

GREENHOUSE GAS EMISSIONS AND SOIL QUALITY IN LONG-TERM INTEGRATED
AND REDUCED TILLAGE ORGANIC SYSTEMS

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Arnab Bhowmik

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By

Arnab Bhowmik

The Supervisory Committee certifies that this *disquisition* complies with
North Dakota State University's regulations and meets the accepted
standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. Larry Cihacek

Co-Chair

Dr. Ann-Marie Fortuna

Co-Chair

Dr. Eakalak Khan

Dr. R. Jay Goos

Dr. Shafiqur Rahman

Approved:

July 7, 2016

Date

Dr. Francis Casey

Department Chair

ABSTRACT

Organic agroecosystems “rely on ecological processes, biodiversity and cycles adapted to local conditions”. Soil health is “the continued capacity of soil to function as a vital living ecosystem that sustains plants, animals, and humans” and can be used to assess agroecosystems. The fertility of organic agroecosystems is dependent upon soil organic matter, an indicator of soil health that supplies much of the nitrogen (N) and carbon (C) in soil. Despite the potential to use soil health as a dynamic measure few data sets compare soil health of different organic systems. My research compares the effects of climate and key best management practices (disturbance, amendment type, and livestock integration) on C sequestration, N cycling and greenhouse gas (GHG) emissions in five organic cropping systems. The data also contribute to our understanding of how microbial community members controlling reactive N (nitrate, nitrous oxide) and C cycling contribute to or reduce GHG as well as the potential of reduced tillage organic systems to lower GHG emissions when N is coupled with C in organic materials. This dissertation research verifies that the types and quantities of N cycling microorganisms can be used as indicators of soil health to assess the impact of short and long-term management on biogeochemical processes (the transformation and cycling of elements between non-living and living matter) that reduce or contribute to global climate change in long-term organic systems. A reduction in GHG emissions benefits the public and may increase the value added of certified organic foods.

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DEDICATION

This dissertation is dedicated to my mom and dad who always encouraged me and supported me and my love Harshita who has always stood by me and believed that I could do it.

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GENERAL INTRODUCTION

Anthropogenic climate change threatens our food supply and natural resources. We are at a crucial moment in the history of our species. Management of agricultural land can reduce or contribute to the effects of climate change. Currently the agriculture sector is a net producer of GHG emissions both directly through conventional farming practices that deplete soil organic carbon (SOC) stocks and nitrogen fertilizer additions that lead to emissions of nitrous oxide (N₂O) and indirectly through land use change (Lal, 2004). The agriculture sector contributes approximately 24% of the total anthropogenic GHG production globally (IPCC, 2014). Since the dawn of agriculture most soils have lost 30-75% of their original SOC (Lal et al., 2007). With the advent of mid-20th century contemporary farming some management practices such as the use of synthetic nitrogen (N) fertilizer, tillage and mono-cropping have accelerated the depletion of soil organic C stocks and have significantly increased the amount of anthropogenic GHGs, carbon dioxide (CO₂) and N₂O, produced (Khan et al., 2007; Lal, 2004). One potential means of shifting agriculture systems from sources of GHGs to sinks is the adaptation of organic agriculture management (Rodale Institute, 2014).

Organic and sustainable agriculture systems have the potential to provide ecological and environmental services that promote soil conservation, reduce GHG emissions and improve soil health but certain organic management practices have the potential to increase net GHG emissions. The National Organic Program Standard states that organic certification requires “An ecological production management system that promotes and enhances biodiversity, biological cycles and soil biological activity. This standard is based on the minimal use of off-farm inputs and management practices that restore, maintain and enhance ecological harmony” (National Organic Program, 2015). Organic management systems typically integrate use of crop rotations,

cover crops and animal amendments to enhance soil fertility by fostering soil quality and mitigating GHGs (Johnson et al., 2007). In organic systems, where N is limited, it is of pivotal importance to conduct research that includes the identification of key management practices which retain carbon (C) and N inputs from plant and animal amendments in soil across climatic conditions, soil type and management.

Since the C and N are coupled in organic agroecosystems, biochemical cycling in these systems is complex and the range of existing organic managements diverse; yet very few studies have been conducted to inventory GHG emissions (N₂O and CO₂). Our knowledge base with respect to cycling of nutrients in organic systems is also limited (Johnson et al., 2007). The primary goal of my research is to fully account for how specific management practices like tillage intensity, amendment type and livestock integration affect soil quality and the biogeochemistry and microbiology underpinning C and N cycling across a range of temperature and moisture regimes. Thus understanding the fundamental processes that drive the release of N from organic sources of C and N will help to achieve the goal of designing sustainable production systems that provide additional ecosystem services by reducing the potential loss of reactive N and soil C.

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LITERATURE REVIEW

Nitrous oxide (N₂O) is not only a potent greenhouse gas but also contributes to the depletion of the stratospheric ozone layer (Ravishankara et al., 2011). It has an average lifetime of 114 years in the atmosphere with 298 times the global warming potential of CO₂ over a period of 100 years (IPCC, 2014). On a global scale, agriculture contributes 65-80% of the total N₂O emissions and is mostly due to increased synthetic fertilizer application. It is predicted that agricultural soils will contribute up to 59% of total N₂O emissions in 2030. There are several biological processes that produce N₂O in soil systems, such as nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), nitrifier denitrification and non-biological chemodenitrification (Butterbach-Bahl et al., 2013; Hu et al., 2015; Wrage et al., 2001; Zhang et al., 2015). However, almost 70% of the global N₂O emissions are due to microbial transformations of ammonium and nitrate by the processes of nitrification and denitrification (Syakila and Kroeze, 2011). Yet, the quantification of these varied N₂O production pathways, and the identification of the role of microbial communities involved in N₂O emission are difficult to determine. Since organic growers rely on the mineralization of organic N sources for N availability to the crops, it is of vital importance to quantify N₂O sources in order to develop a more confident simulation of future N₂O emissions from agroecosystems.

The biogeochemistry and microbiology behind the nitrogen cycle-why is it important?

Globally nitrification is a central component of N cycling and involves the biological aerobic oxidation of ammonium to nitrite and subsequently to nitrate by a group of microorganisms known as nitrifiers. Autotrophic nitrification may be the primary pathway of nitrous oxide production in soil environments (Shaw et al., 2006). Ammonia oxidation, the first step of the nitrification pathway is catalyzed by the enzyme ammonia monooxygenase which is

encoded by the *amoA* gene. In soils, this process is mediated by 2 distinct groups of nitrifiers namely the ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) (Hastings et al., 2000; Venter, 2004). The second step, the conversion of NO_2^- to NO_3^- , is catalyzed by the enzyme, nitrite oxidoreductase produced by nitrite-oxidizing bacteria (NOB) encoded by the *nxrB* gene (Freitag et al., 1987). Bartlett (1981) suggested that non-microbial nitrite to nitrate transformation in soils under acidic soil conditions ($\text{pH} < 6.0$) containing manganese oxides might also be an important mechanism to consider while explaining N dynamics in soils.

Nitrous oxide is produced as a by-product of the nitrification process (Fig. 1). Based on pure culture techniques, Shaw et al. (2006) provided evidence that AOB could directly contribute to soil N_2O emissions during the nitrification process. Recently with the discovery of the *amoA* gene in AOA, studies have also demonstrated the potential of AOA to produce nitrification-related N_2O emissions (Stieglmeier et al., 2014). The ammonia monooxygenase enzyme oxidizes ammonia to hydroxylamine (NH_2OH), and hydroxylamine oxidoreductase (HAO) catalyzes oxidation of hydroxylamine to nitrite (Arp et al., 2002). Structural differences in the archaeal *amo* and bacterial *amo* genes and the absence of genes encoding for HAO and cytochrome c proteins used to recycle electrons suggests there are important differences between bacterial and archaeal ammonia oxidation (Zhalnina et al., 2012). Walker et al. (2010) suggested that nitroxyl (HNO) rather than hydroxylamine may be the intermediate in the ammonia monooxygenase enzymatic reaction in AOA. However, in a recent study by Vajjala et al. (2013), hydroxylamine has been recognized as the most probable product of archaeal monooxygenase homolog in marine archaea. But the mechanisms controlling AOA and AOB N_2O production are unclear and still under debate. The nitrification process in soil ecosystems has the potential to contribute to

almost 80% of the N₂O production from the soil under favorable temperature and moisture conditions (Gödde and Conrad, 1999). The nitrifying bacteria and archaea are also indirectly involved in the denitrification process as they produce an oxygen rich N species NO₃⁻ which is used as a substrate for denitrifiers (Zhu et al., 2013).

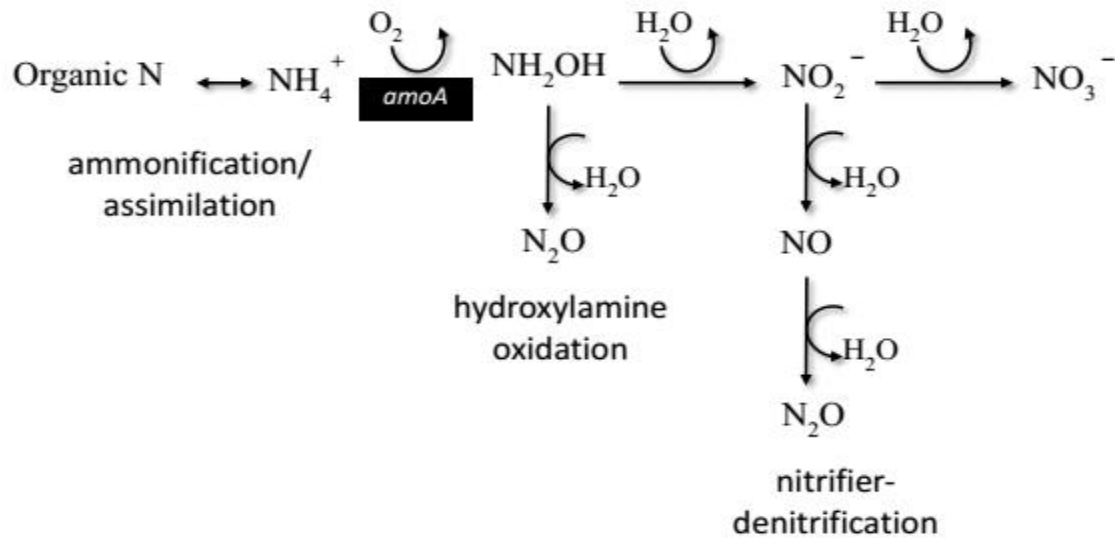


Fig. 1. Outline of N₂O production resulting from the autotrophic nitrification pathway.

Denitrification is the process whereby NO₃⁻ is reduced to N₂ under anaerobic conditions by denitrifiers, and N₂O is a regular intermediate (Philippot et al., 2007) (Fig. 2). Denitrifiers are the dominant producers of N₂O from soil and N₂ gas is the final product of denitrification at 70-80% of WFPS or near saturation (Saggar et al., 2013). The enzymes involved in heterotrophic denitrification are nitrate reductase (encoded by the *narG* or *napA* gene), nitrite reductase (encoded by the *nirK* or *nirS* genes), nitric oxide reductase (encoded by *cnorB* or *qnorB* genes) and nitrous oxide reductase (encoded by the *nosZ* gene) (Philippot et al., 2007; Jones et al., 2013). Substantial N₂O can be released as an intermediate of the denitrification process if the environment lacks oxygen and has abundant NO₃⁻ and organic C.

Recently attempts have been made to correlate rates of nitrification, denitrification and soil N₂O fluxes with the abundance, community composition, and expression of nitrogen-cycling functional genes that include *amoA*, *nirK*, *nirS*, *narG*, and *nosZ* in various ecosystems by using molecular methodologies and isotope signature techniques (Avrahami and Bohannon, 2009; Balsler and Firestone, 2005; Dai et al., 2013; Ma et al., 2008; Philippot et al., 2002; Zhang et al., 2009) in order to determine the source of N₂O emissions. There are instances where both molecular techniques and stable isotope signals have been used contemporarily and gave complementary inferences regarding the N₂O production pathway in agricultural soils. Increase in nitrifier and denitrifier gene copy numbers along with changes in δ¹⁵N-N₂O after a rainfall event confirmed that nitrifier denitrification was the dominant pathway of N₂O production in manure amended soils (Snider et al., 2015). Adair et al. (2013) studied the natural abundance isotope pattern of total soil DNA and found it to be positively correlated to the abundance of archaeal *amoA* gene copy numbers thereby indicating that ammonia oxidizing archaea play an important role in ecosystem N₂O release. Table 1 illustrates the advantages and disadvantages of some of the approaches scientists have been using in order to quantify denitrification in agricultural systems.

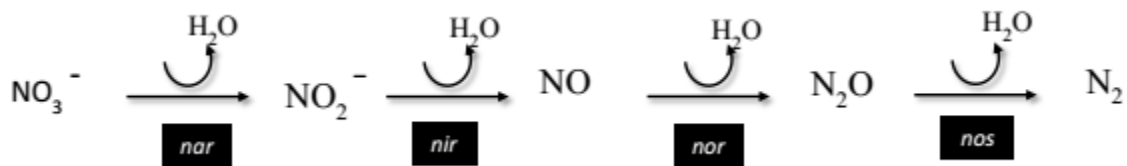


Fig. 2. Functional genes encoding the enzymes in each step of the denitrification process.

Table 1. Summary of denitrification techniques, advantages and disadvantages (Adapted from Saggar et al., 2013).

Denitrification measurement method	Advantage	Disadvantage
<p>Acetylene inhibition (AI) technique</p> <p>Approaches:</p> <p>Calcium carbide (CaC₂) granules.</p> <ul style="list-style-type: none"> ▪ In situ chambers in field. ▪ Static cores 	<ul style="list-style-type: none"> • Simple to conduct • Can run large number of samples at a time • Removes the spatial and temporal variability of denitrification rate • Useful in studying the effect of soil and environmental factors on denitrification and denitrification enzyme activity assay 	<ul style="list-style-type: none"> • Can only be used in NO₃⁻ dominated systems • Inhibits nitrification; can underestimate denitrification • Slow diffusion of C₂H₂ into soil or sediments limits blockage of Nor • Rapid decomposition of C₂H₂ by microbes • Contamination of C₂H₂ with other gases can affect denitrification • Scavenging of NO leading to underestimation of denitrification
¹⁵ N tracer technique	<ul style="list-style-type: none"> • Considered better than AR technique • Gives reliable estimates of denitrification 	<ul style="list-style-type: none"> • Laborious process • Requires costly instruments • Addition of ¹⁵N to the N limiting condition results in overestimation of denitrification
Direct N ₂ quantification	<ul style="list-style-type: none"> • No labelled N or inhibitor is added • Highly sensitive method can even detect low denitrification changes • Can be used to study temperature and moisture effect 	<ul style="list-style-type: none"> • Can only be used in enclosed incubation experiments • Complex and difficult system • Not suitable for longer period. • Challenge to measure N₂ against high atmospheric N₂ concentration

Hole-in-the-pipe conceptual model

The nitrogen cycle is complex and N undergoes multiple biologically driven transformations simultaneously. In order to explain this, a model was proposed by Firestone and Davidson (1989) known as the hole-in-the-pipe conceptual model (HIP model) (Fig. 3). According to this model, gas production and exchange with the atmosphere depend on various factors. These include the factors controlling the amount of nitrogen flowing through the pipe (i.e. those affecting denitrification and nitrification rates, mainly nitrogen availability and temperature) and the size of the holes in the pipe through which nitrogen gases leak. The size of the holes is regulated by factors controlling the partitioning of the reactive nitrogen species to NO, N₂O or more reduced or oxidized forms, while the rate at which nitrogen moves through the pipes determines the importance of the leaks. Lastly soil properties and the length of the path between the production site and the open air play a critical role in determining the release of reactive nitrogen species from the soil to the atmosphere via the hole-in-the-pipe model.

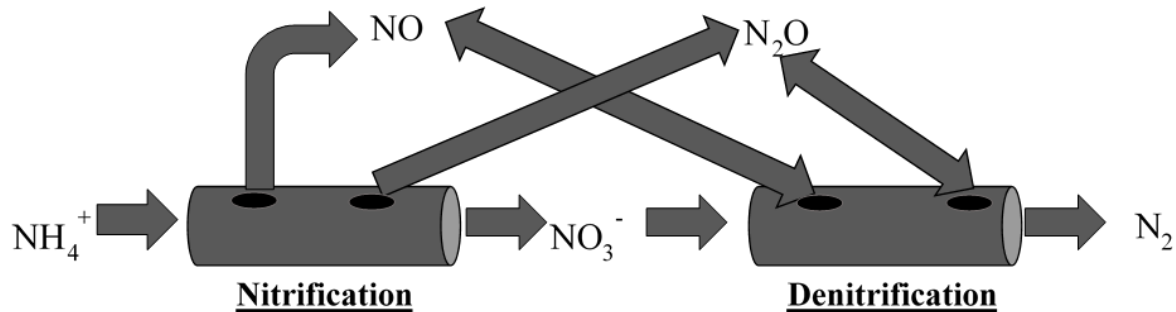


Fig. 3. “Hole-in-the-pipe” model. Adapted from Firestone and Davidson (1989).

Thus, N₂O can be produced by both nitrification (as a byproduct) and denitrification (as an intermediate product) depending on soil biotic and abiotic factors. The abiotic factors are comprised of soil and plant factors like soil mineral nitrogen, carbon availability, oxygen supply and water content and environmental factors like soil pH, temperature, rainfall, soil drying-rewetting and freezing-thawing (Phillips, 2008; Jorgensen and Elberling, 2012; Livesley et al.,

2008). As well as, biotic factors include groups of microorganisms which are responsible for the conversion of nitrogen that include specific genes responsible for nitrification and denitrification.

Factors affecting N₂O production from soil

Temperature and moisture

Climate has a substantial influence on the rate of soil organic matter decomposition, nitrification, denitrification and hence on the N₂O production from the soils. Most studies focus on soil temperatures above 25 °C, which is considered to significantly increase the reactive nitrogen species in soil (Cui et al., 2016). However, the community structures of soil nitrifiers and denitrifiers is influenced by temperatures from 4 °C to 37 °C (Braker et al., 2010). The process of denitrification can occur at a wider range of temperatures sub-zero to 75 °C (Knowles, 1982). Denitrifiers are more tolerant to subzero temperatures than the general bacterial biomass and bacterial nitrifiers (Sharma et al., 2006). Tatti et al. (2015) studied the effect of no tillage vs conventional tillage on denitrifiers over the winter season during which there are prolonged periods at sub-zero temperatures. They found that no tillage had a positive effect on the *nirK* and *nosZ* denitrifier abundance and, surprisingly, the activity of *nirS*, *nirK* and *nosZ* was highest in the winter and was not influenced by the tillage management. Oquist et al. (2004) investigated the potential for N₂O emissions from Swedish boreal forest soils and found that net N₂O production rates at -4 °C equaled those observed at +10 to +15 °C and with moisture contents >60% of the soil's water-holding capacity. They reported denitrification in the anoxic microsites in frozen soils as the source of the N₂O emissions.

In temperate regions, the addition of organic C through incorporation of manures or plant residues in late fall have an impact on the N₂O emissions at low temperatures (below 10°C) (Singurindy et al., 2009). Even at temperatures near the freezing point (-1 °C) and in the presence

of available nitrates and C, stimulation of N₂O emissions and shifts in some nitrifier (AOA and AOB) and denitrifier communities (*nirK*, *nirS* and *Nitrobacter* like bacteria) were observed (Wertz et al., 2013). Seasonal N₂O emissions in winter and late spring can be equivalent to or greater than the N₂O emissions measured during the growing season (van Bochove et al., 2001; Wagner-Riddle et al., 1997). An increase in the N₂O emissions due to higher availability of the C and N substrates after winter freeze-thaw events have been reported (Phillips, 2008). However, there is limited knowledge of how freeze-thaw events affect nitrifier and denitrifier communities and related activities such as N₂O emissions in organic management systems receiving C and N inputs in the form of crop residues and animal manure.

Soil moisture is another major driver of N₂O emissions from soil as in part it regulates the availability of oxygen to aerobic nitrifiers and may limit the activity of anaerobic denitrifiers involved in N cycling processes. Nitrification can account for 55-95% of the N₂O emissions when the water filled pore space (WFPS) is between 40 and 60% (Linn and Doran, 1984). At 70-80% of WFPS denitrifiers are the dominant producers of N₂O from soil by the process of denitrification (Bateman and Baggs, 2005). The nitrous oxide reductase enzyme catalyzes the final step from N₂O to N₂ in the denitrification process. It is encoded by *nosZ* gene which is inhibited by small quantities of oxygen. So, at moisture contents near saturation N₂ is the final product of denitrification (Saggar et al., 2013). Change in soil moisture affects the abundance of the nitrifiers and indirectly regulates N₂O emissions via the nitrification process (Avrahami and Bohannan, 2009). Addition of C substrate in the form of organic residues and manure and enhanced microbial respiration increases the anaerobic microsites which may potentially increase the N₂O emissions from soils.

C and N availability

Depending on the quality of the organic matter added to the soil through diverse crop rotations, cover crops and the integration of livestock in organic agroecosystems, reactive forms of N (ammonium, nitrates) substrates utilized in the process of nitrification and denitrification processes may become immobilized (Dieng et al., 2015). This can result in the reduction of reactive N in the soil system, thereby, minimizing the N₂O emissions from soil (Eagle and Olander, 2012). However, the addition of C substrate might facilitate the heterotrophic denitrification process if there is sufficient nitrate in the soil systems (Mitchell et al., 2013). A positive correlation was observed between soil denitrification capacity and organic C content, especially water-soluble C content (Burford and Bremner, 1975). There are studies where increases in soil C via application of farm effluents such as dairy or other animal wastes have increased heterotrophic denitrification (Bhandral et al., 2007). It is the subtle balance in the water soluble C and the nitrate [$(C_{H_2O})/NO_3^-$] which determines the effect of soil management on the emission of N₂O from the denitrification pathway (Benckiser et al., 2015).

C and N coupling in organic systems

Organic systems attempt to mirror natural ecosystems where C and N are coupled (C and N are bound in organic matter in the form of amino acids, proteins etc.). In natural ecosystems (grassland or forests) where perennial plants create partial permanency of the soil-vegetation interaction, C and N coupling occurs. Such coupling functions in soil through the dynamics of organic matter decomposition and the capacity of the microbes to recycle and recapture N and C. Since different components in the organic matter have different C and N stoichiometry, during microbial decomposition, C fluxes from an ecosystem are linked to N fluxes to a greater extent. Organic material with a wider C to N ratio has a longer mean residence time as compared to

organic material with a narrow C to N ratio (Rasse et al., 2005). The C to N ratio of natural ecosystems vary relative to agroecosystems with tendency to a wider C:N ratio. As a result, N losses to the atmosphere through nitrate leaching or partial denitrification (N_2O) are generally low in natural ecosystems. However, when natural systems are disturbed, or in intensively cultivated soil where large quantities of mineral N are applied, the decoupling of C and N occurs resulting in the accumulation of reactive N which may be lost from soils. USDA organic certified agroecosystems reduce fossil-fuel requirements by refraining from the use of synthetic pesticides and fertilizer inputs that include N which is responsible for the majority of anthropogenic N_2O emissions (Skinner et al., 2014).

Organic management, per se, does not improve soil quality. Increases in soil quality such as increased C sequestration are the result of the long-term adaption of key management practices that include complex rotations, cover crops and animal amendments (Drinkwater et al., 1998; Fließbach et al., 2007). The build-up of organic matter, i.e. carbon sequestration, is directly affected by the amount of plant residue and animal inputs as well as the quality of these materials (Johnson et al., 2007), all of which are influenced by key management practices. West and Post (2002) calculated that converting from moldboard plow to no-till sequestered an additional $0.57 \pm 0.14 \text{ Mg C ha}^{-1} \text{ yr}^{-1}$ of C and complex crop rotations had the potential to sequester an additional $20 \pm 12 \text{ g C m}^{-2} \text{ yr}^{-1}$ of C. Seventeen $\pm 15\%$ of C applied in animal amendments such as poultry manure becomes part of soil organic matter (SOM) (Franzluebbers, 2005; Johnson et al., 2007). Key management practices that retain or return residues to the soil have been shown to insulate and elevate soil temperatures reducing the extremity and frequency of freeze-thaw cycles leading to a reduction in N_2O emissions (van Bochove et al., 2000; Wagner-Riddle and Thurtell, 1998). Systems with living plants (grasslands, agronomic systems

containing winter cover crops or pastures) have reduced GHG emissions compared to bare soil (Wagner-Riddle et al., 1998; Petersen et al., 2011). Most organic management systems integrate several of these key management practices that promote C sequestration and there is a growing body of literature that suggest C sequestration is one of the main ways organic agriculture improves soil quality and fertility (Johnson et al., 2007). Increases in total soil C and N may increase N fertility without necessarily increasing net reactive N and the potential for N₂O emissions relative to conventional N fertilizers (Ghorbani et al., 2010).

These management practices play an important role in changing biotic and abiotic factors in such a way that the N cycling is affected. Management may favor the production of certain reactive N forms which are useful or certain forms that are detrimental. Thus, the effects of different management practices on N₂O production, nitrifier and denitrifier activity and microbial community composition is of pivotal importance to regulating N₂O emissions from soil and increasing the nitrogen-use-efficiency.

Effects of tillage on soil C and N dynamics

Reduced tillage or no till has the potential to increase C stocks in soil but this affect is mainly confined to the top 0-10 cm of soil. At lower depths SOC concentrations in the no till may be equal to or lower relative to mold-board or chisel plow (conventional till). A recent study by Govaerts et al. (2009) showed that across 100 sites, SOC in no till managements were lower in 7, higher in 54 and equal in 39 cases compared with conventional till in the 0- to 30-cm soil depth after 5 years or more of no till. Differences in tillage are often attributed to the magnitude of soil disturbance which may be a major stimulation of nitrous oxide emissions (Wagner-Riddle et al., 2007).

Powlson et al. (2012) studied the effect of reduced tillage and addition of different organic materials (farm manures, digested bio solids, cereal straw, green manure and paper crumble) on soil C stocks and N₂O emissions in UK and northwest Europe. They found that reduced tillage practices increased the annual C stocks compared to conventional tillage. However, this was compensated for increased N₂O emissions under reduced tillage management. The application of green compost that was derived from household and municipal sources had the potential to mitigate GHG emissions by sequestering more C and also reducing the N₂O emissions. Dendooven et al. (2012) conducted an experiment in order to determine the effect of contrasting tillage and residue management on GHG emission in the central highlands of Mexico. They found that no till with crop residue removal and conventional tillage with residue retention or removal were net sources of CO₂, with a positive net GWP ranging from 1.288 to 1.885 Mg CO₂ ha⁻¹ y⁻¹. According to their study, no till when practiced with residue retention had higher N₂O emissions but also increased the C storage to an extent that the systems had net negative GWP. Campbell et al. (2014) reported that long term no till systems (50 years) when combined with crop rotations can lead to improved environmental benefits by reducing the GHG emission and improving soil health. They also highlighted the fact that a corn-soybean rotation yielded lower CO₂ and N₂O compared to a corn-corn rotation irrespective of the tillage management regime. Zhao et al. (2016) conducted a laboratory incubation study to determine the effect of corn straw mixing or ploughing into soil vs corn straw mulching CO₂ emissions in cultivated Chernozems of China. Their results indicated that returning corn straw to the soil along with mixing it reduced the CO₂ emissions and increased the soil organic carbon content thereby improving the composition of micro-aggregate better than straw mulching.

The organic certification standards restrict organic growers from applying herbicides. Thus, organic management systems are primarily dependent upon tillage and physical disturbance to control weeds. This limits the adaptation of no till by growers in organic agroecosystems. Yet, reducing tillage reduces energy consumption, N₂O and CO₂ emissions, soil erosion and increases soil fertility, biodiversity, and water retention (Berner et al., 2008; Holland, 2004). No-till systems have been used for years to improve soil health. Teasdale et al. (2007) found organic practices such as manure addition increased soil organic matter. However, most of the authors examined only conventionally fertilized (synthetic N fertilized) no-till systems and did not examine the combined effects of a range of organic practices and no-till. Recently Armengot et al. (2015) conducted a long term trial to test the feasibility of reduced tillage in organic farming and concluded that a proper diversified crop rotation had the potential to reduce weed infestation when practiced even with reduced tillage. Thomazini et al. (2015) reported that organic no till vegetables implemented with grass/leguminous intercropping and pre-plant compost application had the potential to immobilize C in microorganisms thereby promoting a positive C balance in the soil leading to a C sink and improved soil health in the tropical Atlantic Forest Biome in Brazil. Seidel et al. (2015) compared the ratio between greenhouse gas emissions from inputs and crop output across organic and conventional cropping systems in a field trial at the Rodale Institute in Kutztown, Pennsylvania during 2008-2010. Their data suggests that a legume tilled management exhibited the best ratio (59%) followed by manure tilled (63%), manure no till (65%), legume no till (84%) and conventional till (90%) as a percent of the GHG emissions from conventional no till management.

Crop rotation and cover crop effects on soil C and N dynamics

Crop rotation plays a significant role in determining C stabilization in soil (Chatterjee et al., 2016). Crop rotations can influence carbon storage in the soil due to their potential biomass which in turn determines the quantity of crop residue inputs. Specifically, a mixture of high residue producing crops and covers adds more C and N to the soil as compared to low residue crops (West and Post, 2002). Factors that increase crop yields (fertility, water availability, crop health, growing degree days) will increase the amount of residue available and potentially soil C storage. Kauer et al. (2015) found that application of mineral N fertilizer in a 5 entry point crop rotation i.e. red clover (*Trifolium pratense* L.), winter wheat (*Triticum aestivum* L.), pea (*Pisum sativum* L.), potato (*Solanum tuberosum* L.), barley (*Hordeum vulgare* L.) under sown with red clover (*Trifolium pratense* L.) increased the stable C fraction of SOC to an extent which is comparable to an organic farming system (catch crops and composted manure). Luo et al. (2010) reported that the intensification of cropping systems such as increased number of crops per year, double cropping, and addition of cover crops can result in increased soil C storage particularly under no till management.

Soil C and N dynamics are influenced to a greater degree by quantity rather than quality of plant residues. Gentile et al. (2011) reported that the quality of crop residues effects short term nutrient dynamics and has a less of an impact on C sequestration. Powlson et al. (2012) conducted a study in which they found that plant residues with wider C to N ratios like cereal crops immobilized inorganic N thus making it less prone to loss but in some instance reducing crop productivity. Consistent N immobilization for a number of growing seasons could lead to loss of plant biomass returned thereby reduce the potential for C sequestration. In contrast, plant

residues with narrower C to N ratios such as legumes increased soil N availability which may potentially increase crop yield and or loss of inorganic N.

Rotations often include nitrogen fixing legumes that have low to moderate N requirements and are found in rotation with crops that have higher N fertility requirements. Mixing cereal cover crops with legumes can enhance N cycling because the combination of the two cover crops often provides greater biomass and total N content than legumes alone (Lawson, 2010; Sainju et al., 2005), narrower C to N ratios and more plant available N than monocrop cereals (Kuo and Sainju, 1998; Lawson, 2010; Ranells and Wagger, 1996). Incorporation of legume intercrops or cover crops to the systems along with no till contributed higher C storage as compared to conventional till. This might be due to the fact that the residues in no till are not incorporated which reduces its accessibility to the microorganisms for decomposition process. This results in slower decomposition and wider C to N ratio in the no till systems. Palm et al. (2014) reported that the combined effect of types of crops, intensity of cropping, duration of the cropping systems, the amount of inputs added to the systems in the form of residues and the tillage intensity along with soil properties like soil texture, temperature and moisture determines the overall soil C and N turnover and storage.

Addition of organic amendments and plant residues increase SOM and in turn the contribution of mineralizable N and C to the processes of denitrification and nitrification as sources of N₂O. Mineralization, nitrification and denitrification are stimulated when residues and or animal amendments are returned to the field or at times of high turnover of plant material and senescence in systems such as grassland (Fortuna et al., 2003b). Despite their contributions to pools of soluble C and plant available N, residues and animal amendments can be managed to prevent gaseous N losses (Fortuna et al., 2003b).

Differences in the timing of N release from plant residues and animal manure amendments can be used to regulate N mineralization kinetics such that N release synchronizes with plant uptake. Marinari et al. (2010) found that guano, an organic fertilizer containing 6% N and 32% organic carbon had a lower potential N mineralization rate as compared to DIX10, a mixture of feather meal and dried chicken manure (10% N, 42% organic carbon) when applied to a long term field study in Italy under a three-year crop rotation (pea; durum wheat – *Triticum durum* Desf.; tomato – *Lycopersicon esculentum* Mill.). Their findings indicate that the Guano fertilizer may provide inorganic N to a crop or crops for a longer period of time due to the more gradual release of mineral N observed relative to the DIX10 fertilizer. Thus, the potential mineralization-immobilization processes in soils could be used to synchronize N release from fertilizer source with plant uptake resulting in increased nitrogen use efficiency under different climatic and management systems.

Bowles et al. (2015) found that utilizing diverse organic matter inputs with a wide range of N availabilities in organic Roma-type tomato (*Solanum lycopersicum* L.) operations resulted in high soil N supplies, total and labile soil C and N and crop yields along with tightly coupled C cycling, and low potential N loss. Zhang et al. (2016) demonstrated the use of process based models like the Soil-Plant-Atmosphere Continuum System model to simulate soil organic carbon stocks and soil CO₂ and N₂O emissions in a 20-year wheat-maize intercropping system fertilized with only NPK chemical fertilizer and chemical NPK + pig (*Sus scrofa*) manure. They indicated that the application of chemical fertilizers plus manure could be a suitable management for ensuring crop yield and sustaining soil fertility but the ratio of chemical fertilizers to manure should be optimized to reduce C and N losses to the environment.

Perennial managements, such as grass leys that integrate livestock have the potential to reduce seasonal emissions of N₂O below those of annual vegetable cropping systems due to an overall reduction in N inputs. Additionally, animal manure deposited in pastures emits less N₂O than an equivalent amount of manure on bare soil (Ledgard et al., 2009). Lowered stocking rates of animals in organic systems also limits manure additions reducing the potential for reactive N. The development of modern integrated crop-livestock systems generally encouraged in organic systems involves the close association of grassland systems with cropping systems which reduce environmental fluxes to the atmosphere and hydrosphere due to efficient coupling of C and N within vegetation, soil organic matter and soil microbial biomass (Lemaire et al., 2014) The fundamental role of ecological intensification in integrated crop-livestock systems is to provide future food security and environmental sustainability.

Use of biological indicators of soil health to identify key management practices

Besides producing high quality food, organic agriculture has the potential to provide environmental services that support soil conservation, reduce GHG emissions and improve soil health. Organic agroecosystems, by definition of the International Federation of Organic Agriculture Movements, mimic natural ecosystems “relying on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects” such as synthetic N fertilizers, a major contributor to N₂O production. Although the standard does not refer to soil health per se. the tenants of soil health are inherent in the regulations. Therefore, soil health indicators could be used by growers to verify that they have enhanced biodiversity, biological cycling and soil biological activity through use of proper crop rotations, cover crops, and animal amendments in organic systems to enhance fertility, fosters soil quality and mitigate GHGs due to the potential to sequester C (Franzluebbers, 2005; Johnson et al., 2007).

Soil organic carbon and its fractions as an indicator of soil health

Soil organic C is an indicator of soil health and its maintenance or buildup is a metric to compare a range of management practices that are intended to enhance soil conservation and contribute to climate change mitigation (Fließbach et al., 2007; Johnston et al., 2009). Gattinger et al. (2012) conducted a meta-analysis using data sets from 74 studies from pairwise comparisons of organic vs. non-organic farming systems. They concluded that the SOC stocks and C sequestration rates were significantly higher in the zero net input organic farming systems as compared to non-organic cropping systems by $1.98 \pm 1.50 \text{ Mg C ha}^{-1}$ and $0.07 \pm 0.08 \text{ Mg C ha}^{-1} \text{ y}^{-1}$ (mean \pm 85% confidence interval) respectively. However, most of the data used in the study was utilizing top soils from temperate regions while little data from tropical regions and subsoil horizons was included.

Not all SOC contributes to nutrient cycling and biological activity. Therefore, there are a range of analytical techniques for determining different pool sizes and fractions all of which can be defined and utilized as indicators of soil health that isolate C by physical, chemical and biological means. The most widely used physical methods consist of isolating the particulate organic matter fraction (defined as the sand-sized soil separates remaining on a 53- μm sieve after removal of residues ($>2 \text{ mm}$)), light fraction (a density fractionation using a sodium iodide solution of density 1.7 g cm^{-3}), dissolved organic matter and water extractable carbon all of which measure the quantity but not the quality of each respective C fraction (Alvarez et al., 1998; Ghani et al., 2003). In contrast, chemical and biological methods (acid hydrolysis, C and N mineralization) have the advantage of providing information with respect to both the quality and quantity of organic C in SOM.

There is a plethora of published research illustrating the value of permanganate oxidizable carbon (POXC) as an index of soil health (Culman et al., 2012; Lopez-Garrido et al., 2011; Lopez-Garrido et al., 2014; Melero et al., 2009a; Melero et al., 2009b). The Weil et al. (2003) modification of the Blair et al. (1995) potassium permanganate protocol has provided researchers with a POXC fraction that can serve as an estimate of biologically active soil C (Weil et al., 2003). Previous studies have used a higher concentration of potassium permanganate (0.333M) in order to define the most reactive fractions which are associated with soil health (Blair et al., 1995). But, high molar concentrations of permanganate react with a larger portion of TC that includes the labile C pool and more stable constituents of the slow pool C such as lignin like compounds (Tirol-Padre and Ladha, 2004; Weil et al., 2003). In contrast, the modified method which uses a lower concentration of potassium permanganate (0.02 M) reacts with the portion of the TC most sensitive to changes in soil health and managements (Weil et al., 2003; Culman et al., 2012). The mean residence time (MRT) of the POXC fraction is 2-5 years in comparison to the resistant C fraction which has a turnover rate of several hundreds to thousands of years (USDA NRCS, 2014). There are a number of studies that address the use of the modified POXC method as an indicator of land and soil management (Culman et al., 2012; Culman et al., 2013).

