2019

Nitrous oxide release from agricultural soils under different management practices during freeze-thaw cycles

Julia M. Pupko
University of Vermont

Nitrous oxide release from agricultural soils under different management practices during freeze-thaw cycles

Julia Pupko

Senior thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science
Environmental Sciences Program and Honors College
University of Vermont
2018

Advisors:
Elizabeth (Carol) Adair, Ph.D., University of Vermont
Aimée Classen, Ph.D., University of Vermont
Eric Roy, Ph.D., University of Vermont

Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas (GHG) that has a radiative effect 298 times stronger than carbon dioxide (CO_2) and is a source of ozone-depleting nitrogen oxides (Congreves et al., 2018). Agricultural soils are responsible for 75% of human-induced N_2O emissions and for 23-31% of annual global emissions (Risk et al., 2013). Winter conditions may become more variable as climate change continues, potentially causing more freeze-thaw cycles (FTCs) and driving annual N_2O emissions higher as a result. While both agricultural management practices and FTCs are known to cause large N_2O fluxes, few studies have looked at the interactions between the two. This study built off of an incubation study by Adair et al. (2019) that found agricultural soils from a continuous corn system subject to manure injection had significantly higher N_2O and CO_2 emissions than soils with manure broadcasted and incorporated by plow (broadcast + plow) during thawing and FTC treatments. In this study, we examined mechanistic drivers behind these differences. We anticipated gas fluxes to follow the same trend as the previous study, anticipated higher rates of denitrification and higher microbial biomass to be found in the injection soils, and expected to see differences in extracellular enzyme activity, nitrate, nitrite, and ammonium availability between treatments. We conducted an 8 day incubation study with homogenized soil samples from three injection plot replicates and three broadcast + plow plot treatment replicates. Soil subsamples were placed in air-tight Ball mason jars and were subject to either frozen (-7^oC) or thaw (10^oC) temperature treatments. Half of the jars subject to each temperature treatment had an anaerobic headspace (N_2) to promote denitrification and the other half had an ambient headspace. Half of each of the headspace treatments were inhibited with acetylene to determine total potential denitrification. Our results only partially supported our hypotheses, with inconsistencies between our findings and those of Adair et al. (2019): broadcast + plow soils had marginally higher rates of denitrification and N_2O fluxes were highest from freeze treatment. As predicted, both denitrification and N_2O flux were highest under anaerobic conditions, suggesting that denitrification was the primary source of N_2O flux. Bacterial biomass (gram positive, gram negative, and total bacterial biomass) and total fungal biomass were higher from the injection plots which partially supported our initial hypotheses. Inconsistencies in our results suggest that soil homogenization may have influenced microbial activity and aggregate dynamics.

Acknowledgements

I would first like to thank Dr. Carol Adair for all of the assistance she gave me with selection of the topic and methods, design of the experiment, and data analysis in R. I would also like to thank Kyle Dittmer for his endless support and continual help throughout this process. I would like to thank Dr. Eric Roy and Dr. Aimée Classen for advising me with their invaluable insight into the world of soils. I would like to thank Lindsay Barbieri for lending a hand during the incubation study and for always providing me with guidance whenever I needed it. Lastly, thanks to the GUND Institute for providing funding for this project.

Table of Contents

1. Introduction

The human population is currently at 7.3 billion and is expected to reach 8.5 billion by 2030 (United Nations, 2015). World population has increased by 111% from 1961 to 2005 with a parallel growth in agricultural production due to technological, biological, and chemical advances that began during the Green Revolution (Burney et al., 2010). Agricultural gains (162% increase in total) were primarily intensive: global cropland grew by 27%, but yield increased by 135% (Burney et al., 2010). This is a success in terms of production, but has had unclear environmental implications. Conversion of natural spaces, pesticide pollution, and fertilizer runoff have all added to the anthropogenic footprint. Additionally, agriculture is responsible for 23-31% of global greenhouse gas emissions (GHG), including carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4) (Burney et al., 2010; Charles et al., 2017), which contribute to anthropogenic global climate change. In turn, climate change is adding to the challenges of food security through increasingly severe and unpredictable weather patterns, droughts, and disease (Ren et al., 2018). Unsustainable agricultural practices such as conventional tillage, excessive and untimely fertilizer application, and fallow periods create a positive feedback loop: more intensive agricultural techniques are used to counteract losses due to climate change, but these practices may, in turn, enhance climate change by increasing GHG emissions. To ensure food security and mitigate environmental damages, it is critical to find sustainable agricultural techniques that reduce GHG outputs and pollution.

Agricultural soils are estimated to account for over 75% of anthropogenic N_2O emissions (Inselsbacher et al., 2010; Dong et al., 2015), a GHG 298 times more powerful than carbon dioxide (CO_2) for trapping energy (Koponen et al., 2006; Inselsbacher et al., 2010; Butterbach-Bahl et al., 2013; Risk et al., 2013), which is also a source of ozone-depleting nitrogen oxides (NO and NO_2) (Charles et al., 2017). Large emissions of N_2O have been recorded surrounding freeze-thaw cycles (FTCs) which can account for up to 73% of the annual soil N_2O emissions (Wagner-Riddle et al., 1997; Röver et al., 1998; Wagner-Riddle et al., 2017; Congreves et al., 2018). N_2O emissions result from a combination of biotic and abiotic factors (Wallenstein et al. 2006; Butterbach-Ball et al. 2013; Risk et al., 2013), with microbial processes in soils, sediments, and water bodies being the main source of N_2O emissions. Abiotic sources of nitrous oxide, or chemical reactions such as chemodenitrification (decomposition of nitrite), have been found to be generally small relative to biotic processes (Bremner, 1997; Chang and Hao, 2001; Risk et al., 2013). Microbial processes in soils, which contribute to about 70% of the total known sources of N_2O (Conrad, 1995; Chang and Hao, 2001; Butterbach-Bahl et al., 2013) include: denitrification, nitrification, nitrifier-denitrification, and coupled nitrification-denitrification, where nitrogen-based compounds are oxidized and reduced by microbes during consumption (Phillips, 2008; Risk et al., 2013; Németh et al., 2014). N_2O production in agricultural soils is considered to be largely the result of incomplete denitrification (Duncan et al., 2017), as N_2O is an obligatory intermediate (rather than a byproduct) of denitrification and will be the end product if further reduction to N_2 does not occur (Risk et al., 2013). In comparison, N_2O produced by nitrification is a byproduct of hydroxylamine oxidation (Pathak, 1999; Wrage et al., 2001; Risk et al., 2013) and is not an obligatory intermediate, as in denitrification (See Table 1).

Table 1 Microbial processes

Process	Pathway steps	Associated genes	References
Denitrification	NO_3^- —(Nitrate reductase)→ NO_2^- —(Nitrite reductase)→ NO —(Nitric Oxide reductase)→ N_2O —(N_2O reductase)→ N_2	*nar*G, *nap*A, *nir*K, *nir*S, *nop*Z, *nor*B, *qnor*, *nos*Z	Saggar 2013; Ligi et al., 2014; Chen et al., 2015; Hu et al., 2015
Nitrification	NH_3 —(Ammonia monooxygenase)→ NH_2OH —(Hydroxylamine oxidoreductase)→ NO_2^- —(Nitrite oxidoreductase)→ NO_3^- (N_2O from NH_2OH; N_2O from NO_2^-)	*amo*A, *hao*, *nxr*B, *cnor*B, *qnor*B, *nor*YS	Pathak, 1999; Wrage et al., 2001; Risk et al., 2013; Hu et al., 2015; Heil et al., 2016
Nitrifier denitrification	NH_3 —(Ammonia monooxygenase)→ NH_2OH —(Hydroxylamine oxidoreductase)→ NO_2^- —(Nitrite reductase)→ [NO] —(Nitric oxide reductase)→ N_2O —(N_2O reductase)→ N_2 (N_2O from NH_2OH; N_2O from NO_2^-)	*amo*A, *hao*, *nir*S, *nir*K, *nxr*B, *cnor*B, *qnor*B, *nor*YS	Wrage et al., 2001; Zhu et al., 2013; Hu et al., 2015
Coupled nitrification-denitrification	Nitrification is carried out by nitrifiers, producing nitrate (see "Nitrification" above), which is then reduced by denitrifying microorganisms (see "Denitrification" above). This process occurs in soils where microsites contain conditions that allow nitrification and denitrification to proceed in close proximity. Nitrification can decrease O_2 concentrations in the soil, further promoting coupled nitrification-denitrification.	*amo*A, *nar*G, *nap*A, *nirK*, *nirS*, *nop*Z, *nor*B, *nxr*B, *qnor*, *nos*Z	Cantera and Stein 2007; Kool et al., 2011; Butterbach-Bahl et al., 2013

Table 1: The microbial processes of denitrification, nitrification, nitrifier denitrification, and coupled nitrification-denitrification significantly contribute to the agricultural N_2O budget. Fertilization can increase levels of ammonia (NH_3), ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and organic carbon (C) in the soil, which increases substrate availability for microbial growth and respiration.

Abiotic variables create conditions favoring certain microbial processes over others, which indirectly affect N_2O fluxes. Abiotic variables include soil type, moisture level, temperature, and pH, along with organic matter (OM) content, oxygen (O_2) levels, nutrient type and availability (primarily that of nitrogen, N, and carbon, C), and overwinter conditions (i.e. thick snow layer versus bare ground) (Koponen et al., 2004; Wagner-Riddle et al., 2007; Risk et al., 2013; Makoto et al., 2014). Factors that favor denitrification are fine-grained soils with high availability of organic C and nitrate (NO_3^-), and high soil moisture content (water-filled pore space (WFPS) ≤ 70-80%) under anaerobic conditions (Wrage et al., 2001; Wallenstein et al., 2006; Cai et al., 2010; Butterbach-Bahl et al., 2013; Xue et al., 2013; Dong et al., 2015; Hu et al., 2015; Zhang et al., 2015; Duncan et al., 2017), with organic C and NO_3^- availability being the greatest limiting factors for denitrification in low-O_2 environments (Bremner, 1997; Phillips 2008; Saggar et al., 2013; Hu et al., 2015). The factors that favor nitrification include high ammonium (NH_4^+), ammonia (NH_3), and nitrite (NO_2^-) availability (not organic C, as energy is gained from NH_4^+ oxidation); temperatures between 5°C and 40°C; and aerobic conditions, which means lower soil water content than denitrification (30% < (WFPS) < 60-70%) (Bremner, 1997; Cantera and Stein 2007; Kool et al., 2011; Dong et al., 2015; Hu et al., 2015). In suboxic zones, some nitrifying organisms can produce N_2O through reduction of nitrite during nitrifier denitrification, where NH_3 is reduced to nitrite via hydroxylamine oxidation (nitrification), then reduced to N_2O or dinitrogen (N_2) through denitrification (Wrage et al., 2001; Risk et al., 2013). Nitrification and denitrification are also influenced by pH, with higher N_2O emissions; nitrate,

nitrite, and nitric oxide reductase activity; and reduced *nos*Z activity (discussed later) typically observed in soils with pH < 7 (Hu et al., 2015).

Microbial activity is generally highest in seasons with high soil temperature, however N_2O emissions do not necessarily follow the same trends (Koponen et al., 2004); FTCs can account for some of the highest annual N_2O flux events and for up to 70% of the annual N_2O emission budget in agricultural systems (Wagner-Riddle et al., 1997; Röver et al., 1998; Hao et al., 2001; Koponen et al., 2006). In total, neglecting FTC emissions could underestimate global agricultural N_2O emissions by 17-28% (Wagner-Riddle et al., 2017). There are two primary hypotheses to explain N_2O release from soils upon thaw and/or during FTCs: (1) the physical release of N_2O during thawing which was produced overwinter and trapped under the frozen soil surface and/or within thin films of liquid water surrounding soil colloids and (2) the *in situ* production of N_2O at the onset of the thaw, stimulated by increased biological activity and changes in physical and chemical soil conditions (Risk et al., 2013). Although there is some evidence that physical release may account for a portion of N_2O emissions when soils first thaw (Risk et al., 2014), recent studies (Röver et al., 1998; Chang and Hao, 2001; Wagner-Riddle et al., 2008; Németh et al., 2014; Congreves et al., 2018) point to *in situ* microbial action as the primary mechanism for N_2O emissions during FTCs and thawing.

Overwinter freeze- and FTC-induced changes to soil structure and microbial communities may enhance denitrification by: (1) disrupting soil aggregates, which makes previously unavailable nutrients (organic C and N) available, (2) lysing microbial cells, which mineralizes dissolved organic carbon (DOC) and nitrogen (N), and (3) increasing WFPS when water from melting ice and snow percolates into the soil, leading to reduced oxygen diffusivity (Christensen & Christensen, 1991; Jacinthe et al., 2002; Koponen et al., 2004; Henry 2007; Bruijn et al., 2009; Risk et al., 2013; Makoto et al., 2014; Wang et al., 2014; Congreves et al., 2018). These changes create ideal conditions for denitrification during thawing and FTCs, i.e. high nutrient availability in low O_2 conditions that can quickly become anaerobic due to high microbial growth with limited O_2 diffusion (Teepe et al., 2004; Dusenbury et al., 2008; Bruijn et al., 2009; Koponen and Baath, 2016). As a result of these conditions, denitrification is considered to be the dominant process responsible for large N_2O fluxes during FTCs and thawing (Chen et al., 1995; Prieme´ and Christensen, 2001; Müller et al., 2003; Ludwig et al., 2004; Mørkved et al., 2006; Wagner-Riddle et al., 2008).

In addition, colder freeze temperatures, longer duration of the freeze, and more intense FTCs have been linked to heightened denitrification and increased N_2O emissions, as (1) denitrifiers are very sensitive to changes in temperature, (2) *nos*Z, the gene responsible for the reduction of N_2O to N_2, is inhibited by cold temperatures more than other genes, and (3) increased freeze/FTC duration and/or severity has the potential to increase nutrient availability through increased microbial lysis and soil aggregate disruption (Chen et al., 1995; Christensen & Christensen, 1991; Jacinthe et al., 2002; Koponen et al., 2004; Teepe et al., 2004; Feng et al., 2010; Butterbach-Bahl et al., 2013; Billings and Tienmann, 2014). In summary, increased FTC action within a soil system may increase N and labile C availability and cause large changes to microbial communities, which can lead to heightened N_2O emissions (Song et al., 2017). However, high numbers of FTCs occurring in rapid succession (simulated in lab settings) have been found to limit the available nutrient pool, leaving questions about timing of successive FTCs in relation to nutrient availability (Chen et al., 1995; Grogan et al., 2004; Matzner and Borken, 2008). As climate change continues, some areas will experience reduced snowpack, increased frost intensity, and increased FTC duration and intensity, while other regions may

experience reduced frost intensity (Matzner and Borken, 2008). Both scenarios indirectly impact N_2O emissions by directly impacting microbial lysis and NH_4^+, NO_3^-, and labile C cycling, as microbial lysis and nutrient cycling are, in part, a function of snowpack, frost, and FTC intensity and duration (Jacinthe et al., 2002; Bruijn et al., 2009; Yanai et al., 2004; Buckeridge and Grogan, 2007; Matzner and Borken, 2008; Zhang et al., 2011; Congreves et al., 2018).

