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Abundance and gene expression in nitrifier and denitrifier communities associated with a field scale spring thaw N_2O flux event

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ABSTRACT

Agricultural soils are the main global source of nitrous oxide (N_2O) emissions. Nitrous oxide is produced by nitrifying and denitrifying microbes in soils. In cold regions such as Canada, N_2O emissions during spring thaw can account for the majority of annual emissions. The objectives of this study were: (i) to quantify gene expression in nitrifying and denitrifying communities by targeting key functional gene transcripts in the nitrogen cycle (*amoA*, *nirS*, *nosZ*), and, (ii) to compare microbial activity to timing of N_2O flux in two conventionally tilled corn fields over spring thaw, one field with residues removed (−R), and one with residues returned (+R). In both fields, initial sampling contained populations with *amoA* and *nirS* transcripts, and these quantities did not significantly change over the spring thaw sampling period. In contrast, the −R field had a denitrifying population that transcribed significantly less *nosZ*, and abundance increased significantly over the sampling period, N_2O flux over the spring thaw was inversely proportional to *nosZ* transcription. The combination of anaerobic soil conditions favoring denitrification, nutrient availability, and gene expression in both nitrifiers and denitrifiers present at the onset of the spring thaw provide evidence of *de novo* denitrification. To the best of our knowledge, ours is the first study to relate the analysis of the quantity and activity of N cycling soil microbes *in situ* to the timing of a spring thaw N_2O emission event.

1. Introduction

Nitrous oxide (N_2O) is an environmentally significant gas because of its dual role as a potent greenhouse gas and contributor to the destruction of stratospheric ozone (IPCC, 2007; Ravishankara et al., 2009). Soils, both natural and managed, are considered the primary source of this trace gas in global greenhouse gas budgets (Syakila and Kroeze, 2011). However, the emission of N_2O from soil is highly spatially and temporally variable, resulting in hot spots (small areas) and hot moments (brief periods) that account for the majority of annual N_2O emissions, making measurement, modeling N_2O emissions, and understanding *in situ* processes extremely challenging (Groffman et al., 2009; Butterbach-Bahl et al., 2013).

In regions that experience freeze–thaw cycles (FTC) such as Canada, the highest rates of N_2O emissions over the year often occur during spring thaw, accounting for up to 70% of annual emissions (Lemke et al., 1998; Papen and Butterbach-Bahl, 1999; Wagner-Riddle et al., 2007). Freeze–thaw cycles have a major impact on soil physical and chemical conditions, as well as on soil microbial communities, resulting in a combination of factors that promote N_2O production, recently reviewed by Risk et al. (2013). Physical release of N_2O from subsurface soil layers was initially considered the main mechanism contributing to spring thaw emissions, but most current studies suggest *de novo* production is the source of enhanced fluxes (Risk et al., 2013). Snow and ice melt results in an increase in the amount of water-filled pore space, and availability of anaerobic microsites in the soil (Teepe et al., 2000; Koponen and Martikainen, 2004). In addition, freeze–thaw cycles induce a flush of carbon and nitrogen substrates trapped in soil aggregates (Edwards and Cresser, 1992) and microbial cells (Herrmann and Witter, 2002). Finally, the microbial enzyme, nitrous oxide reductase (*nosZ*), may be impaired by low temperatures causing an increase in the N_2O:N_2 ratio (Holtan-Hartwig et al., 2002).

In general, soil N_2O emissions are attributed predominantly to two microbial processes, autotrophic nitrification and heterotrophic denitrification (Braker and Conrad, 2011). Nitrification is the aerobic production of nitrate from ammonia, with N_2O being produced as a byproduct through the chemical decomposition of hydroxylamine (Firestone et al., 1980). Denitrification, or the

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anaerobic reduction of nitrate to N_2, produces N_2O as an intermediate (Anderson and Levine, 1986). However, the complexity of microbial processes that lead to N_2O formation in the soil is now understood to include nitrifier denitrification, co-denitrification and nitrate ammonification, or dissimilatory nitrate reduction to ammonium (Butterbach-Bahl et al., 2013). Most studies of freeze–thaw N_2O emissions have concluded that denitrification is the dominant process responsible for the large thaw fluxes (Müller et al., 2002; Müller et al., 2003; Ludwig et al., 2004; Oquist et al., 2004; Sehy et al., 2004; Koponen et al., 2006; Mørkved et al., 2006; Wagner-Riddle et al., 2008). However, no studies have characterized functional gene expression in microbial communities associated with freeze–thaw N_2O emissions under field conditions.

A previous study at our research site characterized microbial communities using denaturing gradient gel electrophoresis coupled with PCR (PCR-DGGE), and showed a significant change in the diversity of the microbial communities containing ammonia monoxygenase (*amoA*) and nitrite reductase (*nirS*, *nirK*) associated with a significant spring thaw N_2O emission event (Smith et al., 2010). Although this study demonstrated a structural change in nitrifying and denitrifying communities, it did not quantify corresponding population abundance in the soil environment during the spring thaw event. Recent studies have suggested that the abundance of nitrifying or denitrifying organisms in soil may relate to the level of N_2O emissions from that soil (Hallin et al., 2009; Braker et al., 2010), therefore studies focused on quantifying these communities are important. In a lab based study, Sharma et al. (2006) studied freeze thaw effects on denitrification genes using packed homogenous soil microcosms that were frozen to −20 °C and thawed to a temperature of 10 °C. In their study, transcripts of the denitrification pathway genes *napA* and *nirS*, were quantified with most probable number PCR (MPN-PCR), but no *nirK* or *nosZ* genes could be detected. The community structure was also found to change over the course of thawing, with the most complex community being observed at the end of the N_2O flux event for *nirS* and a decrease in complexity for *napA* at the end of thaw. Laboratory freeze–thaw experiments are limited because they can subject soils to extreme and unrealistic temperatures in a way that may introduce bias (Henry, 2007). In addition, in the field, natural soils are covered by snow and litter layers that provide an insulating effect, and can impact soil physicochemical conditions and consequently N_2O emissions (Henry, 2007). Therefore, it is important to quantify gene expression from microbial populations *in situ* during a spring thaw event. These types of studies are inherently challenging due to the difficulty of predicting weather patterns and thus soil and air temperature changes, and therefore, the timing of a spring thaw N_2O emission event.

