SOIL HEALTH AND SOIL MICROBIAL RESPONSES TO CASH CROP MANAGEMENT IN ONTARIO

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ABSTRACT

Soil health and soil microbial responses to cash crop management in Ontario

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In Ontario, farmers commonly use a MZ (Maize (*Zea mays* L.))-SB (Soybean *(Glycine max)*)/WW (Winter wheat (*Triticum aestivum*)) – CC (mixed cover crop) rotation to maximize economic benefits. This study aimed to investigate the short-term impacts of the crop rotation phases and their associated management practices in this diversified cash crop rotation on soil health and the abundance of nitrogen (N)-cycling soil microbial communities (SMCs). Additionally, the abundance of N-cycling SMCs and plant-available N in both surface (0-5 cm) and rooting zone (5-15 cm) depths were characterized in tile-drained (TD) and non-TD fields. In the present study, soils collected under the CC phase had the highest labile carbon levels (10-17% higher) and water-stable aggregates (35-50% higher) compared to the other two crop phases. Lower nitrifying (*amoA*) gene abundances and soil NO_3^--N levels were observed in the CC phase compared to the MZ and SB-WW phases, suggesting a potential for decreased nitrification in the CC phase. The presence of SB potentially influenced the soil N concentration in the subsequent WW phase likely due to the release of symbiotically fixed N in the SB-WW phase. Further, higher *amoA* abundances and NO_3^--N in the SB-WW phase imply a potential for increased nitrification in the SB-WW phase. Additionally, higher *amoA/nosZI* and *nirS+nirK/nosZI* ratios were observed in the MZ phase than in SB-WW and CC phases, suggesting a potential capacity for increased N_2O emissions from the reactions mediated by N-cycling SMCs in soils

planted to MZ during fall sampling days. In the TD and NTD field study, higher NO_3^--N levels were observed in TD-SB-WW fields at 5-15 cm vs. 0-5 cm depths, which was possibly facilitated by tile drainage. The TD-CC fields displayed higher *nosZI* gene abundances and lower *nirS+nirK/nosZI* abundance ratios, suggesting a greater potential capacity for decreased N_2O emissions in soils planted to CCs during the spring sampling days. When examining changes in plant available N by soil depth, reduced downward movement of NO_3^- through shallow soil depths (0-15 cm depth) was observed in the CC phase compared to cash crops. This short-term study highlights the potential contribution of the CC phase, particularly within TD agricultural fields, for improving soil health and reducing potential N_2O emissions. Together, these results suggest that management-associated differences in crop rotation phases have temporary effects on soil health and the abundance of SMCs. Future studies linking N-cycling SMC's potential activity and field-scale N_2O fluxes will provide a better insight into the longer-term sustainability of Ontario's cash crop management systems.

Keywords: Sustainable agriculture, maize-soybean-winter wheat- cover crop rotation, denitrification, nitrification, nitrate leaching, tile-drainage, soil depth

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Abbreviations

AC	Active Carbon
AOA	Ammonium oxidizing archaea
AOB	Ammonium oxidizing bacteria
BD	Bulk density
C	Carbon
CC	Cover crops
C:N	C to N ratio
DNA	Deoxyribonucleic acid
EC	Electrical conductivity
GHG	Greenhouse gas
KCl	Potassium chloride
$KMnO_4$	Potassium permanganate
KNO_3	Potassium nitrate
MAP	Monoammonium phosphate
MZ	Maize
N	Nitrogen
N_2	Dinitrogen
NH_4^+	Ammonium
NH_2OH	Hydroxylamine
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate

N_2O	Nitrous Oxide
NUE	Nitrogen (N) use efficiency
O_2	Oxygen
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
SB	Soybean
SMC	Soil microbial community
SOC	Soil organic carbon
SOM	Soil organic matter
TB	Total bacteria
TD	Tile drainage
TP	Total Phosphorous
WSA	Water Stable Aggregates
WW	Winter wheat

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Supplementary Fig. 4.3. Daily mean average soil moisture (at 15 cm depth, m^3m^{-3}) throughout the study period in tile-drained (TD) and non-tile drained (NTD) fields, with the black outline indicating the sampling period (Mar -Apr 2022).

Chapter 1: General Introduction

Agricultural lands in Ontario, Canada, are increasingly under pressure as they are intensively utilized to meet the rising demands for food, fuel and fibre associated with a continuously expanding population (Hanke, 2018a; Smith, 2015; Liu et al., 2022a). Agriculture intensification relates to expanding agriculture production through numerous technologies and management practices and increasing inputs such as chemical fertilizers, pesticides, and improved crop varieties to achieve increased output per unit input, maximizing productivity while minimizing resource use (Petersen & Snapp, 2015; Struik & Kuyper, 2017). Furthermore, the intensification had a notable impact on management techniques employed for cash crops, encompassing the use of diversified crop rotations, minimal or conservation tillage approaches, and installation of tile drainage, with increased use of inorganic fertilizers (Tonitto et al., 2006) within agricultural systems (Smith, 2015; Liu et al., 2022). In general, agricultural intensification and sustainable cash crop management practices can contribute to achieving sustainable food production (Smith et al., 2010).

In Ontario, along with the midwestern United States and southern Quebec, farmers primarily rely on simplified corn (MZ - *Zea Mays*) and soybean (SB-*Glycine max*)-rotations as the primary cash crop system (Smith, 2015). Grain corn produced within Ontario is used for feed (60%) and industrial uses (40%) (OMAFRA,2023). In Ontario, over 809,000 ha of SB are grown annually. These SBs are mainly used for food-grade markets, oil production, and livestock feed (OMAFRA,2023). In recent years, diversified crop rotations, including a three-year MZ–SB-winter wheat (*Triticum aestivum*) (WW)-cover crop mixture (CC) rotation, have been increasingly adopted (Bowles et al., 2020a;

Hunter et al., 2019). This rotational cash crop system often uses MZ in year one, followed by SB-WW in year two (Janovicek et al., 2021), with polycultures of CCs planted in the fall. In general, CCs provide a living cover during the winter and early spring (Dabney et al., 2001; Osipitan et al., 2018). The cover crops are often chemically terminated when the following cash crop is planted in the spring (Chim et al., 2022). Farmers consider the diversified rotation to have several advantages over simplified crop rotations, including improved crop yields (Lehman et al., 2017), better control of pests and diseases (Reeves, 2018), decreased weed pressure (Liebman & Dyck, 1993; Weisberger *et al.*, 2019), improved soil productivity (Borase *et al.*, 2020; Hartwig & Ammon, 2002), and nutrient and water use efficiencies (Hartwig & Ammon, 2002; Romdhane et al., 2019). Incorporating cover crops specifically grown to cover bare soils and benefit cropping systems is a sustainable approach to improving soil health (Hobbs et al., 2008). Cover crops function as a living cover to protect soil from erosion while providing organic matter, enhancing soil microbial activity, and improving N cycling (Adeyemi et al., 2020; Mbuthia et al., 2015).

Additionally, more than 70% of agricultural lands in Ontario rely on artificially developed tile drainage (TD) systems, another cash crop management technique used to overcome long-term waterlogging issues in soils (Kokulan, 2019; Hanke, 2018). Tile drainage is a widespread land improvement method for row crop production that lowers soil erosion and restricts surface runoff (Arenas Amado et al., 2017). It involves the installation of a grid of perforated pipes known as tile drains, typically installed 0.6-1.2 m below the soil surface, with tile pipes placed 10-100 m apart (Arenas Amado et al., 2017). Tile drainage was initially introduced around 1800, and during that time, the predominant

type of drainage system was known as the “sole and horseshoe” design (Darby & Steiner Verlag, 1964). The design involved the installation of flat sole tiles as the base, with curved horseshoe-shaped tiles placed on top to create a channel for water drainage (Darby & Steiner Verlag, 1964). Currently, tile pipes are installed to cover entire agricultural fields, with outlets directed to streams or open ditches (Moore, 2016).

Even though tile drainage can increase crop output, TDs can have a negative impact on the environment (Gramlich et al., 2018; Hanke, 2018).One of the primary environmental impacts of TD is the potential alteration of flow patterns and water infiltration within the soil (Arenas Amado et al., 2017; van Esbroeck et al., 2016). These changes in hydraulic dynamics can result in more efficient and expedient transport of NO_3^- and other dissolved nutrients through the drainage system compared to overland flow (Arenas Amado et al., 2017; Hanke, 2018; Moore, 2016). Additionally, TD can change temperature and moisture content in soil (van Leeuwen et al., 2017), potentially leading to changes in the microbial N cycle.

In agricultural systems, the microbial N cycle plays a pivotal role in regulating the availability and movement of N, an essential nutrient for plant growth (Halverson et al., 2014; Kuypers et al.,2018; Wang et al;2020). The N cycle involves a series of interconnected processes mediated by SMCs, and these biologically mediated pathways of N_2O emissions and NO_3^- leaching are integral components of this cycle (Bahl *et al.*, 2013; Hayatsu *et al.*, 2008). The N-cycling SMCs involved in these pathways are responsible for the availability of N for plant growth and for N being lost from agricultural soils in soluble and gaseous forms (Kuypers et al.,2018; Robertson et al.,2006). Furthermore, understanding the impact of cash crop management practices on N-cycling SMCs is

essential for optimizing nutrient management approaches and reducing N losses (Adeyemi et al., 2020; Anas et al., 2020). Nitrifiers and denitrifiers play a crucial role in the N cycle: nitrifiers are involved in the conversion of ammonium to nitrate (NO_3^-) while denitrifiers are involved in the conversion of nitrate to gaseous forms of N, including nitrous oxide (N_2O), which is a long-lived greenhouse gas (GHG). The global warming potential of N_2O is 310 times higher than the global warming potential of CO_2 (IPCC, 1996), and N_2O contributes to ozone depletion in the stratosphere (Risk et al., 2013; Throbäck et al., 2004). In Canada, agriculture is responsible for approximately 10% of the country's national GHG emissions, with about 50% of those emissions from N_2O (Agriculture and Agri-food Canada, 2022).

Nitrate losses from agricultural lands occur during growing and non-growing seasons and can have significant environmental implications due to their potential contribution to water and atmospheric pollution (Fuller et al., 2010; Liang et al., 2020). During the growing season, inadequately formulated and applied nutrient management practices related to N-based fertilizers (Stoddard et al., 2005), and manure amendments (Mkhabela et al., 2008) as well as processes such as mineralization, can result in the leaching of NO_3^--N, which is a highly soluble and mobile form of N (Andraski et al., 2000; Kirchmann et al., 2002).

Nitrate leaching from agricultural soil is a significant cause of concern in aquatic systems, resulting in harmful algal blooms and eutrophication (King *et al.*, 2014; Smith & Schindler, 2009). Water conditions occurring during the non-growing season due to intermittent freeze-thaw cycles provide ideal conditions for NO_3^- leaching into the groundwater (Lapierre et al., 2022). These losses occur after the main crop is harvested and

soils remain devoid of vegetation and protective cover, with excess water in the ground facilitating the movement of NO_3^- underneath the root zone to TD (Lapierre et al., 2022). A wide range of NO_3^--N concentrations in TD outlets have been reported. For example, reported values for corn (MZ) range from less than 5 mgL^{-1} (Mkhabela et al., 2008) , 5-15 $mg\ L^{-1}$(Gordon *et al.*, 2000; Milbum & Richards, 1994), and up to 15-25 $mg\ L^{-1}$ (Stoddard et al., 2005).

1.1 Knowledge gap

Previous studies have examined the impact of diversified crop rotation (Navarro-Noya et al., 2013), cover cropping (Haruna et al., 2020; Hunter et al., 2019; Reeves, 2018) and N fertilizer application on soil characteristics (Chahal et al., 2021; Mbuthia et al., 2015) and SMC abundance and activity in agriculture soils (Liu et al., 2022; Mbuthia et al., 2015). Studies revealed that diversifying crop rotations with cover crops, perennials, and small grain cereals significantly improve soil health indicators and crop productivity (Haruna et al., 2020; Hunter et al., 2019; Reeves, 1997). Furthermore, studies demonstrated that crop rotation has a significant impact on SMC diversity (Borase et al., 2020; Smith et al., 2016; Venter et al., 2016). Maul *et al*. (2019) investigated the abundance of genes associated with denitrification (*nirK, nirS*, and *nosZI*) in different cropping systems during the summer growing season. The results showed that the abundance of *nirK* was primarily driven by the crop rotation phase and the time of the year, and *nosZI* abundance was primarily driven by the different crop rotations. Further, many of these studies were conducted in the US and Canada, representing climatic conditions and soil types different from the present study (Bowles et al., 2020; McDaniel et al., 2014). However, the impacts of cash crop management practices associated with each crop rotation phase in a diversified crop rotation in southern Ontario on N-cycling microbial communities remain a critical knowledge gap.

Several studies have investigated the impact of depth on the vertical distribution of N-cycling SMCs (Chen et al., 2019; Eilers et al., 2012; Hao et al., 2021; Li et al., 2022; Naylor et al., 2022). For instance, Chen et al. (2019) studied microbial properties (biomass

and bacteria: fungi ratio) and C and N mineralization rates in arid and semi-arid grassland ecosystems at four depths (0-20 cm, 20-40 cm, 40-60 cm, and 60-100 cm). The results showed that biomass and bacteria: fungi ratio, C and N mineralization rates, and soil organic C (SOC) decreased with increasing soil depth. Studies have reported decreases in the abundance of nitrogen-cycling functional genes (Stone et al., 2015; Tang et al., 2018) and microbial biomass (Qin et al., 2016; Tang et al., 2018) with increasing depth. Some studies found that different N-cycling microbial groups dominate in different soil layers based on the availability of nitrogen sources (Qin et al., 2016; Tang et al., 2018). However, according to our knowledge, the characterization of N-cycling SMCs in the soil surface (0-5 cm) and the rooting zone (5 -15 cm) in tile-drained fields has not been done in previous studies. This short-term study is conducted to address these gaps to shed light on how cash crop management influences soil health and N-cycling dynamics in a diversified crop rotation.

1.2 Thesis format and research objectives

This thesis is arranged into five main chapters in manuscript style. Due to each study's complimentary but independent nature, specific objectives are stated within each chapter. In this thesis, I aimed to investigate the short-term impacts of cash crop management practices in southern Ontario on N-cycling SMC abundance and soil health. **Chapter 2** is a literature review highlighting how different cash crop management techniques, including diversified crop rotation, cover cropping, and tile drainage, impact soil health and the N cycling SMCs. In **Chapter 3**, I investigated the short-term impact of different crop phases, including CC mixtures in a long-term cropping system under the rotation of MZ-(SB-WW)-CC on soil health parameters and communities of total bacteria (*16S* rRNA),

nitrifying bacteria (*amo*A), and denitrifying bacteria (*nirS, nirK,* and *nosZI*) using quantitative polymerase chain reaction (qPCR). In **Chapter 4,** I characterized the plant available N and the vertical distribution of N cycling SMCs in TD and NTD fields separately. In summary, the scope of this thesis addressed the following objectives:

1. To characterize soil health and the abundance of N-cycling SMCs in different crop phases of a long-term MZ-(SB-WW)-CC diversified crop rotation (**Chapter 3**).
2. To evaluate differences in plant available N and N cycling SMCs within TD vs. NTD fields in a diversified crop rotation **(Chapter 4**).

Chapter 2: Literature Review

2.1 Context

Ontario plays a critical role in Canadian agriculture, with significant contributions to various economic sectors (Census of Agriculture, 2021). This is particularly evident in the province's dominance in key farming commodities such as soybeans, corn for grain, and greenhouse products. According to the Census of Agriculture 2021, Ontario accounted for a significant portion of Canada's farmlands, making up over one-quarter (25.5%) of the total farm area (Chen, 2022). Southern Ontario harbours Canada's largest and most rapidly growing urban population and the continuously growing population in southern Ontario has led to increased demands for food, feedstock, and fibre production, necessitating the intensification of agriculture in the region (Liu *et al.,* 2022; Smith, 2015). Agriculture intensification has significantly impacted cash crop management techniques, particularly the implementation of diversified crop rotation, the adoption of tile drainage in poorly drained agricultural lands, and the increased utilization of inorganic fertilizers (Liu *et al.*, 2022; Smith, 2015). However, agriculture intensification has led to soil degradation and an overall decline in agricultural sustainability (Gaudin 2013; Lehman et al., 2015). Therefore, it is vital to adopt sustainable cash crop management practices that enhance the long-term viability of food production to meet the growing demands of the people (Tahat et al., 2020). Understanding the link between cash crop management and its impact on the soil system is a growing concern for researchers, producers, and consumers (Newton et al., 2020; Tahat et al., 2020).

2.1.1 Soil health and indicators

Soil is a multifaceted, complex, dynamic ecosystem where biotic and abiotic components interact and support numerous societal and ecological services (Lehmann *et al.,* 2020; Prabha *et al.,* 2020; Tahat *et al.,* 2020). The ecological services provided by soil are supporting services such as nutrient and water cycling and soil biological functions; provisioning services such as the production of food, wood, fibre, and raw materials; and regulating services such as flood mitigation, pest control, carbon (C) storage, and regulation of greenhouse (GHG) gases (Bünemann et al., 2018; O'Riordan et al., 2021; Williams et al., 2020). The definition of soil health has evolved over the years, with the most recent definition focusing on the soil's continued capacity to function as an essential living system while sustaining biological productivity and quality of the environment and promoting the health of living organisms, including plants, humans, and animals (Dias et al., 2014; Lehmann et al., 2020; Prabha et al., 2020). Improving soil health improves soil's resilience to climate change (Congreves et al., 2015).

Soil health can be assessed using a combined set of measurable physiochemical and biological attributes (Williams et al., 2020; Allen et al., 2011). Widely used physical parameters include soil texture, soil compaction, infiltration, aggregate stability, rooting characteristics, and plant available water content (Allen et al., 2011; Shah et al., 2017; Sung et al., 2017). Chemical indicators including pH, electrical conductivity, soil organic matter (SOM), labile C and N, total C and Nitrogen (N), available nutrients (including N, phosphorus (P), potassium (K) (Kv et al., 2019; Lehmann et al., 2020; Sung et al., 2017),and biological indicators including respiration, microbial biomass and diversity,

enzyme activities, and N mineralization rates are commonly used soil health indicators (Meena, 2019; Bünemann *et al.,* 2018; Congreves *et al.,* 2015; Allen *et al.,* 2011).

Soil health parameters should fulfil several criteria to be considered good indicators (Williams *et al.,* 2020; Bünemann *et al.,* 2018). The selected parameters should be strongly related to the soil function of interest (Bünemann *et al.,* 2018; Bone *et al.,* 2014), easy to measure, reliable, sensitive to different soil management practices, and cost-effective to adopt (Bünemann *et al.,* 2018; Bone *et al.,* 2014; Oberholzer *et al.,* 2012).

A vital soil health indicator is soil organic carbon (SOC), and it is related to other soil properties, including structural properties, water-holding capacity, and available nutrient content (Liptzin et al., 2022; Nunes et al., 2021; Bongiorno et al., 2019). Changes in SOC occur gradually and measurement of changes during a short study period is difficult; hence, measuring the labile C fraction gives a better understanding of potential short-term SOC changes related to agricultural management (Bongiorno et al., 2019; Meena, 2019). Liptzin *et al.* (2022) suggested a strong positive correlation between SOC and labile carbon concentrations. Moreover, labile carbon is a helpful indicator for most agriculture management systems as it is easily mineralizable and accessible directly to microbial activity (Lawrence et al., 2015; Meena, 2019). The labile C pool originates from various sources, such as plant litter breakdown, root exudates, the hydrolysis of SOM in soils, and microbes and their metabolic processes (Shen et al., 2021).

2.2 Microbial cycling of N

Nitrogen (N) is one of the essential elements for life on earth and is an integral component of many molecules required for the biological functioning of living organisms (Kraiser *et*

al., 2011; Kuypers *et al.,* 2018). Moreover, N is a component in metabolic-related molecules such as amino acids and proteins and genetically related molecules, including nucleotides and nucleic acids (Kuypers *et al.,* 2018; Takai, 2019). In comparison to C, the biological transformation of organic N molecules is less complex (Takai, 2019). In contrast, the inorganic N transformation in the soil is highly complex compared to other essential elements, including C (Takai, 2019).

Two large pools of N can be found on earth: inorganic N, including molecular nitrogen (N_2) in the atmosphere and reactive N in the forms of NO_3^-, NH_4^+, and organic N (Takai, 2019). Inorganic N is available for plant uptake as ammonium nitrogen (NH_4^+-N) and nitrate-nitrogen (NO_3^--N), which enter agricultural systems via inorganic N fertilizer application and through mineralization of organic N in the soil (Chen *et al.,* 2014; Isobe & Ohte, 2014). Organic N bound to complex molecules, such as amino acids, proteins, and nucleotides, are broken down by SMC into simple forms through mineralization, which plants can also take up (Dai *et al.,* 2020; Robertson & Groffman, 2006). Nitrogen gas (N_2) is the most abundant form of N in the atmosphere and comprises 79 % of all atmospheric gases (Jin et al., 2005). However, for atmospheric N_2 to be used by living organisms, it must be transformed into other forms primarily by biological N fixation, which is considered the primary process through which N enters biological systems (Robertson & Groffman, 2006). Additionally, atmospheric N_2 enters terrestrial systems in other ways, including N-based fertilizer applications, atmospheric deposition, and industrial fixation (Dai *et al.,* 2020).

The microbial N cycle is a set of interconnected pathways where N is transformed from one form to another, facilitated by microorganisms and abiotic chemical decomposition of intermediates (Butterbach-Bahl *et al.,* 2013; Hayatsu *et al.,* 2008) (Figure 2.1). The study

of the soil N cycle and the involvement of SMCs in its biogeochemical cycling has a long history, dating back to the recognition of the importance of N for plant growth in the 1800s. (Galloway *et al.,* 2013). Initially, two main pathways were identified, nitrification and denitrification, but later, an understanding of the pathways involved in N transformations expanded to six interlinked processes (Firestone and Davidson, 1989; Kuypers *et al.,* 2018; Stein and Klotz, 2016). In addition to nitrification and denitrification, some pathways provide N assimilation, ammonification, anaerobic ammonium oxidation, and N fixation (Kuypers *et al.*, 2018; Stein and Klotz, 2016).

Mineralization is another critical pathway in the N cycle which transforms organic N into inorganic N forms by heterotrophic SMCs (Robertson & Groffman, 2006; Jansson & Persson, 1982). Mineralization involves three major processes of the N cycle such as ammonization, ammonification, and nitrification (Dai et al., 2020; Jansson & Persson, 1982).As the microbes break down the SOM, NH_4^+-N, and NO_3^--N are released into the soil (Jansson & Persson, 1982). Mineralized NH_4^+ is either taken up by plants or undergoes a series of reactions, which are governed by N-cycling SMCs (Robertson & Groffman, 2006). A wide range of microbial communities are involved in N mineralization (Isobe & Ohte, 2014).

Similarly, immobilization is a crucial microbial process defined as the assimilation of inorganic N forms by SMCs and other organisms (Jansson & Persson, 1982). Mineralization increases plant-available forms of N in the soil solution, while immobilization results in a decrease. Gross N mineralization is the total amount of soluble N produced by SMCs, while gross N immobilization is measured as the total amount of N

consumed. If gross N immobilization exceeds gross N mineralization, inorganic N in the soil decreases (Robertson & Groffman, 2006).

Nitrogen cycling SMCs, particularly nitrifiers and denitrifiers, play significant roles in soil N cycling (Abatenh *et al.,* 2018; Levy-Booth *et al.,* 2014) and can be impacted by agricultural management (Fuller et al., 2010; Hu et al., 2021; Smith et al., 2010). In addition, microbial functional genes involved in N cycling processes are direct biological indicators of N transformation capacity (Levy-Booth *et al.,* 2014).Nitrification and denitrification are the two critical microbial pathways of the N cycle contributing to atmospheric N_2O emissions (Linton *et al.,* 2020; Thompson *et al.,* 2016). Even though denitrification is considered the major pathway of N_2O emissions, a substantial amount of N_2O is produced by nitrification (Chen *et al.,* 2012). Oxygen availability is an essential abiotic driver of nitrification and denitrification processes. Denitrification occurs under O_2-limited conditions, while nitrification proceeds under O_2-available conditions (Barnard *et al.,* 2005). Generally, soils with WFPS < 60 % favour nitrification, while WFPS > 60-90% favour the denitrification process (Braker & Conrad, 2011).