There are numerous studies in the literature that partition SOC into 3 pools, active, slow and resistant with varying size, biological activity and MRT or turnover rate (i.e. decomposition) (Paul et al., 1999). The active pool of carbon consists of easily decomposable carbon compounds (simple sugars, organic acids and microbial metabolites) which have a MRT of days. It is responsible for providing an important source of energy to microorganisms and is sensitive to management practices (Fortuna et al., 2003a; Iqbal et al., 2009). The slow pool of carbon

consists of structural plant residues and physically stabilized carbon. It is responsible for maintaining structure and is also sensitive to soil management practices (Fortuna et al., 2003a). It has a strong influence on the nutrient buffering capacity of the soil and a MRT of 25-50 years (Collins et al., 2000). The active and slow C pool can be estimated from the rate of carbon dioxide (CO₂) evolution from an extended laboratory incubation study (Motavalli et al., 1994; Paul et al., 1999; Fortuna et al., 2003a; Jha et al., 2012). The evolution of CO₂ rates reflects the availability of the substrates to the microbes. The resistant C pool can be measured by the acid hydrolysis procedure (Paul et al., 2006). It is mainly comprised of lignin and chemically stabilized C with an MRT of centuries (1500 years) which from an agronomic perspective contributes negligible amounts of CO₂ mineralization (Buyanovsky et al., 1994; Paul et al., 1999). Management practices such as residue removal, burning, tillage, and crop rotation can have long-term effects on the amount of stable SOM (resistant pool). This pool is responsible for improving the tilth and water holding capacity (Six et al., 2002). The proportion of each of the different C pools present in the soil provides important information with respect to soil health (Fortuna et al., 2003a).

The fertility of organic agriculture systems is determined in part by the size and turnover rates of C and N pools in SOM and their synchronization with C and N additions from organic amendments and crop residues. Fortuna et al. (2003a) found that addition of organic nutrient sources like compost to the soil for more than 6 years has the potential to increase the pools of slow (10% increase) and resistant (30% increase) C and the potential pool of potentially mineralizable N. A study by Jha et al. (2012) suggested that the addition of FYM to soil increased the active C pool to a greater extent as compared to the slow and resistant C pools. Carvalho Leite et al. (2004) simulated the soil organic matter dynamics under no tillage and

different plowed systems using the CENTURY model. They concluded that the active and the slow C pools were more sensitive to management practices as compared to the SOC and resistant C pool.

In a soil health assessment study comparing organic management practices in organic vegetable cropping systems, Pritchett et al. (2011) found that soil organic carbon and bulk density were significantly affected by differences in organic management regimes (tillage, cropping systems and amendments). Soil enzyme activities (e.g. β -glucosidase and dehydrogenase) proved to be a better indicator of changes in organic managements than shifts in microbial communities. Active fractions of soil C such as mineralizable C also responded more quickly to management induced changes in soil C as compared to TOC (Ladoni et al., 2015). Margenot et al. (2015) conducted a study to examine the relationship between SOM functional group composition and labile SOM fractions using diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) from 13 organically managed Roma-type tomato fields. They reported that the differences in the organic functional groups were strongly associated with the differences in the labile SOM fractions thereby indicating the use of absorbance of infrared bands to identify the changes in labile SOC fractions.

Microbiological drivers of nitrification and denitrification as indicators of soil health

The literature is replete with studies which suggests the potential of the nitrifier and denitrifier gene copy numbers to be sensitive indicators of key management practices and are influenced by the amount of available N and C, and climatic factors such as soil temperature and moisture. Berthrong et al. (2013) claimed that long term organic management systems (20 years) induced a change in the soil microbial community structure resulting in an increase in microbial diversity which enhanced microbial activity including N mineralization potential and utilization

of labile substrates. In a 44-year-old grassland fertilizer experiment, Zhou et al. (2015) observed that the *amoA* gene of AOA significantly increased under organic (cattle slurry) additions whereas the AOB population increased with chemical fertilizer N additions. Rudisill et al. (2016) studied the effect of contrasting fertility practices on nitrification potentials in an intensively managed organic vegetable system. They found that organic fertility practices stimulate the nitrification process to a greater extent and that this activity could mainly be attributed to the activity of AOB.

Mitchell et al. (2013) observed that the incorporation of wintering cover crops like winter rye (*Secale cereale* L.) might not be sufficient to immobilize the nitrates in soils where banded N fertilizer is applied. Moreover, the increase in the mineralizable C availability from the cover crop residues might increase the N₂O emissions from soils with high residual nitrates receiving banded N fertilizer applications. Their study indicated that the mineralizable C availability of cover crops could be a determining factor in estimating the N₂O emissions from agricultural soils. The biogeochemical quality of the mulched residues can also be a potential driver of N₂O emissions and might be related to the community structure of denitrifiers (Patra et al., 2006; Philippot et al., 2007). However, in a recent study by Dieng et al. (2015), the denitrification activity was not linked with either the denitrifying community or the soil organic matter quality in maize and peanut residue mulch-based systems.

Previous studies by Wessen et al. (2011) traced nitrogen cycling and nitrate leaching across a 44-ha farm divided into organic and integrated farming systems using the *amoA* gene from functional microbial communities of AOA and AOB as a biological indicator. They suggested that AOA not AOB were contributing mainly to the nitrate leaching by providing substrates for the nitrite oxidizers. The nitrifiers have also proven to be successful indicators of

biogeochemical cycling at large spatial scales such as a Mediterranean watershed (Tsiknia et al., 2015). However, some studies suggest that it is the direct effect of management that regulates nutrient availability in tropical small holder agriculture systems and not necessarily soil microbial activity or diversity (Wood et al., 2015).

A strong interaction in soil abiotic and biotic properties were observed by Fortuna et al. (2012) while studying nitrification potential as well as well as *amoA* gene copy numbers of ammonia-oxidizing bacteria (AOB) and archaea (AOA) in a dairy slurry amended soils of varying soil texture and clay mineralogy. Brenzinger et al. (2015) found that a low soil pH of 5.4 modified the gas kinetics of the *nosZ* denitrifier community thereby affecting the complete reduction of the N₂O to N₂ during the denitrification process in an organic agroecosystem. They found that the activity of the *nirS*, *nirK* and *nosZ* denitrifiers were strongly reduced by low soil pH and even after restoration to neutral soil pH.

Wang et al. (2016) reported that the shifts in the abundance and composition of ammonia oxidizing communities, specifically, were closely linked to soil N₂O emissions when high levels of ammonium-N and nitrate-N were applied in the form of NH₄Cl and NaNO₃ to a subtropical forest in China. Robinson et al. (2014) showed that soil pH regulated the abundance of AOA vs AOB in urine treated soils. The growth of AOA corresponded with N₂O emissions when soil pH decreased. Long term application of organic manure increased the warming induced N₂O production (temperature sensitivity) probably due to shifts in the community abundance and the structure of the *nirS* denitrifiers (Cui et al., 2016). Chen et al. (2013) conducted a study to determine the response of AOA and AOB to N addition and increased precipitation in a typical temperate steppe. They concluded that AOA and AOB had distinct ecological niches and AOB was more sensitive to N and precipitation and was the main driver of nitrification in such soils.

Ouyang et al. (2016) conducted a study to measure the response of ammonia oxidizing nitrifiers in agricultural soils treated with ammonium sulfate or steer waste compost. Although the abundance of AOA was greater than AOB irrespective of the N treatment, the AOB mainly contributed to the potential nitrification activity in these N fertilized soils of varying ammonium availability. A study by Giguere et al. (2015) revealed that the net nitrification rates in a cropped soil was supported more by the AOB population whereas in the non-cropped soil by the AOA populations. Their research indicates the possibility that the AOA and AOB nitrification activities might be managed to improve the N use efficiency in agroecosystems. Banning et al. (2015) reported that *amoA* AOB dominated nitrification activity in semi-arid agricultural soils and were four fold higher than *amoA* AOA gene copies in the 0-10 cm soil layer.

In a 2-year field experiment, Muema et al. (2016) observed that the AOB response was more sensitive to the biochemical quality of organic inputs as compared to AOA. They also observed that the trend was reversed and the AOA population decreased when mineral N fertilizer was solely used or used in combination with organic inputs. Shen et al. (2015) reported that the abundance of archaeal *amoA* genes was significantly increased after the long-term application of organic manures, either with or without mineral NPK fertilizer in an acidic red soil. In contrast, Wang et al. (2014) observed that long term or short term manure fertilization promoted the population of AOB rather than AOA in a paddy soil. Di et al. (2010) reported that high ammonia substrate influenced the AOB whereas under low ammonia substrate conditions AOA were favored in three grassland soils treated with animal urine substrate vs a nitrification inhibitor (dicyandiamide). The ammonia oxidizing populations of AOA and AOB responded to different pasture management (free grazing vs enclosed grazing) on urea addition to semi-arid grasslands implicating their sensitivity to management changes and role in nutrient cycling (Liu

et al., 2016). In N limited systems such as in natural grasslands, AOA are important drivers of nitrification, whereas in N amended grassland soils the contribution of AOB towards gross nitrification is more dominant (Sterngren et al., 2015). Radl et al. (2014) reported that altered environmental conditions created by cattle overwintering led to selection of AOA vs AOB populations. They observed that the AOA numbers dominated that of AOB where there was no grazing whereas the AOB outnumbered AOA in severely grazed sites.

Liu et al. (2015) reported that in the periodically flooded soils where the oxygen concentration was lower, AOA rather than AOB had better adaptability in oxygenated/hypoxic alternant conditions in the water-level-fluctuating zones in the Three Gorges Reservoir. Ke et al. (2015) indicated greater sensitivity of the AOB towards oxygen availability as compared to AOA in rice paddy soils. Both the AOA and AOB populations from a grassland soil that rarely experience drought showed poor resistance and resilience to stress conditions like drought (drying-wetting) in a microcosm based experiment. (Thion and Prosser, 2014). Yarwood et al. (2013) reported the ability of AOA to persist in spite of 12 years of no organic matter input in soil ecosystems. In order to determine the resilience of nitrification activity under increased aridity levels, Sher et al. (2013) studied the ammonia oxidation potentials of AOA and AOB in arid and semi-arid soils. They found that the AOA were associated with the drier periods and higher temperatures whereas the AOB were more predominant in periods with higher precipitation.

Knowledge gap

Previous studies verify that key management practices modify the soil biotic and abiotic factors which in turn regulate the C and N dynamics in soil. However, not much is known about how the long term buildup of these C and N pools or retention of C and N inputs from plant and

animal amendments in soil affect the soil microbial processes that control C and N cycling leading to GHG emissions (CO₂ and N₂O) and their interaction with climatic conditions. Organic management systems are of particular interest because of the diversity of rotations, integration of cover crops, their reliance upon tillage and the possibility of integrating livestock in these diverse systems. My research also investigates the potential to use biological indicators of soil health as dynamic measures to compare different organic management systems that incorporate key management practices in soil across various climatic conditions.

Overall objectives

1. Quantify and model GHG emissions and C storage in long-term and transitional organic systems with varying manure application, crop rotation, and tillage intensity.
2. To link shifts in N₂O fluxes with nitrification and denitrification processes via biochemical-molecular methods that measure nitrification and denitrification processes that are influenced by key management practices and climatic conditions.

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PAPER 1. POTENTIAL CARBON SEQUESTRATION AND NITROGEN CYCLING IN LONG-TERM ORGANIC MANAGEMENT SYSTEMS

Abstract

The fertility and soil health of organic agroecosystems are determined in part by the size and turnover rate of soil carbon (C) and nitrogen (N) pools. Our research contrasts the effects of best management practices (BMP) (reduction in soil disturbance, addition of organic amendments) on C and N cycling in soils from 2 field sites representing five organic agroecosystems. Total soil organic C (SOC), a standard measure of soil health, contains equal amounts of biologically and non-biologically active C that is not associated with release of mineral N. A three-pool first order model can be used to estimate the size and turnover rates of C pools but requires data from a long-term incubation. Our research highlights the use of two rapid C fractions, hydrolysable and permanganate (0.02 M) oxidizable C (POXC), to assess shifts in biologically active C. Adoption of BMPs in organic management systems reduced the partitioning of C to the active pool while augmenting the slow pool C. These pools are associated with potentially mineralizable N supplied by residues, amendments and SOM affecting the concentration and release of mineral N to crops. Our data show that minimizing disturbance (no tillage, pasture) and compost additions have the potential to reduce CO₂ emissions while enhancing slow pool C and or its turnover, a reservoir of nutrients available to the soil biota. Use of these rapid, sensitive indicators of biological C activity will aid growers in determining whether a BMP fosters nutrient loss or retention prior to shifts in total SOC.

Introduction

In addition to producing high quality food, organic agriculture has the potential to provide environmental services that conserve soil, reduce greenhouse gas emissions (GHG) and

improve soil health. Organic agriculture systems typically utilize nitrogen (N) fertilizer sources that are coupled with carbon (C). Additions of animal amendments and the retention of residues from cash and cover crops contribute to soil organic matter (SOM) reserves. Increases in SOM are associated with improvements in soil health such as increased soil C storage which aids in the mitigation of GHGs and serves as a source of mineral N and other nutrients (Gattinger et al., 2012; Lucas et al., 2012). Organic agricultural systems mimic natural ecosystems in that C and N are bound or coupled together in the form of organic constituents that include SOM, the decomposition of which controls a significant proportion of N mineralization and soil fertility (Berthrong et al., 2013).

There is a growing body of literature that suggests soil health and fertility can be improved by adopting organic management practices. Organic agroecosystems are designed to provide a ratio of C to N that promotes biological cycles and soil biological activity such that N and C losses are reduced while N utilization and C sequestration are enhanced (Johnson et al., 2007; Berthrong et al., 2013; Bowles et al., 2015). Increases in total soil organic C (SOC) and N may increase N fertility without necessarily increasing net losses of reactive N (Ghorbani et al., 2010).

Improvements in soil health are the result of long-term adoption of BMP in organic systems such as conservation tillage, complex rotations, cover crops and animal amendments (Drinkwater et al., 1998; Fließbach et al., 2007). Most organic management systems integrate several BMP(s) that promote C sequestration. Therefore, understanding the effects of BMP such as reduced tillage and or additions of organic amendments of variable C, N and biochemical content is needed to predict the release as well as incorporation of C and N from soils in organic systems. In addition, N management in organic production systems is challenging as organic

agricultural systems are typically N limited (Gaskell and Smith, 2007). The fertility of organic agricultural operations is determined in part by the size and turnover rates of C and N pools in SOM and their synchronization with C and N additions. Thus, soil health indicators could be used by organic growers to verify that biological C and N are coupled in a given organic management system.

Stocks of C contained in SOM are often defined as pools that vary in their decomposition rate due to their biochemical composition. Best management practices affect SOC and the further partitioning of C into different pools of variable turnover rates. The $\text{CO}_2\text{-C}$ evolved from long-term 360 d incubations, total SOC and resistant or non-hydrolyzable C can be used to estimate the size of the active pool of C (turnover of days); slow pool C (turnover of years); and resistant pool C (no significant turnover rate) via a three pool nonlinear model (Paul et al., 1999). The proportion of each of the different C pools present in the soil provides important information with respect to soil health and fertility (Fortuna et al., 2003).

The active and slow pools of C in soil are the most directly affected by BMP (Fortuna et al., 2003; Jha et al., 2012; Motavalli et al., 1994; Paul et al., 1999). The active pool of C at a 0-30 cm depth represents $\leq 5\%$ of total SOC, turns over in a growing season and can be equated to seasonal CO_2 emissions in the field. This pool consists of easily decomposable C compounds (simple sugars, organic acids and microbial metabolites) that provide an important source of energy to microorganisms (Fortuna et al., 2003; Iqbal et al., 2009). The slow pool of C at the same depth consists of structural plant residues and physically stabilized carbon and effects the nutrient buffering capacity of soils (Fortuna et al., 2003). The slow C pool contains ~ 40 to 50% of total SOC and turns over in 10-80 years (Paul et al., 2006). The active plus slow C represents hydrolyzable C. The non-hydrolyzable C fraction can be measured via the acid hydrolysis

procedure and contains ~ 40 to 50% of total SOC at a 0-30 cm (Paul et al., 1997). It is mainly comprised of lignin and chemically stabilized C (Buyanovsky et al., 1994; Paul et al., 1999). This fraction improves soil tilth and water holding capacity but does not contribute C for microbial respiration (Six et al., 2002). Despite the broad acceptance of these techniques to estimate C pools and turn-over rates, they are time consuming and require resources that prohibit their use as a standard measure of soil health or fertility index. Therefore, there is a need to correlate other rapid measures of soil C fractions with the information obtained from long-term C incubations. In this paper, the value of correlating several rapid measures of soil C that include SOC, hydrolyzable C and permanganate oxidizable carbon (POXC) measurements with estimates of active and slow pools of C derived from a 360 d incubation is illustrated.

There is a lot of published research illustrating the value and ease of using POXC as an index of soil health (Lopez-Garrido et al., 2011; Lopez-Garrido et al., 2014; Melero et al., 2009a; Melero et al., 2009b;). In contrast, there is a dearth of information with respect to its relationship with other fractions and pools of soil organic C. A current initiative in organic agriculture research is to employ indicators of soil health to reveal useful information about the biological function and fertility of organic farming systems. In furtherance of this goal, a number of research groups have attempted to develop or apply labile soil C tests such as hydrolyzable C and POXC as short-term indicators of long-term changes in soil health and fertility (Culman et al., 2012; Dou et al., 2008; Weil et al., 2003).

The biogeochemical cycling of C and N in organic systems is complex, and the range of existing organic management systems is diverse (Franzluebbers, 2005; Johnson et al., 2007). Although biological indicators of soil health are dynamic measures of biological cycles and soil biological activity, there are few data sets available that compare such measures across different

organic management systems (Azeez, 2009). This study was designed to compare five organic cropping systems, providing a unique opportunity to study the effects of best management practices (tillage intensity, amendment type, and livestock integration) on C sequestration and N cycling in these systems. This study hypothesizes that application of compost and minimizing disturbance (via pasture, no tillage) will enhance C sequestration and foster retention of C in the slow pool which may reduce C and N losses and improve soil fertility. The current study was designed with three objective (i) quantify and model C pools in long-term organic systems of differing animal inputs, cropping systems and tillage intensities; (ii) assess the value of measuring POXC and hydrolyzable C fractions to predict shifts in the size of active and slow pools of C across organic management systems; and (iii) evaluate which BMP in these organic management systems enhanced C stocks, soil health and contributed to climate change mitigation.

Materials and methods

Long-term organic field sites

Two USDA certified organic experimental field sites, The Long-term Organic Tillage Systems (LOTS) study and the Long-term Organic Vegetable Systems Experiment (IFSYS), were used to evaluate the effects of BMP on C and N cycling. The 5 organic management systems chosen from the two experimental sites provided contrasts among treatments receiving varying levels of disturbance and organic C and N additions in the form of animal amendments and cover crops. Growing degree days were calculated for each field crop using the Baskerville-Emin method (Andresen, 2010) using a base temperature of 4.4°C and 7.2°C for LOTS (field pea, *Pisum sativum* L. ssp. *sativum*) and IFSYS (Delicata squash, *Cucurbita pepo*) plots respectively.

The LOTS tillage plots were established in 2010 on Reeder-Farnuf loams (fine-loamy, mixed, superactive, frigid, typic Argiustolls) at the North Dakota State University Dickinson Research and Extension Center, USA (46°53' N, 102°49' W; elevation 760 m). The soil had a pH (H₂O) of 6.7, EC of 0.85 dS m⁻¹ and was loam (30% sand, 47% silt, 23% clay) in texture. Soils were collected in 2014 from 2 treatments i.e. clean tillage and no tillage (8 field plots). The clean tillage plots were cultivated with a tandem disc to a 10-cm soil depth in late summer (August-October, depending on crop) and again the following spring (March-early June), prior to seeding. Seeding with a low-disturbance planter was the only event in the no tillage plots that resulted in soil disturbance, as described in a different study but with identical management of no tillage plots (Carr et al., 2015). The plots were 30 m by 9 m and were arranged as a randomized complete block with each treatment replicated four times.

Weeds were suppressed by pre-plant tillage in clean tillage plots or by a pre-plant application of 20% acetic acid in no tillage plots. Weed management was combined with grazing by sheep (*Ovis aries*) and the use of cover crops, and in the case of no tillage plots, killed cover crop mulch) to suppress weeds after field pea (*Pisum sativum* L. ssp. *Sativum*), navy bean (*Phaseolus vulgaris* L.), proso millet (*Panicum mileaceum* L.), and winter wheat (*T. aestivum* L. emend. Thell.) crops were harvested for grain. In the current study, soils were collected in 2014 where the field pea plots were grazed after harvest, prior to planting hairy vetch (*Vicia villosa* Roth). Detailed information for the crop rotation and other field activities can be found in Appendix Table A1.

The IFSYS organic vegetable systems experiment was established in 2003 as a USDA certified organic field plot at the Washington State University Puyallup Research and Extension Center, USA (47° 11'24" N, 122° 19'48" W; elevation 13 m). The experiment is located on an

alluvial soil classified as a Puyallup fine sandy loam (coarse-loamy over sandy, isotic over mixed, mesic Fluventic Haploxerolls) (Cogger et al., 2016; Pritchett et al., 2011). The soil had a pH (H₂O) of 6.4, EC of 0.46 dS m⁻¹ and was sandy loam (51% sand, 40% silt, 9% clay) in texture. Soils were collected in 2014 from 3 treatments (12 field plots) from the IFSYS experiment. The treatments included: 1) annual fall-seeded cover crop – vegetable rotation, broiler litter amendment; 2) annual fall-seeded cover crop – vegetable rotation, mixed compost amendment; 3) 3-year pasture –vegetable rotation, no amendment. The plots 6.1 m by 15.2 m were arranged as a randomized complete block with four field replicates for each treatment.

The fall cover crop treatment was seeded with a 50:50 mix by seed weight ratio of cereal rye (*Secale cereale* L.) and hairy vetch (Lawson et al., 2012). The fall cover crop plots were tilled twice each year (spring and fall) to a depth of 25 cm using a slow moving rotary spader. The amendments were applied to the plots in spring after the mowing of the cover crop. The N-rich broiler litter (4-6 Mg ha⁻¹ yr⁻¹) consisted mainly of partially composted manure and softwood shavings. The broiler litter was applied after 6-7 weeks of composting in a turned pile. The C-rich mixed compost was applied at 14-18 Mg ha⁻¹ yr⁻¹ and was comprised of dairy manure solids, animal bedding (straw and sawdust with manure), yard debris, and small amounts of broiler litter and fish waste. The mixed compost was applied after 24-28 weeks of composting and curing in an aerated static pile. The available N supplied by both the amendments were comparable (Cogger et al., 2016) (Appendix Table A2). However, the mixed compost supplied 2-5 times more C than the broiler litter. The pasture rotation was 30 months of pasture followed by 6 months of vegetable cash crop. The pasture plots consisted of a fall planted mixture of red clover (*Trifolium pretense*), annual ryegrass (*Lolium multiflorum* Lam.) and perennial ryegrass (*Lolium perenne* L.). Sheep and poultry (*Gallus gallus domesticus*) are raised on the pastures in

the rotational grazing system. The pasture plots were a reduced input system because they have received no amendments in the form of mixed compost or broiler litter since 2005. In the current study when the soil was collected in 2014, the fall-seeded cover plots were in cover crop following Delicata squash, (*Cucurbita pepo*), while the pasture plots were in the 14th month of the pasture phase. Detailed information on the IFSYS plots can be found in Appendix Table A3).

Soil sampling, treatment and storage

Twelve random samples were taken at 2 depths (0-15 and 15-30 cm) from each of the 4 field plot replicates within a 15 m by 6 m area with a soil probe (2.5-cm diam.) at the Puyallup, WA and Dickinson, ND sites in November 2014. The 12 soil cores from each field plot replicate at a given depth were composited and mixed to provide a single sample. The soil bulk density was determined using a hammer driven bulk density core sampler (6 cm deep by 5.4 cm diameter) (Grossman and Reinsch, 2002). Soil samples were analyzed for gravimetric moisture and initial inorganic N content ($\text{NH}_4^+ + \text{NO}_3^-$)-N. Ten g of soil from each field moist composited plot replicate was extracted with 100 mL of 2M KCl. The amount of ($\text{NH}_4^+ + \text{NO}_3^-$)-N in each aliquot was determined with an auto-analyzer (SEAL Analytical Inc, Mequon, WI). A portion of the remaining field moist samples were passed through a 2 mm sieve and used in a 360 d N incubation. The remaining soil was air dried and passed through a 2-mm sieve after which the soil was used to conduct a 360 d C incubation and to determine the SOC, POXC and resistant C for each field plot treatment and depth.

Total soil organic carbon and its fractions

Air dried soil samples were passed through a 100 mesh sieve and total soil C (TC) was measured by dry combustion (Skalar Analytical B.V., Netherlands). The non-hydrolyzable C fraction in soil also referred to as the resistant carbon pool was isolated via acid hydrolysis (Paul

et al., 2001). Twenty-five mL of 6 mol L⁻¹ HCl to each digestion tube containing 1 g of air dried soil (2 mm) was added and vortexed briefly. An aluminium block digester (Bran & Lubbe) was used to heat the samples at 115 °C for 16 hours. After drying the soil samples at 30°C overnight (Gollany et al., 2013) they were ground to pass through a 100 mesh sieve and analyzed for total carbon by dry combustion on the elemental Total Organic Carbon analyzer. The hydrolyzable C fraction was calculated by subtracting the non-hydrolyzable C from the TC (Dou et al., 2008). The permanganate oxidizable organic C (POXC) was determined by a procedure modified by Culman et al. (2012) and derived from the procedure of Weil et al. (2003) according to which 2.5 g of air dried 100 mesh sieved soil was weighed in a centrifuge tube to which 18 mL of deionized water and 2 mL of 0.2 M KMnO₄ (in 0.1 M CaCl₂) was added. The mixture was shaken at 240 oscillations per minute for 2 minutes and allowed to settle for 10 minutes. The supernatant was diluted with deionized water and the absorbance was measured with a SPECTRONIC™ 20D+ spectrophotometer (Thermo Fisher Scientific, WI) at 550 nm (Culman et al., 2012). The PROC GLM procedure of SAS 9.4 (SAS Institute, Cary, NC) was used to determine the effect of treatments on TC, POXC and hydrolyzable C. The LSMEANs procedure was used to compare the least squares' means across different treatments ($P=0.05$ level of significance).

Long-term carbon incubation

Soil samples were incubated for 360 d in a laboratory incubator (Precision™ Low Temperature BOD Refrigerated Incubator, Thermo Fischer Scientific Inc., Waltham, MA) at 25°C and 60% WFPS (Fortuna et al., 2003). Soils from each treatment and depth contained 4 replicates. The total number of samples was 24 (3 treatments × 4 field reps × 2 depths) for IFSYS and 16 (2 treatments × 4 field reps × 2 depths) for LOTS. Samples were incubated in 128

mL sterile specimen vials containing field moist soil equivalent to 50 g on a dry wt. basis was packed to a bulk density of 1.1 g cm³ and 1.0 g cm³ for the LOTS and IFSYS plots, respectively. The microcosms were pre-incubated at 25°C and 30% WFPS for a week before the incubation experiment began. A 7 d pre-incubation was conducted to compensate for the disturbance resulting from sampling, sieving and air drying soil (Fortuna et al., 2003; Collins et al., 2000; Paul et al., 2001). These basic handling procedures do not affect the size and turnover rate of slow pool C but can temporarily artificially elevate CO₂ emissions if soils are not pre-incubated (Collins et al., 2000; Paul et al., 2001).

Specimen vials were placed into 935 cm³ glass canning jars (1 quart) fitted with lids containing septa for gas sampling. The headspace of each replicated treatment was sampled with a 35 mL syringe and subsequently injected into a 20 mL evacuated vial fitted with a 3 mm PTFE-faced butyl crimp seal septum (Supelco Analytical, St. Louis, MO). Successive carbon dioxide (CO₂) measurements were taken at time zero and days 3, 7, 14, 21, 28, 42, 56, 70, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360. Cumulative CO₂ in the headspace at each time interval was well below 6% which verified that aerobic conditions were maintained throughout the 350 d incubation. After each sampling time interval, the headspace in the incubation vessels was flushed (Paul et al., 2001) and the specimen cups were weighed and the moisture content was adjusted to 60% WFPS. The total number of gas samples was 456 (3 treatments × 4 field reps × 2 depths × 19 time intervals) for IFSYS and 304 (2 treatments × 4 field reps × 2 depths × 19 time intervals) for LOTS. The CO₂ samples were run on an Infrared Gas Analyzer (IRGA, LICOR 830). A set of certified CO₂ standards were run and internal standards and blank vials were sampled at each time interval. The PROC GLM procedure was used to determine the effect of organic management treatments on cumulative CO₂-C evolved. The LSMEANS procedure was

used to compare the least squares' means across different treatments ($P=0.05$ level of significance).

Data fitting for determining carbon pools

A three pool nonlinear model in SAS NLIN was fitted in order to estimate the size of the active and slow pools of C and their turnover rates (Fortuna et al., 2003). The active fraction (C_a) was estimated from the model by measuring the amount of CO_2-C evolved from incubation vessels. The non-hydrolyzable C was used to estimate the resistant fraction (C_r) (Paul et al., 2001). A two-pool first-order constrained model, including an interval correction adapted from Ellert and Bettany (1988), was used to estimate the size and turnover rates of individual pools as described below and in Fortuna et al. (2003).

$$C_{(min)} = C_a(e^{-ka*t_1} - e^{-ka*t_1-ka*t_2}) + (C_{t1} - C_{r1} - C_{a1}) \times (e^{-ks*t_1} - e^{-ks*t_1-ks*t_2}) \text{ Eq. (1)}$$

Curve fitting of the CO_2-C evolved per unit time; C (min), was performed using the NLIN procedure of SAS 9.4 (SAS Institute, Cary, NC). C_a , ka = active pool; C_s , ks = slow pool; C_r = resistant pool; t_1 = start of sample interval; t_2 = end of sample interval. The slow pool is defined as $C_s = (C_{t1} - C_{r1} - C_{a1})$ where C_{t1} is the total soil organic C, (C_r) the resistant pool, and C_{a1} , the initial active pool of C at time zero. The resistant fraction (C_r) is equated to the total C content of the residue of acid hydrolysis (Paul et al., 2001). The size of the active pool (C_a), decomposition rate of the active (ka) and slow pools (ks), as well as mean residence times (MRT) of the active ($1/ka$) and slow pools ($1/ks$), was calculated from the model. The laboratory MRT at 25 °C was scaled to an average field temperature (10 °C) by assuming a Q_{10} of 2.8 (Katterer et al., 1998; Paul et al., 1999; Fortuna et al., 2003). The PROC GLM procedure of SAS 9.4 (SAS Institute, Cary, NC) was used to determine the effect of treatments on resistant C. The

LSMEANs procedure was used to compare the least squares' means across different treatments ($P=0.05$ level of significance).

Long-term nitrogen incubation

The total soil N contained in soil samples used in the N incubation was determined using the Kjeldahl method (Bremner, 1960). A 360-d N incubation was conducted in the laboratory at 25°C and 60% WFPS (Fortuna et al., 2003) using fresh field moist soil sieved through a 2 mm mesh. For this purpose, 50 g soil samples in sets of 4 replicates per treatment plot per depth were weighed into 128 g sterile specimen vials and packed to a bulk density of 1.1 g cm³ and 1.0 g cm³ for the LOTS and IFSYS plots, respectively. They were set up to allow for destructive sampling and analysis of inorganic N at 6 separate time intervals: 7, 14, 90, 150, 210 and 360 d (Fortuna et al., 2003). The total number of samples was 144 (3 treatments × 4 field reps × 2 depths × 6 time intervals) for IFSYS and 96 (2 treatments × 4 field reps × 2 depths × 6 time intervals) for LOTS. Soil samples were aerated and maintained at 60% WFPS throughout the 360 d incubation in a Precision™ Low Temperature BOD Refrigerated Incubator (Thermo Fischer Scientific Inc., Waltham, MA).

A completely randomized two-factor factorial analysis of variance (ANOVA) was conducted using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC) to determine the effect of organic managements on the mineralizable N during the 360 d incubation, the mean separations were done using least significant difference with the LSMEANs procedure at the $P=0.05$ level of significance. The linear bivariate relationships and the least square regression lines between the soil C and N pools were graphed using the SPSS 22 software package (IBM Corp., Armonk, NY).

Results and discussion

Use of total carbon as an indicator of soil health in organic management systems

The build-up of organic matter is directly affected by the amount of plant residues and animal inputs as well as the quality of these materials (Johnson et al., 2007), all of which are influenced by management practices. Improved agronomic practices including application of organic fertilizers such as compost and manure products from livestock can increase soil C accumulation and reduce C losses (Diacono and Montemurru, 2010). However, over application of manures might lead to overloading of nutrients such as N in soils which could stimulate nitrous oxide emissions, thereby outweighing the benefits of C sequestration (Conant et al., 2008).

Pasture systems have been shown to promote C accumulation while limiting the availability of inorganic N due to high density of roots, root exudation and lack of physical soil disturbance. Introducing grass species with higher net productivity or legumes in grasslands promoted soil C storage (Soussana et al., 2004). Jones et al. (2006) investigated the effect of different organic and mineral N fertilizer treatments on C storage in temperate grasslands of Scotland. They found that addition of manure increased the C storage whereas mineral N fertilization had minimal effects on C storage. Intensive fertilizer use can result in accelerated mineralization of SOM, enhanced decomposition of SOC and a reduction in soil C stocks.

The soils sampled in this experiment did not contain inorganic C. Therefore, TC has been substituted for soil organic carbon (SOC). In the IFSYS field site (Table 2), soils taken from treatments receiving compost applications had significantly higher amounts of SOC (73.9 Mg C ha⁻¹) relative to the annual system receiving broiler litter (51.5 Mg C ha⁻¹) and the pasture system (45.9 Mg C ha⁻¹) that contained comparable SOC. Soil taken from the mixed compost treatment

in the IFSYS experiment had 30% more SOC relative to the broiler litter treatment and 38% more SOC than the pasture that received ~ a third of the C inputs added to the two annual systems. The greater addition of C inputs that included resistant C from compost additions justifies the higher SOC in the compost treatment and compensates for the tillage disturbance that could result in loss of SOC that was minimized in the pasture management. Teasdale et al. (2007) found that organic management practices that included additions of manure increased the SOC to a greater degree than no tillage systems receiving mineral fertilizer.

Table 2. Measurements of soil organic carbon (SOC), cumulative CO₂ mineralized and total nitrogen (N) for 0-30 cm soil depth.

Field site	Treatment	SOC	Cumulative CO ₂ mineralized		Total N
		Pool size	Pool size	Percent of SOC	
		Mg C ha ⁻¹	Mg C ha ⁻¹	%	Mg N ha ⁻¹
NDSU LOTS	Clean tillage	42.4 a [†]	2.50 b	6 a	2.69 a
	No tillage	45.3 a	3.06 a	7 a	2.90 a
WSU IFSYS	Compost	73.9 A	4.94 A	7 A	4.37 A
	Broiler litter	51.5 B	3.80 B	7 A	2.74 A
	Pasture	45.9 B	3.15 B	7 A	3.29 A

[†]ANOVA was run in SAS PROC GLM (SAS 9.4). Means within a column and field site with different letters are significantly different at $P=0.05$ by the LSMEANS procedure ($P=0.05$ level of significance).

Differences in SOC were not detected between the no tillage (45.3 Mg C ha⁻¹) and clean tillage (42.4 Mg C ha⁻¹) treatments in the LOTS study at $P=0.05$ level of significance. This may be due to the lowered plant biomass returned in the dry climatic conditions of Dickinson, ND and the location of residues on the surface in no tillage plots which could reduce the rate of C decomposition and accumulation. Weil et al. (2003) compared the effect of conventional tillage versus no tillage in a wheat-based rotation at Mandan, ND, and could not find significant differences in SOC. No significant differences in total N were observed among the IFSYS systems or between the LOTS tillage treatments (Table 2).

This study is one of a few experiments that examines the effects of no tillage on organic management systems. There is ample evidence that SOC is higher in soils managed organically that receive nutrient additions like manure as compared to agricultural systems that receive synthetic fertilizer additions such as urea (El-Hage Scialabba and Muller-Lindenlauf, 2010). However, organic producers in some cases have been importing inputs from off farm to supplement their fertility, thereby increasing SOC (Powlson et al., 2011). Yet, studies examining zero net input organic systems like mixed-livestock crop production systems where no imported inputs are utilized have also found that SOC concentrations were significantly higher under organic managed systems as compared to non-organic managed systems (Gattinger et al., 2012).

The SOC is considered to be a key indicator of soil health but SOC alone is not sufficient to understand C cycling in organic systems. Rather it is the cycling of C in soils, i.e. the biological cycling of C and soil biological activity that are critical components of healthy soils and sustainable agricultural farming systems (Janzen, 2006). Approximately half of the SOC for a 0-30 cm plow layer is comprised of C compounds that do not contribute to biologically active C and the remaining half of SOC has a mean residence time of years or decades (Fortuna et al.,

2003; Paul et al., 2006). Therefore, it is not expected to have measurable differences in SOC due to tillage intensity in the LOTS treatments as the no tillage management was implemented for only four years prior to these measurements.