The objectives of this study were to quantify abundance and gene expression in nitrifying and denitrifying communities over spring thaw by targeting key functional genes in the nitrogen cycle (*amoA*, *nirS*, and *nosZ*), and to compare microbial gene expression to timing of N_2O flux in two fields. Using a unique approach of intensive temporal soil sampling, followed by DNA/RNA extraction and quantitative polymerase chain reaction (Q-PCR) and continuous micrometeorological measurements of field-scale N_2O emissions, we were able to enumerate populations of nitrifiers and denitrifiers and gene expression during a spring thaw N_2O emission event *in situ*.

2. Materials and methods

2.1. Site description

The experimental site is located at the Elora Research Station (University of Guelph, Ontario, Canada, 43°38′ N–80°25′ W, Elev 376 m). Soil textural analysis indicated 29% sand, 52% silt, and 19% clay in the 0–15 cm soil layer (Jayasundara et al., 2007). The soil type is an imperfectly drained Guelph silt loam with an average pH of 8, 19 mg kg^{-1} dry soil P, 127 mg kg^{-1} dry soil K. The carbon content is approximately 30 g C kg^{-1} (Jayasundara et al., 2007).

As part of a long term field study a corn–soybean–wheat rotation has been grown under contrasting agricultural management practices on four 150 m by 100 m (1.5 ha) fields located within an aerodynamically homogeneous 30-ha area, a requirement for micrometeorological measurements. The study reported here was conducted on two conventionally-tilled (CT) fields (Fig. 1). From January 2000 to November 2008, the two fields had identical conventional tillage management (CT1 and CT2), which consisted of fall moldboard plowing followed by disking before planting (Jayasundara et al., 2007). Sampling focused specifically on one field year (2010), because of cost constraints and given the fields have consistently produced a significant nitrous oxide flux event during spring thaw (Wagner-Riddle et al., 2007). A study in 2006 indicated that the two CT fields did not differ significantly with respect to soil inorganic nitrogen and microbial community diversity (Smith et al., 2010). In addition, annual N_2O emissions over 2000–2005 were not significantly different and averaged 2.1 ± 0.43 (S.E.) and 2.3 ± 0.50 kg N ha^{-1} yr^{-1} for CT1 and CT2 with 56 and 60% of emissions occurring during Nov to April, respectively.

Starting in November 2008, a differential crop residue management was applied to the two fields with CT1 continuing to have residue returned (+R), and CT2 having residue removed or no residue returned (−R) as part of a larger study investigating the effect of residue removal in CT and no-tillage systems. In this study we did not aim at quantifying the effects of residue removal on N_2O fluxes, but rather we used fields managed in a contrasting way to investigate differences in the relationships between N_2O flux and gene abundance and gene expression.

Measurements for this study were conducted from January to April 2010. Residue return consisted of seed harvest removal and corn residue (leaves, stems, husks) (9.16 ± 0.82 t dry wt ha^{-1}) returned to the soil surface post harvest. The corn residue was mulched with a flail mower to facilitate residue incorporation by

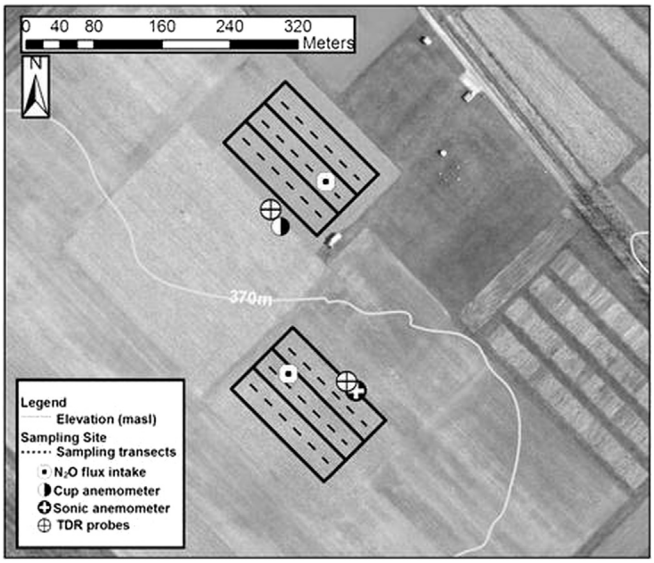

Fig. 1. Layout of experimental site (1.5 ha fields) at Elora, Ontario. Location of N_2O gas sampling intakes, cup anemometers, sonic anemometer are indicated. Soil samples were collected along three replicated transects indicated by dashed lines. Fields were conventionally tilled, and residues were returned (+R) or no residue was returned (−R).

disk cultivation. The residue removal treatment consisted of ensiling corn stalks (9.13 ± 0.82 t dry wt ha^{-1}) approximately 5 cm above the soil with a silage cutter, and removing from the field. The CT fields had post harvest tillage (disking to 10–15 cm depth) applied within 4 h post residue harvest (November 10, 2009). Other relevant management practices applied during the growing season of 2009 consisted of: planting of corn on May 12, and applying 56 kg ha^{-1} (mono-ammonium phosphate 11-52-0) starter fertilizer at planting followed by 118 kg N ha^{-1} side dressing of liquid urea and ammonium nitrate (UAN) (28-0-0) when corn was at the 5–7 leaf stage on June 23.

