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Influences of over winter conditions on denitrification and nitrous oxide-producing microorganism abundance and structure in an agricultural soil amended with different nitrogen sources

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ABSTRACT

Changes in nitrifier and denitrifier abundance, composition and nitrous oxide (N_2O) emissions during the winter period in soils with spring application of nitrogen (N) as mineral fertilizer, cattle manure or poultry manure were evaluated. Soil samples were collected from an agricultural field in Eastern Canada during winters of 2009–2010 and 2010–2011. Surprisingly, N_2O emission rates were up to 150 fold greater in the coldest dates compared to other dates for the two winters, however rates were similar among N sources. Abundance of nitrifiers (*Nitrobacter*-like *nxrA*, archaeal *amoA*) and denitrifiers (*nirK*, *nirS*) increased in March and April compared with other sampling dates. Nitrifier abundance (*nxrA*) was the greatest in soils amended with cattle manure, while denitrifiers (*nirK*, *nirS*) were more abundant in soils amended with poultry manure. Nitrifiers and denitrifiers showed different composition depending on sampling date and N sources in the two winters, suggesting that changes in environmental conditions and N sources both influenced these communities. Our findings demonstrate that N_2O emissions occur at significant rates in snow covered agricultural soils. Moreover, the abundance and diversity of denitrifier and nitrifier communities evolved throughout winter and appeared to be influenced by soil conditions and N sources applied in the previous spring.

1. Introduction

In the past decades, research efforts have increased our knowledge of soil N cycling during the growing season, whereas the non-growing season (NGS) has largely been overlooked in northern countries where freezing conditions and snow cover complicate field investigations. Significant soil N_2O emissions due to denitrification and/or nitrification (Braker and Conrad, 2011) were found during winter or in cold-incubated soils (Chantigny et al., 2002; Öquist et al., 2007; Phillips, 2007) with reports of N_2O emissions accounting for more than 50% of total annual budget (Wagner-Riddle et al., 1997; Virkajärvi et al., 2010).

Soil physical, chemical and biological proprieties are generally affected when temperature drops to ≤0 °C: water freezing in soil pores causes disruption of aggregates, redistribution of liquid water and change in the gas diffusivity and hydraulic conductivity (Kay et al., 1981; Matzner and Borken, 2008). The onset of aforementioned conditions can limit the availability of O_2 thus favouring the use of nitrogen oxidized species as electron acceptors (i.e. denitrification). Moreover, physical destruction of aggregates and microbial cell lysis can mobilize previously inaccessible nutrients, therefore stimulating microbial nitrogen conversions and related N_2O production (Dorland and Beauchamp, 1991; de Bruijn et al., 2009).

Incorporation of animal manures in soil may also impact winter N_2O emissions: in agriculture they constitute one of the most frequently used amendements to increase soil nitrogen and organic matter. Upon decomposition, these organic materials are fragmented and processed by the soil fauna and microbial communities to produce more or less stabilized N and C molecules and dissolved compounds. Interestingly, the addition of organic C has been shown to lower the minimum temperature at which N_2O production occurs in soil (Christensen and Tiedje, 1990). Moreover, losses of N via NO_3^- leaching and N_2O emissions have been reported during the winter months following application of manure and/or mineral fertilizers (Cookson et al., 2002; Gupta et al., 2004; Johnson et al., 2010).

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Table 1
Selected characteristics of the manures used in the experimental plots for 2009 and 2010.

	2009		2010	
Variables	CM	PM	CM	PM
Manure characteristics				
pH	6.5 (0.12)	8.8 (0.09)	6.5 (0.22)	8.9 (0.09)
Dry matter ($g\,kg^{-1}$)	46.8 (5.86)	661.1 (22.8)	47.1 (3.32)	769.0 (22.13)
C:N ratio	9.3 (1.13)	11.7 (0.80)	8.6 (0.63)	11.0 (1.11)
Total N ($g\,kg^{-1}$)	2.4 (0.05)	23.3 (1.95)	2.5 (0.04)	30.2 (2.62)
NH_4^+–N ($g\,kg^{-1}$)	0.7 (0.07)	5.1 (0.39)	1.3 (0.06)	3.7 (0.39)
Application rates				
Manure ($Mg\,ha^{-1}$)	45.7 (0.07)	5.1 (nd)[a]	45.3 (0.13)	4.3 (nd)[a]
Total N ($kg\,ha^{-1}$)	108.3 (2.46)	118.7 (9.93)	113.1 (2.27)	130.6 (11.33)
Total NH_4–N ($kg\,ha^{-1}$)	32.0 (2.98)	26.2 (1.99)	58.4 (2.77)	15.9 (1.67)

CM, cattle manure; PM, poultry manure. Values are means with standard deviation in parenthesis ($n = 6$).
[a] nd, standard deviation was not determined as PM was applied from only one manure pile.

The abundance and composition of denitrifying and nitrifying populations have been shown to regulate N_2O emissions from soil in some instances (Cavigelli and Robertson, 2000; Braker and Conrad, 2011). The majority of studies that examined the effect of cold temperatures on soil denitrifier and nitrifier communities were carried out in microcosms (Avrahami and Conrad, 2003; Sharma et al., 2006; Su et al., 2010; Wertz et al., 2013), whereas field experiments were essentially conducted on Arctic and Antarctic soils (Walker et al., 2008; Yergeau and Kowalchuk, 2008; Männistö et al., 2009). To our knowledge, one study evaluated changes in nitrifier and denitrifier community structures and N_2O emissions overwinter in an agricultural field (Smith et al., 2010). This study showed that community structures are influenced by seasonal variations in environmental factors and by different tillage practice (Smith et al., 2010). Therefore, our understanding of the dynamics, abundance and diversity of N_2O-producing microorganisms in agricultural soils during winter period is, in many respects, limited.

The objective of this study was to quantify overwinter changes in denitrifier and nitrifier abundance, gene expression and diversity, and N_2O emission and denitrification in an agricultural field receiving either mineral fertilizer, poultry manure or dairy cattle manure in the spring. For two consecutive years, five sampling dates were chosen based on their distinctive winter conditions.

It was hypothesized that (1) by providing both available N and C, organic fertilization increases the abundance and diversity of soil nitrifiers and denitrifiers, relative to mineral fertilization. Therefore, denitrification and N_2O production during winter are higher in soils receiving organic fertilizers; (2) changes in environmental conditions over winter (i.e. soil moisture, nutrient availability, temperature) would influence nitrifiers and denitrifiers by lowering both their abundances and expected denitrification and N_2O emissions rates when soil reaches the coldest temperatures.

2. Materials and methods

2.1. Experimental site and design

The experimental site was located at the Laval University Experimental Farm near Saint-Augustin-de-Desmaures, Québec, Canada (46° 44′N, 71° 31′W, altitude 110 m). This site has mean annual air temperature of 4.2 °C, and mean annual precipitation of 1213 mm evenly distributed over the year. Mean precipitation and temperature over the period between November and April is 78.28 mm and −4.2 °C, respectively. The soil was a well-drained sandy loam (170 g clay kg^{-1}, 680 g sand kg^{-1}, 150 g silt kg^{-1}) classified as a mixed frigid typic dystrudept (Canadian classification: orthic dystric brunisol). Before the experiment (2008) the site was cropped to silage corn (*Zea mays* L.). All crop residues were removed at harvest, and the sites were moldboard plowed (0.2–0.25 m depth) in the fall.

The experimental plots were established in May 2009 as a randomized complete block design and were 5 by 7 m in size. Three N sources were selected for the current study (2009–2010 and 2010–2011), mineral N fertilizer (MF), liquid dairy cattle manure (CM), solid poultry manure (PM). The mineral N fertilizer (90 kg N ha^{-1}) was applied as calcium–ammonium–nitrate; organic N sources were applied at target rate of 112.5 kg ha^{-1} total N, considering that on average these organic N sources contain 80% available N (Centre de référence en agriculture et agroalimentaire du Québec (CRAAQ, 2010)). Manure characteristics and actual application rates are presented in Table 1. All N sources were applied in the spring. The mineral N fertilizer was applied, P was applied as triple superphosphate (20 kg ha^{-1} P_2O_5) and K was applied as potassium chloride (20 kg K_2O ha^{-1}) based on the provincial soil recommendation of Quebec (CRAAQ, 2010). Treatments were surface-broadcasted in the spring and all plots were harrowed the same day to a depth of 0.1 m to minimize ammonia volatilization. Manure application and harrowing were performed on May 12th 2009 and May 5th 2010. After harrowing all plots were seeded to spring wheat (*Triticum aestivum* L. cv. AC Brio) using a nine-row, no-till drill seeder (Great Plains Mfg., Inc., Salina, KS) at 450 seeds m^{-2}. Row spacing was 0.18 m. Crop residues were left on the ground at harvest, in mid-August, and plots were not tilled in the fall.

2.2. Soil sampling and analyses

Bulk soil samples were collected (top 7.5 cm) from the experimental plots using a 5-cm diameter stainless steel corer fitted to a toothed knife following the removal of snow and surface vegetation. The corer was driven through the soil using a propane-driven slide hammer (Tippmann Industrial Products, Fort Wayne, IN). Soils were samples on five occasions during NGS: November (end of cropping season; freeze–thaw period), December (early winter: shallow snow cover, soil temperature gradually decreases below 0 °C), January–February (mid-winter: snow cover develops, surface soil frozen for a prolonged period of time), March (late winter: thick snow cover soil temperature around 0 °C), April (spring thaw: freeze–thaw period, large amount of snow and ice water infiltrates in soil). On each sampling date, four soil cores were taken at random within each plot, broken into small clods (<3 cm), and combined into one composite sample. A portion of each soil sample was immediately flash frozen in liquid nitrogen and stored at −80 °C for molecular analysis. The remaining of the soil samples was transferred into plastic bags, put in a cooler, and transported in the laboratory for analysis the same day to avoid any significant shift in soil temperature. Soil mineral N concentration (i.e. nitrate NO_3^- and ammonium NH_4^+) was determined using 1 M KCl (1:5 soil:solution ratio) and extraction for 30 min using a reciprocal shaker. NO_3–N and NH_4–N concentrations were determined in the

extracts using an automated colorimeter (QuickChem 8000 FIA+, Lachat Instruments, Loveland, CO). Soil temperature was monitored at 15 cm depth from November to April using copper-constantan thermocouples fitted to a data logger (model 600–1040; Barnant, Hayward, CA).

