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Season and management related changes in the diversity of nitrifying and denitrifying bacteria over winter and spring

Jillian Smith, Claudia Wagner-Riddle, Kari Dunfield *

Land Resource Science, Ontario Agricultural College, University of Guelph, Guelph, Canada

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ABSTRACT

This study assessed the effects that season and tillage practices have on the diversity of nitrous oxide producing bacteria (nitrifiers and denitrifiers) and to relate this to measured N_2O fluxes at our field site. Large-scale field plots (1.5 ha) were established in Elora, Ontario in 2000, and managed using conventional tillage (CT) or no-tillage (NT). Each field plot was instrumented with micrometeorological equipment to determine N_2O fluxes on a field scale. Soil samples were taken at four time points between the fall of 2005 and the spring of 2006. The diversity of the nitrifier and denitrifier communities was assessed by PCR–denaturing gradient gel electrophoresis (DGGE) using primer pairs targeting the *amoA*, *nirS* and *nirK* gene. Seasonal variation (a combination of soil temperature, available soil moisture, nutrient levels and other potential factors) had the largest influence on the diversity of nitrifier and denitrifier populations; while tillage practice also influenced the diversity of the microbial community at certain time periods. Tillage significantly affected all communities in March and affected denitrifiers on all other dates except for the *nirS* community in February. Further statistical analysis revealed that diversity of the nitrifying and denitrifying populations was the lowest in February, in frozen soils, and rapidly increased in March, corresponding with spring thaw N_2O emissions. Long-term soil nutrient, temperature and N_2O data taken at this site added additional information on the dynamics of the nitrogen cycle.

1. Introduction

Nitrous oxide (N_2O) is emitted from soil as a byproduct of microbial nitrification and denitrification processes (Firestone and Davidson, 1989). While this is a key component of the global N cycle, it is also a concern since N_2O is an important greenhouse gas that also contributes to the destruction of the ozone layer (Cicerone, 1989). In addition, atmospheric N_2O concentration is estimated to be increasing at a rate of 0.25% per year (IPCC, 2001). Agricultural sources account for approximately 90% of the total anthropogenic emissions of N_2O (Duxbury et al., 1993), therefore, agricultural management practices play a role in the amount of N_2O released from a field site. In a study collating data on N_2O emissions under various practices (e.g., crop rotation, N fertilization, manure application, tillage) in Canada, non-manured soils exhibited a linear relationship between the amount of N fertilizer applied and N_2O emissions (Helgason et al., 2005). Furthermore, the authors described a high degree of uncertainty in whether no-till practices reduce N_2O emissions. About half (56%) of the studies indicated lower emissions from no-till (NT) systems, while another 40% indicated higher emissions with no-till (Helgason et al., 2005). Similar uncertainty has been observed in studies outside of Canada (Aulakh et al., 1984; Ball et al., 1999; Bossio et al., 1998; do Carmo et al., 2005; Stres et al., 2004). Generally, a combination of climate, crop history, seasonal variability and agricultural management practice all play a role in the amount of N_2O that is emitted from an agricultural field.

Our field site at the University of Guelph's Elora Research station in Ontario Canada has been studied through micrometeorological methods, and ^{15}N tracer studies. In a long-term assessment measuring N_2O emissions over a 5-year period (January 2000 to April 2005), Wagner-Riddle et al. (2007), determined that using best management practices that included no-tillage and a reduced fertilizer application, decreased N_2O emissions significantly compared to a conventional management practice. Importantly, non-growing season (November–April) emissions were found to account for between 30 and 90% of the annual emissions, occurring mostly during spring thaw. The no-tillage treatment reduced N_2O emissions during thaw by reducing winter soil freezing due to the insulating effects of a larger snow cover when crop stubbles were present, and accounted for 80% of total reduction or 0.63 kg N ha^{-1} y^{-1} averaged over the 5 years (Wagner-Riddle et al., 2007). A follow-up study examining the

* Corresponding author at: Land Resource Science, University of Guelph, 50 Stone Rd. E, Guelph, ON, N1G 2W1, Canada. Tel.: +519 824 4120x58088; fax: +519 824 5730.
E-mail addresses: dunfield@uoguelph.ca, kdunfield@gmail.com (K. Dunfield).