FTCs and other abiotic variables interact with agricultural management practices such as tillage, crop type, fertilizer type, and fertilization application method to influence N_2O emissions by affecting the drivers of the microbial processes that produce N_2O (Wagner-Riddle et al., 2007). Agricultural management practices influence the physical and hydrological state of the soil, timing and distribution of nutrient inputs (Wagner-Riddle et al., 2007), increase nutrient availability (Saggar et al., 2013), and can have long-term impacts on microbial community abundance and N_2O emission potential of the soil (Morales et al., 2010). Untilled soils subject to N-fertilization have been found to have greater N_2O emissions than conventionally tilled fields due to increased bulk density, soil organic matter (SOM) content, and soil compaction; reduced gas diffusivity and porosity; and poor drainage, leading to increased denitrification rates (Ball et al., 2008; Dunsenbury et al., 2008; Rochette et al., 2008; Kong et al., 2009; Wu et al., 2015). However, tillage alone cannot predict N_2O emission levels (Bavin et al., 2009; Kong et al., 2009). Long-term fertilization of fields, especially those fertilized with organic fertilizers or a mixture of organic and synthetic fertilizers, increases N and C availability and can increase the N_2O emission potential of a field (Kaiser and Ruser, 2000; Feng et al., 2010; Inselsbacher et al., 2011; Cui et al., 2016; Dong et al., 2015). Organic fertilizers such as animal slurries, waste waters, and biosolids or mixtures of organic and synthetic fertilizers with a high water content, low C:N ratio, and high mineral N content have the highest potential to increase N_2O emissions, as they create conditions favoring denitrification (Hao et al., 2001; Charles et al., 2017).

Fertilizer slurries are increasingly injected directly into the ground (in contrast to being broadcasted on the surface) to reduce fertilizer loss through NH_3 volatilization or runoff (Dell et al., 2011). Direct injection of a fertilizer slurry into the field can create anaerobic, high nutrient microsites that allow for N loss through denitrification, which can result in significantly higher N_2O emissions from fields subject to slurry injection than those subject to surface application of the same amendment (Flessa and Beese, 2000; Wulf et al., 2002; Dell et al., 2011; Maguire et al., 2011; Duncan et al., 2017). However, studies have conflicting findings on the effect of application techniques on N_2O emissions, with some studies findings showing no correlation between application method and emission rates (Vallejo et al., 2005), while others show increased N_2O emissions from slurry injection (Velthof et al., 2003; Webb et al., 2010), indicating a need for further study to address confounding variables. Additionally, it is possible for nitrification and denitrification to occur simultaneously in microsites of close proximity in complex soil systems through coupled nitrification-denitrification (Iselsbacher et al., 2011), allowing for the NO_3^- created by nitrification to be used by denitrifying microbes (Dell et al., 2011).

While it is known that FTCs and fertilization practices largely contribute to annual agricultural N_2O budgets, much less is known about the interactions between FTCs and agricultural management practices. As the primary microbial process responsible for N_2O emissions in agricultural fields during FTCs is denitrification (Chen et al., 1995; Prieme´ and Christensen, 2001; Müller et al., 2003; Ludwig et al., 2004; Mørkved et al., 2006; Wagner-Riddle et al., 2008), agricultural management practices that promote denitrification, such as slurry injection or fertilization of the field before FTCs, have the potential to significantly

increase N_2O emissions over other management practices, such as broadcast and incorporation by plow. An incubation study conducted by Adair et al. (2019) found that the size of the N_2O emission pulse during a simulated FTC varied with agricultural management treatment, with the largest fluxes from soils subjected to manure injection versus broadcast of manure (no incorporation) or broadcast of manure plus plow incorporation (moldboard plow).

This study builds off of the research done by Adair et al. (2019), seeking to identify the drivers of the difference in emissions previously noted between manure injection versus manure broadcast and incorporation by plow in a continuous corn cropping system. We used a laboratory thaw experiment to test the hypotheses that: (1) soils from the field subject to manure injection would have higher thaw N_2O emissions than the field subject to broadcast + plow, (2) the majority of the N_2O emissions would be due to denitrification, and that (3) N_2O emissions would be driven by increases in microbial biomass and activity during thaw which would be higher under injection soils.

2. Methods

2.1 Site description

Soil samples were collected from the Manure Injection No Till (MINT) farm trial, located at Borderview Farm in Alburgh, VT. Established in May of 2013, the trial was set up in a continuous corn (*Zea mays* L.) field with a winter rye (*Secale cereale*) cover crop. The soils at the site are classified as a Benson rocky silt loam and are somewhat excessively to excessively well-drained with moderate permeability (Soil Survey, 2017). The experimental design is a randomized complete block with a split-split plot arrangement (three blocks and two subplot treatments) with no tillage except for manure incorporation (Fig. 1). Average soil pH is 6.1. In 2013-2015, manure was applied at a rate of 59 m^3 ha^{-1}. In all years, manure was applied between May 12 and 19. Corn was planted at 84,000 seeds ha^{-1} within two days of manure application. The corn crops were harvested for silage in September and an over-winter rye cover crop was planted within two days of corn harvest. Residue was left on the field and corn roots were left undisturbed.

The three replicate blocks had two subplot manure application treatments each: broadcast plus incorporation by plow (broadcast + plow) and closed slot injection (six subplots total) (Fig. 1). Each subplot was 3.7 by 12.2 m. The broadcast + plow manure application treatment used a moldboard plow and disk to incorporate manure to a depth of 15-20 cm. Manure was injected to a depth of 15 to 20 cm, but injection lines were typically filled to the soil surface or just under the soil surface (2–3 cm) with manure. Injection bands were approximately 10 cm wide, with 75 cm spacing between bands.

Block 1	405 Inject	3.7 m
	408 Plow	
Block 2	409 Inject	
	411 Plow	
Block 3	413 Inject	
	415 Plow	
	12.2 m	

Figure 1: MINT field trial experimental design

2.2 Soil sampling

Soils were sampled on April 3, 2018. During this time, the soils were still undergoing freeze-thaw cycles (FTC) from diurnal temperature fluctuations. To ensure that the samples were taken from the correct subplot treatment and to avoid edge effects, samples were only taken from a 2.43 by 7.3 m section in the middle of the subplot (i.e., 2.43 m width buffers and 0.6 m length buffers between subplots). The inner 7.3 by 2.43 section was divided into 24 equal sections. A random number

generator was used to select a section to sample. Within the selected section, a 2.43 m transect was established perpendicular to the direction of corn rows and manure injection lines.

Along each 2.43 m transect, 12 0-20 cm, evenly spaced soil cores were taken, using a 1 cm diameter corer. Six of these samples were taken from "in row" areas, or from a row where corn was planted, and six were taken from "between row" areas, or between the corn rows. Each plot had one polyethylene bag into which all 12 samples from that plot were composited, homogenized, and kept on ice for transport back to the lab. Soil moisture and temperature were taken at the time of sampling, with soil moisture taken four times and temperature taken twice evenly over the area of each section. On average, the soil temperature was within 1.4^{o}C for all plots, with the average soil temperature being 6.^{o}C. Soil moisture was recorded using a soil moisture probe and was also relatively consistent across all plots, with the average period reading being 2708 μs.

2.3 Experimental design

Soil samples from each plot were subjected to two temperature treatments: frozen at -7^{o}C, the mean cold season air temperature from 1980-2014 (Thorton et al., 2016) or thawing at 10^{o}C. We also used two atmosphere treatments: aerobic (ambient air) or anaerobic (N_2 headspace). Additionally, half of the jars under each temperature and atmosphere treatment were treated with acetylene to inhibit nitrification and the final step of denitrification (reduction of N_2O to N_2) (Butterbach-Bah et al., 2013); all N_2O produced in these jars could be assumed to be from denitrification (Fig. 2), yielding total N_2O produced by denitrification.

Soils were incubated in air-tight Ball mason jars. There were 12 jars per temperature by atmosphere treatment (six replicates from the injection subplots and six from the broadcast with plow incorporation subplots). Of these, three from injection and three from broadcast + plow were treated with acetylene, and the others acted as non-acetylene controls (Fig. 2). The soils were subjected to these treatments for eight days. Within each jar, there were four 20 g soil samples in open beakers to allow subsampling throughout the incubation.

2.4 Incubation study

Prior to the incubation study, soils were tested for chemodenitrification. This was done using four, 80 g, soil samples - two from injection soils and two from broadcast + plow soils. One injection sample and one broadcast with plow incorporation sample were autoclaved twice for 20 minutes at 121^{o}C to eliminate biotic controls on N_2O production (2340M Autoclave, Tuttnauer, Hauppauge, NY, USA). The other two jars served as controls. All jars were sealed and the headspace was sampled at time zero and for the next three days (i.e., 0, 24, 48, and 72 hours) using an infrared photoacoustic spectroscopy gas analyzer (Model 1412i, Innova Air Tech Instruments, Ballerup, Denmark). There was no N_2O production from the autoclaved treatments. Therefore chemodenitrification treatments were not included in the experimental design.

	Freeze (N_2)	Freeze (Air)	Thaw (N_2)	Thaw (Air)
Control	3 jars (injection)	3	3	3
	3 jars (Plow)	3	3	3
Acetylene	3	3	3	3
	3	3	3	3

Figure 2: Experimental design of the incubation study.

Before the incubation was initiated, gravimetric soil moisture was taken from each plot's composite sample, and each homogenized sample was divided into 32, 20 g subsamples (see *Experimental design* above), which were placed in glass scintillation vials and frozen at -20ºC until the start of the incubation study.

The incubation study was conducted for eight days, starting on May 30, 2018 (Day 0) and ending June 6, 2018 (Day 7). Aerobic jars were aired on the countertop for five minutes. Jars with an anaerobic headspace were evacuated using a manifold and vacuum pump, then filled with N_2 gas. Evacuation on the manifold occurred for three minutes by inserting a needle on the manifold through a septa in the jar lid. Following evacuation, each anaerobic jar was flushed with N_2 for one minute. This process was repeated four times to ensure that a fully anaerobic headspace was created. The jars subject to acetylene inhibition had acetylene gas added to result in a headspace with 10% acetylene after the correct headspace treatment was restored (aerobic or anaerobic) (Tiedje et al., 1989). Starting at Time 0 on Day 0, a pressure measurement was taken and then 10 mL of gas was removed from each jar's headspace using a sterile syringe for analysis on a GC-2014 Gas Chromatograph (GC) analyzer equipped with a flame ionization detector (FID), electron capture detector (ECD), and a Hayesep N 80/100 Mesh 1/8in. X 1.5M stainless steel pre-conditioned column (Shimadzu Instruments, Kyoto, Japan). Between 22 and 24 hours following the prior sampling, 10 mL of gas was removed from each jar again. On Day 1 (hour 24), Day 2 (hour 48), Day 4 (hour 100), and Day 7 (hour 168), one of the four 20 g subsamples was removed for destructive analysis following gas sampling. After subsample removal, the jars were flushed with air for 5 minutes, the correct headspace and acetylene treatment were restored (as previously described), and a secondary gas sample was taken before returning the jar to its incubation treatment.

To calculate the gas fluxes (CO_2, N_2O, and CH_4) in µg, the slope and y-intercept of the calibration curve from each run was used to convert the peak area output from the GC into µL gas/ µL total gas:

$$\frac{\text{µL gas } (CH_4, N_2O, or\ CO_2)}{\text{µL total gas}} = \frac{(slope * peak\ area) + yintercept}{1000}$$

The amount of gas in µg was calculated using the following series of equations:

$$\frac{\text{µmole gas } (CH_4, N_2O, or\ CO_2)}{\text{µL total gas}} = \left(\frac{\text{µL gas}}{\text{µL total gas}}\right) * \left(\frac{273 + incubation\ temp\ (deg.C)}{0.08205\ L\ atm\ K^{-1}\ mol^{-1}}\right)$$

$$\text{µmole gas } (CH_4, N_2O, or\ CO_2) = \left(\frac{\text{µmole gas}}{\text{µL total gas}}\right) * \left(Jar(ml) - v.loss(mL)\right) * 1000$$

where Jar (mL) is the jar headspace in mL, and v.loss (mL) is the volume change in the headspace of the jar as gas samples were removed. The jar headspace volume was calculated by filling one of the mason jars containing 4 20 g subsamples with water and weighing the jar, then removing the subsamples one at a time and re-weighing the jar to determine how headspace changed over the course of the study. Finally:

$$\text{µg gas } (CH_4, N_2O, or\ CO_2) = \text{ µmole gas} * \text{molecular weight of gas}$$

The flux rates were calculated by fitting a linear regression to the gas concentrations recorded while each jar was closed. The resulting fluxes were in μg gas s^{-1}.

2.5 Soil analyses

Soil subsamples were analyzed for nitrite (NO_2^-), ammonium (NH_4^+), nitrate (NO_3^-), microbial biomass, extracellular enzyme activity, and phospholipid fatty acids (PLFA). Available NO_2^- was determined using 2.5 g of each soil subsample extracted using 15 mL DDI (Hageman and Huckelby, 1971). Available NH_4^+ and NO_3^- were determined using 5 g of each subsample extracted using 0.5 M potassium sulfate (K_2SO_4) (Weatherburn 1967; Doane and Horwath. 2003; Hood-Nowotny et al. 2010). NO_2^-, NH_4^+, and NO_3^- were all analyzed using colorimetric methods on a microplate reader, BioTek Synergy HTX (BioTek Instruments, Inc., Winooski, VT, USA).

To characterize microbial activity during the incubation, we conducted extracellular enzyme assays (EEA) (Bell et al., 2013) on Day 1, 2, and 7 soil samples. Hydrolytic enzyme activity was tested for cellobiohydrolase (CBH or CB), β-glucosidase (BG), β-N-acetylglucosaminidase (NAG), and leucine aminopeptidase (LAP), which underwent fluorometric analysis on a BioTek Synergy HTX (BioTek Instruments, Inc., Winooski, VT, USA). Oxidative enzyme activity was tested for polyphenol oxidase (PPO) and peroxidase (PER), which underwent colorimetric analysis on a BioTek Synergy HTX (BioTek Instruments, Inc., Winooski, VT, USA).