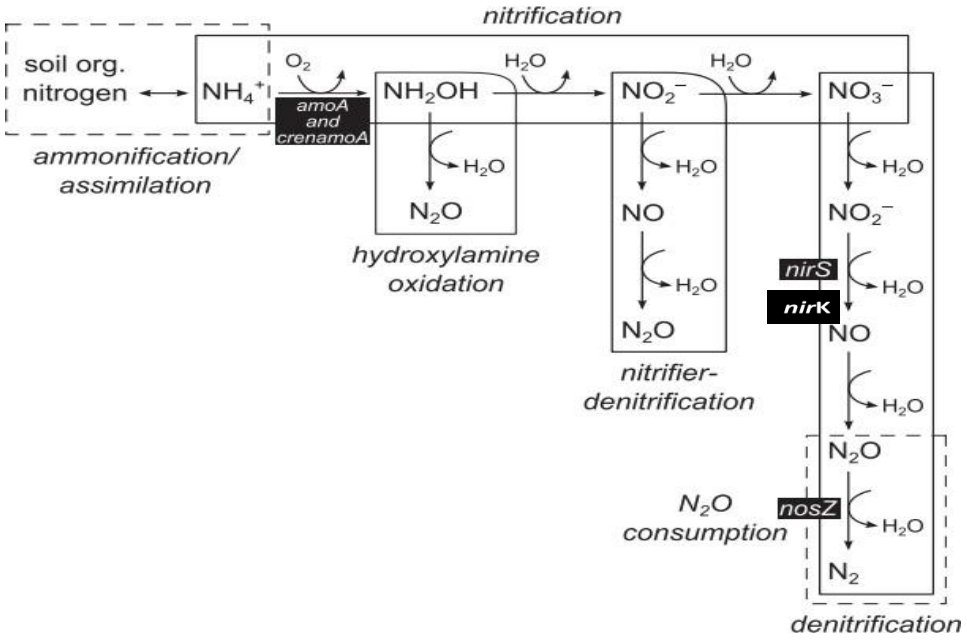


Figure 2.1 Nitrification and denitrification reactions are mediated by nitrifying (*amoA* and *crenamoA*) and denitrifying *(nirS, nirK*, and *nosZI*) soil microbial communities (Snider *et al*. 2015).

2.2.1 Nitrification

Nitrification is the oxidation of reduced forms of nitrogen into nitrite (NO_2^-) and nitrate (NO_3^-), mainly carried out by autotrophic and heterotrophic bacteria and archaea in soil. The autotrophic nitrification process has two steps carried out by ammonia oxidizers and nitrite oxidizers (Robertson & Groffman, 2006), which are chemolithoautotrophic (Frame & Casciotti, 2010). The first step is ammonia oxidation, in which ammonia is oxidized to nitrite by enzymes such as ammonia monooxygenase (encoded by the *amoA gene*) and hydroxylamine oxidoreductase (encoded by the *hao* gene). Then, in the second step, NO_2^- is oxidized to nitrate NO_3^- by nitrite oxidoreductase (encoded by the *nor* gene). Ammonia

oxidizing archaea (AOA) and bacteria (AOB) are responsible for this rate-limiting oxidation step of autotrophic nitrification (Ouyang *et al.,* 2016; Robertson & Groffman, 2006).

Additionally, nitrifier denitrification is a distinct, alternate N transformation pathway from coupled nitrification-denitrification processes. In nitrifier denitrification, ammonia oxidizers are involved in the oxidation of ammonia to nitrite and the subsequent reduction of NO to N_2O (Shaw *et al.,* 2006; Colliver & Stephenson, 2000). The nitrite reduction process occurs when ammonia oxidizers use NO_2^- as the final electron acceptor in O_2-limited conditions and become denitrifying nitrifiers (Shaw *et al.,* 2006; Colliver & Stephenson, 2000). This process is the commonly documented N_2O-releasing pathway associated with nitrification (Barnard *et al.,* 2005; Robertson & Groffman, 2006). However, N_2O can also be released during the ammonia oxidation process due to the chemical decomposition of hydroxylamine (Frame & Casciotti, 2010). N_2O released from this pathway is considerably low (Arp & Stein, 2003).

2.2.2 Denitrification

Denitrification is a dissimilatory reduction process where NO_3^- is sequentially reduced to N_2 by denitrifiers under anaerobic conditions. Denitrification is the primary biological process by which N returns to the atmosphere (Philippot, 2002). The denitrification process has four significant steps that cause the reduction of nitrate into N_2 gas (Figure 2.2) (Philippot, 2002). Denitrifiers can produce N_2O as an intermediate product, which is released into the atmosphere (Németh *et al.,* 2014).

Figure 2.2. The denitrification processes. The intermediate products involved in the pathway are depicted within boxes, and corresponding genes encoding the enzymes responsible for catalyzing these reactions are labelled underneath the arrows.

The functional genes involved in the denitrification process are nitrate reductase (encoded by the *nar* gene), nitrite reductase (encoded by *nir* genes*)*, nitric oxide reductase (encoded by the *nor* gene), and nitrous oxide reductase (encoded by the *nosZ* genes**)** (Isobe & Ohte, 2014; Németh *et al.,* 2014; Bouwman *et al.*, 2002; Philippot, 2002). Among these functional genes, *nir* functional gene is critically important in denitrification as it produces the first gaseous by-product of denitrification (Yao *et al.,* 2022).

The denitrifiers, which contain nitrite reductase enzymes, encoded by *nirS* (Cd-containing cytochrome) or *nirK* (Cu-containing cytochrome), exhibit the same biological functions (Lee & Francis, 2017; Wang et al., 2020). However, they occupy different niches (Maul et al., 2019; Throbäck et al., 2004). However, it is noteworthy that neither type has been reported in a single species (Hu *et al.,* 2021). Zumft (1997) found that nearly three-quarters of denitrifiers that have been cultured have the *nirS* gene, indicating that the *nirS* gene is the dominant form in abundance in the natural environment over the *nirK* gene. Therefore, the *nirS* gene has been widely used to characterize potential nitrate-reducing communities (Deslippe *et al.,* 2014). In contrast, in some studies, authors revealed that in agricultural fields, *nirK* abundance is higher than the *nirS* abundance (Dandie et al., 2011; Hai et al., 2009; Wang et al., 2020; Zhou et al., 2011). Studies also reported that large and

diverse *nirK* denitrifier groups that were previously undetected by widely used PCR primers, were revealed later to enhance the understanding of *nirK*-bearing denitrifiers (Helen *et al.,* 2016; Lee & Francis, 2017; Wei *et al.,* 2015).

The rate-limiting step of the overall denitrification pathway is the conversion of nitric oxide (NO) to nitrous oxide (N_2O) (Yao et al., 2022). Nitric oxide in high concentrations is lethal to bacteria; hence, it must be effectively removed from bacterial cells and converted to N_2O (Yao et al., 2022). After the nitrite reduction step, the next step is the reduction of N_2O to N_2, which is catalyzed by the *nosZ* gene and determines the N_2O/N_2 balance (Thompson *et al.,* 2016). This *nosZ* gene has been classified into two phylogenetic clades, *nosZI* and *nosZII,* based on their sequence similarities and evolutionary relationships (Orellana et al., 2014). The communities bearing the *nosZ* gene engage in the final step of the denitrification process, where N_2 is released into the atmosphere (Thompson *et al.,* 2016). Studies have found that denitrifying community sizes correlate with the denitrification process rates and *in situ* N_2O fluxes (Thompson *et al.,* 2016; Németh *et al.,* 2014), and denitrifying community sizes and denitrifying potential activity have also been correlated (Enwall *et al.,* 2010; Thompson *et al.,* 2018; Throbäck *et al.,* 2004).

In applied field research with multiple plots or fields, obtaining N_2O flux measurements from the field is not always practical or possible. Therefore, instead of N_2O flux measurements, genes and transcripts associated with the N cycle can be quantified to assess the soil's potential to produce N_2O through nitrification and denitrification (Thompson *et al.,* 2018; Thompson *et al.,* 2016; Nemeth *et al.,* 2014). Relative abundances of genes involved in N_2O production *(amoA, nirS,* and *nirK*) and consumption (*nosZI)* have

been previously used to predict system-level N_2O emissions in an organic lettuce production system (Hu *et al.,* 2021; Pereira *et al.,* 2015).

2.3 Impact of agriculture management practices on soil health and Nitrogen dynamics

2.3.1 Diversified crop rotation impacts on soil health and N-cycle dynamics

Crop rotation refers to systematically and repeatedly growing several crop types on the same agricultural land over time (Liebman & Dyck, 1993; Reeves, 2018). MZ-SB/WW-CC rotation is a commonly used diversified cash crop rotation practice in southern Ontario, Canada. Crop rotations incorporating CC mixtures into the rotation, including legumes, grasses, and forbs, are believed to have several ecological benefits over crop rotations without cover crops (Janovicek *et al.,* 2021). When different crop types are in rotation, they can support specific SMCs involved in various N-cycling processes including nitrification, denitrification, N fixation, and N mineralization (Maul et al., 2019). This enhanced diversity and activity of SMCs can lead to improved N transformation, nutrient availability, and overall nutrient cycling efficiently within the system (Agomoh et al., 2021; Borase et al., 2020; Maul et al., 2019; Studdert & Echeverría, 2000). Furthermore, diversified crop rotations consisting of leguminous cover crops can decrease the amount of fertilizer needed for the following cash crop, and this reduction in fertilizer usage contributes to lowering atmospheric CO_2 and N_2O emissions (Hu *et al.,* 2021; Liu *et al.,* 2021).

Diversified crop rotations have been associated with supporting more diverse microbial communities in the soil. This microbial diversity is linked with several beneficial soil characteristics, including better resistance to acidification, lower C: N ratio, higher levels of SOC, and total N (TN) (Borase et al., 2020; Jiang et al., 2021; Zhang et al., 2021), but also

tend to have higher denitrification rates (Jiang et al., 2021; Linton et al., 2020; Borase et al., 2020; Guo et al., 2011). Bowles *et al.* (2020) analysed yield data from 347 sites in 11 experiments conducted across Canada and USA. The authors found that increasing crop rotational diversity significantly improved MZ yields (28 % on average) across a wide range of growing conditions over time, mainly during the years with droughts. Additionally, during drought years, diversified crop rotation resulted in reduced yield losses ranging from 14 %-90 % (Bowles et al.,2020).

Corn (MZ) is usually planted in late spring or early summer, depending on the local climate and soil conditions (Sacks et al., 2010). Corn has a high N requirement and low N use efficiency (NUE) (Adeyemi *et al.*, 2020; Halvorson & Bartolo, 2014). Therefore, adequate nutrient management is essential for MZ growth (Pantoja et al., 2015). Effective weed management minimizes competition and maximizes MZ yield (Chauhan, 2020). The weed management involves pre-emergence herbicide applications, along with practices like crop rotation, tillage, and physical weed management techniques (Chauhan, 2020; Weisberger et al., 2019).

As a leguminous crop, soybean establishes a mutually beneficial symbiotic relationship with a type of soil bacteria known as bradyrhizobia (Gentry et al., 2001). When available concentration is limited in the soil, this relationship fulfils plants' N requirements through biological N fixation (BNF) (Córdova et al., 2019). This symbiotic interaction enables SB plants to have a relatively high accumulation of N, resulting in lower C:N ratios in the tissue residue of SB plants (Córdova et al., 2019). Soybeans typically produce lower quantities of residues than other crop types, leading to low SOC accumulation. Consequently, this reduced accumulation of SOC can negatively impact water-holding

capacity, nutrient retention, and crop yield. Therefore, SB is often incorporated into crop rotations with high C returning crops such as MZ and WW (Agomoh et al., 2021; Piske & Peterson, 2020). Additionally, enhanced SB yields can be obtained when grown in MZ-SB-WW rotations compared to continuous SB or MZ-SB rotations (Frick et al., 2017).

Winter wheat provides a living cover that reduces soil erosion and nutrient leaching during winter (Adhikari *et al.,* 2017). Winter wheat is typically sown in the fall, usually from late September to early October (Mcmaster et al., 2001). This crop generally thrives in various soil conditions if the soil has proper drainage (Frick et al., 2017). However, frequent cycles of freezing and thawing can result in excessive soil heaving, which can be harmful to WW and potentially cause winter kill (Frick et al., 2017). Additionally, soil pH is considered necessary in determining WW growth, particularly regarding nutrient availability. For WW, pH 6.5 is considered the optimal pH level, whereas low pH can hinder plant development, while high pH can cause manganese deficiencies (Frick et al., 2017).

Cover crops are grown between cash crop production within rotation to improve soil health and reduce erosion. Cover crop species and varieties are selected based on the objectives of the farmers and local conditions (Haruna et al., 2020; Kaspar & Singer, 2015a). While CCs remain in the field throughout winter, certain species may experience winter kill (die off), while others can survive until spring (Adhikari *et al.,* 2017). Cover crops are terminated before planting the main crop, and termination methods include mechanical methods, chemical methods, or a combination of both (Bhaskar et al., 2021; Osipitan et al., 2018). Diversifying the MZ-SB rotation by incorporating WW and CC

mixtures is an effective management strategy to achieve high MZ and SB yields while reducing N-based fertilizer usage (Congreves *et al.,* 2015; Gaudin *et al.,* 2015).

2.3.2 Impact of cover cropping on soil health and N-cycle dynamics

Cover crops, called catch crops, living mulches, or green manures, are often grown to cover bare soil, preventing soil erosion, and reducing nutrient leaching (Cai *et al.,* 2019; Kaspar & Singer, 2015; Tani *et al.,* 2017). Incorporating cover crops into crop rotation can increase crop yield without adding excess inorganic N fertilizers (Cai *et al.,* 2019). Cover crops can enhance carbon inputs in agricultural soils, resulting in carbon sequestration (Reeves, 1997). In addition, CCs in rotation can provide other benefits, such as improving water infiltration, improving water quality by reducing the pollutants entering water bodies, and controlling harmful insects (Cai *et al.,* 2019; Dabney *et al.,* 2001).

Several studies have shown that cover crops belonging to the Poaceae family, such as annual ryegrass, are better adapted to water conditions than leguminous cover crops (Blanco-Canqui *et al.,* 2015; Fageria *et al.,* 2005; Hively & Cox, 2001). Due to their high C: N ratio, plant residues decompose more slowly than leguminous CCs, resulting in their persistence in the fields for a more extended period (Blanco-Canqui et al., 2015). A recent study conducted by Hu *et al.* (2021) in a continuous cotton system revealed that N cycling dynamics, particularly *amoA*, *nifH*, *nirK*, *nirS*, and *nosZ* gene abundances, changed considerably under different soil management practices, including cover crops, tillage, and N-based fertilizer application. In this study, the leguminous cover crop hairy vetch promoted the N-cycling gene expression following the termination of the cover crop.

A two-year trial was established in a study to examine 18 cover crop fields of up to 8 species and various combinations of four functional groups, including N fixers, N scavengers, winter hardy, and winter-killed species. Finney & Kaye. (2017) found that incorporating polycultures of cover crops with functional diversity potentially led to agricultural systems with great multifunctionality, which refers to the ability to provide multiple benefits or functions simultaneously. These functions involved N fixation, reduced N leaching, erosion prevention, improvement of soil structure by winter hardy CCs, and weed suppression (Finney & Kaye, 2017; Kaspar & Singer, 2015).

2.3.3 Impact of chemical fertilizer application on soil health and N-cycle dynamics

Chemical fertilizer application is a widely used management practice to enhance soil fertility and increase crop yield (Chen, 2006; Zhen *et al.*,2006; Ayala & Rao, 2002). However, the impact of chemical fertilizers on N-cycling microbial gene abundances varies depending on the N-cycling microbial community being examined (Chen *et al.*, 2012; Sun *et al.*, 2015). For instance, a study was conducted to assess the long-term (35 years) impacts of N fertilization in continuous corn and a MZ-SB rotation on the significant steps of the N-cycle. The study revealed that long-term fertilization impacted BNF by decreasing *nifH* abundance in both systems and increasing the *amoA* genes under continuous corn (Huang *et al.*, 2019). According to this study, long-term fertilization did not impact denitrifier SMC abundance (Huang *et al.*, 2019). The study also found that *amoA* gene abundance largely impacted the variation in the abundance of the N-cycling SMC when comparing different fertilization strategies.

Furthermore, a study was conducted by Hallin et al. (2009) using soil from a 50-year fertilizer trial and the fields contained unfertilized bare fallow, unfertilized with a crop,

and plots with crop fertilized with calcium nitrate, ammonium sulfate, solid cattle manure, or sewage sludge to study the relationship between chemical fertilizer and N-cycling communities. The study findings discovered that the size of functional microbial guilds and the overall bacterial community were significantly influenced by the fertilization fields, mainly sewage sludge and ammonium sulfate. When combined with previously published data on community composition, this study showed that differences in size, rather than composition, were associated with variation in process rates for denitrifiers, ammonia-oxidizing archaea, and total bacteria. In contrast, differences in neither size nor composition were linked to variation in process rates for ammonia-oxidizing bacteria (Hallin et al., 2009). The composition of nitrate-reducing, denitrifying, and total bacterial communities co-varied with primary production and strongly correlated with soil properties (Hallin et al., 2009).

Despite this variability, chemical fertilizers can significantly impact N-cycling microbial community abundances, which are crucial for nutrient cycling and soil health (Chen *et al.*, 2012; Sun *et al.*, 2015). For example, Fan *et al.* (2011) found that NPK fertilizers increased the abundance of AOB and denitrifying SMCs. In a study conducted in a field where WW and summer bean rotation was practiced, Sun et al. (2015) observed long-term NPK fertilizer application significantly increased the size of N-cycling microbial communities; *nirK*, *nosZI*, and bacterial *amoA* gene abundances were higher in fertilized soils compared to unfertilized soils. The fertilizers used in this study were urea (180 kg N ha^{-1} y^{-1}), superphosphate (90 kg P_2O_5 ha^{-1} y^{-1}), and potassium chloride (135 kg K_2O ha^{-1} y^{-1}), respectively. Furthermore, the authors observed that bacterial *amoA* gene abundance

increased significantly, while archaeal abundance decreased when different fertilizer strategies were applied (Sun *et al.,* 2015).

2.3.4 Impact of tile drainage on soil health and N-cycle dynamics

Tile drainage is introduced to agricultural lands where natural drainage is impaired due to soil compaction, low hydraulic conductivity, and poor relief (Arenas Amado *et al.,* 2017). Tile drainage is a widely used land improvement technique in row crop cultivation (Arenas Amado *et al.,* 2017), which limits surface runoff and reduces soil erosion (Gramlich *et al.,* 2018).

Although tile drainage can benefit agricultural fields, TDs can cause several environmental issues (Gramlich *et al.,* 2018; Hanke, 2018). Losses of soluble macronutrients, including P and N, particularly in the non-growing season (October- April) are a significant concern in tile-drained systems (Liu et al., 2022a). High mineralization rates in TD fields, especially during the non-growing season, may contribute to higher soluble nutrient losses during spring thaw (Gentry *et al.,* 1998). In a study conducted in three agricultural fields in southern Ontario, Esbroeck *et al.* (2016) estimated that the annual export of total phosphorus (TP) from tile drains during the non-growing season ranged from 83-97% of the annual combined (surface + tile) runoff P and 84-100% of dissolved P.

Tile drainage is often installed in cash cropping systems that use simplified rotations, often lacking leguminous rotation phases and relying on synthetic N inputs (Chen, 2006; Ayala & Rao, 2002). Applying N-based fertilizers in excessive amounts may increase NO_3^--N concentrations in surface water bodies, e.g., streams and groundwater, due

to leakage from TD (Kladivko *et al.*, 2001). The excess NO_3^--N in the soil, which cannot be taken up by plants, is exported from the fields into the surrounding soil and water primarily through TD (Amado et al., 2017; Gentry et al., 1998). Tile drainage provides a rapid pathway for the export of N compared to the slower natural infiltration through the soil profile into groundwater (Liu et al., 2022; Baker et al., 2014; Randall et al., 1997).

Liu *et al.* (2022) conducted a study in the same area where the present research (chapters 3 and 4) was conducted, consisting of seven sub-watersheds in east-central Ontario, including tributaries flowing into Lake Ontario. In this study site, the row crop area practicing mainly MZ-SB-WW rotation is underlain by TD (Liu *et al.*, 2022). A positive relationship was found between the extent of row crop area within a watershed and NO_3^--N losses. Furthermore, the results of this study suggested that agriculture intensification may be the cause of increasing NO_3^--N concentration in stream water, mainly in the agriculture tributaries draining into Lake Ontario (Liu *et al.*, 2022).

2.4 Spatial distribution of SMCs in the soil profile

Both biotic and abiotic factors, which differ in soil depth, impact the abundance and diversity of SMCs (Li et al., 2022; Chen et al., 2019). Several studies have investigated the impact of soil depth on SMC distribution (Bai et al., 2023; Chen et al., 2019; Eilers et al., 2012; Hao et al., 2021; Li et al., 2022; Naylor et al., 2022). However, the vertical distribution of N-cycling microbial communities and variation in plant-available N in TD agricultural lands have not been studied.

According to Hu *et al*. (2015), the vertical distribution of bacterial communities is primarily driven by soil health parameters, including soil pH, conductivity, and SOC. A

study conducted in MZ and SB fields in Iowa, USA, using *16S* rRNA amplicon sequencing, revealed that the richness and diversity of SMCs decreased with increasing soil depth. Additionally, the composition of the SMCs varied with the crop type only in the top 60 cm of soil and varied vertically only in the top 90 cm of soil (Hao et al., 2021). Sun *et al*.,2018 found that soil depth impacted the soil bacterial community structure more significantly than the soil fungal community structure. Studies have shown that as soil depth increases, there is a decrease in SOM % (Hao et al., 2021b; Liebmann et al., 2020), volumetric water content (VWC) (Zhao et al., 2021), soil temperatures (Zhao et al., 2021), and microbial biomass (Eilers et al., 2012b; Leewis et al., 2022) while soil pH increases (Zhao et al., 2021).

The vertical distribution of SMCs is also affected by differences in O_2/H_2O within different layers influenced by aggregates and roots (Ley et al., 2018). Aggregates bound together by SOM can create microsites with higher O_2 and low H_2O levels, which may favour nitrification (Ley et al., 2018). Conversely, the spaces between aggregates can have lower levels of O_2 and higher levels of H_2O, which may promote denitrification. Roots also play a crucial role in shaping the vertical distribution of SMCs. Roots can release a small amount of O_2 into the rhizosphere by a phenomenon known as “radial oxygen loss” (ROL) (Mei et al., 2014), and this creates distinct zones around plant roots, namely fluctuating aerobic and anaerobic zones, which affect vertical distribution of SMCs (Bai et al., 2023; Mei et al., 2014). In addition, roots can release exudates, which influence soil parameters such as pH, nutrient availability, and redox potential, which can impact the distribution of SMC in different layers (Naylor et al., 2022).

2.5 Summary

Overall, this literature review highlights the importance of adopting cash crop management practices that enhance soil health and N-cycling microbial communities, thereby ensuring the long-term viability of agricultural lands. The review also highlights the need for further research to understand the interrelationships among commonly used cash crop management practices in different crop rotation phases in diversified crop rotations and their impact on soil health and N-cycling SMCs. Furthermore, this literature review highlights a knowledge gap in the characterization of N-cycling microbial communities in agricultural lands with TD systems. Studying the vertical distribution of N-cycling microbial communities and plant available N can enhance our understanding of how commonly used cash crop management practices, such as the use of tile drains impact in-field N-cycling and potential off-field N losses, providing insight into ways to minimize NO_3^- and N_2O losses by crop diversification and incorporating cover crops, thereby mitigating environmental impacts associated with tile drainage. Chapters 3 and 4 of this thesis will contribute to advancing our understanding of these relationships, providing valuable insights into how specific management practices influence soil health and N-cycling SMCs in Ontario agricultural soils.