2.3. Gaseous emissions

Gaseous emissions were measured on the same day soils were sampled. Soils were kept in a cooler during sample handling in order to avoid possible thawing. About 300 g (wet basis) of each composite soil sample was put in a 1-L canning glass jar (Bernardine, Richmond, Canada). Each treatment was done in six replicates. Two jars were prepared for each soil sample to measure total denitrification and N_2O emission rates. The jars were immediately sealed with screw top lids fitted with a rubber septum and put in incubators set at the soil temperature measured in the field the same day. All these precautions were taken to avoid any significant shift in soil temperature during sample handling.

One jar for each soil sample had its headspace (10% of headspace volume) replaced with acetylene (C_2H_2) to quantify total denitrification rate as specified in Groffman et al. (1999). The presence of C_2H_2 inhibits nitrous oxide reductase activity; consequently N_2O emission quantified in presence of C_2H_2 reflects total denitrification ($N_2O + N_2$). The second set of jars had 10% of its headspace volume replaced with compressed air to quantify actual N_2O and CO_2 emission rates. After 1 and 4 h in incubators, 20 mL of headspace gas was collected from each jar and transferred to pre-evacuated 12 mL glass vial (Exetainers; Labco, High Wycombe, UK). Headspace gas samples were analyzed for N_2O and CO_2 concentrations using a gas chromatograph (Model 3800, Varian, Walnut Creek, CA) fitted with an electron capture detector to measure N_2O, a thermal conductivity detector to measure CO_2 and a Combi-PAL Autosampler (CTC Analytics, Zwingen, Switzerland) as described by Miller et al. (2008). N_2O and CO_2 emissions were calculated as the rate of N_2O and CO_2 accumulation in jar headspace between 1 and 4 h of incubation. After incubation, jars were opened and soil sub-sampled to determine soil gravimetric moisture content (weight loss upon drying for 24 h at 105 °C). Soil volume was measured and used to calculate bulk density and then water-filled pored space (WFPS) assuming a particle density of 2.65 g cm^{-3}.

2.4. Nucleic acid extraction

RNA and DNA were co-extracted from 1 g of flash frozen soil samples that were first freeze-dried as described by Henderson et al. (2010). DNA and RNA were quantified using Picogreen and Ribogreen kit (Invitrogen, Burlington, ON, Canada). DNA samples were further purified from co-extracted humic acid using PowerClean DNA Clean-Up Kit (Mobio, Carlsbad, USA) and stored at 4 °C. Immediately following RNA extraction, approximately 500 ng of undiluted RNA were retrotranscribed to cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen) with the addition of 250 ng of T4 gene 32 protein (New England Biolabs, Pickering, Canada) according to the following conditions: 5 min at 25 °C for enzyme activation, 75 min at 42 °C for retrotranscription, and 5 min to 82 °C to inactivate the enzyme. cDNA samples were stored at −80 °C.

2.5. Quantitative PCR

Quantitative PCR (qPCR) targeting archaeal *amoA* (AOA) gene copy number and transcript, and *Nitrobacter*-like *nxrA* gene copy number were performed as described by Wertz et al. (2013), the only exceptions were that denaturation was performed at 94 °C for all cycles and that 2.5 mM $MgCl_2$ (Invitrogen) was added to the reagent mix for amplification of *nxrA* genes. Primers targeting bacterial *amoA*-bearing communities (AOB) failed to amplify both DNA and cDNA while *nirS* and *nirK* failed to amplify cDNA even after extensive optimization of qPCR conditions. Copy numbers of *nirS* and *nirK* denitrifier genes were assessed by qPCR as described by Dandie et al. (2011). qPCR assays were conducted using a Step One Plus Real-Time PCR System (Applied Biosystems Streetsville, ON, Canada) and SYBR Green Master Mix (Applied Biosystems). Standard curves were obtained using three replicates of serial dilutions of linearized plasmids containing cloned nitrifier and denitrifier sequences: the 690 bp sequence from fosmid clone 54d9 (gi|42557759:32578–33234) was used for AOA *amoA* (Treusch et al., 2005), while the 364 bp sequence from *Nitrobacter hamburgensis* X14 was used for *nxrA* (Attard et al., 2010). For denitrifiers, the *nirS* and *nirK* sequences from *Pseudomonas stutzeri* (425 bp) (Thröback et al., 2004) and *Alcaligenes faecalis* (165 bp) (Henry et al., 2004), respectively, were used. Successful amplification of the desired-size fragment was assessed by agarose gel visualization, and specificity of the amplification was verified by performing melting curves. No template controls (NTC) were included for each primer sets. Reactions using not retro-transcribed RNA as template were also included to ensure that contaminating DNA was not present when evaluating *amoA* AOA transcript numbers. DNA and cDNA extracts were tested for the presence of co-extracted inhibitory substances as previously described (Henderson et al., 2010). Template concentrations used were not found to be inhibitory to the qPCR reaction (data not shown). The standard curve descriptors were: for *amoA* of AOA gene copy numbers: slope −3.88 to −3.39, E (efficiency) = 81.6–97.2%, R^2 = 0.984–994 y (intercept) = 35.1–33.8; for *amoA* of AOA transcript copy numbers: slope −3.89 to −3.21, E = 86.6–105.3%, R^2 = 0.995–999 y = 35.4–32.1; for *Nitrobacter*-like *nxrA* gene copy numbers: slope −3.21 to −3.48, E = 93.8–104.7%, R^2 = 0.990–0.999 y = 39.7–40.3; for *nirK* gene copy numbers: slope −3.41 to −3.51, E = 92.7–96.4%, R^2 = 0.997–0.999 y = 42.4–43.7; for *nirS* gene copy numbers: slope −3.38 to −3.57, E = 90.6–97.7%, R^2 = 0.997–0.999 y = 41.6–43.1.

2.6. Terminal restriction fragment length polymorphism analyses

A soil sample from each of the plots was used to analyze changes in the structure of nitrifier and denitrifier communities for a total of three replicates. DNA and cDNA amplification of archaeal *amoA*, DNA amplification of bacterial *amoA* and *Nitrobacter*-like *nxrA* were performed as described by Wertz et al. (2013), and WellRED dye-labelled forward primers (Integrated DNA Technology, Coralville, USA) were used. Due to the low number of AOB in the studied system, bacterial *amoA* was amplified using a semi-nested approach, using unlabelled primers in the first PCR reaction. *nirS* denitrifiers were amplified using WellRED dye-labelled forward primers using the same conditions described by Dandie et al. (2008) while *nirK* denitrifiers were amplified using 0.4 mM WellRED-labelled NirK1F and NirK5R primers (Braker et al., 1998) 2.5 μL of TE buffer 1 mM (Invitrogen) and 0.2 mg mL^{-1} BSA (Roche, Mississauga, Canada). Thermal cycling condition were: 95 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 59.5 °C for 1 min and 72 °C for 1 min, followed by 10 min at 72 °C. Three PCR reactions for each sample were performed then pooled, and amplification specificity confirmed using 1% agarose gel electrophoresis. PCR products were cleaned using MinElute PCR purification Kit (Qiagen, Toronto, Canada). DNA samples (250–500 ng) were digested in separate reactions with *RsaI* (New England Biolabs) for AOA and AOB *amoA*, *TaqI* for *nxrA* and *HhaI* for *nirS* and *nirK*. Digestions were performed in a total volume of 25 μL, including 10 U of enzyme, and were incubated for 3 h at 37 °C. Restriction digests were desalted by precipitation with 100% (v/v) ethanol and 3 M sodium acetate. Labelled DNA was resuspended in 20 μL Sample Loading Solution formamide (Beckman Coulter, Mississauga, Canada). A volume of 0.4 μL of the

600 bp DNA size standard (Beckam Coulter) was added as internal standard to each sample. T-RFLP samples was separated using capillary electrophoresis in the Genetic Analysis System CEQ 8000 (Beckman Coulter, USA) and the default FRAG-3 method and a 33 cm DNA separation capillary. The data, displayed as peaks in the electropherogram files, were analyzed using the CEQ 8000 analysis software. The fragment sizes were determined by comparison with the internal DNA Size Standard, limiting analysis to fragments between 60 and 600 bp. The relative abundances of terminal restriction fragments were standardized in percent by calculating the ratio of a given peak height to the total peak height of each sample. Every peak with less than 5% of total peak height was not considered into further analyses. Terminal restriction fragments that differed by <2 bp were considered identical and merged.

2.7. Statistical analyses

Statistical analyses for soil analytes (NO_3^-, NH_4^+), gas emissions rates, (N_2O denitrification and CO_2) and abundance/transcript numbers of denitrifiers and nitrifiers) were conducted using SYSTAT software (Systat software Inc, Chicago, IL, USA). Non-normal data were log transformed. General Linear Model Analysis of Variance was performed based on a randomized complete design with soil amendments and sampling time as fixed factors. Treatment means in interactions were compared using Tukey adjusted Least Significant (LS) means and treatment means in sample main effects were compared by performing post hoc Tukey Honestly Significant Difference (HSD) tests. Pearson's test (R^2 coefficient) was used to determine possible linear relationships between the following parameters: soil NO_3^-, NH_4^+, CO_2 and N_2O emissions, total denitrification. The differences and correlation coefficients presented were significant at $P < 0.05$ except when otherwise stated in the text.

T-RFLP profiles were analysed as follows. Data matrices consisting of the relative intensity of each DNA band (i.e. ratios of the intensity of each band versus the total band intensity) were calculated. Rank similarity matrices were computed using PRIMER v6 software (Plymouth, United Kingdom). Permutational Multivariate Analysis of Variance (PERMANOVA) was performed from these matrices to test the significance of the effects of soil amendments, sampling times and interactions among these factors on the nitrifier and denitrifier community structures. Two way analysis of similarity (ANOSIM) was performed to determine differences in community structures. Non-metric multidimensional scaling (MDS) representations of similarities in genetic structure were constructed.

Possible monotonic relationships between community abundances or structures and soil inorganic N concentrations (NH_4^+ and NO_3^-) and microbial activities (CO_2 and N_2O emissions, total denitrification) were explored using PRIMER v6 by computing Spearman correlation coefficients (ρ).