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mechanism of N_2O production during spring thaw determined that it was primarily due to the denitrification process. ^{15}N tracer studies determined that was primarily new N_2O production in the surface thawed layer, rather than the release of trapped N_2O in unfrozen soil layers beneath the frozen soil (Wagner-Riddle et al., 2008). Despite the prior conclusions that alterations in denitrification patterns between conventional tillage (CT) and NT fields were responsible for differences in the spring thaw emissions of N_2O, no prior microbial characterization has been undertaken at this site. Studies have shown that the composition of the nitrifying and denitrifying communities is important in regulating the N_2O flux from soil and other ecosystem functioning (Avrahami and Conrad, 2005; Carney et al., 2004; Cavigelli and Robertson, 2000; Cavigelli and Robertson, 2001; Holtan-Hartwig et al., 2000; Munch, 1989). Therefore, it would be reasonable to assume that differences in N_2O emissions at our study site would be associated with different community structure. Tillage can have a significant effect on the soil microbial population. No-till systems can improve the soil habitat for microorganisms, because of increased carbon levels and physical properties of the soil (Kladivko, 2001). Furthermore, soil microbial communities including denitrifiers are known to fluctuate seasonally (Bossio et al., 1998; Bremner et al., 1980; Mergel et al., 2001b; Smit et al., 2001; Wolsing and Priemé, 2004). A change in diversity of nitrifiers and denitrifiers over season or tillage treatment may alter N cycling in soils including the emission of N_2O.

In the current study, micrometeorological nitrous oxide flux measurements were taken over winter (October 2005 to May 2006) in a field study where corn had been grown in the previous growing season under contrasting tillage practices. Using PCR–DGGE analysis we assessed the diversity of the nitrifiers and denitrifiers using functional primers targeting the *amoA* gene for nitrifiers and the *nirS* and *nirK* gene for denitrifiers. The objective of the present study was to investigate changes in the composition of nitrifiers and denitrifiers caused by two different tilling systems over winter and spring. A second objective was to study the composition of denitrifiers and nitrifiers as related to nitrous oxide emissions over winter and spring. It was hypothesized that CT and NT soils will provide different habitats for nitrifiers and denitrifiers and thus lead to significant differences in diversity and community structure. Variation in environmental factors over the winter and spring was also expected to affect diversity and community structure.

2. Materials and methods

2.1. Site description

The field site is located at the University of Guelph's Elora Research Station (ERS) (43°39′N 80°25′W, 376 m elevation), Ontario, Canada. The soil is mapped as an imperfectly drained Guelph silt loam (Canadian Agricultural Services Coordinating Committee, 1998) with an average pH of 7.6 (water), organic carbon of 27 g kg^{-1}, total N of 2 g kg^{-1}, available P of 24 mg $kg^{-1}$and available K of 146 mg kg^{-1}over the 0–15 cm layer. The experimental design was four 100 m × 150 m (1.5 ha) plots, two managed with CT (Plots 1 and 4) and two managed with NT (Plots 2 and 3). The CT treatment included moldboard ploughing in the fall and disking in the spring, and NT received no-tillage. All plots were planted with soybeans in May 2004 and corn in May 2005, no fertilizer was applied in 2004, all plots received the same fertilizer application (urea broadcasted at 150 kg N ha^{-1}) at planting in 2005. The NT plots have been under a best management treatment since 2000, which included a no-tillage treatment, an N fertilizer rate based on a soil N test, and the inclusion of a soil cover crop when possible. The BMP plots were last tilled in May 1999 (Wagner-Riddle et al., 2007). A full description of the timing of different management practices in the 2 systems is given in Jayasundara et al. (2007).