To characterize microbial biomass, we used simultaneous chloroform fumigation (Fierer 2003; Setia et al. 2012) using 5 g of each soil subsample. Unfortunately, the shaking method used (with the tubes upright) did not allow the soil subsample to be fully fumigated, so the results were inaccurate and will not be further discussed. We also used a 3.50 g subsample taken from the last soil sample of the incubation for PLFA (Frey Lab, University of New Hampshire; Bligh and Dyer, 1959).

Soil moisture was tracked over the course of the study to determine if there were substantial losses of water over the 8 day incubation study. Gravimetric soil moisture measurements were taken from each plot's composite sample before the start of the study, and were taken from each subsample when removed for destructive sampling.

2.6 Statistical analyses

2.6.1 Gas fluxes

N_2O and CO_2 fluxes were calculated by fitting a linear regression to N_2O and CO_2 concentrations over time while the jars were sealed. The slope of the line (ppm sec^{-1}) was converted to μg g $soil^{-1}$ hr^{-1} using the amount of dry soil in each jar, which was determined using gravimetric percent moisture performed on subsamples of removed soils.

Daily denitrification, CO_2, and N_2O fluxes were analyzed using a linear mixed model, with jar as a random effect to account for non-independent measurements from the same jar over time and all interactions among atmospheric treatment (N_2 or ambient), temperature (freeze or thaw), and manure application method (inject or broadcast with incorporation by plow). Denitrification and N_2O fluxes were log transformed to meet normality and homogeneity of error assumptions; however, we also included a constant variance function to account for heterogeneous errors among the manure and atmospheric treatments (denitrification fluxes) or for the atmospheric treatment (N_2O fluxes). CO_2 fluxes were not transformed, but the model included a constant variance function to account for heterogeneous errors among the temperature

and atmospheric treatments. Marginal and conditional R^2 values were calculated using the *piecewiseSEM* package in R (Lefcheck, 2015). Marginal R^2 describes the proportion of variance that is explained by fixed factors alone (i.e. atmospheric, manure application, and temperature treatments and interactions), while conditional R^2 describes the proportion of variance explained by fixed and random factors (fixed factors previously listed plus jar; Nakagawa and Schielzeth, 2013). Treatment significance was assessed using X^2 tests. All models were fit using the *nlme* package in R (R Core Team, 2016; Pinheiro et al., 2017).

2.6.2 Nitrate and ammonium

Nitrate and ammonium were analyzed using a linear mixed model that included all interactions among atmospheric treatment (acetylene, N_2, or ambient), temperature treatment (freeze or thaw), and manure application method (injection or broadcast + plow). Non-independent measurements from the same jar over time were accounted for by including jar as a random effect. A constant variance function was included to account for heterogeneous errors among atmospheric treatments for nitrate or among temperature treatments for ammonium.

We again calculated marginal and conditional R^2 values using *piecewiseSEM* (Lefcheck, 2015). Treatment significance was assessed using X^2 tests. All models were fit using the *nlme* package (Pinheiro et al., 2017).

2.6.3 Extracellular enzyme assays (EEA)

Extracellular enzymes were analyzed using a linear mixed model that included the same interactions as listed for nitrate and ammonium (see above) with jar as a random effect to account for non-independent measurements from the same jar over time. Data were untransformed for peroxidase, ln transformed for polyphenol oxidase and BG, or square root transformed for NAG and LAP to meet normality assumptions. CBH activity was 0 in all samples, so CBH was excluded from data analysis. Marginal and conditional R^2 values were calculated as above and all models were fit using the *nlme* package in R (R Core Team, 2016; Pinheiro et al., 2017).

2.6.4 Phospholipid fatty acids (PLFA)

Arbuscular mycorrhizae (AMF), actinomycetes, total fungal biomass, and total bacterial biomass were determined by PLFA analysis (PLFA; Frey Lab, University of New Hampshire; Bligh and Dyer, 1959). For analysis, an ANOVA was run in R (RStudio Team, 2015). Data were untransformed, with the exception of total microbial biomass, which was log transformed to meet normality assumptions. Statistical models included manure, atmosphere, and temperature treatments, along with all interactions. Variance structures were added as needed to meet normality assumptions.

3. Results

3.1 Denitrification and GHG emissions

3.1.1 Denitrification

Despite a marginally significant result for manure and manure by temperature interaction ($P < 0.1$), no substantial difference was found between manure treatments. Denitrification was greater in the thaw treatment than in the freeze treatment, but this difference was only of marginal significance ($P<0.1$; Fig. 3 and 4). Denitrification was higher from the broadcast +

plow soils, but again, this result was only of marginal significance (Table 2; Fig. 3). Denitrification was found to be significantly greater under the N_2 atmosphere than under the ambient atmosphere ($P < 0.05$; Fig. 4). Denitrification was not significant for the manure by atmosphere, temperature by atmosphere, or manure by temperature by atmosphere interactions (Table 2).

	Denitrification (mg N_2O-N g $soil^{-1}$ d^{-1})			N_2O flux (mg N_2O-N g $soil^{-1}$ d^{-1})			CO_2 flux (mg N_2O-N g $soil^{-1}$ d^{-1})		
Variable	X^2	DF	*P*	X^2	DF	*P*	X^2	DF	*P*
Manure	3.41	1	*0.0650*	0.38	1	0.5385	0.10	1	0.7482
Temperature	3.17	1	*0.0752*	0.26	1	0.6095	59.54	1	**<0.0001**
Atmosphere	19.03	1	**<0.0001**	13.26	1	**0.0003**	4.85	1	**0.0277**
Manure*Temp	3.69	1	*0.0547*	1.01	1	0.3155	4.91	1	**0.0267**
Manure*Atm	1.31	1	0.2530	0.02	1	0.8993	0.05	1	0.8308
Temp*Atm	0.75	1	0.3866	12.14	1	**0.0005**	5.39	1	**0.0203**
Manure*Temp *Atm	2.46	1	0.1168	0.00	1	0.9505	0.00	1	0.9520
Conditional R^2	0.1823			0.1757			0.8734		
Marginal R^2	0.1823			0.1757			0.8734		

Table 2 Analysis of variance results (P-values) for denitrification, N_2O flux rates, and CO_2 flux rates for temperature, manure, and headspace incubation treatments. The marginal R^2 value describes the proportion of variance explained by the fixed factors and the conditional R^2 describes the proportion of variance explained by the fixed and random factors. X^2 is the chi square value and DF is degrees of freedom.

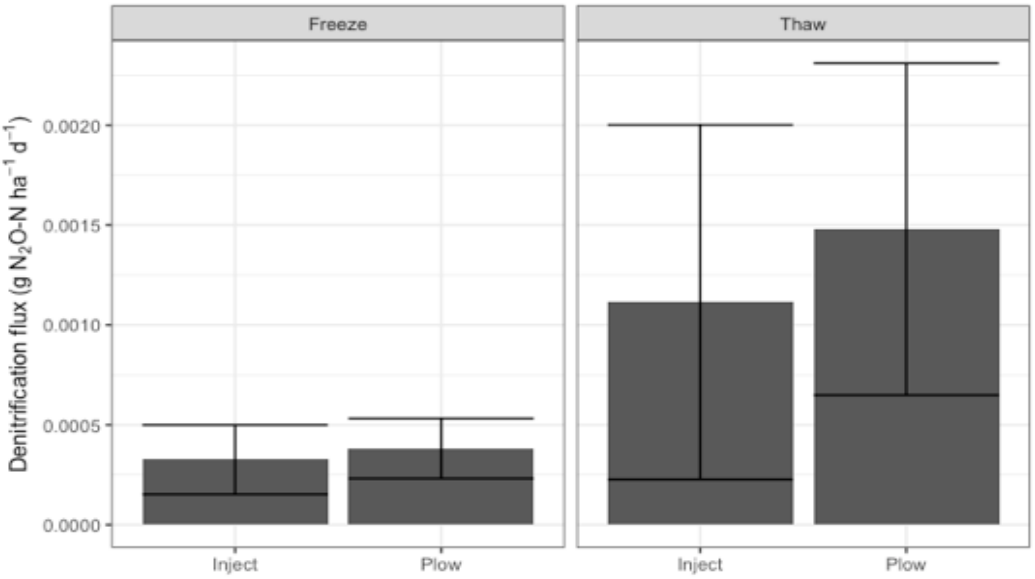


Fig. 3 Denitrification by manure treatment (inject or broadcast + plow) and by temperature treatment (freeze or thaw). Error bars are ± 1 standard error (SE).

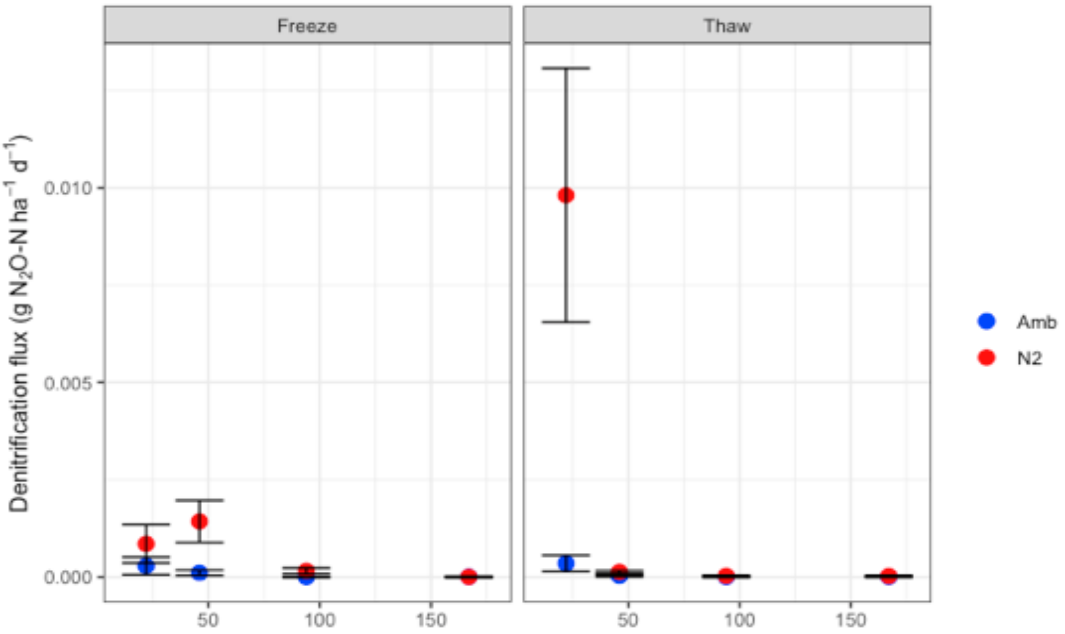


Fig. 4 Denitrification over time (hour) by temperature and atmospheric treatment (N_2 or ambient). Error bars are ± 1 standard error (SE).

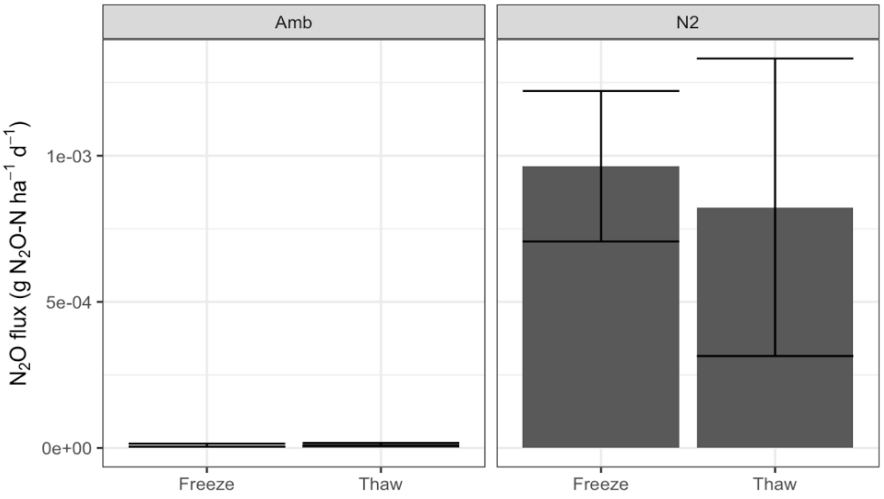


Fig. 5 Average N_2O fluxes by temperature and atmospheric treatment (N_2 or ambient). Error bars are ± 1 standard error (SE).

3.1.2 N_2O Fluxes

No significant differences were found in N_2O fluxes between temperature treatments, manure treatments, or for the manure by temperature, manure by atmosphere, or manure by temperature by atmosphere interactions (Table 2; Fig. 5). However, a significant atmosphere relationship and temperature by atmosphere interaction was found (Fig. 5). N_2O fluxes were higher under the N_2 atmosphere than under the ambient atmosphere, and were slightly higher from the freeze temperature treatment than from the thaw treatment (Fig. 5).

3.1.3 CO_2 fluxes

Fluxes of CO_2 were significantly higher in the thaw than freeze treatment ($P < 0.0001$) and were significantly higher in the ambient versus N_2 atmosphere treatment ($P = 0.0277$) (Table 2; Fig. 7). There were also significant manure by temperature (Table 2, $P = 0.0267$) and temperature by atmosphere (Table 2, $P = 0.0203$) interactions. There were no real differences in CO_2 fluxes between the soils subjected to injection or broadcast + plow in the freeze treatment, but CO_2 fluxes from the broadcast + plow were much higher than CO_2 fluxes from injection under the thaw treatment (Fig. 6; Table 2, significant manure by temperature). The difference between ambient and N_2 treatments was greater in the thaw than freeze treatment (Fig. 7; significant temperature by atmosphere interaction, Table 2), and emissions were the overall highest from soils thawing under an ambient atmosphere. Manure treatment alone and manure by atmosphere had no significant differences in CO_2 fluxes (Table 2).

