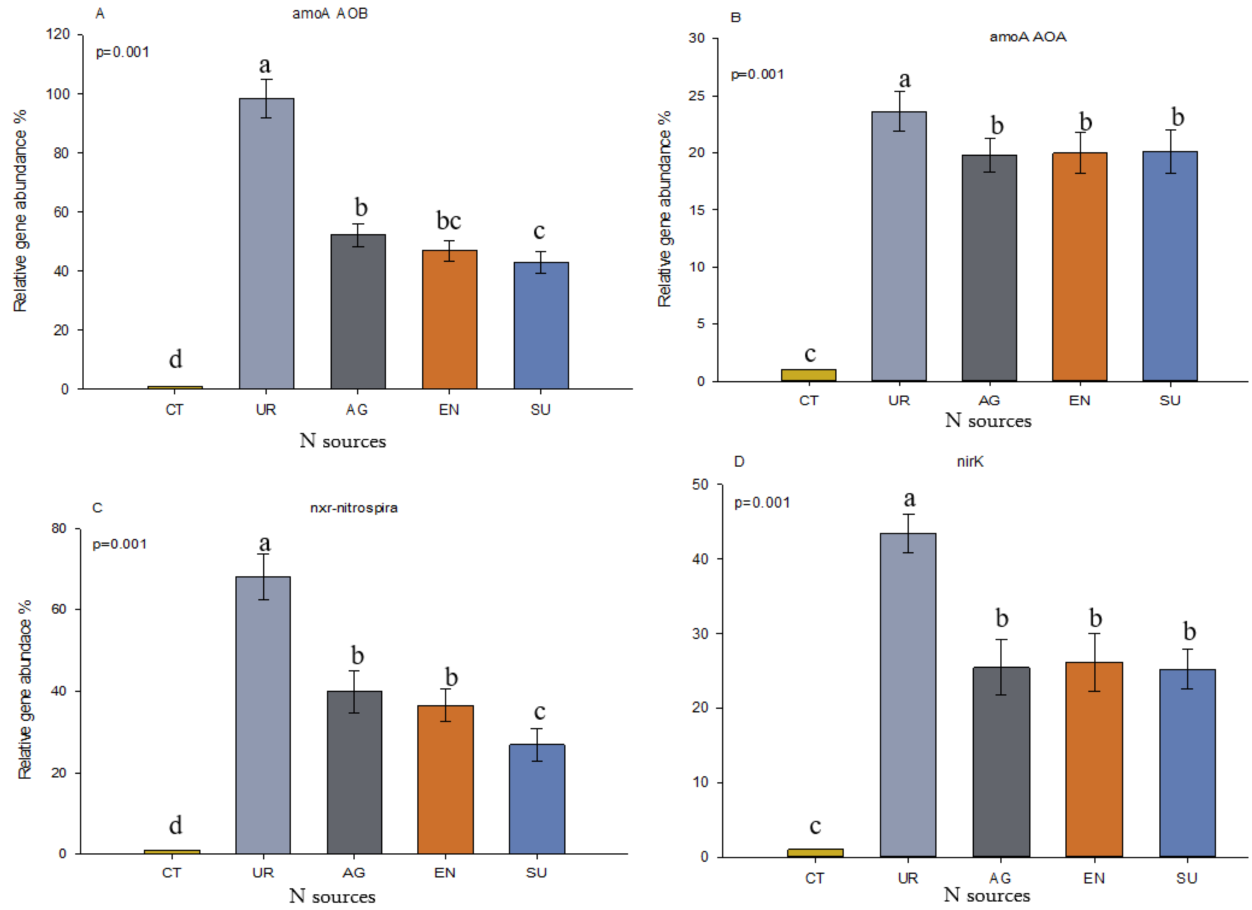

Figure 2.4: Effect of N stabilizers and urea application on relative abundance of (a) AOB *amo*A, (b) AOA *amo*A, (c) *nxr-nitrospira*, (d) *nir*K genes. The means of four independent replicates are provided together with standard error bars. Significant differences between treatments are indicated by different letters ($p < 0.05$, Tukey's post hoc test). CT: control, UR: urea, AG: Agrotain (urease inhibitor), EN: eNtrench (nitrification inhibitor), and SU: Super-U (urease inhibitor + nitrification inhibitor).

Significantly higher (98.3%) relative *amo*A AOB gene abundance of the was observed in UR applied treatment compared to the CT treatment. All N stabilizers significantly reduced relative abundance of the AOB *amo*A gene compared to UR. However, SU conferred 56.3%, 17.6%, 8.3% reduction in relative abundance of AOB *amo*A gene compared to UR, AG, and EN treatments, respectively (Figure 2.5a).

Likewise, significantly higher AOA *amo*A gene abundance was observed in UR applied treatment followed by AG, EN, and SU application. The lowest relative gene abundance of AOA *amo*A was recorded in CT treatment (Figure 2.5b). Likewise, significantly higher *nxr-nitrospira* gene abundance was observed with UR application compared to CT and N stabilizer treatments. SU treatment showed significant reduction in relative abundance of *nxr-nitrospira* genes compared to other N stabilizers and UR. SU application reduced by 60.6%, 32.7%, 26.6%, *nxr-nitrospira* relative gene abundance compared to UR, AG, and EN, respectively (Figure 2.5c).

In case of *nir*K relative gene abundance compared to CT and N stabilizer treatments, UR application showed significantly higher relative gene abundance. N stabilizers application reduced significant count ($p = 0.001$) of relative gene abundance compared to UR treatment, though statistically non-significant with each other (Figure 2.5d).

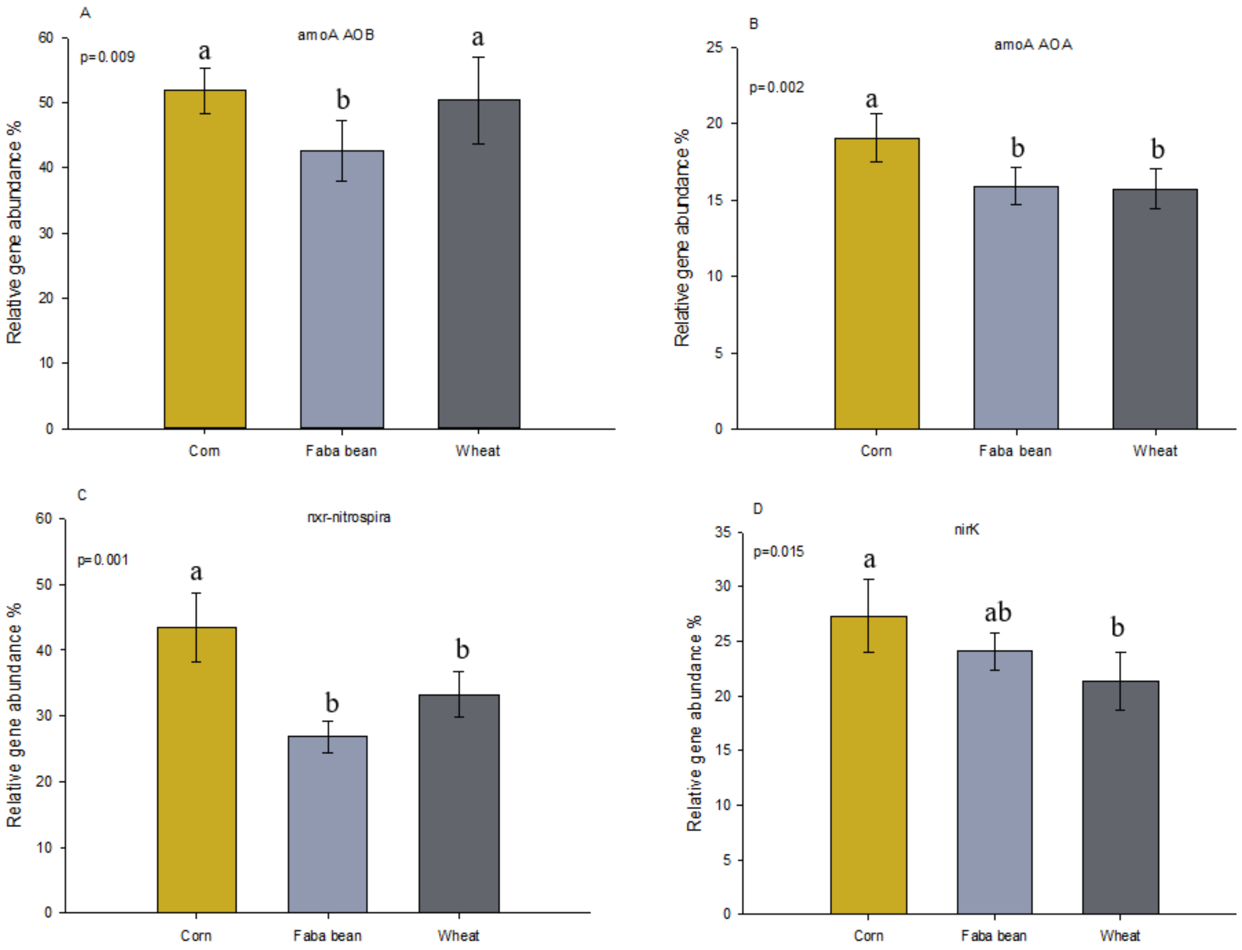

Figure 2.5: Effect of crop rotation on relative abundance of AOB *amo*A (a), AOA *amo*A (b), *nxr-nitrospira* (c), *nir*K genes (d). The means of four independent replicates are provided together with standard error bars. Significant differences between treatments are indicated by different letters ($p < 0.05$, Tukey's post hoc test).

Silage corn, faba bean and wheat had significant effects (p = 0.009; p =0.002; p = 0.001; p = 0.015) on relative abundance of AOB *amo*A, AOA *amo*A, *nxr-nitrospira* and *nir*K gene (Table 2.3). AOB *amo*A gene abundance was significantly lower in faba bean compared to silage corn and wheat. The Relative abundance of AOB *amo*A gene was 51.8% higher in silage corn compared to 42.6% in faba bean and 50.3% in wheat (Figure 2.6a). The Relative abundance of AOA *amo*A gene was significantly higher in silage corn (19%) compared to the lowest in faba bean (16.5%) and wheat (17.6%), respectively (Figure 2.6b). Likewise, higher relative gene abundance of *nxr-nitrospira* (43%) was observed in silage silage corn and lowest (26.8%) was recorded in faba bean, though statistically at par with wheat (33.2%) (Figure 2.6c). Significantly higher relative abundance of *nir*K gene was recorded in silage corn and the lowest was noted in wheat crop. Faba bean *nir*K gene level was similar to that of silage corn and wheat (Figure 2.6d).