Carbon mineralization as CO₂-C during a 360 d laboratory incubation

Long-term incubations have been used by numerous researchers across a wide range of ecosystems to estimate the quantity and rate of C cycling (Fortuna et al., 2003; Paul et al., 1999). In this study, about 66% of the variation in SOC could be explained by the carbon mineralized as CO₂ from the soil during a 360 d incubation (Fig. 4). The unexplained variation could be due to the difference in the extent of physical and chemical protection of C within soil aggregates which is partially dependent upon the soil texture and mineralogy (Benbi et al., 2015). In this study, the percent of SOC mineralized as cumulative CO₂-C during the 360 d incubation ranged from 6-7% (2.50 to 4.94 Mg C ha⁻¹) across all the treatments (Table 2).

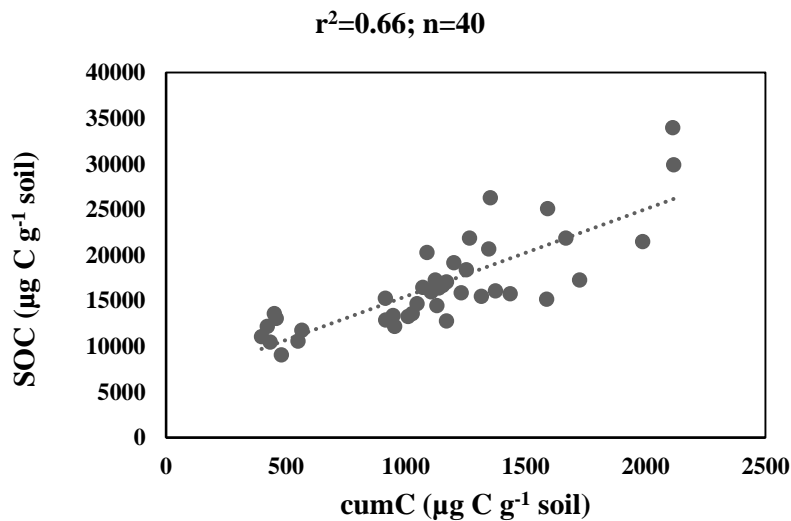


Fig. 4. Interrelationship (linear regression) of cumulative CO₂ evolved at day 360 (cumC) with soil organic carbon (SOC) as measured by coefficient of determination (r^2) across different organic management systems.

The amount of CO₂-C mineralized per unit of SOC during the 360 d incubation, also referred to as the mineralization quotient (Benbi et al., 2015), is an indicator of the efficiency of

microorganisms to consume C rich substrate. No significant difference was observed in the portion of SOC that was mineralized to CO₂-C during the 360 d incubation between soils taken from the 2 tillage managements on the LOTS experiment and among soils taken from the organic IFSYS field site (Table 2). However, the cumulative CO₂-C mineralized during the 360 d incubation was highest in soils collected from the compost amended plots (4.94 Mg C ha⁻¹) in the IFSYS field site (Table 2). The greater amounts of SOC released as CO₂-C from the compost amended soil taken from the IFSYS field site may simply be a function of greater C additions added to the system in the form of compost. Schwendenmann and Pendall (2008) found that 2.9% of the SOC was mineralized as CO₂-C from a tropical grassland soil in Panama during a 6-month incubation experiment. In a separate experiment conducted in a temperate climate, previous applications of compost vs. mineral N fertilizer to cropland for over 10 yr did not have a significant influence on the amount of mineralizable C evolved as CO₂-C (Fortuna et al., 2003). In contrast, Paul et al. (1999) observed higher CO₂ evolution from soils taken from a no tillage, chemically fertilized corn/soybean rotation during a similar long-term incubation experiment. Similarly, mineralizable C increased linearly with 10 yr of additional C inputs from no tillage, intensively cropped systems (wheat/soybean double-crop) on a Fluventic Ustochrept (Franzluebbers et al., 1998). The implementation of no tillage management was too recent and residue inputs were insufficient to have affected the total SOC in this study. But clean tillage (2.50 Mg C ha⁻¹) did significantly affect the amount of soil C lost as cumulative CO₂ compared with no tillage (3.06 Mg C ha⁻¹) in the LOTS field sites.

Estimates of active, slow and resistant C pool sizes and mean residence times (MRT)

Shifts in total SOC concentrations are long-term and are not necessarily directly linked to the biological activity and nutrient cycling of soils (Weil et al., 2003). Cumulative CO₂-C

evolved during the 360 d C incubation was used to estimate the active and slow pools of C. The active and slow C pools are most directly affected by BMPs and constitute approximately half of the C stocks in agronomic soils sampled to a 0-30 cm depth (Fortuna et al., 2003).

During the early stages of the incubation, the C mineralized consisted largely of C from the active pool with a laboratory MRT ranging from 21 to 30 days (Table 3). The active pool comprised only 1% of the SOC in all 5 of the organic management systems and there was no significant difference in size and turnover rate of the active C pools among the treatments. Fortuna et al. (2003) found that the active C pool constituted 1% of the SOC in cropping systems fertilized with compost as compared to 4% of the SOC in cropping systems that were fertilized with mineral N. Lewis et al. (2011) found 14% higher labile C in conservation tillage as compared to conventional tillage organic management systems. In a study conducted on a tropical grassland in Panama, the active pool of C comprised less than 1% of the SOC and had a MRT of 10 days (Schwendenmann and Pendall, 2008). The longer MRT of the active C pool in the organic pasture treatment may partially be due to the slower turnover of organic materials in temperate climates and differences in the methods employed in each experiment (Santruckowa et al., 2000). A small change in the active C pool corresponds to a significant change in the evolution of CO₂, which is a source of greenhouse gas emissions. Thus, adoption of BMPs that reduce disturbance and foster C sequestration in organic management systems could be a means of decreasing the amount of C in the active pool which could reduce the GHGs and provide additional value added to organic commodities and products.

Table 3. Estimates of active, slow and resistant C pools and mean residence times (MRT) [†] for 0-30 cm soil depth as determined by a 360 d laboratory incubation.

Field site	Treatment	Active C pool				Slow C pool				Resistant C pool	
		Size	Percent of total C	Lab MRT	Field MRT [‡]	Size	Percent of total C	Lab MRT	Field MRT	Size	Percent of total C
		Mg C ha ⁻¹	%	days	days	Mg C ha ⁻¹	%	years	years	Mg C ha ⁻¹	%
NDSU LOTS	Clean tillage	0.35 a [§]	1	26 a	73	18.5	44	8 a	22	23.6 a	55 a
	No tillage	0.31 a	1	21 a	59	19.0	42	6 b	17	26.0 a	57 a
WSU IFSYS	Compost	0.47 A	1	25 A	70	35.5	48	8 B	22	38.0 A	51 A
	Broiler litter	0.33 A	1	22 A	62	28.2	55	8 B	22	23.0 B	44 B
	Pasture	0.44 A	1	30 A	84	25.1	55	9 A	25	20.4 B	44 B

[†] Curve fitting of the CO₂-C evolved per unit time was fit with a two-pool first order constrained model using the SAS NLIN procedure (SAS Inst., 1997).

[‡] Field MRTs were estimated from laboratory MRTs using a Q₁₀ of 2.8.

[§] Means within a column and field site with different letters are significantly different at *P*= 0.05 level of significance.

The size of the slow C pool was estimated to contribute 44% (18.5 Mg C ha⁻¹) of SOC in the clean tillage treatment as compared to 42% (19.0 Mg C ha⁻¹) in no tillage treatment in the LOTS plots (Table 3). However, the estimated field MRT of the slow pool C was higher in the clean tillage (22 years) as compared to the no tillage (17 years) treatment. Tillage results in the shredding and incorporation of plant residues into the soil where the majority of microorganisms are located and the environment is more favorable for decomposition of C. As a result, the clean tillage can be assumed to have less of the C rich substrate available to the microorganisms relative to the no tillage. This may explain in part the higher MRT of the slow pool C in the clean tillage relative to the no tillage treatment taken from the LOTS field site.

Addition of compost or broiler litter to the IFSYS plots resulted in the partitioning of 48% (35.5 Mg C ha⁻¹) and 55% (28.2 Mg C ha⁻¹) of SOC to the slow pool, respectively. The pasture had 55% (25.1 Mg C ha⁻¹) of total C in the slow pool with a higher estimated field MRT of 25 years as compared to 22 years in the compost and broiler litter treatments. Similar results were obtained in a temperate grassland of Panama as previously discussed (Schwendenmann and Pendall, 2008). Application of animal derived amendments and reductions in tillage increased the turnover rates of the slow pool in this study.

During the 360 d laboratory incubation all of the C in the active pool and a limited portion of the slow pool was evolved as CO₂-C. The contribution of resistant C to CO₂ evolution during a ~1-yr incubation is negligible (Paul et al., 1999). The resistant pool of C improves the soil physical properties and thus contributes to soil health (Fortuna et al., 2003). Application of compost to the IFSYS field site contributed more to the resistant pool of C (51% of SOC) relative to broiler litter additions (44 % of SOC) and the pasture system (44% of SOC) (Table 3). Other studies found that no tillage (Paul et al., 1999) and compost additions (Fortuna et al., 2003)

contributed more C to the slow and resistant pools relative to the active pool of C. However, there are studies which suggest that organic inputs like the addition of farmyard manure can augment the active pool in comparison to the slow and resistant pools at 0-15 cm soil depth (Jha et al., 2012). Dissimilarities between the results of this study and those of Jha et al. (2012) may be due to differences in the models used to fit the C pools, or variations in nutrient contents and age of the manure between studies which would affect the amount of inorganic N and biologically active C available as well as the biochemical constituents of the manures.

The adoption of organic management practices that include addition of organic amendments (in the form of compost) and no tillage or low disturbance pasture treatments have the potential to reduce CO₂ emission per unit of SOC since these BMPs foster C sequestration and contribute less labile C as a portion of SOC (Dou et al., 2008). In addition, the presence of higher concentrations of resistant C derived from compost additions with MRTs of potentially hundreds or thousands of years (Paul et al., 1997) could reduce C lost as CO₂ in these organic management systems. This three pool model is a valuable tool for assessment of short-term shifts in SOC due to implementation of BMPs. The model used to estimate the first order kinetics of C mineralization allows us to assess both the size and turnover rate of the active and slow pools of C while the rapid assessment methods used in this study, hydrolyzable C, POXC and SOC cannot be used to estimate MRT. Although estimates of the size of slow pool C were comparable between the clean tillage and no tillage LOTS treatments, the MRT of the slow pool varied between the two tillage treatments.

Rapid measures of soil C as a proxy for active and slow pools of C

The three pool first order model is an effective means of partitioning C into pools that cycle on a seasonal and long-term basis but is time and resource intensive. Therefore, relating the

long-term incubation measurements with rapid measures of soil C fractions such as hydrolyzable C and POXC could prove to be useful for comparing different organic management systems. The hydrolyzable C represents the largest labile fraction of C (McLauchlan and Hobbie, 2004). In this study, the application of compost decreased the proportion of hydrolyzable C in SOC (49%) as compared to broiler litter and pasture (56%) treatments (Table 4). Dou et al. (2008) found that hydrolyzable C comprised a larger portion of SOC (59 to 72%) in a tilled wheat field receiving mineral N fertilizer.

Table 4. Measurements of hydrolyzable soil carbon (HC)[†] and permanganate oxidizable carbon (POXC) taken from a 0-30 cm soil depth.

Field site	Treatment	HC		POXC		
		Pool size	Percent of total C	Pool size	Percent of total C	Percent of HC
		Mg C ha ⁻¹	%	Mg C ha ⁻¹	%	%
NDSU LOTS	Clean tillage	18.8 a [‡]	44 a	0.74 a	2 a	4 a
	No tillage	19.4 a	43 a	0.84 a	2 a	4 a
WSU IFSYS	Compost	36.0 A	49 A	2.63 A	4 A	7 A
	Broiler litter	28.6 B	56 B	1.64 B	3 B	6 B
	Pasture	25.5 B	56 B	1.54 B	3 B	6 B

[†] HC is defined as total soil organic carbon – non-hydrolyzable soil carbon.

[‡] ANOVA was run in SAS PROC GLM (SAS 9.4). Means within a column and field site with different letters are significantly different at $P=0.05$ by the LSMEANS procedure ($P=0.05$ level of significance).

Like hydrolyzable C, analysis of the POXC fraction is simple, quick and inexpensive. Weil et al. (2003) developed a modified POXC protocol with the intent of incorporating the measurement into the Natural Resource Conservation Service's soil health test kit. The measure

is a proxy of reactive C and is a sensitive measure of soil health reflecting the effects of BMPs and land use. The fractions of SOM from which POXC originates include fresh organic material, soil microbial biomass, particulate organic matter, and other easily metabolized organic compounds, such as carbohydrates (sugars) and proteins (amino acids), as well as C loosely bound to soil minerals. The MRT of the POXC fraction is short in comparison to the resistant C fraction which has a turnover rate of several hundreds to thousands of years (Weil et al., 2003).

The POXC fraction in this study represented 4-7% of the hydrolyzable C pool (Table 4). In other words, the POXC fraction can be assumed to represent the whole of the active C pool and a portion of the slow C pool estimated by the three pool C model. My data reveal that the POXC fraction targets a more sensitive fraction of the SOC which can act as a proxy for the active and a limited portion of slow pool C (Fig. 5). The mean POXC values in this study were significantly higher in treatments where compost (2.63 Mg C ha⁻¹) was applied as compared to treatments receiving broiler litter (1.64 Mg C ha⁻¹) and the pasture (1.54 Mg C ha⁻¹) system (Table 4). Previous studies have indicated that the application of compost or manure to a crop rotation increases the POXC content in the soil (Culman et al., 2013; Lucas and Weil, 2012). In this study, the POXC varied from 3 to 4% of the total C in the IFSYS plots. However, no significant difference was observed for the POXC fraction in the clean tillage vs. no tillage LOTS plots. Similar results were observed for the POXC fraction with different tillage intensities in a chemically fertilized wheat-sunflower (*Helianthus annuus* L.)-fodder pea cropping system (Lopez-Garrido et al., 2014). However, Weil et al. (2003) reported that the POXC fraction was sensitive to long-term tillage treatments (conventional plow tillage vs. no tillage) in a wheat based rotation in North Dakota.

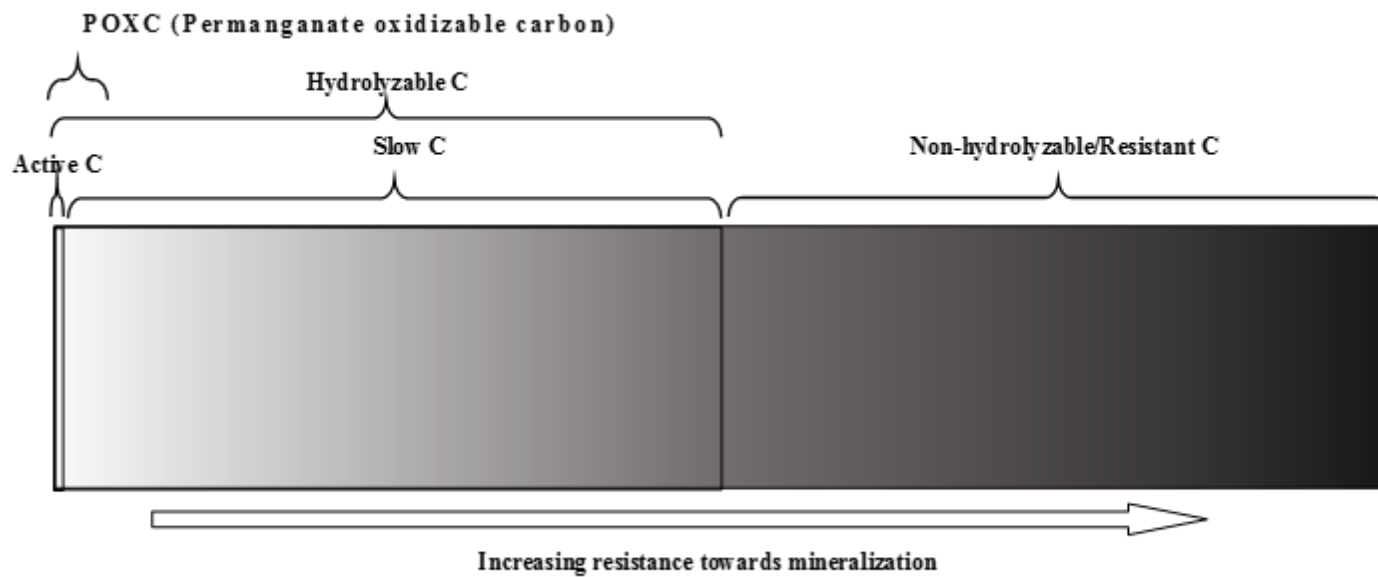


Fig. 5. Diagrammatic representation of the relative size of the various pools in total soil organic carbon (SOC) across different organic management systems.

Growing degree days and N mineralization dynamics

The use of a growing degree day (GDD) calculation allowed for the correlation of incubation time intervals with growth stages of crops grown in the field trials (Lawson et al., 2012) and takes into account the effect of temperature on C and N mineralization. Day 90 of the laboratory incubation approximates the end of a growing season and the optimum potential cumulative inorganic N available for crop growth (Table 5).

Table 5. Correlation of growing degree days (GDD) elapsed in the field from pre-plant to crop maturity and time of laboratory incubation.

Field site	Incubation		Field	
	Days	GDD	Sample	GDD
NDSU LOTS [†]	7	144	2 leaf stage	211
	14	288	4 leaf stage	300
	90	1854	Maturity	1537
WSU IFSYS [‡]	7	124	Planting	125
	14	249	Mid-season	335
	90	1602	Residue incorporation	1415

[†] Calculated on a base temperature of 4.4°C for field pea (*Pisum sativum* L. ssp. *sativum*).

[‡] Calculated on a base temperature of 7.2°C for Delicata squash (*Cucurbita pepo*).

A significant time interval × treatment interaction was observed in the cumulative N mineralized between the two organic tillage treatments in LOTS ($P=0.04$) and among the three IFSYS organic management systems ($P=0.04$) during the 360 d incubation (Table 6). On day 90 of the incubation, the no tillage management (130 kg N ha⁻¹ soil) increased the potentially mineralizable N as compared to the clean tillage (75.9 kg N ha⁻¹ soil) treatments in the LOTS field site. This difference of 54 kg N ha⁻¹ though not statistically significant may be agronomically significant for N uptake in the wheat crop grown in the rotation. The cumulative N mineralized in the first 14 days was statistically at par among the treatments in the IFSYS plots. However, the d 90 cumulative mineralizable N was higher in the compost (215 kg N ha⁻¹

soil) as compared to the pasture treatments (150 kg N ha⁻¹ soil). The compost management contained the highest potentially mineralizable N (245 kg N ha⁻¹ soil) as compared to the broiler litter (222 kg N ha⁻¹ soil) and then to pasture (182 kg N ha⁻¹ soil) treatments averaged across the 360 d incubation in the IFSYS field site ($P < 0.0001$). A recent study on the IFSYS plots revealed that the addition of mixed compost to the soils increased the size (102 ± 17 mg N kg⁻¹) and the MRT (66 ± 30 d) of the long-term pool of mineralizable organic N (Cogger et al., 2016). Similar pattern in soils of this long-term incubation receiving mixed compost additions where the size of the slow pool C (35.5 Mg C ha⁻¹) increased (Table 3) was observed. Previous studies have found increased N mineralization from soils where compost was added in a continuous corn cropping system (Fortuna et al., 2003; Spargo et al., 2011).

Table 6. Cumulative inorganic (NH₄⁺ + NO₃⁻)-N mineralized from a 360 d laboratory incubation taken from a 0-30 cm depth.

Field site	Treatment [†]	Day 7	Day 14	Day 90	Day 150	Day 210	Day 360
		Cumulative (NH ₄ ⁺ + NO ₃ ⁻)- N mineralized					
		kg N ha ⁻¹ soil					
NDSU LOTS	Clean tillage	23.1e [‡]	27.1e	75.9 cde	136 abcde	163 abcd	225 ab
	No tillage	38.0 de	46.9 de	130 bcde	170 abcd	200 abc	265 a
WSU IFSYS	Compost	73.5 G	81.9 G	215 E	336 C	339 C	429 A
	Broiler litter	60.0 G	72.3 G	180 EF	275 D	351 BC	396 AB
	Pasture	59.7 G	62.1 G	150 F	221 E	284 D	318 CD

[†] ANOVA was run in SAS PROC MIXED (SAS 9.4). Time interval * treatment was significant ($P=0.04$).

[‡] Means within a field site with different letters are significantly different at $P=0.05$ by LSMEANS procedure ($P=0.05$ level of significance).

Soil health indicators and the coupling and decoupling of C and N

The application and calibration of a labile soil C test such as hydrolyzable C and POXC could be used as a useful management tool for organic farmers to predict shifts in N fertility and

soil health. The cumulative inorganic N and CO₂-C released on day 90 of the incubation was related to the C fractions that included hydrolyzable C and POXC via a linear regression models. Both hydrolyzable C and POXC contributed significantly to the cumulative CO₂ mineralized at day 90 from soil taken in the LOTS tillage treatments and soil from within the three organic management systems in IFSYS (Figs. 6 a and b, respectively). My data suggests that a significant amount of the C and N mineralized is derived from the hydrolyzable C and POXC fractions on d 90 of the incubation (equivalent to a growing season). Specifically, over 60% of the variation in the hydrolyzable C and POXC fractions could be explained by the quantity of C and N mineralized (Figs. 6 and 7, respectively). The mineralization of C and N have been shown to be good indicators of crop yield and biomass (Culman et al., 2013). Lucas et al. (2012) found that POXC could be used to predict crop yield and response to SOM management when winter rye was planted as a cover crop in a no tillage system. Thus, the hydrolyzable C and POXC can potentially serve as indicators of N availability, CO₂ evolution and crop yield in organic management systems.

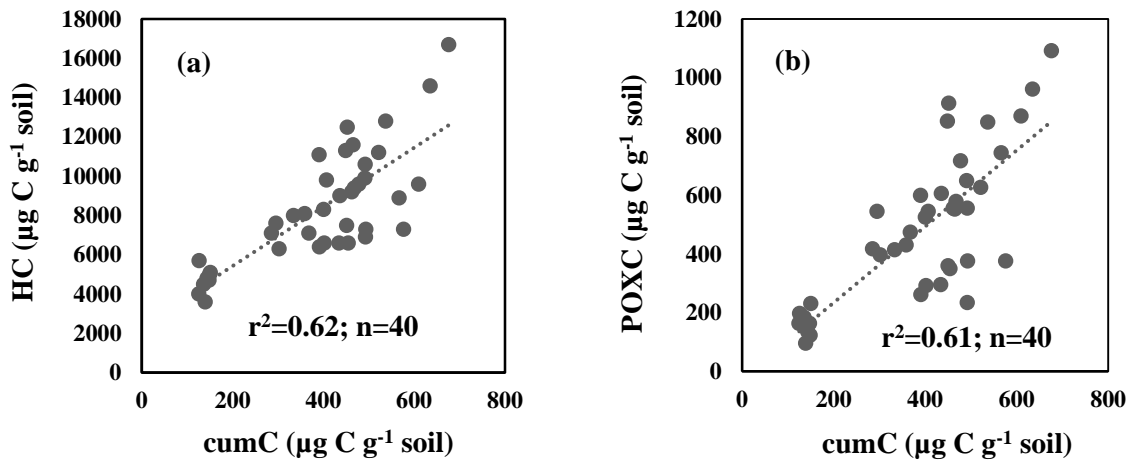


Fig. 6. Interrelationship (linear regression) of cumulative CO₂ evolved at day 90 (cumC) with (a) hydrolyzable soil carbon (HC), and (b) permanganate oxidizable carbon (POXC) measured as coefficient of determination (r^2) across different organic management systems.

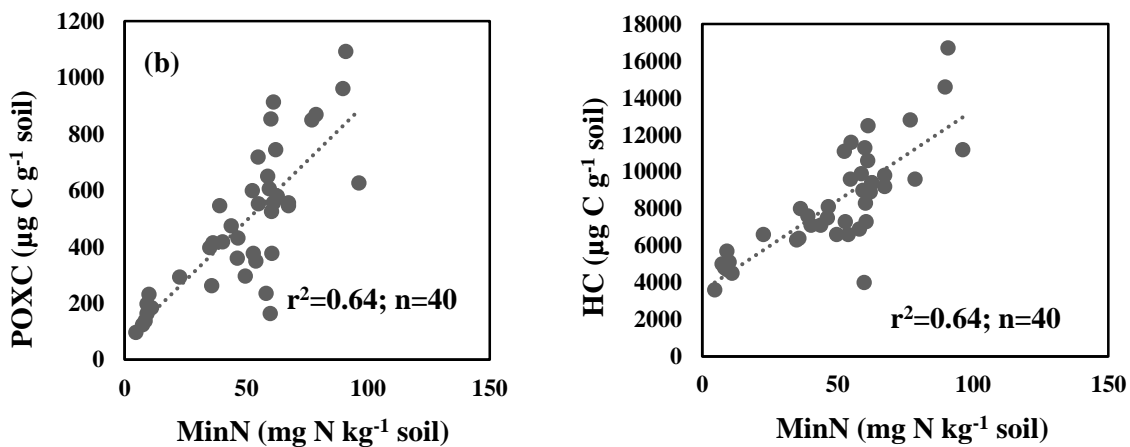


Fig. 7. Interrelationship (linear regression) of cumulative N mineralized at day 90 (MinN) with (a) hydrolyzable soil carbon (HC) and (b) permanganate oxidizable carbon (POXC) measured as coefficient of determination (r^2) across different organic management systems.

The POXC and hydrolyzable C fractions explained over 80% of the variability in SOC measurements ($r^2=0.83$ and $r^2=0.88$) (Figs. 8a and b, respectively). More than 50% of the variability in SOC could be explained by cumulative C mineralized as CO₂ at 90 d ($r^2=0.58$) (Fig. 8 c). A similar relationship between hydrolyzable C and SOC was obtained by Dou et al.

(2008) while examining the sensitivity of labile carbon pools in small grain based cropping systems fertilized with mineral N. The hydrolyzable C may be better related to SOC due to the greater quantity of SOC contained in the hydrolyzable C fraction relatively to that of mineralizable C. Several other studies verify a significant positive interaction between POXC and SOC (Culman et al., 2012; Weil et al., 2003). This might be due to similar methodologies used to estimate SOC and POXC which are based on the oxidation of C fractions contained in both SOC and the POXC fraction (Culman et al., 2013).

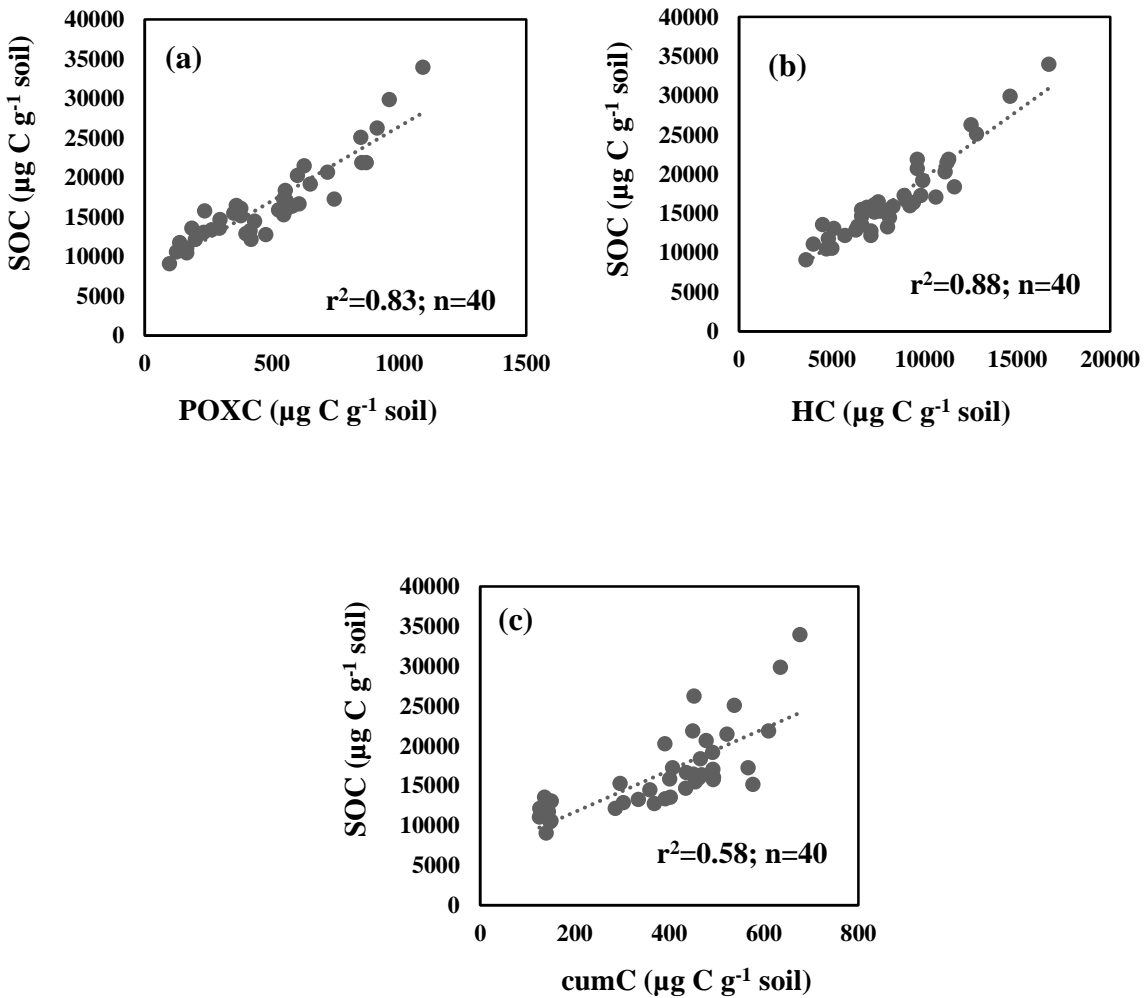


Fig. 8. Interrelationship (linear regression) of total soil organic carbon (SOC) with (a) permanganate oxidizable carbon (POXC), (b) hydrolyzable C (HC) and (c) cumulative CO_2 evolved at day 90 measured as coefficient of determination (r^2) across different organic management systems.

The quantity and quality of organic inputs and level of disturbance in the organic management systems assessed determined the amount of inorganic N mineralized and the $\text{CO}_2\text{-C}$ released via C mineralization. Systems in which slow pool C or the rate of turnover of slow pool C had significantly increased were expected to lead to the coupling of C and N which could reduce the potential of reactive N losses and CO_2 emissions per unit of soil C and N. The amount of cumulative inorganic N and $\text{CO}_2\text{-C}$ released from mineralization on day 90 of the incubation

is depicted in Fig. 9. Although the tillage managements have comparable SOC and CO₂-C released via mineralization, my data suggests that C and N are balanced/coupled in the no tillage system but potentially decoupled in the clean tillage management due to disturbance (Fig. 9 a). This might lead to greater CO₂ emissions in the clean tillage as compared to the no tillage treatments. The C and N appeared to be coupled in the compost and pasture systems (Fig. 9 b). However, the amount of C and N mineralized from the compost amended treatment was greater due to higher SOC and soil N concentrations relative to that of the broiler litter and the pasture system. The soils from broiler litter amended treatments had significantly higher inorganic N mineralized relative to CO₂-C released via mineralization.

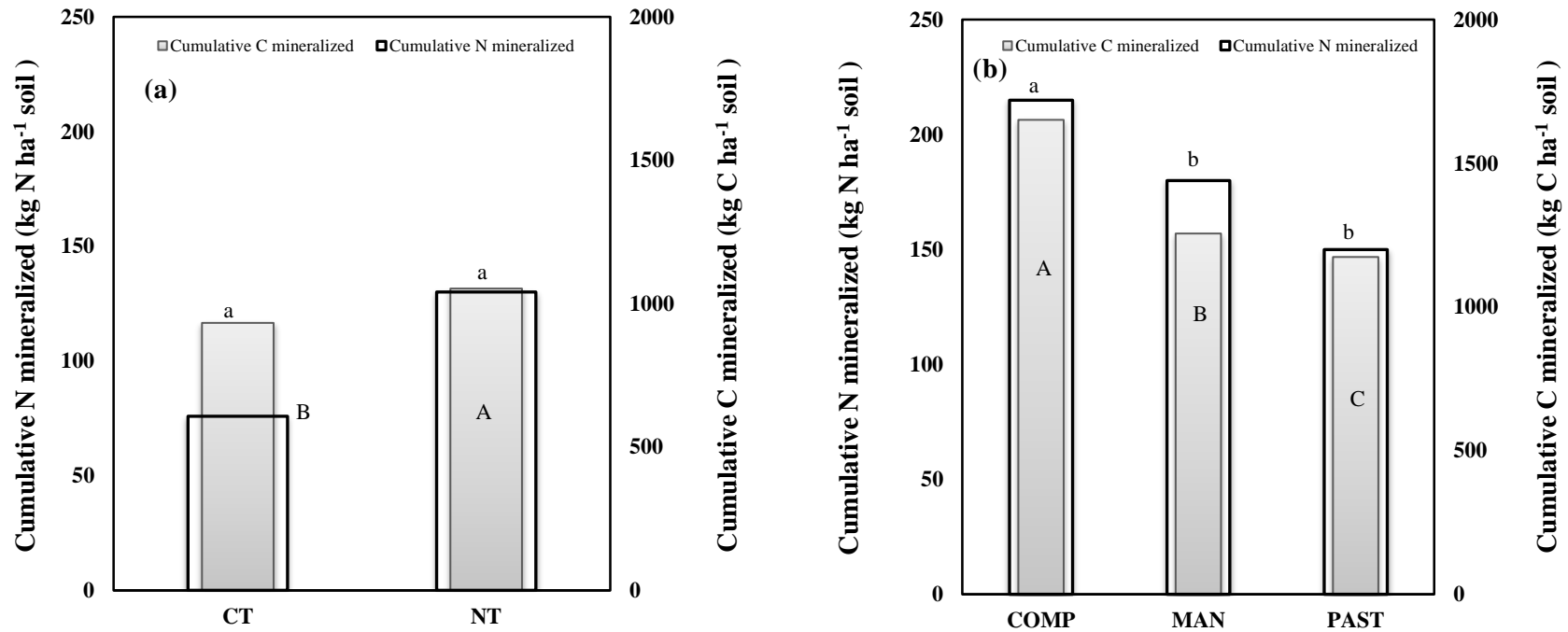


Fig. 9. Cumulative C mineralized ($\text{CO}_2\text{-C}$ evolved) and cumulative N mineralized ($\text{NH}_4^+\text{-N} + \text{NO}_3^-\text{-N}$) at day 90 in (a) Clean tillage (CT) and No tillage (NT) in the LOTS field site and (b) Compost (COMP), Broiler litter (MAN), Pasture (PAST) in the FSYS field site. ANOVA was run in SAS PROC GLM (SAS 9.4). Within a field site, means with different letters and cases (lower case for cumulative C mineralized; upper case for cumulative N mineralized) are significantly different at $P=0.05$ by the LSMEANS procedure ($P=0.05$ level of significance).

Organic management systems reflect the concept that N is stabilized in direct association with C (Bowles et al., 2015; Margenot et al., 2015). Decoupling of C and N that may lead to potential losses of reactive N. Specifically, the ratio of C available for biological activity and cycling relative to inorganic N is a function of organic inputs such as plant residues and manure amendments coupled with biologically available C and mineralizable N in SOM. The addition of readily decomposable C compounds (active C pool) can foster temporary immobilization of mineral N which may reduce losses of reactive N (Dosch and Gutser, 1996). Long-term additions of animal amendments and residue retention as well as reduced disturbance foster increases in N and C pools that cycle more slowly limiting excess mineral N and providing slow release of C for biological activity, potentially reducing the amount of CO₂-C released via the mineralization process. The amount of reactive N and active C are reduced when N is coupled with C of any type but particularly when N is bound with C in humic compounds such as in compost that decomposes more slowly and has a wider C:N ratio. Adoption of BMPs that reduce disturbance and sequester C in organic management systems tends to foster the flow of C into the slow pool (pool of accessible nutrients) while reducing the quantity of C in the active pool (a source of CO₂, a GHG) without fostering losses of reactive N (Fortuna et al., 2003). The enrichment of slow pool C resulting from the adoption of such organic management practices that include compost additions and minimization of disturbance could be used as strategies to improve fertility, soil health and aid in mitigating climate change (Gale et al., 2006).