Chapter 3: Short-term impacts of cash crop management on soil health and N-cycling microbial community abundances

ABSTRACT

Agricultural lands in Ontario are increasingly under pressure as they are intensively used to produce food, fuel, and fibre. Cash crop farmers often use a three-year MZ (Maize (*Zea mays* L.))-SB (Soybean *(Glycine max)*)/WW (Winter wheat (*Triticum aestivum*)) – CC (mixed cover crop) rotation which necessitates varied management depending on the rotation phase. Soil microbial communities (SMCs), particularly nitrifiers and denitrifiers, play a significant role in soil nitrogen (N) cycling. However, the impact of the crop rotation phase and how associated management practices impact the abundances of SMCs responsible for transforming N in the soil is less studied. I selected 11 fields which were managed by a single operator in southern Ontario under a MZ-SB/WW-CC rotation. Soil samples were collected over four days from the 13th of October to the 3rd of November 2021. At sampling, four fields were under CCs, three fields had been planted to MZ (spring 2021), and four fields had been planted to SB (spring 2021) and harvested before being planted to WW in the fall (pre-sampling). Ten soil cores (0-20 cm depth) were collected along each transect (100 m) and composited (n = 4) per field. Soil bulk density, pH, SOM, water-stable aggregates (WSA), active carbon (AC), nitrate (NO_3^--N) and ammonium (NH_4^+-N) concentrations were assessed. DNA was extracted from soils, and quantitative PCR was conducted to enumerate the size of the total bacterial community (*16S* rRNA) and the size of nitrifier (*amoA*) and denitrifier (*nirK*, *nirS*, and *nosZI*) bacterial communities. During the fall sampling period, the highest NO_3^--N and NH_4^+-N were

observed in the SB-WW phase, which may have reflected N inputs from biological N fixation in the SB phase. Additionally, our findings revealed higher *amoA* gene abundances in the SB-WW phase than in the CC phase. Together, these results suggest a potential for an active nitrifier community, which was effectively oxidizing plant available NH_4^+-N to NO_3^--N in the SB-WW phase. The soils collected under the CC phase had the highest levels of labile carbon (10-17% higher), and elevated water-stable aggregates (35-50 % higher) compared to the other crop phases. Further, soils under CCs had lower *amoA/nosZI* and *nirK+nirS/nosZI* ratios compared to the MZ phase, which may be due to CC-induced alterations in the soil environment supporting specific nitrifier and denitrifier populations in the CC phase. Higher *amoA/nosZI* (1.21) and higher *nirK+nirS/nosZI* gene ratios (1.42) were found in the MZ phase compared to the CC (1.14, 1.28 respectively) and SB-WW phases (1.13, 1.20 respectively), suggesting the potential for relatively increased N_2O emissions in the soils planted to MZ. Overall, this short-term study suggests cash crop management practices associated with a diversified cash crop rotation impacted soil health parameters and N-cycling SMCs.

Keywords: Nitrifiers, Denitrifiers, Ontario agriculture, Crop rotation, Cover crops

3.1 Introduction

A commonly used cash crop management technique in southern Ontario is diversified crop rotation, which includes various crop types rotated periodically within the same land in a specific order (Reeves, 2018; Liebman & Dyck, 1993). Cash crop farmers often use a three-year corn (*Zea Mays*) – soybean (*Glycine max*)-winter wheat (*Triticum aestivum*)-cover crop (CC) rotation (Janovicek *et al.*, 2021). Diversified crop rotation is well-known for

enhancing soil health by increasing soil organic matter (SOM) (Garcia et al., 2013; Reeves, 1997; Zhang et al., 2021), enhancing C and N inputs (Zhang et al., 2021), reducing harmful weeds (Weisberger et al., 2019), and improving the functioning of the soil microbial community (SMC) (Maul et al., 2019; Smith et al., 2016; Venter et al., 2016).Moreover, adding CCs in crop rotation has various advantages, including decreasing soil erosion (Dabney et al., 2001; Kaspar & Singer, 2015), enhancing soil structure and nutrient availability (Chavarría et al., 2016; Kim et al., 2020), and improving crop productivity (Fageria *et al.,* 2005; Tonitto *et al.,* 2006). By increasing soil nutrients and reducing the need for fertilizer application, CC can help optimize crop yields (Fageria *et al.,* 2005; Kaspar & Singer, 2015; Thapa *et al.,* 2018).

Different crop phases in a rotation have different nutrient requirements (Janečková *et al.,* 2018). It is necessary to meet each unique crop's nutrient requirements to optimize the benefits (Janečková *et al.,* 2018). For instance, MZ and WW have higher N requirements than SB and CCs (Adeyemi *et al.,* 2020; Ciampitti & Salvagiotti, 2018). Soybeans and leguminous CCs can attain atmospheric N through biological N fixation, thereby fulfilling the N requirements of the plant (Ciampitti & Salvagiotti, 2018). In the context of nutrient management in agriculture, 4R nutrient stewardship is considered an effective way of using nutrients to accomplish benefits related to economic, social, and environmental goals. 4R nutrient stewardship is described as a framework for using the right nutrient source, applied at the right rate, right time, and in the right place, to improve sustainability (Agriculture and Agri-Food Canada, 2022). Furthermore, the right source addresses the selection of nutrient forms that can be easily taken up by plants, ensuring a well-balanced nutrient supply. The right rate implies the application of nutrients in

adequate amounts that meet the crop's requirements for optimal growth and yield (Agriculture and Agri-Food Canada, 2022). The rate should be determined based on the specific needs of the crop while considering yield goals, crop quality-related objectives, and soil nutrient levels. This right rate objective aims to provide sufficient amounts of all limiting nutrients to support the desired crop production without excess or deficiency (Agriculture and Agri-Food Canada, 2022). The right time involves considering the timing of nutrient application based on factors such as crop uptake patterns, soil nutrient availability, leaching risks, and field operations (Agriculture and Agri-Food Canada, 2022). Applying nutrients such as N fertilizers at the appropriate time in the growing season ensures that the crop uses them correctly and efficiently while avoiding harmful environmental effects (Kladivko *et al.*, 2001). The right place focuses on strategically placing nutrients in the soil to optimize the interaction between plant roots and soil. The right place includes considering the movement of nutrients within the soil, variation in nutrient levels across the fields, and the potential to reduce nutrient losses (Agriculture & Agri-Food Canada, 2022).

Nitrogen-cycling SMCs play a crucial role in the pathways of N loss from agricultural systems (Kuypers et al., 2018; Robertson & Groffman, 2006). These SMCs are involved in pathways of N loss from agriculture systems, including N_2O emissions to the atmosphere, NH_3 volatilization, and NO_3^- leaching (Linton *et al.*, 2020; Thompson *et al.*, 2016). Furthermore, microbial functional genes involved in N cycling processes are direct biological indicators of N transformation capacity (Levy-Booth *et al.*, 2014). Varied management practices associated with crop phases can regulate N-cycling SMCs (Kuypers

et al., 2018; Qin et al., 2016; Robertson & Groffman, 2006) which can directly impact the potential to reduce nutrient losses in agricultural soils.

In this study, I investigated the short-term impacts of cash crop management practices associated with different crop phases of a MZ-(SB-WW)-CC rotation within one operational farm. I analyzed soil health parameters and quantified total soil bacterial (*16S* rRNA), nitrifying (*amoA*), and denitrifying *(nirS, nirK, and nosZI*) communities. I predicted that (i) cover crops would have positive impacts within the CC phase on soil health indicators, (ii) that soil N concentrations would be higher in the SB-WW phase compared to the CC and MZ phases, and (iii) that the relative abundances of N-cycling SMCs would differ in soils under different crop phases.

3.2 Material and Methods

3.2.1 Study sites and soil sampling

The study sites (Supplementary Figure 3.1) were located in the Cobourg/Port Hope area in Northumberland County, Southern Ontario, Canada, within the Gages Creek and Cobourg Creek. The prevailing climatic conditions within this region were characterized by a mean annual temperature of 7.1°C and an annual average precipitation of 872 mm (Climate historic data 1981-2010-Coburg station). According to the Canadian soil classification system, the soils in the fields can be classified as Luvisols. All fields were tile-drained and no-till practices were employed by the operator; all fields were last tilled in either 1992 (1 field) or 1998 (remaining 10 fields).

The daily soil temperature data for the sites were collected using probes, which were connected to the HOBO micro station (H21-USB) data logger. These probes were

installed at 15 cm depth at the beginning of the study period (Oct 2021). The loggers were programmed to record hourly soil temperature data downloaded monthly. The probes were installed 50-100 m from the TD outlet in a relatively flat and shade-free area. Average air temperature and average precipitation data (Supplementary Fig 3.2) for the study period were obtained from the historical climate historic data of Canada (Environment and Climate Change Canada, 2022). Supplementary Fig 3.2 displays the daily average soil temperature, mean air temperature, and total precipitation for the study period.

The selected fields for the study were planted with a diversified crop rotation, including maize (MZ), soybean (SB), winter wheat (WW), and polycultures of cover crops (CC) over a three-year period (MZ-SB+WW-CC). One operational farmer managed the selected fields; hence, management was consistent across all fields. Twelve fields were selected for sampling based on the three crop phases being in the field at the time of sampling. During the sampling period, four fields were under CCs, four fields had been planted to MZ (spring 2021) and had not been harvested before sampling, and four fields had been planted to SB (spring 2021) and harvested before being planted to WW in the fall (pre-sampling). Unfortunately, one of the MZ fields was inaccessible due to flooding caused by beaver activity, as a result, the study was carried out with three MZ fields instead of the intended four. In 2021, cover crop mixtures in the fields included oats (*Avena sativa*), rye (*Secale cereal*), turnip (*Brassica rapa*), Daikon radish (*Raphanus sativus longipinnatus*), berseem clover (*Trifolium alexandrinum)*, crimson clover (*Trifolium incarnatum)*, sunflower (*Helianthus sativusannus*), buckwheat (*Fagopyrum esculentum*), hairy vetch (*Vicia villosa*), sorghum (*Sorghum*), fava bean (*Vicia faba*), and pea (*Pisum sativum*). Cover crop mixtures were chosen based on the operator's goal to have at least

one of each functional group, namely cold-season grass, cold-season broadleaf, warm-season grass, and warm-season broadleaf.

The operator applied mono-ammonium phosphate (MAP, 150 lb acre^{-1}) to MZ at planting in the spring and applied 150 lb acre^{-1}potash (a potassium fertilizer) and 50 lb acre^{-1}Kmag (potassium, magnesium, and sulfur fertilizer with low chloride levels and neutral pH) to both MZ and WW fields in the fall after SB harvest and WW planting (early October). At SB harvest/WW planting, fields received 6-24-6 starter (comprised of orthophosphoric acid, potassium hydroxide, and ammonium hydroxide) at 5 gal acre^{-1} and 50 lb acre^{-1}MAP.Cover crops received 150 lb acre^{-1}potash and 50 lb acre^{-1}Kmag in the previous summer (Figure 3.1).

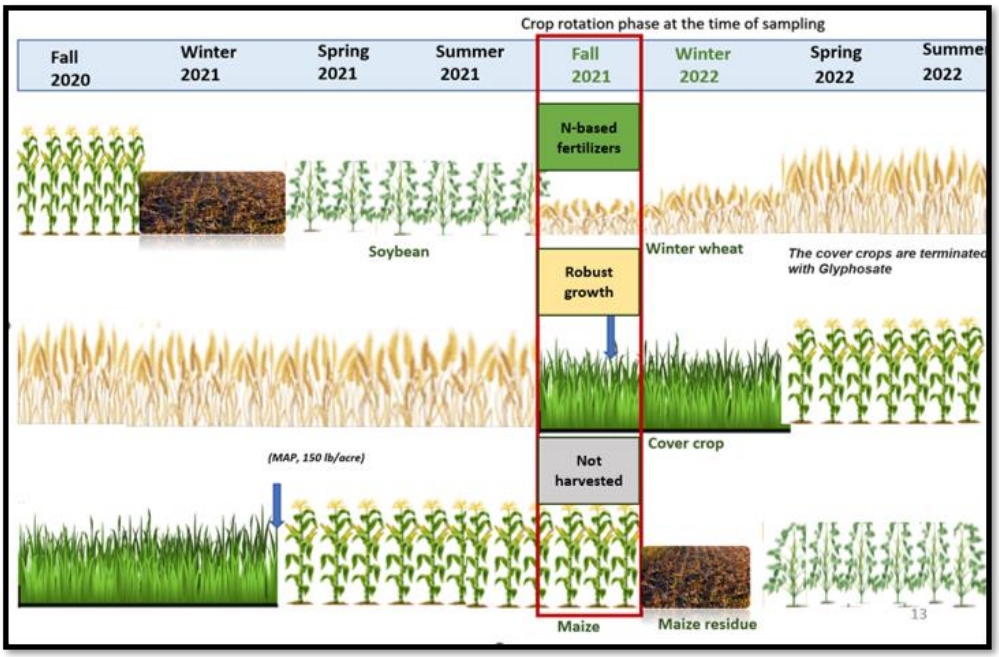


Figure 3.1 Graphical representation of field management practiced in MZ, SB-WW and CC crop rotation phases within one operational farm in MZ-(SB-WW)- CC rotation

In this study, soil samples were collected representing the three different crop rotation phases over four days from the 13th of October to the 3rd of November 2021. During this period, SB had been harvested, and WW was in the initial growth stages with the addition of N-based fertilizer to the WW seedlings. Meanwhile, MZ had not been harvested, and in all CC fields, CCs exhibited robust growth, contributing to considerable plant biomass (Supplementary Fig. 3.3).

Ten soil cores were collected at a depth of 0-20 cm along each 100 m transect with 100 m spacing. The samples were collected in four parallel transects per field at regular intervals and composited (Congreves et al., 2017; Tosi et al., 2020; Németh et al., 2014). Forty-four samples were assessed for each soil parameter and gene target. The soil corer was cleaned and sprayed with ethanol between sampling points to avoid cross-contamination. Composited soil samples were manually homogenized by squeezing and shaking the zip lock bags, and the bags were stored in a cooler containing ice packs before they were transported to the lab. Soil samples were stored at -20°C until further analysis. In each field, electrical conductivity (EC) and soil temperature were measured on each sampling date using a Time Domain Reflectometry (TDR) probe (Model 350, Spectrum Technologies Inc), inserted into the soil at approximately 20 cm depth, and along each transect.

3.2.2 Bulk density

Bulk density (BD) was measured along four 100 m transects in each field. Three cylindrical cores (volume = 231.532 cm^3) were collected along each transect to a 0-5 cm depth, and the samples were bagged and transported to the laboratory for further analysis. Soil samples were weighed before and after oven drying at 105°C for 24 h, and bulk densities were

determined (Eq 1) (Al-Shammary et al., 2018). Volume corrections for coarse fragments were conducted using a water displacement method, in which the volume of water displaced by these materials was measured to determine the actual volume occupied by the soil. This corrected volume was then utilized to calculate the BD of the soil.

$$\text{Bulk density } (gcm^{-3}) = \frac{\text{Weight of the dry soil (g)}}{\text{Total volume of the soil } (cm^3)} \qquad \textbf{Eq 1}$$

3.2.3 Soil pH

Soil pH was determined by adding 10 g of air-dried (<2 mm) soil to a falcon tube and 20 mL of deionized water. Samples were shaken for 30 min, and suspensions were kept at room temperature for one hour. Electrodes of the pH meter (Accumet AB150, Fisher Scientific, Ontario, Canada) were immersed into the soil solution, and readings were recorded once the readings were constant (Prodromou & Pavlatou-Ve, 1998).

3.2.4 Water stable aggregates (WSA)

Water-stable aggregates were measured using a wet sieving apparatus. Field-moist soils were stored in the fridge to avoid freeze-thaw before analysis, sieved (<4 mm) and air-dried. To measure the % WSA, 4.0 g of 4 mm sieved, air-dried soil was placed in small sieves (0.25 mm) in an aggregate stability-water sieving apparatus (Eijkelkamp, Forestry Suppliers Inc., USA). First, pre-moistened aggregates were washed with deionized water and allowed to pass through the sieves. Next, the cans containing the particles and aggregate fragments that passed through the sieves were removed from the apparatus and replaced with cans filled with dispersing solution. The dispersing soil was prepared before

the experiment based on the pH of the soil being tested. For soils with a pH greater than 7, the solution was made by dissolving 2 g of sodium hexametaphosphate/L. For soils with a pH lower than 7, the solution was made by dissolving 2 g of sodium hydroxide/L. Both cans were oven-dried at 110° C until the water evaporated. The weights were recorded, and the % WSA was determined by Eq 2.

$$\%\mathbf{WSA} = \frac{\textbf{Stable soil weight}}{\textbf{Total soil weight}} \times \mathbf{100} \qquad \textbf{Eq 2}$$

3.2.5 Soil Organic Matter (SOM) and Soil Active Carbon Analysis

Soil organic matter (SOM %) was determined by loss on ignition method (Jensen et al., 2018). Initially, crucibles were oven-dried at 105℃ for 1-2 hours to remove residual moisture and the weights of crucibles (W1) were carefully recorded. Field moist soils (5 g, 2 mm sieved) were weighed into crucibles; the samples were oven-dried before igniting, with the oven-dry soil weight recorded (W2). Then the samples were ignited at 550 ℃ for four hours in a muffle furnace, weights were recorded (W3) and the change in weight before and after was used to calculate the SOM % of the soil samples (Eq 3).

$$\%\ \textbf{Soil Organic Matter} = \frac{\textbf{W2}-\textbf{W3}}{\textbf{W3}-\textbf{W1}} \times \mathbf{100} \qquad \textbf{Eq 3}$$

To determine the active carbon content of the soil samples, 2.5 g of air-dried, 2 mm sieved soils were added to reaction tubes. Then, 18.0 mL of de-ionized water and 2 mL of 0.02 M $KMnO_4$ solution were added to each tube. The tubes were placed on an oscillating shaker bed and shaken at 180 rpm for 2 minutes. After shaking, the tubes were allowed to settle for 10 minutes. Next, 0.5 mL supernatant from each sample was taken and diluted

with 49.5 mL of de-ionized water. A standard curve was created from a dilution series prepared from known concentrations of $KMnO_4$ (0.05 M, 0.01 M, 0.015 M, and 0.02 M). The standard curve was run in triplicate on each plate with duplicate unknown samples. The absorbance values of the C oxidized by $KMnO_4$ in unknown samples and standards were measured at 550 nm using an Epoch™ microplate spectrophotometer (Biotech Instruments, Winooski, VT). The active carbon content (POXC) was determined using the following equation (Eq 4) (Weil *et al.*, 2003).

$$\text{POXC (mg kg}^{-1}\text{ soil)} = [0.02\text{ mol/ L} - (a + b \times \text{Abs})] \times (9000\text{ mg C/ mol}) \times (0.02\text{ L rx solution/ Wt}) \quad \textbf{Eq 4}$$

Where: 0.02 mol/L = initial solution concentration

a = intercept of the standard curve

b = slope of the standard curve

Abs = absorbance of unknown

9000 = mg of carbon oxidized by 1 mole of MnO_4

0.02 L = volume of stock solution reacted

Wt = weight of air-dried soil sample in kg

3.2.6 Soil exchangeable NO_3^--N and NH_4^+-N

3.2.6.1 Extraction of NO_3^--N and NH_4^+-N

Soil exchangeable NO_3^--N concentrations in each soil sample were determined by the 2 M KCl extraction method (Nelson, 1983). Frozen soil samples (10 g) were kept at room temperature for nearly 3-4 hours. Then the soils were sieved through 2 mm mesh and placed

into 125 mL flasks, and 100 mL of 2.0 M KCl was added to each flask. The flasks containing soil samples were shaken for one hour in an oscillating shaker bed at 160 rpm, and solutions were allowed to settle and filter through the Whatman no. 42 filter paper (Whatman Plc, ME, USA).

3.2.6.2 Soil exchangeable NO_3^--N analysis

The filtered extracts from 3.2.6.1 were analyzed for NO_3^--N using the Lachat flow injection analyzer (Lachat Instruments, a Hach Company Brand, USA-QuickChem*Method 12-107-04-1-B). A KCl reagent blank was run for every set of four samples (Maynard et al., 1993).To assess extraction efficiency, a triplicated spike test was performed using a 10 mg N/L KNO_3 solution, yielding a recovery percentage of 99.9%.

3.2.6.3 Soil exchangeable NH_4^+-N analysis

The filtered extracts from 3.2.6.1 were analyzed to determine soil exchangeable NH_4^+-N concentrations determined by the salicylate method via a UV-Vis spectrophotometer (Perkin Elmer, Woodbridge, Ontario, Canada). In this method, NH_4^+-N was reacted with salicylate and hypochlorite in an alkaline phosphate buffer, using sodium nitroferricyanide as a catalyst. This reaction resulted in the formation of an emerald-green-coloured solution. The ammonium concentration in the solution was determined at 667 nm and referenced to a standard calibration curve, providing a proportional relationship between the ammonium concentrations and absorbance (Diatloff & Rengel, 2001). In this method, 3 method blanks, 3 reagents blanks, and spike solution (10 mg N/L as NO_3) were carried out through the procedure.

3.2.7 Quantification of total soil bacterial genes and N-cycling soil bacterial genes

Soil DNA was extracted from a 0.250 g subsample obtained from composited field moist soils from each transect (n = 4) per field for 0-20 cm depth according to the manufacturer's protocol using the DNeasy Power Soil Pro Kit (Qiagen laboratories, Toronto, Ontario, Canada). The extracted DNA was quantified, and quality checked using a Nanodrop 8000 UV-Vis Spectrophotometer (ThermoScientific), and aliquots were made and stored at -80^{o}C for downstream analysis.

Quantitative polymerase chain reaction (qPCR) was performed to enumerate bacterial communities by targeting the *16S* rRNA gene, nitrifying communities (*amoA*), and denitrifying communities (*nirS, nirK, and nosZI***).** All DNA extracts were diluted to reduce the impact of inhibition and to increase qPCR reaction efficiency. The qPCR reaction mixtures contained either 10 µL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.) or PowerUp SYBR Green Master Mix (Thermo Fisher Scientific (Mississauga) Inc), 1 µL of 10 µM of each forward and reverse primer, 2 µL of DNA template, and nuclease-free water to a final volume of 20 µL. For *16S* rRNA, *nirS,* and *nirK* SsoAdvanced Universal SYBR Green Supermix was used, and for *amoA* and *nosZI* genes, PowerUp SYBR Green Master Mix was used. All amplifications were performed on a QuantStudio3 Real-Time PCR system (ThermoFisher Scientific, Markham, Ontario, Canada).

In parallel with triplicated unknown samples, triplicate standard curves were included on each qPCR plate, which were constructed by serial dilution of gBlocks gene fragments (Integrated DNA Technologies, Inc.) containing the target genes (10^{1}-10^{8}

copies). The targeted bacterial *16S* rRNA, *amo*A, *nirS*, *nirK,* and *nosZI* standards were created by Integrated DNA Technologies (IDT, Inc.) and were designed based on published genomic DNA sequences of *Clostridium thermocellum* spp., *Nitrosomonas europaea* spp., *Alcaligenes faecalis* spp., *Pseudomonas aeruginosa* spp., and *Pseudomonas fluorescens* spp. respectively (Thompson *et al.*, 2016) (Table 3.1). The qPCR reactions were conducted involving an initiation step of 2 min at 98°C, followed by 35 cycles of denaturation at 98°C for 10 s, and annealing at 55°C (for *16S* rRNA), 57°C (for *amoA* and *nirS*), and 56°C (for *nirK*) for 30 s, and an elongation at 72°C for 30 s. For *nosZI* a touch-down protocol was used (Henry, 2007). Additionally, for assays using PowerUp SYBR Green Master Mix, qPCR reaction conditions involved a Uracil-DNA Glycosylase (UDG) activation step of 50°C before the initiation step.

The qPCR assays were optimized to obtain 92.0%-106.0% reaction efficiencies for *16S* rRNA, *amo*A, *nir*S, *nirK,* and 85.5% -93.0% for *nosZI;* the slopes of the standard curves were between -3.23 to -3.51 with R^2 values between 0.991-0.999. Triplicate no template controls were included in all qPCR assays. Melt curve analysis was used to assess the specificity of the qPCR reactions by confirming the presence of a single qPCR product and the absence of non-specific products or primer dimers. Gel electrophoresis was used to further confirm the presence and correct size of qPCR products. Additionally, the gel can provide information about the purity of the DNA fragments by assessing the presence of any additional bands or smearing, which could indicate the presence of impurities. Amplified DNA fragments were loaded onto a 1% agarose gel matrix and subjected to an electric field, which caused DNA fragments to migrate through the gel matrix. To verify

the proper sequence size of the DNA fragments, a 100bp DNA ladder (NORGEN) was used.