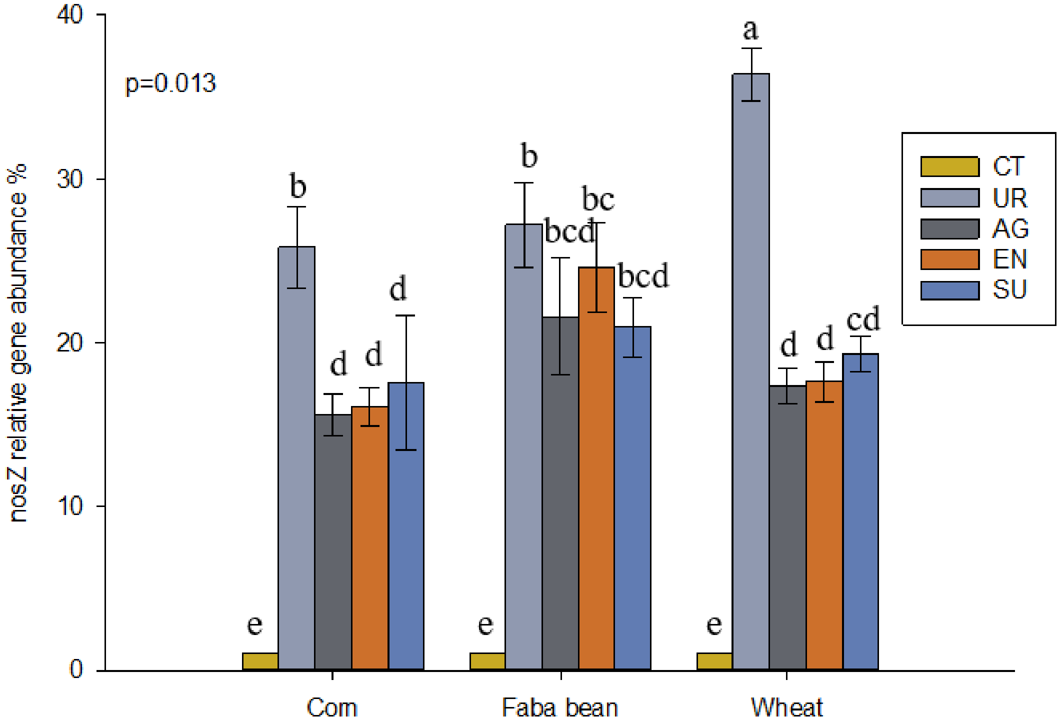

Figure 2.6: Interactive effect of N sources and crop rotations on the relative abundance of the *nos*Z gene. The means of four independent replicates are provided together with standard error bars. Significant differences between treatments are indicated by different letters ($p < 0.05$, Tukey's post hoc test). CT: control, UR: urea, AG: Agrotain (urease inhibitor), EN: eNtrench (nitrification inhibitor), and SU: Super-U (urease inhibitor + nitrification inhibitor).

N sources, crop rotation and their interaction had significant ($p < 0.001$; $p = 0.008$; $p = 0.013$) effects on relative abundance of *nos*Z gene (Table 2.3). Significantly higher relative gene abundance of *nos*Z was observed in UR amended wheat followed by faba bean and silage silage corn. Lower relative abundance of *nos*Z gene was recorded in wheat in CT treatment, however this was not statistically significant when compared with faba bean and silage silage corn in CT. All N stabilizers (AG, EN, and SU) significantly decreased the relative *nos*Z gene abundance as compared to UR in silage corn, and wheat. UR application enhanced relative gene abundance of *nos*Z in faba bean though not significant with N stabilizers and lower level was recorded in CT treatment. Overall, UR application showed significantly higher relative abundance of *nos*Z gene in wheat followed by silage corn compared to N stabilizers and CT treatments whereas, N stabilizers and UR application showed non-significant effects on relative abundance of *nos*Z gene in faba bean (Figure 2.7).

2.5. Discussion

Soil pH is a crucial factor that regulates plant nutrient availability (Oshunsanya 2018). Urea and AG application significantly reduced soil pH compared to control, EN and SU treatments. Urea application can increase soil acidity or decrease soil pH as one OH^- released during hydrolysis to NH_4^+ but two H^+ released during nitrification to NO_3^-. The effect of UI was similar to UR as UI can only delay the UR hydrolysis from 7 to 14 days reported by Zaman et al. (2008), so after 15 days, the effectiveness of UI might be reduced. Furthermore, Matczuk and Siczek (2021) reported that different soil properties (high clay content, organic carbon and moisture content) decrease the effectiveness of UI in soil system. In this research field, high soil moisture condition was also observed in some plots that might contribute to the reduce efficacy of UI. However, soil pH in EN treatments was similar to CT treatment, showing less soil acidification due to delayed ammonia oxidation by suppressing the activity of ammonia oxidizers by binding to the active sites of AMO (ammonia monooxygenase) metallo enzyme involved in the first step of nitrification (Di and Cameron 2002; Menneer et al. 2008; Vogeler et al. 2007). In SU (combination of NI and UI), soil pH was similar to control. This might also be due to high moisture content as explained above. Soil properties like moisture content can affect the efficacy of NI (Adhikari et al. 2021). Results of this study are consistent with the earlier studies which demonstrated that the application of UR and UI significantly decreased soil pH due to the release of extra H^+ during NH_4^+ hydrolysis process (Chen et al. 2019; Fu et al. 2020; Shen et al. 2016; Zhou et al. 2015).

Higher NH_4^+ concentration was observed in AG amended soil in silage corn compared to UR application. Higher NH_4^+ in AG amended treatment can be attributed due to the presence of UI (NBPT) that potentially delayed urea hydrolysis. NBPT can delay urea hydrolyses from7 to 14 days as reported by Zaman et al. (2008), urease is inhibited by blocking its three active sites, which results in the formation of a tridentate bond with two nickel atoms at the center and one oxygen atom from the carbamate bridge connecting the two metals. This improves the N supply cycle by inhibiting hydrolysis (Cantarella et al. 2018). Due to UI mode of action of delaying urea hydrolysis in initial days up to 14 days, after 15 days of fertilizer application the effect of NBPT might be reduced and higher NH_4^+ concentration could be available for plant uptake. Consistent with this study Sigurdarson et al. (2018) reported that NBPT treated soil decreased soil pH, which might have affected the NH_3 and NH_4^+ equilibrium in the soil, reduced NH_3 loss as gas and increased the soil NH_4^+ concentration. However, soil NH_4^+ was higher in N stabilizers (AG, EN, SU) as compared to UR in silage corn and faba bean but the effect was non-significant. This might be due to lack of effectiveness of N stabilizers in high moisture condition. Adhikari et al. (2021) reported that NI efficacy, while Matczuk and Siczek (2021) reported that UI efficacy affected in different soil properties (high moisture content). Soil NH_4^+ was significantly lower in faba bean with N stabilizers and UR application compared to silage corn and wheat, possibly due to lower N fertilizer application rates at 25 kg ha^{-1} in faba bean compared to 115 kg ha^{-1} in silage silage corn and 100 kg ha^{-1} in wheat. Results of this research are consistent with the studies other reports demonstrating that increasing N application rate resulted in increased soil NH_4^+ concentration, due to increased urea hydrolysis (Chen et al. 2019; Shen et al. 2016; Zhou et al. 2015).

Nitrification inhibitors reduced soil NO_3^- concentration specifically with eNtrench application in silage silage corn and wheat, though statistically not significant with UR application. NI suppresses the activity of ammonia oxidizers by binding to the active sites of ammonia monooxygenase, metallo-enzyme involved in catalyzing the first step of nitrification (Di and Cameron 2002; Menneer et al. 2008; Vogeler et al. 2007). This reduction could also be due to limiting rate of substrate availability (NH_4^+) as higher NH_4^+ concentration can also be observed in N stabilizers as compared to UR (Figure 2.3), supporting ammonia oxidation inhibition by NI. NBPT delay the urea hydrolysis process as discussed above which results in slow conversion of urea to NH_4^+ and then NH_4^+ to NO_3^- in the nitrification process as a result NO_3^- concentration was reduced compared to UR application (Figure 2.4). However, there was no difference between N stabilizers and UR on NO_3^- concentration. This might be due to a lack of N stabilizers effectiveness as discussed above. Soil NO_3^- was significantly lower in faba bean cultivated with N stabilizers and UR application compared to silage corn and wheat. Perhaps this occurred due to lower NH_4^+ concentration in faba bean as compared to silage corn and wheat (Figure 2.3), since NH_4^+ can convert to NO_3^- during the nitrification process.

Ammonia oxidizers such as AOB and AOA are important players involved in the first and important step of nitrification in the N cycle (Carey et al. 2016). NH_4^+ oxidized to NO_2^- by AOB and AOA and the gene responsible for this process is ammonia monooxygenase (*amo*A). This process is important as it proceeds to complete nitrification by oxidizing to NO_3^- and then transformed to N_2O and N_2 during the denitrification process (Figure 1.2). The *amo*A gene was higher in UR amended treatments compared to treatments with N stabilizers (Figure 2.5a), possibly due to fast conversion of NH_4^+ to NO_2^-. The lowest *amo*A gene abundance was observed in SU applied treatment which could be linked to the fact that it is the combination of UI and NI. As NI suppresses the ammonia oxidizer activity due to its mode of action of binding to the active sites of AMO and UI delay the UR hydrolysis process. This result in limited (NH_4^+) substrate availability, thus higher NH_4^+ concentration can be observed in NBPT treatment (Figure 2.3). In this way, N stabilizers reduced *amo*A AOB, and *amo*A AOA relative abundance as compared to UR application. Similar to the findings reported in this study, a few other studies have also reported that NI and UI decrease AOB gene copy numbers as compared to UR, this was attributed to decreased (NH_4^+) substrate availability (Fan et al. 2018; Luchibia et al. 2020; Shi et al. 2017).

