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ORIGINAL PAPER

The amplitude of soil freeze-thaw cycles influences temporal dynamics of N_2O emissions and denitrifier transcriptional activity and community composition

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Abstract In agricultural fields at high and mid latitudes, a large percentage of the annual emissions of the greenhouse gas nitrous oxide (N_2O) can occur during freeze-thaw (FT) cycles. The objective of the study was to determine the effects of FT cycles of different amplitudes on N_2O emissions, denitrifier transcriptional activity, and the abundance and composition of present and active denitrifier communities. Soil microcosms amended with NO_3^- (N) and/or NO_3^- plus organic C, i.e., red clover residues (N + RC), were subjected to freezing at −5 °C followed by thawing at either +4 or +15 °C. Peaks of N_2O emission rates following FT were ~2-fold greater in N + RC than in N amended soils. In N + RC amended soils, the maximum rates following FT were similar at +4 and +15 °C. However, thawing at +4 °C resulted in a delay in the peak of emissions, suggesting that denitrification enzymatic activity was reduced at colder temperatures. In general, the abundance and the number of transcripts of *nirS* and *nirK* were similar over time and among treatments with a few exceptions, including, for example, fewer *nirS* transcripts under the cooler thawing temperature. Temperature regimes had a significant effect on the compositions of the present and active *nirS* and present *nirK* denitrifier communities during FT. Changes in these community compositions were correlated with changes in temperature and N_2O emissions, and these variables explained 3.7 to 4.9 % of the changes in community composition. Results indicated that addition of electron donor, i.e., organic C, increased N_2O emissions. Furthermore, the composition of active *nirS* community changed during the peak of N_2O emissions following FT events but not the active *nirK* community. This finding indicated that active *nirS* community better adapted to the changes in conditions has established suggesting a greater role of this group in N_2O production.

Keywords Denitrification · *nirK* · *nirS* · Gene expression · Soil microcosms · Thawing temperature

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Introduction

Nitrous oxide (N_2O), a greenhouse gas and ozone-depleting compound, is the major anthropogenic greenhouse gas produced from agricultural soils (Braker and Conrad 2011). Contrary to previous thought, substantial N_2O emissions can occur during winter, i.e., the non-growing season with up to 50–90 % of the total annual emissions observed in agricultural soils during this period (Virkajärvi et al. 2010; Wagner-Riddle et al. 1997, 2007; Yanai et al., 2011). At high and mid latitudes, agricultural soils experience freeze-thaw (FT) episodes during the non-growing season, mainly in late autumn and early

spring, when soils are not snow covered or have inconsistent snow cover (Henry 2007). Soil freezing may induce a release of nutrients due to physical disruption of aggregates and lysis of microbial cells (van Bochove et al. 2000; Pesaro et al. 2003; reviewed in Wang et al. 2008). During thawing conditions, this release of nutrients, combined with the creation of anoxic microsites due to high soil water content, promote emissions of N_2O (Wang et al. 2008; Risk et al. 2013). Denitrification was reported to be the main soil process involved in N_2O fluxes after thawing using ^{15}N tracers (Ludwig et al. 2004; Malhi and Nyborg 1983; Mørkved et al. 2006; Müller et al. 2003; Nyborg et al. 1997; Wagner-Riddle et al. 2008).

Previous studies examined factors enhancing N_2O emissions during FT (e.g., reviewed by Wang et al. 2008; Risk et al. 2013). Colder and longer freezing periods have caused greater N_2O emissions, likely due to increased disruption of aggregates and death of microorganisms providing a greater release of nutrients (Koponen and Martikainen 2004; Nielsen et al. 2001; Teepe et al. 2004). N_2O emissions were shown to decline with increasing numbers of FT cycles, suggesting decreasing nutrient release over several cycles (Chen et al. 1995; Priemé and Christensen 2001; Pelster et al. 2013; Wagner-Riddle et al. 2008). Greater N_2O emissions were observed with greater soil water content as a result of more anoxic conditions (Koponen and Martikainen 2004; Teepe et al. 2004). Additions of NO_3^- and/or organic matter to soils were also reported to stimulate N_2O emissions by providing electron acceptors and/or donors, respectively, for denitrification (Mørkved et al. 2006; Müller et al. 2003; Singurindy et al. 2009; Tenuta and Sparling 2011). In addition, organic matter amendment can increase N_2O emissions by increasing anaerobic microsites through the stimulation of aerobic respiration and thus O_2 consumption (Mørkved et al. 2006).

Despite extensive research into these factors during FT, there have been no studies examining the effect of thawing temperature on N_2O emissions. Thawing temperatures are lower in autumn, when a general cooling trend occurs (e.g., FT amplitude not exceeding +10 °C in an eastern Canadian soil, Henry 2007), compared to spring, where rapid warming can result in high thaw temperatures (e.g., FT amplitude up to +20 °C in an eastern Canadian soil, Henry 2007). These differences in temperature might be expected to affect N_2O emissions temporal or dynamics due to an increase in denitrification enzymatic activity at warmer soil temperature. Furthermore, thawing temperatures might be changed with climate warming which is predicted to increase the frequency and amplitude of soil FT cycles as a result of reduced snow cover (Groffman et al. 2001; Henry 2008) and/or greater thawing temperatures.

Many studies on FT have tested freezing soil temperatures that were lower than those typically encountered during winter in temperate agricultural soils, e.g., −20 °C (Henry 2007). Moreover, most reports have focused on N_2O emissions and denitrification, and little is known about the effects of FT on denitrifier community abundance and composition, and whether N_2O emissions are related to changes in community composition. Previous studies reported either a decrease in soil denitrifier abundance (Su et al. 2010), or an increase in denitrifier community activity (Yanai et al. 2007) and in the denitrification gene expression with a concomitant flush of N_2O emissions (Sharma et al. 2006) at thaw following a deep freeze at −20 °C.

The objective of the present study was to determine how FTs of different amplitudes affect N_2O emissions, denitrification, denitrifier transcriptional activity, denitrifier abundance, and the composition of the present and active denitrifier communities in an agricultural soil in Canada. The FT cycles were characterized by (i) a freezing temperature of −5 °C, similar to what is encountered during the non-growing season in eastern Canada, and (ii) a thawing temperature representative of either late autumn (+4 °C) or early spring (+15 °C).

We hypothesized that N_2O emissions, denitrification, and denitrifier transcriptional activity would be greater at warmer thawing temperatures and that the denitrifier community composition would differ between the two FT cycles. We also hypothesized that FT cycles would enhance N_2O emissions and denitrification rate compared to unfrozen control soils (i.e., soils incubated at a constant temperature of +4 °C, or at +4 to +15 °C). We predicted that changes in denitrifier community composition during FT would be correlated with changes in N_2O emissions and denitrification rate.

Materials and methods

Soil sampling

Soil samples (0–15 cm depth) for microcosm establishment were collected from four plots (12 m × 6 m) under a potato (*Solanum tuberosum* L.)/red clover (*Trifolium pratense* L.) rotation in Fredericton, New Brunswick, Canada (45°52′N, 66°31′W). Sampling was performed in December 2010. Soil texture (pipette method, Gee and Bauder 1986) was loam with 490 g kg^{-1} sand and 115 g kg^{-1} clay. Soil total N and organic C concentrations were 1.72 and 21.03 g kg^{-1}, respectively (dry combustion: Model CNS-1000, Leco Corp., St Joseph, MI). Soil pH (1:1 water) was 5.97. After sampling, soil from each plot was sieved (4.75 mm diameter mesh) and stored at 4 °C for 2 weeks before the beginning of the experiment. Soil samples from the four plots were treated as four replicates for the preparation of soil microcosms.

Experimental design

Soil microcosms consisted of 322 g equivalent oven dry soil that was adjusted at 60 % water-filled pore space (WFPS) and

amended with (1) 30 mg KNO_3-N kg^{-1} dry soil or (2) 30 mg KNO_3-N kg^{-1} dry soil plus red clover residues at 1000 mg C kg^{-1} dry soil, and were packed to a density of 1 Mg m^{-3} in 1 L canning jars. The jars were covered with Parafilm pierced with holes to ensure sufficient gas exchange. The WFPS chosen represents typical water content during winter in eastern Canada (Tatti et al. 2014). Red clover residues had a C/N ratio of 14:1 and represented forage that is frequently incorporated into agricultural soils in autumn in eastern Canada as a source of organic matter. Concentrations of added NO_3^- and organic C were in the range of concentrations encountered under field conditions following autumn crop residue incorporation (Sanderson and MacLeod 1994; Bolinder et al. 2002).