Conclusions

This research addresses the effects of BMP(s) (tillage, application of organic amendments, livestock integration and land use) on the cycling of C and N in 5 different organic agroecosystems. Adoption of the above BMPs in organic management systems reduced the

partitioning of C to the active pool while augmenting the slow pool C. These pools are associated with the potentially mineralizable N supplied by residues, amendments and SOM affecting the concentration and release of mineral N required for crop N demand. Specifically, hydrolyzable C and POXC were significantly related with the cumulative N mineralized on d 90 of the incubation (the approximate length of the field season in growing degree days). Previous studies in which POXC was used as the sole indicator to predict biologically active C gave less consistent results due to differences in the types of C constituents contained in the POXC fraction. This study couples POXC with hydrolyzable C (C that can undergo hydrolysis reactions) as soil health indicators for rapid (within 2 to 4 yr) assessment of shifts in biologically active C resulting from change in management. Use of hydrolyzable C and POXC as soil health indicators could be employed to assess the impact of BMPs on C sequestration, greenhouse gas emissions and soil fertility prior to shifts in SOC. Both measurements could be completed by a soil testing unit within a few weeks. Future research should include soils from a range of textures, climates and organic agroecosystems in order to assess the value of using POXC and hydrolyzable C fractions to predict shifts in the size of active and slow C pools estimated via a three pool nonlinear model. After careful calibration, POXC and hydrolyzable C may provide guidance to organic growers enabling them to align their organic management practices with that of the USDA-Natural Resource Conservation Service's recommendations for potential future carbon credit payments.

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PAPER 2. USE OF BIOLOGICAL INDICATORS OF SOIL HEALTH TO ESTIMATE REACTIVE NITROGEN DYNAMICS IN LONG-TERM ORGANIC VEGETABLE AND PASTURE SYSTEMS

Abstract

Diverse crop rotations, cover crops and the possibility of integrating livestock make organic systems potentially more sustainable than other agroecosystems. Lower reactive nitrogen (N) in organic systems minimizes the potential for gaseous losses of N. However, addition of organic manures and residues containing mineralizable N and carbon (C) have the potential to enhance nitrous oxide (N₂O) emissions. A 39 d laboratory incubation was conducted to assess the key microbiological drivers controlling nitrification and denitrification in long-term organic vegetable systems during simulated freeze-thaw cycles. Soils were collected from two annual organic vegetable systems receiving 1) mixed-compost, or 2) broiler litter and 3) an organic perennial pasture system cropped to vegetables every third year. Soil microcosms amended with ¹⁵N labelled sugar beet (*Beta vulgaris*) residue or unamended were maintained at 40, 60 and 80% of water filled pore space (WFPS). Significant N₂O was emitted (4287-6138 μg kg⁻¹soil) via denitrification only from amended soil microcosms at 3 °C and 80% WFPS. Archaeal (AOA) and bacterial (AOB) nitrifier *amoA* gene copies were affected by shifts in temperature and reactive N species during freeze-thaws. Long-term organic vegetable cropping systems receiving mixed-compost additions had the potential to accumulate C and immobilize excess reactive soil N (particularly nitrates) thereby improving soil health and reducing N₂O emissions.

Introduction

Almost 70% of global nitrous oxide (N₂O) emissions originate from fertilization of agronomic systems and are the result of microbial transformations of ammonium and nitrate via

the processes of nitrification and denitrification (Syakila and Kroeze, 2011). Nitrous oxide has an average lifetime of 114 years in the atmosphere with 298 times the global warming potential of carbon dioxide (CO₂) over a period of 100 years (IPCC, 2014). The byproduct of the nitrification process is nitrate (NO₃⁻), a substrate required for heterotrophic denitrifiers (Zhu et al., 2013). Ammonia oxidation, the first and rate limiting step of nitrification is catalyzed by the *amoA* gene encoding the α -subunit of the ammonia monooxygenase enzyme. In soils, this process is mediated by 2 distinct groups of nitrifiers, ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) (Hastings et al., 2000; Venter, 2004). Nitrous oxide is an obligate intermediary of the denitrification process whereas the process of ammonia oxidation produces N₂O as a byproduct of nitrification in both AOA (Stieglmeier et al., 2014) and AOB (Lipschultz et al., 1981). There is a growing body of literature which suggests that AOA may be a significant contributor to N₂O emission via the nitrification process particularly in N limited soil systems (Jung et al., 2011).

The primary process of denitrification in agronomic soils is mediated by heterotrophic denitrifiers. This anaerobic form of microbial respiration results in the reduction of NO₃⁻ to elemental nitrogen (N₂) gas via intermediates that include N₂O. The reduction of N₂O to dinitrogen gas is catalyzed by the enzyme nitrous oxide reductase which is encoded in the *nosZ* gene (Braker and Conrad, 2011). Not all denitrifiers possess the complete set of enzymes required to transform nitrate to N₂ (Dandie et al., 2008) and the *nosZ* gene which catalyzes the final step from N₂O to N gas is inhibited by small quantities of oxygen (O₂).

There are several factors that contribute to the partial conversion of NO₃⁻ to N₂ gas that may result in the buildup of N₂O. Primarily N₂O emissions occur when NO₃, organic carbon (C) and O₂ concentrations are not in proper proportion (Firestone and Davidson, 1989). Addition of

C substrate in the form of organic residues and manure can enhance microbial respiration thereby reducing O₂ concentrations in the soil and creating anaerobic microsites in which N₂O is produced. Soil moisture also regulates O₂ concentrations affecting aerobic nitrifiers and anaerobic denitrifiers both involved in key N cycling processes (Firestone and Davidson, 1989). Changes in soil moisture partly determine the abundance of nitrifiers and indirectly regulate N₂O emissions originating from the nitrification process (Avrahami and Bohannan, 2009). Nitrification can account for 55-95% of the N₂O emissions when water filled pore space (WFPS) is between 40 and 60% (Linn and Doran, 1984). Denitrifiers are the dominant producers of N₂O from soil and N₂ is the final product of denitrification at 70- 80% of WFPS or near saturation (Saggar et al., 2013).

Climate and management practices are major drivers influencing mineralization of C and N, nitrification and denitrification that in turn reduce or enhance N₂O production from soils in agroecosystems (Johnson et al., 2007; Livesley et al., 2009). Organic agroecosystems, according to the International Federation of Organic Agriculture Movements, mimic natural ecosystems “relying on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects” such as synthetic N fertilizers, a major contributor to N₂O production. Diverse crop rotations, cover crops and the possibility of integrating livestock make organic systems potentially more sustainable than many other agroecosystems. Specifically, adaption of the above management practices in organic agroecosystems has the potential to provide ecological and environmental services that include the reduction of greenhouse gas emissions (GHG), promotion of soil conservation and improved soil health (National Organic Program, 2015). Lower reactive N and tight nutrient cycling in organic systems minimizes the potential for gaseous losses of N to N₂O (Ghorbani et al., 2010). However, addition of organic

manures and residues containing mineralizable N and C also have the potential to enhance N₂O emissions if not properly managed.

Specific organic management practices may enhance or reduce net reactive N in soil dependent upon whether the managements foster coupling or decoupling of C and N (Bhowmik et al., 2016; Palm et al., 2014). Because organic growers rely on the mineralization of organic N sources to provide inorganic N and other nutrients to crops, organic production systems typically foster coupling of N and C and are highly dependent upon microbial processes. Therefore, there is a critical need to quantify and identify keystone microorganisms that control nitrification and denitrification in different organic farming systems of varying management. Addition of C substrates from residues or animal amendments can facilitate heterotrophic denitrification if there is sufficient NO₃⁻ in a soil system (Mitchell et al., 2013). Diversification of cropping systems, planting of cover crops and the integration of livestock can all be used to augment pools of soil C and N. This enhances the reactive N in the form of inorganic N to be temporarily retained via immobilization but later made available for plant uptake, thereby, reducing the potential for denitrification (Bhowmik et al., 2016; Dieye et al., 2016; Eagle and Olander, 2012). The balance among water content, soluble C and nitrate concentrations [(C_{H2O})/NO₃⁻] can be used to predict the effect of a soil managements on N₂O production originating from heterotrophic denitrification in soil (Benckiser et al., 2015). Inventories of GHG emissions and knowledge with respect to how nutrients cycle in organic agroecosystems is limited, their biogeochemical cycling complex, and the range of existing climatic conditions and managements is diverse (Franzluebbers, 2005; Johnson et al., 2007). Therefore, it is of pivotal importance to study how these complex biogeochemical processes occur via nitrification/denitrification pathways and the influence key management practices (tillage intensity, amendment type, and

livestock integration) have on the potential to mitigate N₂O production during freeze-thaw cycles.

Most studies focus on soil temperatures at or above 25 °C which is considered to significantly increase the reactive nitrogen species in soil (Cui et al., 2016). However, the community structures of soil nitrifiers and denitrifiers is influenced by a wide range of temperature between 4 and 37 °C (Braker et al., 2010). Denitrifiers are more tolerant to subzero temperatures relative to most bacteria including nitrifiers (Sharma et al., 2006) and are active over a wider range of soil temperatures from sub-zero to 75 °C (Knowles, 1982). As a consequence, the addition and incorporation of organic C such as manures or plant residues in late fall in temperate regions is expected to stimulate N₂O emissions even at temperatures in the range of 5-10 °C (Singurindy et al., 2009). Significant shifts in nitrifier and denitrifier communities and increased N₂O production were reported by Wertz et al., (2013) in the presence of NO₃⁻ and C when soil temperatures were as low as -1°C. Thus, seasonal N₂O emissions in winter and late spring can be equivalent to or greater than the N₂O emissions measured during the growing season (van Bochove et al., 2000; Christensen and Tiedje, 1990; Wagner-Riddle et al., 1997). An increase in N₂O emissions due to the release of additional C and N substrates made available by winter freeze-thaw events has been reported previously (Phillips, 2008, Singurindy et al., 2009). Other factors such as how cold the temperature is during a freeze-thaw cycle (amplitude), its duration, and the soil moisture content at the time of the freeze regulates the release of C and N from organic material that may be made available for mineralization, nitrification and denitrification (Zhao et al., 2010). Despite the importance of freeze-thaw events, our knowledge of the effects of freeze-thaw events on nitrifier and denitrifier communities and their contribution to N₂O emissions is still limited particular in organic agroecosystems that relay

on C and N inputs such crop residues and animal amendments that could stimulate N₂O production.

This study examines N dynamics in soils taken from several organic agroecosystems for the purpose of validating estimates of improvements in soil health and environmental services associated with long-term organic management such as the potential for mitigation of GHG emissions. Specifically, this research links shifts in N₂O flux to nitrification and denitrification processes via biochemical-molecular methods that measure nitrifier and denitrifier abundance that are influenced by BMP and climatic conditions. These measurements will provide us with a better understanding of the soil microbiology that controls gaseous N losses and the BMP that influence these microbial processes. This study provides a unique opportunity to study the interaction of multiple abiotic factors (temperature, moisture, C availability, NH₄⁺-N, NO₃⁻-N), their effects on the abundance of genetic markers associated with nitrifier and denitrifier communities and relate the production of N₂O from these communities with the adoption of a range of key organic management practices (tillage intensity, amendment type and livestock integration). The objectives of this study are 1) to assess the microbiological drivers controlling the fluxes of key N cycle processes (nitrification, denitrification) in long-term annual and perennial vegetable systems, 2) Evaluate interactions among nitrifier (*amoA*) and denitrifier (*nosZ*) gene copies, potential biological indicators of soil health, and reactive forms of N (ammonium, nitrate and nitrous oxide) during simulated freeze-thaws and 3) to investigate the potential for niche separation of nitrifier and denitrifier communities on the basis of moisture, temperature and substrate affinity.

Materials and methods

Site description

The Long-term Organic Vegetable Systems Experiment was established at Washington State University (WSU) Puyallup Research and Extension Center, USA (47° 11'24" N, 122° 19'48" W; elevation 13 m) in 2003 as a USDA certified organic field plot. The experimental field is on the alluvial soils of the Puyallup Valley and is a Puyallup fine sandy loam in texture (coarse-loamy over sandy, isotic over mixed, mesic Fluventic Haploxerolls) (Pritchett et al., 2011; Cogger et al., 2016). The soil had a pH (H₂O) of 6.4, EC of 0.46 dS m⁻¹ and was sandy loam (51% sand, 40% silt, 9% clay) in texture.

Soils were collected in 2013 from 3 field treatments (12 field plots): 1) annual fall-seeded cover crop – vegetable rotation, mixed-compost amendment; 2) annual fall-seeded cover crop – vegetable rotation, broiler litter amendment; 3) 3-year pasture – 1-year vegetable rotation. The annual fall-seeded cover crop treatment was planted to cereal rye (*Secale cereale* L.)-hairy vetch (*Vicia villosa* Roth) cover crop mix (50:50 mixtures by seed weight) (Lawson et al., 2012). The three treatments provided contrasts in organic C additions, disturbance (annual vs. perennial) and cover crop management. The plots (6.1 x 15.2 m) were arranged as a randomized complete block with four field replicates for each treatment. The fall cover crop treatment was tilled twice each year (spring and fall) with a rotary spader to a depth of 25 cm. The amendments were added to the plots in spring after the mowing was complete. Soil amendments include C-rich mixed-compost applied at 14-18 Mg ha⁻¹ yr⁻¹ and N-rich broiler litter applied at 4-6 Mg ha⁻¹ yr⁻¹. The mixed-compost was comprised of dairy manure solids, animal bedding (straw and sawdust with compost), yard debris, and small amounts of broiler litter and fish waste. The broiler litter consisted mainly of partially composted manure and softwood shavings. The seasonal N supplied

from both the amendments were comparable, however, the mixed-compost supplied 2-5 times higher C than the broiler litter (Appendix Table A2). The pasture cycle consisted of 30 months of pasture followed by a 6-month vegetable cash crop. The pasture plots consisted of a fall planted mixture of red clover (*Trifolium pretense*), annual ryegrass (*Lolium multiflorum* Lam.) and perennial ryegrass (*Lolium perenne* L.). The pasture plots are grazed by sheep (*Ovis aries*) and poultry (*Gallus gallus domesticus*) on a rotational basis. The pasture rotation treatment has received no amendments (in the form of mixed-compost or broiler litter) since 2005 and was thus a reduced input system. At the time of soil sampling the fall seeded cover crop treatment was in rotation with romaine lettuce (*Lactuca sativa* L.) and the pasture plots were in the 2nd month of the pasture cycle. Detailed information on the IFSYS plots can be found in Appendix Table A3).

Soil sampling

Soils were collected in November of 2013 with push probes (2.5 cm in diameter) from each of the 4 field replicates within a 15 x 6 m area. Seven soil cores were collected to a 10 cm depth and composited from each field plot after the crops were harvested and prior to cover crop planting. The soil bulk density was measured using a hammer driven bulk density core sampler (6 cm deep by 5.4 cm diameter). Field moist soils were passed through 2-mm sieve and soil moisture content was recorded gravimetrically. Sieved soil samples were refrigerated at 4°C for a week prior to incubation. Soil inorganic (NH_4^+ and NO_3^-)-N was extracted from 10 g (field moist soil) with 100 mL of 2 M KCl solution.

Simulated freeze-thaw events

The simulated freeze-thaw conditions imposed during the 39 d laboratory incubation were derived from 10 years of daily average soil temperatures collected at the Puyallup weather station, a part of the AgWeatherNet network maintained by Washington State University

(Appendix Fig. B1). The temperatures maintained during the 39 d incubation included: a pre-freeze maintained at 3°C from time 0 to 13 d with sampling points on days 3 and 13, followed by a 1st freeze at -3°C (duration 4 d) from 13 to 17 d with a sampling point on 17 d, 1st thaw at 4°C (duration 11 d) with sampling points on d 20 and 31 d, a 2nd freeze on 35 d at -2° C (duration 4 d) from 31 to 35 d with a sampling point on 35 d and a 2nd thaw at 4 °C (duration 4 d) from day 35 to 39 with a final sampling point on 39 d.

Microcosm experiments

Two separate but simultaneous 39 d laboratory incubations were conducted with the aim of measuring: 1) N₂O and CO₂ emissions, and 2) soil inorganic (NH₄⁺ + NO₃⁻)-N content and gene copies of nitrifiers (*amoA* genes) and denitrifiers (*nosZ* gene) in soils. For each of the two incubation experiments the total number of samples per time interval was 72 (3 treatments×4 field reps×2 amendments×3 moistures). Only one set of 72 microcosms was required for measuring N₂O and CO₂ emissions in the head space of 72 soil microcosms. In contrast, soil and molecular measurements were destructive requiring a separate set of 72 microcosms for each sampling interval. For this purpose, the equivalent of 50 g (dry wt.) of field moist sieved soils taken from the three organic management systems and their 4 field plot replicates (mixed-compost, broiler litter and pasture; 12 field plots) was weighed into 128 mL sterile specimen vials (diameter 53 mm; height 60 mm), serving as microcosms, and packed to a bulk density of 1.0 g cm³. After the soil microcosms were pre-incubated at 25°C and 30% WFPS for a week, two sets of treatment factors (2 amendments and 3 moisture levels) were added.

One half of the samples (36) were amended with ¹⁵N labelled sugarbeet top residues (1% excess ¹⁵N) and the other half received no N in the form of amendments (control). The sugarbeet tops were harvested at sugar beet maturity but before senescence and air dried at low temperature

(~30 °C) and stored in a sealed plastic bag until use. The material was green and was chopped into a uniform size (1-2.5 cm length) with scissor. Nitrate-N in sugarbeet tops was extracted from 3 replicate 0.25 g plant tissue sample with 15 mL of 2 M KCl solution. The aliquots were run on a SEAL auto analyzer (SEAL Analytical Inc. Mequon, WI) to determine the concentration of NO_3^- -N. The beet residues were applied at a rate of 565 mg per 50 g equivalent dry soil. The beet residues contained 2% of total N and had 21% of the total N was NO_3^- -N. The amendments were mixed with soil to a depth of ~10 mm below the surface of the soil microcosms. Sugar beets were not in the cropping systems but the incorporation of the labeled beet top tissue simulated the effect of tilling residues or cover crops with high N concentrations into a field in late fall. The soil microcosms from each treatment (residue amended (36) and unamended control (36)) were divided into 3 sets of 12 samples and distilled H_2O was added at three rates 40, 60 and 80 % WFPS and run in triplicate.

Soil incubation for N_2O and CO_2 analysis

Each of the 72 soil microcosms were placed in a separate quart canning jar with a 935 cm^3 volume, fitted with a butyl septum to be used as a port for sampling gases Twenty mL of H_2O were placed in the bottom of the canning jars prior to addition of the soil microcosm to be incubated. Jars were sealed to reduce moisture loss and maintain sufficient relative humidity. Three canning jars with 20 mL of water at the bottom but no soil in the incubation vessel served as blanks. Total 72 jars were incubated in a laboratory incubator (Precision™ Low Temperature BOD Refrigerated Incubator, Thermo Fischer Scientific Inc., Waltham, MA) for 39 d while being subjected to simulated freeze-thaw cycles. Thirty mL gas samples were collected from the head space of each canning jar and injected with overpressure into 20 mL vials fitted with a 3 mm PTFE-faced butyl crimp seal (Supelco Analytical, St. Louis, MO). The N_2O and CO_2

samples were taken at time zero and days 3, 13, 17, 20, 31, 35 and 39 using the GRACENet protocol (<http://www.ars.usda.gov/research/GRACENet>). After each gas sampling the headspace of all the canning jars including the blanks was flushed with air (Paul et al., 2001) to avoid anaerobic conditions in the headspace due to the sealing of the jars resulting in a buildup of CO₂ (jars were flushed before CO₂ in the headspace reached 6%), the specimen cups were weighed and the moisture contents were adjusted to 40, 60 or 80% WFPS. The total number of gas samples was 576 (8 time intervals×3 treatments×4 field replicates×2 amendments×3 moistures) + 24 blanks (8 time intervals×3 laboratory rep).

The N₂O and atom % ¹⁵N-N₂O were measured using a ThermoFinniganGasBench + PreCon trace gas concentration system interfaced to a ThermoScientific Delta V Plus isotope-ratio mass spectrometer (Bremen, Germany). Further details of the procedure and working standards can be found in <http://stableisotopefacility.ucdavis.edu/>. The CO₂ samples were run on an Infrared Gas Analyser (IRGA, LI-COR 830). The system is equipped with an infrared ray source, a modulator, an analysis chamber containing the mixture of gases, and a detector together with associated electronic circuits for processing the electric signals delivered by the detector. A set of certified CO₂ (2500, 5,000, 10,000, 20,000 ppm) standards were run. A set of jars containing internal standards and blanks (ambient) were sampled at each time interval. The N₂O and CO₂ readings were converted to μg N₂O kg⁻¹ soil and mg CO₂ kg⁻¹ soil using the ideal gas law equation.

Soil incubation for microbial analysis and inorganic N

For this purpose, soil microcosms were not placed in canning jars but were covered with parafilm and a 0.5 mm hole was placed in the center using a 25-gauge needle, allowing for further gaseous exchange and prevention of rapid moisture loss (Saunders et al., 2012). A total of

504 (7 time intervals×3 treatments×4 field reps×2 amendments×3 moistures) soil microcosm were incubated in a laboratory incubator (Precision™ Low Temperature BOD Refrigerated Incubator, Thermo Fischer Scientific Inc., Waltham, MA) to simulate a typical freeze-thaw cycle (Appendix Fig. B1). Soils were destructively sampled at 7 separate time intervals (3, 13, 17, 20, 31, 35 and 39 d) and stored at -20 °C for inorganic N analysis. Twelve grams of soil from each microcosm (such that it could represent the whole soil microcosm sample) was sub sampled on 13 d (highest N₂O emission event), d 31 (1st freeze-thaw event) and 39 d (2nd freeze-thaw event) for microbial analysis (Saunders et al., 2012). The soils were kept at -80 °C before the genomic DNA was extracted.

Isolation of genomic DNA and quantification of nitrifiers and denitrifiers via quantitative real time PCR

Soil genomic DNA was extracted using a MoBio Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) and stored at -80 °C for qPCR analysis. All three primer sets were synthesized by Invitrogen, Carlsbad, CA, USA. Quantitative Real Time PCR (qPCR) was performed to amplify the ammonia monooxygenase (*amoA*) gene in ammonia oxidizing bacteria (AOB) using forward primer *amoA1F* 5'-GGGGTTTCTACTGGTGGT-3' and reverse primer *amoA2R* 5'-CCCTCKGSAAAGCCTTC TTC-3' (Rotthauwe et al., 1997) and the *amoA* gene in ammonia oxidizing archaea (AOA) using forward primer Arch-*amoAF* 5'-STAATGGTCTGGCTTAGACG-3' and reverse primer Arch-*amoArev* 5'-TTCTTCTTTGTTGCCAGTA-3' (Francis et al., 2005; Wuchter et al., 2006). The forward primer *nosZ1* 5'-WCSYTGTTTCMTCGACAGCCAG-3' and reverse primer *nosZ1R* 5'-ATGTCGATCARCTGVKCRTTYTC-3' were used to amplify the nitrous oxide reductase (*nosZ*) gene in denitrifiers (Henry et al., 2006). All qPCR reactions contained a total volume of 25 µL

that included 10 μ L of SYBR Green master mix (Bio-Rad Laboratories Inc., Hercules, CA), 0.4 μ L of 10 mM forward and reverse primer, 1 μ L of 4 ng of template DNA and brought to volume with RNA/DNA free water (GBiosciences Inc., St. Louis, MO). All samples were run in triplicate in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA). The qPCR thermocycler conditions for quantifying the archaeal *amoA* (AOA) and bacterial *amoA* (AOB) genes were 7 min at 95 °C followed by 40 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s with a data acquisition step, followed by a melt curve analysis (Mao et al., 2011). The standard curve used to determine *amoA* gene copies of nitrifying AOB and AOA were generated using 5 serial dilutions of genomic DNA ranging from 10^3 to 10^7 copy numbers isolated from *Nitrosopumilus maritimus* SCM1 for AOA and from *Nitrosomonas europaea* (ATCC 19718, Manassas, VA, USA) for AOB. The qPCR thermocycler protocol used for quantifying denitrifiers *nosZ* gene copy numbers was 7 min at 95 °C followed by 40 cycles of 95 °C for 45 s, 56 °C for 1 min, and 72 °C for 1 min with a data acquisition step, followed by a melt curve analysis (Mao et al., 2011). The standard curve for the functional gene *nosZ* was generated using 5 serial dilutions of the genomic DNA isolated from *Pseudomonas aeruginosa* PAO-1 (ATCC 47085, Manassas, VA, USA) ranged from 10^3 to 10^7 . An average R^2 value of 0.98-0.99 was obtained from the separate standard curves of known DNA concentrations for all three primer sets.

Statistical analysis

Univariate data were analyzed separately by three-way analysis of variance (ANOVA) in a split block (time)-split plot factorial arrangement in RCBD using the SAS PROC GLM procedure of SAS 9.4 (SAS Institute, Cary, NC) in order to determine the effect of individual treatment and treatment combinations on N_2O , atom % $^{15}N-N_2O$, CO_2 , inorganic N, nitrifier and

denitrifier gene copies. P values <0.05 were considered to be statistically significant. The mean separation was completed using the Fisher LSD post hoc test.

All qPCR data, gene copies g^{-1} dry soil, were log transformed in order to meet the assumptions of non-metric multidimensional scaling (NMDS) analysis performed using PC-Ord (PCORD 6, Gleneden Beach, Oregon) (Kruskal, 1964). The purpose of the log transformation is to normalize, linearize and standardize data to meet the assumptions of a statistical procedure. If the assumptions of a procedure are not met, typically the standard error distribution of a variable is biased. Transforming the data often corrects the skew in the underlying structure of the data set. The use of rank orders in NMDS analysis allows for analysis of data with a distribution that is not multivariate normal. In PC-Ord, the default “slow and thorough” procedure was chosen with a Relative Euclidean distance measure and a random starting point, resulting in 250 runs with real and randomized data. The accuracy of estimated similarities or dissimilarities (distances) between variables is dependent upon the number of comparisons made between variables and the remaining data. A two way PerMANOVA, a nonparametric method based on permutation tests for multivariate analysis of variance (Anderson, 2001), was also run on the multivariate data set to determine treatment differences (WFPS and amendment) and their interactions in PC-Ord (PCORD 6, Gleneden Beach, Oregon). Pairwise comparisons using Bonferonni’s correction were applied to the multivariate data ($P<0.05$) to determine differences due to treatment interactions.

Results

Effects of WFPS, amendment and freeze-thaw cycles on mineralization, nitrification and denitrification

At the start of the incubation experiment baseline soil NH_4^+ -N concentrations were near the detection limit of the instrument. The amount of N released as NH_4^+ -N by the mineralization of sugarbeet tops significantly increased concentrations of NH_4^+ -N during the pre-freeze-thaw period from 3 to 13 d. Amended soil microcosms maintained at 60% (20 mg kg^{-1} dry weight (dw) soil) and 80% (17 mg kg^{-1} dw soil) WFPS contained the highest concentrations of ammonium on 13 d (Fig. 10a). Carbon mineralization from beet amended soil microcosms maintained at all 3 WFPS, estimated via CO_2 evolved, was highest on 13 d. Carbon dioxide evolved from the amended soil microcosms was nearly 10 fold higher (275 mg CO_2 kg^{-1} dw soil) relative to the unamended soil microcosms (19 mg CO_2 kg^{-1} dw soil) (Fig. 10b).

The 1st freeze event at -3 °C began on d 17 of the incubation and reduced NH_4^+ -N concentrations in amended microcosms to 9.3 mg kg^{-1} dw soil at all 3 WFPS. The first thaw event at 4°C from 20 to 31 d of the incubation stimulated N and C mineralization (Figs. 10a and b). As a result, soil NH_4^+ -N concentrations increased at all WFPS but to a greater extent in amended soil microcosms maintained at 60% WFPS (Fig. 10a). By 31 d, soil NH_4^+ -N concentrations decreased in the amended soil microcosms maintained at 60% and 80% WFPS and increased in those maintained at 40% WFPS. The 2nd freeze-thaw event (39 d) had no effect on the NH_4^+ -N concentrations in amended soil microcosms maintained at 80% WFPS but increased the concentrations in soil microcosms maintained at 60% WFPS. Throughout the incubation beet amended soil microcosms maintained at 40% and 60% WFPS tended to have higher NH_4^+ -N concentrations as compared to those maintained at 80% WFPS. Concentrations

of soil $\text{NH}_4^+\text{-N}$ remained constant throughout the incubation in unamended soil microcosms (Fig. 10a).

Baseline soil $\text{NO}_3^-\text{-N}$ concentrations were $\sim 35 \text{ mg kg}^{-1}$ per dw soil for all three organic management systems. The amended soil microcosms had significantly higher $\text{NO}_3^-\text{-N}$ concentrations (77.2 mg kg^{-1} dw soil) as compared to the unamended (35.4 mg kg^{-1} dw soil) on d 3, a time interval during which minimal mineralization and nitrification occurred (Figs. 10a, 10b and 10c). Significant loss of $\text{NO}_3^-\text{-N}$ (67.1 mg kg^{-1} dw soil) from amended soil microcosms maintained at 80% WFPS was observed between 3 and 13 d prior to significant mineralization and nitrification occurring after 20 d. Throughout the incubation (Fig. 10c), soil $\text{NO}_3^-\text{-N}$ concentrations in amended microcosms maintained at 40% WFPS (44.5 mg kg^{-1} dw soil) and 60% WFPS (47.3 mg kg^{-1} dw soil) were significantly higher (\sim double) relative to amended soil microcosms maintained at 80% WFPS (21.8 mg kg^{-1} dw soil). My results suggest that at 80% WFPS nitrification was inhibited and 60% WFPS favored the nitrification process relative to 40% WFPS ($P < 0.0001$).

The amended soil microcosms maintained at 80% WFPS emitted greater N_2O relative to all other treatments (Fig. 10d). The N_2O emissions from the amended soil microcosms maintained at 80% WFPS peaked on 13 d ($3070 \mu\text{g kg}^{-1}$ dw soil), dropped ($330 \mu\text{g kg}^{-1}$ dw soil) during the first freeze at -3°C on 17 d, increased during the thaw at 4°C on 21 d ($1072 \mu\text{g kg}^{-1}$ dw soil) and increased to a lesser amount on 31 d ($645 \mu\text{g kg}^{-1}$ dw soil). However, the 2nd freeze at -2°C on 35 d and thaw event at 4°C on 39 d did not show any significant change in the N_2O emissions from amended or unamended soil microcosms irrespective of the moisture contents.

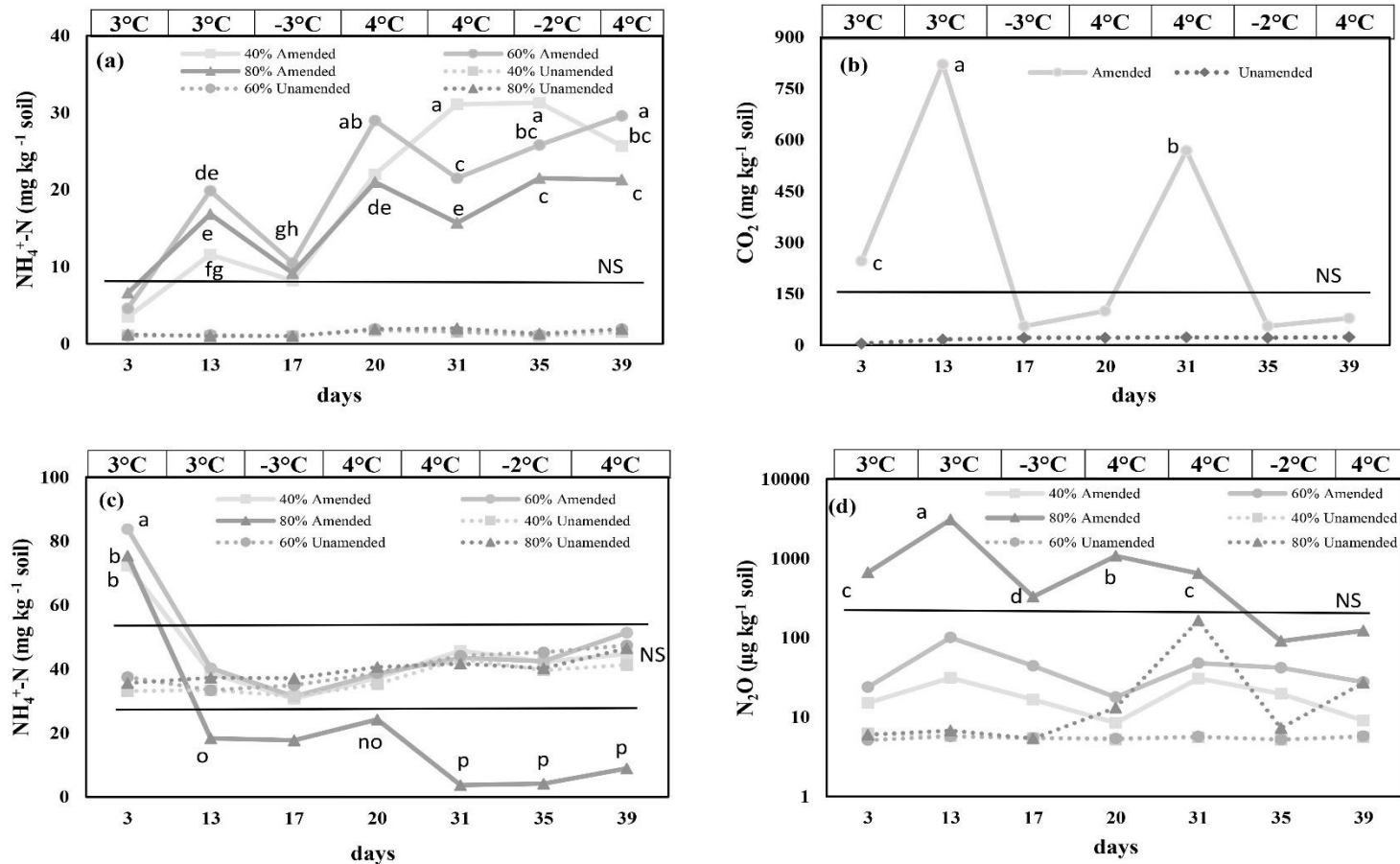


Fig. 10. Variation in reactive nitrogen species and C availability during a 39-day incubation subjected to simulated freeze-thaw events in sugar beet amended and unamended soil microcosms maintained at 40%, 60% and 80% water filled pore space (WFPS). Panels a, c and d show changes in reactive nitrogen species (ammonium, nitrate and nitrous oxide) panel b represents C availability estimated by the amount of carbon dioxide evolved. Statistical significant differences (determined via univariate three-way ANOVA analyses in PROC GLM, *post hoc*: Fischer LSD) are indicated by different letters within each panel. Data points below a solid black line or between two solid black lines are not statistically significant (NS) from each other ($P < 0.05$) within a given panel.

Higher atom % ^{15}N enrichment of the N_2O in the amended soil microcosms maintained at 80% WFPS was observed throughout the incubation experiment (Appendix Table B1). In contrast, amended soil microcosms maintained at 40 and 60% WFPS had significantly lower atom % ^{15}N enrichment of N_2O , particularly after the freeze-thaw events (Appendix Table B1). Soil NO_3^- -N concentrations, a by-product of nitrification, were higher in the amended soil microcosms maintained at 60% and 40% of WFPS relative to amended treatments maintained at 80% WFPS irrespective of prior organic management (Table 7). Amended soil microcosms maintained at 80% WFPS that had previously received long-term mixed-compost applications produced approximately half the N_2O of soils that had received broiler litter or were in pasture management. A similar trend was also observed for N_2O production which was lower in the amended soil microcosms maintained at 80% WFPS in the mixed-compost ($612 \mu\text{g kg}^{-1} \text{ dw soil}$) treatment as compared to broiler litter ($1080 \mu\text{g kg}^{-1} \text{ dw soil}$) and pasture ($877 \mu\text{g kg}^{-1} \text{ dw soil}$) treatments (Table 7).

Table 7. Shifts in the reactive N species in soil microcosms from compost, broiler litter and pasture treatments maintained at 40%, 60% and 80% water filled pore space (WFPS).

Treatment	Compost			Broiler litter			Pasture		
	WFPS								
	40%	60%	80%	40%	60%	80%	40%	60%	80%
	NO₃-N[†]								
	mg kg ⁻¹ soil								
Amended	44.4 bcd	50.2 a	13.1 i	42.6 bcde	43.3 bcd	27.6 h	46.7 abc	48.6 ab	24.5 h
Unamended	41.1 def	44.9 abcd	43.3 bcd	34.0 g	37.2 fg	38.1 efg	35.2 g	38.5 efg	38.2 efg
	N₂O								
	μg kg ⁻¹ soil [‡]								
Amended	14.1 D	41.4 D	612 C	24.3 D	43.3 D	1080 A	17.7 D	45.5 D	877 B
Unamended	5.78 D	5.46 D	79.5 D	5.48 D	5.47 D	6.97 D	5.43 D	5.49 D	12.3 D

[†] Statistical significant differences (univariate three-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different lower case letters for soil nitrate-N; Organic management × WFPS × Amendment, *P*<0.05.

[‡] Statistical significant differences (univariate three-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different upper case letters for soil nitrous oxide (N₂O); Organic management × WFPS × Amendment, *P*<0.05.

Copy numbers of Archaeal (AOA) and bacterial nitrifier (AOB) *amoA* genes and the denitrifier *nosZ* genes

Denitrifier copy numbers estimated via copies of the *nosZ* gene ranged from 9.3×10^5 to 6.1×10^5 gene copies g^{-1} dw soil from the beginning to the end of the 39 d incubation. The *nosZ* gene copy numbers were not significantly affected by freeze-thaw events, previous organic field treatments, WFPS or amendment addition. However, denitrifier copy numbers were 1 to 2 orders of magnitude higher than bacterial and archaeal nitrifier *amoA* gene copies.

In contrast, nitrifier AOB and AOA *amoA* gene copies were affected by temperature and other variables (Table 8, Figs. 11a and 11b). Bacterial nitrifiers increased significantly after the two freeze-thaw cycles across all WFPS and organic treatment (Table 8). Bacterial nitrifier gene copies were highest in soil taken from broiler litter and pasture treatments. Addition of residues to the soil microcosms did not affect *amoA* gene copies of bacterial nitrifiers.