Nxr-nitrospira gene oxidizes NO_2^- to NO_3^- in the nitrification process, where *nir*K is involved in the reduction of NO_2^- to NO during the denitrification process, while *nos*Z is responsible for reduction of N_2O to N_2 during process of denitrification. All N stabilizers significantly reduced *nxr-nitrospira*, *nir*K, and *nos*Z relative gene abundance compared to UR. As UI reduce urea hydrolysis process and the conversion of urea to NH_4^+ whereas, NI inhibits the activity of ammonia oxidizers, resulting in reduced nitrification process (NH_4^+ to NO_2^- then to NO_3^-). This NO_3^- act as a substrate for denitrification process and converted to nitrogenous gases (N_2O and N_2), due to limited substrate (NO_3^-) availability (Figure 2.4) result in less gene abundance in N stabilizers treatments as compared to UR. When N stabilizers in the form of NI inhibit the initial steps of nitrification and UI delay the UR hydrolysis, it results in slowed denitrification due to limited substrate (NO_3^-) availability (Ding et al. 2011). Similar to findings, have been reported where NBPT significantly reduced *nitrospira* due to reduced substrate (NO_3^-) availability, Luchibia et al. (2020). Wang et al. (2017) further reported that DCD and NP (Nis) significantly inhibited the relative abundance of *nir*K genes as well as the denitrification rate due to reduced NO_3^- availability. In present study, crop rotation had significant effects on the relative abundance of all genes involved in nitrification and denitrification. Significantly higher relative genes abundance was observed in silage corn and wheat compared to faba bean (Figure 2.6). This could be due to two reasons: first Congreves et al. (2015) reported, increased in aggregate stability and soil organic matter (SOM), when legume was rotated with silage corn as compared to silage corn monoculture and SOM can directly or indirectly shape nitrifying communities. The interaction between N-cycling bacterial communities and SOM was also observed by Tao et al. (2017), who reported that ammonia oxidizers community increased with addition of organic matter as compared to no fertilizer control. This might be due to changes in SOM as a result of crop rotation, as we did not analyze SOM. Furthermore, Riyo (2018) reported that the conversion of organic N to mineral N can also be affected by crop rotation through changing soil moisture, temperature, and pH. Increase in soil NH_4^+ and NO_3^- was also observed in silage corn and wheat compared to faba bean which appears to regulate the abundance of these genes. A study reported that with increase in N application rate all N-cycle gene abundance significantly increased (Zhu et al. 2016). Linton (2020) reported that in both simple and diverse crop rotation, abundance of nitrifier community was low before addition of N fertilizer, rotation effect was not significant. However, addition of N fertilizer significantly increased *amo*A community after one week. Similar case was also observed by others who reported an increase of *amo*A bacterial abundance after N fertilizer application (Glaser et al. 2010; Liu et al. 2016).

2.6. Conclusion

This study demonstrates that UR and AG application significantly reduced soil pH compared to eNtrench, SU and control treatments. N stabilizers and UR application both significantly enhanced soil NH_4^+ in silage corn and wheat rotation as compared to faba bean. Similarly, significantly higher soil NO_3^- was observed in silage corn - wheat, followed by silage corn - silage corn and lowest was noted in silage corn - faba bean rotation, following either UR or N stabilizers application, respectively. However, silage corn - silage corn rotation significantly increased relative abundance of all genes as compared to silage corn - faba bean crop rotation. Increase in soil mineral N and relative abundance of all genes in c-c rotation as compared to c-fb might to due to different N fertilizer application rate (silage corn 115 kg ha^{-1}, faba bean 25 kg ha^{-1}). N stabilizers significantly decreased the relative gene abundance (*amo*A AOB, *amo*A AOA*, nxr-nitrospira*, *nir*K, and *nos*Z) involved in nitrification and denitrification processes as compared to UR. A significant decrease in the relative abundance of these genes is the key indicator that UI and NI can impede the normal nitrification and denitrification processes instead of UR following cultivation on podzol in boreal climate. Our results conclude that N stabilizers have ability to disturb normal nitrification and denitrification processes by suppressing the microbial activity and can improve N availability for plant uptake.

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Chapter 3

3. Effect of nitrogen sources and crop rotation on soil microbial community structure and abundance in podzolic soils in boreal climate

3.1. Abstract

Crop rotation has numerous advantages for soil health, including increased nutrient cycling and soil tilth, and improving the soil physiochemical and microbial attributes. Microbes are involved in many soil processes including nutrient cycling, which impacts soil quality and health. Anthropogenic activities as a result of nitrogen (N) addition influence soil environment due to more N losses. To evaluate the effect of crop rotation and N sources on soil microbial community structure and abundance, field research was conducted in a randomized complete block design with split plot arrangement and replicated four times with plot size of 3 m × 4 m. The three crop rotations were silage corn-silage corn (c-c), silage corn-wheat (c-w), silage corn-faba bean (c-fb). The five N sources were Control, Urea, Agrotain, Entrench and Super-U. Three soil samples from each plot were collected from 0-20 cm depth, 15 days after fertilization. Extracted DNA samples were sent to the Dalhousie University, NS for amplicon sequencing analysis. Our results revealed that N sources had no significant effect on fungal and bacterial community structure and abundance. However, crop rotation had significant effect on both fungal and bacterial community structure and abundances. Bacterial classes like *Mortierellomycetes* mean relative frequency (MRF) was significantly increased in c-w rotation as compared to c-c rotation, *Leotiomycetes* MRF was significantly increased in c-fb rotation as compared to c-w rotation, while *Tremellomycetes* MRF was significantly increased in c-c rotation when compared to c-w and c-fb rotation. Cereal legume (c-fb) rotation significantly increased fungal classes including *Actinobacteria, Thermoleophilia, Polyangia, Acidimicrobiia* and *KD4-96* MRF as compared to c-w and c-c

rotation in boreal climate. Overall, cereal-legume rotation was a significant factor in shaping the soil microbiome involved in different soil processes in podzolic soil of boreal climate.

3.2. Introduction

N is an essential macronutrient required for improving plant growth, development and crop yield. It enhances leaf area index, photosynthetic rate, plant biomass/yield reported by Kumar et al. (2021), and net assimilation rate (Barraclough et al. 2010). To maintain soil fertility and crop productivity, use of chemical fertilizers especially nitrogenous fertilizers is a common management practice (Shen et al. 2010). Chemical fertilizers like urea are easily available, high in nutrient contents, easy to transport, and rapidly used by the crops (Khan et al. 2014). However, nitrogen use efficiency (NUE) in cropping systems is very low (Giambalvo et al. 2018). For example, more than 70% N fertilizer applied in an agricultural system is lost as volatilization, leaching, and gaseous loss (N_2O) (Haroon et al. 2019).

The N fertilizers stabilizers, Agrotain, eNtrench, and SuperU contain urease inhibitors (UIs) and nitrification inhibitors (NIs) and are known to reduce ammonia volatilization losses by delaying the urea hydrolysis process and nitrification and denitrification processes by suppressing the microbial activities. Nitrification is a biologically mediated process in which ammonium (NH_4^+) is transformed to nitrate (NO_3^-) via nitrite (NO_2^-) by *Nitrosomonas* and *Nitrobacter* (Yao et al. 2016). Denitrification process is carried out by *Thiobacillus denitrificans*, *Micrococcus denitrificans* and some species of *Pseudomonas* that reduce NO_3^- to NO_2^- and then NO_2^- to nitric oxide (NO), and dinitrogen (N_2) (Thies et al. 2019). By inhibiting the ammonia monooxygenase enzyme using copper chelation, NIs prevent ammonia oxidizer activity and consequently the nitrification process is delayed resulting in the reduction of N leaching. It's been observed that NIs target AOB or AOA, which are the two most common ammonia oxidizers (Huang et al. 2012). In general, dicyandiamide (DCD) inhibits AOB rather than AOA as reported by Kawakami et al. (2012), nitrapyrin (NP) inhibited AOA is explained by Ruser and Schulz (2015), and DCD severely hampered AOA in very acidic soils (Huang et al. 2012). N stabilizers had no significant effect on fungal and microbial community composition and structure. However, the effects of UIs and NIs on microbes involved in N cycling along with non-targeted microbial communities have rarely been studied. Furthermore this is non-existent in the context of crop cultivation on podzols used for agriculture production in boreal climate (Shi et al. 2017).

There are various advantages of crop rotation including increased nutrient cycling, soil physical characteristics, and improved weed and disease control (Davis et al. 2012; Gaudin et al. 2015; Munkholm et al. 2013). Rotating cereals with legumes offer an opportunity to maintain crop diversity which not only improves yield stability, as discussed by Gaudin et al. (2013), but also influences bacterial communities, as observed by DeBruyn et al. (2011) across agricultural landscapes. Consequently, rotating legumes with silage corn in a high residue cropping sequence may increase soil organic carbon SOC or residue addition (Ortega et al. 2002). Changes in microbial biomass, diversity, and frequency of microbes are also affected by residue quality, quantity, and recalcitrance levels linked with crop rotations. (White and Rice 2009). Crop rotation significantly decreased AOB abundance in diverse crop rotation as compared to simpler continuous silage corn rotation but did not affect AOA abundance (Munroe et al. 2020). Guo et al. (2020) reported that crop rotation had no significant impact on fungal alpha diversity. Few studies also reported that crop rotation had significant effect on soil fungal community structure (Ai et al. 2018; Cassman et al. 2016). Furthermore, Chamberlain et al. (2020) reported that crop rotation (silage corn-soybean) had significant effect on soil bacteria community structure.

Duff et al. (2022) reported on the effect of different N stabilizers, while Chamberlain et al. (2020) and Ai et al. (2018) reported the effect of different crop rotation on microbial communities in different climates or jurisdictions. However, it is still unclear how N stabilizers and crop rotation affect soil microbial diversity, community, and abundance involved in nitrification and denitrification in podzols used for agriculture production in boreal climate. It was hypothesized that N stabilizers and crop rotation will affect microbial community diversity and abundance. The specific objective of this study was to investigate the effect of crop rotation and N stabilizers on fungal and bacterial community structure and diversity in podzols used for agriculture production in a boreal climate.

3.3. Materials and Methods

3.3.1 Experimental site and treatments

This experiment was comprised of two studies (chapter 2&3) with different research questions and objectives. Hence, the experimental site, treatments, and design was the same. For details, please see section 2.3.1.