The experiment used a randomized complete block design with four treatments including two FT regimes (−5/+4 °C and −5/+15 °C) and two control regimes (+4/+4 °C and +4/+15 °C regimes), six sampling dates, and four replicates. Soil microcosms were pre-incubated for 14 days at +4 °C to allow an acclimatization of soil microorganisms to the amendment treatments. Microcosms were then placed in programmable Sanyo Scientific MIR-553 incubators (Sanyo Scientific, Japan). The entire soil column was subjected to one freeze-thaw event consisting of 3 days at −5 °C followed by 7 days at either +4 or +15 °C (i.e., −5/+4 °C and −5/+15 °C regimes). The chosen temperatures are representative of soil temperatures (5 cm depth) recorded in the field from which soil was collected, which range from −8 to +15 °C between November and April. The −5/+4 °C regime reflects the fluctuation of soil surface temperatures observed in autumn in eastern Canada (i.e., amplitudes of 5–15 °C, Henry, 2007), whereas the −5/+15 °C regime represents larger fluctuations that can occur during spring thaw (amplitudes ≥20 °C, Henry 2007). We also established unfrozen control microcosm treatments that were paired with the FT treatments. Controls of the −5/+4 °C regime were incubated at a constant temperature of +4 °C for the entire 10-day incubation period (i.e., +4/+4 °C regime). Controls of the −5/+15 °C regime were incubated at +4 °C for 3 days, followed by +15 °C for 7 days (i.e., +4/+15 °C regime), in order to distinguish the effects of freeze-thaw from the effects of temperature increase to +15 °C.

Soil microcosms were destructively sampled prior to the freezing period (i.e., at the end of the 14 days of pre-incubation at +4 °C, denoted as PF, prior freezing) and at 0, 1, 2, 4, and 7 days after thaw to measure N_2O and CO_2 emissions, denitrification, and inorganic N concentrations, and to perform molecular analyses as described below. Day 0 sampling started immediately when soil microcosms were thawed for the −5/+4 °C and −5/+15 °C regimes or started at the beginning of incubation at +15 °C for the +4/+15 °C regime (Fig. S1A and B). Because soil took longer to thaw at +4 °C compared to +15 °C, day 0 sampling started (i) 2 h after the temperature started to rise above −5 °C for the −5 °C/+15 °C regime and (ii) 8 h after temperature started to rise above −5 °C for the −5/+4 °C regime (Fig. S1B). The same lag times (2 and 8 h) were applied to the other sampling dates for the −5/+15 °C and −5/+4 °C treatments. Sampling of the +4/15 °C and +4/+4 °C controls was performed using lag times of 2 and 8 h, respectively, similarly to the −5/+15 °C and −5/+4 °C treatments.

Soil temperatures

For each temperature regime, one soil microcosm was dedicated to temperature measurements over the incubation period. Temperatures were monitored at about 2 cm depth in the soil microcosms every 15 min (Fig. S1) using Hobo data loggers and BoxCar Pro 4.3 software (Onset Computer Corporation, MA).

CO_2 and N_2O emissions and denitrification rate

At each sampling date (i.e., prior to freezing and 0, 1, 2, 4, and 7 days after thaw), N_2O and CO_2 emission rates and total denitrification were measured from two sets of microcosms. The microcosms were sealed with screw top lids fitted with a rubber septum. One set of microcosms was used to quantify actual N_2O and CO_2 emission rates, whereas the second set of microcosms had 10 % of headspace volume replaced with acetylene (C_2H_2) and was used to quantify total denitrification ($N_2O + N_2$) rate (Mosier and Klemedtsson 1994). The presence of C_2H_2 inhibits nitrous oxide reductase activity; consequently, N_2O emission quantified in the presence of C_2H_2 reflects total denitrification. At times 1 and 4 h, 10 mL gas samples were collected from all microcosm headspaces and transferred to previously evacuated Exetainers (Labco, Lampeter, UK). Gas samples were then analyzed for CO_2 and N_2O as described by Miller et al. (2008). N_2O emissions, denitrification, and respiration rates were calculated as the change in N_2O or CO_2 concentration in microcosm headspaces over 3 h.

Concentrations of soil inorganic N

At each sampling date (i.e., prior to freezing and 0, 1, 2, 4, and 7 days after thaw), sub-samples of 25 g of fresh soil were taken from the set of microcosms used for measurements of N_2O and CO_2 emissions after completing gas sampling. Concentrations of NH_4^+, NO_2^-, and NO_3^- were determined from the soil sub-samples as described by Miller et al. (2008), except that 0.5 M K_2SO_4 was used as the soil extraction buffer.

DNA and RNA extractions

At each sampling date, soil sub-samples (~10 g) were taken from the microcosms used for measurement of N_2O and CO_2

emissions immediately prior the start of gas emissions measurements. The soil samples were flash frozen in liquid N_2, then stored at −80 °C. Both DNA and RNA were extracted from 1 g of freeze-dried soil using the method of Griffiths et al. (2000) and were further purified using the Power Clean DNA Clean-up kit (Mo-Bio, Carlsbad, CA) for DNA and using a DNase I treatment and the RNeasy mini kit (Qiagen, Toronto, ON, Canada) for RNA. DNA and RNA were quantified using Picogreen and Ribogreen kits (Invitrogen, Burlington, ON, Canada). Reverse transcription of RNA to cDNA was performed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) and 0.2 mg mL^{-1} of BSA using the following conditions: 25 °C for 10 min, 42 °C for 75 min, and 85 °C for 5 min.

Quantitative PCR

Copy numbers of *nirS* and *nirK* denitrifier genes and transcripts were measured by qPCR on DNA and cDNA samples as described by Dandie et al. (2011) with concentration of templates ranging from 2 to 12 ng and 0.2 and 0.4 ng for DNA and cDNA, respectively, using a Step One Plus Real-time PCR system (Applied Biosystems, Streetsville, ON, Canada). Dilutions at 10^{-1} of soil DNA and cDNA had no inhibitory effects on qPCR (data not shown). Absence of DNA contamination in RNA samples was verified by qPCR on non-reverse transcribed RNA for a subset of samples. Standards were linearized plasmids containing cloned *nirS* and *nirK* gene sequences as described in Dandie et al. (2011) and curve descriptors were as follows: for *nirS* copy numbers: slope –3.5 to −3.7, efficiency (E) = 94.7–83.6 %, intercept (Y) = 36.2–39.9, R^2 = 0.996–0.998; and for *nirK* copy numbers: slope –3.7 to −3.8, E = 83.8–80.5 %, Y = 42.7–44.9, R^2 = 0.999–1.

PCR-DGGE analyses

Nested PCR targeting *nirS* and *nirK* were performed on present (DNA) and active (cDNA) microbial communities as described in Dandie et al. (2011), except that nirK1F and nirK5R primers (0.4 μM, Thröback et al. 2004) were used for the first PCR step of *nirK* amplification. Cycling conditions for this PCR step were as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 59.5 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min. The PCR products of *nirS* and *nirK* gene amplifications were analyzed by DGGE using the D-code Universal Mutation Detection System (Bio-Rad) with a 6 % polyacrylamide gel containing a gradient of 35–65 % denaturant, 100 % denaturing solution being defined as 7 M urea, and 40 % formamide.

Gels were run for 4.5 h at 150 V in 1× TAE buffer at 60 °C. The gels were stained with SYBR Green (Invitrogen) then photographed.