2.2. Soil sampling

Samples were taken on four dates throughout 1 year corresponding to post-tillage, pre-spring thaw, post-spring thaw and spring (October 18/05, February 14/06, March 14/06 and May 16/06, respectively). Due to the large size of the plots, six sampling locations were identified on a grid system, and were maintained throughout the study. Therefore a total of 24 locations (4 plots × 6 locations) were sampled. At each of the six sampling locations within each plot, 5 cores were taken to a 10 cm depth and bulked into one composite sample to reduce spatial variability. The composite soil samples were passed through a 4-mm sieve at field moist conditions. A 0.5 g sub-sample was taken and DNA was extracted within 24 h for molecular analysis and the remaining soil sample was stored at −20 °C.

2.3. Soil supporting data

Soil temperature profiles were measured with thermistors (Model#107, Campbell Scientific Inc., Edmonton, AB) inserted at a 5 cm depth (2 probes per depth per plot), and hourly mean temperatures were recorded with a datalogger (21X, Campbell Scientific Inc., Edmonton, AB). Soil water content was measured using reflectometers (Model #CS615, Campbell Scientific, Logan, UT) over the 0–10 cm depth (2 probes per depth per plot).

Soil exchangeable NH_4 and NO_3-N concentrations were determined using a 15 g wet sub-sample from the 0 to 10 cm depth at each sampling location, and extracting with 50 mL 2 M KCl and shaking for 1 h (Keeney and Nelson, 1982).

2.4. Micrometeorological nitrous oxide flux measurements

The N_2O fluxes from the CT and NT plots during the experimental period were measured using a flux-gradient approach (Wagner-Riddle et al., 1996, 2007). Average half-hourly N_2O concentration differences between two sampling heights were obtained for each plot with a tunable laser gas analyzer (TGA 100, Campbell Scientific Inc.). The vertical fluxes of N_2O (F_{N_2O}) from the four plots were calculated using the flux-gradient method, described by the following equation in Wagner-Riddle et al. (2007):

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

where u_* is the friction velocity, k is the von Karman constant (=0.41), ΔC is the nitrous oxide concentration difference between heights z_2 and z_1, d is the displacement height, and ψ_{h_2} and ψ_{h_1} are the integrated Monin–Obukhov similarity functions for heat for both sampling heights. Cup and sonic anemometers were used to obtain the friction velocity and integrated similarity functions for heat for both sampling heights.

Data were filtered according to criteria detailed in Wagner-Riddle and Thurtell (1998) and then used in Eq. (1) to calculate half-hourly nitrous oxide flux for each plot. Due to the sequential air sampling setup used, a maximum of 12 half-hourly flux values were calculated for each plot per day. Daily flux means for each plot were obtained by averaging half-hourly values collected during a day (minimum of two half-hourly values). Mean daily flux for each treatment were compared using a t-test.

2.5. DNA extraction

Total DNA from the soil samples (0–10 cm depth) was extracted according to the manufacturer's protocol using the UltraClean Soil

DNA Isolation Kit (MoBio, Carlsbad, CA, USA). The presence of DNA was confirmed by electrophoresis on a 0.7% agarose gel stained with ethidium bromide. The DNA was stored at −80 °C.

2.6. Polymerase chain reaction (PCR) amplification

A fragment of the *amoA* gene was amplified using primer pairs 1F-2R (Rotthauwe et al., 1997). Fragments of the *nirK* and the *nirS* genes were amplified using degenerate primer pairs F1aCu-R3Cu and cd3aF-R3cd, respectively (Throbäck et al., 2004). Each set of primers contained a GC clamp on the 5′ end of the reverse primer to avoid complete denaturing in the DGGE analysis. The amplification mixtures (final volume 50 μl) contained 5 μl of 5× Buffer, 2.5 mM $MgCl_2$, 100 μM of each deoxynucleoside triphosphate, 10 pmol of forward primer, 10 pmol of reverse primer, and 1.25 U of *Taq* DNA polymerase (Promega, Madison, WI, USA) and 1 μl of extracted sample DNA.