3. Results

3.1. Environmental variables

In both winters, soils experienced freeze–thaw events in November and December, before the snow cover was thick enough to insulate the ground from fluctuations in air temperature (i.e. >20 cm). Soil temperature stabilized during the January–March period, when a thick snow cover was present, and another period of freeze–thaw events occurred in late March–April as snow thawed and soil became exposed to air temperature (Fig. 1E, F). Snow cover was shallower and was present over a shorter of time in the winter 2009–2010 as compared to 2010–2011. In both years significant snow melt events were observed in early January. GWC values significantly changed over time (i.e. significantly higher in January and March) but were similar among N sources in both years (data not shown). Calculated WFPS values showed similar trends as GWC in both years, showing significantly higher values in January and March for both years (i.e. 38% and 43% in 2009–2010 and 39% and 51% in 2010–2011, respectively) compared to November, December and April (25%, 26% and 23% in 2009–2010 and 26%, 23% and 27% in 2010–2011, respectively).

3.2. Soil analyte concentrations

Soil NO_3^- concentrations changed significantly over time but were similar among N treatments in both winters and no interaction was found between the factors (Fig. 1A, B). When averaged across N sources, soil NO_3^- concentration varied from 3.82 to 5.86 mg N kg^{-1} dry soil in the winter of 2009–2010. Although soil NO_3^- concentration appeared relatively stable, values were the lowest in March, whereas they were the highest in April (Fig. 1A). In the following winter (2010–2011), average soil NO_3^- concentrations were 4.9 and 6.8 mg N kg^{-1} dry soil in January and March, respectively, significantly greater ($P < 0.001$) compared with the other sampling dates (average of 2.4 mg N kg^{-1} dry soil).

Time and N source had a significant influence on soil NH_4^+ concentrations. In both winters, NH_4^+ concentrations gradually increased from November through to March, reaching 12.5 and 6.9 mg N kg^{-1} dry soil in March 2010 and 2011, respectively (Fig. 1C, D). The NH_4 concentration declined from March to April to levels close to those measured in November. When averaged across dates, plots fertilized with PM showed NH_4 concentrations 1.6 (2009–2010) and 1.5 (2010–2011) fold higher than with CM and MF, which had similar concentration levels.

3.3. N_2O emissions, denitrification and respiration

N_2O emission rates changed significantly over time, but rates were similar among N sources in both winters (Fig. 2A, B). In November, December and April of both winters, N_2O emissions were low showing an average of 0.37 and 0.34 mg N_2O–N ha^{-1} day^{-1} in 2009–2010 and 2010–2011, respectively. In both winters, N_2O emissions were the highest in January (average across N sources, 34.6 and 29.6 mg N_2O–N ha^{-1} day^{-1} in 2009–2010 and 2010–2011, respectively) and in March (9.3 and 117.8 mg N_2O–N ha^{-1} day^{-1}, respectively).

Denitrification rates showed temporal dynamics similar to N_2O emission rates (Fig. 2C, D), with the lowest rates in November, December and April (average across N sources: 0.44 and 0.9 mg N_2O–N ha^{-1} day^{-1} in 2009–2010 and 2010–2011, respectively) and the highest rates in January (40.8 and 30.3 mg N_2O–N ha^{-1} day^{-1}, respectively) and in March (9.4 and 58.8 mg N_2O–N ha^{-1} day^{-1}, respectively). Interestingly, N_2O emissions and denitrification were higher in January during the first winter then decreased in March, while in the winter of 2010–2011, denitrification and N_2O emission showed significantly higher rates in March compared to January.

Respiration rates also varied over time in both winters with no significant differences among N sources (Fig. 2E, F). In the winter of 2009–2010, respiration rates increased from November to December, and remained constant from December to April (average rate of 5.4 g CO_2–C ha^{-1} day^{-1}). By contrast, in the winter of 2010–2011, rates peaked in January and March (average across N sources: 6.1 and 6.4 g CO_2–C ha^{-1} day^{-1}, respectively) and declined from March to April to levels similar to those recorded previously in November and December (1.8 and 2.5 g CO_2–C ha^{-1} day^{-1}, respectively).

Respiration rates showed a positive increasing linear correlation with N_2O (2009–2010: $R^2 = 0.7$; 2010–2011: $R^2 = 0.71$) and denitrification rates ($R^2 = 0.48$ and 0.75, respectively). In winter 2010–2011, NO_3^- concentrations showed positive increasing correlations with N_2O emissions ($R^2 = 0.67$) and denitrification ($R^2 = 0.87$). Interestingly, NH_4^+ concentrations were positively correlated in both winters with N_2O (2009–2010: $R^2 = 0.54$; 2010–2011: $R^2 = 0.77$), denitrification ($R^2 = 0.33$ and 0.69, respectively) and respiration rates ($R^2 = 0.40$ and 0.60, respectively). No correlation was found between measured gaseous emissions and soil temperature at the time of measurements.

3.4. Denitrifier and nitrifier abundances and nitrification gene mRNA levels

The abundance of *nirS*, *nxrA*, *amoA* AOA and *amoA* AOA transcripts levels did not change significantly over time or among N sources in the winter of 2009–2010 (Figs. 3 and 4) with the exception of *nirK* gene. The abundance of *nirK* gene was significantly higher in soils fertilized with PM (averaged of 9.3×10^6 g^{-1} dry soil across dates) compared with MF and CM which had 3.8×10^6 and 4.2×10^6 copies g^{-1} dry soil g^{-1} dry soil, respectively (Fig. 3C). The abundance of *nirS* and *nirK* genes changed significantly over time and among N sources, and a significant time × N source interaction was observed in winter 2010–2011 (Fig. 3B, D). The *nirS* and *nirK* copy numbers remained stable from November to January (average of 3.6×10^6 and 1.1×10^7 copies g^{-1} dry soil, respectively), and increased in March and April to an average of 7.76×10^6 and 3.1×10^7 copies g^{-1} dry soil, respectively. The PM treatment caused a significant increase in the number of *nirS* copies, compared to MF (average of 8.16×10^6 and 3.84×10^6, respectively), while the abundance of *nirK* copies was higher with PM than with MF and CM treated soil (average of 3.3×10^7, 9.1×10^6 and 1.01×10^6, respectively) (Fig. 3B, D).

In the two studied winters *amoA* AOB abundance in soil could not be assessed by qPCR even after exhaustive optimization of amplification conditions. In 2010–2011 there was a significant effect of time ($P < 0.027$) but not of N source on the abundance of *amoA* AOA (Fig. 4D). The gene copy number of *amoA* significantly increased from an averaged value of 3.9×10^6 copies g^{-1} dry soil in December and January to 6×10^6 *amoA* copies g^{-1} dry soil in March and April. In the 2010–2011 winter,

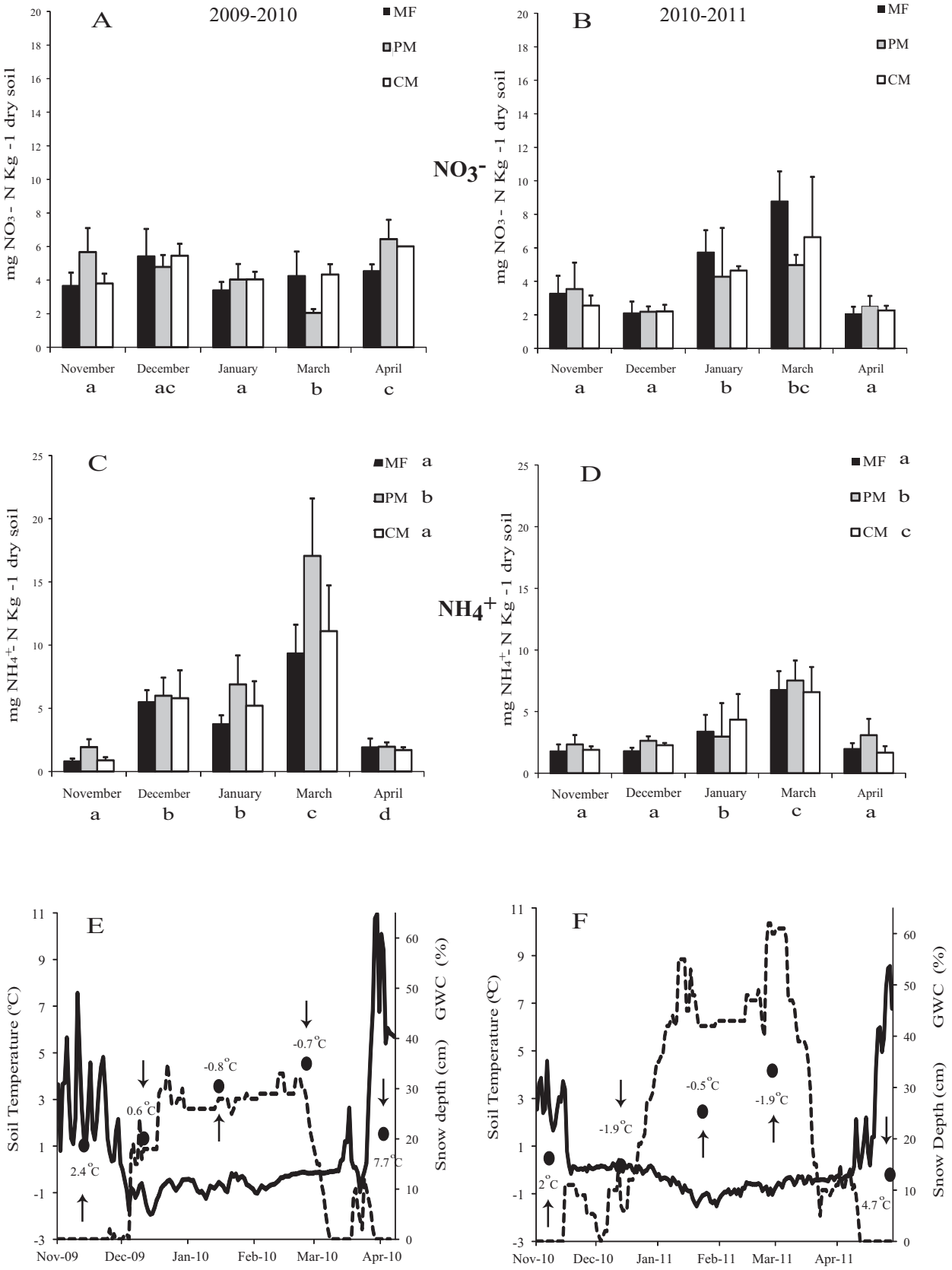

Fig. 1. Concentrations of NO_3^- and NH_4^+ (A, B, C and D) and environmental conditions (i.e. soil temperature, snow depth, GWC, (E and F)) in the experimental plots for winter 2009–2010 and 2010–2011. For NO_3^- and NH_4^+ values are means ($n = 6$) and error bar are standard errors. Significant differences among mean values are represented in two ways based on Tukey's test ($P < 0.05$): (i) significant differences between sampling times are represented by letters under month on the *x*-axis, and (ii) significant differences between N treatments are indicated by letter in the figure legend. For environmental condition black continuous line indicates daily mean soil temperature at 10 cm depth. Dotted black line indicates snow depth in the experimental plot (expressed in cm on *y*-axis). Gravimetric Water content (GWC) is presented as the average across treatments using closed circles (●). Arrows indicate the date of sampling and the related incubation temperature for gaseous emission measurements.