Cloning and sequencing of partial *nirS* and *nirK* sequences

Four *nirS* and four *nirK* bands representing dominant transcripts were excised from DGGE profiles. Each band was excised from profiles of cDNA and DNA soil samples originating from treatments with and without red clover residues, for different sampling dates and temperature regimes. DNA bands were eluted in water overnight at 4 °C and then used as templates for PCR targeting *nirS* and *nirK*. Cloning of PCR products was carried out using pGEM T-Easy vector and competent *Escherichia coli* JM109 cells (Promega, Madison, WI). Clones obtained from each excised band were randomly selected and subjected to PCR. PCR products of clones were loaded on DGGE gels to check that their position matched the original DGGE profile. For each *nirS* and *nirK* band position, 12 clones were selected, for a total of 96 clones sent for sequencing at the Centre d'Innovation Génome Québec et Université McGill (Montréal, QC, Canada).

Clone sequences showing ≥99 % similarity were considered to not be different from each other (Acinas et al. 2005), resulting in 33 different sequences obtained from the 96 clones. Clone sequences were compared to sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/) and were then aligned with closest related sequences using Clustal X (Thompson et al. 1997). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987), and tree topology was evaluated by bootstrap analysis. Sequences generated in this study have been deposited in the GenBank database (accession numbers KJ735412 to KJ735444).

Statistical analyses

SYSTAT software was used to conduct statistical analyses (Systat Software Inc., Chicago, IL). Non-normal data were log-transformed. General linear model analysis of variance was performed based on a completely randomized design with temperature regimes, soil amendments, and sampling date as fixed factors. Treatment means in interactions were compared using Tukey adjusted least significant (LS) means, and treatment means in simple main effects were compared by performing post hoc Tukey honestly significant difference (HSD) tests.

Phoretix software (Total Lab Ltd, Newcastle-Upon-Tyne, UK) was used to obtain the relative intensity of each DNA band (i.e., ratios of the intensity of each band to the total band intensity) from the DGGE profiles. Rank similarity matrices were computed using PRIMER v6 software (Plymouth, UK). Permutational multivariate analysis of variance (PERMANOVA) was performed from these matrices to test the significance of the effects of soil amendments, sampling dates, and temperature regimes on denitrifier community compositions. The effect of the temperature regimes on *nirS* and *nirK*

community compositions was further analyzed using pairwise comparisons. For analysis of denitrifier community compositions, samples were grouped into two different time periods: (i) days 0 and 1 after thaw, in order to examine community changes after FT, and (ii) days 2, 4, and 7 after thaw, in order to examine community changes after several days of incubation at static temperatures of +4 or +15 °C. Relationships between community composition and different factors that could influence and/or correlate with community composition, including temperature, soil inorganic N concentrations (NH_4^+ and NO_3^-), *nirS* and *nirK* abundances and transcriptional activity, gas emissions (CO_2 and N_2O), and denitrification rates, were explored using canonical correspondence analysis (CCA) using the software CANOCO 4.5 (http://www.canoco5.com). CCA was chosen because the unimodal response model gave a better approximation of relationships between denitrifier community composition and the explanatory factors.

CCAs were performed separately in the two different time periods, i.e., days 0 and 1 and days 2 to 7. The explanatory value of the selected factors was determined using forward selection (tested by 499 Monte Carlo permutations). Differences and correlation coefficients were considered significant at $p < 0.05$. Only significant explanatory variables were plotted.

Results

N_2O emissions, denitrification, and respiration

For all temperature regimes, N_2O emissions reached greater maximum rates in soils amended with NO_3^- plus red clover residues (N + RC) compared with soils amended with NO_3^- only (N) (Fig. 1a, b). Increased N_2O emissions in N + RC amended soils were associated with greater depletion of NO_3^- compared to N amended soils (Fig. S2).

In control soils incubated at +4 °C, N_2O emissions were constant over time for both soil amendment types (Fig. 1a, b). In soils subjected to the −5/+4 °C FT regime, N_2O emissions peaked on day 1 after thaw, and declined thereafter (Fig. 1a, b). On day 1 after thawing, N_2O emissions from N + RC amended soils were ~9-fold greater under the −5/+4 °C regime than under the +4/+4 °C control regime in (Fig. 1b), and 1.5-fold greater for N amended soils.

In soils subjected to the −5/+15 °C regime, N_2O emissions peaked on day 0 (i.e., at thaw) in N + RC amended soils. In comparison, this is earlier than soils that only received N and no organic C amendment, for which N_2O emissions peaked on day 2, and for both soil amendments under the −5/+4 °C regime (Fig. 1a, b). No significant differences in N_2O emissions were observed between soils subjected to the −5/+15 °C regime and the +4/+15 °C control regime for any sampling date; however, N_2O emissions were highly variable between replicates of these treatments (Fig. 1a, b).

The peaks of N_2O on day 0 under the +4/+15 °C regime and on day 1 under the −5/+4 °C regime coincided with the detection of NO_2^- in these soils, at concentrations of 0.01 μg N g^{-1} dry soil (Fig. S2).

Denitrification rates were highly variable within replicates of a given temperature treatment. Denitrification rates did not vary over time in soils under the −5/+4 °C and +4/+4 °C regimes (Fig. 1c, d). However, denitrification rates under the −5/+4 °C regime were significantly greater in N + RC than in N amended soils, although this difference due to soil amendments was not seen in any other temperature regime. Whether soil was frozen and thawed did not affect denitrification rates, as rates were not significantly different between soils subjected to FT and their controls for either amendment (Fig. 1c, d). In N amended soils, denitrification rates for the two FT regimes were greater at the higher thaw temperature, as rates under the −5/+15 °C regime were ~34-fold greater on day 2 and ~12-fold greater on day 4 compared with the −5/+4 °C regime. Denitrification rates in N + RC amended soils declined on day 7 for the two temperature regimes that included incubation at 15 °C (Fig. 1d).

Respiration rates were significantly greater in N + RC than N amended soils for all temperature regimes. Whether soils had been frozen and thawed affected respiration rates in N amended soils, as rates on day 0 were higher than their respective controls by ~1.8-fold under the −5/+4 °C regime and by ~1.6-fold under the −5/+15 °C regime (Fig. 1e, f). This effect was not observed in N + RC soils. In N amended soils under the −5/+4 °C regime, respiration rates declined progressively from day 0 to day 7. In N amended soils subjected to the −5/+15 °C regime, respiration rates were greater on days 0, 1, and 2 after thaw compared with rates prior to freezing (Fig. 1e).

Denitrifier abundances and transcriptional activity

Abundance of *nirS* and *nirK* denitrifiers did not vary over time in any temperature regimes with the exception of lower abundance of *nirS* denitrifiers at day 0 compared to day 7 and lower abundance of *nirK* denitrifiers at day 0 compared to day 1 and 7 in soils under the +4/+15 °C temperature regime and amended with N (Fig. S3). The abundance of *nirS* denitrifiers, when averaged over time, was influenced by soil amendments and temperature regimes, while *nirK* abundance did not change. In N amended soils, a lower abundance of *nirS* denitrifiers was observed under the −5/+4 °C regime when averaged over time compared with the other temperature regimes (Fig. 2a). However, in N + RC amended soils, the abundance of *nirS* denitrifiers subjected to the −5/+4 °C regime was higher relative to N amended soils and comparable to the other temperature regimes (Fig. 2a, b).