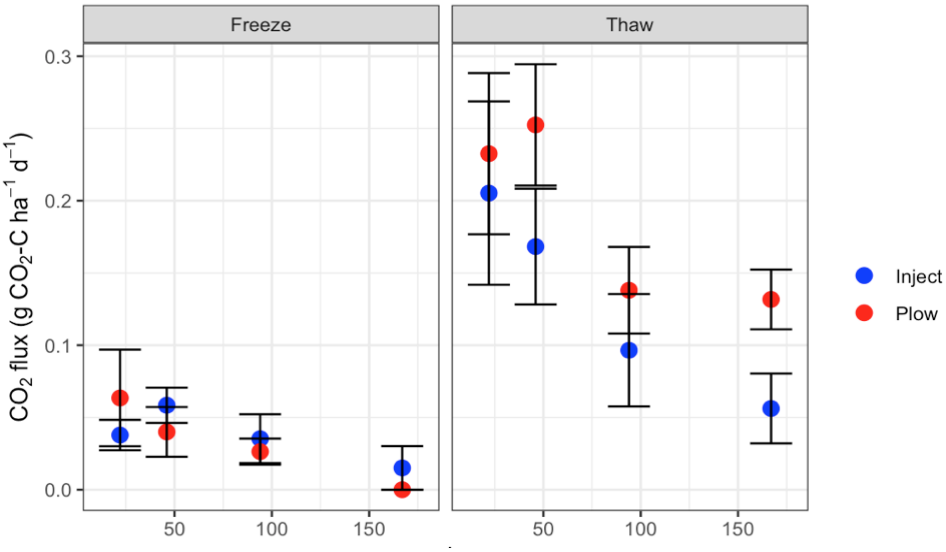


Fig. 6 CO_2 fluxes over time (hour) by temperature and manure treatment. Error bars are ± 1 standard error (SE).

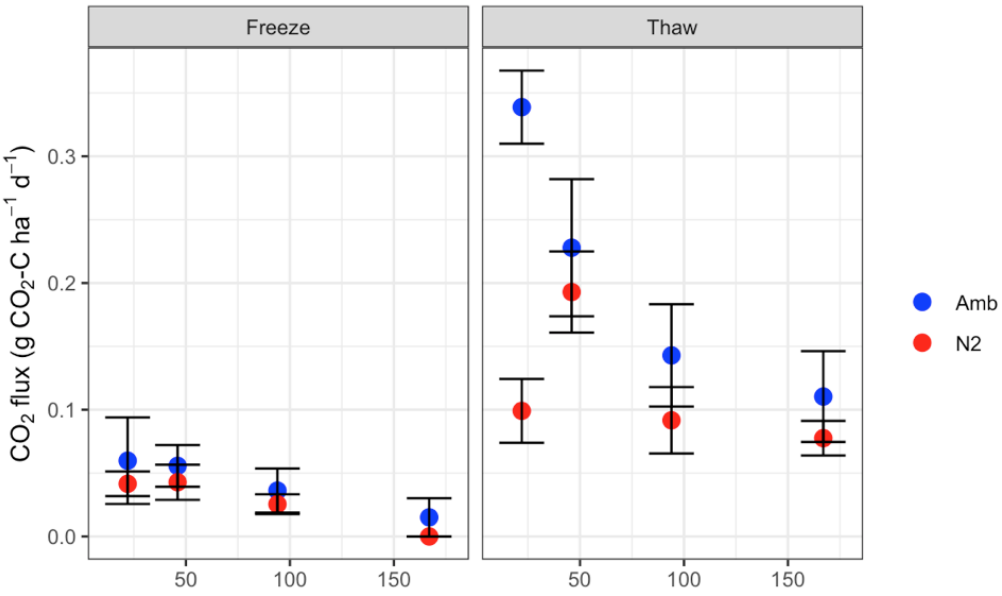


Fig. 7 CO_2 fluxes over time (hour) by temperature and atmospheric treatment. Error bars are ± 1 standard error (SE).

3.2 Soil parameters

3.2.1Physiochemical properties

Soil moisture showed a slight decline over the course of the incubation study (See Appendix 1). At the start of the study, gravimetric soil moisture was, on average, 0.24 (24%, note that all soil moistures are recorded as percentages in decimal form). On average across all plot subsamples, the soil moisture was 0.21 at the end of the study, following the general trend of declining soil moisture, as seen in Appendix 1.

3.2.2 Inorganic N

No significant levels of nitrite were found in any of the soil samples during the incubation study, so nitrite was not analyzed further. No significant results were found for nitrate or ammonium either (see Appendix 2).

3.2.3 Extracellular enzyme assays

There were no significant differences found in enzyme activities (See Appendix 3).

3.2.4 Phospholipid fatty acids

Few statistically significant relationships were found with PLFA analysis: total fungal biomass was found to be significantly higher for manure injection and ambient atmospheric treatments ($P<0.05$, Appendix 4; Fig. 8), gram positive bacteria was found to be significantly higher in the thaw temperature treatment ($P<0.0001$, Appendix 4, Fig. 11), and gram negative bacteria was found to be significantly higher in the injection manure treatment ($P = 0.0053$, Appendix 4; Fig. 11) and significantly higher in the thaw temperature treatment ($P<0.05$, Appendix 4; Fig. 11). Several moderately significant ($P<0.1$, Appendix 3) relationships were found: actinomycetes were moderately higher under the freeze temperature treatment for jars with an ambient headspace, however actinomycetes were higher under the thaw temperature treatment for jars with an N_2 atmosphere (Appendix 4, Temp*Atm interaction; Fig. 9), total bacterial biomass was moderately decreased under the plow treatment and moderately higher under the thaw treatment ($P<0.1$, Appendix 4; Fig. 10), and gram positive bacteria was moderately higher under the injection treatment (Appendix 4, $P<0.1$; Fig. 11).

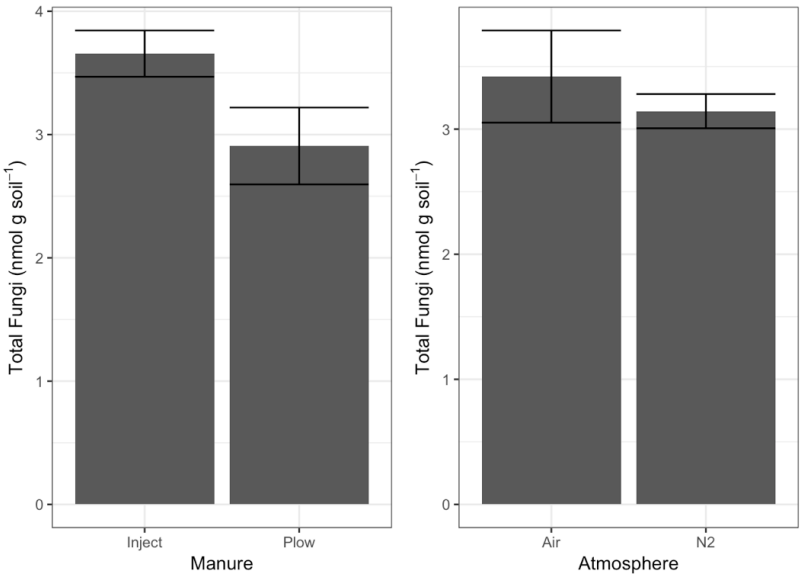


Fig. 8 Total fungal biomass by manure and atmosphere treatment. Significant relationships were found for manure and atmosphere treatments.

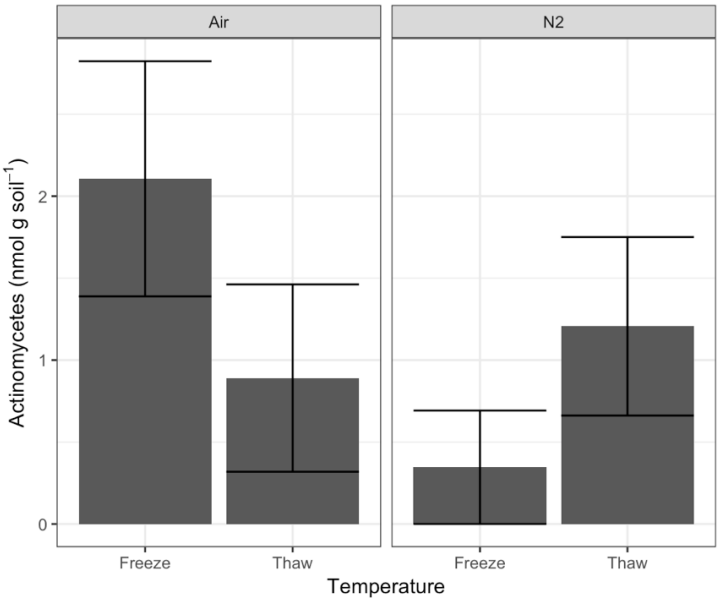


Fig. 9 Actinomycetes by temperature and atmosphere treatments. A moderately significant interaction was found for temperature by atmosphere.

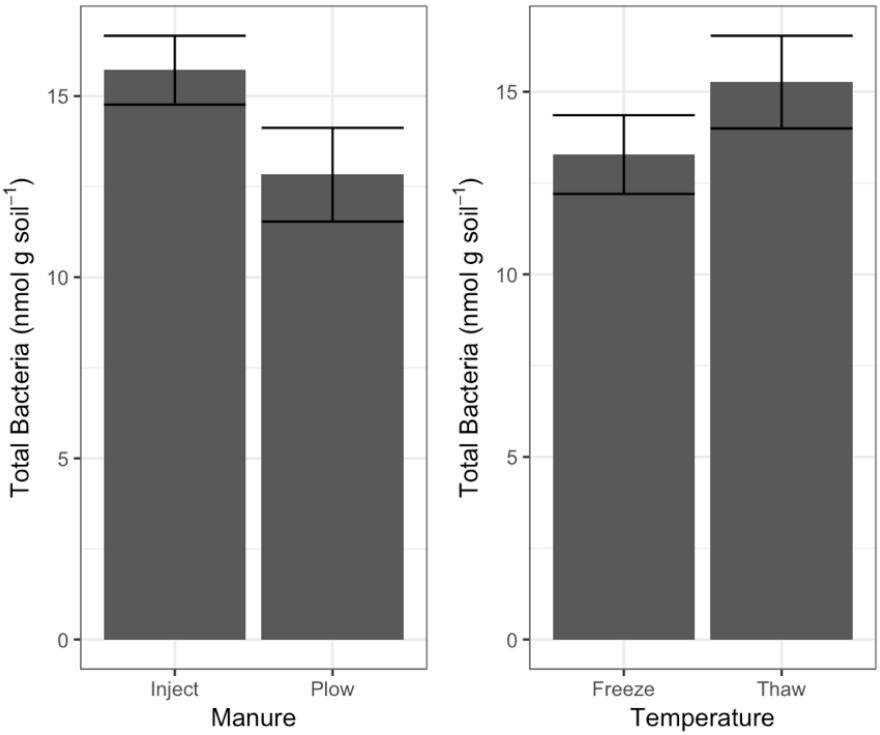


Fig. 10 Total bacterial biomass by manure and temperature treatments. Moderately significant relationships for manure and temperature treatments were found.

4. Discussion

4.1 Denitrification and N_2O flux

We expected to see significant differences in denitrification and N_2O fluxes between manure treatments, temperature treatments, and headspace treatments. We hypothesized that denitrification and N_2O fluxes would follow the same trends, as we hypothesized that the primary source of N_2O emissions from both plots would be from denitrification Furthermore, we expected to find significantly higher rates of denitrification and corresponding N_2O fluxes from the injection treatment than we anticipated finding from the broadcast + plow treatment, as found by Adair et al. (2019), since injection of manure can create anaerobic microsites favorable for denitrification (Flessa and Beese, 2000; Wulf et al., 2002; Dell et al., 2011; Maguire et al., 2011; Duncan et al., 2017; Adair et al., 2019). We anticipated that the anaerobic atmospheric treatment would further promote denitrification and resulting N_2O emissions and expected both denitrification and N_2O fluxes to be highest from the thaw treatment, since thawing promotes microbial growth and respiration, as previously discussed (see Introduction).

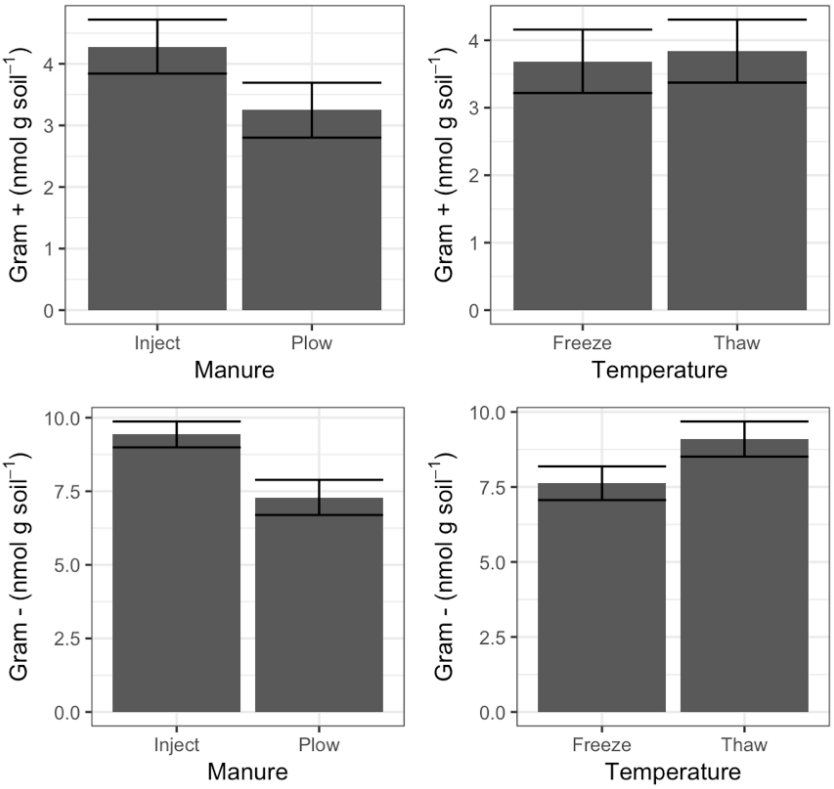


Fig. 11 Gram (+) and (-) bacteria from PLFA analysis by manure and temperature treatments. Significant relationships for manure and temperature treatments.

While our denitrification results supported our atmosphere hypothesis and marginally supported our temperature hypothesis, we were surprised to find that our denitrification results contradicted the manure treatment hypothesis, with the broadcast + plow treatment showing marginally higher rates of denitrification than the injection treatment. Our N_2O results also supported our headspace hypothesis, but surprisingly, the only other significant N_2O relationship or interaction was under the anaerobic atmosphere for the freeze treatment (Table 2), which partially contradicted our hypothesis and the findings by Adair et al. (2019).

Higher denitrification and N_2O fluxes from the N_2 atmosphere suggests that denitrification was the primary process resulting in N_2O emissions in this study (Phillips 2008; Saggar et al., 2013; Hu et al., 2015), which supports our second hypothesis. However, we have no way to be certain that all N_2O emissions were from denitrification alone, given the relatively low moisture content of our soils. Nitrifier denitrification can occur in anoxic conditions, but is

not limited to higher moisture conditions like denitrification (Yu et al., 2010; Wrage-Mönnig et al., 2018). Therefore, some of the denitrification activity and N_2O flux may have been from nitrifier denitrification.