Table 8. Abundance of *amoA* ammonia oxidizing archaea (AOA) and *amoA* ammonia oxidizing bacteria (AOB) *amoA* gene copy numbers in soil microcosms from compost, broiler litter and pasture treatments maintained at 40%, 60% and 80% water filled pore space (WFPS) during selected time intervals in the incubation.

Nitrifier type	Pre-freeze [†]			1 st Freeze-thaw			2 nd Freeze- thaw		
	WFPS								
	40%	60%	80%	40%	60%	80%	40%	60%	80%
Compost									
gene copy numbers g ⁻¹ soil									
AOA [‡]	1.9 × 10 ⁵ C			1.9 × 10 ⁴ DE			6.8 × 10 ³ E		
AOB [§]	6.1 × 10 ³ k	6.9 × 10 ³ jk	6.8 × 10 ³ jk	1.1 × 10 ⁴ hij	1.3 × 10 ⁴ ghi	1.3 × 10 ⁴ ghi	1.4 × 10 ⁴ fghi	1.3 × 10 ⁴ ghi	1.7 × 10 ⁴ cdefg
Broiler litter									
AOA	3.7 × 10 ⁵ A			5.5 × 10 ⁴ D			1.4 × 10 ⁴ E		
AOB	7.8 × 10 ³ jk	1.0 × 10 ⁴ ijk	1.3 × 10 ⁴ ghi	1.9 × 10 ⁴ bcd	1.8 × 10 ⁴ bcde	2.2 × 10 ⁴ abc	2.5 × 10 ⁴ a	2.3 × 10 ⁴ ab	1.6 × 10 ⁴ defg
Pasture									
AOA	2.6 × 10 ⁵ B			3.4 × 10 ⁴ DE			1.3 × 10 ⁴ E		
AOB	8.2 × 10 ³ jk	1.1 × 10 ⁴ hij	1.1 × 10 ⁴ hij	1.6 × 10 ⁴ defgh	1.9 × 10 ⁴ bcd	1.8 × 10 ⁴ cdef	1.8 × 10 ⁴ cdef	1.9 × 10 ⁴ bcd	1.5 × 10 ⁴ efghi

[†] Pre-freeze- sampling time point 13 d at 3 °C; 1st Freeze-thaw- sampling time point 31 d (freeze at -3 °C, thaw 4 °C); 2nd Freeze-thaw- sampling time point d 39 (freeze at -2 °C, thaw 4 °C).

[‡] Statistical significant differences (univariate three-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different upper case letters for AOA; Sampling time point × WFPS × organic management, *P*<0.05.

[§] Statistical significant differences (univariate three-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different lower case letters for AOB; Sampling time point × WFPS × organic management, *P*<0.05.

Prior to the first freeze-thaw (13 d) *amoA* AOA abundance was highest in soil microcosms taken from the broiler litter treatment (3.7×10^5 gene copies g^{-1} dw soil) followed by pasture (2.6×10^5 gene copies g^{-1} dw soil) and mixed-compost (1.9×10^5 gene copies g^{-1} dw soil) treatments (Table 8). On d 13 the soil microcosms from the mixed-compost treatment had 2 orders of magnitude greater archaeal nitrifiers (1.9×10^5 gene copies g^{-1} dw soil) relative to bacterial nitrifiers (6.6×10^3 gene copies g^{-1} dw soil). In contrast, the AOA *amoA* gene copies on 13 d were only 1 order of magnitude greater relative to those of AOB in soil microcosms taken from broiler litter and pasture treatments. The archaeal *amoA* gene copies in soil microcosms from all three organic treatments decreased by one order of magnitude from 13 to 31 d during the 1st freeze-thaw event and remained significantly lower than the pre-freeze samples through the end of the incubation (39 d). This decrease in AOA *amoA* gene copies and simultaneous increase in the AOB *amoA* gene copies was more distinct after the 1st as compared to the 2nd freeze-thaw event (Table 8).

Archaeal *amoA* gene copies were significantly higher in the unamended microcosms in soil taken from broiler litter (2×10^5 gene copies g^{-1} dw soil) as compared to pasture (1.4×10^5 gene copies g^{-1} dw soil) and mixed-compost (9.4×10^4 gene copies g^{-1} dw soil) treatments (Fig. 11a). Archaeal *amoA* gene copies were highest in the unamended soil microcosms (1.4×10^5 gene copies g^{-1} dw soil) relative to the amended soil microcosms (7×10^4 gene copies g^{-1} dw soil) (Fig. 11a) which corresponded to lower concentrations of soil NH_4^+ -N in unamended relative to amended soil microcosms (Fig. 10a).

Soil WFPS did not have a significant effect on the archaeal nitrifier copy numbers on either amended or unamended soil microcosms after 13 d (the end of the pre-freeze). Gene copies of archaeal *amoA* were not significantly different in amended and unamended soils

maintained at 40% WFPS (Fig. 11b). In contrast, unamended soils maintained at 60 and 80% WFPS contained higher *amoA* gene copies than amended soils of the same WFPS (Fig. 11b). The abundance of AOA (*amoA* gene) was highest on 13 d in unamended soil microcosms maintained at 80% WFPS (4.1×10^5 gene copies g^{-1} dw soil) as compared to 3.5×10^5 gene copies g^{-1} dw soil at 60% WFPS and 3×10^5 gene copies g^{-1} dw soil at 40% WFPS. The higher abundance of nitrifier archaea in amended and unamended soil microcosm on 13 d corresponded well with low concentrations of $\text{NH}_4^+\text{-N}$ at the end of the pre-freeze. This suggests that the *amoA* AOA organisms have high affinity towards $\text{NH}_4^+\text{-N}$ substrate at low $\text{NH}_4^+\text{-N}$ concentrations.

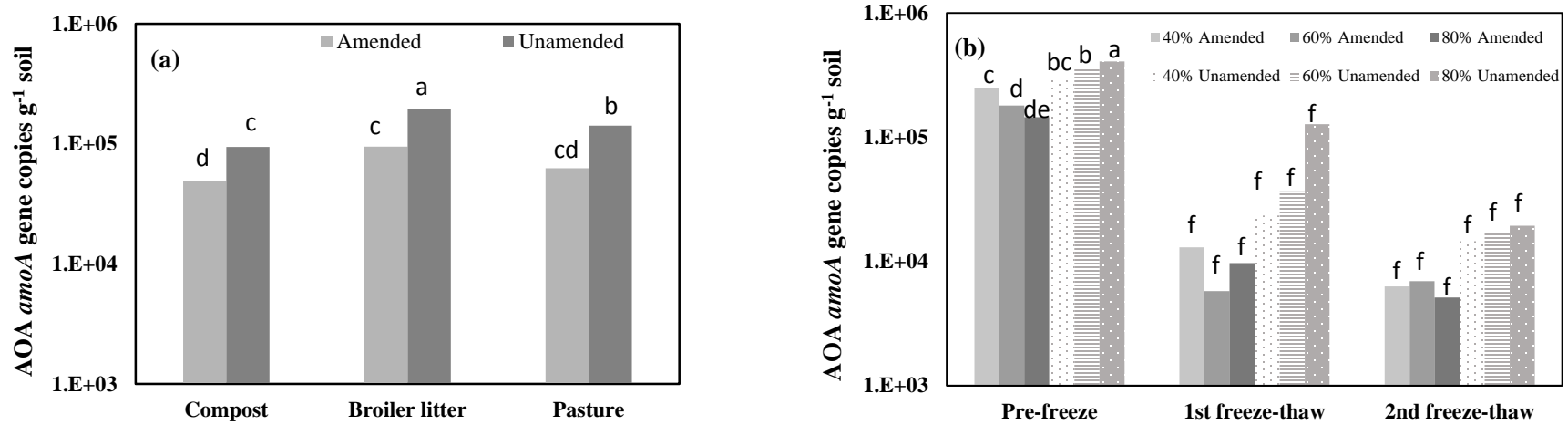


Fig. 11. Variations in archaeal *amoA* gene copies per gram dry soil equivalent in amended and unamended soil microcosms taken from (a) compost, broiler litter and pasture treatments and (b) maintained at 40%, 60% and 80% water filled pore space (WFPS) at selective time intervals representative of freeze thaw events during the 39-day incubation. Statistical significant differences (univariate two-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different letters. In panel b, the increase in the intensity of darkness in the bars represent increasing WFPS.

Effect of the freeze-thaw and multivariate treatments on the soil properties

Distinct clustering patterns representing WFPS and amendment were observed for the pre-freeze (13 d), 1st freeze-thaw (31 d) and the 2nd freeze-thaw (d 39) (Figs. 12, 13 and 14) using NMDS. The ordination analysis reduced the variation explained by each of the three data sets to two axes. The two dimensional solution (selected axes) explained the majority of the variation in the data and was significantly different from random ($p < 0.05$). Interpretations were based upon distances among data points, points that are closer together are more similar whereas points that are further apart are dissimilar. An absolute r value of 0.5 or above was used as the criteria to determine the measurements to be used as explanatory variables for the structure of the ordination. The final stress for the two dimensional solutions ranged from 1.1 to 4.2 and an instability of 0.1×10^{-5} was reached.

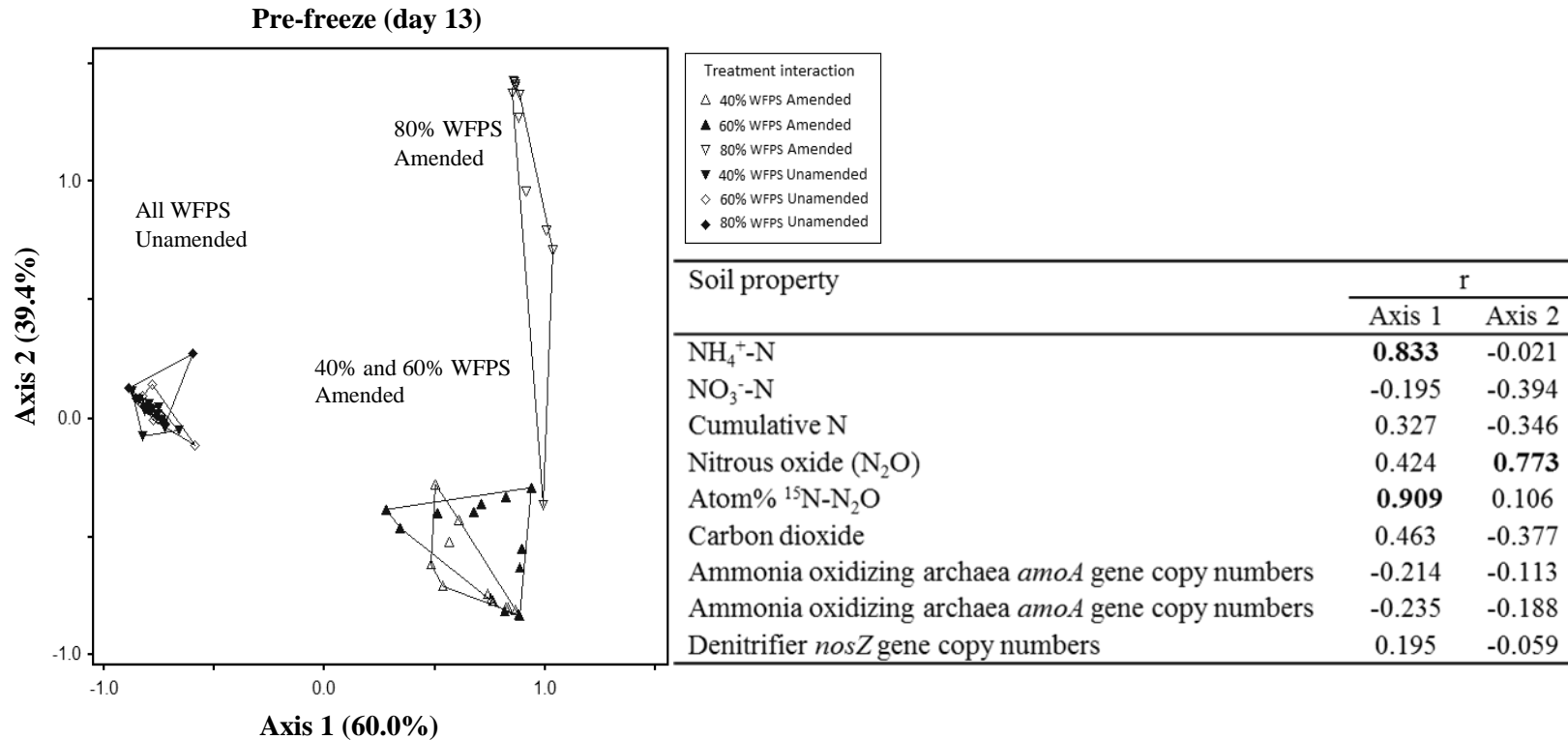


Fig. 12. Non-metric multidimensional scaling analysis (NMS) of amendment and water filled pore space (WFPS) effects on reactive N species, C mineralization and archaeal *amoA*, bacterial *amoA* and *nosZ* gene copies at the end of the pre-freeze (13 d; 3 °C). The percent of variation explained by the 2 NMS axes are represented by the proximity of the measured variables to each of the 2 axes. The chart below represents the correlation coefficient (r) and correlation results between individual variables and each NMS axis. A two-way PerMANOVA, pairwise comparison using Bonferroni correction ($P < 0.0002$) was performed to determine which interactions between WFPS and amendment were significant. The shape of the symbols and color coding represent all possible 2 way interactions between the WFPS and the addition/no addition of amendment treatments.

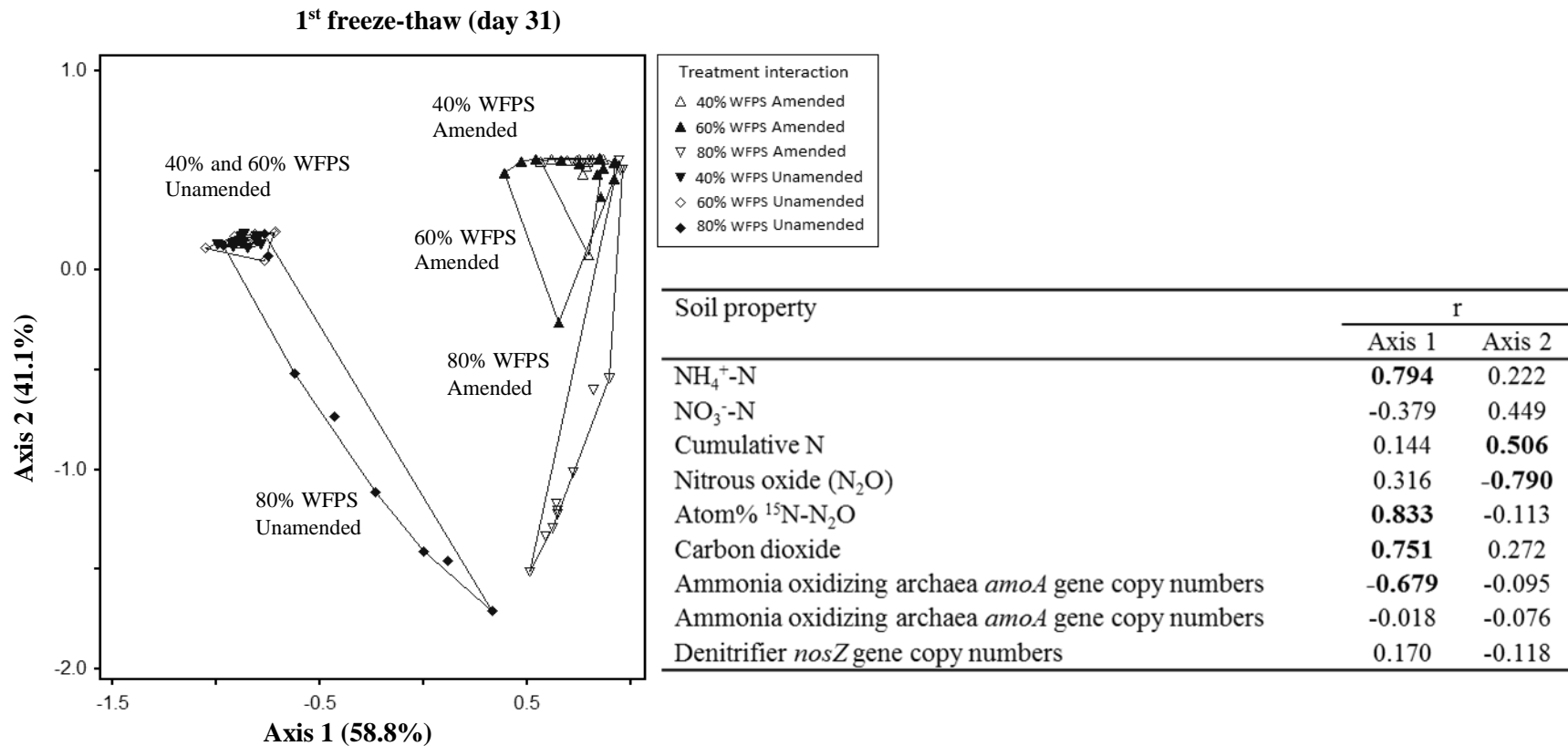


Fig. 13. Non-metric multidimensional scaling analysis (NMS) of amendment and water filled pore space (WFPS) effects on reactive N species, C mineralization and archaeal *amoA*, bacterial *amoA* and *nosZ* gene copies at the end of the 1st freeze-thaw (4 d freeze -3 °C, 14 d thaw 4 °C) on day 31. The percent of variation explained by the 2 NMS axes are represented by the proximity of the measured variables to each of the 2 axes. The chart below represents the correlation coefficient (r) and correlation results between individual variables and each NMS axis.

A two-way PerMANOVA, pairwise comparison using Bonferroni correction ($P < 0.0002$) was performed to determine which interactions between WFPS and amendment were significant. The shape of the symbols and color coding represent all possible 2 way interactions between the WFPS and the addition/no addition of amendment treatments.

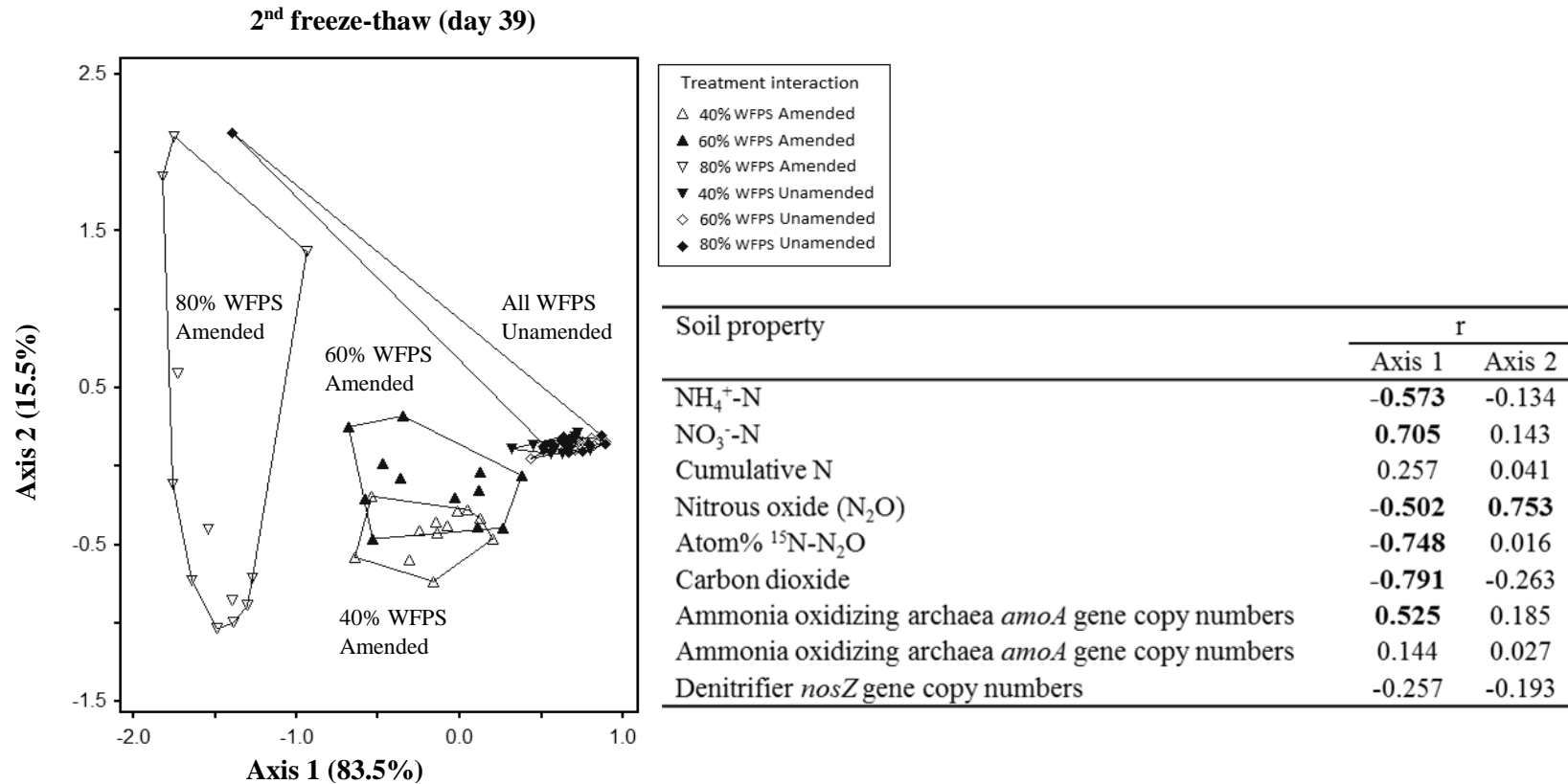


Fig. 14. Non-metric multidimensional scaling analysis (NMS) of amendment and water filled pore space (WFPS) effects on reactive N species, C mineralization and archaeal *amoA*, bacterial *amoA* and *nosZ* gene copies at the end of the 2nd freeze-thaw (4 d freeze -2 °C, 4 d thaw 4 °C) on day 39. The percent of variation explained by the 2 NMS axes are represented by the proximity of the measured variables to each of the 2 axes. The chart below represents the correlation coefficient (r) and correlation results between individual variables and each NMS axis.

A two-way PerMANOVA, pairwise comparison using Bonferroni correction ($P < 0.0002$) was performed to determine which interactions between WFPS and amendment were significant. The shape of the symbols and color coding represent all possible 2 way interactions between the WFPS and the addition/no addition of amendment treatments.

About 99.4% of the variability was explained via axis 1 (60.0%) and axis 2 (39.4%) on d 13 (pre-freeze event) (Fig. 12). Soil $\text{NH}_4^+\text{-N}$ and atom% $^{15}\text{N-N}_2\text{O}$ were positively correlated with axis 1 whereas the N_2O was positively correlated with axis 2. Non-parametric PerMANOVA analysis for the d 13 data set indicated that amended soil microcosms maintained at 80% WFPS grouped separately from all other WFPS*amendment combinations ($P=0.0002$). No significant differences were observed between the amended soil microcosms maintained at 40% or 60% WFPS or among the unamended soil microcosms maintained at 40%, 60% or 80% WFPS.

On 31 d (1st freeze-thaw) 99.9% of the variation was explained by axis 1 (58.8%) and axis 2 (41.1%) (Fig. 13). Axis 1 was positively correlated with soil $\text{NH}_4^+\text{-N}$, atom% $^{15}\text{N-N}_2\text{O}$, CO_2 and negatively correlated with ammonia oxidizing archaea *amoA* gene copy numbers whereas axis 2 was positively correlated with cumulative soil inorganic N and negatively correlated with N_2O . The PerMANOVA analysis revealed that amended soil microcosms maintained at 80% WFPS were significantly different from the amended microcosms at 40% and 60% WFPS ($P=0.0002$). Unamended soil microcosms maintained at 80% WFPS were also grouped differently from unamended microcosms at 40% and 60% WFPS.

On 39 d (2nd freeze-thaw) 99% of the variation was explained by axis 1 (83.5%) and axis 2 (15.5%) (Fig. 14). Axis 1 was negatively correlated with soil $\text{NH}_4^+\text{-N}$, N_2O , atom% $^{15}\text{N-N}_2\text{O}$, CO_2 and positively correlated with soil $\text{NO}_3^-\text{-N}$ and ammonia oxidizing archaea *amoA* gene copies. Axis 2 was positively correlated with N_2O . Significant differences among treatments were observed via PerMANOVA analysis between the amended soil microcosms maintained at 40%, 60% and 80% WFPS ($P=0.0002$). The unamended soil microcosms maintained at 80% WFPS grouped separately from the unamended soil microcosms maintained 40% and 60% WFPS ($P=0.0002$).

Effect of long-term organic treatments and short-term effects (WFPS, amendments and freeze-thaw cycles) on global warming potential

Although not statistically significant, the cumulative N₂O emissions from the unamended soil microcosms maintained at 80% WFPS taken from the mixed-compost treatment was 10 fold higher (556 μg kg⁻¹ dw soil) relative to pasture (85.9 μg kg⁻¹ dw soil) and broiler litter (48.8 μg kg⁻¹ dw soil) treatments (Table 9). In contrast after addition of sugar beet top residues to the soil microcosms, the cumulative N₂O emissions from the broiler litter (7561 μg kg⁻¹ dw soil) and the pasture (6138 μg kg⁻¹ dw soil) treatments were significantly higher as compared to the mixed-compost (4287 μg kg⁻¹ dw soil) treatments. About 1.1% of the total N supplied by the residues was lost to N₂O-N in the microcosms with the soil taken from the mixed-compost treatment as compared to 2% and 1.6 % in the soil microcosms taken from broiler litter and pasture treatments, respectively. Significantly greater NO₃⁻ originating from sugarbeet residues was transformed into N₂O in the microcosms with soil taken from the broiler litter (9.7%) and pasture treatments (7.8%) relative to the mixed-compost treatment (5.5%). Addition of amendment and type of prior organic management had a significant effect on global warming potential (GWP) estimates (cumulative CO₂ + cumulative N₂O) resulting from the simulated freeze-thaw. The total GWP was significantly higher in the amended soil microcosms as compared to the unamended soil microcosms. Although the total GWP was similar for the amended microcosms from mixed-compost, broiler litter and pasture treatments, the contribution of N₂O to the GWP from the broiler litter and pasture systems was nearly double that of the mixed-compost amended systems. No significant difference in the amended systems were observed with respect to the contribution of CO₂ to the total GWP from simulated freeze-thaw.

Table 9. Amounts of N added by residues as total N and NO₃⁻-N, nitrous oxide (N₂O) emissions, N lost as a % of total N and NO₃⁻-N and contribution of carbon dioxide (CO₂) and nitrous oxide (N₂O) to the simulated winter freeze-thaw global warming potential (GWP) from the compost, broiler litter and pasture treatments.

Management	N added by residues [†]		Cumulative N ₂ O				GWP		
	Total N	NO ₃ -N	N ₂ O	N ₂ O-N	Total residue N	Residue NO ₃ -N	N ₂ O	CO ₂	Total GWP
	mg kg ⁻¹	mg kg ⁻¹	µg kg ⁻¹	mg kg ⁻¹	%	%	kg CO ₂ eq ha ⁻¹		
	Amended[‡]								
Compost	226	47	4287 b	2.6 b	1.1 b	5.5 b	290 b	352 a	642 a
Broiler litter	226	47	7561 a	4.5 a	2.0 a	9.7 a	511 a	210 a	721 a
Pasture	226	47	6138 a	3.7 a	1.6 ab	7.8 ab	415 a	264 a	679 a
	Unamended								
Compost	-	52§	556 c	0.33 c	-	0.63 c	37.6 c	31.2 b	68.8 b
Broiler litter	-	46	48.8 c	0.03 c	-	0.06 c	3.3 c	30.4 b	33.7 b
Pasture	-	41	85.9 c	0.05 c	-	0.12 c	5.8 c	29.9 b	35.7 b

[†] Total N and nitrate N in sugar beet top residues.

[‡] Statistical significant differences (univariate two-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different lower case letters within each column; Amendment × organic management interaction (P<0.05).

§ Soil NO₃-N in case of unamended soil microcosms.

Discussion

Nitrogen cycling and reactive N species in organic management systems

Mineralization, nitrification and denitrification are stimulated when residues are returned to the field or at times of high turnover of plant material and senescence in perennial systems and natural prairie plantings (Cookson et al., 2002; Fortuna et al., 2003b). Shifts in soil moisture, available C and nitrates have also been shown to modify rates of nitrification, denitrification and N₂O emissions (Ginting et al., 2003; Mendum et al., 1999; Wallenstein et al., 2006). On day 3 of this incubation higher NO₃⁻-N in the amended soil microcosms was likely due to the leaching of NO₃⁻ from sugar beet residue tops (47 mg kg⁻¹ dw soil). High initial NO₃⁻-N and low NH₄⁺-N concentrations in the amended soil microcosms were the result of negligible mineralization of residues and nitrification on day 3 at a temperature as low as 3° C. The limited ¹⁵N fractionation in N₂O suggests that the primary source of NO₃⁻ for N₂O production was nitrate leached from sugar beet residue and native soil NO₃⁻. My data suggests that denitrification was the primary process from which N₂O was produced in the amended soil microcosms maintained at 80% WFPS. The addition of sugarbeet residues to the microcosms in this laboratory study simulated addition of legumes or cover crops in organically managed soils during late fall or winter when soil temperatures are as low as 3° C and precipitation leads to excess soil moisture, 80% WFPS. This study indicates that NO₃⁻ in residue additions may be lost to denitrification at temperatures low enough to inhibit nitrification if sufficient organic C and saturated soil conditions exist. The control microcosms contained sufficient NO₃⁻ to stimulate denitrification but the resulting conditions did not produce significant amounts of N₂O even at 80% WFPS. My results and those of others indicate that labile C as well as O₂ concentrations and not soil NO₃⁻ limited denitrification in these organic management systems (Bhowmik et al., 2016; Saunders et al.,

2012). Mitchell et al. (2013) reported higher N₂O emissions from agricultural soils fertilized with inorganic N and planted to a winter cover crop of hairy vetch. Their study showed that increases in mineralizable C resulting from the cover crop rather than increases in NO₃⁻ from N fertilization stimulated N₂O emissions.

The decline in the NO₃⁻-N concentrations from 3 to 13 d in the sugar beet top residue amended soil microcosms may in part be the result of immobilization of inorganic N by microorganisms in addition to loss via denitrification. Others have shown that cover crops have the potential to reduce inorganic N in soil via immobilization during residue decomposition (Thorup-Kristensen et al., 2003). My findings support those of others who have also shown that long-term applications of organic amendments have the potential to augment pools of soil C and N such that available reactive nitrogen species (NH₄⁺ and NO₃) are temporarily immobilized thereby reducing potential losses of inorganic N to denitrification and leaching (Bhowmik et al., 2016; Fortuna et al., 2003a Vigil and Kissel, 1991). Recent studies by Rudisill et al. (2016) have reported that application of partially composted animal and green manure to soils in an intensively managed pepper crop increased potential nitrification activity relative to soils receiving urea or unfertilized at 25° C.

Addition of sugarbeet top residues to soil microcosms in this experiment did not stimulate nitrification. This incubation simulated freeze-thaw cycles at temperatures well below optimum (25° C) for bacterial and ammonia oxidizer activity and the incubated soils contained lower total soil N and larger pools of C that cycled more slowly allowing for immobilization of inorganic N leached or mineralized from residue additions (Bhowmik et al., 2016). My data suggests that there was some nitrification in the amended soil microcosms maintained at 40% and 60% WFPS which was supported by the depletion in ¹⁵N- N₂O that was mainly due to the

dilution of ^{15}N labeled NO_3^- with that of the unlabeled baseline soil NO_3^- and to a lesser degree possibly due fractionation effect associated with nitrification (Yoshida, 1988) (Appendix Table B1). Higher NH_4^+ -N concentrations along with higher CO_2 evolution on 13 d indicate mineralization of the residues did not lead to increased nitrate concentrations. The lower NO_3^- -N concentrations in the amended soil microcosms maintained at 80% WFPS may be due to the loss of NO_3^- -N through the process of denitrification and a reduction in nitrification rates resulting from lowered temperature and O_2 concentrations.

The 1st freeze event at -3°C on 17 d decreased microbial activity which reduced the processes of mineralization, nitrification and denitrification resulting in decreased production of NH_4^+ , NO_3^- , CO_2 , and N_2O in amended soil microcosms maintained at 40 and 60% WFPS. In contrast, continued emission of N_2O from soils maintained at temperatures below freezing for 4 days at 80% WFPS may be due to the activity of denitrifiers in soil water (H_2O) that may have been only partially frozen. Soil pore spaces containing H_2O retain less O_2 due to the lowered diffusion rate of O_2 in H_2O relative to air. Water filled pores could also remain at higher temperatures relative to air filled pore spaces due to the higher thermal conductivity of H_2O relative to air given the short duration of the freezes that persist for only a few days (Teepe et al., 2004). Freeze-thaw cycles often augment the supply of organic C for microbial processes as temperatures below freezing cause the lysing of cells and disintegration of soil aggregates that result in a burst of mineralization upon soil thawing (Zhao et al., 2010). Similar mechanisms that explain increases in N_2O after freeze-thaw events include freeze-thaw release of C from soil aggregates (Matzner and Borken, 2008; Schimel and Mikan, 2005), cold temperature death of microbial biomass followed by release of soluble C that is utilized by cold tolerant heterotrophic denitrifiers (Matzner and Borken, 2008; Schimel and Mikan, 2005), water phase changes that

regulate O₂ concentrations (Smith et al., 2010; Yanai et al., 2004), and diffusion of previously respired N₂O from broken aggregates (Phillips, 2008). My results show that the increase in the frequency of the freezing and thawing events has no effect on the C and N mineralization or denitrification. This is likely due to the exhaustion of C substrate available for the microorganisms with increasing numbers of freeze-thaw cycles as NO₃⁻ concentrations were not limited (Yanai et al., 2004).

The multivariate analysis confirmed that the factors controlling reactive N species and nitrifier and denitrifier gene copies were WFPS and sugar beet top residue addition. This indicates that seasonal or short-term effects of WFPS, temperature and amendment at a given interval of the incubation had a greater influence on N cycling in soil relative to long-term organic management. However, differences in long-term management, application of mixed-compost vs. broiler litter and annual vs. perennial organic systems, determined the size and turnover rate of soil N and C pools affecting the processes of nitrification and denitrification.

The amount and biochemical characteristics of added organic amendments are reported to partially control N cycling processes such as mineralization, nitrification and losses of reactive N from soil systems (Kunlanit et al., 2014; Rasche and Cadisch, 2013). The seasonal N supplied from both animal manure treatments were comparable and the pool of potentially mineralizable soil N in the mixed-compost treatment (102 mg kg⁻¹) differed only slightly from that of the broiler litter (87 mg kg⁻¹) and pasture (86 mg kg⁻¹) treatments (Cogger et al., 2016). In contrast, pools of soil C were significantly different among the three organic management systems. The size of the slow pool of C was greater in the organic systems treatments receiving long-term mixed-compost applications relative to those receiving broiler litter applications or under pasture management (Bhowmik et al., 2016; Cogger et al., 2016). The increase in slow pool C that

occurred in all long-term organic systems resulted in the temporary immobilization of reactive N species present in the soil.

Therefore, lowered NO_3^- availability in amended soil microcosms taken from organic vegetable systems receiving mixed-compost applications had the potential to reduce inorganic N by immobilization leading to lower reactive N concentrations (Bhowmik et al., 2016; Fortuna et al., 2003a). In particular, reactive species of N released from sugarbeet residue additions (used to mimic seasonal C and N inputs from green manures) were immobilized reducing the potential for denitrification. The greater humic fraction, low labile N concentrations and wider C to N ratios in compost favored immobilization of available NO_3^- and NH_4^+ (Cogger et al., 2016; Fortuna et al., 2003a). Higher NO_3^- -N concentrations coupled with low N_2O emissions in the amended and unamended soils taken from mixed-compost treatments maintained at 60% WFPS supports the concept that these systems have the potential to supply adequate N for plant growth while providing environmental services by reducing greenhouse gas emissions.

Broiler litter treatments contained smaller pools of slow pool C and sufficient inorganic N which may have contributed to higher N_2O emissions (Pelster et al., 2012). The presence of higher NO_3^- and low O_2 concentrations suppressed the reduction of N_2O into N_2 during the denitrification process. Such conditions increase the ratio of $\text{N}_2\text{O}:\text{N}_2$ produced during the denitrification process. However, immobilization of the nitrates reduces the $\text{N}_2\text{O}:\text{N}_2$ production ratio. This may be the cause of lower N_2O emissions from the mixed-compost treatment irrespective of additions of sugarbeet residues containing high nitrates.

Denitrifiers and nitrifier communities

Addition of amendments and crop inputs have been shown to support larger numbers of nitrifiers and denitrifiers (Kravchenko et al., 2002). Organic treatments in this study had high

denitrifier *nosZ* abundance. The *nosZ* gene encoding the N₂O reductase enzyme which catalyzes the final step in the denitrification process was stable throughout the incubation and was not affected by treatments. However, long-term application of animal manures and reduced disturbance could increase the *nosZ* denitrifier gene copies due to increases in organic carbon content, an important factor used to explain variations in denitrifier abundance (Cui et al., 2016; Tatti et al., 2014). The *nosZ* gene copy numbers did not vary due to freeze-thaw events in this study, thus, supporting others findings that the *nosZ* gene copies in denitrifiers are more resilient to the adverse effects of freeze-thaw conditions. In a recent study evaluating the effect of tillage on the denitrifier community abundance, Tatti et al. (2015) found that the denitrifier population was constant and did not change with prolonged sub-zero winter temperatures. Other researchers have shown that cold sensitive denitrifier species can be replaced by cold resistant species fairly quickly (Grogan et al., 2004; Sharma et al., 2006). During an incubation experiment, Wertz et al. (2013) observed shifts in the denitrifier community structure in frozen and unfrozen soils within two days. In general, denitrifiers are more tolerant to subzero temperatures and are more active in anaerobic microsites relative to total soil microbial biomass and bacterial nitrifiers (facultative aerobes) (Avrahami and Conrad, 2003; Sharma et al., 2006; Szukics et al., 2010).