3.3.2. Soil sampling and processing

Two bulk soil samples from each experimental treatment/plot were randomly collected after 15 days of N stabilizer and urea fertilizer application. In total 120 samples were collected: 5 treatments, 3 crops, 4 replicates, and 2 soil samples. Soil sampling was done from 0-20 cm depth using soil tubes (JMC Backsaver N-2 Handle, USA) and were packed in ziploc bags. To avoid contamination in samples the soil sampling tube was washed with distilled water followed by 70% ethanol and then cleaned with paper towel. All soil samples were kept on ice and transported immediately to Boreal Ecosystem Research Facility (BERF), Grenfell Campus Memorial University. Soil samples were sieved with 2 mm mesh immediately upon arrival in the lab; 2

subsamples of 5-g each were transferred into 15 mL falcon tubes and were stored at -80˚C to conduct further analyses on microbial and genes analysis.

3.3.3. DNA isolation

The DNase PowerSoil™ DNA Isolation Kit (Qiagen, Netherland) was used to isolate total genomic DNA from 0.25 g of soil according to the instructions of manufacturer. DNA quality of 280/230 and 260/230 ratios was checked by Nanodrop (Nanodrop 2000c spectrophotometer by ThermoFisher Scientific) and gel electrophoresis. DNA was further purified by using DNA cleanup kit (DNA clean & concentrator-10, catalog no. D4011) by ZYMO Research (Cedarlane Laboratories, Ltd., Canada) according to manufacturer protocol with a modification of centrifuge speed at 16000 x g.

Table 3.1 Primers used for amplicon sequencing of bacteria V6-V8 and fungi

Primers target region	**Primer**	**Sequence**	**Reference**
Bacteria-specific, 16S rRNA, V6-V8 region	B969F	ACGCGHNRAACCTTACC	(Srinivasan et al. 2015)
	BA1406R	ACGGGCRGTGWGTRCAA	
Fungi-specific, ITS2	ITS86(F)	GTGAATCATCGAATCTTTGAA	(Blaalid et al. 2013)
	ITS4®	TCCTCCGCTTATTGATATGC	

3.3.4. DNA sequencing

Ten µL of DNA samples were sent to the Dalhousie University, Nova Scotia, CGEB-Integrated Microbiome Resource (IMR) (http://cgeb-imr.ca/) for ITS2 and V6-V8 16s RRNA (16S) library preparation and sequencing. Samples were sequenced on an Illumina MiSeq with paired-end 300 + 300 bp reads after being multiplexed using a dual-indexing approach. The specifics of all PCR techniques, primers, and Illumina sequencing were as detailed in (Comeau et al. 2017). The DNA amplicon sequence was done for fungi-specific ITS2 genes as well as for prokaryotic V6-V8 16S r RNA (16S), details of all primers are given above (Table 3.1) (Blaalid et al. 2013; Srinivasan et al. 2015).

Sequence Processing: The overlapping paired-end forward and reverse reads were stitched together using Paired-End read mergeR (PEAR) Paired-End read mergeR (PEAR) was used to stitch together the overlapping paired-end forward and reverse reads (Zhang et al. 2014) before exporting them into QIIME2 (Bolyen et al. 2019). Using the Cutadept plug-in for QIIME2, the sequences were trimmed of their primers (Comeau et al. 2017; Martin 2011). Using QIIME2’s q-score-joined function, low-quality sequences were filtered from the dataset. The sequences were grouped into amplicon sequence variants (ASV), high resolution genomic groupings, using QIIME2's Deblur plug-in (Amir et al. 2017; Callahan et al. 2017; Comeau et al. 2017). Amplicon Sequencing Variant (ASV), which made up less than 0.1 percent of the total sequences, were removed to allow for potential MiSeq bleed-through between runs (estimated by Illumina to be less than 0.1 percent). Using the naive-Bayes scikit-learn function of QIIME2 and the SILVA databases (an online source for RRNA quality check and aligned sequence data), taxonomic classifications of the ASV were assigned (Bokulich et al. 2018; Quast et al. 2012). ASV associated with mitochondria and chloroplasts was additionally screened out. (Comeau et al. 2017).

3.3.5. Data analysis

Shannon indices (alpha diversity) and UniFrac matrices were calculated using QIIME2's diversity function (beta diversity) (Kim et al. 2017; Lozupone et al. 2011). In order to determine how sample groupings relate to microbial community structure, QIIME was used to assess variations in sample groupings described by UniFrac beta-diversity distances (Adonis tests, 999 permutations). Using the Vegan R package, non-metric multidimensional scaling (NMDS) of bacterial populations was carried out on Bray-Curtis matrices (Oksanen et al. 2016). Using ALDEx2, differential abundances of bacterial species were determined (Fernandes et al. 2014) with the Kruskal-Wallace test's Benjamini-Hochberg corrected p value ($p < 0.05$), ggplot2 was used to make the graphics (Wickham et al. 2016).

3.4. Results

3.4.1. Effects of N sources and crop rotation on bacterial and fungal communities

A total of 1,467,271 high-quality ITS2 sequences covering 1,089 features were obtained from 120 samples, with a mean frequency of 12,227 reads/sample, and a median frequency of 9,962 reads/samples. 381,330 reads were kept after normalisation to a depth of 3,345 reads (the depth of the smallest dataset after the six lowest samples was eliminated). The distribution of these reads was across 1,088 features. The most relative abundant fungal taxa in a total microbiome were *Mortierellomycetes, Sordariomycetes, Leotiomycetes, Dothideomycetes, Tremellomycetes,* and *Agaricomycetes* represented by 26%, 24%, 16%, 11, 9%, and 7% of total ITS reads, respectively (Figure 3.1, A).

A total of 2,819,661 high-quality 16S sequences covering 9,649 features were obtained from 120 samples, with 23,497 reads/sample mean frequency and 21,501 reads/samples median frequency. 1,093,161 readings were kept after normalization to a depth of 10,029 reads (the depth of the smallest dataset after the 11 lowest samples were eliminated). These reads were distributed across 9,649 features. The most abundant bacterial taxa in total microbiome were *Abditibacteria, Acidobacteriota, AT-s3-28, Acidobacteriae, Blastocatellia* and *Holophagae* represented by 18%, 15%, 11%, 6.5%, 5.4%, and 5% of total 16S reads, respectively. (Figure 3.1B).

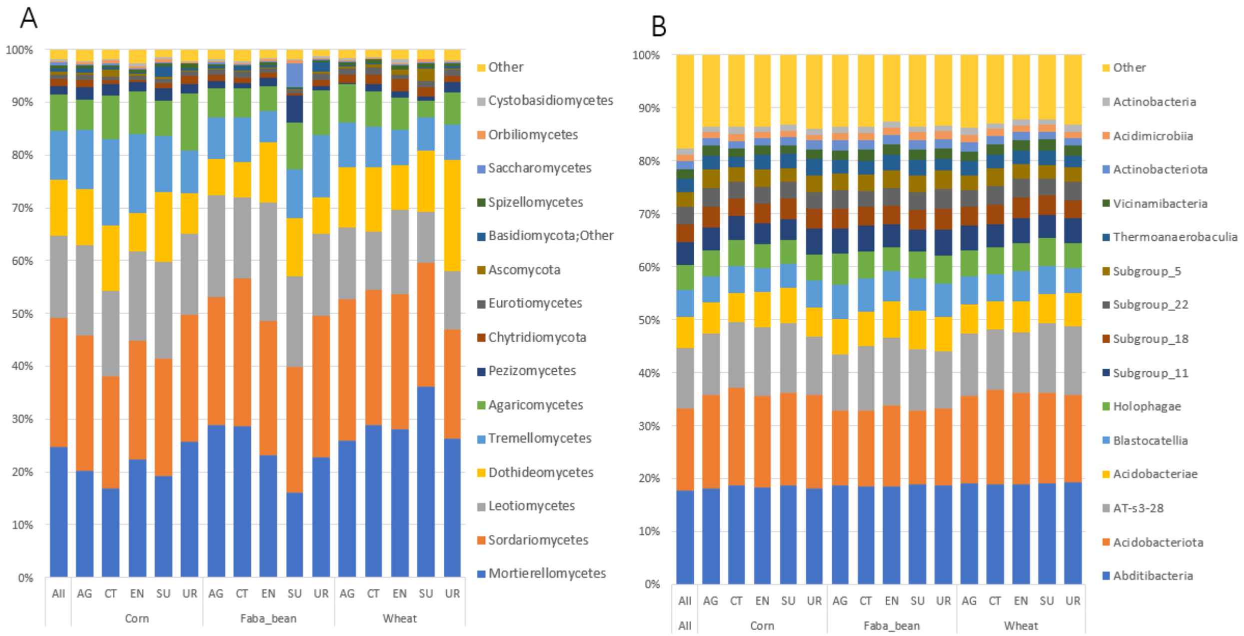

Figure 3.1: The top 15 most relatively abundant fungal taxa (A) and bacterial taxa (B) in silage corn, faba bean and wheat rotated with silage silage corn in podzolic soil in boreal climate.

3.4.2. Effect of N sources and crop rotation on fungal and bacterial community structure

The visualization of differences between fungal communities based on type in N application did not reveal a visible trend in beta diversity (Figure 3.2, A). The type of N treatment was not a significant factor influencing the fungal community, according to the analysis of the sample groupings' strength and statistical significance ($R^2 = 0.02$, $p > 0.05$). However, we detected some visual separation between fungal microbiomes grouped by crop rotation (Figure 3.2, B). The study of the statistical significance and strength of the sample groupings showed that the crop was a significant factor influencing the fungal community ($R^2 = 0.06$, $p < 0.05$), supporting the visual community separation.

Similar to fungal community, N sources did not reveal a visible trend in beta diversity visualization of dissimilarity between bacterial communities (Figure 3.2, C) and N sources was not a significant factor shaping bacterial community ($R^2 = 0.02$, $p > 0.05$) based on the analysis of strength and statistical significance of sample groupings. However, crop rotation was also a significant factor in shaping a bacterial community ($R^2 = 0.08$, $p < 0.05$), which was also confirmed by some visual community separation (Figure 3.2, D).