Table 3.1: Gene targets, primers, and annealing temperatures

Gene	Target	Primers	Oligo Sequence (5'-3')	Annealing Temp (°C)	Reference
16S **rRNA**	Total bacteria	338f 518r	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	55	Fierer *et al.*, 2005),
amoA	Ammonium monooxygenase	*amoA-1F* *amoA-2R*	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	57	Rotthauwe *et al.*, 1997
nirS	nitrite reductase	*nirS-cd3af* *nirS-R3cd*	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	57	Throbäck *et al.*, 2004
nirK	nitrite reductase	*nirK-F1aCu* *nirK-R3Cu*	ATCATGGTSCTGCCGCG TTGGTGTTRGACTAGCTCCG	56	Hallin and Lindgren, 1999
nosZI	Nitrous oxide reductase	*nosZI-1F* *nosZI-1R*	WCSYTGTTCMTCGACAGCCAG ATGTCGAT- CARCTGVKCRTTYTC	Touch down protocol	Henry, 2006

3.2.8 Statistical analysis

All statistical analyses were conducted in the R version 4.2.1 (R core Team 2023 - Integrated Development Environment for R. R Studio, PBC, Boston, MA). Before parametric tests, normality and homoscedasticity were assessed using the Shapiro-Wilks and Bartlett's tests respectively to check model assumptions. Data were log-transformed

when necessary to meet parametric assumptions and then back-transformed for data presentation. All gene abundances were normalized to $\log_{10}$ gene copy numbers per gram of dry soil to meet the model assumptions regarding residual variance.

Analysis of variance (ANOVA) type III sum of squares (SS) using a linear mixed effect model was conducted through the '*lmer*' function in the '*lme4'* package. This analysis was performed on each soil parameter, *16S* rRNA total bacterial gene abundance and each N-cycling gene abundance separately. Within each data set, the independent fixed variable was the crop rotation phase, while the fields and the transects were random variables. Individual least-square means (least square means = emmeans) within the data set were compared with Tukey's HSD adjustment using the '*emmeans'* function in the '*emmeans*' package. Significant differences among and between least-square means were determined by p values, where the null hypothesis (Ho) was rejected at $p < 0.05$.

Pearson's correlation analysis was conducted to assess the statistical associations between gene abundances and soil health parameters tested. Finally, to visually demonstrate the multiple relationships between soil health parameters, total bacterial gene abundance, and N-cycling SMC gene abundances, a principal components analysis (PCA) was carried out in R Studio software. In the PCA, a scree plot was examined for breaks, and PC components with eigenvalues ≥ 1 (PC1 and PC2) were retained in the 2-dimensional plot for visual analysis.

3.3 Results

3.3.1 Site description

All the fields considered in this chapter were tile-drained and no-till fields with high SOM (4.13 ±1.05% - 5.35 ±1.08%). The soil texture of the selected fields was fine (silty clay loam or silt loam soils) with a low slope (average of nearly 4%) (OMAFRA,2019). The fields from the same crop rotation phase were considered replicates based on their similarities in soil texture, slope and SOM.

3.3.2 Active Carbon (AC)

Active Carbon levels were significantly different among crop phases. The highest AC levels were in soils under CC (781.0 ± 26.0 mg kg^{-1} soil), and the lowest AC levels were in soils under SB-WW (669.0 ± 17.0 mg kg^{-1} soil) and MZ (712.0 ± 22.0 mg kg^{-1} soil) crop phases. Soil AC levels were not significantly different between SB-WW and MZ phases **(**Table 3.2).

3.3.3 Water stable Aggregates (WSA)

The WSA values significantly differed among crop rotation phases, and WSA% decreased in the order of CC (52.1 ± 2.1%) > SB-WW (45.6 ± 1.8%) > MZ (34.0 ± 4.1%) (Table 3.2).

3.3.4 Soil pH and EC

Both pH and EC were significantly different among crop rotation phases. The highest pH values were found in MZ fields (7.1 ± 0.09), while the lowest values were found in both CC (6.3 ± 0.08) and WW fields (6.4 ± 0.08), which were not different from each other. MZ (280 ±10 μS cm^{-1}) and WW (290 ±10 μS cm^{-1}) fields had significantly higher EC values than CC fields (150 ± 10 μS cm^{-1}) **(**Table 3.2).

3.3.5 Soil exchangeable NO_3^--N and NH_4^+-N

The soils under SB-WW had significantly higher NO_3^--N levels (5.2 ± 1.14 μg N g^{-1} dry soil) compared to fields under CC (1.0 ± 1.1 μg N g^{-1} dry soil) and MZ (2.5 ± 1.2 μg N g^{-1} dry soil) phases at the time of sampling (Table 3.2). The lowest NH_4^+-N levels were found in the CC phase (2.60 ±1.16 μg N g^{-1} dry soil), while comparatively high NH_4^+-N levels were found in MZ (10.07±1.17 μg N g^{-1} dry soil) and SB-WW (9.73 ± 1.72 μg N g^{-1} dry soil) (Table 3.2).

Table 3.2. Mean values of BD, pH, EC µS cm^{-1}, WSA (%), SOM (%), AC mg kg^{-1}soil, NO_3^--N µg N g^{-1} dry soil, and NH_4^+-N µg N g^{-1} dry soil among fields under CC, MZ and SB-WW crop rotation phases in an operational farm in southern Ontario (October-November 2021). The data are least-square means (n =9) ± SE; parameters indicated as NS are not significantly different. Lowercase letters following data indicate significant differences between crop phases within each column ($p < 0.05$).

Crop phase	BD (NS)	pH	EC	WSA	SOM (NS)	AC	NO_3^--N	NH_4^+-N
CC	1.33±0.01	6.3^{b}±0.08	150^{b} ±0.01	52.1^{a}±2.1	4.13 ±1.05	781^{a} ±26	1.02^{b} ±1.15	2.60^{b} ±1.16
MZ	1.30±0.02	7.1^{a} ±0.09	280^{a} ±0.01	34.0^{c} ±4.1	5.35 ±1.08	712^{b} ±22	2.50^{b} ±1.17	10.07^{a} ±1.17
WW	1.29±0.01	6.5^{b}±0.08	290^{a} ±0.01	45.6^{b}±1.8	4.92±1.07	669^{b} ±17	5.20^{a}±1.14	9.73^{a} ±1.72
P value	0.1998	<.0001	<.0001	<.0001	0.3735	0.0058	0.009	<.0001
F-value†	$0.81_{2,40}$	$0.86_{2,40}$	$1.72_{2,40}$	$18.96_{2,40}$	$1.007_{2,40}$	$5.84_{2,40}$	$8.74_{2,40}$	$21.29_{2,40}$

†F value subscript indicates the numerator and denominator degrees of freedom, respectively.

3.3.6 Soil microbial community analysis

3.3.6.1 Total soil bacterial and N-cycling gene abundances

Total bacterial gene abundances (*16*S rRNA) were not significantly different among crop phases **(**$\log_{10}$ 9.75 – $\log_{10}$ 10.35 gene copies g^{-1} dry soil) (Table 3.3). Similarly, denitrifying *nirS* communities did not differ in abundance among the three crop rotation phases ($\log_{10}$ 7.2 – $\log_{10}$ 7.5 gene copies g^{-1} dry soil). However, *nirK* gene abundances were significantly higher in CC ($\log_{10}$ 7.95 ± 0.18 gene copies g^{-1} dry soil) and MZ ($\log_{10}$ 8.27 ± 0.21 gene copies g^{-1} dry soil) soils compared to WW ($\log_{10}$ 7.41 ± 0.18 g^{-1} dry soil) soils. In contrast, *nosZI* genes were highest in abundance in WW soils ($\log_{10}$ 6.2 ± 0.01gene copies g^{-1} dry soil), while the lowest *nosZI* abundances were found in CC ($\log_{10}$ 5.7 ± 0.07 gene copies g^{-1} dry soil) and MZ fields ($\log_{10}$ 5.8 ± 0.08 gene copies g^{-1} dry soil). Nitrifying *amoA* communities were significantly larger in MZ ($\log_{10}$ 7.0 ± 0.10 gene copies g^{-1} dry soil) and WW fields ($\log_{10}$ 6.9 ± 0.08 gene copies g^{-1} dry soil) compared to CC fields ($\log_{10}$ 6.5 ± 0.09 $\log_{10}$ gene copies g^{-1} dry soil) (Table 3.3). Higher *amoA/nosZI* (1.21) and higher *nirK+nirS/nosZI* ratios (1.42) were found in the MZ phase compared to CC (1.14, 1.28 respectively) and SB-WW phases (1.13, 1.20 respectively).

Table 3.3

Mean values of total (*16S* rRNA), nitrifying (*amoA*), and denitrifying (*nir*S, *nirK*, and *nosZI*) bacterial gene abundances, *amoA* / *nosZI* ratios and *nirS+nirK*/ *nosZI* ratios and the results of ANOVA among fields under CC, MZ, and (SB-WW) crop rotation phases in an operational farm in southern Ontario (October-November 2021). Gene abundances are presented in $\log_{10}$ gene copies per gram dry soil. The data are least-square means (n = 9) ± SE; parameters indicated as NS are not significantly different. Lowercase letters following data indicate significant differences between crop phases within each column ($p < 0.05$).

Crop phase	***16S* rRNA (NS)**	***amoA***	***nirS* (NS)**	***nirK***	***nosZI***	***amoA/ nosZI***	***nirS+nirK/ nosZI***
CC	9.75 ± 0.2	6.5^{b} ± 0.09	7.5 ± 0.20	7.9^{a} ± 0.18	5.7^{b} ± 0.07	1.14^{b}	1.28^{b}
MZ	10.67 ± 0.3	7.0^{a} ± 0.10	7.4 ± 0.10	8.2^{a} ± 0.21	5.8^{b} ± 0.08	1.21^{a}	1.42^{a}
WW	10.35 ±0.22	6.9^{a} ± 0.08	7.2 ± 0.20	7.4^{b} ± 0.18	6.2^{a} ± 0.01	1.13^{b}	1.20^{b}
P value	0.057	<.0001	0.204	0.040	0.0002	0.004	<.0001
F-value†	$4.16_{2,40}$	$10.66_{2,40}$	$1.65_{2,40}$	$4.89_{2,40}$	$10.42_{2,40}$	$6.19_{2,40}$	$8.40_{2,40}$

†F value subscript indicates the numerator and denominator degrees of freedom, respectively.

3.3.7 Pearson Correlation analysis and principal component analysis (PCA) for soil health parameters and Microbial Responses

Pearson's correlation analysis showed a strong positive correlation between *amoA* community size and pH ($p < 0.05$, $r = +0.53$) and between *amoA* gene copies and EC ($p < 0.05$, $r = +0.69$) (Fig 3.2). Similarly, *nirK* gene copies were positively correlated with soil moisture ($p < 0.05$, $r = +0.37$) and pH ($p < 0.05$, $r = +0.63$). *amoA* gene abundance and NO_3^--N ($p < 0.05$, $r = +0.48$) and *amoA* gene abundance and NH_4^+-N ($p < 0.05$, $r = +0.53$) were also positively correlated (Fig 3.2).

A PCA loading plot was created to visualize multivariate relationships between soil health parameters and SMCs. The soil health parameters used to build the PCA plot were BD, pH, EC μS cm^{-1}, WSA (%), SOM (%), AC mg kg^{-1}soil, and NO_3^--N μg g^{-1} dry soil, and NH_4^+-N μg g^{-1} dry soil. SMC parameters were total bacteria *16*S rRNA, nitrifying (*amo*A), and denitrifying (*nir*S, *nirK*, and *nosZI*) gene abundances (log_{10} gene copies g^{-1} dry soil). The first two principal components, PC1 and PC2, accounted for 30.4% and 22.3% of the data set variance, respectively.

The loading plot indicated that parameters including EC, NO_3^--N μg g^{-1} dry soil, and NH_4^+-N μg g^{-1} dry soil, and *16S* rRNA and *amoA* gene abundances (log_{10} gene copies g^{-1} dry soil) were predominantly loaded on the first principal component (PC1). Meanwhile, parameters including AC, MC, pH, and *nirK* and *nosZI* gene abundances loaded on the second principal component (PC2) (Fig. 3.3).

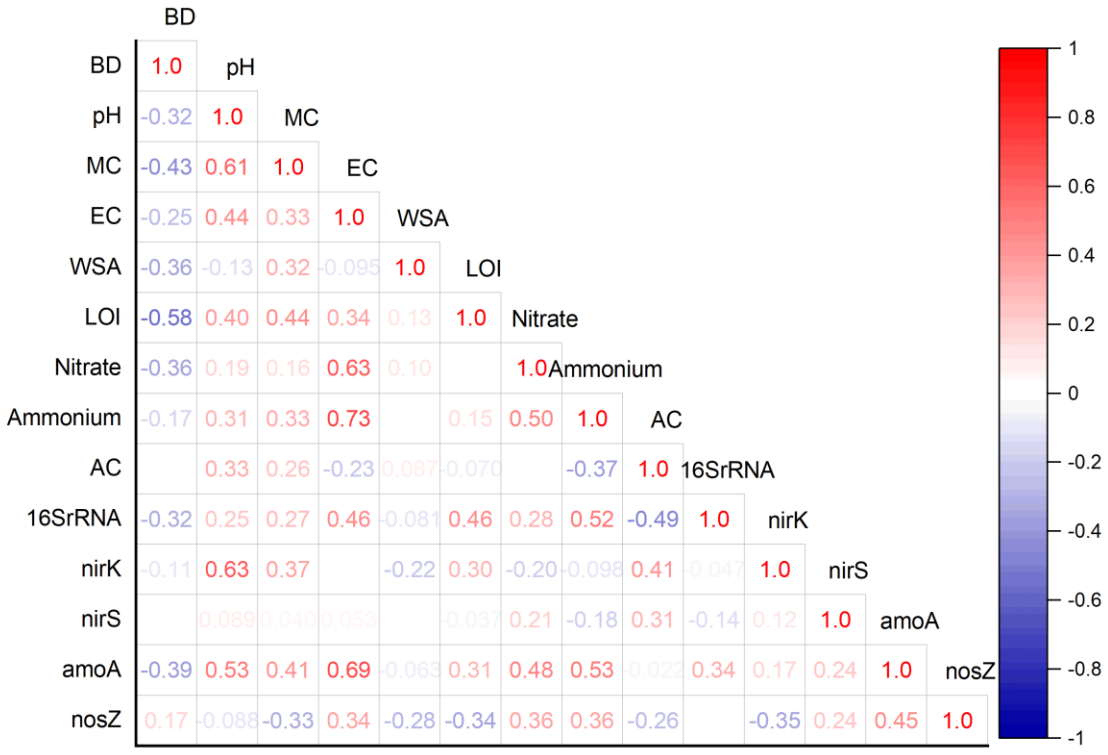


Figure 3.2 Pearson's correlation of soil health parameters: BD, pH, MC, EC µS cm^{-1}, WSA (%),SOM (LOI) (%), AC mg kg^{-1} soil, NO_3^--N µg N g^{-1} dry soil, and NH_4^+-N µg N g^{-1} dry soil, and total (*16S* rRNA), nitrifying (*amoA*), and denitrifying (*nirS*, *nirK*, and *nosZI*) bacterial gene abundances in soil samples collected from an operational field in southern Ontario (October – November 2021).

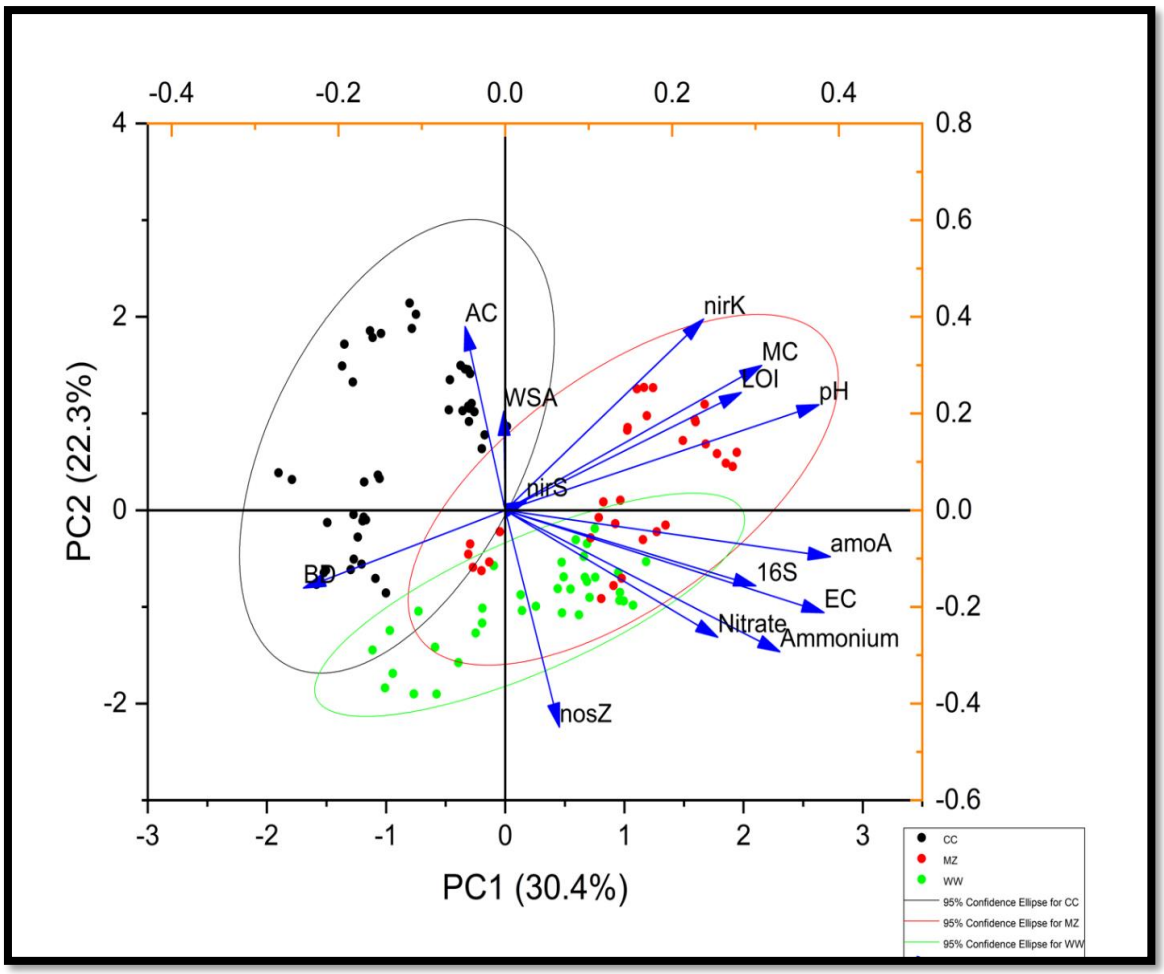


Figure 3.3 Principal component analysis (PCA) of soil health parameters: BD, pH, EC µS cm $^{-1}$, WSA %, SOM (LOI) %, AC mg kg^{-1} soil, NO_3^--N µg g^{-1} dry soil, and NH_4^+-N µg g^{-1} dry soil, and total (*16S* rRNA), nitrifying (*amoA*), and denitrifying (*nirS*, *nirK*, and *nosZI*) bacterial gene abundances in soil samples collected from maize (MZ), winter wheat (WW), and cover crops (CC) phases in a MZ-SB-WW-CC rotation in an operational field in southern Ontario (October – November 2021).

3.4 Discussion

In this study, I collected soils from 11 fields, managed by a single operator, to study the impact of cash crop management practices associated with different crop rotation phases in MZ-SB/WW-CC crop rotation. Further, in this chapter, I characterized selected physical, chemical, and biological parameters of soil health in soils from each crop rotation phase with varied management practices. The selected soil health parameters reflect specific aspects of soil functioning relevant to the present study (Bünemann *et al.*, 2018; Bone *et al.*, 2014). Soil bulk density is an indicator of soil compaction, porosity, and overall soil structure (Logsdon and Carlon,2004). It influences water retention, aeration, and root penetration which are crucial for plant growth and SMC growth (Al-shammary et al., 2018). Soil pH is a fundamental factor influencing nutrient availability and SMCs (Lehmann et al., 2020; Sung et al., 2017), affecting the solubility of minerals and the activity of soil enzymes, thereby impacting soil health and plant nutrient uptake (Prodromou & Pavlatou-Ve, 1998). Soil organic matter is vital in nutrient recycling, water retention, and support of soil biota (Garcia et al., 2013; Liang et al., 2019). Measuring SOM provides insights into the soil's capacity to support plant growth and SMCs (McDaniel et al., 2014). Active carbon represents the portion of SOC actively involved in microbial heterotrophic functions, such as decomposition, nutrient transportation, and SOM turnover (Weil *et al.*, 2003). Water-stable aggregates are beneficial in many ways including C sequestration, nutrient retention, soil aeration, erosion control, and providing microenvironments for SMCs (Blankinship et al., 2016; Upton et al., 2019). Additionally, WSA is also an indicator of biological activity because they are formed by root and microbial exudates (Denef et al., 2002). Therefore, the fraction of WSA is used as one of the key parameters to determine soil health (Denef et al., 2002). Measuring soluble nitrogen in the form of

soil nitrate and ammonium is essential to understand soil nitrogen availability for crops and the potential for nitrogen losses (Maynard et al., 1993; Wang et al., 2020).I quantified the total bacterial community (*16S* rRNA), and bacterial nitrifier (*amoA*) and denitrifier (*nirK*, *nirS*, and *nosZI*) communities in each crop rotation phase which represent the capacity for key biological N transformations in soils (Levy-Booth *et al.*, 2014), allowing a more targeted assessment of the microbial process involved in N cycling (Abatenh *et al.*, 2018; Levy-Booth *et al.*, 2014).

Based on the findings of the present study, CC had higher (10-17% higher) AC levels compared to SB-WW and MZ phases. Active carbon, or the labile fraction of SOC, mainly originates from SOC decomposed by SMC, root exudates, and microbial necromass (Liptzin *et al.*, 2022; Nunes *et al.*, 2021; Bongiorno *et al.*, 2019). Accumulation of microbial necromass makes up around 30-60% of the SOM in topsoil (Liang et al., 2019). At the time of sampling, CC mixtures were well established with relatively good soil coverage and large biomass (supplementary figure 3.3) likely resulting in increased C returns to these soils during the CC phase. It may have resulted in the observed higher AC levels in the CC phase compared to the other two phases.

Moreover, plant roots release primary root exudates into the soil, and SMCs use these compounds as nutrient sources or signalling molecules (Huang *et al.*, 2019; Weil *et al.*, 2003). The root exudates are a mixture of organic compounds released by plant roots into the soil (Steinauer *et al.*, 2016). They include sugars, organic acids, enzymes, amino acids, carbohydrates, secondary metabolites, and hormones which provide different functions such as facilitating nutrient uptake, providing substrates to the microbial community, and suppressing pathogens with antimicrobial properties (Badri & Vivanco, 2009; Jia *et al.*, 2014; Steinauer *et al.*, 2016). Interestingly, the composition, quantity, and quality of C entering the soil through root exudation depends on the plant

species and fluctuating biotic and abiotic factors (Chavarría et al., 2016; Hooper et al., 2000; Maurer et al., 2021).In the CC phase, the observed large plant root biomass of CC mixtures may have contributed to greater production of root exudates compared to the MZ and WW phases (Chavarría et al., 2016).

Water stable aggregates (WSA) are the fraction of soil aggregates resistant to disruption or breakage of the bonds within the aggregates by water (Angers. *et al.*, 2006). Soil texture, structure, organic matter content, land management practices, and cover cropping greatly influence aggregate stability (Al-Kaisi *et al.*,2014; Angers *et al.*, 2006). The distribution of plant roots varies greatly among crop species (Reeves, 1998) and can influence aggregate stability. Monroe & Kladivko (1987) reported no significant differences in aggregate stability among MZ, SB and WW crops. Nouwakpo *et al.* (2018) demonstrated the increased ability of MZ residues to improve soil aggregation compared to SB residues. In contrast, some studies reported that, in some instances, MZ roots can reduce aggregate stability due to crop management practices adopted in the fields (Reid *et al.*, 1982; Reid & Goss, 1982). In the present study, the application of glyphosate for the termination of CCs in spring before planting MZ may have negatively impacted WSA and AC levels in the MZ phase soils compared to the CC phase soils. The study findings by Reid *et al* (1982) and Reid & Goss. (1982) aligns with our results, which found significantly lower WSA (%) in MZ compared to CC soils. The significantly higher WSA values in CC fields (35-50% higher) compared to the other two phases may be linked with increased organic matter inputs, which can bind soil particles from aggregates. Cover crops contribute to changing soil environment by returning aboveground biomass, enhancing soil aggregation, and stimulating the activity of SMCs (Haruna et al., 2020; Kaspar & Singer, 2015a; Kim et al., 2020; Koudahe et al., 2022). Additionally, high CC biomass contributes to canopy

coverage, reducing the potential for erosion (Castellano-Hinojosa *et al.,* 2021; Haruna *et al.,* 2020; Saleem *et al.,* 2020). Furthermore, CCs can increase water infiltration via root penetration into deeper layers, which can further enhance microbial activity and promote aggregate formation (Castellano-Hinojosa *et al.,* 2021; Haruna *et al.,* 2020).