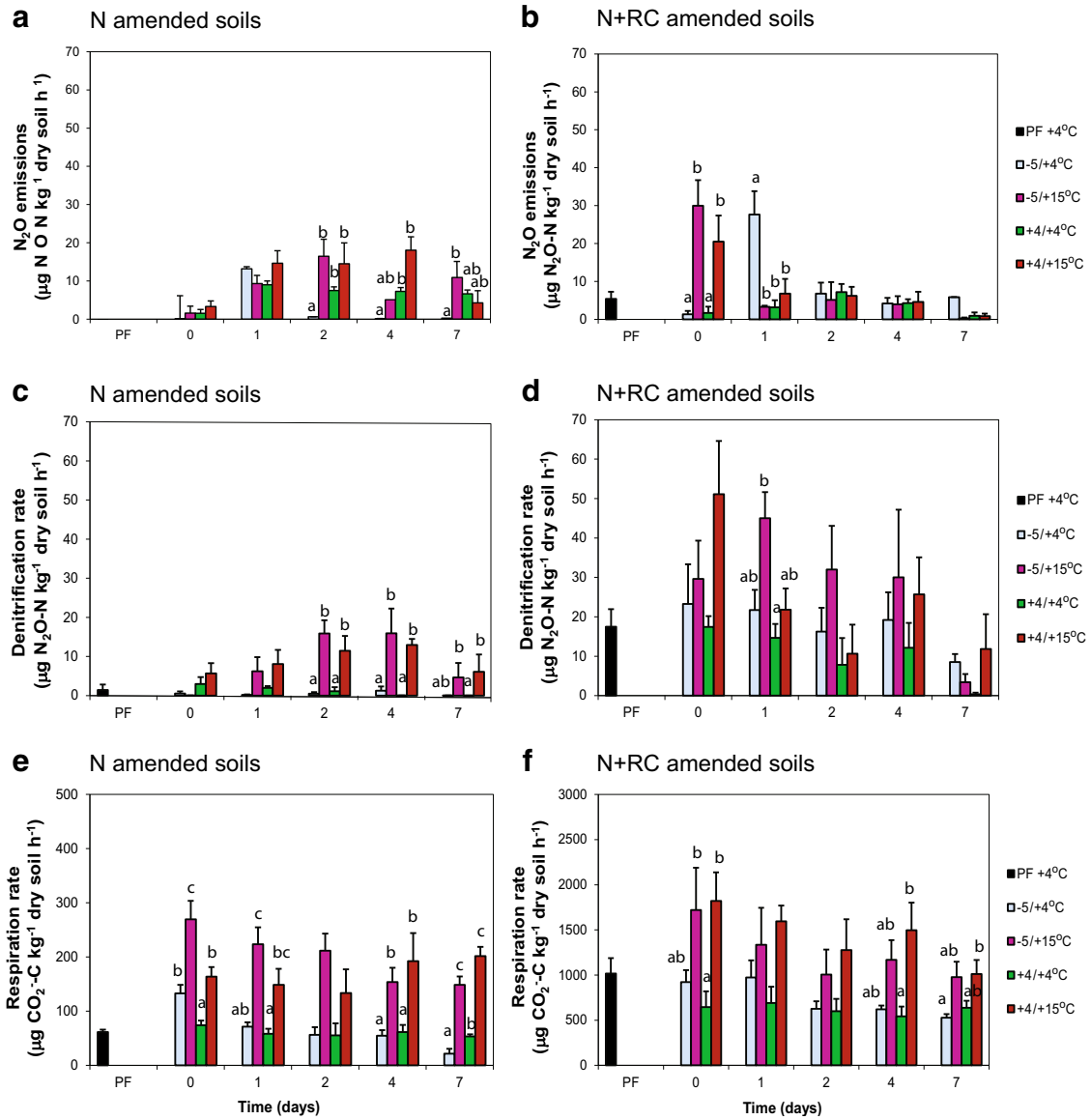

Fig. 1 N_2O emissions (**a**, **b**), denitrification rates (**c**, **d**) (i.e., N_2O emissions in presence of acetylene) and respiration rates (**e**, **f**) (i.e., CO_2 emissions) in soils amended with NO_3^- (N) or with NO_3^- plus red clover residues (*N+RC*) and subjected to −5/+4 °C, −5/+15 °C, +4/+4 °C, or +4/+15 °C temperature regimes. Measurements were performed prior to the freezing period (*PF*) and at 0, 1, 2, 4, and 7 days after thaw. Values are means ($n = 4$) and *errors bars* are standard errors. Treatment means within an individual sampling date with the same letter are not significantly different at $p < 0.05$. Note that the range of values on the *y*-axis for respiration rates differs between N and N+RC amended soils

There were no significant changes in number of *nirS* and *nirK* transcripts per gene copies (calculated as the ratios between the number of transcripts divided by the number gene copies) over time probably because the data was highly variable (Fig. S3). The number of *nirS* transcripts per gene copies was greater in N + RC than N amended soils, and transcriptional activity of the *nirK* gene could only be detected in N + RC amended soils (Fig. 2c, d). The number of *nirS* transcripts per gene copies varied among temperature regimes. In N amended soils, the lower abundance of *nirS* denitrifiers observed under the −5/+4 °C regime compared with the other temperature regimes resulted in a greater number of transcripts per gene copies under this temperature regime. In N + RC amended soils, the number of *nirS* transcripts per gene copies were greater in soils incubated at −5/+15 °C compared to soils incubated at −5/+4 °C (Fig. 2d).

Denitrifier community composition

The compositions of the present (genes) and active (cDNA) denitrifier communities were assessed by DGGE analysis of *nirS* and *nirK* genes and transcripts (see Fig. S3 for examples of DGGE profiles). Effects of temperature regime, soil amendment, sampling date, and the interactions among these factors

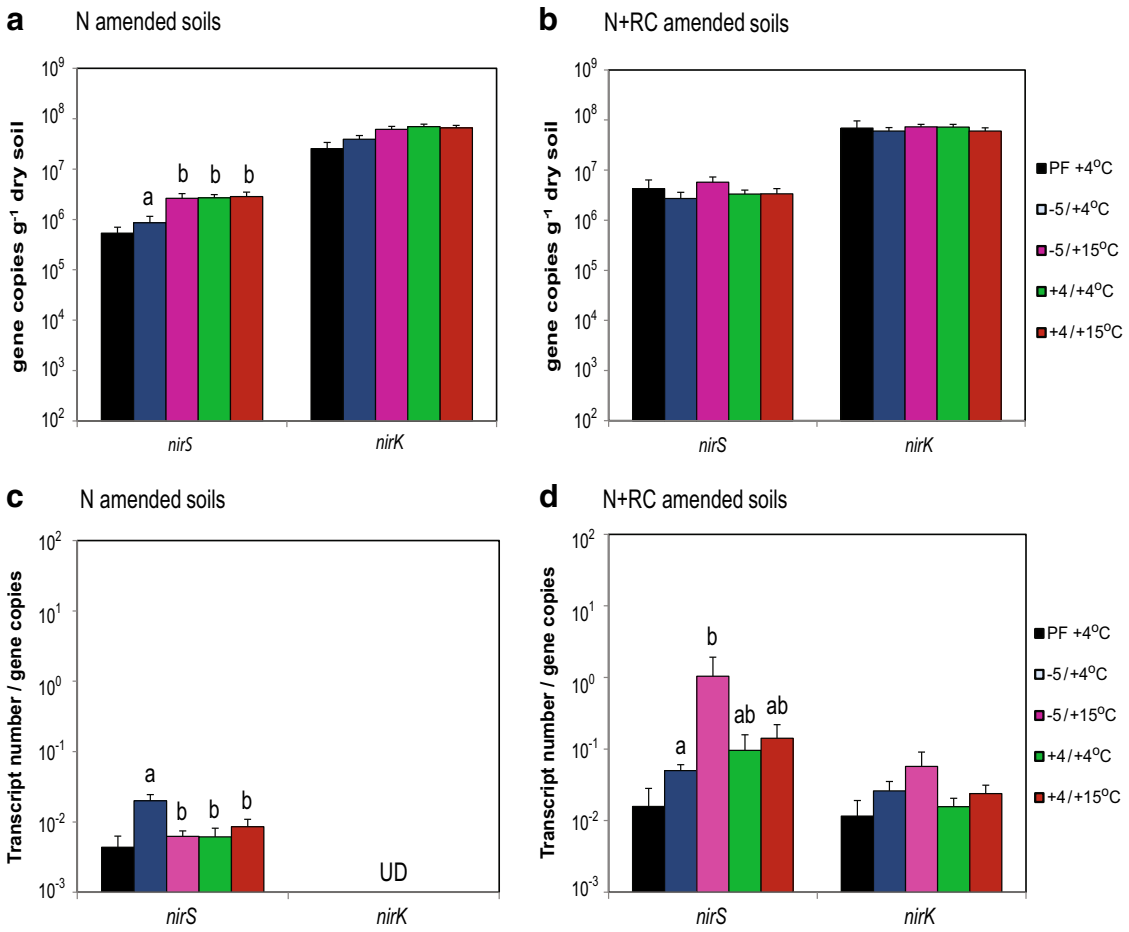

Fig. 2 Abundances of *nirS* and *nirK* genes (**a**, **b**) and the number of *nirS* and *nirK* transcripts per gene copies (calculated as the ratios between the number of transcripts divided by the number gene copies) (**c**, **d**) in soils amended with NO_3^- (*N*) or with NO_3^- plus red clover residues (*N+RC*) and subjected to −5/+4 °C, −5/+15 °C, +4/+4 °C, or +4/+15 °C temperature regimes. Values are averaged across sampling dates ($n = 20$) and *errors bars* are standard errors. *Treatment means with the same letter* are not significantly different at $p < 0.05$. *UD*, under detection level, i.e., transcript number could not be quantified

were assessed by PERMANOVA analyses for days 0 and 1 to assess the immediate effect after FT, and for days 2 to 7 to assess the effect of incubation temperature (Tables 1 and 2). Community compositions did not differ between N and N+RC amended soils on the date "prior freezing" (data not shown). For the other sampling dates, red clover residue amendment affected community compositions (Table 1). The compositions of the present and active denitrifier communities differed between sampling dates (Table 1), except for the active *nirK* denitrifier community on days 4 and 7 (data not shown).