2.2. Surface nitrous oxide flux measurements

The half-hourly flux of N_2O per field (F_{N_2O}) was measured using the flux gradient method (Wagner-Riddle et al., 1996):

$$F_{N_2O} = \frac{u^* k \Delta C}{\left[\ln\left(\frac{z_2-d}{z_1-d}\right) - \psi_{h_2} + \psi_{h_1}\right]} \quad (1)$$

where u^* is the friction velocity, k is the von Karman constant (=0.41), ΔC is the difference of N_2O concentration [N_2O] between sample heights z_2 and z_1, d is the displacement height, and ψ_{h_2} and ψ_{h_1} are the integrated Monin–Obukhov similarity functions for heat for each sampling heights. The friction velocity and the integrated Monin–Obukhov similarity functions for heat were obtained using sonic anemometers. Cup anemometers were used to estimate u^* when sonic anemometer data were not available. A sampling tower was setup in each field (Fig. 1) and air was continuously directed to a sampling manifold located in a centrally located instrumentation trailer through the use of a vacuum pump (Wagner-Riddle et al., 2007). Half-hourly averages of N_2O concentration differences between the lower and upper sampling heights were determined on sampled air using a tunable diode laser trace gas analyzer (TGA 100, Campbell Scientific Inc., Campbell Scientific Inc., Logan, UT) housed in the instrumentation trailer. Each of four fields was sequentially sampled; therefore each field had a maximum of 12 half-hourly flux values per day. Data presented here refer to two of the fields as described above. The half-hourly data from each field was filtered according to criteria described in Wagner-Riddle et al. (2007), including fetch criteria which ensured measured fluxes were integrated over each of the field's area. Daily flux means were calculated by averaging half-hourly F_{N_2O} values using a minimum of two data points.

2.3. Collection of soil samples

The timing of main spring thaw is quite variable in the study region and can occur anytime between mid-February and early April depending on weather conditions. Hence, identifying when the main spring thaw was occurring was critical as budget constraints did not allow for continuous sampling. Based on previous year's thaws it was determined that the main N_2O flux event usually occurs at the time of fast snow melting and when surface temperature is > 0 °C (Wagner-Riddle et al., 2010). Surface temperature was measured remotely using an infrared thermometer installed 1.5 m above and pointing at the soil surface on each field. Using surface temperature data, and weather forecasts of air temperature as well as real-time micrometeorological measurements, intensive field sampling was started on March 7, and soil samples were taken approximately every 48 h for two weeks (March 7, 9, 11, 13, 15, 17, 19). Multiple mechanisms likely lead to high spring thaw N_2O emissions. N_2O may be formed in unfrozen water films in frozen soil and released upon soil thaw, or from increased microbial production of N_2O in thawing soil (*de novo*) (Koponen and Martikainen, 2004; Oquist et al., 2004; Sharma et al., 2006). We wished to target the *de novo* microbial production of N_2O, therefore, we sampled the soil thaw layer. To quantify microbial communities in the soil thaw layer, soil was collected with sterilized sampling tools from the soil surface (variable depth between 0 and 0.5 cm depth). In an attempt to account for spatial variability within the footprint (1.5 ha) of our flux measurements, a systematic soil sampling design was used (Pennock et al., 2007). Soil was sampled along three (150 m) transects (replicates), 8 subsamples were randomly collected at regular intervals along each transect and combined into a composite sample (Fig. 1). There were a total of 42 soil samples (2 fields × 7 dates × 3 reps) collected. Soil samples (*ca.* 2 g) were placed immediately into a pre-weighed sterile collection tube containing (1 mL g^{-1} soil) LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc. Carlsbad, CA) to stabilize nucleic acids *in situ* for transportation to the laboratory, as described previously in Yarwood et al. (2013). Samples were stored on ice in a cooler and transported to the lab. In the lab, exact soil weights used in each extraction were determined by re-weighing each tube with soil, then converting to gram dry weight using soil gravimetric water content. Samples were frozen (−20 °C) and extracted within 12–28 d.

2.4. Soil physicochemical analysis

Within each field, volumetric liquid water content (VWC, m^3 m^{-3}) was measured using three Time Domain Reflectometry (TDR) probes (Model CS616, Campbell Scientific Inc., Logan), installed at approximately 5 cm depth, and 18 m apart on each field.

Soil temperature was measured using thermocouples built in house. Three sensors were installed in each field at approximately 3–5 cm depth close to the TDR probe location. Half-hourly mean VWC and soil temperature were recorded with a datalogger (21X Campbell Scientific Inc., Edmonton, AB).

Dissolved organic carbon (DOC) and soil exchangeable NH_4^+–N and NO_3^-–N were measured from a composite of 10 cores per transect per field (0–15 cm soil depth) collected prior to, and near the end of the spring thaw event (March 4 and 17, 2010). Soil DOC was determined by taking 80 g dry soil and extracting with 150 mL 0.5 M K_2SO_4 followed by shaking for 1 h. Filtered extractant was analyzed using a TOC-5000 analyzer (Shimadzu, Kyoto, Japan). Exchangeable NH_4^+–N and NO_3^-–N were extracted using 10 g wet sieved soil and 50 mL 2.0 M KCl and shaken for 1 h (Maynard et al., 2007). Filtered extract was analyzed for NH_4^+-N (Salicylate QuikChem® Method 12-107-06-2-A), and for NO_3^-–N (Cadmium-copper reduction, QuikChem® Method 12-107-04-1-B) using a Lachat AutoAnalyzer (Lachat Instruments, Loveland, Co, USA). Standards for NH_4^+–N and NO_3^-–N were in the range of (0.0–5.0 and 0.0–10.0 ppm N, respectively). Analyzed NH_4^+–N and NO_3^-–N results were converted to mg N kg^{-1} dry soil.