We speculated that the higher N_2O fluxes from the freeze treatment (significant temperature by atmosphere interaction, Table 2) were a result of methodological error. Our soil subsamples were very small and uninsulated, so the time out of the freezer for gas sampling and headspace restoration may have been great enough to allow the samples to thaw. Additionally, both incubation treatment temperatures rose between 5 and 10^{o}C each day during sampling from opening the incubators multiple times while removing and replacing jars, so jars were not immediately restored to the correct incubation temperature following sampling. If the soil samples from the freeze incubation thawed enough, they would have experienced FTC-like conditions, which could explain the higher N_2O flux rates from the freeze treatment (Matzner and Borken, 2008; Zhang et al., 2011; Congreves et al., 2018). This may also explain the lack of significant temperature results for both denitrification and N_2O flux, since thawing during a simulated FTC in the frozen jars may cause bursts of microbial activity (Risk et al., 2013; Congreves et al., 2018). As a result, we were not comparing denitrification and N_2O flux between a frozen control and a thawing soil, but instead were comparing denitrification and N_2O flux between soils experiencing FTC and those that were continuously thawing.

Other aforementioned contradictions between our findings and that of Adair et al. (2019) and the rejection of several of our hypotheses may be a result of our soil homogenization procedure, which caused soil matrix and aggregate disruption. Intact soils are heterogeneous, and can have high variability in mineral and organic content, nutrient availability, nutrient transport, porosity, and water and gas diffusivity (Six et al., 2004; Ebrahimi and Or, 2016; Wang et al., 2019). Microbial and nutrient dynamics exist within and between macro- and micro-aggregates and are influenced by aggregate size, soil physical properties, and changes to the soil environment, such as FTC disruption or fertilization (Or et al., 2007; Kuzyakov and Blagodatskaya, 2015; Bach et al., 2018). Microbial activity is constrained to so-called "hotspots," or microsites within or between soil aggregates that contain adequate conditions for growth (Kuzyakov and Blagodatskaya, 2015). These microsites are not necessarily connected and are as heterogeneous as the soil matrix they exist in, resulting in high variation in microbial community composition and dynamics on both the aggregate and soil matrix levels (Sexstone et al., 1985; Six et al., 2004; Kremen et al., 2005; Kuzyakov and Blagodatskaya, 2015; Ebrahimi and Or, 2016; Hagemann et al., 2017). In turn, microbial communities actively shape aggregate properties and dynamics through modification of nutrient, water, and O_2 availability, creating a feedback loop (Rabot et al., 2018). For example, aggregate and microbial dynamics may indirectly influence N_2O emissions as anaerobic "hotspots" for denitrification can be created within an aerobic matrix (van Bochove et al., 2000; Manucharova et al., 2001; Six et al., 2004; Henry, 2007; Ebrahimi and Or, 2016; Bocking and Blyth, 2018; Wang et al., 2019). Biotic and abiotic changes can cause major shifts in microbial communities and pore-scale interactions by affecting inter- and intra-aggregate dynamics, which are delicate and highly sensitive to soil changes (Or et al., 2007; Helgason et al., 2010; Wang and Or, 2010; Bach et al., 2018).

Studies analyzing aggregate-related effects on microbial activity and N_2O emissions are inconsistent (Kværnø & Øygarden, 2006; Matzner and Borken, 2008; Makoto et al., 2010; Risk

et al., 2014; Li et al., 2015), but seem to be most strongly correlated to available C and soil moisture (Kuzyakov and Blagodatskaya, 2015; Ameloot et al., 2016; Bach et al., 2018; Bocking and Blyth, 2018). In unfrozen soils, microbial movement, growth, and nutrient uptake relies heavily on the existence of water or aqueous films within and between aggregates (Sehy et al., 2004; Or et al., 2007; Wang and Or, 2010; Kuzyakov and Blagodatskaya, 2015). When soils freeze, solutes are concentrated into thin films of water around and within aggregates, which can remain liquid at temperatures below 0^{o}C (Jefferies et al., 2010; Risk et al., 2014). Microbial survival during freezing has been positively correlated with the existence of these films and with the water-holding capacity of the soil upon freezing (Yanai et al., 2004; Matzner and Borken, 2008; Jefferies et al., 2010; Phillips et al., 2010; Congreves et al., 2018; Wang et al., 2019), and could be particularly important for denitrifying microbes, as there is limited O_2 diffusion in these aqueous films (Wang et al., 2008; Ebrahimi and Or, 2016).

In summary, microbial activity only occurs in small pockets (hotspots) within the greater soil matrix, and is constrained by complicated biotic and abiotic dynamics within and between aggregates, specifically by substrate availability and WFPS. We speculated that homogenization altered the aforementioned soil properties, disrupting interactions and dynamics in the soil by breaking up aggregates, water films, and high-quality microsite habitats (Kuzyakov and Blagodatskaya, 2015). For example, if homogenization separated many previously-interacting communities, nutrient dynamics such as coupled nitrification-denitrification could be changed (Khalil et al., 2004; Sey et al., 2008; Cantera and Stein 2007; Kool et al., 2011; Butterbach-Bahl et al., 2013), a portion of the soil microbes may have been rendered inactive due to a lack of microsites, and low soil moisture may have impeded restoration of nutrient flow through cell- and pore-interactions (Stevens et al., 1997; Six et al., 2004; Or et al., 2007; Wang and Or, 2010; Ebrahimi and Or, 2016; Hagemann et al., 2017).

Additionally, our samples were frozen at -20^{o}C for 57 days (April 3, 2018 to May 30, 2018), and likely froze rapidly due to their small size. This could be problematic for three reasons: (1) samples were frozen to a temperature that is highly unlikely to occur *in situ*, reducing microbial survival and potentially shifting community composition, (2) destruction of aggregates and aqueous films in low moisture conditions may have reduced nutrient exchange, microbial mobility, and the number of habitable sites upon freezing, and (3) freezing low-moisture soils can lead to further drying of the soil (Six et al., 2004; Sharma et al., 2006; Henry, 2007; Or et al., 2007; Wang and Or, 2010; Wang et al., 2011; Ebrahimi and Or, 2016; Thornton et al., 2016). Almost all jars saw a reduction of soil moisture over the course of the study (Appendix 1), which may have further suppressed denitrification-related N_2O emissions (Wrage et al., 2001; Wallenstein et al., 2006; Butterbach-Bahl et al., 2013).

It is possible that homogenization could have impacted the injection soils and the broadcast + plow soils differently, which may explain the marginally higher denitrification rates from the broadcast + plow treatment soils over the injection soils. Reduced tillage has been found to increase N, labile C, aggregate stability, occurrence of anaerobic microsites, microbial populations, and shift community composition to have higher numbers of anaerobic microbes, while reducing frost depth and O_2 diffusivity (van Bochove et al., 2000; Phillips, 2008; Helgason et al., 2010; Zhang et al., 2018). Given that microbial communities are very sensitive to soil conditions, major differences can be found in microbial richness and diversity between adjacent

soils that share all but a few characteristics (Mangalassery et al., 2013; Ebrahimi and Or, 2016; Sun et al., 2016; Bach et al., 2018). Radical alteration of aggregate properties, nutrient availability, microbial community composition, and microbial activity can occur with changes in management practices, including tillage (Jacinthe et al., 2002; Helgason et al., 2010; Blaud et al., 2012; Li et al., 2015; Bocking and Blyth, 2018), which may translate to the effects of homogenization. In the field, microbial communities in the soils subject to the injection treatment experience a relatively undisturbed soil matrix, likely with higher aggregate stability, while the microbial communities in the broadcast + plow plots experience annual aggregate disruptions during manure incorporation. As a result, the microbial communities from the injection plots may have been more sensitive to homogenization, destruction of microsite conditions, and a rapid freeze than the soils from broadcast + plow plots, thus leading to lower denitrification and N_2O emissions from the injection soils than from the broadcast + plow soils.

Methodological differences between the current incubation study and that of Adair et al. (2019) further support our speculation that low soil moisture combined with high soil structure disruption affected our results. Adair et al. (2019) used intact soil cores, thereby retaining *in situ* soil structure, aggregates, and aqueous microsites. Denitrification can occur in lower moisture conditions if there is adequate labile C (Wang et al., 2008; Hagemann, 2017). Since soil moisture was very similar between the two studies but results were very different, the use of intact cores may have better preserved microsites, supporting denitrifier communities and activity, particularly within the injection treatment samples. Additionally, soil cores used by Adair et al. (2019) were insulated, reducing the speed at which the cores could freeze and thaw, and simulating unilateral (top down) FTCs as they would occur in the field (Hu et al., 2006).

4.2 CO_2 fluxes

Unlike N_2O fluxes and denitrification, CO_2 fluxes were responsive to all three treatments. CO_2 fluxes are a measure of C mineralization and soil respiration, influenced by temperature, C availability, soil moisture, microbial abundance, C storage, aggregate structure, and aggregate stability (Raich and Tufekciogul, 2000; Sey et al., 2008; Ferrara et al., 2017), and are largely from aerobic respiration (Bridgham and Richardson, 1992). Higher CO_2 fluxes under thaw conditions could be a result of higher mineralized C, or higher rate of microbial decomposition from those that had lysed during freeze (Six et al., 2004; Makoto et al., 2010). As expected, general microbial respiration was the highest under ambient conditions, but denitrification and N_2O fluxes were higher under anaerobic conditions, suggesting that CO_2 fluxes were not caused by the same mechanisms as N_2O fluxes.

4.3 Microbial activity and nutrient availability

The majority of both nitrifying (e.g. *Nitrosomonas* and *Nitrobacter*) and denitrifying (e.g. *Pseudonomas* and *Alcaligenes*) bacteria in soils are gram negative bacteria, which are generally the smallest bacteria and very sensitive to drought and water stress (Gamble et al., 1977; Mosier et al., 1983; Tiedje, 1988, Ji et al., 2015). Actinomycetes are a type of gram positive (larger than gram negative and more resistant to water stress) denitrifying bacteria that is typically anaerobic, requiring moist soils and a relatively neutral pH (Ji et al., 2015; Barka et al., 2016).

Significantly higher gram positive, gram negative, and total bacterial biomass under the thaw treatment supported our hypothesis that microbial growth would occur as the soils thawed and provided more favorable temperatures and physiochemical soil conditions (Kim et al., 2012; Risk et al., 2013). Since actinomycetes are denitrifiers, higher actinomycete biomass under the anaerobic conditions for the thaw treatment supported our hypothesis, but higher actinomycete occurrence under ambient conditions for the freeze treatment did not support our hypothesis, and we were unable to explain this occurrence. It is possible that there were changes in and interactions between microbial and fungal communities over the course of the study (Bach et al., 2018), but we have no valid way to quantify or predict these potential changes or interactions, given data limitations.

Significantly higher amounts of gram positive, gram negative, and total bacterial biomass were found in the injection soils. The occurrence of higher numbers of the aforementioned bacterial communities under the injection treatment was surprising when compared to marginally higher denitrification rates and significantly higher CO_2 fluxes occurring in the broadcast + plow soils. These results affirm that there were higher numbers of these microbes in the injection soils, which could suggest that there were high numbers of inactive microbes in the injection soils that did not add to denitrification or flux rates. Although microbial biomass in the injection soils conflicted with denitrification and N_2O flux levels, these results support other studies that have found reducing tillage and aggregate disruption in nutrient-rich sites to increase microbial biomass, particularly that of denitrifying microbes (Helgason et al., 2010; Blaud et al., 2012; Zhang et al., 2013; Zhang et al., 2018), and support our speculation about the effects of homogenization on our results. Significantly higher total fungal biomass in the injection soils and under ambient conditions (Fig. 8; Appendix 4), supports findings that plowing can reduce fungal activity by breaking up fungal hyphae (Helgason et al., 2010; Li et al., 2015).

EEA provided no significant results, suggesting that there was very little microbial activity occurring at the time of sampling (Appendix 3; Bell et al., 2013). Furthermore, levels of NH_4^+ and NO_3^- were relatively low (approximately 4 mg N kg soil^{-1}), NO_2^- levels were near zero, and no significant treatment results were found for NO_3^-, or NH_4^+ (Appendix 2). Low levels of inorganic N may partially explain the low levels of microbial activity, as NO_3^- is a limiting factor for denitrification and NO_2^- and NH_4^+ are limiting factors for nitrification (Phillips 2008; Saggar et al., 2013; Hu et al., 2015), and the duration of microbial activity is linked to labile nutrient availability (Kuzyakov and Blagodatskaya, 2015). Low nutrient levels and extreme freeze temperatures, combined with disruption of the soil matrix, aggregates, and aqueous microsites may have placed too much stress on the microbial communities, which may have caused microbes to become inactive or a die-back during freeze with few nutrients and viable sites to support high population activity or growth upon thaw (Or et al., 2007; Hao et al., 2001; Wang and Or, 2010; Makoto et al., 2014).