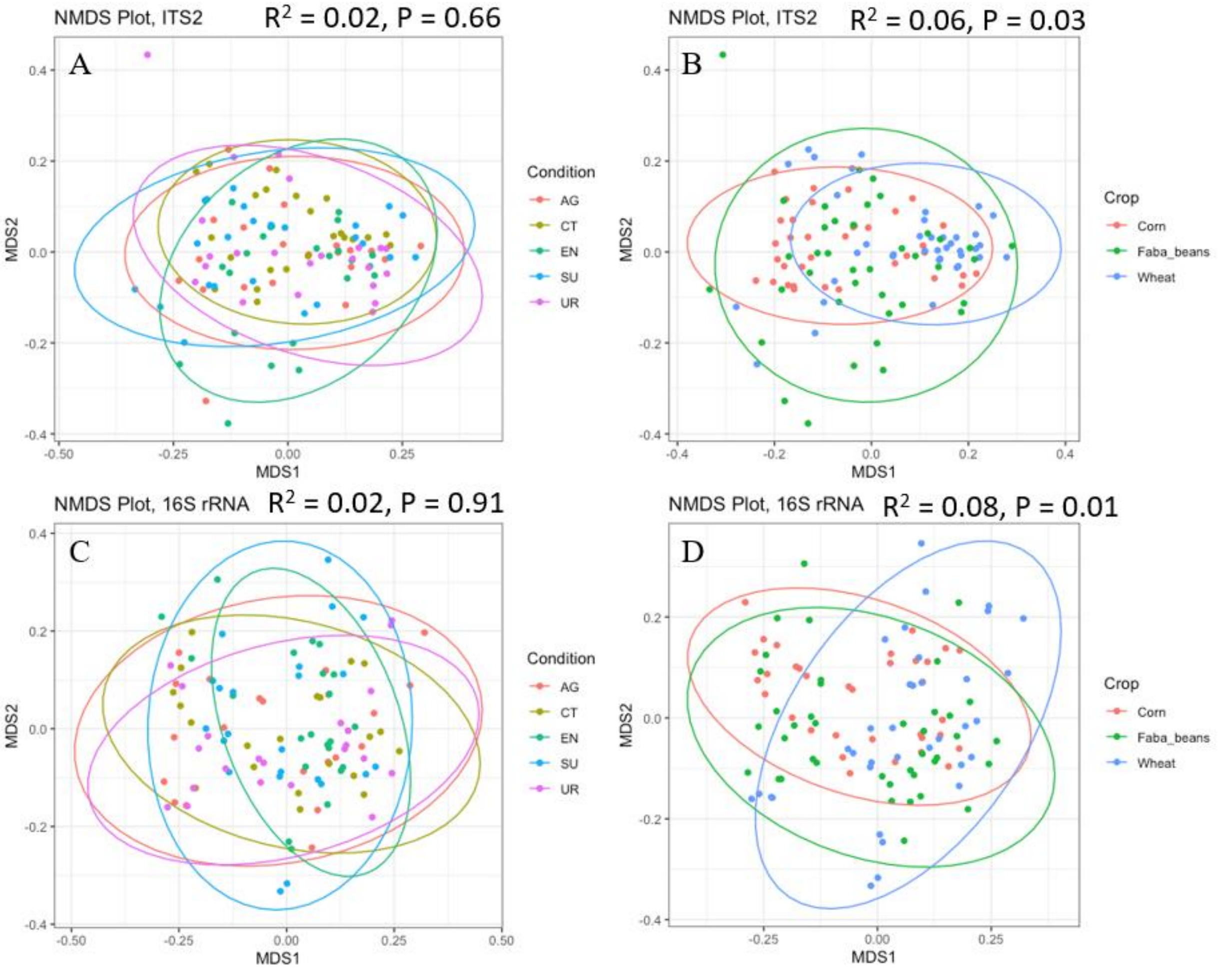

Figure 3.2: Variation in sample groupings as explained by weighted UniFrac dissimilarity distances and Non-metric multidimensional scaling (NMDS) of bacterial and fungal communities. Communities' differences based on Bray-Curtis distances. Effect of N sources (A and C) and crop rotation (B and D) on fungal (A and B) and bacterial (C and D) community structure. To determine whether beta-diversity is connected to sample groupings, 999 permutations, R2 –Adonis tests were used. The importance of sample grouping was determined using weighted UniFrac beta-diversity distances. AG – Agrotain (urease inhibitor), CT – Control, EN – eNtrench (nitrification inhibitor),

SU – Super-U (combination of urease and nitrification inhibitors), UR – Urea and crop rotation silage corn, faba bean and wheat.

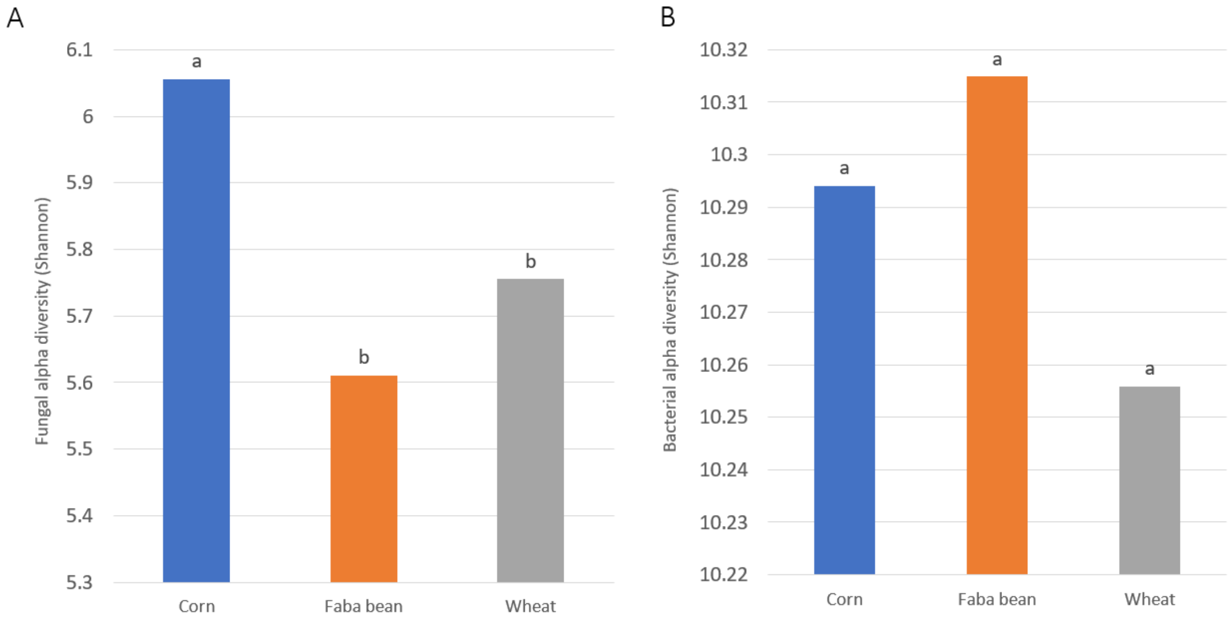

Figure 3.3: Estimated Shannon diversity. Fungi (A) and bacteria (B). Different letters showing significant difference according to Kruskal-Wallis pairwise test ($P < 0.05$) in silage corn, faba bean and wheat.

We did not observe any change due to use of different types of N application on fungal Shannon diversity within each crop rotation group. We also did not detect any difference in the alpha-diversity between crops for any type of N application. However, when all N treatments were combined together the crop significantly affected the fungal alpha-diversity. Fungal microbiomes associated with silage corn rotation exhibited an increase in Shannon diversity, compared to those associated with faba bean and wheat rotation (Figure 3.3, A). However, no significant change was detected between different types of N application within each crop rotation group as well as between crops for any type of N application on bacterial Shannon diversity (Figure 3.3, B).

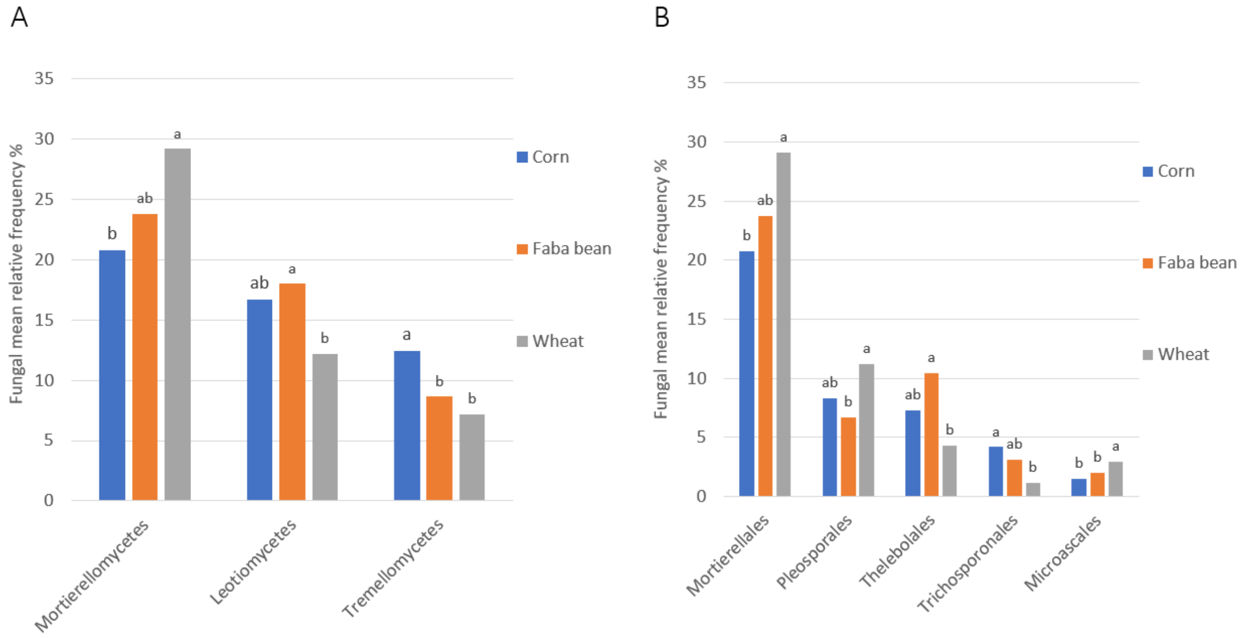

Figure 3.4: Fungal taxa that were differentially represented between soils under silage corn, fab bean and wheat rotation. Based on Kruskal-Wallace test with Benjamini-Hochberg multiple test corrected P value <0.05. A – class level, B – order level.