Cover crop mixtures, including N fixers, N scavengers, winter hardy, and winter-killed crops, are considered a good strategy for nutrient scavenging and the timely release of nutrients to successive crops (Finney *et al.,* 2016). Additionally, leguminous cover crops and grasses are frequently grown together. This practice aims to decrease the overall C: N ratio of CC residues returned to the soil. By lowering the C:N ratio, the decomposition of CC residue is accelerated, allowing for quicker decomposition, and reducing the potential for excessive nutrient accumulation (Nielsen *et al.,* 2015; Sainju *et al.,* 2005). Muzangwa *et al.* (2015) demonstrated that a blend of oat and vetch regulates the decomposition and availability of nutrients for future cash crops. Finney *et al.* (2016) found extensive N scavenging with cover crop mixtures, and determining mixtures should be done based on their potential to achieve specific objectives such as reducing nutrient runoff, weed suppression, enhancing soil fertility, erosion control and improving biodiversity. In our study, the CC phase consists of cover crop mixtures of leguminous and non-leguminous cover crops, which might act as NO_3^--N scavengers, potentially leading to lower NO_3^--N (-80% and -60% respectively) and NH_4^+-N (-73% and -74% respectively) in CC phase compared to SB-WW and MZ phases.

Maize and SB-WW soils exhibited higher EC values than CC fields during the sampling period. This observation in the SB-WW phase could be attributed to the excessive application of synthetic fertilizers beyond the natural nutrient requirements

of the crop being cultivated, which can lead to elevated nutrient concentration in the soil, potentially impacting soil pH and EC (Dabney *et al.*, 2001; Jacobs & Timmer, 2005). Additionally, the proximity of field sites, mainly MZ fields, to Highway 401 might be another reason for EC differences among fields, especially due to the road salt being applied on the highway. The impact is mainly due to the salt's chloride component; these salts can be transported to nearby fields through runoff and impacting EC (Equiza et al., 2017; Shannon et al., 2020). Even though three CC fields were in proximity to Highway 401, and road salt can be impacted, interestingly, the EC values recorded in the CC fields were comparatively lower than those of the SB-WW and MZ fields.

Although the abundance of total bacteria *16S* rRNA did not significantly differ among the three crop rotation phases, the observed p-value of 0.057 suggests that the crop phase could have a biological or ecological impact on the size of the total bacterial community. The p-value indicates a possible relationship that may warrant further investigation. Moreover, several studies have demonstrated that an increase in SOM provided by root exudates and plant residues in the CC phase corresponds to increased SMC abundances, indicating improved nutrient cycling (Graaff *et al.,* 2010; Navarro-Noya *et al.,* 2013; Ramirez-Villanueva *et al.,* 2015). However, it is essential to note that total bacterial *16S* rRNA abundance is not an absolute quantifier, as the number of gene targets per genome can vary per microbial species (Fogel *et al.,* 1999). These results suggest that while changes in *16S* rRNA abundance can provide insights into bacterial community dynamics, it does not directly measure the absolute number of bacterial cells.

The highest NO_3^--N and NH_4^+-N concentrations were observed in the SB-WW phase compared to the other two phases. These results can be explained by the release of N, which was symbiotically fixed by the leguminous SB, the previous crop phase, before being planted to WW. Moreover, our results indicated a higher *amoA* gene abundance in WW fields (+58%) compared to the CC phase. High NO_3^--N and NH_4^+-N concentrations together with the high *amoA* abundance in the SB-WW phase imply a potential for increased nitrification. Additionally, at the planting of WW, ammonia-based fertilizer and N:P:K starter was applied, and these applications were done very close to the sampling days. This fertilization application could result in higher NH_4^+-N concentration, a substrate for the nitrification process, and higher *amoA* abundance in the SB-WW phase compared to the CC phase. Further, Sun *et al.* (2015) conducted a study on the long-term application of NPK fertilizers in a WW and summer bean crop rotation. Their findings revealed a strong relationship between fertilizer application and *amoA* gene abundance. These results aligned with our study findings, demonstrating a high *amoA* abundance in SB-WW, close to fertilizer application. In this study, according to Pearson's correlation analysis and PCA, *amoA* gene abundance showed a positive correlation with NO_3^--N, and NH_4^+-N in the SB-WW phase. Together, these results suggest a potential for increased nitrification in the soils planted to SB-WW. In contrast, CC fields had lower *amoA* gene abundance and lower NO_3^--N values, suggesting a potential for decreased nitrification in the soils planted to CC.

According to the findings, no significant differences in the abundance of the *nirS* gene were observed across three crop rotation phases, while *nirK* abundances were higher in the CC (+216%) and MZ (+530%) phases compared to the SB-WW phase. Furthermore, in the present study, the *nirK* gene abundance in the CC phase was higher (+58%) than the *nirS* gene abundance in the CC phase. Romdhane *et al.* (2019) and

Schmidt *et al.* (2019) have also reported higher *nirK* abundance in soils treated with CC compared to *nirS* abundance. Denitrifiers bearing the *nirK* or *nirS* gene differ in their enzyme structure, substrate specificity, and response to environmental conditions, leading to variation in their ecological roles and responses (Helen et al., 2016; Lee & Francis, 2017). According to correlation analysis, *nirS* gene abundances were not correlated with any of the soil characteristics; however, *nirK* gene abundances showed a positive correlation with soil pH and soil moisture. According to Helen *et al.* (2016), denitrifiers possessing the *nirS* gene are typically dominant in regions with fluctuating oxygen concentrations and high nitrate availability. In agricultural soils, *nirK* gene abundances have been reported to be higher than *nirS* abundances (Dandie et al., 2011; Hai et al., 2009; Wang et al., 2020; Zhou et al., 2011). Even though denitrifiers carrying these enzymes share similar functions, these two types occupy different niches (Hu *et al.,* 2021).

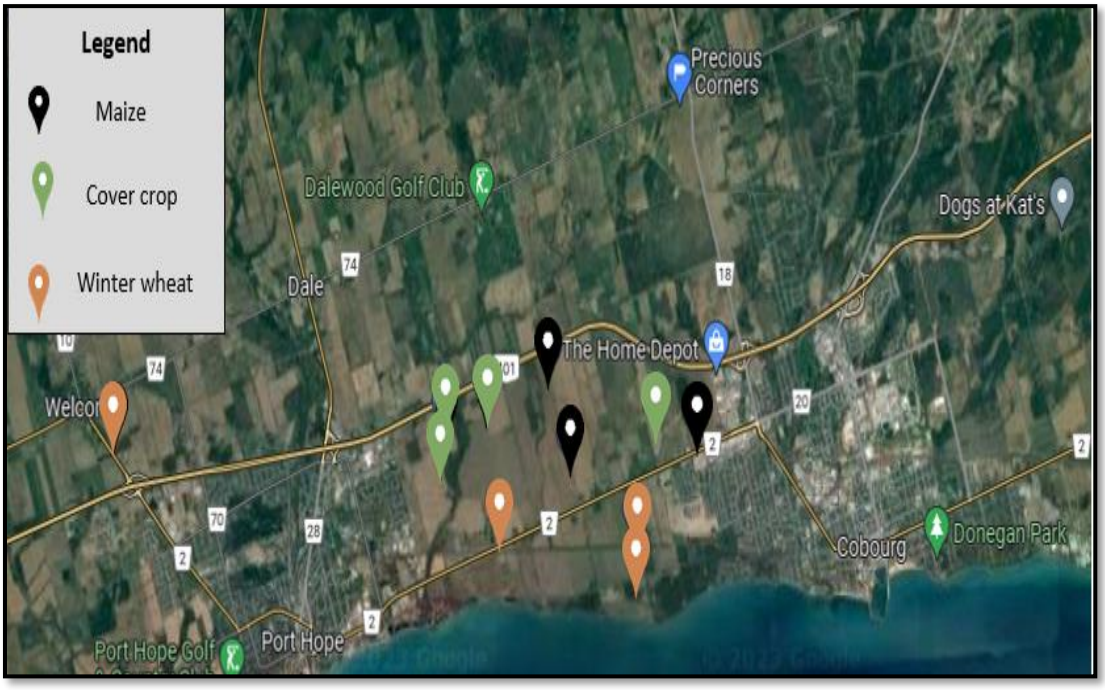
In the denitrification process when the denitrification is incomplete, N_2O can be released into the atmosphere (Kuypers et al., 2018; Robertson & Groffman, 2006). Moreover, in investigating the factors influencing N_2O emissions, Daniel *et al.* (2014) found that although N_2O emissions depend on environmental conditions and inherent cell regulatory mechanisms, emissions are primarily governed by *nosZI* functional genes in the denitrifying community. In general, high N_2O emission potential is associated with high *amoA/nosZI* and *nirK+nirS/nosZI* ratios (Saarenheimo *et al.,* 2015; Wan *et al.,* 2023; Xiang *et al.,* 2022). Therefore, high *amoA/nosZI and nirK+nirS/nosZI* ratios in the MZ phase imply the potential for higher N_2O emissions from the soils planted to the MZ compared to the soils planted to SB-WW and CCs.

3.5 Conclusion

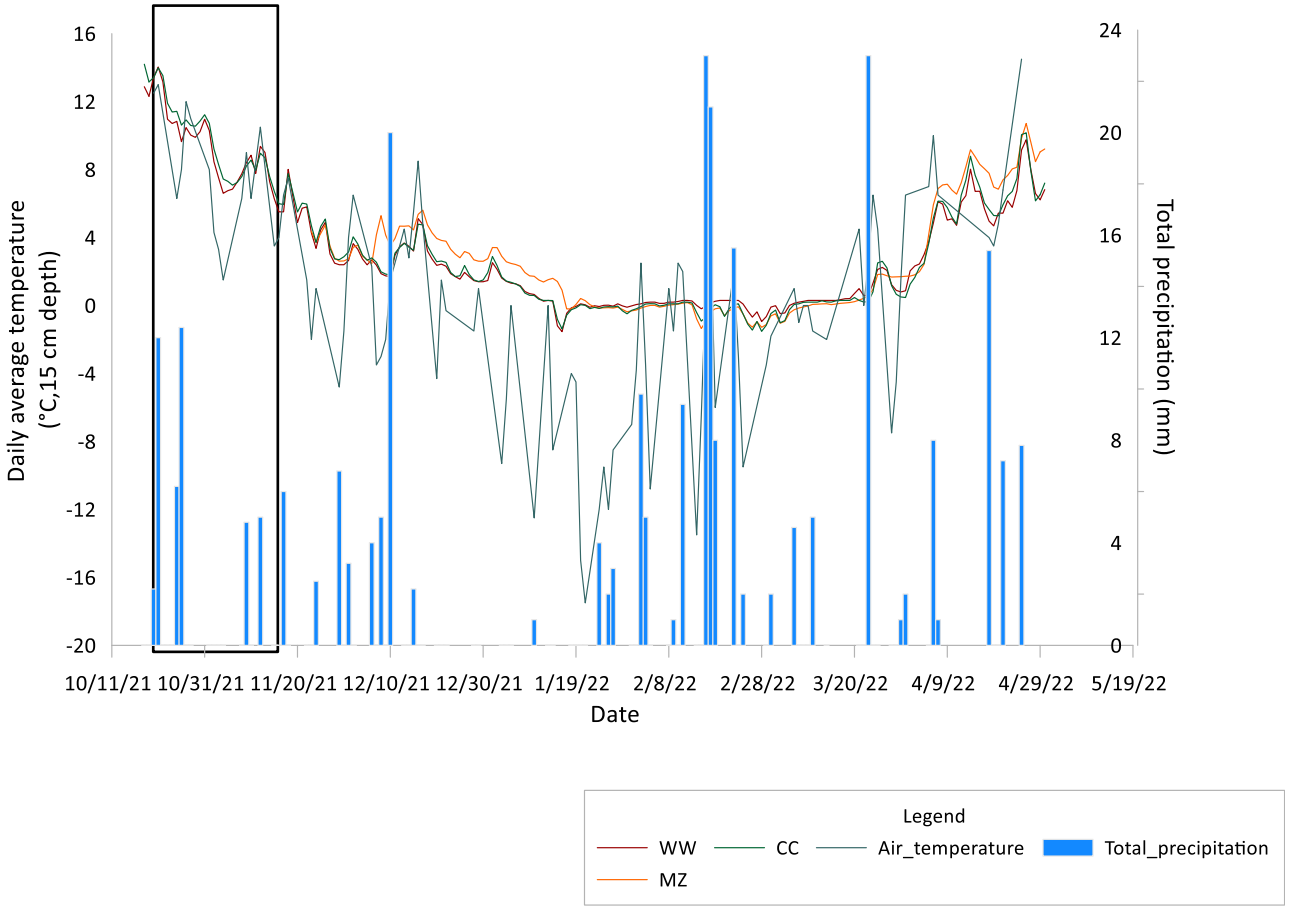
In this study, I examined the short-term impacts of different phases in a diversified MZ-(SB-WW)-CC crop rotation in southern Ontario on soil health parameters and soil N-cycling SMC abundances associated with nitrification (*amoA*) and denitrification (*nirS, nirK,* and *nosZI*). I discovered that the soils collected from the CC phase had higher AC and WSA than the soils collected from the MZ and SB-WW phases, likely due to the CC's potential to change soil structure, retain moisture in soils, build SOM and release primary root exudates by abundant living root biomass in CC mixtures consisting of leguminous and non-leguminous CCs which potentially contribute to higher AC and soil aggregation. However, while it is conceivable that the observed effects could result from the immediate influence of the current cover crop phase, it is also crucial to acknowledge the potential compounding effects of previous crop phases that may have impacted these results. In the soil collected during the fall sampling period, *nirS* abundance did not vary among crop rotation phases, while *nirK* abundance varied among SB-WW and the other two phases. These different trends for the *nirS* and *nirK* bearing community abundances suggest that different environmental and ecological factors could have contributed to their abundances. Higher *nirK* abundances in the MZ and CC phases compared to the SB-WW phase suggest a potential for increased denitrification potential in the soils planted to the MZ and CC phases. The reduced NO_3^--N concentration during the CC phase is likely a consequence of the CC's ability to change the soil environment, fostering conditions favourable for specific N-cycling SMCs. For instance, the CC phase had the lowest nitrifier (*amoA*) gene abundance, and highest denitrifier (*nirK*) abundance suggesting low nitrification potential and high denitrification potential during the fall sampling period. Conversely, the SB-WW phase had higher soil NO_3^--N and NH_4^+-N levels, which may be associated with higher *amoA* abundance and lower *nirK* abundance

in the SB-WW phase compared to the CC phase. Moreover, the highest levels of NO_3^--N and NH_4^+-N were observed in the SB-WW phase and can be explained by the presence of SB in the previous crop phase. Additionally, N-based fertilizer application to the SB-WW phase close to sampling days could be another reason for higher NO_3^--N and NH_4^+-N levels in the SB-WW phase compared to the CC phase. Significantly higher *amoA/nosZI* and *nirK+nirS/nosZI* ratios were observed in the MZ phase compared to the other two phases, suggesting a potential for increased N_2O emission from the soils in the MZ phase.

Appendix 1



Supplementary Fig. 3.1. The locations of sampling fields with MZ, SB-WW, and CC crop rotation phases. Google Maps. (2023). [Map of study sites for the fall sampling]. Retrieved August 2023, from https://www.google.com/maps/@43.9676529, 78.2348833,2689m/data=!3m1!1e3?entry=ttu.



Supplementary Fig. 3.2. Daily mean average soil temperature (15 cm depth, °C), daily mean air temperature (°C) and total daily precipitation (mm) throughout the study period in MZ, WW, and CC phases, with the black outline indicating the sampling period (Oct-Nov 2021).

Supplementary Fig. 3.3. Cover crop phase at the time of sampling (Oct-Nov 2021).

Chapter 4: Characterization of N-cycling microbial communities and plant available N in tile-drained and non-tile-drained fields

ABSTRACT

Tile drainage (TD) is often a necessary management approach to improve yields in imperfectly drained soils and has been installed extensively throughout Ontario's agricultural landscapes. Yet, N-cycling soil microbial community (SMCs) abundance that transforms N in tile-drained soil has not been characterized extensively. To address this gap, I selected 12 fields: six tile-drained (TD) and six non-tile-drained (NTD) fields, which were managed by a single operator in southern Ontario under a MZ (Maize (*Zea mays* L.))-SB (Soybean *(Glycine max)*)/WW (Winter wheat (*Triticum aestivum*))–CC (mixed cover crop) rotation. Soil samples were collected over five days from the 21st of March to the 8th of April 2022. At the time of sampling, four fields were under CCs, four fields had been planted to MZ (spring 2021) and recently harvested, and four fields had been planted to SB (spring 2021) and harvested before being planted to WW in the fall (pre-sampling). Three transects were set up in each field (100 m), and ten soil cores were collected at regular intervals at two depths: 0-5 cm and 5-15 cm, and composited (n = 3) per depth within each field. Soil pH, soil organic matter (SOM), nitrate (NO_3^--N), and ammonium (NH_4^+-N) concentrations were assessed. DNA was extracted from soils, and quantitative PCR was conducted to enumerate the size of the total bacterial community (*16S* rRNA) and the size of nitrifier (*amoA*) and denitrifier (*nirK*, *nirS*, and *nosZI*) communities. In TD fields, characterized by finer texture soils, elevated SOM was observed, ranging from 3.63 ± 0.10% to 5.82 ± 0.05%. Conversely, in NTD fields characterized by coarse-textured soils, SOM ranged from 2.40 ± 0.01 to 3.69 ± 0.03. In the MZ and CC phases of both TD and NTD fields, NH_4^+-N

concentrations and nitrifier gene (*amoA*) abundances were higher in the 0-5 cm vs. 5-15 cm suggesting a potential for increased nitrification in the surface soils. The highest NO_3^--N levels (4.16 ± 0.17 μg N g^{-1} dry soil) were found in TD-SB-WW fields at 5-15 cm vs. 0-5 cm, while in NTD-SB-WW fields, the NO_3^--N concentrations were not significantly different in two depths. The results could be due to tile drainage, which may have facilitated the accumulation of root zone NO_3^--N. During the study period, CC phase exhibited lower soil NO_3^--N levels in both TD and NTD fields at 5-15 cm vs. 0-5 cm. In this study, higher ratios of *amoA/nosZI* and *nirK+nirS/nosZI* genes were observed in the TD-MZ soils compared to the other two crop phases in TD fields, implying a potential for increased N_2O emissions in TD fields planted to MZ. During the study period, higher *nosZI* abundances and lower *nirK+nirS*/*nosZI* ratios were observed in TD-CC fields at both depths compared to other crop phases in TD fields, suggesting a potential for decreased N_2O emissions from TD-CC soils compared to the cash crop-TD fields. This chapter highlights the potential role of the CC phase, particularly within TD agricultural fields, in reducing N losses during the specific short-term study period.

Keywords: Nitrifiers, Denitrifiers, Ontario agriculture, Crop rotation, Soil health

4.1 Introduction

In Ontario, the expansion of area under row crops resulting from agricultural intensification has often been accompanied by tile drainage as a land improvement technique (Hanke, 2018; Kokulan, 2019). Approximately 14% of farmland in Canada (ICID, 2018) and 45% of fields in southern Ontario, Canada (ICID, 2018; Kokulan, 2019) are managed using TD systems. The diversity of the region's land, including soil type, topography, and climate variability in the region, can create water-logged

conditions, making natural drainage more challenging (Kompanizare *et al.,* 2023). Therefore, in agricultural lands where natural drainage can be impacted by soil compaction, low hydraulic conductivity, and poor relief, TD is considered a good option (Arenas Amado et al., 2017) to manage excess water and improve soil conditions for crop growth (Hanke, 2018; Kokulan, 2019). Additionally, reduced water logging in agricultural fields enhances crop growth and makes fields more accessible for farmers (Cordeiro & Ranjan, 2012; Kokulan, 2019).

Subsurface tile drainage facilitates the removal of excess water from the soil using perforated tubes placed beneath the soil surface and allows for easier field management (Arenas Amado *et al.*, 2017; Kokulan, 2019). Tile drains allow water to drain to a certain depth before being collected by a series of perforated tile pipes and delivered to drainage ditches or streams downstream of the field (Moore, 2016). Additionally, the timing of water movement through TD varies across different regions of Canada (Kokulan, 2019). For instance, in Ontario, water flows through TD during the non-growing season, while water often does not flow significantly during growing seasons (Moore, 2016). In comparison to Ontario, in the Canadian prairies water flows through tile drains rarely in summer, while there is limited or no flow in the winter due to either cold or dry conditions (Cordeiro & Ranjan, 2012).

Installation of TD in agricultural lands has a significant impact on soil and water quality, particularly concerning NO_3^--N loading into the stream water (Gentry *et al.,* 1998; Kompanizare *et al.,* 2023). Additionally, with subsurface drainage, the main pathway for the movement of excess precipitation changes from surface runoff to subsurface flow, leading to increased leaching of NO_3^--N from agricultural soil compared to soils without TD (Dinnes *et al.,* 2002a; Grossel *et al.,* 2016). In row crop agriculture, the loss of NO_3^--N has intensified in regions of high TD density (Smith,

2015b). For instance, Tiemeyer *et al*. (2008) and Boles *et al.* (2015) found that 89% to 95% of NO_3^--N losses in a ditch catchment were attributed to agricultural sources and transported through the TD system. Wang *et al*. (2015) conducted research in Iowa, USA and the study suggests a higher potential for water quality issues from N losses through TD. The risk is due to the increased installation of TD in agricultural lands, leading to increased mineralization rates with rising temperatures due to climate change.

The major issues associated with the loss of NO_3^--N from TD agricultural lands are eutrophication and harmful algal blooms in surface water bodies (Dinnes *et al.,* 2002; Kokulan, 2019). Even though N and P are essential nutrients when the excess N and P enter freshwaters, algal growth increases along with the decomposition rates and biological oxygen demand, resulting in fish die-offs and dead zones (Li et al., 2021; Smith et al., 1999; Smith & Schindler, 2009).This process of excess nutrient enrichment is known as eutrophication. Agriculture lands are recipients of N and P inputs through fertilizers and therefore contribute to the exacerbation of this issue (Li et al., 2021). The excessive use or inefficient management of fertilizers leads to increased nutrient runoff into surface water bodies, initiating the eutrophication process (Li et al., 2021; Smith & Schindler, 2009). Eutrophication and harmful algal blooms pose a massive threat to aquatic life through the deterioration of aquatic systems and can impact human health (Smith & Schindler, 2009; Wolfe & Patz, 2002). Furthermore, chronic NO_3^--N loadings in streams can cause declines in ecosystem health and impact sensitive species in the ecosystem (Camargo & Alonso, 2006; Smith & Schindler, 2009; Thornton *et al.*, 2013).

Cover crops which are also referred to as catch crops, green manure, or living mulches are grown as living covers to protect soil from erosion while providing organic matter to enhance soil health (Cai et al., 2019; Clark, 2018; Hobbs et al., 2008; Mbuthia

et al., 2015). Considering the environmental issues associated with TD, incorporating CCs into crop rotations on TD fields emerges as a potential solution to mitigate the adverse impacts of TD. Cover crops are considered a highly cost-effective approach for mitigating greenhouse gas emissions, with the potential climate benefits estimated to be achievable at < CAD 50/Mg CO_2e (Drever et al., 2021). According to a meta-analysis conducted by Drever et al. (2021), Ontario has the highest potential to reduce net GHG emissions using cover crops compared to other provinces in Canada, mainly due to land and resource availability. Mitigation of GHGs by cover crops occur by increasing soil C, mitigating direct N_2O emissions, preventing N leaching, and reducing indirect N_2O emissions (Drever et al., 2021). Studies found that incorporating cover crop mixtures containing species with high functional diversity potentially led to agricultural systems with great benefits (Finney & Kaye, 2017; Kaspar & Singer, 2015). These benefits involved N fixation, reduced N leaching, erosion prevention, improvement of soil structure by winter hardy CCs, and weed suppression (Finney & Kaye, 2017; Kaspar & Singer, 2015).