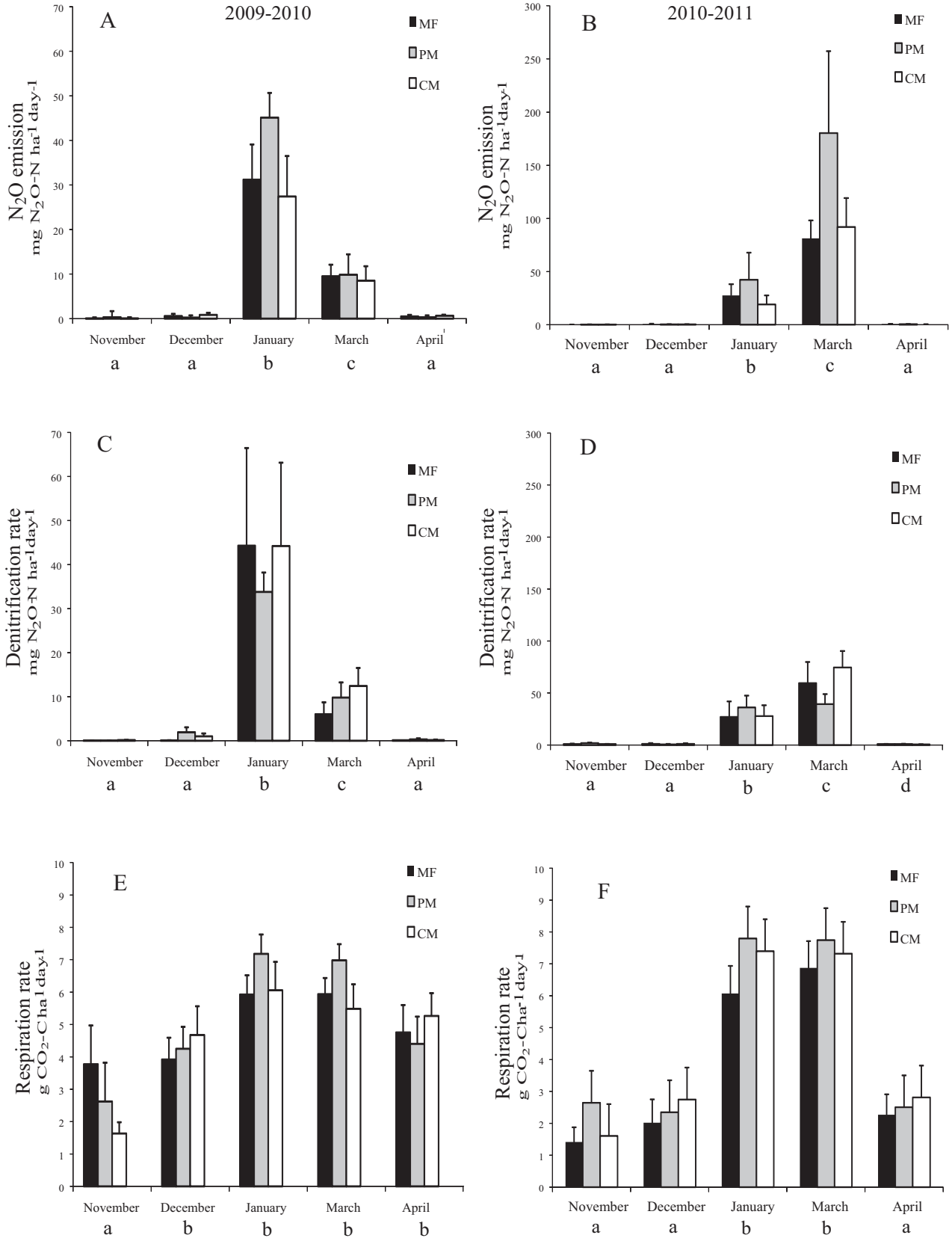

Fig. 2. N_2O emission (A, B), denitrification rate (C, D) and respiration rate (E, F) in soil cores incubated at field temperatures for winters 2009–2010 and 2010–2011. Value are means ($n = 6$) and error bars are standard error. Significant differences among mean values are represented based on Tukey's test ($P < 0.05$): significant differences between sampling times are represented by letters under month on the *x*-axis.

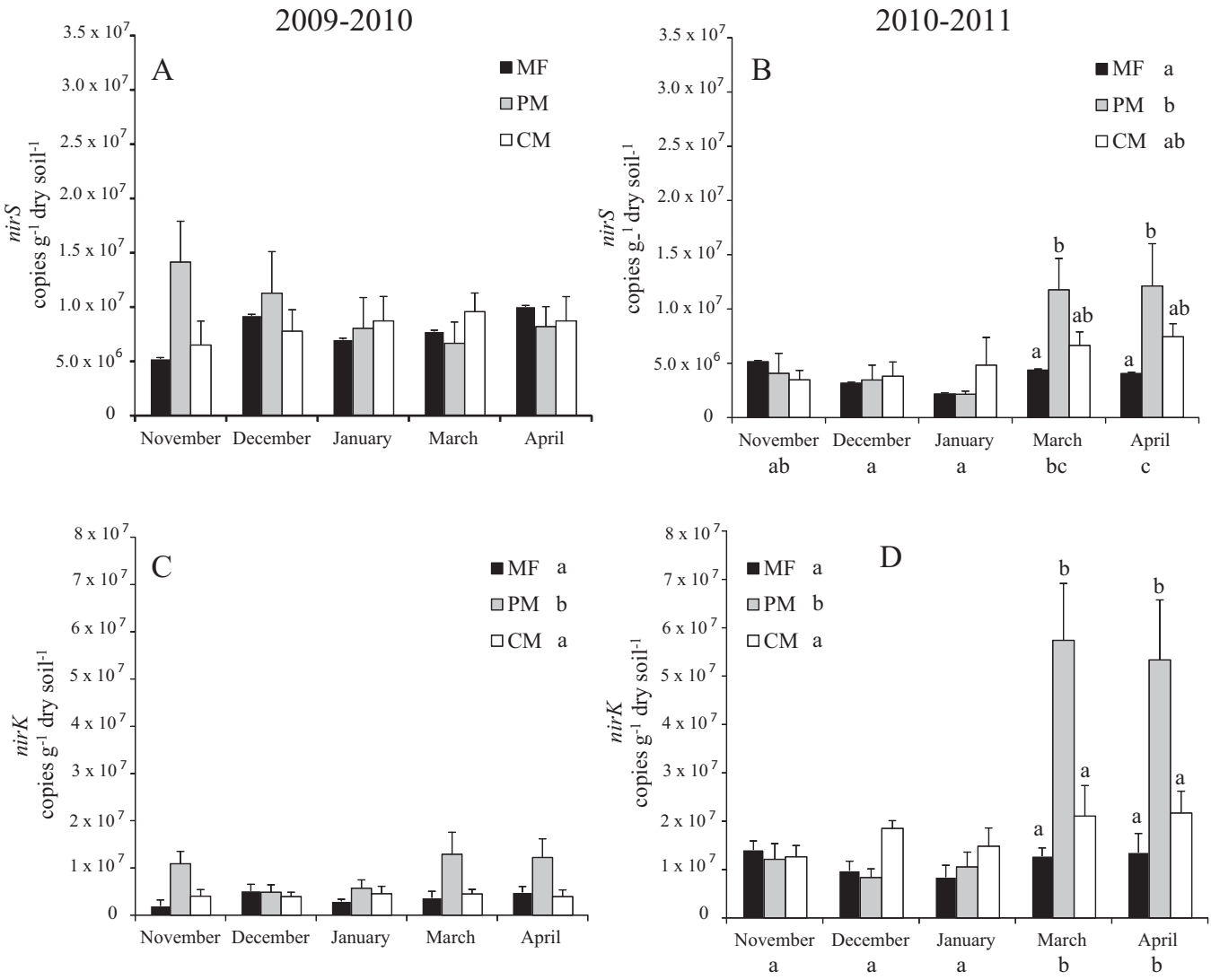

Fig. 3. Abundance of *nirS* and *nirK* genes in winters 2009–2010 and 2010–2011 obtained by qPCR. Values are means ($n = 6$) and error bars are standard error. Significant differences among mean values are represented in three ways based on Tukey's test ($P < 0.05$): (i) significant differences between sampling times are represented by letters under month on the *x*-axis (ii) significant differences between N treatments are indicated by letter in the figure legend (iii) differences among time × treatment interactions are indicated by letter over the specific bar.

Nitrobacter-like *nxrA* copy number were influenced by time ($P < 0.010$), N source, and interaction between the two factors (Fig. 4B). In March 2011, the abundance of *Nitrobacter*-like *nxrA* increased from 1.1 to 2.8×10^7 copies g^{-1} dry soil. The abundance in March was more pronounced in the CM (4.5×10^7 copies g^{-1} dry soil) than in PM or MF treatments (2.1 and 1.5×10^7 copy g^{-1} dry $soil^{-1}$ respectively).