Silage corn, faba bean and wheat crop rotation had significant effect on fungal mean relative frequency (MRF) at class level. *Mortierellomycetes* MRF was significantly increased in wheat as compared to silage corn by 28.7% though statistically at par with faba bean. While MRF of *Leotiomycetes* was significantly increased in faba bean as compared to wheat by 32.3% though statistically at par with silage corn. *Tremellomycetes* MRF was significantly increased in silage corn when compared to faba bean and wheat by 30.5% and 42.3%, respectively (Figure 3.4, A). Silage corn, faba bean, and wheat rotation had significant effect on fungal MRF at order level. *Mortierellales* MRF significantly increased in wheat as compared to silage corn by 28.7% though statistically at par with faba bean. *Pleosporales* MRF significantly increased in wheat as compared to faba bean by 40.4% though statistically at par with silage corn. MRF of *Thelebolales* significantly increased in faba bean as compared to wheat by 58.6% though statistically at par with silage corn. *Trichosporonales* MRF significantly increased in silage corn as compared to wheat by 71.8% though statistically at par with faba bean. While *Microascales* MRF significantly increased in wheat when compared to silage corn and faba bean by 50% and 32%, respectively (Figure 3.4, B).

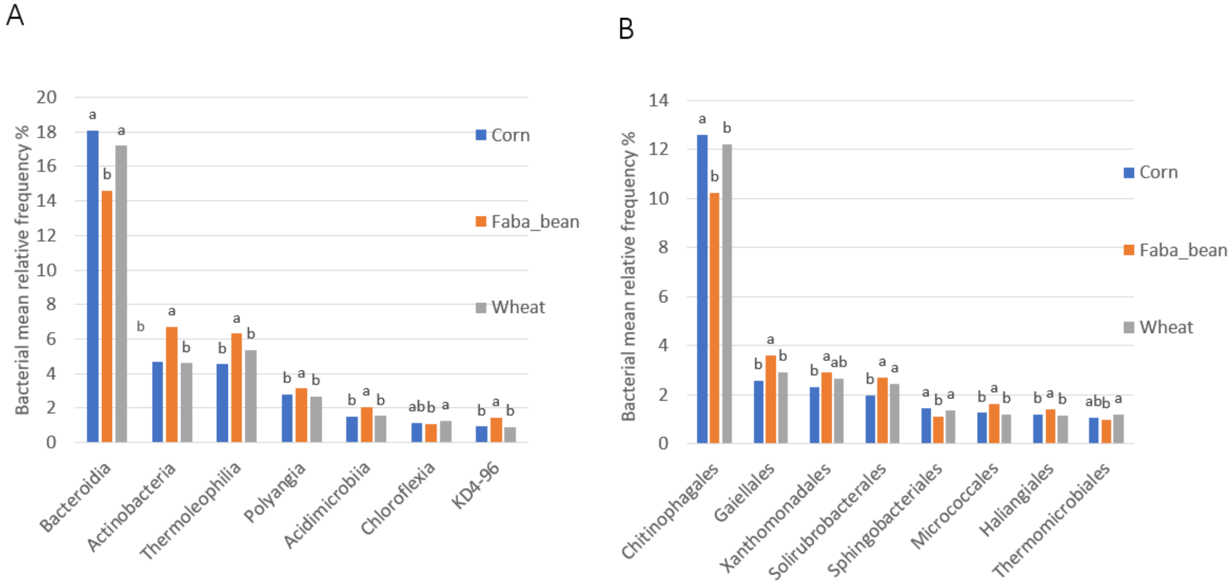

Figure 3.5: Bacterial taxa that were differentially represented between soils under silage corn, fab bean and wheat rotation with silage silage corn. Based on ALDEx2 Benjamini-Hochberg corrected P value <0.05 of Kruskal-Wallace test. A – class level, B – order level.

Crop rotations silage corn-silage corn; silage corn-faba bean, and silage corn-wheat had significant effects on bacterial mean relative frequency (MRF) at class level. *Bacteriodia* MRF was significantly higher (19%) in silage corn-silage corn compared to silage corn-faba bean though statistically at par with silage corn-wheat crop rotation. *Actinobacteria, Thermoleophilia, Polyangia, Acidimicrobiia* and *KD4-96* MRF were significantly increased by 30%, 28.2%, 11.7%, 25.3%, and 36.3%, in silage corn-faba bean rotation, and 31.6%, 15.2%, 15.2%, 24.3%, and 37.6%

in silage corn-wheat rotation compared to continuous silage corn rotation respectively. However, *Chloroflexia* MRF was significantly increased by 17% in silage corn-wheat compared to silage corn-faba bean though statistically at par with continuous silage corn rotation (Figure 3.5, A).

Likewise, crop rotation had a significant effect on bacterial MRF at order level. Significantly higher *Chitinophagales* MRF was observed in silage corn-silage corn compared to the lowest observed in silage corn-faba bean but was statistically on par with silage corn-wheat rotation. Whereas silage corn-faba bean rotation exhibited significantly higher *Gaiellales, Micrococcales,* and *Haliangiales* MRF and the lowest *Sphingobacteriales* compared to silage corn-wheat and silage corn-silage corn rotation. Silage corn-faba bean rotation showed significantly higher MRF of *Xanthomonadales*, and *Solirubrobacterales* compared to lower levels in continuous silage corn, though statistically on par with silage corn-wheat. Silage corn–wheat rotation showed significantly higher MRF of *Thermomicrobiales* compared to silage corn-faba bean which showed the lowest MRF (Figure 3.5, B).

3.5. Discussion

Soil microbes play a significant role in many soil processes including N and carbon cycling, organic material decomposition, conversion of plant residues to SOM, and are also involved in various soil biophysical processes (Critter et al. 2004; Van Der Heijden et al. 2008). Soil properties, seasonality or different management practices may affect the soil microbial communities composition (Garbeva et al. 2004). Crop rotation is a type of agricultural management that has been proven as one of the most effective ways to increase the diversity of soil microbes (Benitez et al. 2017; D'Acunto et al. 2018; Peralta et al. 2018).

In this study 3 different agronomic factors including N sources, crop rotation and different N fertilizer rate (silage corn 125 kg ha^{-1}, wheat 100 kg ha^{-1}, faba bean 25 kg ha^{-1}) were studied. These fertilizer rates were recommended based on local soil tests due to crop requirement. The lowest fertilizer application rate was in faba bean because it is a legume crop, and it can fix atmospheric N and add to the soil system. However, as bulk soil samples were taken after 15 days of fertilizer application, it is possible that N fixation via a legume might have not started by the soil sampling time. Results of this research showed that there was non-significant effect of N sources while it was significant for crop rotation (specially between silage corn and faba bean) on soil fungal and bacterial community structure and composition, that might be due to different fertilizer rates between silage corn and faba bean. Furthermore, in literature there were different reasons reported explaining the effect of different crop rotations on soil rhizosphere fungal and bacterial communities, which are discussed below.

Several fungal taxa were differentially represented between rotational groups (Figure 3.4 A & B). The relative abundances of *Leotiomycetes* were significantly increased in silage corn-faba bean rotations as compared to silage corn-wheat rotations and the relative abundance of *Tremellomycetes* were significantly increased in silage corn-silage corn rotations as compared to silage corn-wheat and silage corn-faba bean rotations. *Leotiomycetes* are know to decompose organic matter (Baschien et al. (2013), and *Tremellomycetes* play role in mineralization and utilization of recalcitrant carbon (Yu et al. 2018). It has been documented that the root exudates produced by the host plant select rhizosphere microbiota, depend on the quantity and chemical composition of root exudates which affect the presence and structure of the related microorganisms. (Hu et al. 2018). It appears that plant secondary metabolites released through roots may persist in the soil and along with plant residues which influence the soil microbial community structure. Therefore, the variations in fungal composition or fungal taxa found in our study could be attributed to plant-host specific symbionts and pathogens (Maarastawi et al. 2018).

Furthermore, few studies also explained that successional changes in microbial community composition in bulk soil induced by residue decomposition (Bastian et al. 2009; Conrad et al. 2012; Tardy et al. 2015). Furthermore, Schloter et al. (2018) reported that microbial diversity in bulk soils may be more important as it is considered to be the rhizosphere's resource library, hence the impact of plant roots is frequently transient in rhizosphere and can be moved to bulk soil.

Moreover, supply of organic carbon compounds which affect fungal composition differ to some extent due to different rotating crops. It is further observed by Maarastawi et al. (2018) that maize monocropping results in enrichment of the host-specific plant microorganisms, while host specific plants have specific fungal or bacterial regime under different crop rotation. Berg and Smalla (2009) and Ladygina and Hedlund (2010) reported that plant specific rhizodeposition processes develop the host plant specific rhizosphere communities. In this way, the difference among fungal classes could be due to different plant-host specific symbionts in cereal and legume rotation. It has been reported that plant diversity has significantly and positively correlated with soil fungal alpha diversity (Shen et al. 2021). In the present study, soil fungal alpha diversity significantly increased in silage corn-silage corn rotation as compared to silage corn-wheat and silage corn-faba bean rotation (Figure 3.3A). A study reported that plant growth stage strongly influence the fungal alpha diversity in rhizosphere than in the bulk soils (Wang et al. 2017). Moreover, it has been reported that depending on their stage of development and health status, plants can choose certain taxa and have some influence over their microbiomes. (Bulgarelli et al. 2012; Vujanovic et al. 2007). As samples were taken during early stage (emergence), that might result in selection of specific taxa resulting in the significant differences in fungal alpha diversity between cereal and legume rotation. Findings of this study are consistent with the studies reported by Ai et al. (2018) and Cassman et al. (2016) that crop rotation had significant effect on soil fungal community structure, while other researchers reported that short-term nutrients addition did not have a significant effect on soil microbial community structure and activity (Geisseler and Scow 2014; Roberts et al. 2011; Stark et al. 2007).