The bulk density from six soil cores per field were collected (2 cores per transect) at 3–10 cm depth using a 4.3 cm cylindrical aluminum core sampler, on March 4 and March 17. Cores were bagged, and transported to the laboratory and weighed before and after drying in an oven (24 h at 105 °C) (Maynard et al., 2007). Liquid water-filled pore space (WFPS) over March 4–17 was calculated using VWC, measured bulk density and an assumed particle density of 2.65 g cm^{-3}.

2.5. Nucleic acid extraction

The total DNA and RNA contents of soil samples were co-isolated according to the manufacturer's protocol using the RNA PowerSoil® Total RNA Isolation Kit and DNA Elution Accessory Kit (MoBio Laboratories, Inc. Carlsbad, CA). Both RNA and DNA were eluted in

RNase and DNase-free water to a final volume of 50 μL, and stored at −80 °C. The quality and purity of DNA and RNA were checked by 1% agarose gel electrophoresis and NanoDrop 8000 spectrophotometry (NanoDrop Technologies, Wilmington, DE).

2.6. Reverse transcription

Quantitative conversion of mRNA to single stranded cDNA was completed using High Capacity cDNA Reverse Transcription (Applied Biosystems, Life Technologies Corp., Carlsbad, California). Extracted RNA was converted to cDNA in triplicate. Briefly, 10 μL of RNA was used in a reverse transcription reaction using random primers. To verify the absence of DNA, a control reaction was performed with nuclease-free water instead of reverse transcriptase. cDNA was stored at −80 °C until further analysis.

2.7. Quantitative analysis of genes and transcripts

A quantitative polymerase chain reaction assay (qPCR) was used to enumerate the starting quantities of *amoA*, *nirS* and *nosZ* genes and transcripts using a Bio-Rad iQ5 detection system (Bio-Rad Laboratories, Mississauga, ON). Preliminary experiments targeting the *nirK* gene indicated that this gene was present below detection limits for all samples, so *nirK* was excluded from subsequent work (data not shown). Primer pairs targeting a segment of the *amoA*, *nirS* and *nosZ* genes were used following previously described reaction conditions (Rotthauwe et al., 1997; Throbäck et al., 2004; Henry et al., 2006). Quantitative PCR analysis used optimized cycling profiles for each primer set, and initial quantification assay of gene copy numbers, achieved by measuring fluorescent intensity dye during each of the 40 cycles. For each 1 μL template (approx. 5 ng of DNA, 1.5 ng cDNA), reactions were performed in a total volume of 25 μL per sample, with 12.5 μL of 1x SYBR Green Supermix (12.0 μL for *amoA*), 10 μM (10 pmol μL^{-1}) each forward and reverse primers, 1 μL T4g32, and RNase and DNase free water. To reduce inhibitory effects of soil humic acid during the qPCR process, 150 ng μL^{-1} T4 gene 32 protein (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA) was included (Kreader, 1996). A melt curve analysis was used to confirm amplicon authenticity, and size and presence of a single band was confirmed on 1% agarose gel electrophoresis. For each primer pair, the no-template control reactions in each run had undetectable amplification. Each measurement was performed in triplicate.

Serial dilutions of linearized plasmid DNA (10^6–10^1) containing the targeted genes were used to construct the standard curves. Standard curves were prepared by cloning *amoA*, *nirS* and *nosZ* genes from genomic DNA from *Nitrosomonas europaea* spp., *Pseudomonas aeruginosa* spp., and *Pseudomonas fluorescens* spp., respectively. PCR products using primers, as described above, were cloned into One Shot® DH5α™-T1R, Top10 *Escherichia coli* competent cells using a TOPO TA Cloning® kit (Invitrogen, Burlington, ON, Canada). Cloned plasmid DNA was extracted using a PureLink™ Quick Plasmid Miniprep Kit (Quagen Inc., Mississauga, ON, Canada) and verified by sequencing. Standard curves were sensitive to quantify an abundance of 10 gene copy numbers. PCR efficiencies and slopes for *amoA*, *nirS*, *nosZ* amplifications were 98%, −3.35; 99.4%, −3.33; and 93%, −3.46, respectively, with R^2 values of >0.91 for *amoA*, and >0.991 for *nirS*, *nosZ* genes.

2.8. Statistical analysis

The probability distribution of N_2O fluxes has been shown to follow log normal or highly skewed (e.g. reverse J shaped) distributions (Yates et al., 2006). This was also the case for daily flux values observed in this study. Hence, treatment effects were tested by paired comparison of daily means using the Wilcoxon signed rank test in SAS 9.2 (SAS Inst., Cary, NC, USA) (Wagner-Riddle et al., 2007). This statistical test was performed using measured values, that is, without considering the interpolated daily means, so as not to bias treatment comparisons.

For soil physicochemical and microbial variables there were three replicate observations per field. The Shapiro–Wilks test was used to test for normality of data ($P < 0.05$). Studentized residuals were checked for outliers. Gene abundance data were normalized to gene copy number (g^{-1} dry soil) and $\log_{10}$ transformed when required. All data tested conformed to assumptions for ANOVA. Soil physicochemical data and microbial data were analyzed using Minitab for Windows version 16.2.3 (Minitab Inc, State College, PA). Gene and transcript abundance were analyzed using a repeated measures two-factor ANOVA (Field × Date), in a general linear model (GLM) with two between-subjects factor (+R and −R) and a seven-level within-subjects factor (sampling date). Soil physicochemical data was analyzed using a repeated measures two-factor ANOVA (Field × Date), in GLM with two between-subjects factor (+R and −R) and a two-level within subjects factor (sampling date). Means were compared using a Tukey's adjustment. Statistical significance was accepted at $P < 0.05$, unless otherwise noted, in cases where it is important to see biological trends that may be marginally significant. Treatment means and standard errors presented in the figures are calculated from nontransformed data. A Pearson correlation analysis was applied to evaluate relationships between N_2O flux and microbial variables.