No amplification of *nirS*, *nirK*, *nxrA* and *amoA* of AOB cDNA was obtained, even after extensive optimization of qPCR conditions. The number of archaeal *amoA* transcripts was similar across time and treatments in winter 2009–2010 (Fig. 4E). The mRNA levels of archaeal *amoA* showed a significant effect of time but not of N source in winter 2010–2011 (Fig. 4F). A constant number of transcripts was detected from November to March (1.7×10^5 transcripts g^{-1} dry soil), while a significant increase was found in the month of April (4.2×10^5 transcripts g^{-1} dry soil). Interestingly, the number of AOA *amoA* transcripts increased significantly while the concentration of NH_4^+ decreased in March and April of both winters (Figs. Fig. 1C, D and Fig. 4E, F). However, no significant correlations were observed between denitrifiers and nitrifiers abundance or nitrification mRNA gene and soil inorganic N concentrations, or with N_2O emissions, denitrification or respiration (data not shown).

3.5. Nitrifiers and denitrifiers community analysis

Community composition of *nirK* and *nirS* denitrifiers, *Nitrobacter*-like nitrite oxidizing bacteria (NOB), AOB and AOA nitrifiers were characterized for the two winters. Due to the similarity in trends in the datasets of the two winters, results from winter 2009–2010 is presented herein while the results of 2010–2011 are presented as supplementary data (Figs. S1 and S2). PERMANOVA analysis showed that the community composition of denitrifiers, AOA and *Nitrobacter*-like NOB were influenced significantly by time, treatment and by the time × treatment interaction (Table 2). Community compositions of *nirK* and *nirS* bearing denitrifiers were distinct between all sampling dates (ANOSIM $R = 0.99$ and 0.7, $P < 0.001$, respectively) and treatments (ANOSIM $R = 0.9$ and 0.6, $P < 0.001$ respectively) (Fig. 5A, B). The bacterial nitrifier community structures of AOB and *Nitrobacter*-like NOB (involved in both steps of the nitrification pathways) were different between all sampling dates (ANOSIM $R = 0.6$ and 0.83, $P < 0.001$, respectively) (Fig. 6A, B). Interestingly, community structure of *Nitrobacter*-like NOB showed a clear separation in two distinct cluster (November–December and January–March–April) (Fig. 6A), highlighting a greater temporal effect ($R = 0.83$, $P < 0.001$) rather than an amendment effect ($R = 0.64$, $P < 0.001$) on community structure. Community structure of AOB varied significantly ($P < 0.001$) among sampling dates but was not influenced by amendments (Fig. 6B).

The *amoA* gene of AOA structure was analyzed for the present (DNA) and active (cDNA) community (Fig. 6C, D). nMDS analysis revealed a good temporal separation of both present and active AOA community structures (ANOSIM $R = 0.79$ and 0.64, $P < 0.001$), with all the sampling dates significantly different in their structure. While community structure of AOA present exhibited a significant difference between different N treatments ($P < 0.001$, ANOSIM $R = 0.65$) (Fig. 6C), the structure of active AOA community was not significantly different between CM and MF-treated soil, therefore showing a weaker separation across profiles (ANOSIM $R = 0.41$). Peaks from T-RFLP profiles of archaeal *amoA* genes and cDNA (i.e. derived from *amoA* transcripts) were analyzed and compared. T-RFLP profiles of archaeal *amoA* genes showed four major peaks with different relative abundance (in brackets) (78 bp (15%), 86 bp (5%) 100 bp (20%) and 379 bp (10%)) that were observed in November, December, January, March and April. Moreover, peaks 106 and 445 bp (10% and 12% of relative

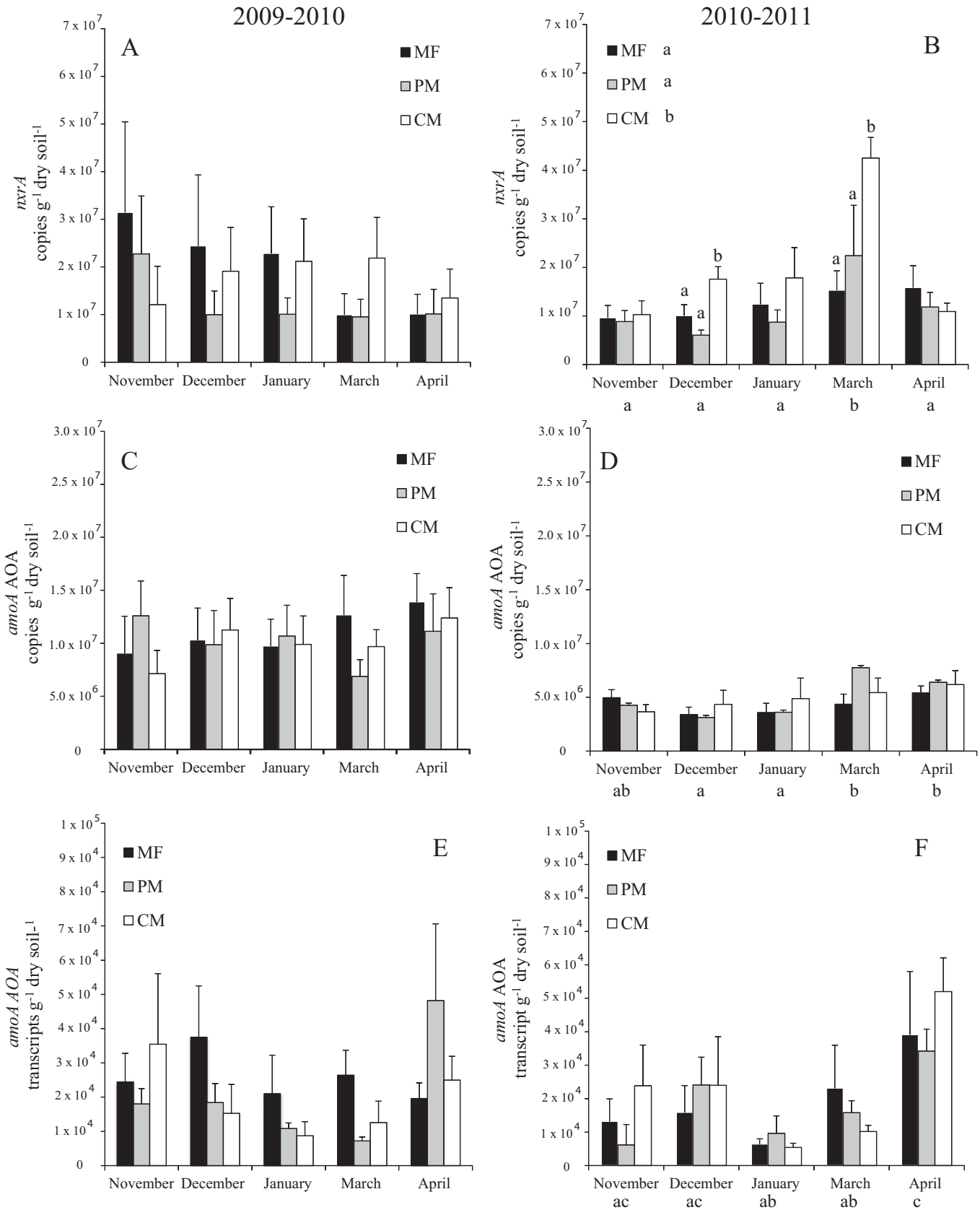

Fig. 4. Abundance of *Nitrobacter*-like *nxrA*, *amoA* of AOA genes and *amoA* of AOA transcripts in winters 2009–2010 and 2010–2011 obtained by qPCR. Values are means ($n = 6$) and error bars are standard error. Significant differences among mean values are represented in three ways based on Tukey's test ($P < 0.05$): (i) significant differences between sampling times are represented by letters under month on the *x*-axis (ii) significant differences between N treatments are indicated by letter in the figure legend (iii) differences among time × treatment interactions are indicated by letter over the specific bar.

Table 2
Result of PERMANOVA testing the significance of the effect of time, soil treatments and interaction among these factors on the denitrifier and nitrifier community structure for winter 2009–2010. *P* values are indicated.

2009–2010	*nirS*	*nirK*	*nxrA*	*amoA* AOB	*amoA* AOA	*amoA* AOA cDNA
Time	0.001	0.001	0.001	0.001	0.001	0.001
Treatment	0.001	0.001	0.001	0.28	0.005	0.006
Ti × Tr	0.001	0.001	0.001	0.02	0.001	0.001

abundances) were present on all sampling dates except March and January respectively. T-RFLP profiles of archaeal *amoA* transcripts showed 5 major peaks (90, 106, 148, 200 and 379 bp) having the highest relative abundance with 5%, 10%, 15%, 15% and 40% respectively and were observed on all sampling dates. In addition, peak 388 bp (showing an average abundance of 5%) was present in T-RFLP profiles of archaeal *amoA* transcripts on all sampling dates with the exception of November. Interestingly the peaks 90, 200 and 388 bp were not revealed in the T-RFLP profiles of archaeal *amoA* genes.

4. Discussion

4.1. Overwinter N_2O emission, denitrification and CO_2 emission rates

N_2O emissions had the greatest rates in January and March in both winters, showing an average increase of N_2O emissions up to 150 fold compared to the other sampling times. Soil water content consistently increased in January and March in both years, possibly due to snowmelt events that occurred during these periods, as revealed by the reduction in snow depth (Fig. 1E, F). The presence of liquid water in soil probably facilitated the diffusion of nutrients and soil analytes which may arise from soil freeze–thaw events in early winter, which can increase the availability of substrates by soil aggregates disruption, death and lysis of microbial biomass and exposure of new reactive mineral surfaces (Phillips, 2007). The January and March fluxes of N_2O were coupled with increased rates of CO_2 emission, further suggesting an increase in available substrates in soil. Respiration has been shown to occur at low temperatures in both microcosm and field studies (Chantigny et al., 2002; Drotz et al., 2010; Wertz et al., 2013). The simultaneous increase in water content, caused by snow melt, and respiration may restrict O_2 diffusion and create favourable conditions for denitrification (de Bruijn et al., 2009). Moreover, part of the soil water present in January and March in both winters may have been partially frozen, which has been reported to increase nutrient concentrations in the remaining unfrozen water (Staähli and Stadler, 1997; Arenson and Sego, 2006) N_2O gaseous emission rates in the NGS were not positively correlated with soil temperature at the day of measurement, as shown for unfrozen soil (Keeney et al., 1979; De Klein and Van Logtestijn, 1996). It can be hypothesized that in partly frozen soils access to substrate, as mainly driven by water availability, is more determinant to microbial activities than the actual temperature, as observed by Öquist et al. (2007).