5. Conclusions

In summary, N_2O emissions during FTCs are a function of microbial activity and nutrient availability, which are related to aggregate physiochemical properties, such as C and N levels, porosity, water content, and O_2 diffusion (Or et al., 2007; Risk et al., 2014; Kuzyakov and Blagodatskaya, 2015; Bach et al., 2018). While it is known that both manure application method

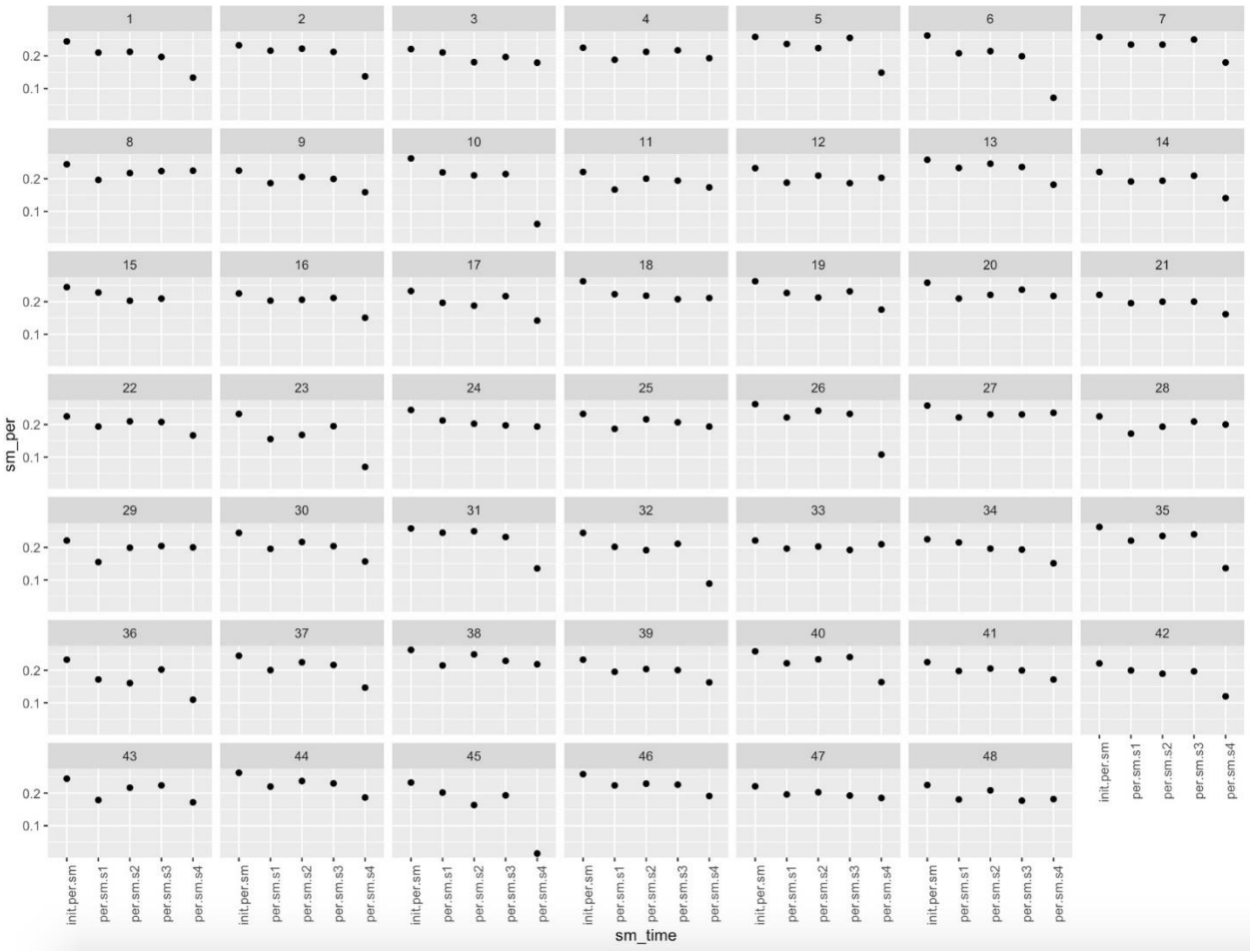
and FTCs can heavily influence N_2O fluxes independently, large knowledge gaps still exist surrounding interactions between the two. This study built off of the previous work by Adair et al. (2019), exploring the driving mechanisms that caused soils subject to fertilizer injection to have higher N_2O fluxes during simulated thawing and FTCs than those from soils subject to broadcast + plow incorporation.

Our findings were inconsistent, with some supporting our hypotheses and others contradicting both our hypotheses and the findings of Adair et al. (2019). Our findings suggest that homogenization of samples under low soil moisture conditions may have been detrimental to microbial community survival, microbial activity, and aggregate dynamics (Or et al., 2007; Li et al., 2015). While injection soils were found to generally have higher microbial biomass, denitrification was higher from broadcast + plow, suggesting that microbial communities in the injection soils may have been less tolerant of aggregate disturbance, causing them to become more inactive than those in the broadcast + plow soils. FTC action may have occurred in the freeze treatment soils during sampling, explaining the higher flux of N_2O from the freeze treatment.

In future studies, several steps can be taken to mitigate potential effects of soil structure destruction on incubation studies. First of all, use of undisturbed soil cores is recommended, allowing for laboratory study of the soil that best retains *in situ* aggregate and matric dynamics (Or et al., 2007; Kuzyakov and Blagodatskaya, 2015; Li et al., 2015). Second, collecting soils closer to the initiation of the incubation study and freezing them at the minimum temperature occurring *in situ* can minimize the potential for unrealistic levels of cell death that may be caused by extreme freezer temperatures (Henry, 2007). Finally, insulation of soil cores and unilateral freezing and thawing is recommended, as this would most closely represent FTC dynamics occurring in the field (Hu et al., 2006). Future research examining interactions between aggregate dynamics, soil moisture, microbial biomass and activity, and N_2O emissions during FTCs may help to solve unanswered questions that remain surrounding FTC-induced N_2O emissions (Wang et al., 2019). Studies examining the effects that homogenization may have on laboratory incubation results and on potential relationships between field management (i.e. tillage versus no tillage) and homogenization-induced changes in soil samples may be useful to improve the accuracy of laboratory incubation study methodologies and results.

Appendix

Appendix 1 Soil moisture over time



Appendix 1 The plots show soil moisture as a fraction of total sample weight over the course of the study. Each plot represents one of the 48 jars in the study, with the points showing the change in subsample soil moisture within each jar over time. The first point on each plot represents the initial soil moisture of the composite sample from which the jar's subsamples came from, and the four points following the initial point show the soil moisture of the first, second, third, and fourth subsamples within the jar, respectively. Gravimetric soil moisture of the subsamples was determined upon destructive sampling, which occurred on Days 1, 2, 4, and 7.

Appendix 2 Nitrate and ammonium analysis

Variable	X^2	DF	P	R^2
Nitrate (mg N kg soil^{-1})				
Manure	0.164	1	0.6851	
Temperature	0.328	1	0.5670	
Atmosphere	2.577	1	0.1085	
Manure*Temp	0.087	1	0.7678	
Manure*Atm	0.007	1	0.9342	
Temp*Atm	0.014	1	0.9059	
Manure*Temp*Atm	0.172	1	0.6781	
Marginal R^2				0.0683
Conditional R^2				0.0683
Ammonium (mg N kg soil^{-1})				
Manure	1.1546	1	0.2826	
Temperature	0.0024	1	0.9613	
Atmosphere	0.0621	1	0.8032	
Manure*Temp	0.0373	1	0.8469	
Manure*Atm	0.0515	1	0.8204	
Temp*Atm	0.1593	1	0.6898	
Manure*Temp*Atm	0.0106	1	0.9182	
Marginal R^2				0.0460
Conditional R^2				0.5716

Appendix 3 The table shows analysis of variance results (P-values) for nitrate and ammonium levels of all soil subsamples. The marginal R^2 value describes the proportion of variance explained by the fixed factors and the conditional R^2 describes the proportion of variance explained by the fixed and random factors. X^2 is the chi square value and DF is degrees of freedom. No significant results were found for any relationships or interactions.

Appendix 3 Extracellular enzyme ANOVA

Enzyme (nmol g soil^{-1})	Variable	X^2	DF	P	Marginal R^2	Conditional R^2
BG	Manure	0.070	1	0.7921	0.0359	0.3618
	Temperature	0.352	1	0.5531		
	Atmosphere	0.002	1	0.9641		
	Manure:Temperature	0.045	1	0.8313		
	Manure:Atmosphere	0.328	1	0.5668		
	Temperature:Atmosphere	0.634	1	0.426		
	Manure:Temperature:Atmosphere	0.148	1	0.7009		
LAP	Manure	0.059	1	0.8074	0.0605	0.1341
	Temperature	0.001	1	0.9789		
	Atmosphere	0.554	1	0.4568		
	Manure:Temperature	1.647	1	0.1994		
	Manure:Atmosphere	0.017	1	0.8971		
	Temperature:Atmosphere	0.308	1	0.5792		
	Manure:Temperature:Atmosphere	1.371	1	0.2416		
NAG	Manure	1.281	1	0.2578	0.0925	0.0925
	Temperature	1.180	1	0.2773		
	Atmosphere	0.002	1	0.9654		
	Manure:Temperature	1.231	1	0.2671		
	Manure:Atmosphere	0.842	1	0.3589		
	Temperature:Atmosphere	2.589	1	0.1076		
	Manure:Temperature:Atmosphere	0.114	1	0.7355		
Polyphenol oxidase	Manure	1.003	1	0.3167	0.0401	0.0401
	Temperature	1.144	1	0.2849		
	Atmosphere	0.000	1	0.9926		
	Manure:Temperature	0.251	1	0.6162		
	Manure:Atmosphere	0.726	1	0.3943		
	Temperature:Atmosphere	0.024	1	0.8771		
	Manure:Temperature:Atmosphere	0.209	1	0.648		
Peroxidase	Manure	0.386	1	0.5343	0.0347	0.0347
	Temperature	0.021	1	0.8852		
	Atmosphere	0.036	1	0.8497		
	Manure:Temperature	0.205	1	0.6505		
	Manure:Atmosphere	0.989	1	0.3201		
	Temperature:Atmosphere	0.672	1	0.4124		
	Manure:Temperature:Atmosphere	0.245	1	0.6206		

Appendix 3 The table shows analysis of variance results (P-values) for extracellular enzyme (BG, LAP, NAG, polyphenol oxidase, and peroxidase) levels of all soil subsamples. The marginal R^2 value describes the proportion of variance explained by the fixed factors and the conditional R^2 describes the proportion of variance explained by the fixed and random factors. X^2 is the chi square value and DF is degrees of freedom. No significant results were found for any relationships or interactions.

Appendix 4 ANOVA table for PLFA variables

Microbial pool (nmol g soil^{-1})	Variable	X^2	DF	P	R^2
AMF	Manure	0.061	1	0.8042	0.0701
	Temperature	0.393	1	0.5307	
	Atmosphere	2.601	1	0.1068	
	Manure*Temp	0.520	1	0.4709	
	Manure*Atm	1.539	1	0.2147	
	Temp*Atm	2.687	1	0.1012	
	Manure*Temp*Atm	0.933	1	0.3342	
Actinomycetes	Manure	0.147	1	0.7016	0.0623
	Temperature	0.098	1	0.7543	
	Atmosphere	1.616	1	0.2037	
	Manure*Temp	0.461	1	0.4970	
	Manure*Atm	2.271	1	0.1318	
	Temp*Atm	***3.337***	***1***	***0.0677***	
	Manure*Temp*Atm	0.599	1	0.4389	
Total fungi	**Manure**	**7.623**	**1**	**0.0058**	0.3160
	Temperature	0.157	1	0.6924	
	Atmosphere	**6.898**	**1**	**0.0086**	
	Manure*Temp	0.478	1	0.4895	
	Manure*Atm	0.415	1	0.5193	
	Temp*Atm	0.408	1	0.5232	
	Manure*Temp*Atm	0.827	1	0.3632	
Total bacteria	***Manure***	***2.905***	***1***	***0.0883***	0.2472
	Temperature	***3.296***	***1***	***0.0695***	
	Atmosphere	0.000	1	0.9889	
	Manure*Temp	0.171	1	0.6790	
	Manure*Atm	0.001	1	0.9762	
	Temp*Atm	0.875	1	0.3497	
	Manure*Temp*Atm	1.326	1	0.2495	
Gram + bacteria	***Manure***	***3.258***	***1***	***0.0711***	0.2593
	Temperature	**47.143**	**1**	**<0.0001**	
	Atmosphere	0.003	1	0.9576	
	Manure*Temp	0.583	1	0.4451	
	Manure*Atm	0.569	1	0.4508	
	Temp*Atm	1.953	1	0.1623	
	Manure*Temp*Atm	0.637	1	0.4249	
Gram - bacteria	**Manure**	**7.758**	**1**	**0.0053**	0.4391
	Temperature	**5.293**	**1**	**0.0214**	
	Atmosphere	0.941	1	0.3320	
	Manure*Temp	0.006	1	0.9367	
	Manure*Atm	0.099	1	0.7535	
	Temp*Atm	0.322	1	0.5706	
	Manure*Temp*Atm	0.264	1	0.6073	

Appendix 4 The table shows analysis of variance results (P-values) for the microbial pool (AMF, actinomycetes, total fungal biomass, total bacterial biomass, gram positive (+) bacteria, and gram negative (-) bacteria) tested for using PLFA in the Day 7 soil subsamples. The marginal R^2 value describes the proportion of variance explained by the fixed factors and the conditional R^2 describes the proportion of variance explained by the fixed and random factors. X^2 is the chi square value and DF is degrees of freedom.

Citations

Adair, C., Barbieri, L., Schiavone, K., Darby, H. 2019. Nutrient management practices impact greenhouse gas emissions during wintertime thaws. Journal of Environmental Quality.

Ameloot, N., Maenhout, P., De Neve, S., & Sleutel, S. (2016). Biochar-induced N2O emission reductions after field incorporation in a loam soil. *Geoderma*, *267*, 10-16.

Bach, E. M., Williams, R. J., Hargreaves, S. K., Yang, F., & Hofmockel, K. S. (2018). Greatest soil microbial diversity found in micro-habitats. *Soil Biology and Biochemistry*, *118*, 217-226.

Ball, B. C., Crichton, I., & Horgan, G. W. (2008). Dynamics of upward and downward N2O and CO2 fluxes in ploughed or no-tilled soils in relation to water-filled pore space, compaction and crop presence. *Soil and Tillage Research*, *101*(1-2), 20-30.

Barka, E.A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.P., Clément, C., Ouhdouch, Y. and van Wezel, G.P., (2016). Taxonomy, physiology, and natural products of Actinobacteria. *Microbiol. Mol. Biol. Rev.*, *80*(1), 1-43.

Bavin, T. K., Griffis, T. J., Baker, J. M., & Venterea, R. T. (2009). Impact of reduced tillage and cover cropping on the greenhouse gas budget of a maize/soybean rotation ecosystem. *Agriculture, ecosystems & environment*, *134*(3-4), 234-242.

Bell, C. W., Fricks, B. E., Rocca, J. D., Steinweg, J. M., McMahon, S. K., & Wallenstein, M. D. (2013). High-throughput fluorometric measurement of potential soil extracellular enzyme activities. *Journal of visualized experiments: JoVE*, (81).

Billings, S. A., & Tiemann, L. K. (2014). Warming-induced enhancement of soil N 2 O efflux linked to distinct response times of genes driving N_2O production and consumption. *Biogeochemistry*, *119*(1-3), 371-386.

Blaud, A., Lerch, T. Z., Chevallier, T., Nunan, N., Chenu, C., & Brauman, A. (2012). Dynamics of bacterial communities in relation to soil aggregate formation during the decomposition of 13C-labelled rice straw. *Applied soil ecology*, *53*, 1-9.

Bligh, E.G., Dyer, W.J., 1959. A rapid method for total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37 911–917.

Bocking, C. R., & Blyth, M. G. (2018). Oxygen uptake and denitrification in soil aggregates. *Acta Mechanica*, *229*(2), 595-612.

Bremner, J. M. (1997). Sources of nitrous oxide in soils. *Nutrient cycling in Agroecosystems*, *49*(1-3), 7-16.