In the present study, silage corn-faba bean (legume) rotation significantly affected bacterial composition at class level and order levels (Fig. 3.5) compared to continuous silage corn cropping (monocropping) and silage corn-wheat rotation. Several bacterial classes including *Actinobacteria, Thermoleophilia, Polyangia, Acidimicrobiia* and *KD4-96* MRF significantly increased in silage corn-faba bean rotation as compared to silage corn-wheat rotation and continuous silage corn rotation. Actinobacteriota phylum consisting of classes *Actinobacteria, Thermoleophilia* and *Acidimicrobiia* are involved in breading down of wide range of plant debris in decomposition process (Schaechter 2009). *KD4-96* class belonging to phylum Chloroflexi may have possible role in carbon cycling in respiration by sugars and CO_2 fixation via acetogenesis reported by Schmidt (2019). A recent study conducted in the Canadian prairies reported that inclusion of a pulse crop (legume) in the rotation with wheat altered the rhizosphere bacterial composition (Hamel et al. (2018) or the fungal community (Borrell et al. 2017). Diverse crop rotation can alter the composition of soil microbial community, diversity and richness via root exudates and other rhizodeposits as reported by Dennis et al. (2010), Soman et al. (2017) and Ridl et al. (2016). Additionally, root exudation varies among cultivars, plant species, or even their developmental stage (Papik et al. 2020). Some of the phyla like *Actinobacteria* and *Acidobacteria* were observed more abundant in arable soils (Delgado-Baquerizo et al. 2018; Janssen 2006). Some of these bacteria are known as copiotrophic bacteria (low C use efficiency and fast growing) and were observed to be more abundant in rhizosphere soil and respond to root exudation (Fierer et al. 2007; Leff et al. 2015). Furthermore, Zhao et al. (2015) reported that humus content which affect soil properties that determine soil bacterial community significantly reduced in long-term maize monoculture. Results of this study are consistent with the studies which reported that crop rotation (silage corn-soybean) had a significant effect on soil bacterial community structure (Chamberlain et al. 2020; Soman et al. 2017).

The higher diversity of plant residues in the organic mixture during silage corn-wheat and silage corn-faba bean rotations may encourage the diversification of the microbiota (Santschi et al. 2018). Furthermore, compared to graminoid plants (grasses) with labile organic carbon compounds (higher C/N ratio and cellulose and lignin content), nitrogen-fixing plants, such as legumes (faba bean), which can fix atmospheric N and add in the soil system, can produce different chemical compounds in their plant residues (Cartenì et al. 2018). In general, decomposition rate is faster when high N crop residues combined with low lignin content, result in specific selection of saprotrophic microbiota (Bonanomi et al. 2019). A deeper knowledge of the function that crop rotation plays in terms of plant tissue chemistry would be a vital step in generating reliable guidelines for proper crop rotation management because crop residues and root turnover are the main sources of organic carbon for microbiota. This research explains that silage corn-faba bean rotation significantly enhanced bacterial and fungal diversity and richness compared to continuous silage corn crop rotation (monocropping). This suggests that faba bean and wheat caused a strong microbial selection and signalling due to root exudates which reshaped the microbial community, diversity and abundance in soil system. However, more research is required to determine whether rhizodeposits have an ongoing impact on the composition of the soil's microbial community structure and diversity in boreal climate.

3.6. Conclusion

This study reveal that N sources had no significant on fungal and bacterial community structure and abundance, however, crop rotation significantly affected both fungal and bacterial community structure and abundance. Silage corn-silage corn rotation significantly increased fungal Shannon alpha diversity as compared to c-w and c-fb, but crop rotation had no significant effect on the bacterial alpha-diversity (Shannon). Crop rotation significantly affected bacterial composition at class level. *Mortierellomycetes* mean relative frequency (MRF) was significantly increased in c-w rotation as compared to c-c rotation, *Leotiomycetes* MRF was significantly increased in c-fb rotation as compared to c-w rotation, while *Tremellomycetes* MRF was significantly increased in c-c rotation when compared to c-w and c-fb rotation. Fungal composition at class level also significantly affected by crop rotation, c-fb rotation significantly increased fungal classes including *Actinobacteria, Thermoleophilia, Polyangia, Acidimicrobiia* and *KD4-96* MRF as compared to c-w and c-c rotation. Cereal legume (c-fb) rotation significantly increased fungal classes (*Actinobacteria, Thermoleophilia, Polyangia, Acidimicrobiia* and *KD4-96*) MRF when compared to c-w and c-c rotation in boreal climate. Overall, this study concludes that crop rotation was a significant factor in shaping soil microbiome and resulted in change in fungal and bacterial composition in podzolic soil in a boreal climate. However, long term studies are required to better understand the effects of crop rotation and fertilization on fungal and bacterial community structure and abundance in a boreal climate.

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Chapter 4

4. General discussion and conclusion

Overall, objectives of this study were to investigate the effect of N stabilizers and crop rotation on:

i. Soil pH, EC and mineral N dynamics in podzolic soils in boreal climate.
ii. Relative gene abundance involved in nitrification and denitrification processes.
iii. Microbial community structure and abundance in podzolic soils in boreal climate.

To achieve these objectives, field experiments were conducted which are described in Chapter 2 and 3. Effect of N stabilizers and crop rotation on soil pH, EC, mineral N dynamics and relative gene abundance in nitrification and denitrification have been described in chapter 2. Whereas effect of N fertilizer stabilizers and crop rotation on soil microbial community structure were discussed in chapter 3.

4.1. Effect of nitrogen sources and crop rotation on soil pH, EC and mineral nitrogen

4.1.1. Effect of N sources and crop rotation on soil pH and EC

Soil pH is considered as an important factor that regulates plant nutrient availability (Oshunsanya 2018). However, soil pH could be influenced by few factors. For example, urea application increases the soil acidity or decreases soil pH as one OH^- release during hydrolysis to NH_4^+ but subsequent nitrification to NO_3^- releases two H^+, resulting in decrease soil pH and increased soil acidification. The response of UR and AG application was similar, both significantly reduced soil pH compared to control and other N stabilizer amended treatments. AG is a urea coated with NBPT (UI) which delays urea hydrolyses, however, different soil factors like high soil moisture content decrease the effectiveness of UI in soil system (Matczuk and Siczek 2021). In this study high moisture condition in some plots were also observed that might the efficacy of UI reduced. Other N stabilizers such as EN and SU application didn’t decrease soil pH due to delayed ammonia oxidation by NI and UI. In SU (combination of NI and UI), soil pH was also similar to control. Adhikari et al. (2021) reported that soil properties like moisture content can affect the efficacy of NI. Results of this study supported by studies, who reported that the application of UR and UI significantly decreased soil pH due to release of extra H^+ during NH_4^+ hydrolysis process (Chen et al. 2019; Fu et al. 2020; Shen et al. 2016; Zhou et al. 2015). N sources, crop rotation and their interaction (N × crop rotation) had no significant effect on soil EC.

4.1.2. Effect of N sources and crop rotation on soil NH_4^+ and NO_3^- concentration

Plant growth and crop productivity is strongly determined by N availability. Plants use N in different forms including NH_4^+ and NO_3^- (Hachiya and Sakakibara 2017). Soil NH_4^+ is less dominant form of mineral N with low concentration in soil less taken up by the plants as compared to NO_3^- (Li et al. 2013).

Higher NH_4^+ concentration was observed in AG amended treatment in silage corn compared to UR but not statistically significant, it could be due to the presence of urease inhibitor (NBPT) that is play role in delaying urea hydrolysis process. Zaman et al. (2008) reported that UI can delay urea hydrolysis up to 14 days, due to its mode of action by blocking the three active sites of the urease enzyme inhibiting hydrolysis and increasing the N supply (Cantarella et al. 2018). After 15 days of fertilizer application higher NH_4^+ concentration could be available for plants due to the reduced effect of NBPT. Results of this research are in agreement with Sigurdarson et al. (2018) reported that NBPT treated soil showed lower pH compared to UR alone, which might have affected the NH_3 and NH_4^+ equilibrium in the soil, which might result in reduced NH_3 loss as gas and increased the soil NH_4^+ concentration. However, Soil NH_4^+ was higher in N stabilizers (AG, EN, SU) as compared to UR in silage corn and faba bean but effect was non-significant, because high moisture content affect the effectiveness N stabilizers. Matczuk and Siczek (2021) reported that UI efficacy, while Adhikari et al. (2021) reported that NI efficacy affected under different soil properties like high moisture content. Soil NH_4^+ was significantly higher in silage corn and wheat as compared to faba bean with N stabilizers and UR application, possibly due to higher N fertilizer application rates 115 kg ha^{-1} in silage silage corn and 100 kg ha^{-1} in wheat compared to 25 kg ha^{-1} in faba bean. Results of present study are consistent with the studies who reported that increasing N application rate result in increased soil NH_4^+ concentration due to increased UR hydrolysis (Chen et al. 2019; Shen et al. 2016; Zhou et al. 2015).

Nitrification inhibitors reduced soil NO_3^- concentration in silage silage corn and wheat, though statistically non-significant with UR application. In nitrification process, NH_4^+ converted to NO_3^- via NO_2^-. This reduction could be due to limiting rate of substrate availability (NH_4^+) as we can observe higher NH_4^+ concentration in N stabilizers as compared to UR (Figure 2.3), showing ammonia oxidation inhibition by NI and UI. Slow conversion of urea to NH_4^+ and then NH_4^+ to NO_3^- in the nitrification process might be due to as NBPT can delay the UR hydrolysis process due to its mode of action as discussed above, which result in reduced NO_3^- concentration compared to UR application (Figure 2.4). Additionally, NI suppresses the ammonia oxidizers activity by binding to the ammonia monooxygenase' active sites, which result in the slow conversion of NH_4^+ to NO_3^- in the nitrification process (Shi et al. 2017). However, there was no significant difference between N stabilizers and UR on NO_3^- concentration might be due to lack of N stabilizers effectiveness as discussed above. Soil NO_3^- was significantly higher in silage corn and wheat with N stabilizers and UR application compared to faba bean due to higher NH_4^+ concentration in silage corn and wheat as compared to faba bean (Figure 2.3), as NH_4^+ convert to NO_3^- in the nitrification process.

4.1.3. Effect of N sources and crop rotation on relative gene abundance involved in nitrification and denitrification

AOA are responsible for the oxidation of NH_4^+ to NO_2^- in first and rate-limiting step of nitrification and the gene responsible for this process is ammonia monooxygenase (*amo*A). This process is important as it proceeds to complete nitrification by oxidizing to NO_3^- and then transformed to denitrification process of N cycle. N stabilizers significantly reduced the *amo*A relative gene abundance as compared to UR (Figure 2.5a), while the lowest *amo*A gene abundance was observed in SU. As SU is the combination of NI and UI, NI suppresses the ammonia oxidizer activity due to its mode of action by blocking the active sites of *amo*A gene. While UI delays the UR hydrolysis by blocking the active sites of urease enzymes and enhance the N supply in the form of NH_4^+ in the soil system which lead to slow conversion to NO_3^- resulting in slowing down the nitrification process and reduced the *amo*A relative gene abundance, which can be observed. Consistent to findings of this study, few studies have also reported that NI and UI decreased (NH_4^+) substrate availability as compared to UR which result in decrease AOB gene copy numbers (Fan et al. 2018; Luchibia et al. 2020; Shi et al. 2017).