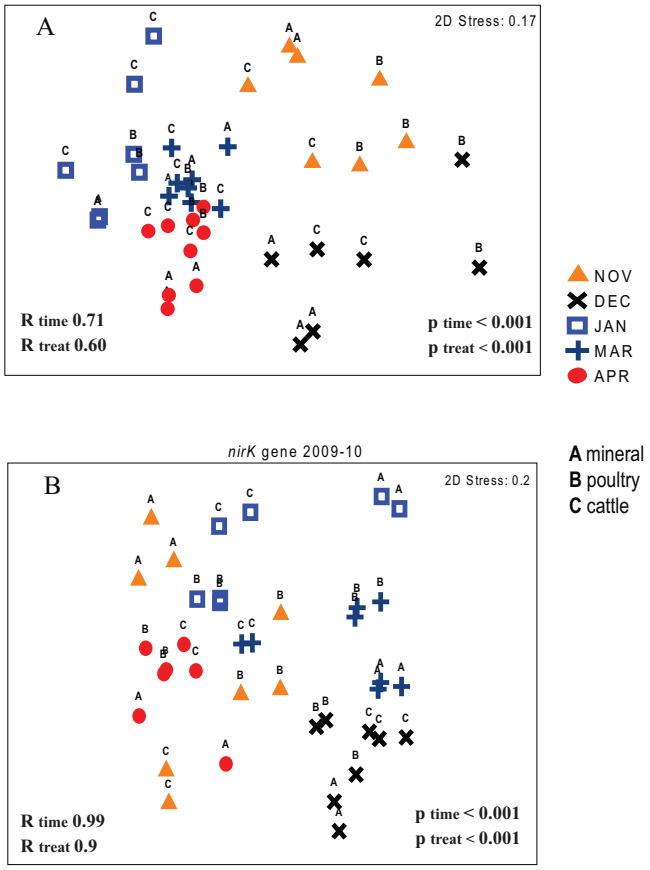

Fig. 5. nMDS representations of *nirS* (A) and *nirK* (B) denitrifier community structures in winter 2009–2010 obtained by T-RFLP. Similarities or differences among sampling times and treatments are indicated by symbols and letters above symbols, respectively, as described in the legend.

High N_2O emission rates have been reported during soil freeze–thaw events in early winter and spring (Wagner-Riddle et al., 1997; Rover et al., 1998; Dörsch et al., 2004; Furon et al., 2008; Virkajärvi et al., 2010). The chosen sampling date interval did not permit to observe freeze–thaw induced N_2O, which may be short (Christensen and Tiedje, 1990). The fact that N_2O fluxes were detected in January and March in all plots and for two consecutive winters suggests that N_2O emissions could be present over extended periods of time in winter, possibly due to the favourable environmental soil conditions provided by snow cover (insulation), high soil water content, and the absence of competition from plants for nitrates (Brooks et al., 1997). Denitrification rates showed that most of the total emissions were accounted for by N_2O in the two winters. The predominance of N_2O over N_2 supports the hypothesis that N_2O reductase is inhibited at temperatures below 5 °C (Holtan-Hartwig et al., 2002). Greater N_2O emission over denitrification rates were previously reported in different type of soil (Dörsch and Bakken, 2004) and in Canadian agroecosystem over winter (Wagner-Riddle et al., 2010). The linear and positive correlation found between N_2O and CO_2 emission rates suggests that denitrification was the main source of N_2O in our system in winter, similarly to other studies (Müller et al., 2002; Mørkved et al., 2006). However, soil conditions suggest that nitrification could also have contributed to N_2O fluxes including: (a) the calculated WFPS values in January and March for both years never exceeded 50%, suggesting a favourable oxic environment for nitrification, (b) an increase in soil NH_4^+ the substrate of nitrification, throughout winter, and (c) the production of NO_3 overwinter and larger N_2O emission than denitrification rates. Furthermore, there was a positive linear correlation between soil NH_4^+ and N_2O emission rates for the two winters. Öquist et al. (2007) indicated denitrification as the main source of N_2O emission in a frozen boreal forest soils, but also proposed nitrification to fuel NO_3^-, thus sustaining denitrification for a prolonged period of time. Clark et al. (2009) reported that both net mineralization and nitrification occur at significant level in agricultural soil down to −2 °C. On the other hand, Mørkved et al. (2006)

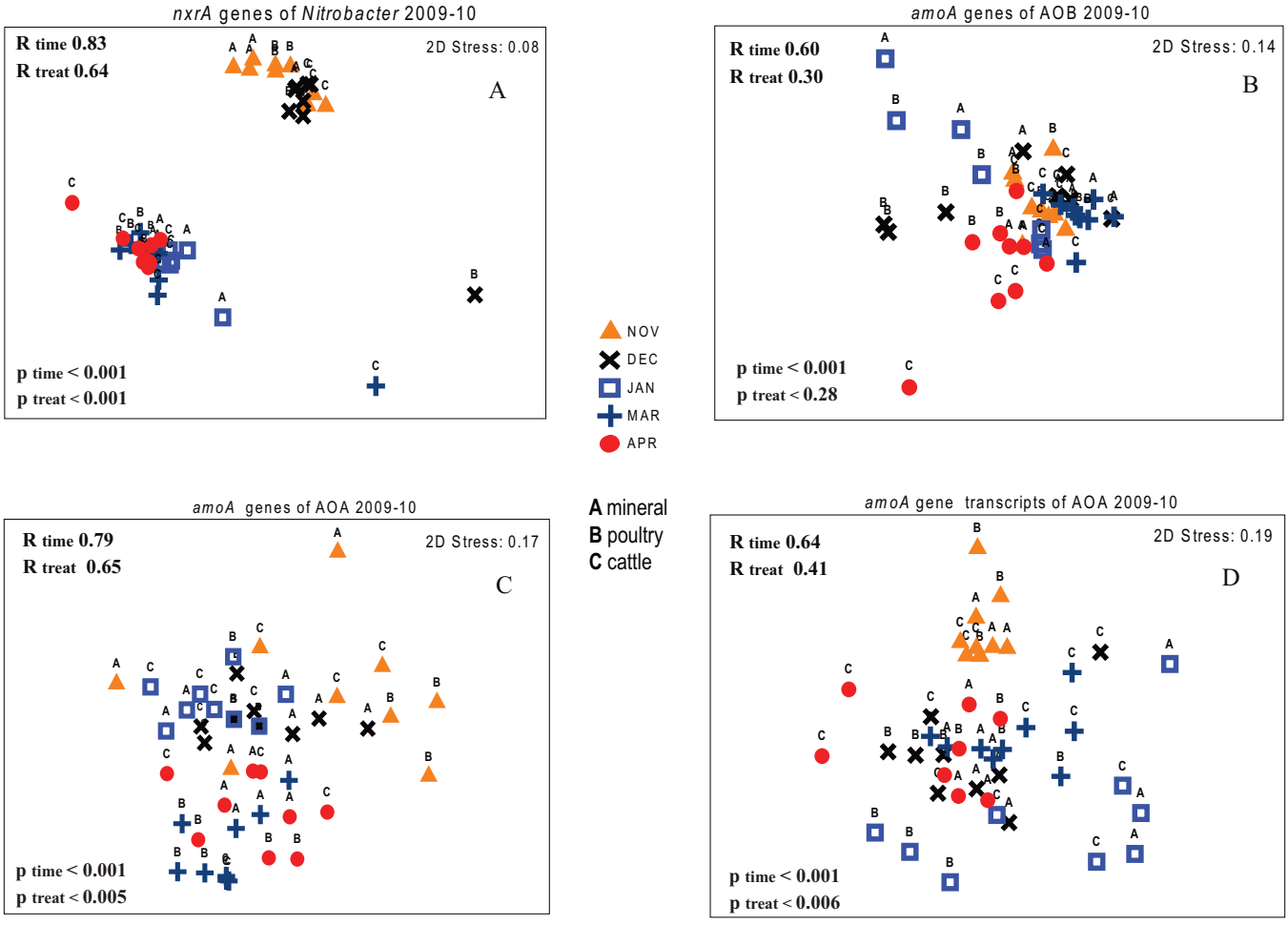

Fig. 6. nMDS representations of *nxrA* (A), AOB (B), AOA (C) and gene transcripts of AOA community structure (D) and winter 2009–2010 obtained by T-RFLP. Similarities or differences among sampling times and treatments are indicated by symbols and letters above symbols, respectively, as described in the legend.

reported nitrification contributed up to 4.4% of total measured N_2O during freeze–thaw in incubated agricultural soil.

Interestingly, overwinter N_2O emission, denitrification, and CO_2 emission rates were not influenced by the different N sources. Generally, most N_2O emissions derived from fertilizer and amendment treatments are concentrated in the first 4–8 weeks following application, with little emissions thereafter (Chantigny et al., 2002; Rover et al., 1998). Therefore, being the N source applied 5 months before investigation of NGS emissions started, it may be hypothesized that the direct effect of N input of N_2O production likely faded out during summer, leaving negligible influence in the fall (Jayasundara et al., 2010).

4.2. Denitrifier and nitrifier abundance and community composition

Abundance of nitrifiers and denitrifiers did not change significantly over the winter 2009–2010, perhaps because the N sources were applied a single time at that site (spring of 2009), which had no former history of manure application, and/or due to winter conditions of 2009–2010 that had colder temperatures in November and April and shorter period with snow cover, thus soil experienced longer exposure to freeze–thawing events compared to the winter of 2010–2011. Abundance of nitrifiers and denitrifiers averaged across treatments in the 2010–2011 winter generally did not change in November, December and January despite presence of freeze–thaws cycles, decreasing temperatures, and freezing soil, which can kill a portion of the microbial biomass. It has been reported that freeze–thaw events lead to the establishment of cold-resistant microorganisms (Groffman et al., 2001; Yanai et al., 2007) thus the growth of the cold adapted microorganisms might have maintained constant the abundance of studied functional groups in November, December and January. *nirK*- and *nirS*-bearing denitrifiers and *nxrA*-bearing nitrifiers abundance increased in March and/or April of winter 2010–2011 probably indicating that the disruption of soil aggregates following freeze–thaw cycles and deep soil freezing may have released aggregate-protected C (Edwards and Cresser, 1992).