Bridgham, S. D., & Richardson, C. J. (1992). Mechanisms controlling soil respiration (CO2 and CH4) in southern peatlands. *Soil Biology and Biochemistry*, *24*(11), 1089-1099.

Bruijn, A. M. G., Butterbach-Bahl, K., Blagodatsky, S., & Grote, R. (2009). Model evaluation of different mechanisms driving freeze–thaw N2O emissions. *Agr. Ecosyst. Environ*, *133*(3-4), 196-207.

Buckeridge, K. M., & Grogan, P. (2008). Deepened snow alters soil microbial nutrient limitations in arctic birch hummock tundra. *applied soil ecology*, *39*(2), 210-222.

Burney, J. A., Davis, S. J., & Lobell, D. B. (2010). Greenhouse gas mitigation by agricultural intensification. *Proceedings of the national Academy of Sciences*, *107*(26), 12052-12057.

Butterbach-Bahl, K., Baggs, E. M., Dannenmann, M., Kiese, R., & Zechmeister-Boltenstern, S. 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls?. *Philosophical Transactions of the Royal Society B.*, *368*(1621), 2013.01.22.

Cai, Y., Ding, W., Zhang, X., Yu, H., & Wang, L. (2010). Contribution of heterotrophic nitrification to nitrous oxide production in a long-term N-fertilized arable black soil. *Communications in soil science and plant analysis*, *41*(19), 2264-2278.

Cantera, J. J. L., & Stein, L. Y. (2007). Role of nitrite reductase in the ammonia-oxidizing pathway of Nitrosomonas europaea. *Archives of microbiology*, *188*(4), 349-354.

Chang, C., & Hao, X. 2001. Source of N2O Emission from a Soil During Freezing and Thawing. *PHYTON-HORN-*, *41*(3), 49-60.

Charles, A., Rochette, P., Whalen, J. K., Angers, D. A., Chantigny, M. H., & Bertrand, N. (2017). Global nitrous oxide emission factors from agricultural soils after addition of organic amendments: A meta-analysis. *Agriculture, Ecosystems & Environment*, *236*, 88-98.

Chen, Y., Tessier, S., MacKenzie, A. F., & Laverdiere, M. R. 1995. Nitrous oxide emission from an agricultural soil subjected to different freeze-thaw cycles. *Agriculture, ecosystems & environment*, *55*(2), 123-128.

Chen, Z., Wang, C., Gschwendtner, S., Willibald, G., Unteregelsbacher, S., Lu, H., Kolar, A., Schloter, M., Butterbach-Bahl, K. and Dannenmann, M. (2015). Relationships between denitrification gene expression, dissimilatory nitrate reduction to ammonium and nitrous oxide and dinitrogen production in montane grassland soils. *Soil Biology and Biochemistry*, *87*, 67-77.

Christensen, S., & Christensen, B. T. (1991). Organic matter available for denitrification in different soil fractions: effect of freeze/thaw cycles and straw disposal. *European Journal of Soil Science*, *42*(4), 637-647.

Congreves, K. A., Wagner-Riddle, C., Si, B. C., & Clough, T. J. (2018). Nitrous oxide emissions and biogeochemical responses to soil freezing-thawing and drying-wetting. Soil Biology and Biochemistry, 117, 5-15

Conrad, R. (1995). Soil microbial processes involved in production and consumption of atmospheric trace gases. In *Advances in microbial ecology* (pp. 207-250). Springer, Boston, MA.

Cui, P., Fan, F., Yin, C., Song, A., Huang, P., Tang, Y., Zhu, P., Peng, C., Li, T., Wakelin, S.A. and Liang, Y. (2016). Long-term organic and inorganic fertilization alters temperature sensitivity of potential N2O emissions and associated microbes. *Soil Biology and Biochemistry*, *93*, 131-141.

Dell, C. J., Meisinger, J. J., & Beegle, D. B. (2011). Subsurface application of manures slurries for conservation tillage and pasture soils and their impact on the nitrogen balance. *Journal of environmental quality*, *40*(2), 352-361.

Doane, T. A., & Horwath, W. R. (2003). Spectrophotometric determination of nitrate with a single reagent. *Analytical letters*, *36*(12), 2713-2722.

Dong, Z., Zhu, B., Hua, K., & Jiang, Y. (2015). Linkage of N2O emissions to the abundance of soil ammonia oxidizers and denitrifiers in purple soil under long-term fertilization. *Soil science and plant nutrition*, *61*(5), 799-807.

Dörsch, P., Palojärvi, A., & Mommertz, S. (2004). Overwinter greenhouse gas fluxes in two contrasting agricultural habitats. *Nutrient Cycling in Agroecosystems*, *70*(2), 117-133.

Duncan, E. W., Dell, C. J., Kleinman, P. J. A., & Beegle, D. B. 2017. Nitrous oxide and ammonia emissions from injected and broadcast-applied dairy slurry. *Journal of environmental quality*, *46*(1), 36-44.

Dusenbury, M. P., Engel, R. E., Miller, P. R., Lemke, R. L., & Wallander, R. (2008). Nitrous oxide emissions from a northern great plains soil as influenced by nitrogen management and cropping systems. *Journal of environmental quality*, *37*(2), 542-550..

Ebrahimi, A., & Or, D. (2016). Microbial community dynamics in soil aggregates shape biogeochemical gas fluxes from soil profiles–upscaling an aggregate biophysical model. *Global Change Biology*, *22*(9), 3141-3156.

Feng, W. A. N. G., Gao, S. B., Zhang, K. Q., Li, H. B., Bai, L. J., Huang, Z. P., & Mi, C. H. (2010). Effects of nitrogen application on N2O flux from fluvo-aquic soil subject to freezing-thawing process. *Agricultural Sciences in China*, *9*(4), 577-582.

Ferrara, R.M., Mazza, G., Muschitiello, C., Castellini, M., Stellacci, A.M., Navarro, A., Lagomarsino, A., Vitti, C., Rossi, R. and Rana, G. (2017). Short-term effects of conversion to no-tillage on respiration and chemical-physical properties of the soil: A case study in a wheat cropping system in semi-dry environment. *Ital. J. Agrometeorol*, *1*, 47-58.

Fierer, N. 2003. Stress Ecology and the Dynamics of Microbial Communities and Processes in Soil. University of California, 226 pages.

Flessa, H., & Beese, F. (2000). Laboratory estimates of trace gas emissions following surface application and injection of cattle slurry. *Journal of environmental quality*, *29*(1), 262-268.

Gamble, T. N., Betlach, M. R., & Tiedje, J. M. (1977). Numerically dominant denitrifying bacteria from world soils. *Appl. Environ. Microbiol.*, *33*(4), 926-939.

Grogan, P., Michelsen, A., Ambus, P., & Jonasson, S. (2004). Freeze–thaw regime effects on carbon and nitrogen dynamics in sub-arctic heath tundra mesocosms. *Soil Biology and Biochemistry*, *36*(4), 641-654.

Hageman, R. H., & Hucklesby, D. P. (1971). Nitrate reductase from higher plants. In *Methods in enzymology* (Vol. 23, pp. 491-503). Academic Press.

Hagemann, N., Harter, J., Kaldamukova, R., Guzman-Bustamante, I., Ruser, R., Graeff, S., Kappler, A. and Behrens, S., (2017). Does soil aging affect the N2O mitigation potential of biochar? A combined microcosm and field study. *Gcb Bioenergy*, *9*(5), 953-964.

Hao, X., Chang, C., Carefoot, J. M., Janzen, H. H., & Ellert, B. H. (2001). Nitrous oxide emissions from an irrigated soil as affected by fertilizer and straw management. *Nutrient Cycling in Agroecosystems*, *60*(1-3), 1-8.

Heil, J., Vereecken, H., & Brüggemann, N. (2016). A review of chemical reactions of nitrification intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil. *European journal of soil science*, *67*(1), 23-39.

Helgason, B. L., Walley, F. L., & Germida, J. J. (2010). No-till soil management increases microbial biomass and alters community profiles in soil aggregates. *Applied Soil Ecology*, *46*(3), 390-397.

Henry, H. A. (2007). Soil freeze–thaw cycle experiments: trends, methodological weaknesses and suggested improvements. *Soil Biology and Biochemistry*, *39*(5), 977-986.

Hood-Nowotny, R., Umana, N. H. N., Inselbacher, E., Oswald-Lachouani, P., & Wanek, W. (2010). Alternative methods for measuring inorganic, organic, and total dissolved nitrogen in soil. *Soil Science Society of America Journal*, *74*(3), 1018-1027.

Hu, H. W., Chen, D., & He, J. Z. (2015). Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. *FEMS microbiology reviews*, *39*(5), 729-749.

Hu, Q. C., Van Bochove, E., Warland, J., Kay, B., & Wagner-Riddle, C. (2006). New method to simulate soil freezing and thawing cycles for studying nitrous oxide flux. *Soil Science Society of America Journal*, *70*(6), 2106-2113.

Inselsbacher, E., Wanek, W., Ripka, K., Hackl, E., Sessitsch, A., Strauss, J., & Zechmeister-Boltenstern, S. (2011). Greenhouse gas fluxes respond to different N fertilizer types due to altered plant-soil-microbe interactions. *Plant and Soil*, *343*(1-2), 17-35.

Jacinthe, P. A., Dick, W., & Owens, L. 2002. Overwinter soil denitrification activity and mineral nitrogen pools as affected by management practices. *Biology and fertility of soils*, *36*(1), 1-9.

Jefferies, R. L., Walker, N. A., Edwards, K. A., & Dainty, J. (2010). Is the decline of soil microbial biomass in late winter coupled to changes in the physical state of cold soils?. *Soil Biology and Biochemistry*, *42*(2), 129-135.

Ji, B., Yang, K., Zhu, L., Jiang, Y., Wang, H., Zhou, J., & Zhang, H. (2015). Aerobic denitrification: A review of important advances of the last 30 years. *Biotechnology and bioprocess engineering*, *20*(4), 643-651.

Kaiser, E. A., & Ruser, R. (2000). Nitrous oxide emissions from arable soils in Germany—An evaluation of six long-term field experiments. *Journal of Plant Nutrition and Soil Science*, *163*(3), 249-259.

Khalil, K., Mary, B., & Renault, P. (2004). Nitrous oxide production by nitrification and denitrification in soil aggregates as affected by O2 concentration. *Soil Biology and Biochemistry*, *36*(4), 687-699.

Kim, D. G., Vargas, R., Bond-Lamberty, B., & Turetsky, M. R. 2012. Effects of soil rewetting and thawing on soil gas fluxes: a review of current literature and suggestions for future research. *Biogeosciences*, *9*(7), 2459.

Kong, A. Y., Fonte, S. J., van Kessel, C., & Six, J. (2009). Transitioning from standard to minimum tillage: Trade-offs between soil organic matter stabilization, nitrous oxide emissions, and N availability in irrigated cropping systems. *Soil and Tillage Research*, *104*(2), 256-262.

Kool, D. M., Wrage, N., Zechmeister-Boltenstern, S., Pfeffer, M., Brus, D., Oenema, O., & Van Groenigen, J. W. (2010). Nitrifier denitrification can be a source of N2O from soil: a revised approach to the dual-isotope labelling method. *European Journal of Soil Science*, *61*(5), 759-772.

Kuzyakov, Y., & Blagodatskaya, E. (2015). Microbial hotspots and hot moments in soil: concept & review. *Soil Biology and Biochemistry*, *83*, 184-199.

Kool et al. 2011. Nitrifier denitrification as a distinct and significant source of nitrous oxide from soil. Soil Biology and Biochemistry. 43:174-178.

Koponen, H. T., Flöjt, L., & Martikainen, P. J. (2004). Nitrous oxide emissions from agricultural soils at low temperatures: a laboratory microcosm study. *Soil Biology and Biochemistry*, *36*(5), 757-766.

Koponen, H. T., Jaakkola, T., Keinänen-Toivola, M. M., Kaipainen, S., Tuomainen, J., Servomaa, K., & Martikainen, P. J. (2006). Microbial communities, biomass, and activities in soils as affected by freeze thaw cycles. *Soil Biology and Biochemistry*, *38*(7), 1861-1871.

Koponen, H. T., & Bååth, E. (2016). Soil bacterial growth after a freezing/thawing event. *Soil Biology and Biochemistry*, *100*, 229-232.

Kremen, A., Bear, J., Shavit, U., & Shaviv, A. (2005). Model demonstrating the potential for coupled nitrification denitrification in soil aggregates. *Environmental science & technology*, *39*(11), 4180-4188.

Kværnø, S. H., & Øygarden, L. (2006). The influence of freeze–thaw cycles and soil moisture on aggregate stability of three soils in Norway. *Catena*, *67*(3), 175-182.

Lefcheck, J.S. 2015. piecewiseSEM: Piecewise structural equation modeling in R for ecology, evolution, and systematics. Methods Ecol. Evol. 75:573–579. doi:10.1111/2041-210X.12512

Li, N., Yao, S. H., Qiao, Y. F., Zou, W. X., You, M. Y., Han, X. Z., & Zhang, B. (2015). Separation of soil microbial community structure by aggregate size to a large extent under agricultural practices during early pedogenesis of a Mollisol. *Applied Soil Ecology*, *88*, 9-20.

Ligi, T., Truu, M., Truu, J., Nõlvak, H., Kaasik, A., Mitsch, W. J., & Mander, Ü. (2014). Effects of soil chemical characteristics and water regime on denitrification genes (nirS, nirK, and nosZ) abundances in a created riverine wetland complex. *Ecological Engineering*, *72*, 47-55.

Ludwig, B., Wolf, I., & Teepe, R. (2004). Contribution of nitrification and denitrification to the emission of N2O in a freeze-thaw event in an agricultural soil. *Journal of Plant Nutrition and Soil Science*, *167*(6), 678-684.

Maguire, R.O., P.J.A. Kleinman, C.J. Dell, D.B. Beegle, R.C. Brandt, J.M. McGrath, et al. 2011. Manure application technology in reduced tillage and forage systems: A review. J. Environ. Qual. 40:292–301. doi:10.2134/ jeq2009.0228

Makoto, K., Kajimoto, T., Koyama, L., Kudo, G., Shibata, H., Yanai, Y., & Cornelissen, J. H. C. (2014). Winter climate change in plant–soil systems: summary of recent findings and future perspectives. *Ecological research*, *29*(4), 593-606.

Mangalassery, S., Sjögersten, S., Sparkes, D. L., Sturrock, C. J., & Mooney, S. J. (2013). The effect of soil aggregate size on pore structure and its consequence on emission of greenhouse gases. *Soil and Tillage Research*, *132*, 39-46.

Manucharova, N. A., Stepanov, A. L., & Umarov, M. M. (2001). Microbial transformation of nitrogen in water-stable aggregates of various soil types. *Eurasian soil science*, *34*(10), 1125-1131.