Temperature regime significantly influenced *nirS* and *nirK* community compositions with the exception of active *niK* denitrifier community composition (Table 1). The compositions of the present and active denitrifier communities under the −5/+4 °C regime differed from the other regimes for both time periods (i.e., on days 0 and 1 and on days 2, 4, and 7 after thaw, Table 2). The compositions of the present and active *nirS* denitrifier community varied between the −5/+15 °C regime and its +4/+15 °C control on days 2, 4, and 7 after thaw (Table 2).

CCA showed that among the several parameters correlating with the present and active *nirS* denitrifier community compositions, three factors were consistently observed to be important in both time periods: *nirS* transcriptional activity, temperature, and N_2O emissions (Fig. 3a, b and Table S1), whereas NH_4^+ was correlated with the present *nirS* denitrifier community compositions only. The present *nirK* community composition was correlated with *nirK* abundance and temperature in both time periods (Fig. 4a, b, Table S1). In contrast, the active *nirK* denitrifier community composition was correlated with fewer parameters than the present *nirK* community composition, and there were no common factors between the present and active *nirK* community compositions (Table S1). Interestingly, temperature and N_2O emissions were correlated with the present and active *nirS* and the present *nirK* community composition, although not the active *nirK* denitrifier community composition (Table S1).

Table 1 Results of permutational multivariate analysis of variance (PERMANOVA) testing the significance of the effects of temperature regime, soil amendment, sampling date, and interactions among these factors on the present and active *nirS* and *nirK* denitrifier community compositions. PERMANOVA was performed on communities after FT (i.e., on days 0 and 1 after thaw) and after several days of incubation at +4 or +15 °C (i.e., on days 2, 4, and 7 after thaw). *P* values are indicated

	nirS genes		*nirS* transcripts		*nirK* genes		*nirK* transcripts	
	Days 0 and 1	Days 2, 4, and 7	Days 0 and 1	Days 2, 4, and 7	Days 0 and 1	Days 2, 4, and 7	Days 0 and 1	Days 2, 4, and 7
Temperature (T)	0.001	0.0001	0.004	0.0001	0.0001	0.0001	0.052	0.42
Amendment (A)	0.001	0.0001	0.0001	0.0001	0.0001	0.0001	0.002	0.004
Date (D)	0.001	0.0001	0.0001	0.0001	0.006	0.0001	0.004	0.02
T × A	0.001	0.0008	0.69	0.0007	0.01	0.01	0.08	0.11
T × D	0.5	0.007	0.16	0.01	0.003	0.002	0.02	0.10
A × D	0.001	0.0001	0.0001	0.01	0.0001	0.0001	0.02	0.31
T × A × D	0.05	0.0001	0.21	0.45	0.008	0.03	0.16	0.08

Phylogenetic analyses of *nirS* and *nirK* sequences

Some of the dominant bands in the DGGE profiles of *nirS* and *nirK* transcripts, i.e., representing major active denitrifiers under all FT and control regimes, were selected for phylogenetic analysis: i.e., bands 6, 8, 12, and 16 for *nirS* and 6, 7, 8, and 9 for *nirK* (Fig. S3). Partial *nirS* and *nirK* sequences representative of the selected DGGE band positions were more similar to sequences obtained from uncultured bacteria retrieved from soils than from isolated strains (Figs. 5 and 6). Among *nirS* DGGE profiles, sequences of bands 8, 12, and 16 were spread in several clusters of the phylogenetic tree, whereas sequences from band 6 belonged to one cluster with sequences of *Pseudomonas mandelii* PD8 and *Pseudomonas lini* PD 28 (Fig. 5). Among *nirK* DGGE profiles, sequences of bands 6 and 7 belonged to closely related but distinct clusters. Sequences of *Pseudomonas mendocina*, *Rhizobiales bacterium* D205, and *Proteobacterium* D248a were the closest to the sequences of these bands (Fig. 6). Sequences of bands 8 and 9 belonged to distinct clusters. Sequences 8-1 and 9-1 had high homology with *Sinorhizobium* sp. R-32546 while sequences 9-2, 9-3, and 9-4 were homologous to *Bradyrhizobiaceae* sequences (Fig. 6).

Discussion

N_2O emission, denitrification, and respiration rates following freeze-thaw cycles

The present study demonstrates that the timing of N_2O emissions from thawed soils in a FT cycle depends on thawing temperature, a factor that had not previously been investigated. Maximum rates of N_2O emissions in N + RC amended soils were observed at day 0 in soils thawed at +15 °C compared to day 1 for soils thawed at +4 °C. A possible explanation is that where the thawing temperature was high, microbial aerobic respiration was increased, which rapidly depleted O_2 supply, leading to anoxic conditions and a faster induction of denitrification and associated N_2O emissions (i.e., on day 0). In contrast, at the cooler thawing temperature, respiration rates were lower and more time was required to deplete O_2 supply, delaying the peak in maximum N_2O emissions. This hypothesis is supported by the fact that respiration rates were positively correlated to N_2O emission rates ($R^2 = 0.34$) and to denitrification rates ($R^2 = 0.24$) in N + RC amended soils. Pelster et al. (2013) similarly showed a 24 to 48 h delay in the appearance of the N_2O emission peak in soils

Table 2 Results of pairwise comparisons between temperature regimes for the *nirS* and *nirK* denitrifier community compositions for which temperature regime effect was significant (tested by PERMANOVA, Table 1). Pairwise comparisons were performed for communities after FT (i.e., on days 0 and 1 after thaw) and after several days of incubation at +4 or +15 °C (i.e., on days 2, 4, and 7 after thaw). *P* values are indicated

	nirS genes		*nirS* transcripts		*nirK* genes	
	Days 0 and 1	Days 2, 4, and 7	Days 0 and 1	Days 2, 4, and 7	Days 0 and 1	Days 2, 4, and 7
−5/+4 °C versus −5/+15 °C	0.007	0.0001	0.01	0.003	0.035	0.0001
−5/+4 °C versus +4/+4 °C	0.01	0.0001	0.02	0.002	0.034	0.0007
−5/+4 °C versus +4/+15 °C	0.03	0.001	0.001	0.0001	0.033	0.0001
−5/+15 °C versus +4/+4 °C	0.78	0.053	0.54	0.53	0.66	0.48
−5/+15 °C versus +4/+15 °C	0.73	0.004	0.30	0.01	0.54	0.58
+4/+4 °C versus +4/+15 °C	0.84	0.19	0.20	0.12	0.66	0.65