In TD agricultural lands, N-cycling SMCs are involved in driving N losses through nitrification and denitrification processes which are mediated by specialized groups of SMCs, the nitrifiers and denitrifiers (Gentry et al., 1998). Nitrifiers play a crucial role in converting NH_4^+ to NO_3^- (Barnard et al., 2005; Sahrawat, 2008; Xue et al., 2006). Denitrifiers are responsible for the reduction of NO_3^- sequentially into N_2 under anaerobic conditions (Miller et al., 2008a; Wan et al., 2023). When the denitrification is incomplete, N_2O can be emitted into the environment (Kuypers et al., 2018; Robertson & Groffman, 2006). Therefore, loss of N via N_2O emissions and leaching of NO_3^- can be considered as the two major pathways of N loss in TD agricultural lands (Bowles et al., 2018; Gentry et al., 1998; Moore, 2016).

Moreover, various soil properties such as moisture content, nutrient availability, and SOM interact to influence the vertical distribution of N-cycling SMCs in TD agricultural lands (He *et al.,* 2022; Naylor *et al.,* 2022; Tang *et al.,* 2018). In addition, hydrological conditions that occur in TD agriculture lands vs non-TD agriculture lands (Gramlich *et al.,* 2018; King *et al.,* 2014) may further impact the vertical distribution of SMCs. For instance, the fluctuating water table and changes in soil moisture content associated with TD could create distinct niches for SMCs at different depths (Gramlich *et al.,* 2018; King *et al.,* 2014).

Despite the extensive use of TD systems in agricultural lands, mainly in Canada and the USA, there has been limited research on the N-cycling SMCs associated with TD agricultural lands that drive field-level N losses (Gentry *et al.,* 1998). In this chapter, soil characteristics including soil pH, soil organic matter (SOM), nitrate (NO_3^--N), and ammonium (NH_4^+-N) concentrations were assessed. Quantitative PCR was conducted to enumerate the size of the total bacterial community (*16S* rRNA) and the size of nitrifier (*amoA*) and denitrifier (*nirK*, *nirS*, and *nosZI*) bacterial communities.

In this chapter, I predicted (i) higher *amoA* abundances on the surface, at 0-5 cm compared to the 5-15 cm rooting zone in all three crop rotation phases due to nitrifiers being associated with the oxygen-rich environment in the surface, and (ii) in TD-CC fields, I predicted higher *nosZI* abundances and lower *nirS+nirK/nosZI* ratios compared to the TD-MZ and TD-SB-WW phases. Moreover, (iii) I predicted lower NO_3^--N at 5-15 cm vs. 0-5 cm depth in the CC phase in both TD and NTD fields.

4.2 Material and Methods

4.2.1 Study Site description and soil sampling

The study site (Supplementary Fig 4.1) was in the Cobourg/Port Hope area in Northumberland County, Southern Ontario, Canada, within the Gages Creek and Cobourg Creek watershed. The prevailing climatic conditions within this region were characterized by a mean annual temperature of 7.1°C and an annual average precipitation of 872 mm (Climate historic data 1981-2010 -Coburg station. The soils in the study sites are classified as Luvisolic soils according to the Canadian system of classification. The samples were collected from the 21st of March 2022 to the 8th of April 2022. The daily soil temperature and soil moisture data were collected in study sites using a HOBO micro station (H21-USB). These probes were installed at a depth of 15 cm at the beginning of the study period in the fall (Oct 2021). The loggers were programmed to record hourly soil temperature and soil moisture, and data was downloaded monthly. The probes were installed 50-100 m from the tile drain outlet in a relatively flat and shade-free area within each field. Supplementary Fig. 4.2 displays the daily average soil temperature, mean air temperature, and total daily precipitation for the study period collected at 15 cm depth. Average air temperature and average precipitation data for the study period were obtained from the climate historic data of Canada (Environment and Climate Change Canada, 2022) (Supplementary Fig. 4.2). Supplementary Fig. 4.3 displays the daily average moisture content in TD and NTD fields.

The selected fields for the study were planted with a diversified crop rotation, including maize (MZ), soybean (SB), winter wheat (WW), and polycultures of cover crops (CC) over a three-year period (MZ-SB+WW-CC). The study sites consisted of

six tile-drained (TD) fields and six non-tile-drained fields (NTD) belonging to one operational farm (Table 4.1). Bulk density did not differ between fields, with mean values ranging from 1.41 ± 0.03 – 1.45 ± 0.025 g/cm^3 (at 0-5 cm depth).

At the time of sampling, four fields were planted for each crop rotation phase, with two on TD fields and two on NTD fields for each rotation phase cover crops (CCs), maize (MZ), and winter wheat (WW). Tile drains were installed in the SB-WW and CC fields in 1980 or 1981, while tile drains were installed in the MZ field in 2002. The depth of the tile drains was 0.76-0.91 m and spacing for old tiles (installed in 1980 or 1981) was 15 m, while for the new tiles (installed in 2002), it was 12 m. In the 12 fields, consisting of six TD fields and six NTD fields, the operator employed no-till practices. All fields were last tilled in either 1998 or 2002.

Three 100 m parallel transects were established in each field (100 m spacing), approximately 50-100 m distance from the tile drainage outlet. Ten soil cores were collected at regular intervals at both 0-5 cm and 5-15 cm depths along each transect (3 transects per field) and composited (Bork et al., 2020; Ingram et al., 2008; Tosi et al., 2020). In this chapter, a total of 72 samples were analyzed for each soil characteristic, while each gene target abundance was assessed using 72 samples. Gloves and sampling probes were sterilized using 70% ethanol to collect samples aseptically for molecular analysis. Collected soil samples were manually homogenized by squeezing and shaking the Ziplock bags, and the bags were stored in a cooler containing ice packs before they were transported to the lab. Soil samples were stored at -20^{o}C until analysis.

4.2.2 Bulk density (BD)

Bulk density was measured along three 100 m transects in each field. Three cylindrical cores (volume = 231.532 cm^3) were collected along each transect at 0-5 cm depth, and

the samples were bagged and transported to the laboratory for further analysis. Collected samples were weighed before and after oven drying at 24 h at 105°C, and BD was determined (Eq 1). Volume corrections for coarse fragments were conducted using a water displacement method, in which the volume of water displaced by these materials was measured to determine the actual volume occupied by the soil. This corrected volume was then utilized to calculate the BD of the soil.

$$\text{Bulk density } (gcm^{-3}) = \frac{\text{Weight of the dry soil (g)}}{\text{Total volume of the soil } (cm^3)} \quad \textbf{Eq 1}$$

4.2.3 Soil organic matter (SOM)

Soil organic matter (SOM %) was determined by loss on ignition method (Jensen et al., 2018). Initially, crucibles were oven-dried at 105°C for 1-2 hours to remove residual moisture and the weights of crucibles (W1) were carefully recorded. Field moist soils (5 g, 2 mm sieved) were weighed into crucibles; the samples were oven-dried before igniting, with the oven-dry soil weight recorded (W2). Then the samples were ignited at 550 °C for four hours in a muffle furnace, weights were recorded (W3) and the change in weight before and after was used to calculate the SOM % of the soil samples (Eq 2).

$$\%\ \textbf{Soil Organic Matter} = \frac{W2-W3}{W3-W1} \times 100 \quad \textbf{Eq 2}$$

4.2.4 Soil exchangeable NO_3^--N

Soil exchangeable NO_3^--N concentrations in each soil sample were determined by the KCl extraction method (Nelson, 1983). Soil samples (10 g of field-moist soil) were sieved through 2 mm mesh and placed into 125 mL flasks, and 100 mL of 2.0 M KCl

was added to each flask. The flasks containing soil samples were shaken for one hour on an oscillating shaker bed at 160 rpm, and solutions were allowed to settle and filtered through Whatman no. 42 filter paper. The filtered extracts were analyzed for NO_3^--N using the Lachat flow injection analyzer (Lachat Instruments, a Hach Company Brand, USA-QuickChem*Method 12-107-04-1-B). A KCl reagent blank was run for every set of four samples. To assess extraction efficiency, a triplicated spike test was performed using a 10 mg N/L KNO_3 solution, yielding a recovery percentage of 99.9%. Moisture content for all the soil samples was measured and the final NO_3^--N concentrations were calculated in μg N g^{-1} dry soil.

.4.2.5 Soil exchangeable NH_4^+-N

Soil exchangeable NH_4^+-N concentrations were determined by the salicylate method via a UV-Vis spectrophotometer (Perkin Elmer, Woodbridge, Ontario, Canada). Soil samples (10 g of field-moist soil) were placed into 125 mL flasks, and 100 mL of 2.0 M KCl was added to each flask. Samples were shaken for 1 hour. Solutions were allowed to settle and filtered through the Whatman no. 42 filter paper (Nelson, 1983). Ammonium was reacted with salicylate and hypochlorite in an alkaline phosphate buffer, using sodium nitroferricyanide as a catalyst. This reaction resulted in the formation of an emerald-green-coloured solution. In parallel, a triplicate standard curve was constructed by performing a serial dilution of 5 mM ammonium sulfate to a final concentration of 50 μM, 100 μM, 150 μM, 200 μM, and 300 μM. The ammonium concentration in the solution was determined at 667 nm and referenced to the standard calibration curve, providing a proportional relationship between the ammonium concentration and absorbance (Diatloff & Rengel, 2001). Moisture content for all the

soil samples were measured and the final NH_4^+-N concentrations were calculated in µg N g^{-1} dry soil.

4.2.6 Quantification of total soil bacterial genes and N-cycling soil bacterial genes

Soil DNA was extracted from a field moist soil sample (approximately 0.250 g) per transect (n=3) per field at both depths (0-5 cm and 5-15 cm) using the manufacturer's protocol using the DNeasy Power Soil Pro Kit (Qiagen laboratories, USA). The extracted DNA was quantified, and quality checked using Nanodrop 8000 UV-Vis Spectrophotometer (ThermoScientific), and aliquots were made and stored at -80ºC for downstream analysis.

Quantitative polymerase chain reaction (qPCR) was performed to enumerate bacterial communities by targeting the *16S* rRNA gene, nitrifying communities (*amoA*), and denitrifying communities (*nirS, nirK, and nosZI*). All DNA extracts were diluted to reduce the impact of inhibition and to increase qPCR reaction efficiency. The qPCR reaction mixtures contained either 10 µL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.) or PowerUp SYBR Green Master Mix (Thermo Fisher Scientific (Mississauga) Inc), 1 µL of 10 µM of each forward and reverse primer, 2 µL of DNA template, and nuclease-free water to a final volume of 20 µL. For *16S* rRNA, *nirS,* and *nirK* SsoAdvanced Universal SYBR Green Supermix was used, and for *amoA* and *nosZI* genes, PowerUp SYBR Green Master Mix was used. All amplifications were performed on a QuantStudio3 Real-Time PCR system (ThermoFisher Scientific, Markham, Ontario, Canada). In parallel with triplicated unknown samples, triplicate standard curves were included on each qPCR plate, which were constructed by serially diluting gBlocks gene fragments (Integrated DNA Technologies, Inc.) containing the target genes (10^1-10^8 copies).

The targeted bacterial *16S* rRNA, *amo*A, *nirS, nirK,* and *nosZI* standards were created by Integrated DNA Technologies (IDT, Inc.) and were designed based on published genomic DNA sequences of *Clostridium thermocellum* spp., *Nitrosomonas europaea* spp., *Alcaligenes faecalis* spp., *Pseudomonas aeruginosa* spp., and *Pseudomonas fluorescens* spp. respectively (Thompson *et al.,* 2016). The qPCR reactions were conducted involving an initiation step of 2 min at 98°C, followed by 35 cycles of denaturation at 98°C for 10 s, and annealing at 55°C (for *16S* rRNA), 57°C (for *amo*A and *nirS*), and 56°C (for *nirK*) for 30 s, and elongation at 72°C for 30 s. For *nosZI,* a touch-down protocol was used (Henry, 2007). Additionally, for assays using PowerUp SYBR Green Master Mix, qPCR reaction conditions involved a Uracil-DNA Glycosylase (UDG) activation step of 50°C before the initiation step.

The qPCR assays were optimized to obtain 93.0%-106.0% reaction efficiencies for *16S* rRNA, *amoA, nirS, nirK,* and 86.0% - 93.0% for *nosZI;* the slopes of the standard curves were between -3.24 to -3.58 with R^2 values between 0.991-0.999. Triplicate no template controls were included in all qPCR assays.

Melt curve analysis was used to assess the specificity of the qPCR reactions by confirming the presence of a single qPCR product and the absence of non-specific products or primer dimers. Gel electrophoresis was used further to confirm the presence and correct size of qPCR products. Amplified DNA fragments were loaded onto a 1% agarose gel matrix and subjected to an electric field, which caused DNA fragments to migrate through the gel matrix. To verify the proper sequence size of the DNA fragments, a 100bp DNA ladder (NORGEN) was used.

4.2.7 Statistical analysis

All statistical analyses were conducted in the R version 4.2.1 (R core Team 2023) Integrated Development Environment for R. R Studio, PBC, Boston, MA). Before parametric tests, normality and homoscedastic were assessed using the Shapiro-Wilks and Bartlett's tests respectively to check model assumptions. Data were log-transformed when necessary to meet parametric assumptions and then back-transformed for data presentation. All gene abundances were normalized to $\log_{10}$ gene copy numbers per gram dry soil to meet model assumptions regarding residual variance.

Analysis of variance (ANOVA) type III sum of squares (SS) using a linear mixed effect model (LME) was conducted through the '*lmer*' function in the '*lme4'* package. LME was used to assess both fixed effects and random effects in the study. This analysis was performed on each soil parameter, *16S* rRNA total bacterial gene abundance, and each N-cycling gene abundance separately. Within each data set, the fixed variables were the crop rotation phase and sampling depth (D1:0-5 cm, and D2:5-15 cm) in the fields with the presence/absence of tile drainage (tile drained, "TD", and non-tile drained, "NTD"), while the fields and transects were the random variables. Individual least-square means (least square means= emmeans) within the data set were compared with a post-hoc Tukey's HSD adjustment using the '*emmeans'* function in the '*emmeans'* package with confidence intervals adjusted using the Sidak method. Significant differences among and between least-square means were determined by p values, where the null hypothesis (H_o) was rejected at $p < 0.05$.

To visually demonstrate the multiple relationships between soil health parameters, total bacterial gene abundances, and N-cycling SMC gene abundances, Pearson's correlation and a principal components analysis (PCA) were carried out

including all the variables considered in this study. In the PCA, a scree plot was examined for breaks, and PC components with eigenvalues ≥ 1 (PC1 and PC2) were retained in the 2-dimensional plot for visual analysis.

4.3 Results

4.3.1 Site description

The soil texture of the TD fields was fine texture (silty clay loam or silt loam soils) with a smaller slope (nearly 4%) than NTD fields. All the NTD fields considered in the study were coarse-textured (sandy loam) with a higher slope (nearly 12%) compared to TD fields. The NTD fields are naturally sandier and better drained. They are also more sloped than the southern TD fields. The volumetric moisture values from probes indicated that the NTD soils are always drier than the TD fields (Supplementary Fig. 4.3). The temperature and moisture were also consistently different in TD vs. NTD fields (Supplementary Fig. 4.2, 4.3). Due to the differences between TD and NTD fields including soil texture, slope, and inherent characteristics of the fields, response variables in TD and NTD fields were analyzed separately.

4.3.2 Soil Organic Matter (SOM %)

High SOM % were observed in finer texture TD fields (3.63 ± 0.10-5.82 ± 0.05), while low SOM % were observed in coarse texture NTD fields (2.40 ± 0.01- 3.69 ± 0.03).
In TD fields, higher SOM % was observed at 0-5 cm vs. 5-15 cm depth in MZ (5.82 ± 0.05 vs. 4.03 ± 0.19) and CC (4.47 ± 0.11 vs. 3.63 ± 0.10) rotation phases (Table 4.1). In NTD fields, higher SOM % was observed at 0-5 cm vs. 5-15 cm depth in SB-WW (3.65 ± 0.12 vs. 2.45 ± 0.04) and CC (3.69 ± 0.03 vs. 2.40 ± 0.01) rotation phases (Table 4.1).

4.3.3 Plant available NO_3^--N

In TD fields the highest NO_3^--N concentrations were found in TD-SB-WW soils at 5-15 cm (4.16 ± 0.17 μg N g^{-1} dry soil) vs.0-5 cm (2.58 ± 0.07 μg N g^{-1} dry soil). In the TD-CC phase higher NO_3^--N concentrations were observed at 0-5 cm (3.11 ± 0.67 μg N g^{-1} dry soil) vs. 5-15 cm depth (2.00 ± 0.13 μg N g^{-1} dry soil) (Table 4.1).

In the NTD-CC phase, as seen in the TD-CC phase, higher NO_3^--N concentrations were observed at 0-5 cm (2.13 ± 0.10 μg N g^{-1} dry soil) vs. 5-15 cm depth (1.45 ± 0.01μg N g^{-1} dry soil). Overall, TD fields had higher NO_3^--N concentrations (2.00 ± 0.13-4.16 ± 0.17 μg N g^{-1} dry soil) relative to NTD fields (1.45 ± 0.01-2.13 ± 0.10 μg N g^{-1} dry soil).

4.3.4 Plant available NH_4^+-N

The NH_4^+-N concentrations were significantly higher in shallow depth (0-5 cm) compared to deeper depth (5-15 cm) in both TD and NTD fields in MZ and CC crop rotation phases. In TD-MZ soils, NH_4^+-N concentrations at 0-5 cm (7.00 ± 0.47) were higher than NH_4^+-N concentrations at 5-15 cm (3.28 ± 0.40 μg N g^{-1} dry soil). Similarly, in NTD-MZ soils, the NH_4^+-N concentrations at 0-5 cm (7.50 ± 0.25 μg N g^{-1} dry soil) were higher compared to NH_4^+-N concentrations at 5-15 cm (3.95 ± 0.17 μg N g^{-1} dry soil). In both TD and NTD SB-WW fields, NH_4^+-N concentrations were not significantly different between the two depths (0-5 cm and 5-15 cm) (Table 4.1).

Table 4.1. Mean pH, SOM (%), NO_3^--N μg N g^{-1} dry soil, and NH_4^+-N μg N g^{-1} dry soil and the results of ANOVA in spring sampling sites. The data are least-square means (n = 6) ± SE; parameters indicated as NS are not significantly different. Lowercase letters and uppercase letters following data within each column indicate significant differences in TD and NTD fields respectively ($p < 0.05$).

Fixed variables			Soil parameters			
Drainage	**Crop rotation**	**Depth**	**pH**	**SOM (%)**	**NO_3^--N (μg N g^{-1} dry soil)**	**NH_4^+-N (μg N g^{-1} dry soil)**
TD	MZ	0-5	$6.41^{f} \pm 0.36$	$6.54^{a} \pm 0.05$	$2.49^{bc} \pm 0.33$	$7.00^{ab} \pm 0.47$
		5-15	$7.21^{ab} \pm 0.10$	$4.03^{cd} \pm 0.19$	$2.89^{bc} \pm 0.22$	$3.28^{c} \pm 0.40$
	SB-WW	0-5	$6.53^{ef} \pm 0.06$	$4.85^{b} \pm 0.08$	$2.58^{bc} \pm 0.07$	$4.52^{bc} \pm 0.42$
		5-15	$6.44^{f} \pm 0.03$	$4.15^{bcd} \pm 0.12$	$4.16^{a} \pm 0.17$	$4.67^{bc} \pm 0.56$
	CC	0-5	$6.50^{ef} \pm 0.04$	$4.47^{bc} \pm 0.11$	$3.11^{b} \pm 0.67$	$7.45^{a} \pm 0.46$
		5-15	$7.13^{abcd} \pm 0.07$	$3.63^{d} \pm 0.10$	$2.00^{c} \pm 0.13$	$5.10^{bc} \pm 0.09$
NTD	MZ	0-5	$6.79^{C} \pm 0.07$	$3.49^{AB} \pm 0.24$	$1.61^{AB} \pm 0.08$	$7.50^{A} \pm 0.25$
		5-15	$7.09^{B} \pm 0.05$	$3.38^{B} \pm 0.14$	$1.90^{AB} \pm 0.07$	$3.95^{BC} \pm 0.17$
	SB-WW	0-5	$6.96^{BC} \pm 0.03$	$3.65^{AB} \pm 0.12$	$1.92^{AB} \pm 0.19$	$4.90^{B} \pm 0.25$
		5-15	$7.44^{A} \pm 0.03$	$2.45^{C} \pm 0.04$	$1.80^{AB} \pm 0.10$	$4.80^{B} \pm 0.41$
	CC	0-5	$6.83^{BC} \pm 0.01$	$4.15^{A} \pm 0.03$	$2.13^{A} \pm 0.10$	$6.94^{A} \pm 0.67$

	5-15	$7.62^{A} \pm 0.01$	$2.40^{C} \pm 0.01$	$1.45^{B} \pm 0.01$	$4.53^{B \pm} 0.01$

TD/NTD	Effects (Interactions)	pH		SOM (%) (P>F)		NO_3^--N (P>F)		NH_4^+-N (P>F)	
		P	F†	P	F†	P	F†	P	F†
TD	Crop rotation	0.0010	$2.40_{2,15}$	0.0007	$12.10_{2,15}$	0.3237	$1.21_{2,15}$	0.1841	$1.89_{2,15}$
	Depth	<0.0001 **	$5.58_{1,15}$	<0.0001 **	$3.15_{1,15}$	0.2924	$1.12_{1,15}$	0.1097	$2.61_{1,15}$
	Crop rotation *depth	<0.001**	$6.63_{2,15}$	<0.0001 **	$6.34_{2,15}$	<0.0001 **	$9.29_{2,15}$	<0.0001 **	$8.71_{2,15}$
NTD	Crop rotation	0.0228	$4.91_{2,15}$	0.1620	$2.05_{2,15}$	0.8990	$0.10_{2,15}$	0.9796	$0.02_{2,15}$
	Depth	<0.0001 **	$3.57_{1,15}$	<0.0001 **	$8.31_{1,15}$	0.0338	$4.64_{1,15}$	<0.0001 **	$3.97_{1,15}$
	Crop rotation *depth	<0.0001 **	$2.05_{2,15}$	<0.0001 **	$2.88_{2,15}$	<0.0001 **	$12.74_{2,15}$	<0.0001 **	$6.30_{2,15}$

Significant effects are indicated by P<0.05=* and P<0.01=**

†F value subscript indicates the numerator and denominator degrees of freedom, respectively.

4.3.5 Soil microbial community analysis

4.3.5.1 Total soil bacterial *16S* rRNA abundance

16S rRNA gene abundances were higher at shallow depths (0-5 cm) vs. deeper depths (5-15 cm) in all TD and NTD fields except for TD-MZ fields ($\log_{10}$ 9.60 ± 0.03 - $\log_{10}$ 9.83 ± 0.04 gene copies per gram dry soil). *16S* rRNA gene abundances were lowest in NTD-CC fields at 5-15 cm ($\log_{10}$ 8.84 ± 0.04 gene copies per gram dry soil), compared to remaining NTD fields at both depths (Table 4.2).

4.3.5.2 N-cycling gene abundances

In all TD fields, *amoA* gene abundances were higher at shallow depths (0-5 cm) vs. deeper depths (5-15 cm) in all three crop rotation phases (0-5 cm: $\log_{10}$7.24 ± 0.02 - $\log_{10}$7.40 ± 0.10 gene copies per gram dry soil vs. 5-15 cm: $\log_{10}$ 6.65±0.04 - $\log_{10}$ 6.95 ± 0.07 gene copies per gram dry soil) (Table 4.2). In NTD fields, *amoA* gene abundances were higher at shallow depths (0-5 cm) vs. deeper depths (5-15 cm) in MZ ($\log_{10}$ 7.42 ± 0.03 vs. $\log_{10}$ 6.71 ± 0.04 gene copies per gram dry soil) and CC crop rotation phases ($\log_{10}$ 6.92 ± 0.03 vs. $\log_{10}$ 6.01 ± 0.10 gene copies per gram dry soil).