Matzner, E., & Borken, W. (2008). Do freeze-thaw events enhance C and N losses from soils of different ecosystems? A review. *European Journal of Soil Science*, *59*(2), 274-284.

Morales, S. E., Cosart, T., & Holben, W. E. (2010). Bacterial gene abundances as indicators of greenhouse gas emission in soils. *The ISME Journal*, *4*(6), 799.

Mosier, A. R., Parton, W. J., & Hutchinson, G. L. (1983). Modelling nitrous oxide evolution from cropped and native soils. *Ecological Bulletins*, 229-241.

Mørkved, P. T., Dörsch, P., Henriksen, T. M., & Bakken, L. R. (2006). N2O emissions and product ratios of nitrification and denitrification as affected by freezing and thawing. *Soil Biology and Biochemistry*, *38*(12), 3411-3420.

Müller, C., Kammann, C., Ottow, J. C. G., & Jäger, H. J. (2003). Nitrous oxide emission from frozen grassland soil and during thawing periods. *Journal of Plant Nutrition and Soil Science*, *166*(1), 46-53.

Nakagawa, S., and H. Schielzeth. 2013. A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods Ecol. Evol. 4:133–142. doi:10.1111/j.2041-210x.2012.00261.x

Németh, D. D., Wagner-Riddle, C., & Dunfield, K. E. (2014). Abundance and gene expression in nitrifier and denitrifier communities associated with a field scale spring thaw N2O flux event. *Soil Biology and Biochemistry*, *73*, 1-9.

Or, D., Smets, B. F., Wraith, J. M., Dechesne, A., & Friedman, S. P. (2007). Physical constraints affecting bacterial habitats and activity in unsaturated porous media–a review. *Advances in Water Resources*, *30*(6-7), 1505-1527.

Pathak, H. 1999. Emissions of nitrous oxide from soil. *Current science*, 359-369.

Phillips, R. L. (2008). Denitrification in cropping systems at sub-zero soil temperatures. A review. *Agronomy for sustainable development*, *28*(1), 87-93.

Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar, and R.C. Team. 2017. nlme: Linear and nonlinear mixed effects models. R package version 3.1-128.

Priemé, A., & Christensen, S. (2001). Natural perturbations, drying–wetting and freezing–thawing cycles, and the emission of nitrous oxide, carbon dioxide and methane from farmed organic soils. *Soil Biology and Biochemistry*, *33*(15), 2083-2091.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rabot, E., Wiesmeier, M., Schlüter, S., & Vogel, H. J. (2018). Soil structure as an indicator of soil functions: a review. *Geoderma*, *314*, 122-137.

Raich, J. W., & Tufekciogul, A. (2000). Vegetation and soil respiration: correlations and controls. *Biogeochemistry*, *48*(1), 71-90.

Ren, X., Weitzel, M., O'Neill, B.C., Lawrence, P., Meiyappan, P., Levis, S., Balistreri, E.J. and Dalton, M., (2018). Avoided economic impacts of climate change on agriculture: integrating a land surface model (CLM) with a global economic model (iPETS). *Climatic Change*, *146*(3-4), 517-531.

Risk, N., Snider, D., & Wagner-Riddle, C. (2013). Mechanisms leading to enhanced soil nitrous oxide fluxes induced by freeze—thaw cycles. *Canadian Journal of Soil Science*, *93*(4), 401-414.

Risk, N., Wagner-Riddle, C., Furon, A., Warland, J., & Blodau, C. (2014). Comparison of simultaneous soil profile N 2 O concentration and surface N 2 O flux measurements overwinter and at spring thaw in an agricultural soil. *Soil Science Society of America Journal*, *78*(1), 180-193.

Rochette, P., Angers, D. A., Chantigny, M. H., & Bertrand, N. (2008). Nitrous oxide emissions respond differently to no-till in a loam and a heavy clay soil. *Soil Science Society of America Journal*, *72*(5), 1363-1369.

Röver, M., Heinemeyer, O., & Kaiser, E. A. 1998. Microbial induced nitrous oxide emissions from an arable soil during winter. *Soil Biology and Biochemistry*, *30*(14), 1859-1865

RStudio Team. 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL: http://www.rstudio.com/.

Ruan, L., & Robertson, G. P. (2017). Reduced snow cover increases wintertime nitrous oxide (N 2 O) emissions from an agricultural soil in the upper US midwest. *Ecosystems*, *20*(5), 917-927.

Saggar, S., Jha, N., Deslippe, J., Bolan, N.S., Luo, J., Giltrap, D.L., Kim, D.G., Zaman, M. and Tillman, R.W. 2013. Denitrification and N2O: N2 production in temperate grasslands: processes, measurements, modelling and mitigating negative impacts. *Science of the Total Environment*, *465*, 173-195.

Sehy, U., Dyckmans, J., Ruser, R., & Munch, J. C. (2004). Adding dissolved organic carbon to simulate freeze-thaw related N2O emissions from soil. *Journal of Plant Nutrition and Soil Science*, *167*(4), 471-478.

Setia, R., Verma, S. L., & Marschner, P. (2012). Measuring microbial biomass carbon by direct extraction–comparison with chloroform fumigation-extraction. *European journal of soil biology*, *53*, 103-106.

Sey, B. K., Manceur, A. M., Whalen, J. K., Gregorich, E. G., & Rochette, P. (2008). Small-scale heterogeneity in carbon dioxide, nitrous oxide and methane production from aggregates of a cultivated sandy-loam soil. *Soil Biology and Biochemistry*, *40*(9), 2468-2473.

Sharma, S., Szele, Z., Schilling, R., Munch, J. C., & Schloter, M. (2006). Influence of freeze-thaw stress on the structure and function of microbial communities and denitrifying populations in soil. *Appl. Environ. Microbiol.*, *72*(3), 2148-2154.

Six, J., Bossuyt, H., Degryze, S., & Denef, K. (2004). A history of research on the link between (micro) aggregates, soil biota, and soil organic matter dynamics. *Soil and Tillage Research*, *79*(1), 7-31.

Soil Survey Staff, N. R. C. S., United States Department of Agriculture. 2017. Web Soil Survey.

Song, Y., Zou, Y., Wang, G., & Yu, X. (2017). Altered soil carbon and nitrogen cycles due to the freeze-thaw effect: A meta-analysis. *Soil Biology and Biochemistry*, *109*, 35-49.

Stevens, R.J., R.J. Laughlin, L.C. Burns, J.R.M. Arah, and R.C. Hood. 1997. Measuring the contributions of nitrification and denitrification to the flux of nitrous oxide from soil. Soil Biol. Biochem. 29: 139-151. doi:10.1016/S0038-0717(96)00303-3

Sun, B., Jia, S., Zhang, S., McLaughlin, N.B., Zhang, X., Liang, A., Chen, X., Wei, S. and Liu, S. (2016). Tillage, seasonal and depths effects on soil microbial properties in black soil of Northeast China. *Soil and Tillage Research*, *155*, 421-428.

Teepe, R., Brumme, R., & Beese, F. (2001). Nitrous oxide emissions from soil during freezing and thawing periods. *Soil Biology and Biochemistry*, *33*(9), 1269-1275.

Thornton, P. E., Thornton, M. M., Mayer, B. W., Wilhelmi, N., Wei, Y., Devarakonda, R., & Cook, R. B. (2014). *Daymet: Daily Surface Weather Data on a 1-km Grid for North America, Version 2*. Oak Ridge National Lab.(ORNL), Oak Ridge, TN (United States).

Tiedje, J. M. (1988). Ecology of denitrification and dissimilatory nitrate reduction to ammonium. *Biology of anaerobic microorganisms*, *717*, 179-244.

Tiedje, J. M., Simkins, S., & Groffman, P. M. (1989). Perspectives on measurement of denitrification in the field including recommended protocols for acetylene based methods. *Plant and Soil*, *115*(2), 261-284.

United Nations. (2015). World population projected to reach 9.7 billion by 2050.

van Bochove, E., Prevost, D., & Pelletier, F. (2000). Effects of freeze–thaw and soil structure on nitrous oxide produced in a clay soil. *Soil Science Society of America Journal*, *64*(5), 1638-1643.

Vallejo, A., Garcı´a-Torres, L., Dı´ez, J.A., Arce, A., Lo´ pez-Ferna´ ndez, S., 2005. Comparison of N losses (NO3, N2O, NO) from surface applied, injected or amended (DCD) pig slurry of an irrigated soil in a Mediterranean climate. Plant Soil 272, 313–325.

Velthof, G. L., Kuikman, P. J., & Oenema, O. (2003). Nitrous oxide emission from animal manures applied to soil under controlled conditions. *Biology and Fertility of Soils*, *37*(4), 221-230.

Wang, B., Brewer, P. E., Shugart, H. H., Lerdau, M. T., & Allison, S. D. (2019). Soil aggregates as biogeochemical reactors and implications for soil–atmosphere exchange of greenhouse gases—A concept. *Global change biology*, *25*(2), 373-385.

Wang, J., Song, C., Hou, A., Miao, Y., Yang, G., & Zhang, J. (2014). Effects of freezing–thawing cycle on peatland active organic carbon fractions and enzyme activities in the Da Xing'anling Mountains, Northeast China. *Environmental earth sciences*, *72*(6), 1853-1860.

Wang, L., Sun, X., Cai, Y., Xie, H., & Zhang, X. (2008). Relationships of soil physical and microbial properties with nitrous oxide emission affected by freeze-thaw event. *Frontiers of Agriculture in China*, *2*(3), 290-295.

Wagner-Riddle, C., Thurtell, G. W., Kidd, G. K., Beauchamp, E. G., & Sweetman, R. (1997). Estimates of nitrous oxide emissions from agricultural fields over 28 months. *Canadian Journal of Soil Science*, *77*(2), 135-144.

Wagner-Riddle, C., Furon, A., Mclaughlin, N.L., Lee, I., Barbeau, J., Jayasundara, S., Parkin, G., Von Bertoldi, P. and Warland, J.O.N., (2007). Intensive measurement of nitrous oxide emissions from a corn–soybean–wheat rotation under two contrasting management systems over 5 years. *Global Change Biology*, *13*(8), 1722-1736.

Wagner-Riddle, C., Q.C. Hu, E. van Bochove and S. Jayasundara. 2008. Linking nitrous oxide flux during spring thaw to nitrate denitrification in the soil profile Soil Sci. Soc. Am. J. 72: 908-916. doi:10.2136/sssaj2007.0353.

Wagner-Riddle, Claudia, Katelyn A. Congreves, Diego Abalos, Aaron A. Berg, Shannon E. Brown, Jaison Thomas Ambadan, Xiaopeng Gao, and Mario Tenuta. 2017. Globally important nitrous oxide emissions from croplands induced by freeze–thaw cycles. *Nature Geoscience*, *10*(4), 279.

Wallenstein, M. D., Myrold, D. D., Firestone, M., & Voytek, M. (2006). Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. *Ecological applications*, *16*(6), 2143-2152.

Wang, G., & Or, D. (2010). Aqueous films limit bacterial cell motility and colony expansion on partially saturated rough surfaces. *Environmental microbiology*, *12*(5), 1363-1373.

Webb, J., Pain, B., Bittman, S., & Morgan, J. (2010). The impacts of manure application methods on emissions of ammonia, nitrous oxide and on crop response—a review. *Agriculture, Ecosystems & Environment*, *137*(1-2), 39-46.

Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical chemistry*, *39*(8), 971-974.

Wrage, N., Velthof, G. L., Van Beusichem, M. L., & Oenema, O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. Soil biology and Biochemistry, 33(12-13), 1723-1732.

Wrage-Mönnig, N., Horn, M. A., Well, R., Müller, C., Velthof, G., & Oenema, O. (2018). The role of nitrifier denitrification in the production of nitrous oxide revisited. *Soil Biology and Biochemistry*, *123*, A3-A16.

Wu, T., Milner, H., Díaz-Pérez, J. C., & Ji, P. (2015). Effects of soil management practices on soil microbial communities and development of southern blight in vegetable production. *Applied Soil Ecology*, *91*, 58-67.

Wulf, S., Maeting, M., & Clemens, J. (2002). Application technique and slurry co-fermentation effects on ammonia, nitrous oxide, and methane emissions after spreading. *Journal of environmental quality*, *31*(6), 1795-1801.

Xue, Y., van Es, H. M., Schindelbeck, R. R., Moebius-Clune, B. N., Melkonian, J. J., Graham, C., & Yang, P. (2013). Effects of N placement, carbon distribution and temperature on N2O emissions in clay loam and loamy sand soils. *Soil Use and Management*, *29*(2), 240-249.

Yanai, Y., Toyota, K., & Okazaki, M. (2004). Effects of successive soil freeze-thaw cycles on nitrification potential of soils. *Soil Science and Plant Nutrition*, *50*(6), 831-837.

Yu, R., Kampschreur, M. J., Loosdrecht, M. C. V., & Chandran, K. (2010). Mechanisms and specific directionality of autotrophic nitrous oxide and nitric oxide generation during transient anoxia. *Environmental Science & Technology*, *44*(4), 1313-1319.

Zhang, X., Bai, W., Gilliam, F. S., Wang, Q., Han, X., & Li, L. (2011). Effects of in situ freezing on soil net nitrogen mineralization and net nitrification in fertilized grassland of northern China. *Grass and Forage Science*, *66*(3), 391-401.

Zhang, J., Müller, C., & Cai, Z. (2015). Heterotrophic nitrification of organic N and its contribution to nitrous oxide emissions in soils. *Soil Biology and Biochemistry*, *84*, 199-209.

Zhang, S., Li, Q., Zhang, X., Wei, K., Chen, L., & Liang, W. (2012). Effects of conservation tillage on soil aggregation and aggregate binding agents in black soil of Northeast China. *Soil and Tillage Research*, *124*, 196-202.

Zhang, S., Li, Q., Lü, Y., Zhang, X., & Liang, W. (2013). Contributions of soil biota to C sequestration varied with aggregate fractions under different tillage systems. *Soil Biology and Biochemistry*, *62*, 147-156.

Zhang, X., Xin, X., Zhu, A., Yang, W., Zhang, J., Ding, S., Mu, L. and Shao, L., (2018). Linking macroaggregation to soil microbial community and organic carbon accumulation under different tillage and residue managements. *Soil and Tillage Research*, *178*, 99-107.

Zhu, X., Burger, M., Doane, T. A., & Horwath, W. R. (2013). Ammonia oxidation pathways and nitrifier denitrification are significant sources of N2O and NO under low oxygen availability. *Proceedings of the National Academy of Sciences*, *110*(16), 6328-6333.