Some studies have suggested that nitrifying archaea are more tolerant to cooler temperatures than nitrifying bacteria while other experiments suggest nitrifying bacteria dominate in cooler regions (Sahan and Muyzer, 2008). The number of ammonia monooxygenase operon per genome in AOA is comparable to AOB that range between 1-3 copies per genome (Norton et al., 2002). However, there are additional factors that regulate the population of archaeal and bacterial nitrifiers in agricultural soils. Land-use and management history select for different ratios of AOA to AOB and influence the size of the nitrifier communities (Giguere et

al., 2015; Lehtovirta et al., 2009; Taylor et al., 2010; Webster et al., 2005). Overall higher archaeal *amoA* nitrifier gene copies in the unamended soil microcosms as compared to the amended soil microcosms during the pre-freeze (13 d, 3 °C) was observed. This might be related to higher NH₄⁺-N concentrations on 13 d in the amended soil microcosms due the mineralization process. Lower ammonium concentrations in the unamended soils provided a favorable niche for the archaeal nitrifiers. Also long-term application of organic amendments may have facilitated the growth of mixotrophic archaeal nitrifiers (Habteselassie et al., 2013). However, in a recent study by Ouyang et al. (2016), the AOA abundance fluctuated but did not respond to organic N fertilizers as compared to the AOB populations. Research conducted by Taylor et al. (2010) found higher AOA abundance in N limited grassland systems whereas AOB population dominated in agronomic systems. The archaeal nitrifiers have very high substrate affinity and higher concentrations of ammonium inhibit the archaeal nitrification process (Martens-Habbena and Stahl, 2011). Higher numbers of archaeal nitrifier gene copies than bacterial nitrifier gene copies in the soil microcosms were found during the pre-freeze at 3 °C in the unamended soils taken from mixed-compost, broiler litter and pasture treatments. Addition of organic amendments such as swine (*Sus scrofa domesticus*) manure have been reported to stimulate the AOB population with little or no effect on the AOA population (Schauss et al., 2009). Long-term (24 yr) application of organic manure has been reported to increase archaeal *amoA* gene copies as compared to urea fertilization (Shen et al., 2015).

Best management practices influence the amount of available N and C, soil temperature, and water content. The C:N ratio of organic inputs have also been reported to be a good indicator of the AOA and AOB abundance dynamics (Muema et al., 2016; Strauss et al., 2014; Wessén et al., 2011). This study found higher AOA *amoA* gene copies in soil microcosms taken from the

broiler litter treatments. These results may be partly due to the higher labile C content of broiler litter applications. Previous studies have shown that AOA are mixotrophs that can utilize organic C as well as CO₂ (Hallam et al., 2006; Prosser and Nicol, 2012). Hai et al. (2009) reported that the AOA populations were stimulated by application of sorghum straw and cattle manure. However, other studies have reported that dairy slurry application to soil had no effect on AOA *amoA* gene copies and did not always increase AOB *amoA* gene copies (Fortuna et al., 2012).

This study is one of the few to illustrate the effects of a defined set of key management practices and simulated freeze-thaw conditions on *amoA* gene copies of archaeal and bacterial nitrifiers in agronomic systems via an incubation technique. These results show that archaeal and bacterial nitrifier *amoA* gene copies responded differently to simulated freeze-thaw events. The freeze-thaw cycles had a negative effect on the archaeal nitrifiers as their *amoA* gene copies decreased after the freeze-thaw events. The significant decrease in the abundance of archaeal nitrifier gene copies may also be explained partially by the increase in mineralization that resulted in higher NH₄⁺-N concentrations after the freeze-thaw events. However, lower NH₄⁺-N concentrations in the unamended soil microcosms did not stimulate the archaeal *amoA* gene copies. The frequency of freeze-thaw did not appear to affect the archaeal nitrifier population.

In contrast to AOA, bacterial nitrifiers (AOB) *amoA* gene copies significantly increased after the freeze-thaw events. This result may be attributed to freeze-thaw events enhancing the mineralization process in the amended soil microcosms. Previous studies have reported that AOB abundance increases as NH₄⁺-N concentrations increase in soils whereas AOA growth remained constant in agricultural and managed grassland soils receiving no N inputs (Di et al., 2010; Jia and Conrad, 2009; Ke and Lu, 2012; Verhamme et al., 2011). In this study the archaeal and bacterial nitrifier gene copy numbers could be correlated to the NH₄⁺-N concentrations.

However, in a similar study by Wertz et al. (2013), no significant correlation was found among *amoA* archaeal and bacterial nitrifier gene copies and ammonium concentrations when soil microcosms were maintained at temperatures near the freezing point.

The activity of nitrifier and denitrifier communities is directly related to soil temperature and O₂ concentrations (Avrahami et al., 2002; Szukics et al., 2010) both of which are directly influenced by the amount of water in the system. In this study neither the AOA nor the AOB population appeared to be affected by WFPS alone. Although recent studies have reported that AOA populations are more tolerant to hypoxic conditions relative to AOB populations (Liu et al., 2015; Pett-Ridge et al., 2013).

Effect of long-term organic agroecosystems to reduce global warming potential

Key management practices have a vital impact on N cycling in organic systems. Best management practices (BMPs) that retain or return residues and other organic amendments to the soil have been shown to insulate and elevate soil temperatures reducing the extremity and frequency of freeze-thaw cycles that foster N₂O emissions (Bochove et al., 2000; Wagner-Riddle and Thurtell, 1998). In this study, the isotopic composition of N₂O indicated that most of the N₂O derived from the soil microcosms amended with 1% ¹⁵N labelled sugar beet top residues was from nitrates contained in the labelled residues that underwent denitrification. Even when conditions fostered N₂O loss via denitrification (i.e. 80% WFPS), the amended soil microcosms taken from mixed-compost treatments emitted less N₂O relative to soils taken from broiler litter amended and pasture systems. The contribution of CO₂ to the total freeze-thaw GWP was comparable among the three organic treatments. However, total organic soil C was higher in the soils with mixed-compost treatment (27.7 g kg⁻¹) as compared to the soils with broiler litter (18.3 g kg⁻¹) treatment and pasture systems (17.2 g kg⁻¹) (Bhowmik et al., 2016). Thus, the estimated

contribution of CO₂ to the GWP per unit C added to the systems was lower in mixed-compost as compared to the organic broiler litter treatment and pasture systems. This accumulation of C in the mixed-compost amended system interacts with and immobilizes the reactive N. Therefore, the contribution of N₂O to GWP from mixed-compost treated organic systems is lower as compared to broiler litter and pasture systems. This research illustrates that key organic management practices like long term additions of animal manures to soil have the potential to build up soil health by increasing C accumulation thereby providing environmental services that include C sequestration and a reduction in GHG emissions.

In conclusion, freeze-thaw events, sub-zero temperatures that persist for a few days, (particularly under saturated conditions) do not reduce N cycling microorganisms or the processes they perform sufficiently to diminish N₂O and CO₂ emissions but release additional labile C and reactive N from soil and organic amendments that fosters GHG production. This study simulated seasonal variations in C and N by addition of sugarbeet top residues, rainfall by maintaining soil microcosms at 40%, 60% and 80% WFPS and winter conditions by mimicking freeze-thaw events in an incubator. The AOA, *amoA* gene copies were significantly lower after freeze-thaw cycles relative to gene copies of AOB. Changes in AOA and AOB *amoA* gene copies verifies the existence of different temperature niches for each nitrifier community. Higher copies of AOA, *amoA* genes in unamended soils substantiates the preference of AOA for low concentrations of NH₄⁺ (oligotrophic, differing affinities for ammonium substrate) relative to AOB. My results indicate that gene copies of keystone species serve as biological indicators of soil health revealing useful information about biogeochemical processes and losses of reactive N in different organic farming systems. Under conditions favorable for the denitrification process, the long-term organic mixed-compost systems had the potential to minimize N losses to N₂O by

immobilizing excess available nitrates in soil, increasing in C stocks and or C as compared to the broiler litter and pasture systems. These management practices could be adopted by organic growers to improve soil health and fertility as well as a means to mitigate climate change.

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**PAPER 3. USE OF LABORATORY INCUBATION TECHNIQUES TO ESTIMATE
GREEN HOUSE GAS FOOTPRINTS FROM CLEAN AND NO TILLAGE ORGANIC
AGROECOSYSTEMS**

Abstract

Organic agroecological systems “produce goods using methods that preserve the environment” but can be substantial sources of greenhouse gases (GHG) if not managed properly. The objective of this experiment was to monitor nitrogen (N) and carbon (C) transformations resulting from previous management (clean tillage (CT) vs. no tillage (NT)), simulated freeze-thaws, water filled pore space (WFPS) and amendments. Soils incubated for 149 d were unamended or amended with ¹⁵N labelled urea or sugar beet residue and maintained at 40, 60 and 80% WFPS. Non-metric multidimensional scaling verified that amended soils (beet, urea) clustered separately in ordination space. A two way PerMANOVA analysis confirmed a significant interaction between WFPS and N amendment (p=0.0002). Tillage, amendment and WFPS had a significant effect on GHG emissions of soil taken from two USDA certified organic five-year small grain rotations with mixed legume cover crops. At 40% WFPS, soil from the NT system amended with beet residues emitted more N₂O and CO₂ relative to soil from the CT system. At 60% WFPS, soil from the CT system amended with beet residues emitted greater N₂O and less CO₂ relative to soil from the NT system. Our research indicates that climate, carbon stocks and duration of tillage management, rather than organic management, determined the potential of no-till to reduce GHG emissions during a simulated freeze-thaw event. Growers should note that at an average air temperatures of 10°C, the presence of N in the form of residues or urea may lead to significant production of N₂O.

Introduction

The agriculture sector contributes up to 10-12% of the total global anthropogenic greenhouse gas (GHG) production. A reduction in GHG emissions from the agricultural sector has the potential to reduce the radiative forcing of GHGs by 1.15–3.3 Pg C equivalents per year (Cole et al., 1997). Tillage is one of the major agronomic activities that influence CO₂ emissions and regulates soil organic carbon (SOC) stocks. Conservation tillage practices such as no-tillage (NT) have been promoted as agricultural practices that have the potential to prevent or reduce soil erosion and degradation of soil structure (Petersen et al., 2011), increase water stable aggregates (Fernandez et al., 2010; Zotarelli et al., 2007), enhance accrued and sequestered C (Gollany et al., 2012; West and Post, 2002), mitigate GHG emissions (Kong et al., 2009) and improve biological activity (Helgason et al., 2010).

Conservation tillage often augments stocks of C and N in soil which also affect N₂O emissions. In no-till systems receiving synthetic N fertilizer applications such as urea, gains in soil C (or reduced CO₂ emissions) resulting from NT may be countervailed by greater nitrous oxide (N₂O) emissions resulting from N fertilizer applications (Stockle et al., 2012; Skinner et al., 2014). Increased N₂O emission under NT might be due to the fact that tillage modifies the physico-chemical and hydro-thermal regime in the soil which can lead to the creation of anaerobic microsites with increased microbial activity leading to greater competition for oxygen (O₂) stimulating denitrification (Mangalassery et al., 2014). These emissions are of concern because N₂O is a potent GHG with a 298 times the global warming potential (GWP) as compared as of CO₂ based on a 100-year time horizon (Six et al., 2004). So, it is imperative that while taking into account the CO₂ mitigation potential of NT management, that we do not

overlook the potential for N₂O emissions, a major contributor to the overall GHG budget (Beaulieu et al., 2011).

An alternative to NT farming systems with manufactured fertilizers and other synthetic inputs are certified organic management systems that disallow addition of synthetic N fertilizers and are dependent upon organic amendments “mimicking natural ecosystems” in which C and N are coupled (e.g. bond in the form of crop residues, cover crops, animal amendments) (Cavigelli et al., 2013; Lyon and Hergert, 2014). Organic agroecosystems are also different from many other agricultural systems because they are low in N and high in organic C inputs. Therefore, these systems may improve soil health, increase pools of soil N and C, relative to other agroecosystems. Some studies have also reported that implementing NT in organic systems can increase the SOC by 9% after 2 years and by 21% after 6 years (Carr et al., 2013; Gadermaier et al., 2011). Previous studies have reported that organic systems provide ecosystem services by reducing nutrient losses (Cavigelli et al., 2013; Drinkwater et al., 1998) and GHGs, thereby, lowering the GWP of organic agroecosystems (Cavigelli et al., 2013). Thus, it seems that after an initial transition period of some years, NT organic agroecosystems foster a ‘win–win’ scenario by enhancing agricultural sustainability while reducing CO₂ emissions.

However, there is a degree of uncertainty within the published literature with respect to N₂O emissions from NT organic management systems. Specifically, with regards to their potential to reduce rather than increase N₂O emissions. A number of NT cropping systems that are not organic certified have been found to emit less CO₂ but more N₂O relative to moldboard plow systems (Powlson et al., 2012). However, the coupling of C and N in organic systems often leads to tight nutrient cycling minimizing the potential for N₂O loss from some organic agroecosystems. In contrast, addition of organic amendments may provide mineralizable C and

N which may act as substrates for CO₂ and N₂O emissions if not managed properly (Johnson et al., 2012).

There are several drivers of N₂O from soil of which soil inorganic N (ammonium and nitrate), labile C, oxygen concentration, moisture and climate are most important (Groffman and Tiedje, 1991; De Klein et al., 2001; Saggar et al., 2013). Soil nitrates are regulated by microbial processes such as ammonification, nitrification and denitrification all of which are dependent upon the N source (s) added. The availability of labile C is also dependent on the quality and quantity of organic matter added to the soil. The soil oxygen concentration is controlled in part by the water filled pore space (WFPS) and microbial respiration in soil. All these factors are interrelated and in turn are partially regulated by key management practices.

Specific management practices such as the timing of application of N source(s) to the soil along with their quality (C to N ratio) and quantity determined by the types of animal amendments, cover crop and crop residue incorporated via reduced tillage or conventional tillage. (Saggar et al., 2013). Most of the N in organic fertilizers must be converted to inorganic N before it is available for plant uptake. When not taken up by a crop or in the absence of a crop (non-growing season), reactive N forms are mobile and are susceptible to loss. Nitrogen applied in excess of crop needs is particularly susceptible to loss if not immobilized. Thus it is of vital importance to regulate the availability of reactive forms of N in soil which might otherwise be lost as N₂O (Thapa et al., 2015).

Differences in tillage intensity are attributed to the degree of disturbance(s) the soil is subject to and is a major driver of N₂O production, liberating available C for heterotrophs that include denitrifiers (Wagner-Riddle et al., 2008). However, the effect of tillage on N₂O emissions is variable because it influences numerous soil biophysical factors that affect N₂O

flux. Other studies have also reported that NT can increase N₂O emissions by reducing soil aeration, increasing soil C and N, and soil moisture within the surface layers, leading to higher denitrification rates compared with tilled soils (Ball et al., 1999; Rochette, 2008). Alternatively, no-till can improve soil structure, lower soil temperature, and reduce N₂O loss (Gregorich et al., 2008; Mosier et al., 2006; Six et al., 2002; Venterea et al., 2011).

Climate determines temperature and rainfall events which in turn partially regulates N₂O emissions from soil. Most of the studies conducted to date focus on N₂O emissions during the growing season during which prevailing temperatures are well above 10°C. Regions of mid to higher-latitude are characterized by long winters during which soil is frozen or snow-covered for 5 to 6 months of the year. Despite these seemingly harsh conditions, 39–90% of annual N₂O emissions can occur during winter (van Bochove et al., 2000; Jayasundara et al., 2007; Teepe et al., 2004; Yanai et al., 2011). Fall application of N fertilizer or manures before soils freeze is a common practice in colder regions due to long-standing assumptions that temperatures below 10°C are too low for nitrification and that microorganisms are inactive over winter due to low temperatures (Phillips, 2007). However, increases in N₂O emissions from soils in winter have been reported in the literature after freeze-thaw events release C and N substrates suitable for microbial metabolism (Nemeth et al., 2014; Wagner-Riddle et al., 2008). In a meta-analysis study, Kessel et al. (2013), determined that the combined effect of dry climate and NT on N₂O emissions, reported that NT significantly reduced N₂O emissions in dry climates if practiced for more than 10 years and that the reduction was a result of low biomass production in such systems. Six et al. (2004) observed a tendency toward increased N₂O emissions during the first 10 years after conversion from CT to NT because of a transient yet significant N deficiency which lowered crop yields in recently established NT systems, but thereafter N₂O fluxes tended

to decrease. However, this reduction in N₂O emissions following long-term NT was only significant in humid climates.

There is a gap in knowledge regarding how tillage management, climate, cropping system and duration of a given tillage by cropping system regime effects the GHG footprint of organic agroecosystems. A recent study conducted by Bhowmik et al. (2016b) estimated reactive forms of N losses including N₂O emissions from long term organic management systems (12 years of USDA certified organic plot). They reported that addition of mixed compost and pasture management for 10 yr augmented slow pool soil C (turnover in years, ~40 to 50% of soil C) reducing the potential for gaseous losses of N as N₂O likely due to the temporary immobilization of inorganic N. The study above indicated that climate and duration of organic management practices that include addition and decomposition rates of crop biomass and/or organic amendments determines N₂O and CO₂ emissions. Specifically, organic agroecosystems have the potential to contribute significant amounts of N₂O during winter freeze-thaw cycles of short duration (4 d). These cycles release C and N substrates that can lead to N₂O production when soil moisture is $\geq 60\%$ of WFPS and temperatures are at or below 10°C, a temperature range at which microbial communities are still active, allowing for turnover of C and N. Under such conditions reactive N can be lost as N₂O if stores of SOC are not sufficient to immobilize inorganic N. If no till organic management systems have only been in place for a few years and organic inputs, such as residue biomass returned are low and pools of soil C will likely be insufficient to immobilize inorganic N. As a result, recently established NT organic agroecosystems may have the potential to emit significant amounts of N₂O over winter when fertilized with reactive N in the form of organic inputs or urea in fall and early spring. The main objective of this study was to determine the effects of previous tillage management (clean-till/no-till), WFPS and a

simulated winter freeze of 70 d duration followed by a thaw on reactive forms of nitrogen (NH_4^+ -N, NO_3^- -N, N_2O , NH_3), GHG emissions (N_2O , CO_2) and the potential GHG footprint of two (CT and NT) USDA certified organic five-year small grain rotations with mixed legume cover crops using laboratory incubations techniques.

Materials and methods

Site description, soil sampling and storage

The Long Term Organic Tillage Study (LOTS) was established in 2010 and managed as a USDA certified organic field on Reeder-Farnuf loams (fine-loamy, mixed, superactive, frigid, typic Argiustolls) at the North Dakota State University Dickinson Research and Extension Center, USA (46°53' N, 102°49' W; elevation 760 m) (Bhowmik et al., 2016a). The soil had a pH (H_2O) of 6.7, EC of 0.85 dS m^{-1} and was loam (30% sand, 47% silt, 23% clay) in texture. Soil samples (0-30 cm) were collected from clean and no tillage (CT, NT) plots in November of 2013. The plots were arranged in a randomized complete block design containing 4 field replicates per treatment (30 m x 9 m). The CT plots were cultivated with a tandem disc to a soil depth of 10 cm in late summer (August to October of each year) and again the following spring (March-early April) prior to seeding in June. No soil disturbance except by a low-disturbance planter at seeding occurred in NT plots. No further tillage was applied to these plots. Pre-plant tillage (CT plots) or a pre-plant application of 20% acetic acid in water (NT plots only) combined with grazing and cover crops (in the case of NT plots, killed cover crop mulch) was used to suppress weeds. Nutrients were cycled when sheep (*Ovis aries*) grazed the plots before or after harvest. Proso millet (*Panicum mileaceum* L.) and navy bean (*Phaseolus vulgaris* L.) plots were grazed prior to harvest; field pea (*Pisum sativum* L. ssp. *Sativum*) plots were grazed after seed was harvested but before planting hairy vetch (*Vicia villosa* Roth) while proso millet was

grazed after grain was harvested but before winter rye (*Secale cereale* L.) was planted. Wheat (*T. aestivum* L. emend. Thell.) plots were grazed in the fall after grain was harvested and the cover crops were planted. Detailed information for the cover crop and other field activities can be found in Appendix Table A1.

For this study, the soils were collected in November of 2013 from the plots seeded into navy beans in 2013 after the navy beans were harvested and sheep had grazed the plots but prior to planting of hairy vetch. One composite sample consisted of 10 random samples taken from 0-30 cm with a soil probe (2.5-cm diam.) from each of the 4 field plot replicates. The soil bulk density was determined using a hammer driven bulk density core sampler (6 cm deep by 5.4 cm diameter) (Grossman and Reinsch, 2002). The visible crop residues were removed from soil samples and the field moist soil samples were passed through a 2-mm sieve. A portion of the sieved soil samples was analyzed for gravimetric moisture and initial inorganic N content. Soil inorganic N (NH_4^+ and NO_3^-)-N was extracted from 10 g (field moist soil) with 100 mL of 2 M KCl solution. The aliquots were run on a SEAL auto analyzer (SEAL Analytical Inc. Mequon, WI) to determine the concentration of NH_4^+ -N and NO_3^- -N. The remaining field moist soil was pre-incubated at 4°C for 1 wk.

Incubation experiment

Simulated winter freeze-thaw temperature cycle

In order to mimic the winter freeze-thaw cycle (November to March) in North Dakota 30 years of average daily soil temperatures obtained from the North Dakota Weather Network (NDAWN) were used. Based on this data a freeze-thaw cycle for the Dickinson, ND field site (Appendix Fig. C1) was designed. A total of 7 sampling time intervals were taken during a 149 d incubation broken into 4 sampling time intervals during a six-week pre-freeze period at 10°C, 1

sampling time interval after a 2 month and 10 d freeze at -5°C , 1 sampling time interval after a one-month thaw period at 10°C and a final sampling time interval after a week at 25°C .

Microcosm experiments

Three simultaneous 149 d laboratory incubations were conducted in order to measure 1) the amount of N_2O , and CO_2 evolved, 2) ammonia volatilization and 3) inorganic N (NH_4^+ + NO_3^-) mineralization. The vessels used as microcosms during the incubation were 128 mL sterile specimen vials (diameter 53 mm; height 60 mm) containing 60 g of moist soil (50 g dry weight) taken from each of the 4 replicates of the CT and NT treatments (8 field plots). Samples in the vials were packed to a bulk density of 1.1 g cm^{-3} . The microcosms were pre-incubated at 25°C and 30% WFPS for a week before the incubation experiment began. A pre-incubation is conducted to compensate for the disturbance that occurs when taking a sample from the field and sieving (Paul et al., 2001). These basic handling procedures artificially elevate CO_2 emissions for approximately one week and will introduce an artifact into the cumulative CO_2 data if not pre-incubated. The total number of samples for the GHG measurements and ammonia volatilization incubation experiments was 72 (2 treatments \times 4 field reps \times 3 amendments \times 3 moistures), for a total of 144 samples. The third incubation contained 576 samples (2 treatments \times 4 field reps \times 3 amendments \times 3 moistures \times 8 time intervals) due to the destructive nature of soil sampling required to obtain inorganic N concentrations at each of the 8 time intervals. Amendments and distilled water (dH_2O) were added to the microcosms after the pre-incubation. The amendments represent N sources with and without carbon added to the soil microcosms. To one-third of the samples ^{15}N (5% excess ^{15}N) labelled urea (51.5 mg of urea per 50 g equivalent dry soil) was applied, and ^{15}N labelled sugar beet residues (1% excess ^{15}N) was added at a rate of 565 mg of beet residues per 50 g equivalent dry soil to the other one-third. The beet residues contained 2%

of total N and had 21% of the total N was NO_3^- -N. No nitrogen in the form of amendments was added to the remaining 1/3 of the soil microcosms (control).

The N rates of urea and sugar beet top residues simulated the fall application of synthetic fertilizers and biomass returned of a cover crop/residue incorporation which is a common practice in this region. The sugar beet top residues were harvested at sugar beet maturity but before senescence and air dried at low temperature ($\sim 30^\circ\text{C}$) and stored in a sealed plastic bag until use. The material was green in color and was chopped into a uniform size (1-2.5 cm length) with sterile scissor. The amendments were uniformly broadcast on the surface (NT) or broadcast and mixed uniformly with soil to a depth of ~ 10 mm below the surface of the soil microcosms (CT) to simulate the effect of tillage in the laboratory. The soil microcosms from the GHGs and ammonia volatilization incubations were divided into 3 sets of 8 samples each (urea amended (24), residue amended (24) and unamended control (24)) and distilled H_2O was added at three rates 40, 60 and 80 % WFPS and run in triplicate. Treatment of the soil microcosms for the N incubation were divided into 3 sets of 64 samples each due to the destructive sampling required to measure soil inorganic N. Soil microcosms from each treatment and 8 time intervals were amended with urea (24×8), residue (24×8) or were left unamended control (24×8) and distilled H_2O was added at three rates 40, 60 and 80 % WFPS and run in triplicate.

Soil incubation for N_2O and CO_2 analysis

Each of the 72 soil microcosms were placed in 1 quart (935 mL) canning jar (1 vial. per jar) with 935 mL head space for gas sampling. The lids of the jars had rubber septa attached to them which acted as a sampling port for both N_2O and CO_2 (Supelco Analytical, St. Louis, MO). Twenty mL of dH_2O was pipetted into the bottom of the canning jars prior to placing the incubation vessels (soil microcosms) into the jars and sealing the jars. Water was added to the

bottom of each canning jar to maintain sufficient relative humidity inside the jars preventing moisture loss from the vials. Blanks were also maintained and consisted of 3 canning jars with 20 mL di H₂O at the bottom of each jar but no soil in the incubation vessel. The 72 jars were incubated in a laboratory incubator (Precision™ Low Temperature BOD Refrigerated Incubator, Thermo Fischer Scientific Inc., Waltham, MA) for a period of 149 d under simulated winter freeze-thaw conditions as previously discussed. The gas samples (30 mL) were collected from the headspace of the jar and over pressured into 20 mL vials fitted with a 3mm PTFE-faced gray butyl crimp seal septum.

Gas samples for N₂O and CO₂ were taken at 0, 7, 14, 28, 42, 112, 142 and 149 d using the GRACENet protocol (<http://www.ars.usda.gov/research/GRACENet>). The headspace of the canning jar was flushed with air after each gas sampling and soil microcosms were weighed and moisture was adjusted to 40%, 60% and 80% WFPS if necessary. The total number of gas samples was 576 (8 time intervals×2 treatments×4 field replicates×3 amendments×3 moistures) + 24 blanks (8 time intervals×3 laboratory replicates). Gas samples for N₂O underwent N¹⁵ isotope analysis using a ThermoFinniganGasBench + PreCon trace gas concentration system interfaced to a ThermoScientific Delta V Plus isotope-ratio mass spectrometer (Bremen, Germany). Further details of the working procedure can be found at <http://stableisotopefacility.ucdavis.edu/>. The CO₂ samples were measured with an infrared gas analyzer (IRGA, LI-COR 830, Lincoln, NE). The system is equipped with an infrared ray source, a modulator, an analysis chamber containing the mixture of gases, and a detector together with associated electronic circuits for processing the electric signals delivered by the detector. A set of certified CO₂ (2500, 5,000, 10,000, 20,000 ppm) standards were run. The N₂O and CO₂ readings were converted to μg N₂O kg⁻¹ soil and mg CO₂ kg⁻¹ soil using the ideal gas law equation.

Soil incubation for ammonia volatilization

Ammonia volatilization from soil microcosms was measured by using the method of Conway, a micro-diffusion incubation which was modified for soil by Bremner and Krogmeier (1989). A second set of 72 canning jars was used for the ammonia volatilization experiment. A quartz glass petri dish with a diameter of 6 cm was filled with 10 mL of 2% boric acid and placed at the bottom of each jar. A netted mesh was placed carefully over the petri dish and the soil microcosm (vial) was placed on the mesh to avoid any direct contact with the boric acid. Jars with boric acid on the petri dishes but no soil served as blanks. The total number of samples for the ammonia volatilization experiment was 576 (8 time intervals×2 treatments×4 field replicates×3 amendments×3 moistures) + 24 blanks (8 time intervals×3 laboratory replicates). Samples were incubated in a laboratory incubator (Precision™ Low Temperature BOD Refrigerated Incubator, Thermo Fischer Scientific Inc., Waltham, MA) for a period of 149 d under simulated winter freeze-thaw conditions. At each time interval boric acid traps were titrated with 0.005 N HCl using 2-3 drops of the N-Point indicator (VWR International, Radnor, PA, USA). The petri dishes were subsequently acid washed and refilled with fresh boric acid for the next time interval.

Soil incubation for inorganic N analysis

For this experiment instead of placing the soil microcosms in sealed jars microcosms were covered with parafilm having a 5 mm hole in the center to allow for gaseous exchange while preventing rapid loss of moisture (Saunders et al., 2012). The parafilm allows for diffusion of O₂ but prevents loss of H₂O. The total number of soil microcosms incubated for this purpose was 576 (8 time intervals× 2 treatments× 4 field reps× 3 amendments× 3 moistures). At each time interval soils were destructively sampled for inorganic N (NH₄⁺ and NO₃⁻)-N analysis. Ten grams of field moist soil was shaken for 30 min at 180 rpm with 100 mL of 2M KCl. An additional 10 g soil

sample was weighed to adjust data for soil moisture content. The filtrate was collected after passing through a Whatman No.1 filter paper and stored at -20°C until analysis. The aliquots were run on a SEAL auto analyzer (SEAL Analytical Inc. Mequon, WI) to determine the concentration of (NH₄⁺ and NO₃⁻)-N.

Statistical analysis

Changes in individual variables (univariate data), N₂O, atom% ¹⁵N-N₂O, CO₂ and inorganic N, due to the effects of individual treatments and treatment combinations was analyzed using a three-way analysis of variance in a completely randomized factorial analysis using the SAS PROC GLM procedure of SAS 9.4 (SAS Institute, Cary, NC) and Fisher LSD test ($P < 0.05$). In order to determine the effects of treatments and treatment combinations on multivariate data which considers all variables simultaneously (N₂O, atom% ¹⁵N-N₂O, CO₂ and inorganic N), a non-metric multidimensional scaling (NMDS) analysis was applied using PC-Ord (PCORD 6, Gleneden Beach, Oregon) (Kruskal, 1964). Log transformation normalizes, linearizes and standardizes a data set to meet the assumptions of a statistical procedure that when not met typically suggest that the standard error distribution of a variable is biased. Transformation of the data can correct for skew in the structure of the data set. The NMDS analysis allows for analysis of data with a distribution that is not multivariate normal by employing rank orders. In PC-Ord, the default “slow and thorough” procedure was chosen with a Relative Euclidean distance measure and a random starting point, resulting in 250 runs (the recommended number of iterations and the default for the program) with real and randomized data. The accuracy of estimated similarities or dissimilarities (distances) between variables is dependent upon the number of comparisons made between variables and the remaining data. One-way and two-way PerMANOVA permutation tests ($n=499$)

were used to compute the statistical significance of treatments or treatment combinations during the 149 d incubation experiment in PC-Ord (PCORD 6, Gleneden Beach, Oregon).

Results

Effect of tillage, amendment (N source) and freeze-thaw event on reactive N species ($\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ and N_2O) and C mineralization

No significant ammonia volatilization was detected from the soil microcosms during the 149 d freeze-thaw incubation. The beet top residue amended soil microcosms from the CT and NT treatments had higher C mineralization estimated via cumulative CO_2 evolved relative to the urea amended or unamended soil microcosms (Fig. 15a). The soil microcosms from NT treatments had higher CO_2 emissions as compared to the soil microcosms from CT treatments. The amount of CO_2 emitted increased from ($442 \text{ mg kg}^{-1} \text{ dw soil}$) on 7 d to ($566 \text{ mg kg}^{-1} \text{ dw soil}$) on 14 d at 10°C from residue amended soil microcosms taken from NT plots. Thereafter, CO_2 emissions decreased to ($471 \text{ mg kg}^{-1} \text{ dw soil}$) and ($286 \text{ mg kg}^{-1} \text{ dw soil}$) on 28 d and 42 d at 10°C . A steep decrease in CO_2 emissions was observed due to the prolonged freeze of 70 d at -5°C (112 d). The CO_2 released from the C mineralization of the residue amended soil microcosms taken from the CT treatment was highest on 7 d ($460 \text{ mg kg}^{-1} \text{ dw soil}$) and thereafter gradually decreased till 112 d ($36 \text{ mg kg}^{-1} \text{ dw soil}$). Upon thawing at 10°C for a month, the residue amended soil microcosms from the CT and NT treatments increased on 142 d and then decreased on 149 d at 25°C . No significant differences in CO_2 emissions were observed between the CT and NT treatments after the freeze-thaw event (Fig. 15a).

The $\text{NH}_4^+\text{-N}$ concentrations were significantly higher in the urea amended microcosms relative to the residue amended soil microcosms (Fig. 15b). The unamended soil microcosms had almost negligible $\text{NH}_4^+\text{-N}$ throughout the 149 d incubation. Soil microcosms amended with urea

from the CT and NT treatments had similar $\text{NH}_4^+\text{-N}$ concentrations during the incubation with the exception of 28, 42 and 112 d on which the urea amended soil microcosms from the NT treatment had significantly higher $\text{NH}_4^+\text{-N}$ concentrations relative to the urea amended soil microcosms taken from the CT treatment. The residue amended soil microcosms taken from the CT treatments had higher $\text{NH}_4^+\text{-N}$ concentrations on 28 d at 10 °C ($93 \text{ mg kg}^{-1} \text{ dw soil}$) as compared to the residue amended soil microcosms from NT soils ($57 \text{ mg kg}^{-1} \text{ dw soil}$). No effect of freeze-thaw event on the $\text{NH}_4^+\text{-N}$ concentrations was observed in the residue, urea amended or unamended soil microcosms taken from the CT and NT treatment.

No significant differences in the $\text{NO}_3^-\text{-N}$ concentrations were observed in the urea amended soil microcosms collected from CT and NT treatments during the first 14 d of the incubation at 10 °C (Fig. 15c). Thereafter the $\text{NO}_3^-\text{-N}$ concentrations increased on 28 and 42 d at 10 °C in the urea amended soil microcosms taken from CT and NT treatments. During the freeze maintained at -5 °C, $\text{NO}_3^-\text{-N}$ concentrations decreased in the urea amended soil microcosms taken from NT treatments. No effect of freezing temperature was observed in the urea amended soil microcosms taken from the CT treatment. Increases in $\text{NO}_3^-\text{-N}$ concentrations were observed during the thaw period at 10 and 25 °C on 142 and 149 d in both urea amended soil microcosms taken from CT and NT treatments.

The $\text{NO}_3^-\text{-N}$ concentrations in the residue amended soil microcosms taken from the CT treatment remained stable from 7 through 112 d and then increased to ($81.8 \text{ mg kg}^{-1} \text{ dw soil}$) and ($105.4 \text{ mg kg}^{-1} \text{ dw soil}$) on 142 and 149 d respectively. In contrast the $\text{NO}_3^-\text{-N}$ concentrations in the residue amended soil microcosms taken from the NT treatment remained stable from 7 to 42 d and thereafter increased until the end of the incubation ($137 \text{ mg kg}^{-1} \text{ dw soil}$).

The N₂O emissions from soil microcosms amended with residues was 4 and 8 times higher relative to urea amended and unamended soil microcosms regardless of the fact that the urea was applied at 100 kg N ha⁻¹ whereas the residue was applied at 50 kg N ha⁻¹ (Fig. 15d). Nitrous oxide emissions from the residue amended microcosms with soil taken from the NT treatment were highest on 7 d then decreased to (896 μg kg⁻¹ dw soil) on 14 d (2nd highest N₂O spike). Thereafter, a steep decline in N₂O production was observed in the residue amended microcosm with soil taken from the NT treatment on 28 and 42 d. The N₂O emissions decreased after the 70 d freeze at -5 °C on 112 d and then increased to (260 μg kg⁻¹ dw soil) after the 1-month thaw at 10 °C on d 142 and (206 μg kg⁻¹ dw soil) after the 1-week thaw at 25 °C on 149 d. The N₂O emissions from the residue amended microcosms with soil taken from the CT (1175 μg kg⁻¹ dw soil) and NT (1211 μg kg⁻¹ dw soil) treatments were highest on 7 d. The N₂O production from the residue amended microcosms with soils collected from the CT treatment decreased on 14 d (342 μg kg⁻¹ dw soil) then remained constant on 28 d at 10 °C. The 2nd spike in N₂O emissions from the CT residue amended soil occurred on 42 d at 10 °C. Nitrous oxide emissions increased to 653 μg kg⁻¹ dw soil on 42 d and thereafter decreased during the freeze at -5 °C on 112 d. The thaw event at 10 °C did not significantly increase the N₂O emissions. However, higher N₂O was emitted from the residue amended soil microcosms of the soil taken from the CT treatments during the thaw when temperatures were increased from 10 °C to 25 °C for a week from 142 to 149 d.