Interestingly, denitrifier (*nirS* and *nirK*) and *nxrA*-bearing nitrifier abundances showed a significant effect of N treatment in March and/or April of the winter 2010–2011. The abundance of *nirS*- and *nirK*-bearing denitrifiers increased only in PM treated soil, while *nxrA*-bearing nitrifiers increased in CM-treated soil compared with other N treatments. The greater dry matter content of PM may have favoured the growth of heterotrophic denitrifiers. As some *Nitrobacter* strains have the ability to grow under mixotrophic conditions (Degrange et al., 1997), the abundance of *Nitrobacter*-like NOB in soils treated with CM may have been increased by combination of high N content (greater than in PM) and organic C.

Seasonal variations had a strong impact on the composition of nitrifier and denitrifier populations. According to obtained PERMANOVA and ANOSIM *R* values, community structures of *nirK*- and *nirS*-bearing denitrifiers and of AOA showed significant difference

and clear separation between different sampling times, suggesting that members of these communities may be constantly adapting to the evolving winter conditions. Change in season brings important difference in environmental factors that can shape microbial communities in soil over time. Previous works have reported that the soil microbial community structure was strongly controlled by seasonal dynamics including soil temperature and moisture (Waldrop and Firestone, 2006) rather than overlaying vegetation type or agricultural practices (Cruz-Martinez et al., 2009; Tatti et al., 2012, respectively).

Interestingly, *nxrA*-bearing community structure showed a different response to overwinter conditions in both winters, with a separation in two distinct groups (November–December *versus* January–March–April) suggesting a main community composition shift and the subsequent establishing of a stable cold-adapted population after the onset of frozen-soil conditions. Results from Cookson et al. (2002) indicated that gross nitrification was inhibited at 5 or 7 °C causing an accumulation of NH_4^+however NO_3^- subsequently accumulated suggesting that nitrifying bacteria took longer to acclimatize to the cold conditions than ammonifying microorganisms. Moreover, Wertz et al. (2013) showed a clear shift in *Nitrobacter*–*nxrA* community composition from unfrozen to frozen soil conditions.

The different N treatments clearly influenced nitrifiers and denitrifiers community composition and the changes could be observed on all sampling dates for two consecutive winters with the exception of AOB in winter 2009–2010, where no effect of treatment was revealed on the community. This result suggests that the spring application of different treatments may have diversified organic C content in analyzed plots, thus influencing the denitrifier and nitrifier composition. Moreover, this effect could still be observed overwinter. Other studies reported changes in nitrifier and denitrifier communities after plant residues application in frozen soil microcosm experiment (Wertz et al., 2013) and following different tillage practices but only in certain period of time overwinter in an agricultural field (Smith et al., 2010).

Notably, the data from abundance and composition of AOA and AOB communities suggested a different role of these two functionally equivalent populations during winter. In the two analyzed winters, AOB were always below detection limit: AOB have been reported to be negatively affected by extensive land management (Bru et al., 2011) and the stress caused by cold soil conditions (Su et al., 2010) while AOA have been indicated as more resistant to environmental stress (Valentine, 2007). Moreover, archaeal *amoA* transcripts were measured for two consecutive winters, while bacterial *amoA* mRNA were not detected. This data suggests that the AOA might be actively participating to nitrification overwinter being therefore more significant than AOB in this system. The actual overwinter involvement of archaeal ammonia oxidizers in N cycle in the soil system is supported by the concurrent increase of archaeal *amoA* transcripts and decrease of NH_4^+ level in March and/or April. Considering that mRNA may be extremely labile (Hambraeus et al., 2003; Selinger et al., 2003), the results on the active AOA *amoA* transcript numbers or active populations reflect the situation at the moment of the sampling time and could change significantly over time. However, the AOA *amoA* transcripts were constantly measured at each sampling times for two consecutive years further suggesting that this microbial group is active over winter. In addition, some of the active AOA populations (i.e. obtained T-RFLP peaks 90, 148, 200 and 389 bp) were observed over the five sampling dates and the two winters suggesting that these populations were maintained over the winter.

The analysis of both the present and active AOA populations revealed that 40% of the populations were both present abundantly and active. It may be hypothesized that the AOA present but not active i.e. where *amoA* gene transcripts could not be detected were actually not strong contributors to nitrification possibly due to a poor adaptation to winter conditions. Similarly, in a microcosm study Sharma et al. (2006) reported that the most active *nirS* denitrifiers were not the most abundant ones following a freeze–thaw event. Moreover, some of the most active AOA were not detected on the T-RFLP profiles of present AOA suggesting that these metabolically active AOA were not among the dominating species.

Although some studies suggested a role of abundance or composition of denitrifier and nitrifier communities (Cavigelli and Robertson, 2000; Braker and Conrad, 2011) on N_2O emissions or denitrification, correlations between the biological component and soil process rates remain elusive (Dandie et al., 2011; Henry et al., 2008; Wertz et al., 2013; Smith et al., 2010). In this study, there were no correlations between nitrifier and denitrifier community abundance or composition and N_2O and denitrification. It cannot be ruled out that the limited number of sampling times do not allow a good representation of the N budget over the winter. Smith et al. (2010) measured N_2O emissions continuously and measured changes of communities over winter however there was also no correlation between denitrifier and nitrifier communities and the emissions. These results might not be surprising considering that N_2O emissions and denitrification/nitrification are controlled by environmental conditions (soil moisture, soil pH, temperature and presence of substrates) that will favors these soil processes, resulting in high temporal and spatial variability in emissions (Groffman, 2012). Furthermore, the environmental conditions controlling the abundance and composition of denitrifier and nitrifier communities might differ from the key factors influencing denitrification and nitrification rates and N_2O emissions in most instances. More investigations are required to understand the role of the microbial ecology on N_2O emissions and soil N process rates and the partitioning between the different microbial sources of N_2O under winter conditions.

5. Conclusions

The results of this field study showed that significant N_2O emissions can be measured in the middle of winter in frozen soils. The temporal dynamics N_2O fluxes appeared to increase during winter as NO_3 and NH_4 were gradually accumulating in soils. However, the observed changes in nitrifier and denitrifier community structure let us conclude that the high N_2O fluxes measured in the middle of winter may be caused by the development of cold-adapted microbes. The abundance of nitrifiers and denitrifiers were increased in late winter, especially in soils amended with animal manure in spring, suggesting that organic amendments may further support the development of cold-adapted microbial populations in late winter. However, the application of animal manure did not result in increased N_2O emissions and denitrification in this study, further supporting the idea that temporal dynamics of N_2O emissions during winter was more closely related to the actual composition rather than the abundance of nitrifier and denitrifier populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.agee.2013.10.021.

References

Arenson, L.U., Sego, D.C., 2006. The effect of salinity on the freezing of coarse-grained sands. Can. Geotech. J. 43, 325–337.

Attard, E., Poly, F., Commeaux, C., Laurent, F., Terada, A., Smets, B.F., Recous, S., Roux, X.L., 2010. Shifts between nitrospira- and nitrobacter-like nitrite oxidizers underlie the response of soil potential nitrite oxidation to changes in tillage practices. Environ. Microbiol. 12, 315–326.

Avrahami, S., Conrad, R., 2003. Patterns of community change among ammonia oxidizers in meadow soils upon long-term incubation at different temperatures. Appl. Environ. Microbiol. 69, 6152–6164.

Braker, G., Fesefeldt, A., Witzel, K.P., 1998. Development of PCR primer system for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. Appl. Environ. Microbiol. 64, 3769–3775.

Braker, L., Conrad, R., 2011. Diversity, structure and size of N_2O-producing microbial communities in soils-what matters for their functioning? Adv. Appl. Microbiol. 75, 33–70.

Brooks, P.D., Schmidt, S.K., Williams, M.W., 1997. Winter production of CO_2 and N_2O from alpine tundra: environmental controls and relationship to inter-system C and N fluxes. Oecologia 3, 403–413.

Bru, D., Ramette, A., Saby, N.P.A., Dequidt, S., Ranjard, C., Jolivet, D., Arrouays, L., Philippot, L., 2011. Determinants of the distribution of nitrogen-cycling microbial communities at the landscape scale. ISME J. 5, 532–542.

Cavigelli, M.A., Robertson, G.P., 2000. The functional significance of denitrifier community composition in a terrestrial ecosystem. Ecology 81, 1402–1414.

Chantigny, M.H., Angers, D.A., Rochette, P., 2002. Fate of carbon and nitrogen from animal manure and crop residue in wet and cold soils. Soil Biol. Biochem. 34, 509–517.

Christensen, S., Tiedje, J.M., 1990. Brief and vigorous N_2O production by soil at spring thaw. J. Soil Sci. 41, 1–4.

Clark, K., Chantigny, M.H., Angers, D.A., Rochette, P., Parent, L.E., 2009. Nitrogen transformation in cold and frozen agricultural soil following organic amendments. Soil Biol. Biochem. 41, 348–356.

Cookson, W.R., Cornforth, I.S., Rowarth, J.S., 2002. Winter soil temperature (2–15 °C) effects on nitrogen transformation in clover green manure amended or unamended soil: a laboratory and field study. Soil Biol. Biochem. 34, 1401–1415.

CRAAQ. 2010. Guide de référence en fertilisation. 2nd ed. (In French.) Centre de référence en Agriculture et Agroalimentaire du Québec, Québec, QC, Canada.

Cruz-Martinez, K., Suttle, K.B., Brodie, E.L., Power, M.E., Andersen, G.L., Banfield, J.F., 2009. Despite strong seasonal responses, soil microbial consortia are more resilient to long-term changes in rainfall than overlying grassland. ISME J. 3, 738–744.

Dandie, C.E., Burton, D.L., Zebarth, B.J., Henderson, S., Trevors, J.T., Goyer, C., 2008. Changes in bacterial denitrifier community abundance over time in an agricultural field and their relationship with denitrification activity. Appl. Environ. Microbiol. 74, 5997–6005.

Dandie, C.E., Wertz, S., Leclair, C.L., Goyer, C., Burton, D.L., Patten, C.L., Zebarth, B.J., Trevors, J.T., 2011. Abundance, diversity and functional gene expression of denitrifier communities in adjacent riparian and agricultural zones. FEMS Microb. Ecol. 77, 69–82.

de Bruijn, A.M.G., Butterbach-Bahl, K., Blagodatsky, S., Grote, R., 2009. Model evaluation of different mechanisms driving freeze–thaw N_2O emissions. Agric. Ecosyst. Environ. 133, 196–207.