3. Results

3.1. Environmental conditions at spring thaw

Between January 1 and March 12, 2010, fields had variable snow cover of up to 11 cm and the average snow depth did not differ between the two fields (~5 cm) (Fig. 2B). The average liquid VWC at 5 cm depth over Jan to Feb was 0.035 ± 0.005 m^3 m^{-3} for +R compared to 0.044 ± 0.009 m^3 m^{-3} for −R at (Fig. 2A). Low liquid water conditions indicated water freezing in the soil surface layer, as also supported by temperatures at 3 cm depth of −2.4 ± 0.3 °C for both fields over the Jan–Feb period (Fig. 2A). Soil remained frozen in both fields until March 5. Rapid snow melting occurred between March 4 and March 12, 2010 (Supplemental Fig. S1). Water content at 5 cm depth increased sharply during this warming event, averaging 0.213 ± 0.001 m^3 m^{-3} for +R and 0.433 ± 0.017 m^3 m^{-3} for −R over March 11–14 (Fig. 2A). No significant differences in daily soil temperature at 5 cm depth were observed between fields with >0 °C values after March 10 and mean of 3.0 ± 0.35 °C for March 11–19 (Fig. 2A, Supplemental Fig. S2).

3.2. Surface nitrous oxide emissions

Daily micrometeorological N_2O fluxes showed a two-week period of increased N_2O fluxes from mid to end of January, which coincided with some soil surface warming and decreased in snow cover (Fig. 2B). This was followed by a period with low fluxes (<10 ng N m^{-2} s^{-1}) during early February. The major N_2O flux event occurred during the main spring thaw period (March 4–March 17, 2010) (Fig. 2A). During this time significant differences were observed between fields with lower N_2O fluxes from the +R (14.8 ng N m^{-2} s^{-1}) compared to the −R field (67.0 ng N m^{-2} s^{-1}) (Table 2, Fig. 3a, b).

3.3. Soil chemical dynamics during thaw

During the March 4 and 17 sampling dates, the +R field had an average DOC level of approximately 65.3 mg kg^{-1} dry soil, while

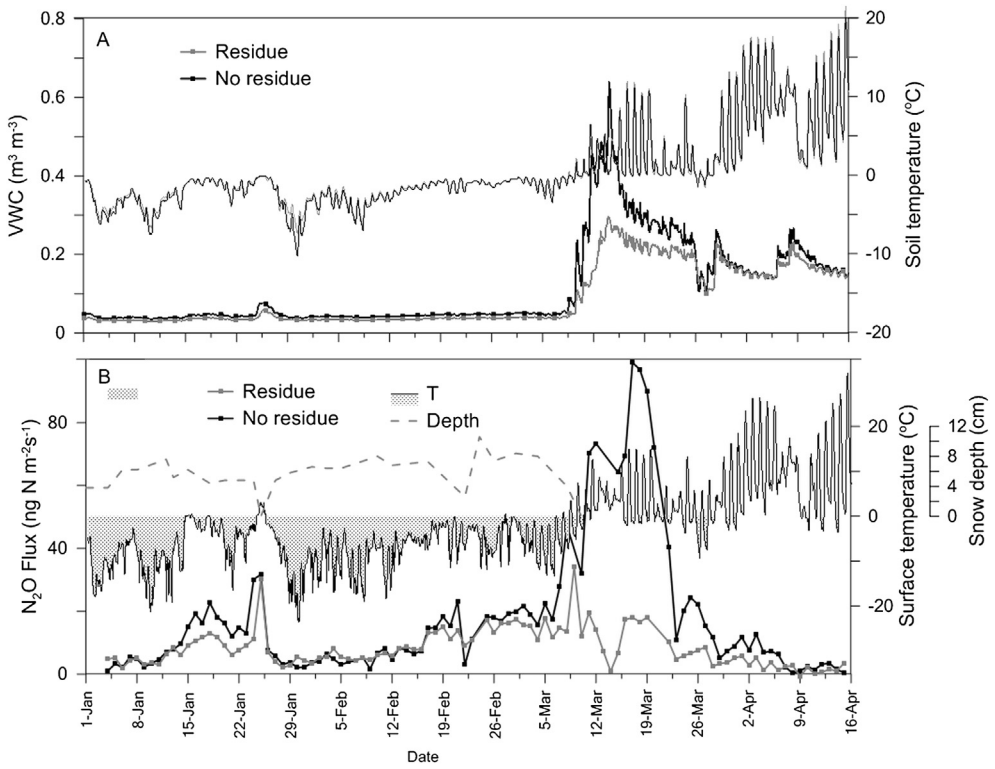

Fig. 2. Environmental conditions and daily N_2O flux for conventionally tilled fields with residue returned (+R) (black lines) and no residue returned (−R) (gray lines). A. Volumetric water content (VWR, lines with symbols) and soil temperature (line) both at 5 cm depth; B. Average surface temperature (solid line) and snow depth (dashed line) for both fields, and N_2O flux (lines with symbols).

significantly lower levels between 39.8 mg kg^{-1} dry soil were observed for −R (Table 1). Soil NH_4^+−N was also significantly greater in the +R compared to the −R field (Table 2). However, soil NO_3^-−N concentrations in the field with no residue were highest on March 4 decreasing to a value 3 times smaller by March 17. Low bulk density values, characteristic of frozen soils (<1 g cm^{-3}) were observed on March 4 and with the thawing increased to ~1.3 g cm^{-3} by March 17. The liquid WFPS increased significantly in both fields over the sampling period, reaching higher values in −R compared to +R (64.4% vs. 47.6%; Table 2).