The PCR was performed with an automated thermal cycler (Mastercycler Epgradient S, Eppendorf, Hamburg, Germany). The *amoA* fragments were amplified using the following PCR conditions: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 46 s, annealing at 55 °C for 60 s and extension at 72 °C for 90 s. Cycling was completed at 72 °C for 15 min. The *nirS* and *nirK* fragments were amplified using a touchdown PCR: an initial denaturation step at 94 °C for 5 min, followed by 7 cycles of denaturation at 94 °C for 45 s, annealing gradient of 61–57.5 °C for 60 s, and extension at 72 °C for 90 s. This was then followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 59 °C for 60 s and extension at 72 °C for 90 s. Cycling was completed at 72 °C for 15 min.

PCR products were visualized under UV light on a 1% agarose gel stained with ethidium bromide to ensure that the amplified DNA was the right size for the targeted sequence and were approximately the same concentration.

2.7. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed with a D-Code system (BioRad, Hercules, CA, USA) for all PCR products as described in Muyzer et al. (1993). DGGE gels containing 8% acrylamide were poured with a gradient of 30–70% denaturant. The gel was left to set for 10 min and then a 0% denaturant stack was poured above this gel to minimize denaturing of the PCR product while in the wells. The PCR product was added to the wells in the stack (30 μl per well) and run at 20 mV until the temperature reached 65 °C and then the gels were run at 75 mV for 17 h. The gels were stained with 0.02× SYBR Green for 15 min, UV illuminated and digitally photographed using GeneSnap (Syngene, Cambridge, UK). These digital images were then imported into GeneTools (Syngene, Cambridge, UK), for analysis.

3. Statistical analysis of DGGE profiles

3.1. Multivariate analysis

The images of the DGGE gels were analyzed with GeneTools software (Syngene, Cambridge, UK) and corrected visually to eliminate marks on the gel that the software identified as bands. The corrected DGGE profiles from GeneTools (Syngene, Cambridge, UK) were imported into GeneDirectory (Syngene, Cambridge, UK) and compared to each other based on clustering via the unweighted pair group method with mathematical averages (UPGMA: Dice coefficient of similarity). The position tolerance was set at 3%. For each time period a similarity matrix was created in GeneDirectory (Syngene, Cambridge, UK). This matrix was analyzed with non-metric multidimensional scaling (MDS) in Primer v.6 (Primer-E Ltd., Plymouth, UK). The MDS algorithm constructs a map with points whose distances have the same rank order as the corresponding matrix. A 2D stress value is created, which indicates how faithfully the high dimensional relationships among the samples are represented in two-dimensional space. A stress value closest to 0 indicates excellent representation of the points in 2D, whereas a stress value greater than 0.3 indicates that the placement of the points on the plot is close to arbitrary.

Microbial community profiles were statistically analyzed for differences due to tillage, time period, and for tillage by time period interactions using analysis of similarity (ANOSIM) (Primer v.6, Primer-E Ltd., Plymouth, UK). An ANOSIM value (R) ranges between 0 and 1; with 0 indicating no differences in community structure, and 1 indicating complete difference in community structure.

3.2. Univariate analysis

The population diversity of the microbial community was examined by the Shannon's index of general diversity ($H' = -\sum(ni/N)\log(ni/N)$), Margalef's richness index ($d = (S-1)/\log(N)$) and Pielou's evenness index ($J = H'/\log(S)$). H', d and J were calculated on the basis of densitometric curves created by GeneTools (Syngene, Cambridge, UK) in which the intensity of the band is transformed into a peak on the curve, as described in (Costa et al., 2006). Therefore H', d and J are calculated by knowing the peak height of each individual band (ni) and the total peak heights of all bands in the track (N) as well as the total number of bands (S). Variance analysis was performed using proc mixed repeated measures in the SAS statistical package v.9.1 (SAS Institute, Cary, NC) to determine variation due to time, tillage treatment and tillage treatment within each time period. Means comparison were done using Tukey's adjustment. A type II error level of 0.05 was used throughout the study. Although the use of diversity indices with DGGE studies has been contested (Bent et al., 2007) our results showed the same patterns whether we used diversity indices or just a count of the number of bands so we felt very confident in using diversity indices to describe our results. The purpose here is to have a comparative measure of diversity, not an absolute measure.