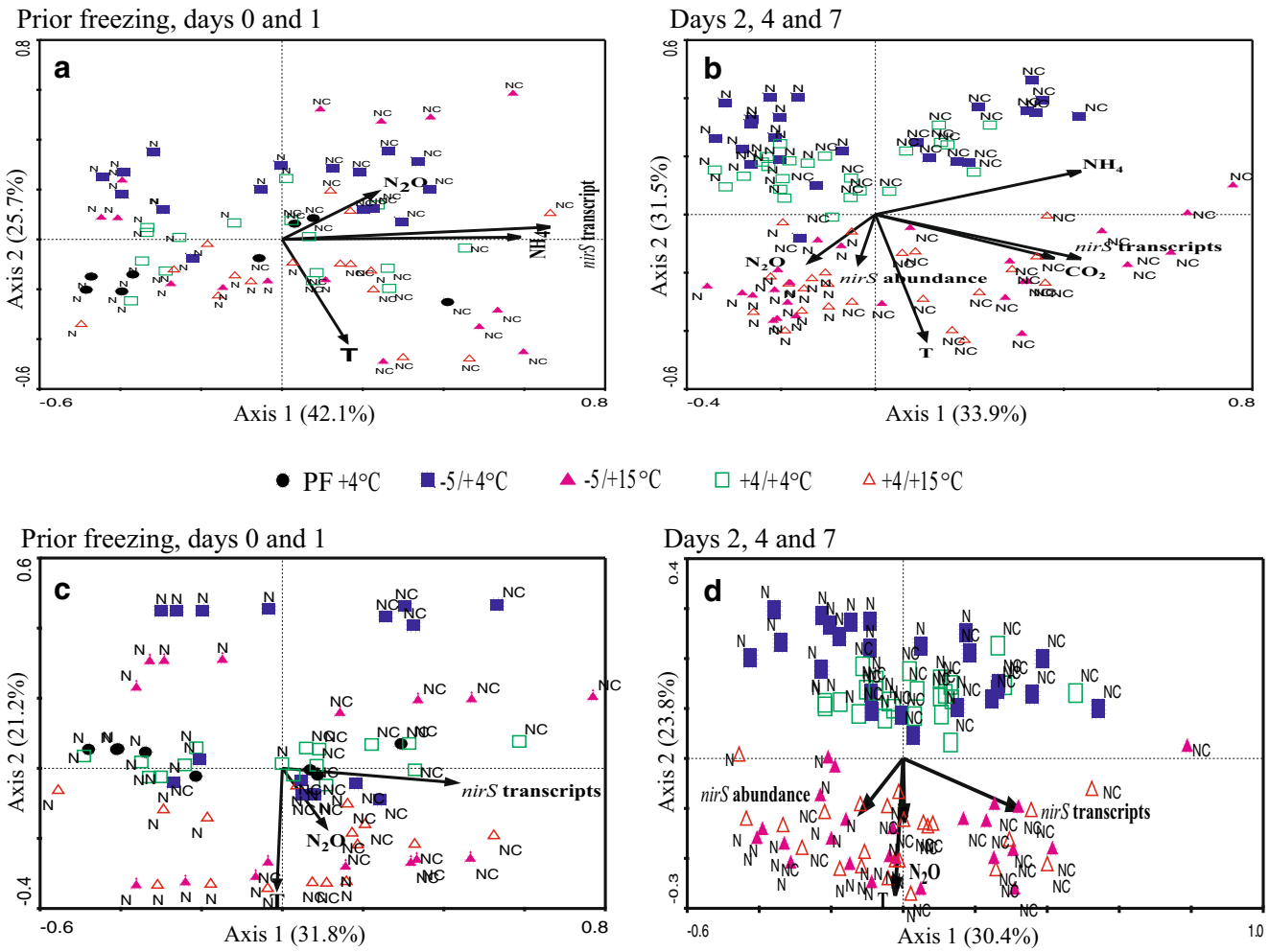

Fig. 3 Canonical correspondence analysis (CCA) of the present (**a**, **b**) and active (**c**, **d**) *nirS* denitrifier community compositions obtained from DGGE analysis in relation to temperature, soil inorganic N concentrations (NH_4^+ and NO_3^-), *nirS* abundances and transcriptional activity, gas emission (CO_2 and N_2O), and denitrification rates. *Letters above the symbols* represent soil amendment, i.e., NO_3^- (*N*) or NO_3^- plus red clover residues (*NC*)

thawed at +1 °C after freezing at −7 °C compared with freezing at −3 °C, suggesting that thawing and freezing temperatures can influence the temporal dynamics of N_2O emissions. Maximal N_2O emissions were observed one day earlier in soils amended with N + RC compared to those amended with N alone in the −5/+15 °C temperature regime. It further suggests that the increased respiration rate might affect the timing of N_2O emissions. The increase in organic C through the addition of RC stimulated aerobic respiration, as observed by Miller et al. (2008), and this may have more rapidly induced anoxic conditions favorable for denitrification. In addition, RC residues is a source of NO_3^- and could have stimulated mineralisation resulting in greater NO_3^- availability thus increasing denitrification in soils amended with RC + N compared to those with soils amended with N .

Nitrous oxide emissions and respiration rates were not always enhanced by FT, depending on the thawing temperature and the applied soil amendment. Respiration rates were increased by both FT regimes relative to the control regimes in N amended soils immediately after thaw, but not in N + RC amended soils. Mørkved et al. (2006) observed a similar increase in respiration induced by FT relative to unfrozen controls in unamended soils, but not in soils amended with plant residues. Freeze-thaw may have induced a release of substrates, such as organic C, due to soil aggregate disruption and/or death and lysis of microbial biomass (van Bochove et al. 2000; Pesaro et al. 2003; reviewed in Wang et al. 2008), which may have stimulated respiration in N amended soils. However, in N + RC amended soils, it is likely that there was sufficient organic C added through RC amendment to stimulate respiration and that any additional organic C released during FT had no effect on respiration.

In contrast with respiration rates, N_2O emission rates were not increased under the FT of −5/+15 °C compared to its control +4/+15 °C in both N and N + RC amended soils. This suggests that the release of substrates induced by freezing at −5 °C had

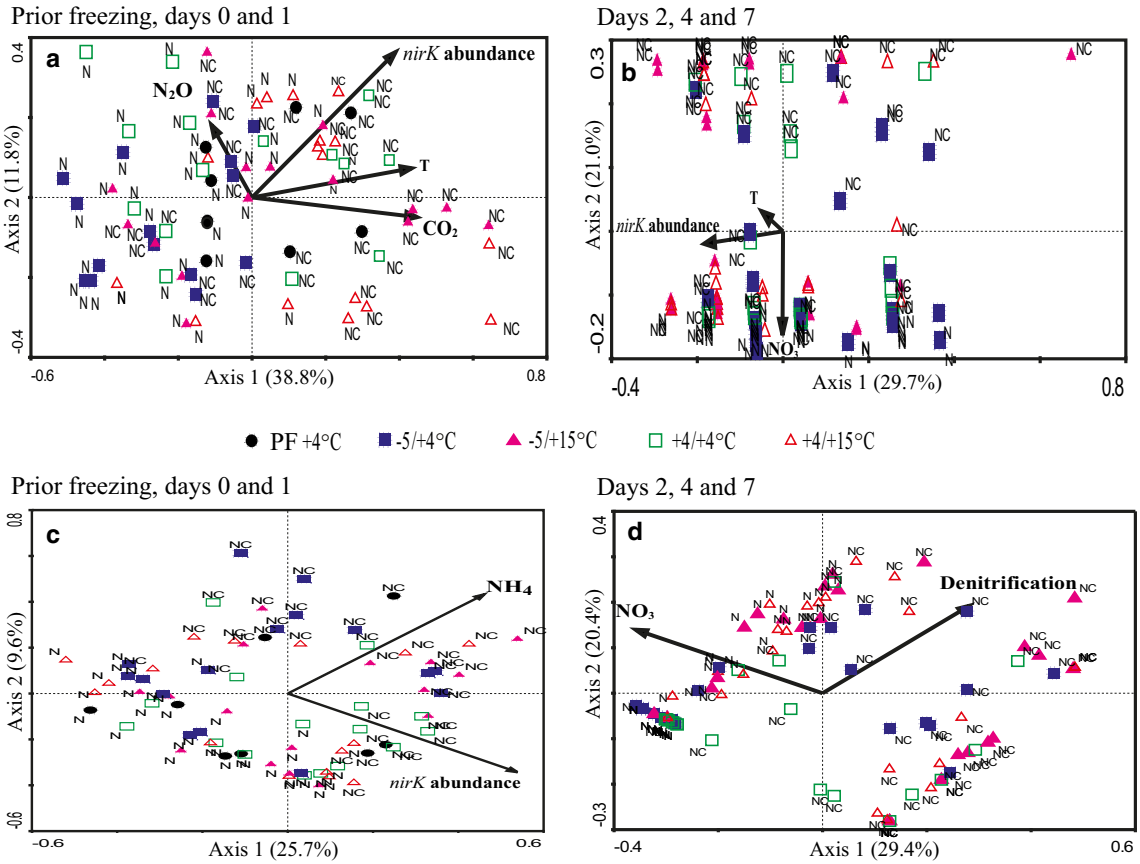

Fig. 4 Canonical correspondence analysis (CCA) of the present (**a**, **b**) and active (**c**, **d**) *nirK* denitrifier community compositions obtained from DGGE analysis in relation to temperature, soil inorganic N concentrations (NH_4^+ and NO_3^-), *nirK* abundances and transcriptional activity, gas emissions (CO_2 and N_2O), and denitrification rates. *Letters above the symbols* represent soil amendment, i.e., NO_3^- (*N*) or NO_3^- plus red clover residues (*NC*)