In TD fields, *nirK* abundances were higher at 0-5 cm depths compared to 5-15 cm depths in MZ ($\log_{10}$ 8.64 ± 0.06 vs. $\log_{10}$ 8.16 ± 0.03 gene copies per gram dry soil) and SB-WW ($\log_{10}$ 8.69 ± 0.06 vs. $\log_{10}$ 8.43 ± 0.07 gene copies per gram dry soil) crop rotation phases (Table 4.2). Similarly, in NTD fields *nirK* abundances were higher at 0-5 cm depths compared to 5-15 cm depths in MZ ($\log_{10}$ 8.52 ± 0.13 vs. $\log_{10}$7.95 ± 0.03 gene copies per gram dry soil) and SB-WW ($\log_{10}$ 8.55 ± 0.09 vs. $\log_{10}$ 7.95 ± 0.04 gene copies per gram dry soil) crop rotation phases (Table 4.2).

In TD fields *nirS* abundances were higher at 0-5 cm depths compared to 5-15 cm depths in both SB-WW ($\log_{10}$7.11 ± 0.02 vs. $\log_{10}$ 6.79 ± 0.02 gene copies per gram dry soil) and MZ ($\log_{10}$ 7.23 ± 0.07 vs. $\log_{10}$ 6.79 ± 0.09 gene copies per gram dry soil). In NTD fields *nirS* abundances were not different between the two depths across three crop rotation phases (Table 4.2).

The nitrous oxide-reducing communities (*nosZ1* gene abundance) were largest in TD-CC fields at both 0-5 cm ($\log_{10}$ 6.58 ± 0.02 gene copies per gram dry soil) and 5-15 cm ($\log_{10}$ 6.03 ±0.03 gene copies per gram dry soil) depths compared to all other TD fields. In NTD fields, the highest *nosZI* gene abundances were observed in CC fields at 5-15 cm depth (Table 4.2). In TD fields the lowest *amoA/nosZI* and *nirK+nirS/nosZI* ratios were found in CC fields at both depths, while in NTD fields these ratios in CC fields were not different from other crop phases.

Table 4.2. Mean values of total (*16S* rRNA), nitrifying (*amoA*), and denitrifying (*nir*S, *nirK*, and *nosZI*) bacterial gene abundances, *amoA* / *nosZI* ratios and *nirS+nirK*/ *nosZI* ratios and the results of ANOVA at spring sampling sites. SMC gene abundances are presented in $\log_{10}$ gene copies per gram dry soil. The data are least-square means (n = 6) ± SE; parameters indicated as NS are not significantly different. Lowercase letters and uppercase letters following data within each column indicate significant differences in TD and NTD fields respectively ($p < 0.05$).

Fixed variables			**Soil microbial community abundance**						
Drainage	**Crop rotation phase**	**Depth (cm)**	**TB *16S* rRNA ($\log_{10}$ gene copies g^{-1} soil)**	***amoA*** **($\log_{10}$ gene copies g^{-1} soil)**	***nirK*** **($\log_{10}$ gene copies g^{-1} soil)**	***nirS*** **($\log_{10}$ gene copies g^{-1} soil)**	***nosZI*** **($\log_{10}$ gene copies g^{-1} soil)**	***amoA/ nosZI***	***nirS+ nirK/ nosZI***
TD	MZ	0-5	$9.83^{ab} \pm 0.04$	$7.40^{a} \pm 0.10$	$8.64^{ab} \pm 0.06$	$7.23^{ab} \pm 0.07$	$5.53^{d} \pm 0.06$	1.34^{ab}	2.87^{b}
		5-15	$9.60^{bc} \pm 0.03$	$6.68^{b} \pm 0.03$	$8.16^{c} \pm 0.03$	$6.97^{cd} \pm 0.09$	$5.13^{e} \pm 0.03$	1.32^{ab}	2.95^{b}
	SB-WW	0-5	$9.77^{ab} \pm 0.01$	$7.31^{a} \pm 0.11$	$8.69^{a} \pm 0.06$	$7.11^{abc} \pm 0.02$	$5.79^{c} \pm 0.02$	1.26^{b}	2.73^{c}
		5-15	$9.36^{c} \pm 0.04$	$6.95^{b} \pm 0.07$	$8.43^{bc} \pm 0.07$	$6.79^{d} \pm 0.02$	$4.92^{e} \pm 0.04$	1.40^{a}	3.09^{a}
	CC	0-5	$9.86^{ab} \pm 0.05$	$7.24^{a} \pm 0.02$	$8.35^{abc} \pm 0.05$	$7.13^{bc} \pm 0.03$	$6.58^{a} \pm 0.02$	1.10^{c}	2.35^{e}
		5-15	$9.43^{c} \pm 0.05$	$6.65^{b} \pm 0.04$	$8.28^{abc} \pm 0.02$	$7.02^{c} \pm 0.05$	$6.03^{b} \pm 0.03$	1.10^{c}	2.54^{d}
						(NS)		**(NS)**	
NTD	MZ	0-5	$9.73^{AB} \pm 0.05$	$7.42^{A} \pm 0.03$	$8.52^{A} \pm 0.13$	7.50 ± 0.10	$5.23^{BC} \pm 0.05$	1.19	2.58^{C}
		5-15	$9.36^{C} \pm 0.08$	$6.71^{BC} \pm 0.04$	$7.95^{BC} \pm 0.03$	7.34 ± 0.11	$5.45^{BC} \pm 0.05$	1.27	2.81^{B}
	SB-WW	0-5	$9.81^{A} \pm 0.03$	$7.21^{AB} \pm 0.05$	$8.55^{A} \pm 0.09$	7.29 ± 0.06	$5.70^{BC} \pm 0.03$	1.27	2.78^{B}
		5-15	$9.41^{BC} \pm 0.04$	$7.15^{AB} \pm 0.07$	$7.95^{BC} \pm 0.04$	7.12 ± 0.01	$4.98^{D} \pm 0.05$	1.25	3.16^{A}

CC	0-5	$9.55^{ABC} \pm 0.03$	$6.92^{BC} \pm 0.03$	$7.86^{BC} \pm 0.03$	7.83 ± 0.03	$6.19^{A} \pm 0.03$	1.12	2.54^{C}
	5-15	$8.84^{D} \pm 0.04$	$6.01^{D} \pm 0.10$	$7.48^{C} \pm 0.06$	7.46 ± 0.06	$5.10^{CD} \pm 0.11$	1.18	2.95^{B}

TD/NTD	**Effects (Interactions)**	**TB *16S* rRNA (P>F)**		***amoA* (P>F)**		***nirK* (P>F)**		***nirS* (P>F)**		***nosZI* (P>F)**		***amoA/nosZI* (P>F)**		***nirS+ nirK/ nosZI* (P>F)**	
		P	**F†**	**P**	**F†**	**P**	**F†**	**P**	**F†**	**P**	**F†**	**P**	**F†**	**P**	**F†**
TD	Crop rotation	0.3600	$1.07_{2,15}$	0.8193	$0.20_{2,15}$	<0.2193	$1.68_{2,15}$	0.5838	$2.40_{2,15}$	<0.0001 **	$3.40_{2,15}$	<0.0001 **	$8.84_{2,15}$	<0.0001 **	$1.53_{2,15}$
	Depth	<0.0001 **	$3.40_{1,15}$	<0.0001 **	$0.16_{,15}$	<0.0001 **	$9.77_{1,15}$	<0.0001 **	$4.28_{1,15}$	<0.0001 **	$9.19_{1,15}$	<0.008	$3.02_{1,15}$	<0.0001 **	$6.78_{1,15}$
	Crop rotation *depth	<0.0001 **	$11.54_{2,15}$	0.0202	$1.62_{2,15}$	<0.0001 **	$4.98_{2,15}$	0.5521	$12.85_{2,15}$	<0.0001 **	$5.74_{2,15}$	<0.0001 **	$16.36_{2,15}$	<0.0001 **	$24.90_{2,15}$
NTD	Crop rotation	0.006	$7.26_{2,15}$	0.1136	$1.17_{2,15}$	<0.005 **	$7.52_{2,15}$	0.092	$2.79_{2,15}$	<0.0001 **	$9.67_{2,15}$	<0.0030**	1.17_{215}	<0.0001 **	$1.12_{2,15}$
	Depth	<0.0001 **	$8.62_{1,15}$	<0.0001 **	$8.27_{1,15}$	<0.0001 **	$8.50_{1,15}$	<0.0001 **	$7.63_{1,15}$	<0.0001 **	$2.95_{1,15}$	<0.0001 **	$1.89_{1,15}$	<0.0001 **	$5.28_{1,15}$
	Crop rotation *depth	<0.0001 **	$4.41_{2,15}$	0.03442	$12.49_{2,15}$	<0.03	$3.50_{2,15}$	<0.0001 **	$8.10_{2,15}$	<0.0001 **	$7.93_{2,15}$	<0.5644	$0.57_{2,15}$	<0.0001 **	$13.0_{2,15}$

Significant effects are indicated by P<0.05=* and P<0.01=**

†F value subscript indicates the numerator and denominator degrees of freedom, respectively.

4.3.6 Correlation and Principal component analysis (PCA) for soil health parameters and abundances of total bacteria abundance and N-cycling SMC abundance

SOM (%) and *16S* rRNA abundances were positively correlated according to Pearson's correlation analysis ($p < 0.05$, $r = +0.59$). SOM % was also positively correlated with *amoA* abundances ($p < 0.05$, $r = +0.62$), *nirK* abundances ($p < 0.05$, $r = +0.55$), and *nosZI* abundances ($p < 0.05$, $r = +0.30$). Furthermore, SOM (%) showed a positive correlation with NO_3^--N μg g^{-1} dry soil ($p < 0.05$, $r = +0.35$). pH showed a negative correlation with *16S* rRNA ($p < 0.05$, $r = -0.47$), *amoA* ($p < 0.05$, $r = -0.49$), *nirS* ($p < 0.05$, $r = -0.27$), and *nosZI* ($p < 0.05$, $r = -0.53$) (Fig 4.1).

PCA loading plots were created to visualize multivariate relationships between soil parameters and microbial gene abundances. The soil parameters used to build the PCA plot were pH, SOM (%), NO_3^--N μg g^{-1} dry soil, and NH_4^+-N μg g^{-1} dry soil; SMC parameters were total bacterial (*16S* rRNA), nitrifying (*amoA*), and denitrifying (*nirS, nirK* and *nosZI*) gene abundances. The first two principal components, PC1 and PC2, accounted for 40% and 22% of the data set variance, respectively. The loading plot indicated that parameters including pH, SOM,*16S* rRNA, *amoA, nirK, nosZI* loaded into PC1 and NO_3^--N μg g^{-1} dry soil, and NH_4^+-N μg g^{-1} dry soil loaded into PC2 (Fig 4.2).

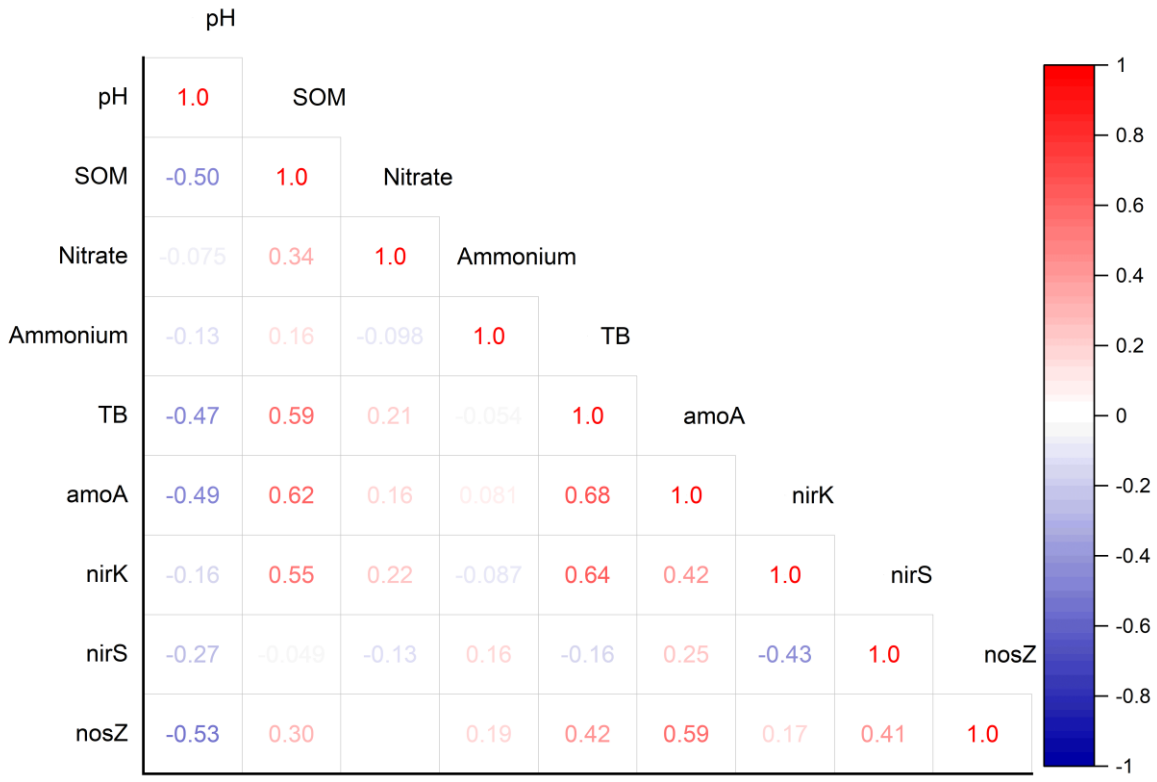


Fig. 4.1. Pearson's correlation of soil health parameters: pH, SOM (%), NO_3^--N µg N g^{-1} dry soil, and NH_4^+-N µg g^{-1} dry soil, and total (*16S* rRNA "TB"), nitrifying (*amoA*), and denitrifying (*nirS*, *nirK*, and *nosZI*) bacterial gene abundances in soil samples collected from an operational farm in southern Ontario (March – April 2022).

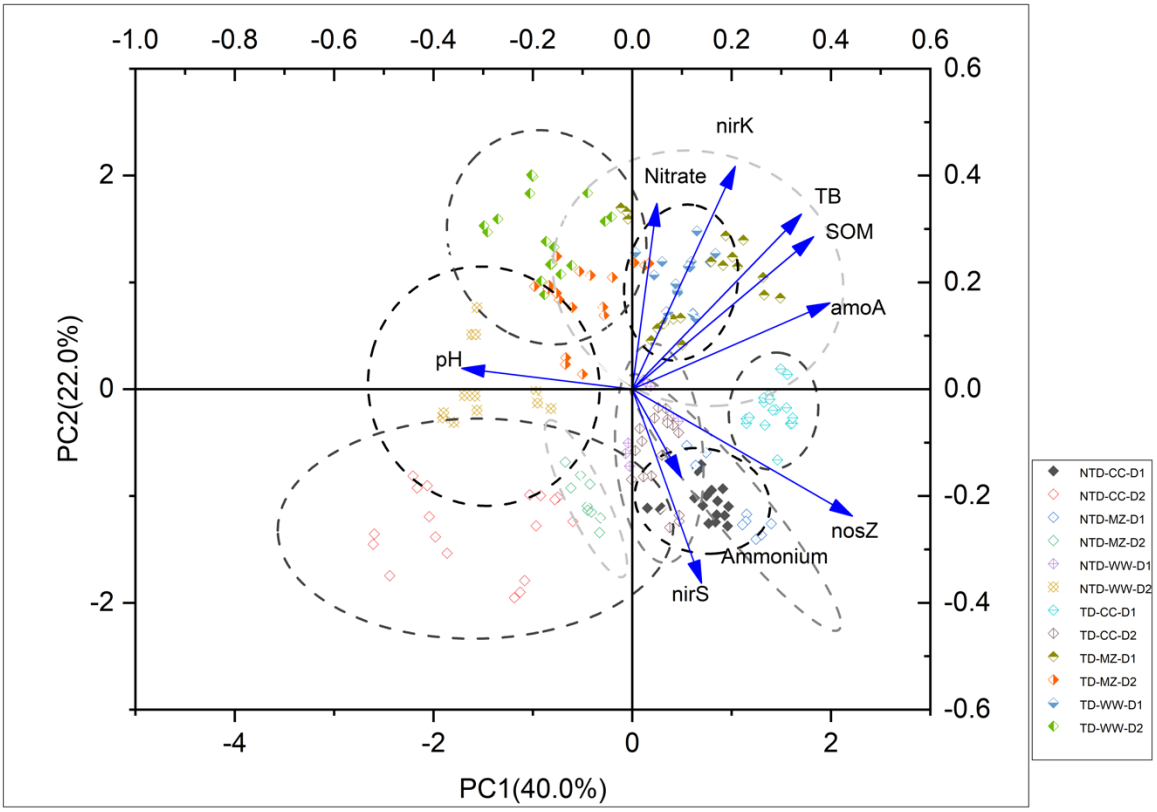


Fig 4.2. Principal component analysis (PCA) of soil health parameters: pH, SOM (%), NO_3^--N µg N g^{-1} dry soil, and NH_4^+-N µg g^{-1} dry soil, and total (*16S* rRNA), nitrifying (*amoA*), and denitrifying (*nirS*, *nirK*, and *nosZI*) bacterial gene abundances in soil samples collected from fields with TD and non-TD (NTD) under maize (MZ), winter wheat (WW), and cover crops (CC) phases in a MZ-SB-WW-CC rotation, in an operational farm in southern Ontario (March – April 2022). The samples were collected at two different sampling depths (0-5 cm (D1) and 5-15 cm (D2)).

4.4 Discussion

In Ontario, tile drainage is often a necessary management approach to improve yields in poorly drained soils and has been installed extensively throughout Ontario's agricultural landscapes (ICID, 2018; Kokulan, 2019). In this study, I characterized the plant available N and the abundance of SMCs, particularly the N-cycling microbial communities, at two depths (0-5 cm and 5-15 cm) in TD and NTD fields across three crop rotation phases.

I focused on the shallow depth range of 0-15 cm, as this depth includes the rooting zone which exhibits higher microbial activity and higher microbial abundance than the deeper layers (Chen et al., 2015; Eilers et al., 2012b; Seuradge et al., 2017). The 0-15 cm depth range was chosen due to its sensitivity to agricultural management practices (Eilers et al., 2012b; Seuradge et al., 2017). Moreover, focusing on the 0-15 cm depth allowed for practical and effective sampling. Although TD systems are typically installed around 1 m below the surface, analyzing shallower depths (0-15 cm) is crucial for capturing differences in plant available N and N-cycling SMCs as influenced by TD in soil depths, where most of the annual crop roots can be found.

4.4.1 Variation of soil health and SMC variables across two depths

The NH_4^+-N concentrations were higher in 0-5 cm vs. 5-15 cm depths in both TD and NTD fields under MZ and CC crop rotation phases. Soil microbial communities play a vital role in the breakdown of SOM and the release of NH_4^+ through mineralization processes (Dai *et al.*, 2020; Muruganandam *et al.*, 2009; Xue *et al.*, 2006; Jansson & Persson, 1982). The increased microbial activity may have led to higher rates of NH_4^+ production and

subsequent accumulation in the 0-5 cm depth. In addition, organic residue decomposition in CC and MZ fields may have contributed to the accumulation of organic matter in the 0-5 cm depth (De Graaff *et al.*, 2010; Vachon & Oelbermann, 2011). As these residues decompose, releasing SOM and nitrogenous compounds can lead to a higher accumulation of NH_4^+-N.

In the present study, significantly higher nitrifying (*amoA*) and denitrifying (*nirS*, *nirK*, and *nosZI*) bacterial gene abundances were observed at 0-5 cm compared to 5-15 cm across crop rotation phases in TD and NTD fields. In previous studies related to the vertical distribution of SMCs in agricultural lands, authors reported the abundance of SMCs varied between different depths and attributed this to several factors, such as nutrient availability and moisture content (Liu *et al.*, 2018; van Leeuwen *et al.*, 2017), SOM (Zhao *et al.*, 2021), and microbial interactions (Naylor *et al.*, 2022; van Leeuwen *et al.*, 2017). Moreover, roots can release root exudates (Huang *et al.*, 2019; Steinauer *et al.*, 2016), which influence soil parameters such as pH, SOM %, nutrient availability, and redox potential, which in turn can impact the distribution of SMCs across depths (Naylor *et al.*, 2022; Huang *et al.*, 2019).

The strength of the environmental gradient present within the soil profile is demonstrated by the fact that microbial communities within the same profile, even communities that may be only separated by 10-20 cm apart in depth, can be as different from one another as soil communities from entirely different biomes separated by thousands of kilometres (Eilers et al., 2012). A study conducted in Xilingo, China (Zhao *et al.*, 2021) investigated the variation in physical, chemical and SMC diversity using high-throughput sequencing at different soil depths (10 cm, 20 cm, and 30 cm). Researchers revealed that soil depth played a critical role in determining the relative abundances of

bacteria, with the highest SMC abundances reported at 10 cm depth when compared to 20 cm and 30 cm depths (Zhao *et al.,* 2021).

Microbial-mediated soil biogeochemical processes vary across different soil depths, influencing the N cycle (Naylor *et al.,* 2022). Several studies have reported variation in N-cycling functional genes across distinct soil layers, indicating the dominance of specific microbial groups based on the availability of various N sources, particularly in the rooting zone (Eilers *et al*., 2012; Tang *et al.,* 2018; Stone *et al.,* 2015). Furthermore, alterations in the relative abundances of nitrifiers and denitrifiers in the two depths (0-5 cm and 5-15 cm) (Castellano-Hinojosa *et al.,* 2018; Liu *et al.,* 2018; Zhao *et al.,* 2021) could be due to the decrease in N availability with increasing soil depth. This decrease in N availability can, in turn, impact enzymatic process rates carried out by N-cycling SMCs, as N plays a crucial role in enzyme synthesis (Naylor *et al.,* 2022).

The lower *amoA* gene abundances in deeper depth (5-15 cm) vs. shallow depth (0-5 cm) in both TD and NTD fields across MZ and CC crop rotation phases can be attributed to the differences in ammonium concentrations and oxygen concentration between the two depths. Nitrifiers are typically associated with the surface layer of the soil, where oxygen and ammonium concentrations are higher (Jones *et al.,* 2018; Stone *et al.,* 2015). Additionally, although residue effects were not investigated in the present study, the presence of residues on the surface (0-5 cm) in these study sites, where no-till management was adopted, may have impacted the higher NH_4^+-N concentrations in the shallow depth vs. deeper depth in MZ and CC fields. These NH_4^+-N concentrations may have potentially created favourable conditions for *amoA*-bearing nitrifiers (Ramirez-Villanueva *et al.,* 2015; Chen *et al.,* 2014; Miller *et al.,* 2008;), particularly in MZ and CC fields. In contrast, at

5-15 cm depth, reduced oxygen, nutrients, and SOM % availability compared to 0-5 cm depth could potentially limit the abundance of *amoA* nitrifiers.

4.4.2. Characterization of plant available N and SMC abundances in TD and NTD fields

Tile drainage improves soil aeration by reducing excess water and preventing water logging. Adequate O_2 availability in TD systems may promote microbial activity and facilitate nitrification, resulting in higher NO_3^--N concentrations in TD-SB-WW (+130%) soils at 5-15 cm depth relative to NTD-SB-WW soils at 5-15 cm depth (Dinnes *et al.,* 2002).Tile drains provide a direct and efficient pathway for water and water-soluble nutrients, including NO_3^--N, to bypass the soil and flow directly into surface waters or drainage outlets (Arenas Amado *et al.,* 2017; Dinnes *et al.,* 2002; Kokulan, 2019), which can lead to rapid spring NO_3^--N leaching from TD agricultural lands, particularly in saturated conditions. Consequently, NO_3^--N can be exported more rapidly from the TD system compared to if it went through the natural drainage processes and reached the groundwater system more gradually (Arenas Amado *et al.,* 2017; Dinnes *et al.,* 2002; Kokulan, 2019). The quick transport facilitated by tile drains can lead to a higher rate of NO_3^--N leaching before nitrate has a chance to be taken up by plants or undergo biological transformations that could mitigate its loss (Arenas Amado *et al.,* 2017; Hanke, 2018; Vivekananthan Kokulan, 2019). This explanation suggests the trade-off between tile drainage and the potential for NO_3^--N losses.