4. Results

4.1. N_2O emissions and soil data

Micrometeorological N_2O flux measurements indicated that there was a flush of N_2O that was higher in CT plots during a spring thaw event between our two sampling dates of February 14th, 2006 and March 14th, 2006 (Fig. 1A), however, immediately after sampling, N_2O flux measurements were higher in the NT plots. An additional thaw related N_2O event was also observed in early January for CT before winter soil sampling. Large N_2O emissions were not seen in the fall 2005, however, there was more N_2O emitted from NT plots between October 2005 and January 2006. During the entire sampling period, higher accumulated N_2O emissions were observed from soils under CT compared to NT (486.8 ng N m^{-2} s^{-1} vs. 211.1 ng N m^{-2} s^{-1}) and during the spring thaw sampling period (158.3 ng N m^{-2} s^{-1} vs. 92.5 ng N m^{-2} s^{-1}) (Fig. 1A).

Soil NH_4^+-N was low in October (0.18 and 0.91 mg kg^{-1} soil for CT and NT, respectively) and built up over winter with the highest concentrations at the March sampling point (3.31 and 5.76 mg kg^{-1} soil for CT and NT, respectively) and then became nearly undetectable in May (Fig. 1B). Significantly more NH_4^+-N was present in the NT plots, in all but the May sampling date. In contrast, soil NO_3^--N levels decreased over winter and built up in the late spring (Fig. 1C). During the May sampling period there

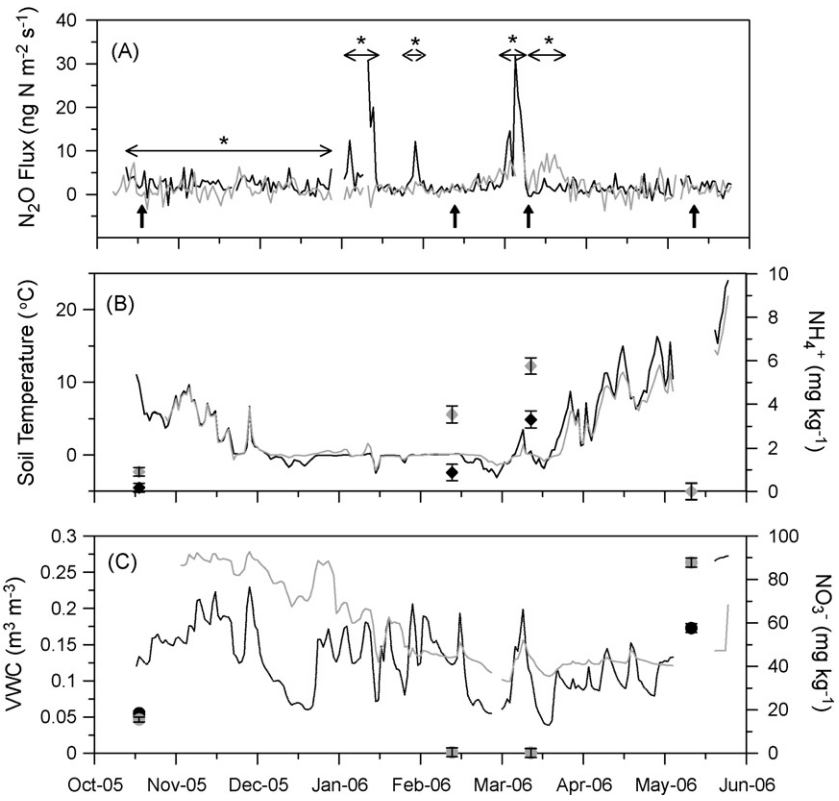

Fig. 1. Fluxes and environmental conditions for the plots subjected to conventional tillage (black) and no-tillage (grey) from October, 2005 to May 2006: (A) mean daily N_2O flux, (B) mean daily soil temperature at the 5 cm depth, and soil NH_4^+-N (mg kg^{-1}) (◆), (C) mean daily volumetric water contents over the 0–10 cm depth, and soil NO_3^--N (mg kg^{-1}) (●). *Periods where the mean daily flux is significantly different in CT and NT treatments ($P < 0.05$). Arrows indicate date of sampling, October, 18/05, February 14/06, March 14/06 and May 16/06. Bars indicate the standard error of the means.

were differences in the amount of nitrate present between CT (57.6 mg kg^{-1} soil) and NT (87.9 mg kg^{-1} soil) plots.