3.4. Quantification of microbial communities during spring thaw

Over the spring thaw, targeted nitrifying and denitrifying genes (*amo*A, *nir*S, *nos*Z) were successfully quantified for all sampling dates using qPCR (Fig. 3). In addition, gene expression in populations of nitrifiers and denitrifiers was assessed through the quantification of *amoA*, *nirS* and *nosZ* gene transcripts. As expected, due to the large size of the fields (1.5 ha), there was high variability across the field transects in copies of transcripts for all genes analyzed. However, despite variability, significant differences between fields were detected (Table 2, Fig. 3). Gene quantity of nitrifiers (*amo*A) was higher in the field with residues (+R). The *amo*A transcript, was detected, but did not differ between fields and remained consistent over time (Table 2, Fig. 3c, d). Gene quantities of *nirS* and abundance of *nirS* transcripts indicated that a population of denitrifiers containing the nitrite reductase gene was present and being transcribed at similar levels in both fields, and remained consistent over time (Fig. 3e, f). Significant differences in the population of denitrifiers containing *nosZ* were evident between the two fields, with *nosZ* gene copies being more abundant in +R field ($P > 0.0001$). Furthermore, *nosZ* transcript was significantly higher in the +R field compared to the −R field (Table 2, Fig. 3g, h). In addition, the quantity of the *nosZ* gene in the −R plot

Table 1
Characterization of soil physicochemical properties before (March 4, 2010) and after (March 17, 2010) spring thaw.

	Dissolved organic carbon (DOC) (mg C kg^{-1} dry soil)	Ammonia (NH_4^+−N) (mg N kg^{-1} dry soil)	Nitrate (NO_3−N) (mg N kg^{-1} dry soil)	Liquid water-filled pore space (%)	Bulk density (ρ_b) (g cm^{-3})
Residue (+R)					
March 4	69.2a[a]	3.6a	2.4a	13.9c	0.70b
March 17	61.3a	4.0a	0.9a	47.5b	1.26a
Mean (+R):	**65.3A**[b]	**3.7A**	**1.7A**	**30.6A**	**0.98A**
No residue (−R)					
March 4	35.9a	1.5a	5.4a	12.2c	1.06ab
March 17	43.6a	0.4a	0.9a	64.4a	1.27a
Mean (−R):	**39.8B**	**0.9B**	**3.1A**	**38.3A**	**1.17A**

[a] Means followed by the same small case letter within one column are not statistically different according to Tukey's means comparison, $P < 0.5$, $n = 3$.
[b] Means followed by the same capital letter within one column for both dates together are not significantly different according to Tukey's means comparison, $P < 0.5$, $n = 6$.

Table 2
Average N_2O flux and abundance of *amo*A, *nir*S and *nos*Z gene and transcript over spring thaw sampling dates (March 7–19).

	N_2O flux (ng N m^{-2} s^{-1})	Soil nitrifying bacteria (gene/transcript copy g^{-1} soil)		Soil denitrifying bacteria (gene/transcript copy g^{-1} soil)			
		amoA DNA	*amoA* RNA	*nirS* DNA	*nirS* RNA	*nosZ* DNA	*nosZ* RNA
Residue (+R)	14.8a[a]	1.00×10^8a[b]	2.88×10^3a	6.46×10^6a	1.17×10^3a	1.07×10^6a	1.70×10^3a
No residue (−R)	67.0b	1.07×10^7b	1.78×10^3a	3.39×10^6a	2.95×10^2a	3.72×10^5b	2.18×10^2b

[a] Mean N_2O flux values followed by the same letter are not significantly different according to a Wilcoxon signed rank test.
[b] Means followed by the same letter within one column are not significantly different according to Tukey's means comparison, $P < 0.5$, $n = 21$ maximum.

significantly increased over the sampling period, increasing from 8.17×10^4 to 1.36×10^6 *nos*Z gene copies g^{-1} dry soil (Fig. 3h). Daily average N_2O emission was correlated with *amo*A DNA gene copies ($R = -0.496$; $P < 0.002$), *nir*S DNA gene copies ($R = -0.324$; $P < 0.051$), and *nos*Z transcript ($R = -0.343$; $P < 0.044$) in the soil during spring thaw (March 7–19) (Table 3).

4. Discussion

Peak micrometeorological N_2O fluxes occurred with thawing of the soil surface, and when surface temperature was above 0 °C (Fig. 2B). Our strategy was effective in identifying the main thaw period as it unfolded and hence, the timing of soil sampling