negligible effects on N_2O emissions compared to the stimulation caused by the increase in temperature to +15 °C, which may have induced an increase in microbial respiration and growth and O_2 depletion as explained above. However, N_2O emission rates were greater under the FT of −5/+4 °C compared to its control +4/+4 °C in N + RC amended soils, although not in N amended soils. This indicates that greater organic C availability following FT in soil amended with N + RC was required to increase N_2O emissions in −5/+4 °C compared to its control. The greater organic C availability was expected to induce microbial activity and growth resulting in greater respiration rate in −5/+4 °C compared to its control. However, the respiration data was similar between FT temperature regimes and their controls in N + RC amended soils. Several studies reported an absence of respiration increase following FT (Grogan et al. 2004; Koponen and Martikainen 2004; Wang and Bettany 1993). Koponen and Martikainen (2004) hypothesis was that the relative increase in organic C following FT could be negligible when aerobic respiration was high, but essential for denitrifiers competing for this organic carbon. Furthermore, it is possible that FT increased mineralisation and nitrification in soils amended with N + RC resulting in greater NO_3^- availability for denitrification. An increase in mineralisation following FT in soils amended with N + RC is supported by a significantly greater NH_4^+ concentration in soils at day 2 and a trend in greater NH_4^+ concentrations at day 0, 1, and 4 in soils submitted to a FT compared to their controls in soils amended with N + RC. Several studies have reported an increase in mineralisation following soil FT (Deluca et al. 1992; Herrmann and Witter 2002).

Denitrification rates did not differ from N_2O emission rates under the various temperature regimes, except in the +4/+4 °C regime at days 0 and 1 in N + RC treatments, for which denitrification rates were greater than N_2O emissions. These results suggest that gaseous N was generally emitted in the form of N_2O in most of our treatments. These high N_2O emissions can reflect a reduction of the activity of nitrous oxide reductases due to the low temperatures imposed in our study (Holtan-Hartwig et al. 2002) and/or non-limiting NO_3^- concentrations in the soils (Miller et al. 2008).

The water content imposed in our study (60 % WFPS) and the presence of NH_4^+ and NO_3^- in the soils may have allowed both nitrification and denitrification to occur (Braker and Conrad 2011), and thus both processes may have been contributing to N_2O emissions. In previous studies, denitrification

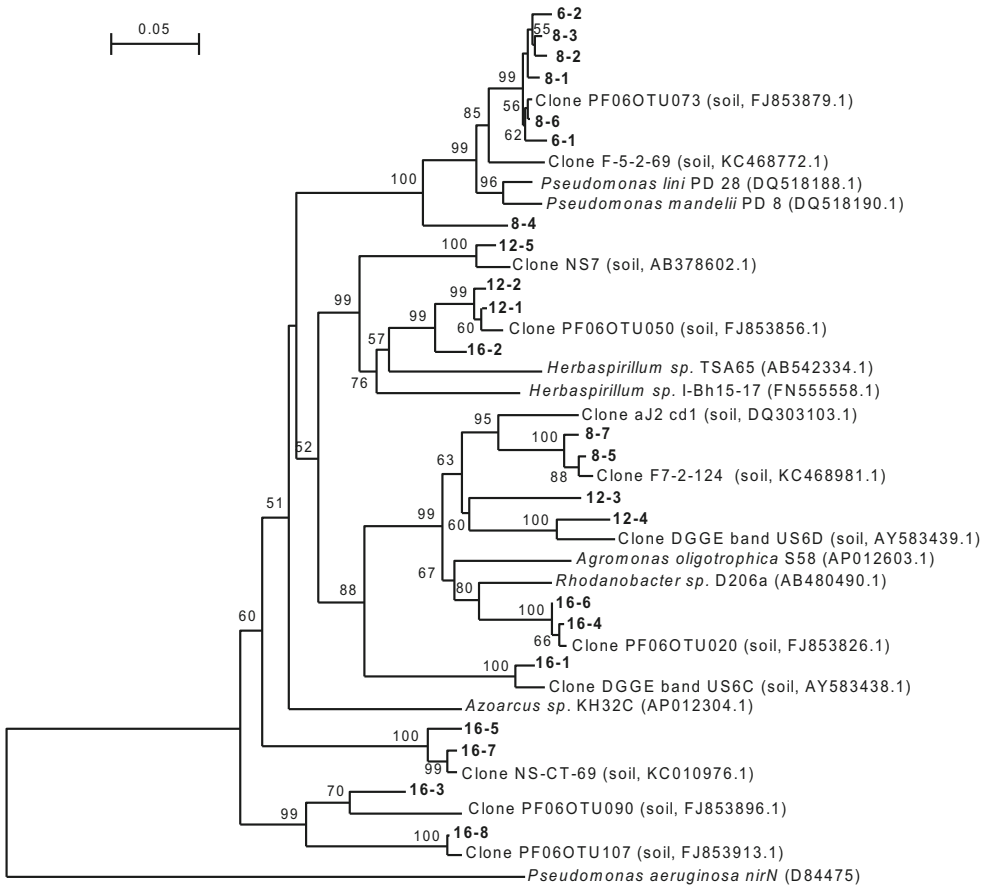

Fig. 5 Neighbor-joining phylogenetic tree of partial *nirS* sequences of clones representative of selected DGGE band positions in relation to *nirS* sequences of the closest uncultured and cultured relatives. Clone sequences from this study are labeled by numbers in *bold*. The *first number* corresponds to the DGGE band position and the *second number* corresponds to clones with identical DGGE band position but different sequences. The accession numbers of the closest relatives are indicated in *brackets*. The *scale bar* indicates 5 % nucleotide substitutions. Bootstrap values greater than 50 % (500 replicates) are reported at the nodes. The sequence of *nirN* from *Pseudomonas aeruginosa* served as an outgroup to root the phylogram

was reported to be the major process producing N_2O emissions during FT at high water content (81 % WFPS, Ludwig et al. 2004) as well as in soils under a range of O_2 concentrations (from 0 to 21 %, Mørkved et al. 2006). Since acetylene blocks nitrification, the similarity between N_2O emissions (obtained from soil microcosms without acetylene) and denitrification rates (obtained from soil microcosms with acetylene) suggest that denitrification was a major contributor to N_2O emissions under the conditions used in this study. It cannot be ruled out that dissimilatory nitrate reduction to ammonium (DNRA) could also be involved; however, there is no information available currently on the importance of DNRA over winter or following freeze-thaw events.

Effects of freeze-thaw events on denitrifier community transcriptional activity and composition

In this study, *nirS* and *nirK* genes of denitrifier communities, coding for the two forms of nitrite reductases, were targeted to evaluate the impact of FT cycles on soil denitrifier communities because (i) the majority of the denitrifiers harbor one of the two nitrite reductase genes and (ii) the nitrite reduction is the rate-limiting step in denitrification since this is the first step of the pathway that yields a gaseous product (Zumft 1997). Furthermore, the nitrite reductase gene is tightly and coordinately regulated with the nitric oxide gene presumably to maintain nitric oxide at a low steady-state level and to limit the toxic effects of this radical (Moir 2011).