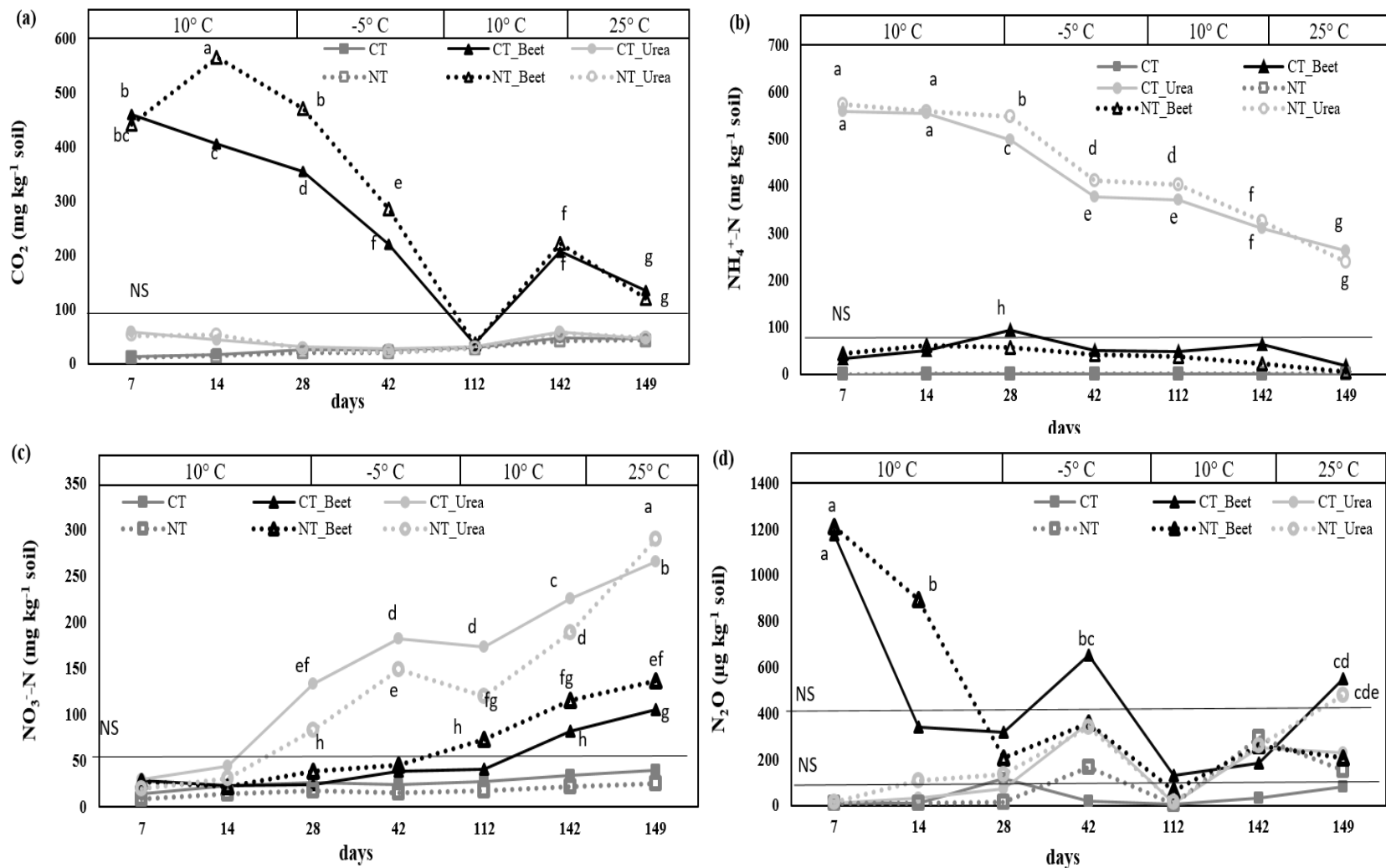


Fig. 15. Variation in (a) C availability estimated by the amount of cumulative carbon dioxide (CO₂) evolved, (b) ammonium-N (NH₄⁺-N), (c) nitrous oxide (N₂O) and (d) nitrate-N (NO₃⁻-N) during a 149 day incubation subjected to simulated freeze-thaw event in unamended, sugar beet top residue amended and urea amended soil microcosms collected from clean tillage (CT) and no tillage (NT) organic management systems.

The N₂O from urea amended soil microcosms taken from the CT and NT treatments spiked on 42 d at 10 °C and on 142 d after the freeze-thaw event. During the thaw period of 1 week at 25 °C the N₂O emissions from the urea amended microcosm with soil taken from the NT treatments were significantly higher (480 µg kg⁻¹ dw soil) than urea amended soil taken from the CT treatment (230 µg kg⁻¹ dw soil). The unamended soil microcosms taken from the CT treatment emitted higher in N₂O emission on 28 d (116 µg kg⁻¹ dw soil) but did not respond significantly to the freeze-thaw event during the 149 d incubation. A lag in the N₂O emission was observed from the unamended soil microcosms collected from the NT plots (N₂O highest on 42 d) as compared to unamended soil microcosms taken from the CT plots (N₂O highest on 28 d).

Effect of water filled pore space (WFPS), N source and freeze-thaw event on the dynamics of reactive nitrogen

No significant differences in NH₄⁺-N concentrations were observed among the residue amended soil microcosms maintained at 40, 60 and 80% WFPS during the pre-freeze period (7, 14, 28 and 42 d at 10 °C) or during the freeze (112 d at -5 °C) (Table 10). However, during the thaw period (142 d at 10 °C and d 149 at 25 °C), the NH₄⁺-N concentrations in the residue amended soil microcosms maintained at 60% WFPS (6.77 mg kg⁻¹ dw soil) were significantly lower as compared to the residue amended soil microcosms maintained at 80% (42.6 mg kg⁻¹ dw soil) WFPS. The NH₄⁺-N concentrations in the residue amended soil microcosms maintained at 40% and 60% WFPS were significantly higher during the pre-freeze as compared to the thaw period.

Table 10. The dynamics of reactive N species, ammonium-N ($\text{NH}_4^+\text{-N}$), nitrate-N ($\text{NO}_3^-\text{-N}$) and nitrous oxide (N_2O) under different treatment combinations during a simulated freeze-thaw incubation.

Time†	Unamended			Beet top residue			Urea		
	WFPS								
	40%	60%	80%	40%	60%	80%	40%	60%	80%
$\text{NH}_4^+\text{-N}$									
mg kg ⁻¹ soil									
Pre-freeze, 10 °C	0.07 k	0.13 k	0.07 k	60.6 g	46.1	54.3 gh	499 b	510 b	534 a
Freeze, -5 °C	0.30 k	0.54 k	0.57 k	49.3 ghi	34.8 hi	41.9 ghi	413 c	370 d	377 d
Thaw, 10 & 25	0.18 k	0.24 k	0.13 k	29.3 ij	6.77 jk	42.6 ghi	300 e	273 f	279 ef
$\text{NO}_3^-\text{-N}$									
mg kg ⁻¹ soil									
Pre-freeze, 10 °C	17.1 l	18.3 kl	18.7 kl	40.3 ij	38.7 ijk	14.4 l	71.5 gh	96.9 f	84.4 fg
Freeze, -5 °C	21.5	23.9 jkl	23.1 jkl	53.0 hi	78.0 fg	40.0 ij	90.8 fg	163 d	186 c
Thaw, 10 & 25	28.7	32.5 ijkl	29.9 jkl	124 e	134 e	72.7 gh	212 b	252 a	265 a
N_2O									
μg kg ⁻¹ soil									
Pre-freeze, 10 °C	10.4 h	15.6 gh	69.3 gh	176 defgh	579 b	1182 a	45.4 gh	74.9 fgh	284 cdef
Freeze, -5 °C	7.45 h	7.34 h	7.58 h	18.4 gh	49.1 gh	223 defg	8.47 h	9.39 h	20.0 gh
Thaw, 10 & 25	24.8	56.6 gh	342 cd	128 efgh	99.1 efgh	674 b	145 defgh	300 cde	471 bc

†Statistical significant differences (univariate three-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different lowercase letters within each reactive N species; Organic management × WFPS × Amendment, $P < 0.05$.

The urea amended soil microcosms during the pre-freeze had significantly higher NH_4^+ -N concentrations as compared to the freeze and thaw periods. During the pre-freeze, the urea amended soil microcosms maintained at 40% (499 mg kg^{-1} dw soil) and 60% (510 mg kg^{-1} dw soil) WFPS had significantly lower NH_4^+ -N concentrations as compared to the urea amended soil microcosms maintained at 80% WFPS (534 mg kg^{-1} dw soil). However, lower NH_4^+ -N concentrations were observed during the freeze in the urea amended soil microcosms maintained at 60% and 80% WFPS as compared to 40% WFPS. The thaw resulted in higher NH_4^+ -N concentrations in the urea amended soil microcosms maintained at 40% WFPS as compared to urea amended soil microcosms maintained at 60% WFPS. The NH_4^+ -N concentrations in the unamended soil microcosms were barely detectable.

The NO_3^- -N concentrations in the residue (47 mg NO_3^- -N kg^{-1} dw soil) amended soil microcosms maintained at 40% (40.3 mg kg^{-1} dw soil) and 60% WFPS (38.7 mg kg^{-1} dw soil) were significantly higher as compared to the residue amended soil microcosms maintained at 80% WFPS (14.4 mg kg^{-1} dw soil) during the pre-freeze period (Table 10). During the freeze at -5 °C on 112 d, the residue amended soil microcosms maintained at 60% WFPS had significantly higher NO_3^- -N concentrations as compared to residue amended soil microcosms maintained at 40% and 60% WFPS. During the thaw period significantly higher NO_3^- -N concentrations in the residue amended soil microcosms maintained at 40% and 60% as compared to 80% were observed. Overall the NO_3^- -N concentrations in residue amended soil microcosms maintained at 40%, 60% or 80% WFPS increased significantly during the thaw event as compared to the pre-freeze or freeze events. The pre-freeze at 10°C resulted in higher NO_3^- -N concentrations in the urea amended soil microcosms at 60% WFPS as compared to 40% WFPS. During the freeze, the urea amended soil microcosms maintained at 80% WFPS had significantly higher NO_3^- -N

concentrations as compared to urea amended soil microcosms maintained at 60% WFPS and at 40% WFPS. Higher NO_3^- -N concentrations were observed in the urea amended soil microcosms maintained at 60% and 80% WFPS as compared to 40% WFPS. The urea amended soil microcosms had higher NO_3^- -N concentrations during the thaw as compared to the pre-freeze and freeze periods.

The N_2O emissions were highest from the residue amended soil microcosms maintained at 80% WFPS ($1182 \mu\text{g kg}^{-1} \text{ dw soil}$) followed by 60% WFPS ($579 \mu\text{g kg}^{-1} \text{ dw soil}$) and then by 40% ($176 \mu\text{g kg}^{-1} \text{ dw soil}$) WFPS during the pre-freeze period (Table 10). The urea amended soil microcosms maintained at 80% WFPS had significantly higher N_2O emissions as compared to 40% and 60% WFPS during the pre-freeze period. No significant effect of moisture was observed during the freeze on the N_2O emission from the amended or unamended soil microcosms. During the thaw, the residue amended and unamended soil microcosms maintained at 80% WFPS emitted higher N_2O as compared to 40% and 60% WFPS. In the case of soil microcosms amended with urea, the microcosms maintained at 80% had higher N_2O emissions as compared to 40% WFPS during the thaw. The freezing and thawing of the urea amended soil microcosm and the unamended soil microcosms significantly increased the N_2O emissions as compared to the pre-freeze, whereas the highest N_2O emissions from the residue amended soil microcosms was observed during the pre-freeze period (first 42 d at 10°C after the application of residues to the soil microcosm).

Soil moisture and the time of incubation did not have any significant effect on the ^{15}N – N_2O fractionation in the unamended soil microcosms (Table 11). Limited ^{15}N – N_2O fractionation was observed after the freeze-thaw event on 142 d in residue amended soil microcosms (1 atom% ^{15}N enriched) maintained at 60% WFPS. My data suggests that the 60% WFPS allowed

more of the nitrates in the beet top residues to leach out into the soil thereby directly contributing to the N_2O emissions through the denitrification process and bypassing nitrification. The atom% $^{15}\text{N}-\text{N}_2\text{O}$ was lower in the urea (5 atom% ^{15}N enriched) amended soil microcosms during the initial days of incubation but gradually increased up to 42 d. The freeze at $-5\text{ }^\circ\text{C}$ decreased the atom% $^{15}\text{N}-\text{N}_2\text{O}$ values in urea amended soil microcosms irrespective of the WFPS. However, on thawing, the urea amended soil microcosms' values of $^{15}\text{N}-\text{N}_2\text{O}$ increased to about 4.4 atom% $^{15}\text{N}-\text{N}_2\text{O}$. Limited fractionation in the $^{15}\text{N}-\text{N}_2\text{O}$ was observed in the urea amended soil microcosms maintained at 60% or 80% WFPS as compared to 40% WFPS on 28 and 142 d of the incubation.

Table 11. The atom % $^{15}\text{N-N}_2\text{O}$ concentrations in soil microcosms under different treatment combinations during a simulated freeze-thaw incubation.

Time†	Unamended†			Beet top residues			Urea		
	WFPS								
	40%	60%	80%	40%	60%	80%	40%	60%	80%
$^{15}\text{N-N}_2\text{O}$									
days	Atom %			Atom %			Atom %		
7				0.69 e	0.93 ab	0.89 b	0.92 k	0.96 k	0.83 k
14				0.94 a	0.96 a	0.89 b	1.50 i	1.80 h	2.29 g
28				0.88 bc	0.97 a	0.91 b	3.36 e	3.72 d	3.70 d
42	————	0.36-0.38	————	0.91 b	0.97 a	0.94 a	4.19 abc	4.40 ab	4.30 ab
112				0.65 e	0.87 bc	0.99 a	1.06 k	1.33 j	2.89 j
142				0.95 a	0.83 d	0.95 a	4.18 bc	4.35 ab	4.42 a
149				0.99 a	0.88 bc	0.97 a	4.39 ab	4.42 a	4.00 c

†Statistical significant differences (univariate two-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different lower case letters within each column; time of incubation × water filled pore space interaction ($P < 0.05$).

Non-metric multidimensional scaling ordination

The different time points in the incubation experiment are represented by individual NMDS ordination analysis that correlated all measured variables and reduced the variation along the two axes (Fig. 16). The selected axes for each ordination analysis were significantly different from random ($p < 0.05$). The explanatory variables used to represent the 2 axes had an absolute r value of 0.5 or more. The final stress for the 2 dimensional solution was less than 5 and an instability of 0.1×10^{-5} was attained.

Axis 1 (75.5%) and axis 2 (16.9%) accounted for 92.4% of the variation on 7 d at 10 °C. The data clustered into distinct patterns according to the WFPS and N source (Fig. 16a). Two-way PerMANOVA confirmed that the multiple measured variables were significantly affected by WFPS and N source ($p=0.0002$) (Table 12). Measurements from the residue amended soil microcosms maintained at 80% WFPS clustered to the right on axis 1 whereas the variables from the urea amended soil microcosms clustered to the left on axis 1. Axis 1 was positively correlated with measurements of CO_2 and N_2O and negatively correlated to $\text{NH}_4\text{-N}$ and cumulative N. Thus, the ordination diagram revealed that the residue amended soil microcosms maintained at 80% WFPS had the highest N_2O and CO_2 emissions whereas the urea amended soil microcosms had the highest $\text{NH}_4\text{-N}$ concentrations. The soil variables on 14, 28 and 42 d are represented in a single NMDS ordination diagram (Fig. 16b). Axis 1 explained the largest proportion of the variation (65.8%) and was positively correlated with atom % $^{15}\text{N-N}_2\text{O}$, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ and cumulative N concentrations and negatively correlated with CO_2 emissions. Axis 2 represented only 24.7% of the variation and was positively correlated with N_2O emissions. There was separate and distinct clustering separating urea amended soil microcosms and residue amended or unamended soil microcosms. A two-way PerMANOVA confirmed that the soil

variables were significantly affected by WFPS and N source ($p=0.0004$) (Table 12). My data suggests that from 14 to 42 d the N from the urea underwent hydrolysis and was nitrified thereby increasing the $\text{NO}_3\text{-N}$ concentrations in the urea amended soil microcosms.

During the freeze event at $-5\text{ }^\circ\text{C}$ for 70 d, axis 1 (60.8%) and axis 2 (37.6%) represented 98.4% of the total variation (Fig. 16c). The axis 1 was positively correlated to the atom % $^{15}\text{N-N}_2\text{O}$, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ and cumulative N concentrations whereas axis 2 was negatively correlated with CO_2 and N_2O emissions. The urea amended soil microcosms and the unamended soil microcosms were clustered on the right and the left of axis 1 respectively. Only the residue amended soil microcosms maintained at 80% WFPS contributed to the low CO_2 and the N_2O emissions during the freeze event. Two-way PerMANOVA confirmed that the soil variables were significantly affected by WFPS and N source ($p=0.0002$) (Table 12).

After thawing at $10\text{ }^\circ\text{C}$ for a month on 142 d, about 97% of the variability was explained via axis 1 (62.3%) and axis 2 (34.7%) (Fig. 16d). The atom % $^{15}\text{N-N}_2\text{O}$, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ and cumulative N concentrations were positively correlated with axis 1 and negatively correlated with axis 2. Soil N_2O emissions were negatively correlated with both axis 1 and 2. A more distinct clustering pattern was observed between the urea amended soil microcosms as compared to the residue amended and unamended soil microcosms. One-way PerMANOVA confirmed that the soil variables were significantly affected by the N source ($p=0.0002$) (Table 12).

During the thaw at $25\text{ }^\circ\text{C}$ on 149 d, axis 1 (65.5%) and axis 2 (34.1%) represented about 99.7% of the variation in the data (Fig. 16e). Axis 1 was positively correlated with N_2O whereas axis 2 was positively correlated to atom % $^{15}\text{N-N}_2\text{O}$, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ and cumulative N concentrations. Soil variables were clustered into distinct groups according to N treatment. The urea amended soil microcosms formed a separate cluster as compared to the unamended and

residue amended soil microcosms and suggests that the thawing of the soils contributed to N₂O emissions. One-way PerMANOVA confirmed that the soil variables were significantly affected by the N source (p=0.0004) (Table 12).

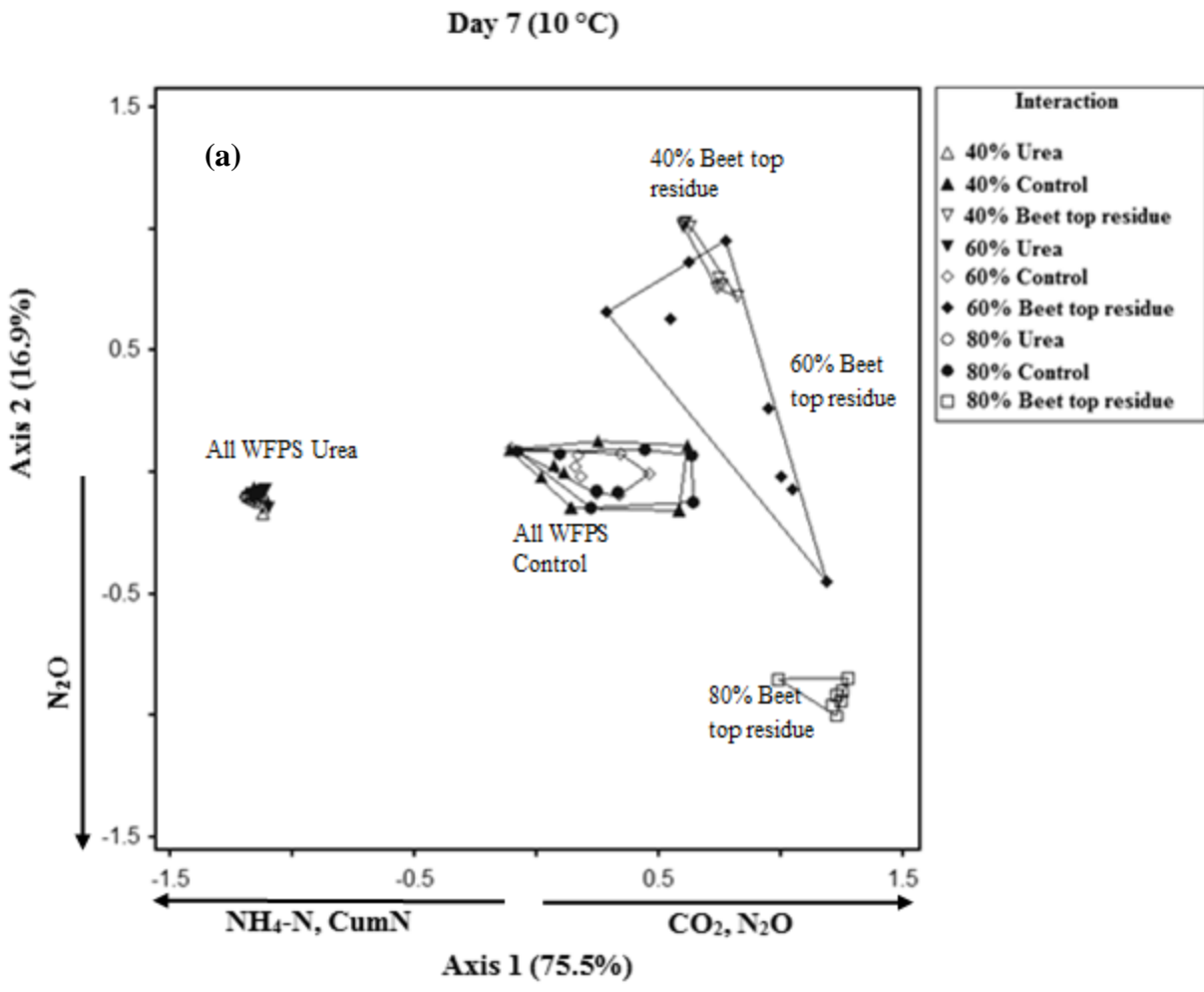


Fig. 16. Non-metric multidimensional scaling analysis (NMS) of amendment (N source) and water filled pore space (WFPS) effects on reactive N species and C mineralization on (a) 7 d at 10 °C (pre-freeze), (b) 14, 28 and 42 d at 10 °C (pre-freeze), (c) 112 d at -5 °C (freeze), (d) 142 d at 10 °C (thaw) and (e) 149 d at 25 °C (thaw). The proportion of total variance in the parameter matrix represented by Axes 1 and 2 is indicated in parentheses. Linear correlations between axis scores and variables significant at $p=0.05$ are indicated in the form of arrows along the 2 NMS axes.

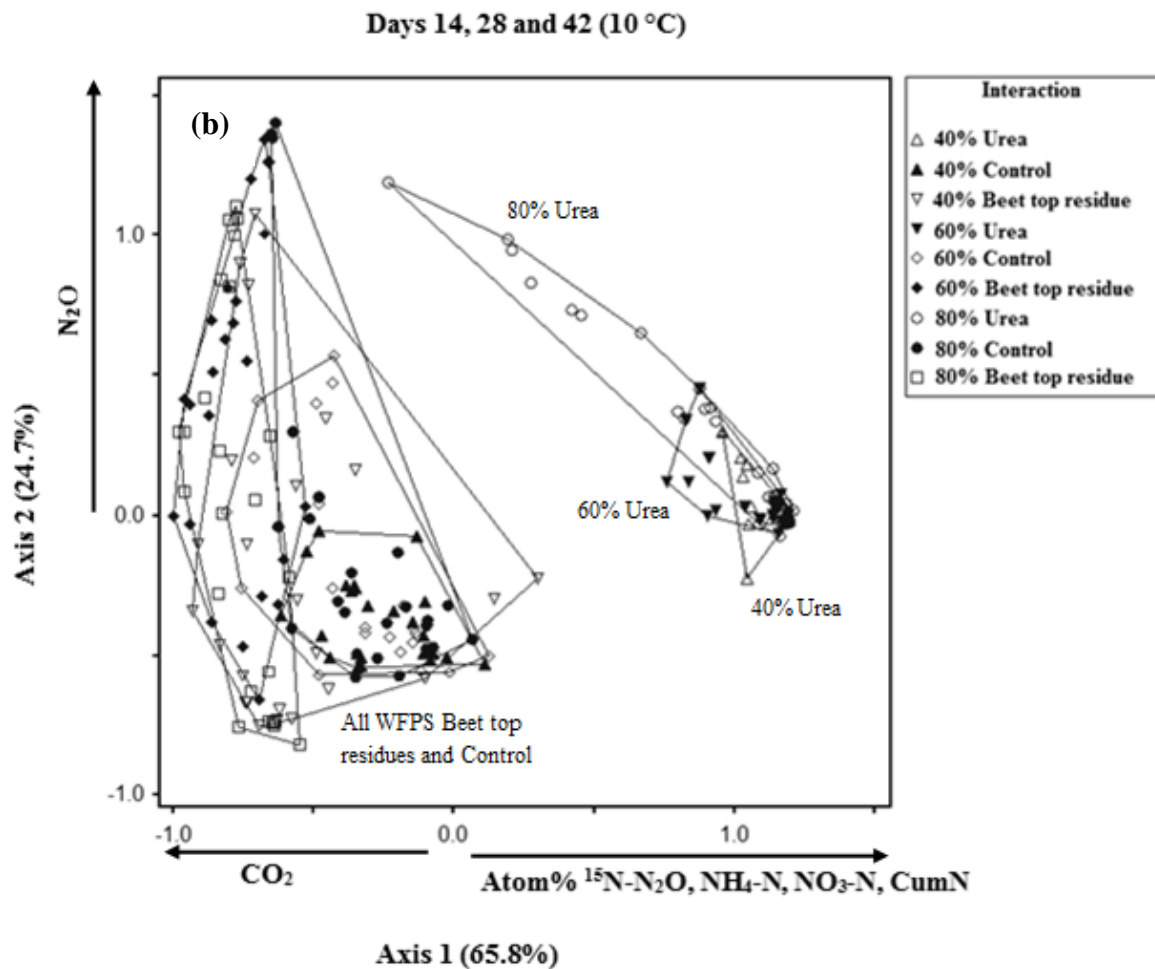


Fig. 16. Non-metric multidimensional scaling analysis (NMS) of amendment (N source) and water filled pore space (WFPS) effects on reactive N species and C mineralization on (a) 7 d at 10 °C (pre-freeze), (b) 14, 28 and 42 d at 10 °C (pre-freeze), (c) 112 d at -5 °C (freeze), (d) 142 d at 10 °C (thaw) and (e) 149 d at 25 °C (thaw) (continued). The proportion of total variance in the parameter matrix represented by Axes 1 and 2 is indicated in parentheses. Linear correlations between axis scores and variables significant at $p=0.05$ are indicated in the form of arrows along the 2 NMS axes.

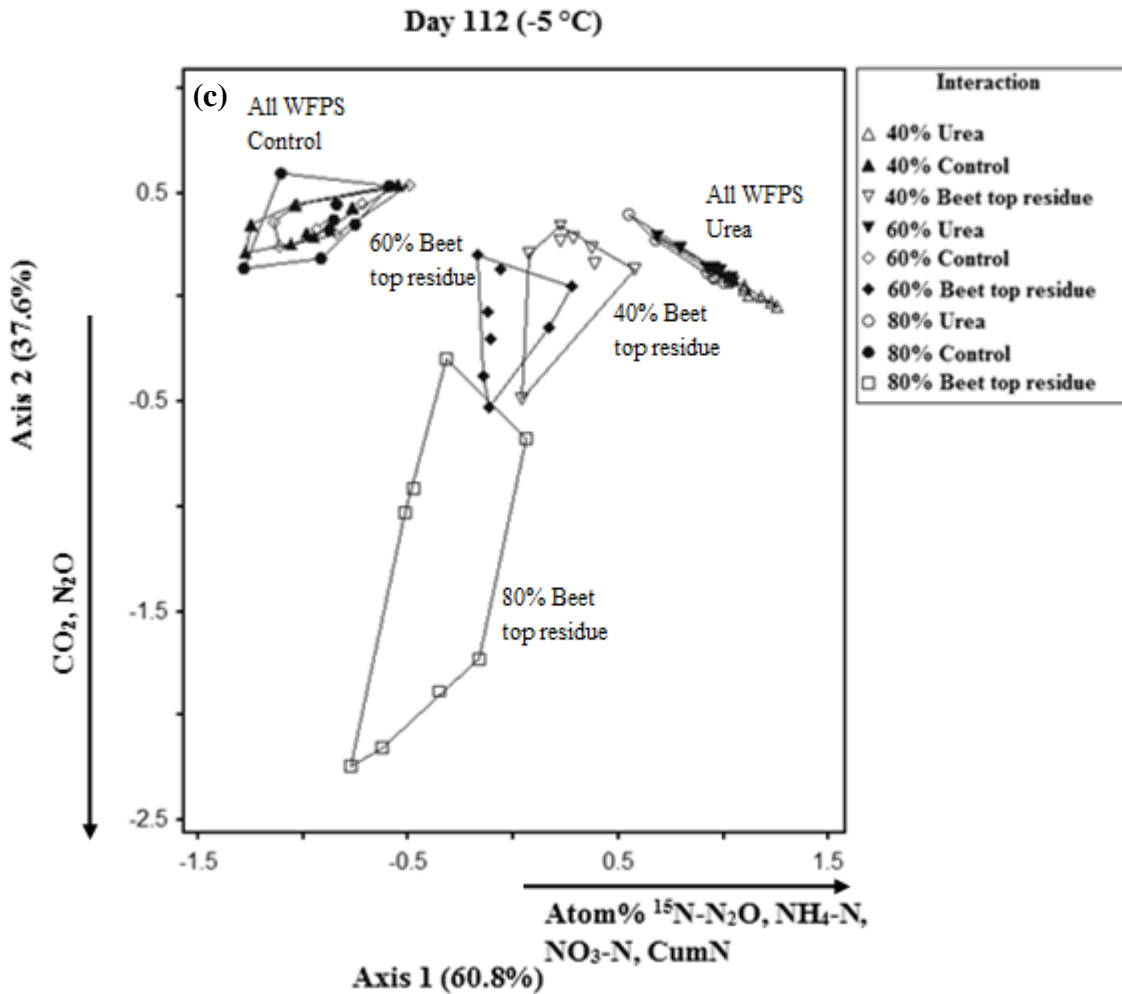


Fig. 16. Non-metric multidimensional scaling analysis (NMS) of amendment (N source) and water filled pore space (WFPS) effects on reactive N species and C mineralization on (a) 7 d at 10 °C (pre-freeze), (b) 14, 28 and 42 d at 10 °C (pre-freeze), (c) 112 d at -5 °C (freeze), (d) 142 d at 10 °C (thaw) and (e) 149 d at 25 °C (thaw) (continued). The proportion of total variance in the parameter matrix represented by Axes 1 and 2 is indicated in parentheses. Linear correlations between axis scores and variables significant at $p=0.05$ are indicated in the form of arrows along the 2 NMS axes.

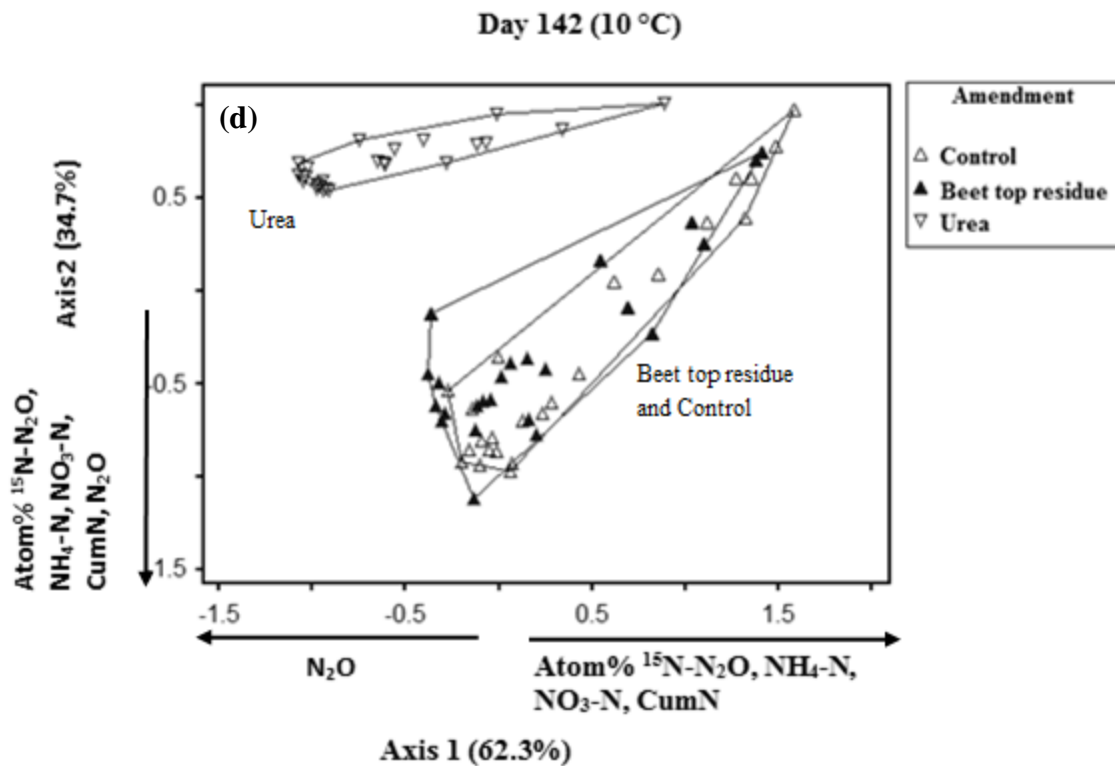


Fig. 16. Non-metric multidimensional scaling analysis (NMS) of amendment (N source) and water filled pore space (WFPS) effects on reactive N species and C mineralization on (a) 7 d at 10 °C (pre-freeze), (b) 14, 28 and 42 d at 10 °C (pre-freeze), (c) 112 d at -5 °C (freeze), (d) 142 d at 10 °C (thaw) and (e) 149 d at 25 °C (thaw) (continued). The proportion of total variance in the parameter matrix represented by Axes 1 and 2 is indicated in parentheses. Linear correlations between axis scores and variables significant at $p=0.05$ are indicated in the form of arrows along the 2 NMS axes.

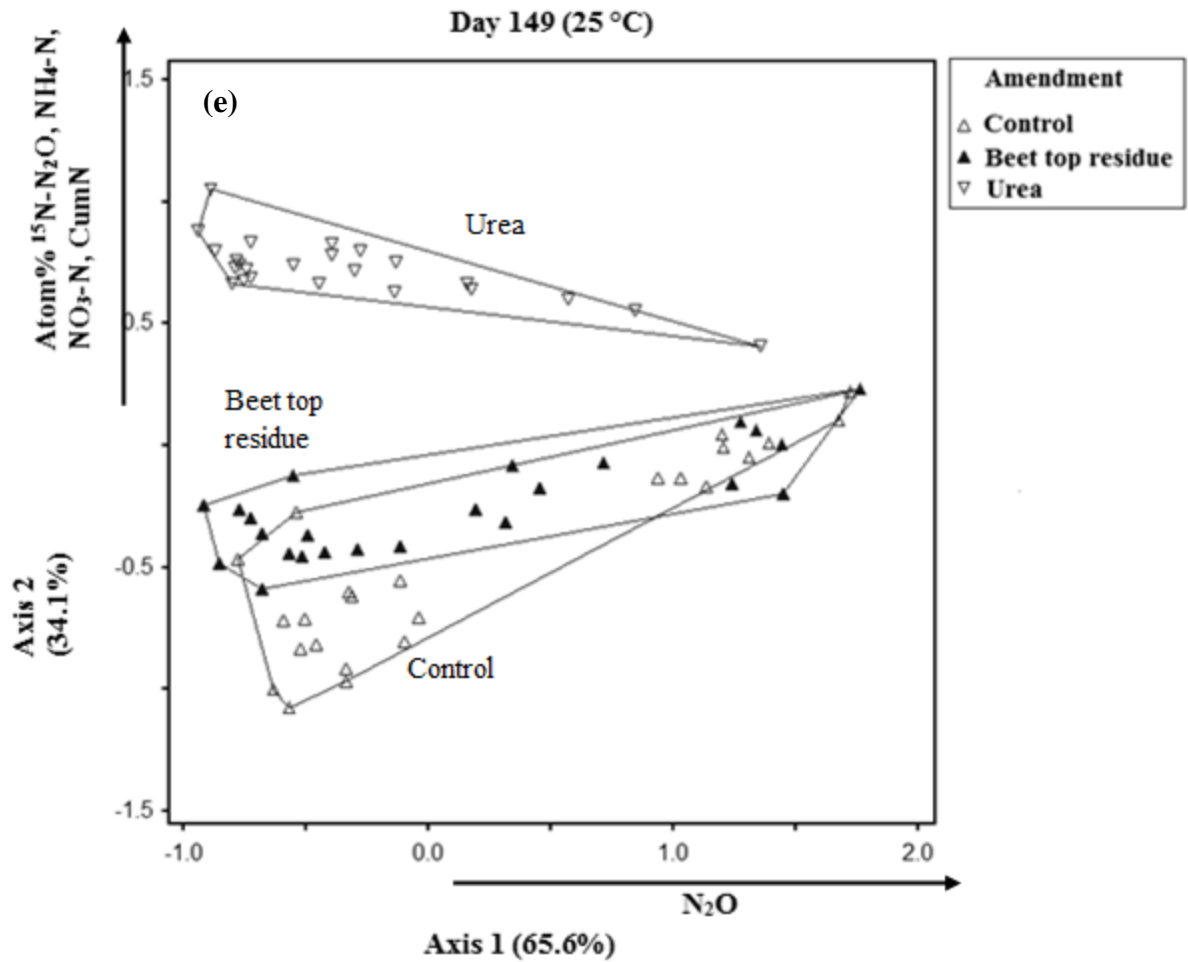


Fig. 16. Non-metric multidimensional scaling analysis (NMS) of amendment (N source) and water filled pore space (WFPS) effects on reactive N species and C mineralization on (a) 7 d at 10 °C (pre-freeze), (b) 14, 28 and 42 d at 10 °C (pre-freeze), (c) 112 d at -5 °C (freeze), (d) 142 d at 10 °C (thaw) and (e) 149 d at 25 °C (thaw) (continued). The proportion of total variance in the parameter matrix represented by Axes 1 and 2 is indicated in parentheses. Linear correlations between axis scores and variables significant at $p=0.05$ are indicated in the form of arrows along the 2 NMS axes.