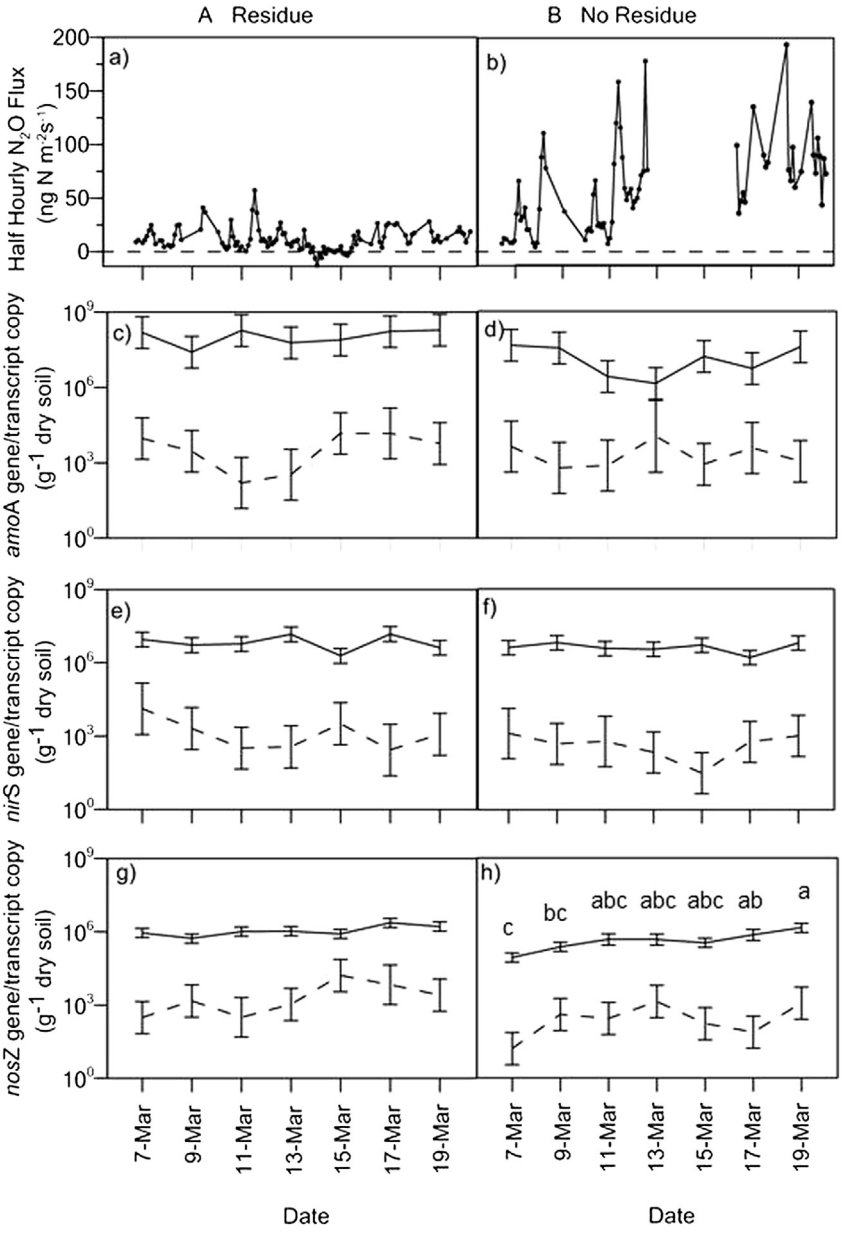

Fig. 3. Half-hourly N_2O flux and microbial abundance during spring thaw (March 7–19, 2010) in fields with residue returned (+R), and no residue returned (−R). Gene copy (solid line) (gene copies g^{-1} dry soil) and transcript copy (dotted line) (transcripts g^{-1} dry soil) of *amoA* (c, d), and *nirS* (e, f), and *nosZ* (g, h). Bars indicate the standard error of the mean. Significant differences between sampling dates within each field are indicted with different letters. Means were compared with a Tukey's adjustment, $P < 0.5$, $n = 3$ maximum.

coincided with the period of enhanced N_2O fluxes due to soil thawing. High temporal resolution in N_2O flux measurement was an important factor in the success of capturing the significant N_2O emission event, because the timing of enhanced fluxes during the thaw period lasted only a few days (Fig. 2). Moreover, temporal sampling of fields provided an opportunity to relate gene copies and transcripts with N_2O fluxes under changing soil conditions over a spring thaw period (Supplemental Figs. S1, S2). Field studies of the mechanisms leading to heightened N_2O fluxes induced by freezing are challenging due to the lack of control over determining factors, such as soil and air temperatures (Risk et al., 2014). On the other hand, laboratory freeze thaw experiments cannot replicate realistic field conditions such as insulation provided by snow and litter layers and changes in soil physicochemical conditions (Henry, 2007). Our study provides insight into gene abundance and expression associated with a spring thaw N_2O emission event, under changing field conditions over a spring thaw period. However, the processes involved need to be investigated over multiple sites and multiple years in order to fully understand the complexity of microbial processes associated with freeze–thaw associated N_2O emissions.

The two fields under different crop residue management (+R, −R) provided contrasting edaphic conditions over the sampling period (Supplemental Figs. S1 and S2). The removal of crop residues resulted in significantly lower DOC and lower inorganic nitrogen (NH_4^+–N) (Table 1). All nitrifying and denitrifying genes (*amoA*, *nirS*, and *nosZ*) were successfully quantified in both fields throughout the study. Copies of the *amoA* gene were higher in the +R compared to the −R fields (Table 2). In a study examining spatial variability of ammonia oxidizing bacteria and archaea (AOB, AOA) in agricultural soils, Wessén et al. (2011) also found that AOB abundance was positively influenced by soil carbon and inorganic nitrogen content. We also quantified populations of denitrifiers throughout the study, with no difference in the *nirS* gene copies between the fields, but significantly higher *nosZ* gene copies in the +R field. The abundance of *nosZ* communities was lower in the −R field at the start of the sampling period (March 7); however, increased significantly from 8.17×10^4 to 1.36×10^6 gene copies g^{-1} dry soil over the sampling period (Fig. 3h). The *nirS* and *nosZ* communities have previously been shown to respond differently to organic carbon amendments in a microcosm study, where the addition of plant residues had no significant impact on *nirS* bearing denitrifiers, but resulted in a significant increase in the abundance of *nosZ* bearing denitrifiers (Henderson et al., 2010).

DNA gene copies were analyzed to determine the abundance of each gene; however the DNA-based method does not distinguish between active and inactive cells. To capture active gene expression during the spring thaw event, transcript was preserved *in situ* as field conditions changed. Results show that *amoA*, *nirS* and *nosZ* genes were expressed throughout the study, providing evidence that both nitrification and denitrification was occurring in the surface soil layer. In a microcosm study examining the impact of cold temperature incubation on nitrifier and denitrifier abundance, Wertz et al. (2013) also detected both communities in soil maintained at 55% WFPS. Combined increases in soil water from snow melt, a significant rainfall event on March 12–13, and reduced infiltration rates due to a frost layer, significantly increased the volume of water content in the 0–5 cm soil surface over the time period of our study (Table 1). Braker and Conrad (2011) suggest that nitrification is the preferential source of N_2O flux in soils with WFPS <60% while denitrification dominates in wet soil (60–90% WFPS), therefore the conditions of our fields by the end of the sampling period (WFPS: 48% (+R) to 64% (−R)) (Table 1) would permit both processes to occur simultaneously.