De Klein, C.A.M., Van Logtestijn, R.S.P., 1996. Denitrification in grassland soils in the Netherlands in relation to irrigation, N-application rate, soil water content and soil temperature. Soil Biol. Biochem. 28, 231–237.

Degrange, V., Lensi, R., Bardin, R., 1997. Activity, size and structure of a *Nitrobacter* community as affected by organic carbon and nitrite in sterile soil. FEMS Microb. Ecol. 24, 173–180.

Dorland, S., Beauchamp, E.G., 1991. Denitrification and ammonification at low soil temperature. Can. J. Soil Sci. 71, 293–303.

Dörsch, P., Bakken, F., 2004. Low-temperature response of denitrification: comparison of soils. Eurasian Soil Sci. 37 (Suppl. 1), S102–S106.

Dörsch, P., Palojärvi, A., Mommertz, S., 2004. Overwinter greenhouse gas fluxes in two contrasting agricultural habitat. Nutr. Cycl. Agroecosyst. 70, 117–133.

Drotz, S.H., Sparrman, T., Nilsson, M.B., Schleucher, J., Öquist, M.G., 2010. Both catabolic and anabolic heterotrophic microbial activity proceed in frozen soil. Proc. Natl. Acad. Sci. U. S. A. 107, 21046–21051.

Edwards, A.C., Cresser, M.S., 1992. Freezing and its effect on chemical and biological proprieties of soil. Adv. Soil Sci. 18, 59–79.

Furon, A.C., Wagner-Riddle, C., Smith, C.R., Warland, J.S., 2008. Wavelet analysis of wintertime and spring thaw CO_2 and N_2O fluxes from agricultural fields. Agric. Forest Meteorol. 148, 1305–1317.

Groffman, P.M., Holland, E., Myrold, D.D., Robertson, G.P., Zou, X., 1999. Denitrification. In: Robertson, G.P., Bledsoe, C.S., Coleman, D.C., Sollins, P. (Eds.), Standard Soil Methods for Long Term Ecological Research. Oxford University Press, New York, pp. 272–288.

Groffman, P.M., Driscoll, C.T., Fahey, T.J., Hardy, J.P., Fitzhugh, R.D., Tierney, G.L., 2001. Colder soil in a warmer world: a snow manipulation study in a northern hardwood forest ecosystem. Biogeochemistry 56, 135–150.

Groffman, P., 2012. Terrestrial denitrification: challenges and opportunities. Ecol. Process 1, 11.

Gupta, S., Munyankusi, E., Moncrief, J., Zvomuya, F., Hanewall, M., 2004. Tillage and manure application effects on mineral nitrogen leaching from seasonally frozen soils. J. Environ. Qual. 33, 1238–1246.

Hambraeus, G., von Wachenfeldt, C., Hederstedt, L., 2003. Genome-wide survery of mRNA half-lives in *Bacillus subtilis* identifies extremely stable mRNAs. Mol. Genet. Genomics 269, 706–714.

Henderson, S.L., Dandie, C.E., Patten, C.L., Zebarth, B.J., Burton, D.L., Trevors, J.T., Goyer, C., 2010. Changes in denitrifier abundance, denitrification gene mRNA levels, nitrous oxide emissions, and denitrification in anoxic soil microcosms amended with glucose and plant residues. Appl. Environ. Microbiol. 76, 2155–2166.

Henry, S., Baudoin, E., Lopez-Gutierrez, J.C., Martin-Laurent, F., Brauman, A., Philippot, L., 2004. Quantification of denitrifying bacteria in *nirK* gene targeted real time PCR. J. Microbiol. Methods 59, 327–335.

Henry, S., Texier, S., Hallet, S., Bru, D., Dambreville, C., Cheneby, D., Bizouard, F., Germon, J.C., Philippot, L., 2008. Disentangling the rhizosphere effect on nitrate reducers and denitrifiers: insight into the role of root exudates. Environ. Microbiol. 10, 3082–3092.

Holtan-Hartwig, L.H., Dörsch, P., Bakken, L.R., 2002. Low temperature control of soil denitrifying communities: kinetics of N_2O production and reduction. Soil Biol. Biochem. 34, 1797–1806.

Jayasundara, S., Wagner-Riddle, C., Parkin, G., Lauzon, J., Fan, M.Z., 2010. Transformation and losses of swine manure ^{15}N as affected by application timing at two contrasting sites. Can. J. Soil. Sci. 90, 55–73.

Johnson, J.M.F., Archer, D., Barbour, N., 2010. Greenhouse gas emission from contrasting management scenarios in the Northern Corn Belt. Soil Sci. Soc. Am. J. 74, 396–406.

Kay, B., Fukuda, M., Izuta, H., Sheppard, M.I., 1981. The importance of water mitgration in the measurement of the thermal conductivity of unsaturated frozen soils. Cold Regions Sci. Technol. 5, 95–106.

Keeney, D.R., Fillery, I.R., Marx, G.P., 1979. Effect of temperature on the gaseous nitrogen products of denitrification in a silt loam soil. Soil Sci. Soc. Am. J. 43, 1124–1128.

Männistö, M.K., Tiirola, M., Häggblom, M.M., 2009. Effect of freeze–thaw cycles to microbial community of arctic tundra soil. Microb. Ecol. 58, 621–631.

Matzner, E., Borken, W., 2008. Do freeze–thaw events enhance C and N losses from soils of different ecosystems? A review. Eur. J. Soil. Sci. 59, 274–284.

Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L., Goyer, C., Trevor, J.T., 2008. Crop residue influence on denitrification, N_2O emissions and denitrifier community abundance in soil. Soil Biol. Biochem. 40, 2553–2562.

Mørkved, P.T., Dörsch, P., Henriksen, T.M., Bakken, L.R., 2006. N_2O emissions and product ratio of nitrification as affected by freezing and thawing. Soil Biol. Biochem. 38, 3411–3420.

Müller, C., Martin, M., Stevens, R.J., Laughlin, R.J., Kammann, C., Ottow, J.C.G., Jäger, H.J., 2002. Processes leading to N_2O emissions in grassland soil during freezing and thawing. Soil Biol. Biochem. 34, 1325–1331.

Öquist, M.G., Petrone, K., Nilsson, M., Klemedtsson, L., 2007. Nitrification controls N_2O production rates in a frozen boreal forest soil. Soil Biol. Biochem. 39, 1809–1811.

Phillips, R.L., 2007. Denitrification at sub-zero temperatures in arable soils: a review. Agron. Sust. Dev. 28, 87–93.

Rover, M., Heinemeyer, O., Kaiser, E.A., 1998. Microbial induced nitrous oxide emissions from an arable soil during winter. Soil Biol. Biochem. 14, 1859–1865.

Selinger, D.W., Saxena, R.M., Cheung, K.J., Church, G.M., Rosenow, C., 2003. Global RNA half-life analysis in *Escherichia coli* reveals positional patterns of transcript degradation. Genome Res. 13, 216–223.

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

Smith, J., Wagner-Riddle, C., Dunfield, K., 2010. Season and management related changes in the diversity of nitrifying and denitrifying bacteria over winter and spring. Appl. Soil. Ecol. 44, 138–146.

Staähli, M., Stadler, D., 1997. Measurement of water and solute dynamics in freezing soil columns with time domain reflectometry. J. Hydrol. 195, 352–369.

Su, M., Kleineidam, K., Schloter, M., 2010. Influence of different litter quality on the abundance of genes involved in nitrification and denitrification after freezing and thawing of an arable soil. Biol. Fertil. Soils 46, 537–541.

Tatti, E., Decorosi, F., Viti, C., Giovannetti, L., 2012. Despite long-term compost amendments seasonal changes are main drivers of soil fungal and bacterial population dynamics on a Tuscan vineyard. Geomicrobiol. J. 29, 506–519.

Throbäck, I.N., Enwall, K., Jarvis, A., Hallin, S., 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbial. Ecol. 49, 401–417.

Treusch, A.H., Leininger, S., Kleztin, A., Schuster, S.C., Klenk, H.P., Schleper, C., 2005. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarcheota in nitrogen cycling. Environ. Microbiol. 7, 1985–1995.

Valentine, D.L., 2007. Adaptations to energy stress dictate the ecology and evolution of Archaea. Nat. Rev. Microbiol. 5, 316–323.

Virkajärvi, P., Maljanen, M., Saarijärvi, K., Haapala, J., Martikainen, P.J., 2010. N_2O emissions from boreal grass and grass-clover pasture soils. Agric. Ecosyst. Environ. 137, 59–67.

Wagner-Riddle, C., Thurtell, G.W., King, K.M., Kidd, G.K., Beauchamp, E.G., 1997. Nitrous oxide and carbon dioxide fluxes from a bare soil using a micrometeorological approach. J. Environ. Qual. 25, 898–907.

Wagner-Riddle, C., Rapai, J., Warland, J., Furon, A., 2010. Nitrous oxide fluxes related to soil freeze thaw periods identified using heat pulse probes. Can. J. Soil Sci. 90, 409–418.

Waldrop, M.P., Firestone, M.K., 2006. Seasonal dynamics of microbial community composition and function in oak canopy and open grassland soils. Microb. Ecol. 52, 470–479.

Walker, J.K.M., Egger, K.N., Henry, G.H.R., 2008. Long-term experimental warming alters nitrogen-cycling communities but site factors remain the primary drivers of community structure in high arctic tundra soils. ISME J. 2, 982–995.

Wertz, S., Goyer, C., Zebarth, B.J., Burton, D.L., Tatti, E., Chantigny, M.H., Filion, M., 2013. Effects of temperatures near the freezing point on N_2O emissions, denitrification and on the abundance and the structure of nitrifying soil communities. FEMS Microbiol. Ecol. 83, 242–254.

Yanai, Y., Toyota, K., Okazaki, M., 2007. Response of denitrifying communities to successive soil freeze–thaw cycles. Biol. Fertil. Soils 44, 113–119.

Yergeau, E., Kowalchuk, G.A., 2008. Responses of Antarctic soil microbial communities and associated functions to temperature and freeze–thaw cycle frequency. Environ. Microbiol. 10, 2223–2235.