Table 12. Results of permutational multivariate analysis of variance (PerMANOVA) testing the significance of the effects of water filled pore space (WFPS), N source (amendment) and interactions among these factors during a simulated freeze-thaw incubation.

Day 7; 10 °C					
Source	df	SS	MS	Pseudo-F	P (Perm)
WFPS	2	2.24	1.12	34.639	0.0002
N source	2	23.223	11.61	359.11	0.0002
WFPS*N source	4	4.2592	1.0648	32.931	0.0002
Residual	63	2.0370			
Total	71	31.759			
Day 14, 28 and 42; 10 °C					
WFPS	2	1.8460	0.92298	8.1081	0.0002
N source	2	53.799	26.900	236.31	0.0002
WFPS*N source	4	1.7041	0.42601	3.7424	0.0004
Residual	207	23.564	0.11383		
Total	215	80.913			
Day 112; -5 °C					
WFPS	2	1.0174	0.50872	13.443	0.0002
N source	2	12.395	6.1977	163.78	0.0002
WFPS*N source	4	1.9887	0.49717	13.138	0.0002
Residual	63	2.3841	0.0378		
Total	71	17.786			
Day 142; 10 °C					
N source	2	9.1462	4.5731	26.571	0.0002
Residual	69	11.875	0.17211		
Total	71	21.021			
Day 149; 25 °C					
N source	2	6.3833	3.1916	16.817	0.0002
Residual	69	13.095	0.18978		
Total	71	19.478			

Effect of tillage management, soil moisture and N source on the GWP potentials

Total GWP ($\text{N}_2\text{O} + \text{CO}_2$) was calculated taking into consideration N_2O and CO_2 , GHG fluxes from the various tillage and amendment interaction at 40, 60 and 80% WFPS. The cumulative N_2O -N lost during the 149 d incubation under simulated winter freeze-thaw conditions at 40% WFPS was very low and ranged from 0.06-0.89 mg N_2O -N kg^{-1} soil (Table 13). Thus, N_2O emissions did not contribute much to the total GWP except for the residue amended soil microcosms from the NT treatments which had the highest N_2O contribution (100 kg CO_2 eq ha^{-1}). The main contribution to the simulated winter freeze-thaw GWP was due to CO_2 emissions from the soil microcosms maintained at 40%. The GWP from the residue amended microcosms with soil taken from the NT treatment were higher (576 kg CO_2 eq ha^{-1}) as compared to the residue amended microcosms with soil taken from CT treatment (440 kg CO_2 eq ha^{-1}). Tillage did not have a significant effect on the total GWP from unamended and urea amended soil microcosms maintained at 40%.

Although tillage management did not affect the total GWP during the incubation at 60% WFPS, the amount of CO_2 and N_2O emitted varied due to tillage. With the residue amended microcosms, soil taken from the CT plots had higher cumulative N_2O -N emissions (2.12 mg N_2O -N kg^{-1} soil, ~1% of the total N supplied through residues) as compared to the residue amended microcosms with soil taken from the NT plots (0.95 mg N_2O -N kg^{-1} soil, ~0.4% of the total N supplied through residues) (Table 14). Almost 5% of the nitrates supplied by the residues were converted to N_2O in the residue amended soil microcosms from the CT plots. The contribution of N_2O to the total GWP was higher in the residue amended microcosms with soil taken from CT treatment as compared to NT, whereas, the residue amended soil microcosms

with soil taken from the CT treatment contributed less CO₂ to the total GWP as compared to the residue amended microcosms with soil from the NT treatment.

Overall the unamended and urea amended microcosms with soil taken from the NT plots maintained at 80% WFPS had higher simulated winter freeze-thaw total GWP as compared to their CT counterparts at 80% WFPS over the 149 d laboratory incubation (Appendix Table C1). The N₂O contribution to the simulated winter freeze-thaw GWP was significantly higher in the unamended and urea amended soil microcosms collected from the NT as compared to the CT treatment maintained at 80% WFPS. The residue amended soil microcosms collected from the NT treatment, although not statistically significant, also had higher trends of N₂O emissions as compared to the CT treatment. In contrast, the contribution of CO₂ to the GWP regardless of being unamended or urea and residue amended in the soil microcosms did not vary with the tillage treatment.

Table 13. Percent of total N applied in urea or sugar beet residues and as NO_3^- -N in sugar beet residues lost to nitrous oxide (N_2O) emissions and the contribution of carbon dioxide (CO_2) and N_2O emissions from soil at 40% water filled pore space (WFPS) to global warming potential (GWP) during a simulated winter freeze-thaw.

Amendment	Management	Total N	Cumulative	Total N	Nitrates [†]	N_2O [‡]	CO_2	Total
		mg kg ⁻¹	N_2O -N	as N_2O	as N_2O	kg CO_2 eq ha ⁻¹	GWP [§]	
Unamended	Clean tillage	-	0.06	-	-	32.8 c	208 d	240 d
	No tillage	-	0.06	-	-	32.8 c	189 d	222 d
Urea	Clean tillage	452	0.21	0.05	-	115 b	312 c	427 c
	No tillage	452	0.21	0.05	-	115 b	299 c	414 c
Beet	Clean tillage	226	0.28	0.12	0.60	154 b	1980 b	2134 b
	No tillage	226	0.89	0.39	1.89	485 a	2310 a	2795 a

[†]sugar beet top residues contained 47 mg NO_3^- -N kg⁻¹ soil.

[‡]Statistical significant differences (univariate two-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different lower case letters within each column; amendment × organic tillage management interaction (P<0.05).

[§] Total GWP= GWP due to both N_2O and CO_2 .

Table 14. Percent of total N applied in urea or sugar beet residues and as NO₃⁻-N in sugar beet residues lost to nitrous oxide (N₂O) emissions and the contribution of carbon dioxide (CO₂) and N₂O emissions from soil at 60% water filled pore space (WFPS) to global warming potential (GWP) during a simulated winter freeze-thaw.

Amendment	Management	Total N	Cumulative	Total N	Nitrates [†]	N ₂ O [‡]	CO ₂	Total
		mg kg ⁻¹	N ₂ O-N	as N ₂ O	as N ₂ O	kg CO ₂ eq ha ⁻¹	GWP [§]	
Unamended	Clean tillage	-	0.12	-	-	65.6 d	229 d	294 c
	No tillage	-	0.09	-	-	49.2 d	193 d	242 c
Urea	Clean tillage	452	0.54	0.12	-	295 c	331 c	626 b
	No tillage	452	0.55	0.12	-	300 c	312 c	612 b
Beet	Clean tillage	226	2.12	0.94	4.51	1158 a	1825 b	2983 a
	No tillage	226	0.95	0.42	0.89	519 b	2475 a	2994 a

[†]sugar beet top residues contained 47 mg NO₃⁻-N kg⁻¹ soil.

[‡]Statistical significant differences (univariate two-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different lower case letters within each column; amendment × organic tillage management interaction (P<0.05).

[§] Total GWP= GWP due to both N₂O and CO₂.

Discussion

C mineralization

In this incubation study higher CO₂ mineralized from the residue amended soil microcosms as compared to the urea amended or unamended soil microcosms is due to the supply of C from the sugar beet top residues that simulate the incorporation of a green manure cover crop or crop residue during late fall when the soil temperature is low (around 10 °C). Higher CO₂ mineralization from the residue amended microcosms with soil taken from the NT treatment might be due to the higher mineralizable C pools in the NT (3.06 Mg C ha⁻¹) as compared to CT (2.50 Mg C ha⁻¹) which could have partly mineralized during the first 42 d of the incubation at 10 °C (Bhowmik et al., 2016a). Dou et al. (2008) reported that placement of residues near the surface in NT as compared to CT might result in higher dissolved organic carbon concentrations that influence CO₂ emissions. Decreased microbial activity due to soil freezing at -5 °C for 70 d resulted in decreased CO₂ emission during the freeze period. The peak in CO₂ emissions observed during the thaw resulted from the release of C and N from soil, microbial biomass and sugarbeet tissue (where applicable) during the freeze. This research and those of others have shown that microbial activity increases at higher temperatures during thaws and may be augmented by the release of C and N substrates previously released from lysed cells and other sources during freezing (Prieme and Christensen, 2001; Schimel and Clein, 1996). Kurganova et al. (2007) also measured elevated CO₂ emissions from soil during a thaw period in a study determining the effects of winter freeze-thaw cycles on forested and arable land (under winter barley) in Germany. Almost negligible CO₂ emission from the unamended and urea amended soil microcosms confirmed that the C substrate was supplied by the sugarbeet residue, a source of carbon for heterotrophic microorganisms. Previous companion experiment verified

that biologically active C is limited in these systems and that soil organic C pools have not yet shifted after due to 3 yr of variable tillage management (CT vs NT) (Bhowmik et al., 2016a).

N mineralization, nitrification and denitrification

Higher NH_4^+ -N concentrations in the urea amended soil microcosms was result of hydrolysis and conversion of amide N in urea to NH_4^+ -N. This process cannot be considered to be mineralization in a true sense. The slightly higher NH_4^+ -N concentration in the urea amended NT soil microcosms may be the result of the trend of higher potentially mineralizable N in soil taken from NT treatments as compared to urea amended soil taken from the CT treatment (Bhowmik et al., 2016a). Increased urea hydrolysis due to the higher moisture content in the urea amended soil microcosms maintained at 80% WFPS resulted in higher NH_4^+ -N as compared to 40% and 60% WFPS urea amended soil microcosms. The NH_4^+ -N concentrations in the urea amended and residue amended soil microcosms were higher during the pre-freeze (7 to 42 d) and decreased during the freeze-thaw which may in part be due to the conversion of NH_4^+ -N to NO_3^- -N via nitrification.

The NO_3^- -N concentrations in the urea amended and residue amended soil microcosms increased 28 d after application of the N source. The time required for nitrification in this study is longer than other studies probably due to the fact that the maximum growth rate of nitrifiers decreases by more than half when the temperature decreases from 14 to 6 °C (Gujer, 2010). The lower amounts of soil NO_3^- -N in the urea amended NT soils might be potentially due to greater immobilization of the nitrates as compared to urea amended CT soils. The greater size along with high turnover rates of the slow pool C in the NT soils in this study might have aided the immobilization as compared to the CT soils (Bhowmik et al 2016a). Veldkamp et al. (1999) studied the effect of NT on restored pasture and determined that unmanaged pastures had higher

NH_4^+ -N concentrations but lowered NO_3^- -N concentrations and low rates of nitrification. The thawing of soils increased the NO_3^- -N concentrations in both the urea amended and the residue amended soils taken from either tillage management due to the fact that increases in temperature favor the growth and activity of nitrifiers in the soil (Gujer, 2010). Kushwaha et al. (2000) found that both tillage reduction and residue retention increased the proportion of total N present in the soil organic matter as microbial biomass. The effects of WFPS on nitrification in the residue amended soil microcosms was more prominent during the thaw period. My results show that nitrification is inhibited in residue amended soil microcosms maintained at 80% WFPS probably due to the fact that anaerobic conditions do not favor nitrification.

High input of NO_3^- -N without significant inputs of available C or soil C in urea amended soil microcosms led to an initial decrease in the ratio of NO_3^- -N / available C in the soil. This incubation data indicates that labile C, reduced conditions and low initial nitrates limited denitrification in these systems. Further evidence that C and NO_3^- were limited and could have reduced the potential for denitrification is that while urea contained double the inorganic N the residues contained higher initial NO_3^- and C. Higher N_2O emissions from the residue amended soil microcosms might be due to the supply of labile C, an energy source supplied by the beet top residues and potentially utilized by heterotrophic denitrifiers. In addition, the C added as residues increased all heterotrophic microbial activity (soil respiration) thereby creating more anaerobic sites that favor potential denitrification. Groffman and Crawford (2003) reported that CO_2 efflux and denitrification activity may be positively correlated. The N_2O emissions in the urea amended soil microcosms were significantly lower than the residue amended soil microcosm.

The N_2O emissions from the residue amended soil microcosms were high on 7 and 14 then decreased on 24 d which may indicate that the labile C from the beet top residue was

consumed by the heterotrophs as CO₂ emissions also began to drop prior to 24 d. The mean residence time of active pool C in the soils in the CT and NT treatments was estimated to be ~23 d at 25 °C. Thus, with time, soil C and turnover of C in microbial biomass may have mineralized and served as a source of energy for the heterotrophic denitrifiers which resulted in a second N₂O spike on 42 d from the residue amended soil microcosms at 10 °C (Bhowmik et al., 2016a). A similar reason might explain the increase in N₂O. On 42 d in the urea amended soil microcosms. The difference in the time corresponding to the increase in N₂O from the unamended microcosms with soil collected from NT and CT plots is probably due to the higher turnover rate of slow pool C in soil taken from the NT as compared to soil from the CT treatment which may have resulted in a lag in the N₂O emissions (Bhowmik et al. 2016a).

Freezing soil for 70 d decreased the microbial activity resulting in decreased N₂O emissions on 112 d. The increase in N₂O after the freeze-thaw may be the result of anaerobic microsites in soil that facilitate denitrification (Wagner-Riddle et al., 2008) physical release of N₂O trapped by ice or snow cover upon thawing (thus primarily influences the timing of flux) (Risk et al., 2013), and increased availability of labile organic C and mineral N in soil (due to absence of plant N uptake, disaggregation of soil aggregates upon freeze–thawing, microbial lysis due to low temperatures) (Schimel and Clein, 1996). The 5-fold increase in the N₂O emissions in the unamended soil microcosms maintained at 80% WFPS as compared to the 2-fold decrease in the residue amended soil microcosms maintained at 80% WFPS might emphasize the importance of returning plant residues to the soil when temperatures drop during winter freeze-thaw conditions. Bouwman et al. (2002) reported that in low N input systems including organic systems, the N₂O released by mineralization of soil organic matter and subsequent denitrification (background emission) may be substantial. On 149 d, the urea

amended NT soil microcosms had comparable or more N₂O emissions relative to the residue amended soil microcosms which suggest that the freeze-thaw may have released C from the slow C pool in the NT soil treatment providing an organic C source for heterotrophic denitrifiers to produce N₂O (Bhowmik et al., 2016a). In a study conducted by Senbayram et al. (2009), addition of N through synthetic N fertilizer (ammonium sulfate) revealed a pulse of N₂O emissions immediately after fertilizer application. The N₂O emissions increased for the next 2 d and then sharply decreased after 4 d. This might be due to the fact that their soils were NO₃-N limited and not C limited and that nitrification was not inhibited. The N₂O emissions from the soil microcosms increased as the moisture contents increased from 60% to 80% WFPS. The higher moisture contents can also create a greater shrink-swell effect during the freeze-thaw releasing more C and N at higher WFPS (Kurganova and Gerenyu, 2010). This supports earlier findings that increasing moisture levels increases the restriction in O₂ diffusion and availability (Chen et al., 2010; Menéndez et al., 2012). This also supports the fact that factors driving the denitrification process are redox potential, substrate and oxygen diffusion which strongly depend on water availability and free pore space in soil (Bateman and Baggs, 2005).

Determining the source of N in N₂O by isotopic ¹⁵N-N₂O values

The ¹⁵N-N₂O atom% values indicate that after addition of 5% ¹⁵N labelled urea to the soil microcosms most of the N source for the denitrification process was derived from soil nitrates and not urea fertilizer. However, gradually with time the N from urea was nitrified and the ¹⁵N-N₂O atom% values indicate that the source of N lost as N₂O was mostly from urea compared to native soil N. My data verifies that by the end of the pre-freeze event on 42 d almost all the N₂O was derived from fertilizer N in the urea amended soil microcosms. The increase in the atom% ¹⁵N-N₂O corresponded well with the increase in NO₃-N concentrations in the urea amended soil

microcosms with time and followed the same trend after the freeze-thaw event on 142 and 149 d, respectively. The high atom% ^{15}N - N_2O values close to 0.9% in the 1% ^{15}N labelled residue amended soil microcosms indicated that during the pre-freeze NO_3^- leached from the beet top residues contributed to N_2O emissions. This trend in N_2O emissions also explains why NO_3^- -N levels were low during the pre-freeze-thaw. However, after the freeze-thaw event on 142 d, ^{15}N - N_2O fractionation in the residue amended soil microcosms maintained at 40% and 60% WFPS was observed. This suggests that the N contributing to the N_2O emissions was partly derived from NO_3^- in beet residues and the mineralization of organic N in beet residues that underwent the process of nitrification. The scope of this study does not allow us to directly determine if the source of the N_2O is nitrification or denitrification. Senbayram et al. (2009) in a labelled N isotope study, showed that almost all N_2O emissions were derived from synthetic N fertilizer (ammonium sulfate) in soils maintained at 76% WFPS. Thus, according to Bateman and Baggs (2005), soil microsites become predominantly anaerobic at ≥ 60 percent WFPS and promote greater N_2O emissions through the denitrification process because of limited O_2 availability. Therefore, it can be assumed the higher N_2O emissions from soil microcosms maintained at 80% WFPS is primarily from the process of denitrification.

Tillage and moisture effects on GHGs and its contribution to simulated winter freeze-thaw GWP

One of the major objectives of this study was to simulate the effect of cover crops/crop residue or urea fertilizer application to soils of differing tillage management during late fall when temperatures are low in the Northern Great Plains and the potential for GHG emissions has not been sufficiently determined or estimated. In this study the soil microcosms maintained at 40% WFPS would simulate moisture conditions near field capacity (FC). Higher N_2O emissions in the

NT residue amended soil microcosms as compared to CT suggest that residues added on the surface of the soil microcosms might have retained more moisture thereby creating more anaerobic microsites for N₂O emission. The soil microcosms maintained at 60% WFPS would reflect field conditions after a ~ 15 cm rainfall event. The amount of N added to the urea amended soil was double the N supplied by the beet top residues. However, the cumulative N₂O-N emissions were more than double in the residue amended soil microcosms as compared to the urea amended soil microcosms. This emphasizes the fact that the rate of N fertilization alone does not always allow us to predict the magnitude of N₂O emission. Specifically, at 60% WFPS which is considered to be optimum for C mineralization, the availability of labile C which serves as an energy source for the denitrifiers is crucial in estimating the N₂O emissions from agroecosystems containing limited active C. The higher N₂O emissions in the residue amended CT as compared to NT soil microcosms might be due to the fact that incorporation of beet tops to the soil microcosms increases its contact with soil and increases the availability of NO₃⁻ and C available to denitrifiers resulting in higher cumulative N₂O emissions.

Higher trends in cumulative N₂O emitted from soil taken from the NT treatment as compared to the CT treatment in microcosms maintained at 80% WFPS was measured. However, the climatic conditions and soil properties in the region suggest that having an 80% WFPS in the field would be a rare phenomenon as the soils are well drained and light textured. Even after a heavy rainfall these conditions would not last longer except if the downward flow of water was hindered by a restrictive layer (e.g. an argillic horizon).

The most important factors in determining N₂O emissions are duration of NT management, climate (Six et al., 2004) and organic material returned to the soil. Based on a literature review by Six et al. (2004), during the first 10 years after conversion from CT to NT

the N₂O fluxes were higher in NT as compared to CT, managements regardless of the climate. However, after 20 years, climate played a significant role in determining the effect of NT on N₂O emissions. Nitrous oxide emissions in humid climates were lower in NT than CT systems whereas in dry climates no effect of tillage systems were observed. Regina et al. (2010) reported that 5-7 years of NT management in Finnish soils resulting in 21 to 86% higher N₂O emissions as compared to tilled soil. There are studies which indicate that even after 30 years of NT management the in situ N₂O flux increased by 39% (Oorts et al., 2007). The highly variable results found by researchers would not be surprising as tillage affects a number of biophysical and chemical characteristics which regulate the N₂O emissions (Mangalassery et al., 2014; Snyder et al., 2009) and tillage alone does not take into account biomass of cropping systems returned or addition of animal amendments.

In this study, the certified organic plots were under NT management for only 3 years. Similar increases in N₂O fluxes were also observed following adoption of NT in recently established NT systems (Vetsch and Randall, 2000) where increased N₂O emissions were associated with observed N deficiencies (Linn and Doran, 1984). Keller et al. (1993) reported threefold higher N₂O-fluxes in recently formed pastures than in the original forest, but 10 yr after conversion N₂O-fluxes were lower in the pasture than in the original forest.

Trends of higher CO₂ emission were observed in the residue amended soil microcosms from NT as compared to CT. However, one would expect that the decomposition of plant residues in NT due to reduced soil-residue contact would result in lower CO₂ emissions as compared to CT practices where the residues are fully incorporated (Drury et al., 2006). However, Abdalla et al. (2013) measured no consistent trend in CO₂ emissions due to tillage effect under dry/cool climatic conditions. In this study, total SOC had a higher trend in the NT

treatment ($45.3 \text{ Mg C ha}^{-1}$) as compared to CT ($42.4 \text{ Mg C ha}^{-1}$) treatment whereas the CO_2 emissions per unit SOC was lower in NT as compared to CT which shows a greater potential of the NT soil to accrue C and reduce CO_2 emissions. Several studies have reported minimal C sequestration with adoption of NT in the North American Great Plains Region if NT is not coupled with high inputs of crop biomass or crop intensification (Cavigelli et al., 2013; Halvorson et al., 2002; Norton et al., 2012).

The NT and CT organic management systems in this study were low C input system as they mainly contained small grains that did not return sufficient biomass to augment C and N stocks. Bhowmik et al. (2016a) in a recent study on the same plots reported that there was no significant difference in the total SOC and slow pool C in the NT as compared to the CT organic systems. They also concluded that the turnover rates of the slow pool in the NT organic systems were higher than the CT organic systems. Adoption of NT in dry climates has been reported to lead to an initial loss of C from the 0-30 cm soil layer for the first 5 years (Hendrix, 1997). This early trend might be due to slower incorporation of surface residues into the soil through soil faunal activity as compared to corresponding CT systems where residues are incorporated through plowing. Thus, it is not necessarily surprising to assume that these NT organic systems are in a transitional phase and have not attained a steady state. The time required to reach a steady state varies with respect to climate, soil types and management practices and it would be interesting to see if at steady state this NT organic agroecosystem might reduce GHG emissions under these dry/cool climatic conditions.

In conclusion, this study takes into account the combined effects of climate and biomass inputs on GHG emissions in 2 USDA certified organic agroecosystems with varying tillage management during a simulated winter freeze thaw (November to March) in the Northern Great

Plains. My results indicate that dry, cool climates coupled with low biomass inputs does not allow the buildup of stores of C that can potentially immobilize reactive N and reduce the loss of N as N₂O. Moreover, the duration of NT management in the 2 USDA certified organic systems was relatively short (3 yr). Therefore, one can assume the tillage systems are in the transition phase which may limit the benefits associated with no till. Due to the relatively low inputs of C in this 5 yr rotation dominated by small grains and mixed cover crops and arid, frigid climate, these systems may take more than a decade to attain an equilibrium or steady state.

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GENERAL CONCLUSIONS

This research addresses the effects of best management practices (BMP) (tillage, application of organic amendments, livestock integration and land use) and climate on the cycling of carbon (C) and nitrogen (N) in 5 different organic agroecosystems. This study simulates factors such as temperature i.e. changes in the duration and intensity of the freeze and thaw during winter (in an incubator), seasonal variations in C and N (by addition of sugarbeet top residues or urea) and rainfall (by maintaining soil microcosms at 40%, 60% and 80% WFPS) that modify the niches of microbiological indicators like ammonia oxidizing bacterial and archaeal nitrifiers as well as reactive N species and CO₂ emissions from soils. This study verifies the potential application and value of using permanganate oxidizable organic carbon and hydrolyzable C (C that can undergo hydrolysis reactions) as soil health indicators for rapid (within 2 to 4 yr) assessment of shifts in biologically active C resulting from change in organic management. More importantly the shifts in these indicators can be strongly correlated with C and N inputs and the microbiological drivers that regulate N cycling in these soils. Multiple short duration freeze-thaw events, sub-zero temperatures that persist for a few days, (particularly under saturated conditions) do not reduce the N cycling microorganisms or the processes they perform sufficiently to diminish N₂O and CO₂ emissions but release additional labile C and reactive N from soil and organic amendments that fosters greenhouse gas production.

Archaeal and bacterial nitrifier *amoA* gene copies varied due to shifts in reactive N species during freeze-thaws. Under conditions favorable for the denitrification process, the long-term organic mixed-compost systems displayed the potential to minimize N losses to N₂O by immobilizing excess available nitrates due to increases in C pools or total soil C as compared to the broiler litter and pasture systems. The data also indicate that the potential of organic

management systems to immobilize the reactive N and reduce the loss of N as N₂O in dry/cool climates receiving low biomass inputs is limited as these systems do not allow the buildup of stores of C even with reduced disturbance.

This research applied novel indicators of soil health that included hydrolyzable C and permanganate (0.02 M) oxidizable C (POXC) both of which represent shifts in biologically active C as well as nitrifier gene copies that partly control the process of nitrification that produces N₂O as a byproduct and NO₃⁻ as a final product which is also used as an energy source by heterotrophic denitrifiers. These indicators of soil health were successfully employed to determine the impact of BMP (annual vs perennial, NT vs CT, animal manure additions) on C and N cycling and GHG emissions. Organic systems in climates that support diverse crop rotations that include cover crops, integration of animals and or their manure and reduced disturbance were found to play a pivotal role in mitigating climate change through the buildup and retention of C and N pools which in turn was shown in some instance to reduce GHG emissions (N₂O and CO₂) by immobilizing inorganic N in soil. The two BMP that augmented soil C and N pools and reduced decomposition of organic matter were addition of organic inputs (plant biomass, animal manure) and minimizing disturbance (annual pasture, no till). This study also suggests that by reducing disturbance alone in the form of tillage without addition of sufficient organic inputs the potential of an organic agroecosystems to mitigate GHG emissions and foster soil health is minimized and could lead to higher GHG emissions from organic N inputs such as residue relative to synthetic fertilizers such as urea. Thus, a holistic approach is of pivotal importance to determine the management practices that could be adopted by organic growers to improve soil health and fertility as well as a means to mitigate climate change by reducing the GHG footprint of a given organic agroecosystem.

Table A1. Important field operation dates in the LOTS plots, 2011-2013.

Crop	2011	2012	2013
Planting date			
Winter wheat	13 Sep 2010	30 Sep 2011	30 Sep (2012); reseed with spring wheat 07 May
Proso millet/Oat-Field Pea-Radish-Turnip (polyculture) cover crop (when fall moisture supported seeding)	29 June	--	26 Aug
Hairy vetch cover crop	13 Aug 2010	See 2011	Reseed with spring pea 07 May
Winter rye cover crop	13 Sep 2010	See 2011	See 2012
Buckwheat†	24 June	--	--
Navy bean	--	25 May (CT) 21 June (NT)	11 June (CT) 25 June (NT)
Flax/Lentil (intercrop)‡	15 June	--	--
Spring pea	--	23 April	8 May
Millet	24 June	24 May	7 June
Harvesting date			
Winter wheat	25 Aug	20 July	16 Aug
Proso millet/Oat-Field Pea-Radish-Turnip (polyculture) cover crop (when fall moisture supported seeding)	9 Aug (mowed)	Not seeded (too dry)	26-27 Aug
Buckwheat†	7 Sep (swathed); hvstd 19 Sep	--	--
Flax/Lentil (intercrop)‡	7 Sep (swathed); hvstd 19 Sep	--	--
Spring pea	--	19 July	12 Aug
Navy bean	--	03 Oct (CT)	Not recorded
Millet	15 Sep (swathed); hvstd 23 Sep	15 Sep (swathed); hvstd 21 Sep	Not recorded
Other field operations			
Terminate cover crops	HV: 7 & 20 July	HV: 14 & 29 June; Rye: 15 & 29 June	HV: 18 July; Rye: 6 June & 21 July
Tillage	--	21 June & 09 July – CT, row cultivation (navy bean)	1 July – CT, row cultivation (navy bean)
Plant fall cover crop	HV – 8 Sep, rye 15 Sep	HV -15 Aug, rye 30 Sep	HV – 6 Sep rye - 28 Sep 26-30 Aug (pea); Not recorded (wheat & millet)
Grazing	--	Not recorded	

† In 2012, buckwheat was replaced by navy bean/winter rye cover crop.

‡ In 2012, flax/lentil was replaced by field pea/hairy vetch cover crop.

Table A2. Carbon and nitrogen content of the amendments added in the IFSYS plots, 2003-2013.

Year	NH₄-N	C:N ratio	Application rate	Total C applied	Total N applied
	mg kg ⁻¹		-----Mg ha ⁻¹ -----		kg ha ⁻¹
Broiler litter					
2003	3324	11	4.0	1.9	170
2004	-	12	5.0	2.2	180
2005	5405	12	6.0	2.9	240
2006	3432	13	6.0	2.1	167
2007	8725	10	5.6	1.9	201
2008	1685	10	6.7	2.2	218
2009	9207	12	5.2	1.9	154
2010†	5380	17	3.4	1.2	68
2012	5903	11	4.3	1.3	113
2013	1411	12	5.7	1.2	98
Mixed-compost					
2003	998	15	30	7.9	540
2004	-	15	31	7.6	500
2005	213	16	39	9.6	630
2006	770	14	38	10.0	738
2007	1520	15	22	7.4	488
2008	2140	14	19	5.6	403
2009	5120	14	14	4.1	291
2010	6360	18	16	5.5	316
2012	94	12	18	4.9	425
2013	564	13	24	4.5	396

†No amendments were applied in 2011.

Table A3. Vegetable planting, harvest and field operation dates in the IFSYS plots, 2006-2013.

Crop	2006	2007	2008	2009	2010	2012	2013
Planting date							
Broccoli	11 May	22 May	21 May	5 June	1 June	17 May	-
Winter squash	29 May	11 June	2 July	8 June	8 June	18 May	-
Snap bean	29 May	25 May	-	-	-	-	-
Spinach	15 Aug	8 Aug	-	-	-	-	-
Lettuce 1	-	-	23 May	19 May	19 May	4 June	24 June
Lettuce 2	--	-	11 July	21 July	21 July	7 Aug	7 Aug
Harvest date							
Broccoli	3 July	13 July	16 July	29 July	20 Aug	23 July	-
Winter squash	21 Sept	25 Sept	6 Oct	3 Sept	21 Sept	12 Sept	-
Snap bean	7 Aug	Aug	-	-	-	-	-
Spinach	29 Sept	21 Sept	-	-	-	-	-
Lettuce 1	-	-	9 July	30 June	8 July	17 July	30 July
Lettuce 2	-	-	25 Aug	24 Aug	27 Aug	11 Sept	12 Sept
Field activities							
Terminate cover crops	10 Apr	9 Apr	2 Apr	4 May	30 Apr	23 Apr	13 May
Apply amendments	12 Apr	11-12 Apr	8 Apr	7 May	11-12 May	26-27 Apr	15 May
Plant fall cover crop	6 Sept	5 Sept	29 July	12 Aug	†	28 Aug	3 Oct
Plant pasture	4 Oct	27 Sept	4 Oct	18 Sep	†	17 Oct	3 Oct

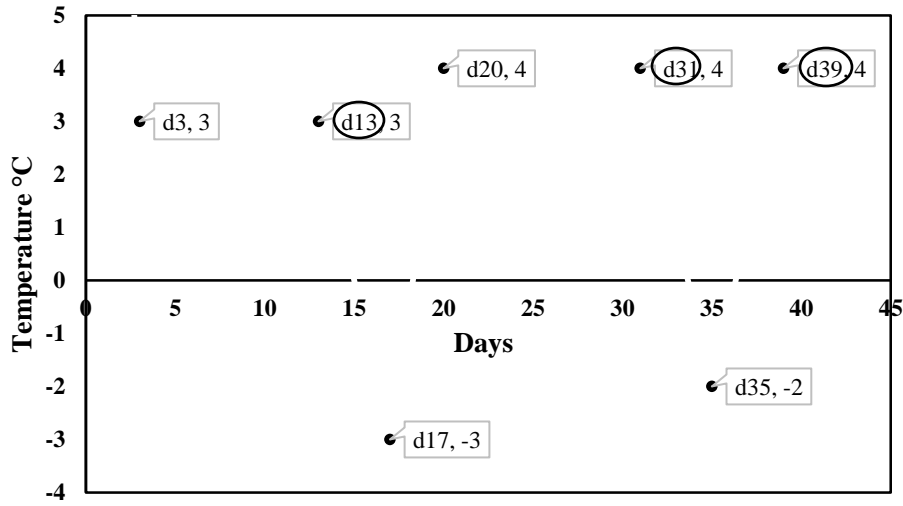
†Winter wheat was planted in the fall of 2010 in place of cover crops.

APPENDIX B. THE SUPPLEMENTARY DATA FOR PAPER 2

Table B1. The atom % ¹⁵N-N₂O concentrations in amended and unamended soil microcosms maintained at 40%, 60% and 80% (Water filled pore space) WFPS during the incubation.

Time	Amended			Unamended		
	WFPS					
	40%	60%	80%	40%	60%	80%
	¹⁵ N-N ₂ O†					
days	atom %					
3	0.69 def	0.75 bcd	0.78 abc			
13	0.68 efg	0.78 abc	0.79 ab			
17	0.62 gh	0.73 cdef	0.80 ab			
20	0.51 i	0.62 gh	0.80 ab		0.37-0.40 j	
31	0.60 h	0.69 def	0.83 a			
35	0.66 fg	0.74 bcde	0.74 bcde			
39	0.53 i	0.72 cdef	0.73 cdef			

† Statistical significant differences (univariate three-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different lower case letters; Incubation time × WFPS × Amendment, *P*<0.05.



• represents the time intervals when soil and gas sampling was conducted

○ represents the time intervals when soil DNA was extracted

Fig. B1. Distribution of sampling time points for the IFSYS incubation experiment.

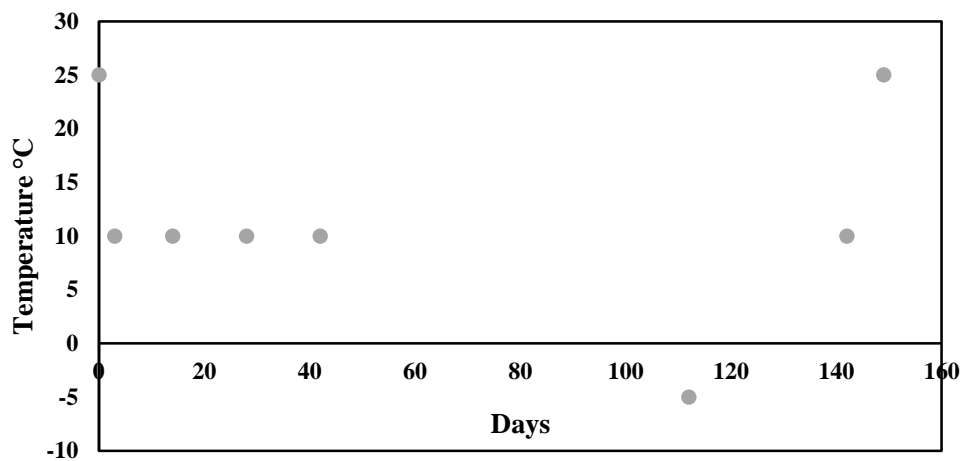
Table C1. Percent of total N applied in urea or sugar beet residues and as NO_3^- -N in sugar beet residues lost to nitrous oxide (N_2O) emissions and the contribution of carbon dioxide (CO_2) and N_2O emissions from soil at 80% water filled pore space (WFPS) to global warming potential (GWP) during a simulated winter freeze-thaw.

Amendment	Management	Total N	Cumulative	Total N	Nitrates [†]	N_2O [‡]	CO_2	Total
		mg kg ⁻¹	N_2O -N	as N_2O	as N_2O	kg CO_2 eq ha ⁻¹	GWP [§]	
Unamended	Clean tillage	-	1.03	-	-	563 c	234 c	797 c
	No tillage	-	0.13	-	-	71.0 d	200 c	271 d
Urea	Clean tillage	452	1.00	0.22	-	546 c	330 b	876 c
	No tillage	452	1.52	0.34	-	830 b	321 b	1152 b
Beet	Clean tillage	226	3.64	1.61	7.74	1989 a	2135 a	4124 a
	No tillage	226	3.92	1.73	8.34	2142 a	2218 a	4360 a

[†]sugar beet top residues contained 47 mg NO_3^- -N kg⁻¹ soil.

[‡]Statistical significant differences (univariate two-way ANOVA in PROC GLM, *post hoc*: Fischer LSD) are indicated by different lower case letters within each column; Amendment × organic tillage management interaction ($P < 0.05$).

[§] Total GWP= GWP due to both N_2O and CO_2 .



● represents the time intervals when gas sampling was conducted

Fig. C1. Distribution of sampling time points for the LOTS incubation experiment.