In the spring sampling period, both *nirS* and *nirK* genes showed no significant difference in abundance among crop rotation phases in TD fields. Even though the

denitrifiers containing the genes *nirS* and *nirK* carry the same biological function, they occupy different ecological niches (Maul et al., 2019; Throbäck et al., 2004). In agricultural soils, *nirK* gene abundances have been reported to be higher than *nirS* abundances (Dandie et al., 2011; Hai et al., 2009; Wang et al., 2020; Zhou et al., 2011). As seen in Chapter 3, higher *nirK* abundances were observed than *nirS* abundances across the three crop rotation phases, particularly in TD fields.

4.4.3 Role of cover crops in potentially mediating tile drainage impacts on soil health and N-cycling SMCs

Incorporating cover crops into the diversified crop rotations to mitigate the adverse impacts of cash crop management is a widely used approach (Fageria et al., 2005; Haruna et al., 2020; Kaspar & Singer, 2015a; Muhammad et al., 2019). The cover crop phase had higher SOM % at 0-5 cm compared to 5-15 cm in both TD and NTD fields. These findings, consistent with previous studies (Fageria *et al.*, 2005; Haruna *et al.*, 2020; Kaspar & Singer, 2015), suggest that CCs have the potential to act as a strong driver of increases in SOM, particularly in the 0-5 cm layer. Moreover, the presence of CCs mitigates soil erosion in agricultural lands, consequently enhancing SOM (Haruna et al., 2020; Mccormick, 2013). Soil organic matter at the surface layer is important, as it can positively impact soil structure, nutrient availability, and water retention (Liang et al., 2019; Liebmann et al., 2020; McDaniel et al., 2014; Reeves, 1997).

Interestingly, our results also indicated an enhanced SOM (+62%) in TD-MZ fields at 0-5 cm vs. 5-15 cm. This enhancement could be due to the carryover effect of SOM retention from the previous CC phase in the same fields (Jarecki et al., 2018; Kaspar & Singer, 2015b; McDaniel et al., 2014). The positive influence of CCs on SOM might have

persisted into the subsequent crop phase in diversified crop rotation (Garcia et al., 2013; Kaspar & Singer, 2015b; McDaniel et al., 2014) , contributing to the observed increase of SOM in TD-MZ fields. Additionally, in the CC phase, lower NO_3^--N levels were observed at the 5-15 cm vs. 0-5 cm depth in both TD and NTD fields. This intriguing finding suggests CCs may have acted as efficient N scavengers, reducing the downward movement of NO_3^--N through the soil profile.

In the TD-CC soils, there were higher *nosZI* gene abundances at both 0-5 cm and 5-15 cm depths, compared to all other TD fields. One plausible explanation for larger *nosZI* communities observed in TD-CC soils can be due to the alteration of oxygen and the availability of labile C and N facilitated by the presence of living root biomass and primary root exudates associated with CCs (Naylor et al., 2022). Simultaneously, enhanced SOM availability due to the incorporation of CC in TD fields can promote *nosZI* gene abundance in TD-CC soils (Rocha *et al.*, 2020; Romdhane *et al.*, 2019). Furthermore, the presence of TD can influence changes in oxygen levels (Barnard et al., 2005; Ley et al., 2018) in the soil by changing water movement and aeration (Ley et al., 2018). According to the results of the present study, incorporating CCs appears to create favourable conditions for larger nitrous oxide-reducing (*nosZI*) communities, thereby increasing the potential capacity for complete denitrification by denitrifier communities.

However, it is important to acknowledge potential trade-offs associated with the incorporation of CCs into cash crop rotations, including increasing labour and economic costs, and the potential for unintended environmental impacts, such as increased P losses particularly during cold seasons (Liu et al.,2019). Most importantly, it is necessary to select

diverse cover crop mixtures suitable to farmers' management capacities and site-specific conditions to avoid adverse impacts (Finney & Kaye, 2017; Kaspar & Singer, 2015).

4.4.4 Insights into System Sustainability

In the context of system sustainability, studies have linked high *amoA/nosZI* and *nirK+nirS/nosZI* ratios to the potential for high N_2O emissions (Saarenheimo *et al.*, 2015; Wan *et al.*, 2023; Xiang *et al.*, 2022). Combining analyses of N_2O measurements and denitrification genes in a lake ecosystem, Saarenheimo *et al.* (2015) found that when the *nir/nosZ* ratio was higher than 1:1 the microbial community had a higher potential capacity to produce N_2O than to reduce it. Wan et al. (2023) found a significant positive correlation between N_2O flux and *amoA/ nosZ* ratios in an agricultural field. In the same study, results of a random forest analysis showed that the ratio of *amoA/ nosZ* gene abundances was the best predictor for N_2O emissions. Wang et al. (2022) and Castellano-Hinojosa et al.(2022) revealed a strong link between *nosZI* than *nosZII* genes with the total N_2O consumption.

In our study, particularly in TD fields, we observed higher *amoA/nosZI* and *nirK+nirS/nosZI* ratios at both depths in the MZ and SB-WW phases compared to the CC phase. These findings suggest a potential capacity for increased N_2O emissions by SMCs in soils in TD-MZ and TD-SB-WW fields. Additionally, the higher *amoA/nosZI* and *nirK+nirS/nosZI* at 5-15 cm (rooting zone) vs. 0-5 cm (surface) in the SB-WW phase suggest a potential capacity for increased N_2O production at the rooting zone by bacterial nitrifying and denitrifying communities. In contrast, the TD-CC fields displayed higher *nosZI* abundance and lower *nirK+nirS/nosZI* compared to all other TD fields. These

results suggest a greater potential capacity for soil bacterial N_2O consumption (mitigation) in CC fields compared to the cash crop phases.

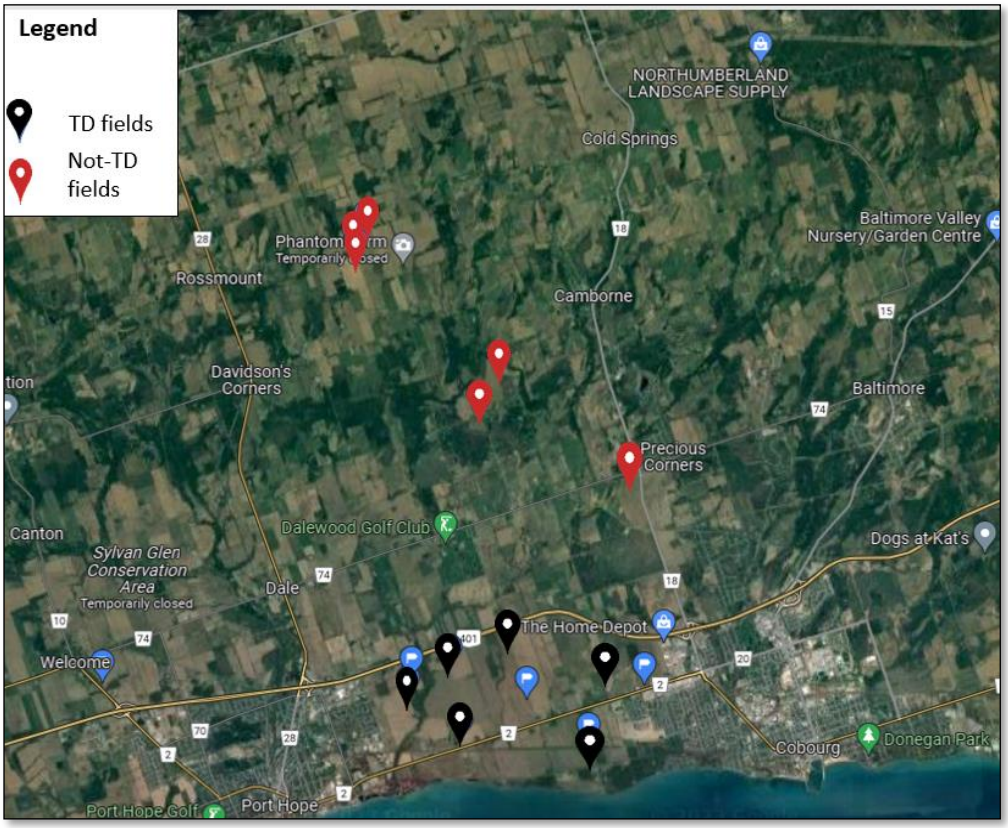
However, to further investigate these findings, more studies involving multiple cropping cycles in diversified crop rotation are required. Investigating the longer-term effects of CCs on SOM, plant available N, and N-cycling SMCs in various soil types, climates, and crop rotations can provide a more comprehensive understanding of CC's role as a best management practice over extended periods.

4.5 Conclusion

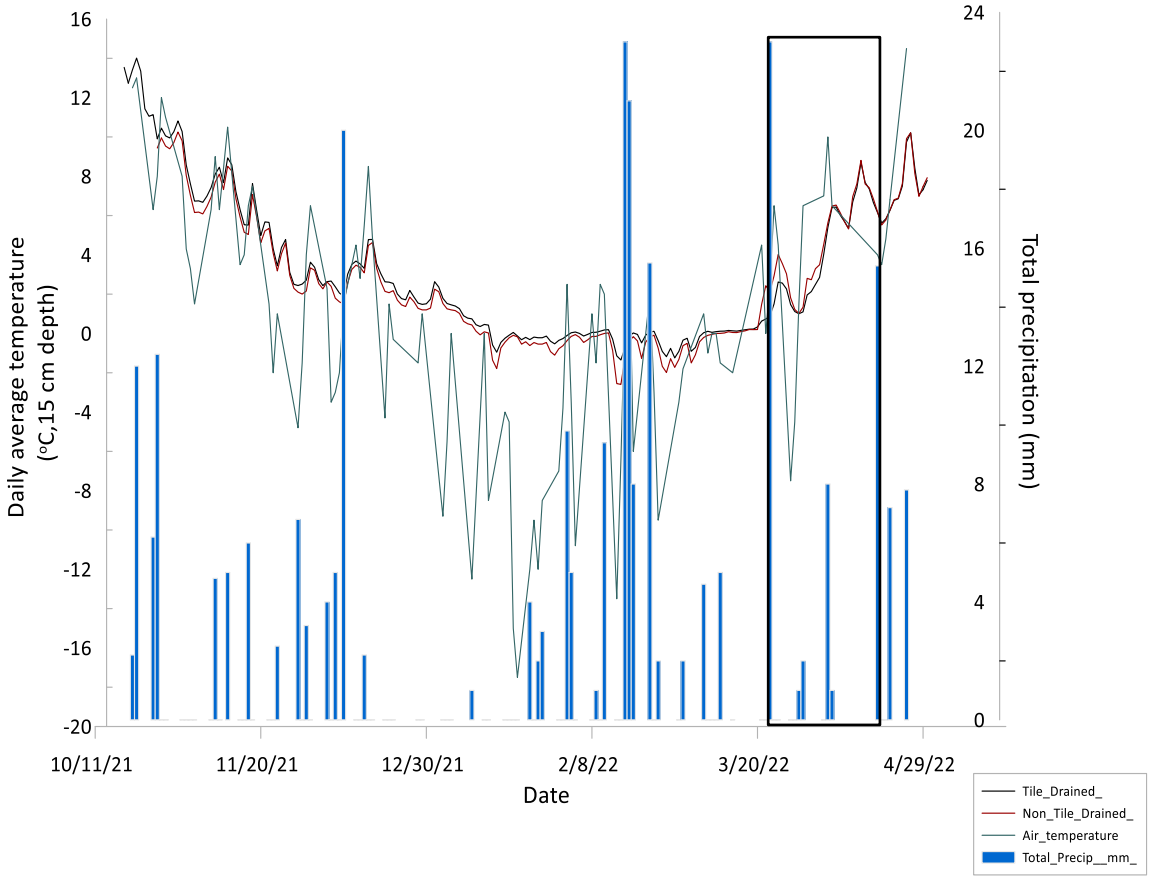
This study aimed to contribute valuable insights to the knowledge gap regarding the characterization of N-cycling SMCs in TD and NTD fields in Southern Ontario. In the MZ and CC phases of both TD and NTD fields, NH_4^+-N concentrations and nitrifier gene (*amoA*) abundances were higher in the 0-5 cm vs. 5-15 cm suggesting a potential for increased nitrification in the surface soils. In CC fields, lower NO_3^--N at 5-15 cm vs. 0-5 cm depths suggests a potential for decreased downward movement of NO_3^--N in TD and NTD fields. The results could be due to CC's potential to change the soil environment by changing resource availability, moisture, soil structure, and thereby providing niches to different functional groups. Therefore, the soil under CC will foster conditions favourable for specific N-cycling SMCs. In TD-CC fields, the highest *nosZI* abundances and lowest *nirK+nirS/nosZI* could be due to the changes in oxygen status and carbon and nitrogen inputs via abundant living root biomass and relatively high levels of primary root exudates produced by cover crop mixtures. In contrast, in TD fields, the MZ phase at 5-15 depth and SB-WW fields at both depths showed the highest *amoA/nosZI* and *nirK+nirS/nosZI*

suggesting the highest N_2O emission potential. In NTD fields, the MZ phase at 5-15 cm depth showed the highest *nirK+nirS/nosZI*, while, in NTD fields, *amoA/nosZI* ratios, were not significantly different. Overall, this study provides insight into the existing knowledge gap in Ontario regarding the N-cycling SMC abundances in TD agricultural lands. To expand the understanding of the vertical distribution of SMCs in TD fields, future studies should focus on more depths in the rooting zone and below the rooting zone. Additionally, measuring microbial potential activity, and community structure in different soil depths would provide a broader understanding of SMC functionality and their interactions in different soil depths. This would equip farmers and policymakers with the knowledge needed to make informed decisions about the implementation of tile drainage on agricultural lands while minimizing negative impacts.

Appendix 2



Supplementary Fig. 4.1. The locations of TD fields (6 fields) and NTD fields (6 fields). Google Maps. (2023). [Map of study sites for the spring sampling]. Retrieved August 2023, from https://www.google.com/maps/@43.9676529,78.2348833,2689m/data=!3m1!1e3?entry=tt



Supplementary Fig. 4.2. Daily mean average soil temperature (15 cm depth, °C), daily mean air temperature (°C) and daily total precipitation (mm) throughout the study period in TD and NTD fields, with the black outline indicating the sampling period (Mar -Apr 2022).

Supplementary Fig. 4.3. Daily mean average soil moisture (at 15 cm depth, m^3/m^3) throughout the study period in tile-drained (TD) and non-tile drained (NTD) fields, with the black outline indicating the sampling period (Mar -Apr 2022).

Chapter 5: General Discussion and Conclusions

Cash crop management practices aim to bolster cash crop yields to maximize economic benefits (Belfry et al., 2017; Gentry et al., 2013; Jiang et al., 2021). However, the intensification of cash crop management practices has led to environmental degradation, mainly through decreasing SOM (Kopittke et al., 2019; Van et al., 2019), and increasing NO_3^- leaching (Andraski et al., 2000; Fuller et al., 2010; Kirchmann et al., 2002), and GHG emissions (Benckiser et al., 2015; Miller et al., 2008). There are limited studies on how the cash crop management practices associated with different crop rotation phases in a diversified crop rotation impact N-cycling SMCs specifically involved in NO_3^- leaching and N_2O emissions. Cultivating CCs during fallow periods and over winter has proven instrumental in safeguarding soil from erosion (Dabney et al., 2001; Kaspar & Singer, 2015) and mitigating nutrient loss due to nitrification and denitrification (Ekwunife et al., 2022; Fernandez Pulido et al., 2023). Growing cover crops has significantly improved soil health indicators and overall crop productivity (Fernandez et al., 2023; Haruna et al., 2020; Hunter et al., 2019; Reeves, 2018).

Tile drainage is a cash crop management practice widely used in Ontario to address the challenges in natural drainage systems in agricultural lands (Arenas Amado et al., 2017). Tile drainage emerges as a preferred solution in agricultural lands with soil compaction and uneven terrains that hinder natural drainage (Arenas-Amado et al., 2017; Kompanizare et al., 2023; Kokulan, 2019). However, tile drainage can pose environmental issues, including the efficient transportation of NO_3^--N off-field with tile water, causing algal booms and subsequent eutrophication in water bodies (Jacinthe et al., 2001; Kokulan, 2019). In Ontario, even though tile drainage has been extensively implemented in

agricultural fields (Kokulan, 2019; Hanke, 2018), the characterization of N-cycling SMCs in tile-drained soils, particularly in the rooting zone (0-15 cm), remains an important knowledge gap that may improve our understanding of how to manage these lands to minimize N losses.

In this thesis, I examined the short-term impacts of cash crop management practices associated with a MZ-(SB-WW)-CC rotation on soil health and N-cycling SMCs within one operational farm. Specifically, I quantified total bacterial (*16S* rRNA), nitrifying (*amoA*), and denitrifying (*nirS, nirK,* and *nosZI*) communities across three crop rotation phases in a MZ-(SB-WW)-CC diversified crop rotation. Additionally, I characterized plant available N and the abundance of N-cycling SMCs in the three crop rotation phases (MZ, SB-WW, and CC) in both the surface (0-5 cm) and rooting zone (5-15 cm) in TD and NTD fields.

In **Chapter 3**, I collected soils from fields planted to a diversified MZ-(SB-WW)-CC rotation and managed by the same farm operator over four days in the fall 2021. According to the findings of the present study, soils collected under the CC phase had the highest levels of labile carbon (10-17% higher) and water-stable aggregates (35-50% higher) compared to the other two crop phases. These results are likely due to the CC's potential to change the soil environment by changing soil structure (Haruna et al., 2020; Kaspar & Singer, 2015a), increasing resource availability (Chavarría et al., 2016; Kim et al., 2020), building SOM (Hartwig & Ammon, 2002; Haruna et al., 2020; Reeves, 2018), and releasing root exudates through abundant living root biomass (Haruna et al., 2020; Steinauer et al., 2016). Moreover, these alterations in the soil environment can create a heterogeneity of niches, promoting specific SMCs (Schimel & Schaeffer, 2012;

Vukicevich et al., 2016), which may have contributed to higher labile carbon levels (De Graaff et al., 2010) and soil aggregation (Liu et al., 2005; Shen et al., 2021) in the CC phase compared to other cash crop phases. During the fall sampling period, the highest levels of NO_3^--N and NH_4^+-N were observed in the SB-WW phase. These findings suggest that the presence of SB in the previous crop phase has potentially influenced the soil N concentration in the subsequent WW phase. This influence is likely due to the release of N, which was symbiotically fixed during the SB phase (Ciampitti & Salvagiotti, 2018; Córdova et al., 2019). Further, we found higher *amoA* gene abundance and higher soil NO_3^--N in the SB-WW phase than in the CC phase. These results imply a potential for a more active nitrifier community which is effectively oxidizing plant-available NH_4^+ to NO_3^- in the SB-WW phase (Nunes-Alves, 2016). Additionally, in the SB-WW phase, ammonium-based fertilizer (MAP) and N:P:K starter fertilizer were applied close to sampling dates (fall sampling), which may have contributed to higher soil NO_3^--N levels in the SB-WW phase than the other two phases. In CC soils, low NO_3^--N levels and low *amoA* abundances were found, implying net nitrification rates may be lower in the CC vs. SB-WW phase. Additionally, the higher *nirK* gene abundances and lower NO_3^--N levels in the CC phase compared to the SB-WW phase may suggest the potential for increased denitrification in the soils under CCs. The CC and SB-WW phases had lower *amoA/nosZI* and *nirK+nirS/nosZI* ratios compared to the MZ phase. These results imply the potential for decreased N_2O emissions in the soils planted to CC and SB-WW while suggesting a potential for increased N_2O emissions from soils planted to MZ during the fall sampling days.

In **Chapter 4**, I conducted a short-term field study to evaluate the differences in the vertical distribution of N-cycling SMCs and plant-available N in TD and NTD fields. In my study, tile-drained fields were characterized by finer texture and higher SOM % relative to NTD fields, characterized by coarse texture and lower SOM %. In both TD and NTD fields across MZ and CC crop rotation phases, NH_4^+-N concentrations and *amoA* gene abundances were significantly lower in the rooting zone (5-15 cm) compared to the surface zone (0-5 cm) suggesting a potential for increased nitrification in the surface zone. Higher NO_3^--N levels and lower *amoA* abundances were found in TD-SB-WW fields at the rooting zone vs. surface zone, while NO_3^--N concentrations and *amoA* gene copies were not significantly different in the two depths in NTD fields. In TD-SB-WW fields, these results may be attributed to the rapid downward transport of NO_3^--N facilitated by efficient water movement promoted by tile drains (Gentry et al., 1998; Kokulan, 2019), potentially leading to increased movement of NO_3^--N to the rooting zone. During the study period, higher *nosZI* abundances and lower *nirK*+*nirS*/*nosZI* ratios were observed in TD-CC fields at both depths compared to other crop phases in TD fields, suggesting a potential for decreased N_2O emissions from TD-CC soils compared to the cash crop-TD fields.

In the soil collected during the fall sampling period, *nirS* abundances did not differ among crop rotation phases, while *nirK* abundances were higher in SB-WW and CC phases compared to the MZ phase. However, during the spring sampling period, the abundances of both *nirS* and *nirK* genes were not significantly different among crop rotation phases in TD fields. Even though the denitrifiers containing the genes *nirS* and *nirK* carry the same biological function, they occupy different ecological niches (Maul et al., 2019; Throbäck et al., 2004). Additionally, the abundances of *nirS*-encoding and *nirK*-encoding denitrifiers

differ in their responses to seasonal variation (Lee & Francis, 2017; Shi et al., 2019; Wang et al., 2020). In agricultural soils, *nirK* gene abundances have been reported to be higher than *nirS* abundances (Dandie et al., 2011; Hai et al., 2009; Wang et al., 2020; Zhou et al., 2011). Similarly, in Chapters 3 and 4, higher *nirK* abundances were observed than *nirS* abundances across the three crop rotation phases.

However, it is important to underscore the limitations of the present study. The short-term nature of both field studies is a major limitation. In Chapter 3, even though it is plausible to attribute the observed differences in soil health parameters and SMC abundances to the immediate effect of the crop phase at the time of sampling, compounding effects of previous crop phases could have impacted the analysis. In both chapters, a major challenge was to find comparable sites (fields) due to inherent differences in soil characteristics. Although I selected fields based on their similarity in soil texture, slope, and SOM, in-field variability in soils and microtopography exist. Additionally, trade-offs associated with CCs, such as P losses, should be considered in future studies when assessing the benefits of integrating cover crops into agricultural systems.

Given the short-term nature of the study design, the results of both chapters suggest that incorporating CCs into crop rotation has an effect in the short-term on some soil health parameters and promotes SMC abundances, particularly in tile-drained agricultural lands. Furthermore, the observed effects during the CC phase may primarily stem from multi-species CC mixtures' ability to alter the soil environment at the micro-scale through diverse root growth. These alterations include changes in soil structure (Haruna et al., 2020; Kaspar & Singer, 2015a), increased resource availability (Haruna et al., 2020), increased moisture retention (Acharya et al., 2019; Gabriel et al., 2021), and the provision of niches for diverse

functional microbial groups (Kim et al., 2020; Vukicevich et al., 2016). In line with our prediction regarding CCs, the CC phase promotes certain N-cycling SMCs. Moreover, in this study, larger nitrous oxide-reducing (*nosZI*) SMCs were observed in the CC phase than in cash crop phases (MZ and SB-WW) in both TD and NTD fields. Chapters 3 and 4 consistently revealed that soils collected under the CC phase exhibited the lowest relative ratios of *amoA/nosZI* and *nirK+nirS*/*nosZI*. These results suggest a potential for decreased N_2O emissions from soils planted to CC compared to cash crops.

The combined results of the two chapters highlight the potential contribution of CCs in enhancing soil health parameters and mitigating potential N-losses, particularly within the cash crop rotations commonly used in tile drained Ontario soils . It is important to note that these conclusions were drawn from a short-term study conducted over a limited time during the fall (2021) and spring (2022). Therefore, further research spanning an extended period is crucial to establish more comprehensive insights. The findings of this study set a stage for more comprehensive studies on diverse cash crop management practices, with a specific focus on understanding their implications for soil health and N-cycling SMCs in Ontario's agricultural lands. As the study was conducted over a short period, broadening the research scope of this study to assess the potential activity of N-cycling SMCs and monitoring field-scale N_2O emissions across growing and non-growing seasons is vital to understanding the relationship between N-cycling SMCs and N losses in agricultural soils. Furthermore, expanding this study to include a more comprehensive depth profile, incorporating analyses of SMCs in layers beyond the rooting zone is recommended to provide valuable insight to better understand how cash crop management choices can influence soil health and N-cycling dynamics.

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