The abundance of ammonium oxidizers was significantly higher in +R compared to −R fields and corresponded to a lower daily N_2O flux. Although an inverse relationship was observed, there was no difference in the *amoA* transcripts between the fields, and NH_4^+–N did not decrease significantly over the course of the study, suggesting that although nitrification likely contributed to N_2O fluxes at the site, it did not lead to significant difference in emissions between the two fields. An earlier study at our field site suggested that both nitrifiers and denitrifiers were involved in spring thaw N_2O fluxes since the diversity of both the *amoA* and *nirS*-containing microbial communities changed significantly after a spring thaw emission event (Smith et al., 2010). The current study indicates that denitrifier populations were present and transcribing *nirS*, and *nosZ* genes in the surface soil thaw layer, and that the proportion of the denitrifying population containing *nosZ* increased temporally over the sampling period suggesting that *de novo* denitrification contributed to the spring thaw N_2O flux over the sampling period. Using ^{15}N tracers, in a study at our site, Wagner-Riddle et al. (2008) also detected *de novo* N_2O production in the thawed (0–5 cm) layer above a frozen soil layer.

The ratio of $N_2O/N_2 + N_2O$ is often used as a mechanism to quantify the amount incomplete denitrification occurring in a system. It is important to consider this ratio because lower N_2O emissions from soils can be a result of either a decline in the total N denitrified, or an enhancement of the final reduction of N_2O to N_2 (Cayuela et al., 2013). In our field study, we could not measure N_2 emissions; however, there were significant differences in the magnitude of the N_2O produced during spring thaw. Data suggest that the N_2O variation between fields was driven mostly by changes in *nosZ* gene and transcript abundances, as *nirS* did not change significantly with time, or between fields (Fig. 3). In association with a lower N_2O flux, both the population of *nosZ* denitrifiers and *nosZ* transcription was significantly higher in the field with residues (+R), suggesting that lower N_2O was a result of an enhancement of the reduction of N_2O to N_2, rather than a decline in the amount of N denitrified. In a lab study, Henderson et al. (2010) reported a significant increase of both the abundance of *nosZ* bearing denitrifiers and *nosZ* gene RNA transcript numbers in anoxic microcosms soils amended with plant residue and, similar to our study, they found no significant effect of plant residue addition on *nirS* bearing denitrifiers or RNA. Henderson et al. (2010) could show no link between expression of *nosZ* and N_2O emissions, but suggested that the *nosZ* gene would be expected to be negatively correlated with N_2O emissions, due to an increased reduction of N_2O reduction to N_2, as we see in the current study. It is important to consider that we were able to see a marginal ($R = -0.343$; $P < 0.044$) correlation in our study because of the contrasts in *nosZ* transcripts and N_2O flux between our two fields of study. Although the population of *nosZ* denitrifiers increased significantly over the time of our sampling period in the −R field, no correlation was found between N_2O emissions and *nosZ* DNA or *nosZ* RNA within individual fields. Studies showing a correlation between increases in the *nosZ*

Table 3
Pearson correlation between daily average N_2O emission (ng N m^{-2} s^{-1}) and abundance of *amo*A, *nir*S and *nos*Z gene and transcript (log gene copy g^{-1} dry soil, log transcript g^{-1} dry soil) over spring thaw sampling dates (March 7–19).

	Soil nitrifying bacteria		Soil denitrifying bacteria			
	amoA DNA	*amoA* RNA	*nirS* DNA	*nirS* RNA	*nosZ* DNA	*nosZ* RNA
	$n = 42$	$n = 42$	$n = 42$	$n = 42$	$n = 42$	$n = 42$
Pearson correlation	−0.496	−0.134	−0.324	−0.211	−0.232	−0.343
P-value	0.002	0.480	0.051	0.231	0.180	0.044

denitrifier community and N_2O emissions are rare, despite the fact that NosZ is the only enzyme known to date that utilizes N_2O as a primary substrate (Jones et al., 2013). Recently, a second phylogenetic clade of *nosZ* sequences has been identified that could also play a role in our system (Jones et al., 2013). Our study focused on the two primary microbial processes associated with N_2O emissions, autotrophic nitrification and heterotrophic denitrification (Braker and Conrad, 2011); however, it is important to note that other microbial processes can lead to N_2O formation in the soil, such as, nitrifier denitrification, co-denitrification and dissimilatory nitrate reduction to ammonium (DNRA) (Butterbach-Bahl et al., 2013), and their role in our study is unknown. Finally, spring thaw N_2O emission at our study site was recently shown to be comprised of two soil processes, the physical release of N_2O built-up within the soil profile and released after ice melt, and fluxes due to *de novo* production at the time of melting (Risk et al., 2014). We quantified microbial processes within the soil thaw layer, in order to assess the occurrence of *de novo* N_2O production; however, the physical release could still contribute to the total N_2O flux measured by micrometeorological equipment, therefore, complicating the ability to link N_2O with microbial community measurements.

5. Conclusions

To the best of our knowledge, ours is the first study to relate the analysis of the quantity and gene expression of N cycling soil microbes *in situ* to the timing of a spring thaw N_2O emission event. The results identify both changing abundance of microbial populations in the soil environment, and provide ecologically meaningful associations between changes in gene expression and N_2O emissions *in situ*. These results support measuring changes in N-cycling microbial activity as a useful feedback of how agriculture management practices affect climate-influencing N_2O emissions. Most climate models start from a satellite top- down approach, but these studies provide bottom-up ecosystem data of detailed microbial processes and environmental gas flux. Bridging soil ecology and climatology is critical in both fields of study. Integrating data on soil microbial functioning with climate models will allow future models to better predict the scale of microbially-mediated soil processes, and the impacts of anthropogenic activities.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.02.007.

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