Oxidation of NO_2^- to NO_3^- in the nitrification process is done by *nxr-nitrospira* gene, while reduction of NO_2^- to NO in denitrification process is done by *nir*K, and *nos*Z is responsible for the reduction of N_2O to N_2 in the denitrification process. All N stabilizers significantly reduced relative gene abundance of *nxr-nitrospira*, *nir*K, and *nos*Z compared to UR due to their mode of action as explained above. As a result N stabilizers reduced nitrification process (NH_4^+ to NO_2^- then to NO_3^-) and then NO_3^- to nitrogenous gases in denitrification process due to limited substrate (NO_3^-) availability (Figure 2.4) result in less gene abundance as compared to UR. Similar to findings of this study, Luchibia et al. (2020) reported that NBPT (UI) significantly reduced nitrospira in their incubation experiment due to reduced substrate (NO_3^-) availability; Wang et al. (2017a) reported that DCD and nitrapyrin (NIs) significantly inhibited relative abundance of *nir*K genes and then denitrification rate due to less NO_3^- availability.

Microbial biomass and activities as well as soil fertility can be maintained by crop rotation (Munkholm et al. 2013; Munroe et al. 2020). N mineralization rate can be affected by crop rotation, through changing soil temperature, moisture, pH (Kladivko 2001). Additionally, rotation with legume crop has ability to fix atmospheric contribute N in the soil profile (Gaudin et al., 2013). Crop rotation specifically with legume had significant effect on the relative gene abundance involved in nitrification and denitrification (Linton 2020; Munroe et al. 2020; Xu et al. 2018). In present study, crop rotation had significant effects on relative abundance of all genes involved in nitrification and denitrification process. Significantly lower relative genes abundance was observed in faba bean as compared to silage corn and wheat (Figure 2.6). There could be two possible reasons reported in the literature, Congreves et al. (2015) reported that when legume was rotated with silage corn as compared to silage corn monoculture result in increased soil aggregate stability and SOM which can directly or indirectly shape nitrifying communities. Another study also explained the interaction between N-cycling bacterial communities and SOM, that ammonia oxidizers community increased with addition of OM as compared to control (Tao et al. 2017). This might be due to change in SOM as a result of nature of crop rotation. Furthermore, Riyo (2018) reported that rate of the change of organic N to mineral N can also affected by crop rotation through changing soil moisture, temperature, pH. All above explained factors were reported in the literature affecting the microbes involved in nitrification and denitrification.

4.2. Effect of N sources and crop rotation on fungal and bacterial community structure and composition

Soil microbes play key role in many soil processes including N and carbon cycling, organic material decomposition, soil organic matter conversion through plant residues, and also involve in various soil biophysical processes (Critter et al. 2004; Van Der Heijden et al. 2008). Soil microbial communities composition may affected by soil properties, seasonality or different management practices (Garbeva et al. 2004). It has been reported that there are so many agricultural management practices but crop rotation has been considered as one of the most favorable practices that affect soil microbial diversity (Benitez et al. 2017; D'Acunto et al. 2018; Peralta et al. 2018). In this study, N sources had non-significant effect while crop rotation had significant effect on fungal community structure and composition. Fungal composition at class and order level had significantly affected by crop rotation (Figure 3.4 A & B). The relative abundances of *Leotiomycetes* were significantly increased in silage corn-faba bean rotation as compared to silage corn-wheat rotation. Different response of fungal classes were observed in different crop rotation, the relative abundance of *Tremellomycetes* were significantly increased in silage corn-silage corn rotation as compared to silage corn-wheat and silage corn-faba bean rotation. Baschien et al. (2013) reported that *Leotiomycetes* play role in decomposition of organic matter while *Tremellomycetes* play role in mineralization and utilization of recalcitrant carbon (Yu et al. 2018). These variations in fungal composition or fungal taxa could be attributed to plant-host specific symbionts and pathogens (Maarastawi et al. 2018). Moreover, fungal composition affected by the supply of organic carbon compounds which differ to some extent due to different crop rotations. Further observed by Maarastawi et al. (2018) that maize mono-succession result in enrichment of host-specific plant microorganisms, while host specific plants have specific fungal or bacterial regime under different crop rotation. Berg and Smalla (2009) and Ladygina and Hedlund (2010) reported that plant specific rhizodeposition processes develop the host plant specific rhizosphere communities. In this way, the difference among fungal classes could be due to different plant-host specific symbionts in cereal and legume rotation. Furthermore Schloter et al. (2018) observed that microbial diversity in bulk soils which is considered as the rhizosphere's resource library may be more important, thus the effect of plant roots is often temporary in rhizosphere and can be shift to bulk soil. As we also used the bulk samples in this study, so bulk soil could be more important evaluating the microbial diversity. However, Shen et al. (2021) reported that soil fungal alpha diversity has significantly and positively correlated with plant diversity. In this study, soil fungal alpha diversity significantly increased in silage corn-silage corn rotation as compared to silage corn-wheat and silage corn-faba bean rotation (Figure 3.3A). A study explained that plant growth stage strongly influence the fungal alpha diversity in rhizosphere than in the bulk soils due to root exudation (Wang et al. 2017b). Moreover, It has been reported that, plants select for specific taxa an exert some control over their microbiomes depending on plant growth stage and health status (Bulgarelli et al. 2012; Vujanovic et al. 2007). As we took samples during early stage (emergence), that might result in selection of specific taxa resulting in the significant differences in fungal alpha diversity between cereal and legume rotation.

It has been proven that the host plant produce root exudates which select rhizosphere microbiota, their quantity and the chemical composition influence the abundance and structure of the microbes (Hu et al. 2018). It appears that plant secondary metabolites released through roots may persist in the soil and together with plant residues which influence the soil microbial community structure. Findings of present study are consistent with the studies reported by Ai et al. (2018) and Cassman et al. (2016) that crop rotation had significant effect on soil fungal community structure, while other reported that short-term nutrients addition had always non-significant effect on soil microbial community structure and activity (Geisseler and Scow 2014; Roberts et al. 2011; Stark et al. 2007).

In current study, cereal-legume (silage corn-faba bean) rotation significantly affected bacterial composition at class level and order level (Fig. 3.5) compared to continuous silage corn cropping (monocropping) and cereal-cereal (silage corn-wheat) rotation. Most of the fungal classes including *Actinobacteria, Thermoleophilia, Polyangia, Acidimicrobiia* and *KD4-96* MRF significantly increased in silage corn-faba bean rotation as compared to silage corn-wheat rotation and continuous silage corn rotation. Fungal classes including *Actinobacteria, Thermoleophilia* and *Acidimicrobiia* belong to Actinobacteriota phylum are involved in breading down of wide range of plant debris in decomposition process (Schaechter 2009). However, phylum Chloroflexi consisting of class *KD4-96* may have possible role in carbon cycling in respiration by sugars and CO_2 fixation via acetogenesis discussed by Schmidt (2019). In a recent study it was reported that inclusion of a pulse crop (legume) in the rotation with wheat altered the rhizosphere bacterial composition in Canadian prairie observed by Hamel et al. (2018) or the fungal community (Borrell et al. 2017). Diverse crop rotation can alter the soil microbial composition, diversity and richness via root exudates and other rhizodeposits as reported by Dennis et al. (2010), Soman et al. (2017) and Ridl et al. (2016). Additionally, Papik et al. (2020) explained that root exudation varies among plant species, cultivars or even their developmental stage. However, some of the phyla like *Actinobacteria* and *Acidobacteria* were observed more abundant in arable soils (Delgado-Baquerizo et al. 2018; Janssen 2006). Some of these bacteria are known as copiotrophic bacteria (low C use efficiency and fast growing) were observed more abundant in rhizosphere soil and respond to root exudation (Fierer et al. 2007; Leff et al. 2015). Furthermore, Zhao et al. (2015) reported that humus content were significantly reduced with long-term maize monoculture which affect soil properties that determine soil bacterial community. Consistent with this study other also reported that crop rotation (silage corn-soybean) had significant effect on soil bacterial community structure (Chamberlain et al. 2020; Soman et al. 2017).

In cereal-legume (silage corn-faba bean), and cereal-cereal (silage corn-wheat) rotation, diversification of the microbiota can be promoted by greater plant residue diversity (Santschi et al. 2018). Moreover, the chemistry of plant residues can differ depending on nitrogen-fixing plants, like legumes (faba bean), which can fix atmospheric nitrogen and add to the soil profile, in comparison to graminoid plants (grasses), which have labile organic carbon compounds and a higher C/N ratio as well as a higher content of cellulose and lignin. (Cartenì et al. 2018). In general, when high N crop residues combined with low lignin content result in faster decomposition rate, further result in specific selection of saprotrophic microbiota (Bonanomi et al. 2019). In light of the fact that crop residues and root turnover serve as the main sources of organic carbon for microbiota, a deeper comprehension role of crop rotation plays in terms of plant tissue chemistry would be a crucial first step in creating trustworthy guidelines for proper crop rotation management. In this study, there could be 3 different factors that can affect soil microbial community including N sources, crop rotation and different N fertilizer rate (silage corn 125 kg ha^{-1}, wheat 100 kg ha^{-1}, faba bean 25 kg ha^{-1}). These fertilizer rates vary among crops due to crop requirement. Lowest rate was in faba bean because it is a legume crop, and it can fix atmospheric N and add to the soil system. However, as we did bulk soil sampling after 15 days of fertilizer application so at that time it is possible that N fixation by legume was not started at the time of sampling. Results of this research show that there was non-significant effect of N sources while significant effect of crop rotation (specially between silage corn and faba bean) on soil fungal and bacterial community structure and composition, that might be due to different fertilizer rate between silage corn and faba bean.

This study reveals that cereal-legume (silage corn-faba bean) rotation significantly increased bacterial and fungal composition as compared to continuous silage corn crop rotation (monocropping). This suggests that cereal and legume rotation caused a strong microbial selection and signalling due to root exudates which might be responsible for reshaping microbial community, diversity and abundance. However, more research is required to identify a potential long-lasting impact of rhizodeposits on the soil microbial community structure and diversity in podzolic soils of boreal climate.

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