Thawing temperature affected both denitrifier transcriptional activity and the composition of the present and active denitrifier communities. In fact, the number of *nirS* transcripts per gene copies were lower under the −5/+4 °C regime than under the −5/+15 °C regime in N + RC amended soils, which suggests, similarly to the results of N_2O emission rates, a lower induction of denitrification activity upon thawing at +4 °C compared to +15 °C. Interestingly, different factors were influencing the denitrifier community compositions between the two time periods, i.e., during FT (PF, days 0 and 1) and 2, 4, and 7 days after thaw as indicated by the CCA analysis. During FT, changes in the composition of the *nirS* denitrifier communities present and active and the *nirK* denitrifier community present correlated with changes in temperature and N_2O emissions and transcriptional activity, although these factors explained only 3.7 to 10.3 % of the changes in community composition. On days 2, 4, and 7 after thaw, denitrifier community compositions correlated with

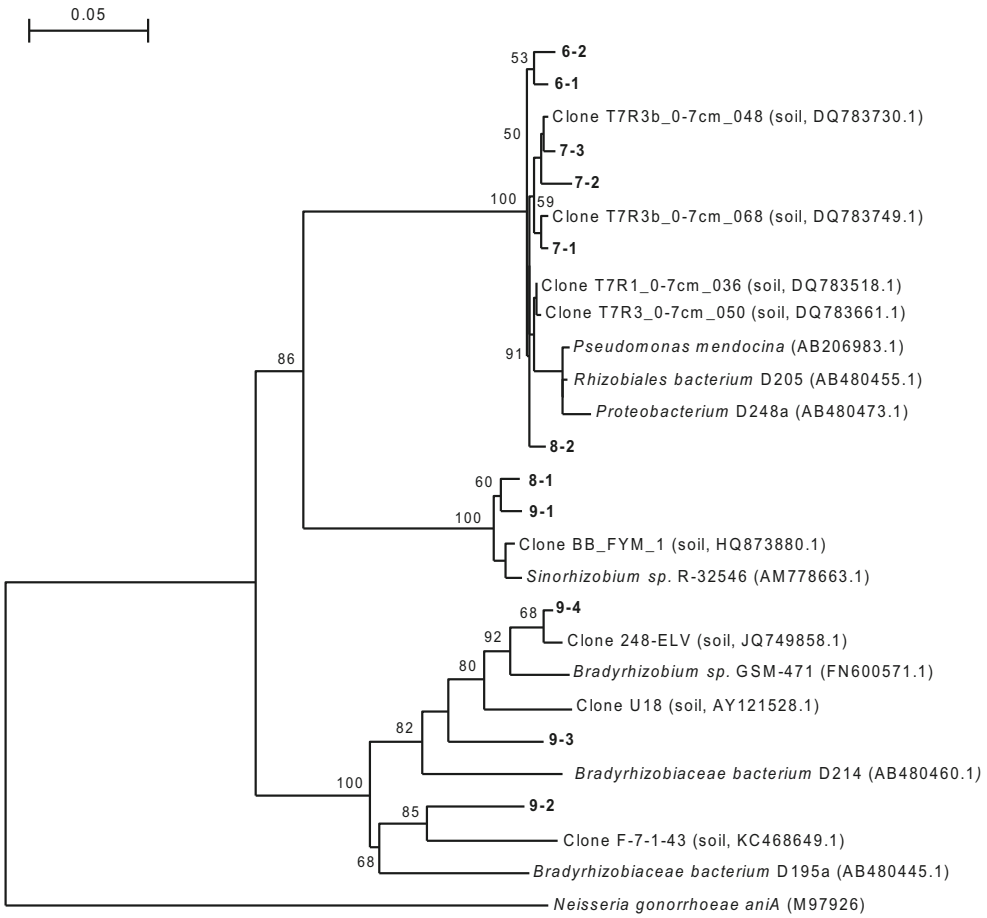

Fig. 6 Neighbor-joining phylogenetic tree of partial *nirK* sequences of clones representative of some DGGE band positions in relation to *nirK* sequences of the closest uncultured and cultured relatives. Clone sequences from this study are labeled by numbers in *bold*. The *first number* corresponds to the band position and the *second number* corresponds to clones with identical DGGE band position but different sequences. The accession numbers of the closest relatives are indicated in *brackets*. The *scale bar* indicates 5 % nucleotide substitutions. Bootstrap values greater than 50 % (500 replicates) are reported at the nodes. The sequence of *aniA* from *Neisseria gonorrhoea* served as an outgroup to root the phylogram

NH_4^+ or NO_3^- concentrations or CO_2 emissions, suggesting that changes in nutrient levels were influencing the denitrifier community composition at this point.

Temporal changes in denitrifier transcriptional activity during FT was possible since FT can induce denitrification by releasing organic C that is used as electron donor in this process and by increasing respiration thus decreasing oxygen level. A previous study reported that denitrification gene transcripts increased over time after freezing at −20 °C (Sharma et al. 2006). In the current study, denitrifier transcriptional activity did not change over time in soils subjected to FT, probably due to the shorter FT amplitude that did not result in a significant release of organic C. Similarly, under field conditions, *nirS* transcripts remained constant over spring thaw events (Németh et al. 2014). Despite this lack of change in denitrifier transcript number per gene copies, there were some differences between changes in the composition of the denitrifier communities present and active in the FT treatment (i.e., on sampling dates: prior freezing and on days 0 and 1 after thaw) versus its control when thawing occurred at +4 °C (except for active *nirK* denitrifiers). In contrast, denitrifier community compositions in the FT treatment did not vary from its control when thawing occurred at +15 °C, which corresponds with the lack of differences in N_2O emissions observed between the −5/+15 °C FT and its control. It is possible that the colder thawing temperature following FT favored the development of cold-adapted denitrifier communities. Similar to the current study, the composition of the *nirS* denitrifier community present in an agricultural field was shown to change between pre and post spring thaw that had a FT amplitude of −5 to +4 °C (Smith et al. 2010).

Phylogenetic analyses of dominant *nirS* and *nirK* transcripts revealed that some of the major *nirS* and *nirK* denitrifiers contributing to N_2O under FT and control temperature regimes had sequences close to strains belonging to several genera. Wide phylogenetic diversity of active denitrifiers in agricultural soils was also reported in previous studies (Wertz et al. 2009; Wertz et al. 2013; Pastorelli et al. 2011; Yoshida et al. 2012). FT might have been expected to select for cold-adapted active genera or to cause a decrease in diversity. However, our results suggest that there is high functional diversity during winter and FT, similarly to other time periods with warmer soil temperatures.

N_2O emission rates were not significantly correlated with the number of *nirS* or *nirK* transcripts (data not shown). This suggests that changes in N_2O emissions may have been mainly due to variations in the activity of denitrification enzymes (such as the ones encoded by the *nirS* and *nirK* genes targeted), rather

than due to a variation in the pool of enzymes. The absence of correlation between heterotrophic denitrifying bacteria, i.e., *nirS* and *nirK* transcripts, and N_2O emissions may also result from the additional contributions to N_2O emissions of other microorganisms, i.e., fungal denitrifiers, nitrifiers, nitrate ammonifiers, and the process of chemodenitrification (Braker and Conrad 2011). Furthermore, N_2O emissions are also controlled by nitrous oxide reduction. Indeed, Németh et al. (2014) observed no relationship between the number of *nirS* transcripts and N_2O emissions in agricultural fields during spring thaw, but showed a correlation between the number of *nosZ* transcripts and N_2O emissions.

In conclusion, the cooler thawing temperature, representing temperatures that may occur in fields in late autumn as opposed to early spring, induced a delay in the appearance of a N_2O peak and decreased transcription of *nirS* genes. These results indicate that the cooler thawing temperature led to conditions that were less favorable for denitrification, possibly through slower depletion of O_2 by microbial respiration and growth. With the exception of active *nirK* denitrifiers, the composition of the present and active denitrifier communities varied between the two FT regimes, and between FT and control regimes when thawing occurred at the cooler temperature. N_2O emissions were consistently explaining some of the variability in most present and active denitrifier community composition suggesting some importance of the community composition although the explanatory power was low (3.7 to 4.9 %). Although an effort was made in this study to use conditions that closely mimic natural in situ conditions, further experiments in the field during FT periods will be required to support the findings of this study and validate the results obtained. In addition, taking into account additional potential contributors to N_2O emissions such as nitrifiers, fungal denitrifiers, and nitrate ammonifiers will be necessary to characterize a wider range of microorganisms actively involved in N_2O emissions during FT.

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