The soil temperature under CT and NT systems differed significantly where CT plots had significantly lower temperatures between December 8 to January 14, and February 19 to March 7, and significantly higher temperatures after March 25 (Fig. 1B). Soil temperatures under CT changed more quickly and to a larger degree than the soil temperatures in NT plots. Variation of soil temperatures was seen during the spring thaw sampling period with daily mean temperatures ranging between −3.1 and +3.5 °C in CT plots and between −1.4 and +1.3 °C in NT plots. The lower winter soil temperatures in CT systems are also reflected by lower levels of liquid water during the winter, due to more freezing in these plots (Fig. 1B and C). Liquid volumetric water content (VWC) was highest in the fall and lowest during the winter with an average of 0.125 m^3 m^{-3} for CT and 0.174 m^3 m^{-3} for NT over the entire sampling period and an average of 0.103 m^3 m^{-3} for CT and 0.126 m^3 m^{-3} for NT over the spring thaw period and an average of 0.142 m^3 m^{-3} for CT and 0.252 m^3 m^{-3} for NT over the fall period (Fig. 1C).

4.2. Seasonal impacts on community composition of nitrifiers and denitrifiers

The *nirS* and *nirK* genes were successfully amplified from denitrifier communities for all sampling dates from the majority of soil samples ($n = 71/96$ for *nirS* and $n = 89/96$ for *nirK*). Bacteria containing the *amoA* gene fragments were also successfully amplified for October, March and May soil samples ($n = 66/72$), but we were not able to amplify any nitrifiers for the February sampling date. All positive PCR samples were separated using DGGE, gels were scored and analysed by UPGMA (Fig. 2).

When comparing DGGE banding patterns from all sampling dates, UPGMA and MDS plots revealed seasonal clustering for all genes tested (Figs. 2 and 3). A two-way crossed analysis of similarity (ANOSIM) was used along with MDS plots to test for variation due to season. The global *R*-values for season were 0.52 ($P = 0.01$) for *amoA*, 0.50 ($P = 0.01$) for *nirS* and 0.35 ($P = 0.01$) for *nirK*. The closer the *R*-value is to 0 indicates more similarity between communities, therefore, the analysis showed significant seasonal effects on community structure for each community. Ecological indices also indicated that season had a significant impact on diversity (*H*) and richness (*d*) for all genes tested (Tables 1 and 2). The lowest diversity and richness for *nirS* and *nirK* occurred in the mid-winter, pre-thaw (February) sampling. Between February and March, pre- and post-spring thaw, were the most significant changes seen in the community structure. ANOSIM values comparing February and March were 0.63

Table 1
Means of indices for diversity (*H′*), richness (*d*) and evenness (*J*) for the nitrifying community (*amoA* gene) on sample dates from October 2005 to May 2006 for conventional tillage (CT) and no-tillage (NT) treatments.

Gene	Date	Trt	*H′*	*d*	*J*
amoA	October	CT	0.24 *c*	0.50 *c*	0.86 *b*
amoA	February	CT	*	*	*
amoA	March	CT	1.13 *a*	0.35 *c*	0.76 *b*
amoA	May	CT	1.23 *ab*	1.01 *ab*	1.69 *ab*
amoA	October	NT	0.37 *c*	0.59 *bc*	1.10 *ab*
amoA	February	NT	*	*	*
amoA	March	NT	0.91 *b*	0.57 *bc*	0.95 *b*
amoA	May	NT	1.30 *a*	1.18 *a*	1.81 *a*

Means followed by the same letter within one column for both treatments together are not significantly different according to Tukey's, $P < 0.05$. $n = 12$ maximum.
*Could not be detected.

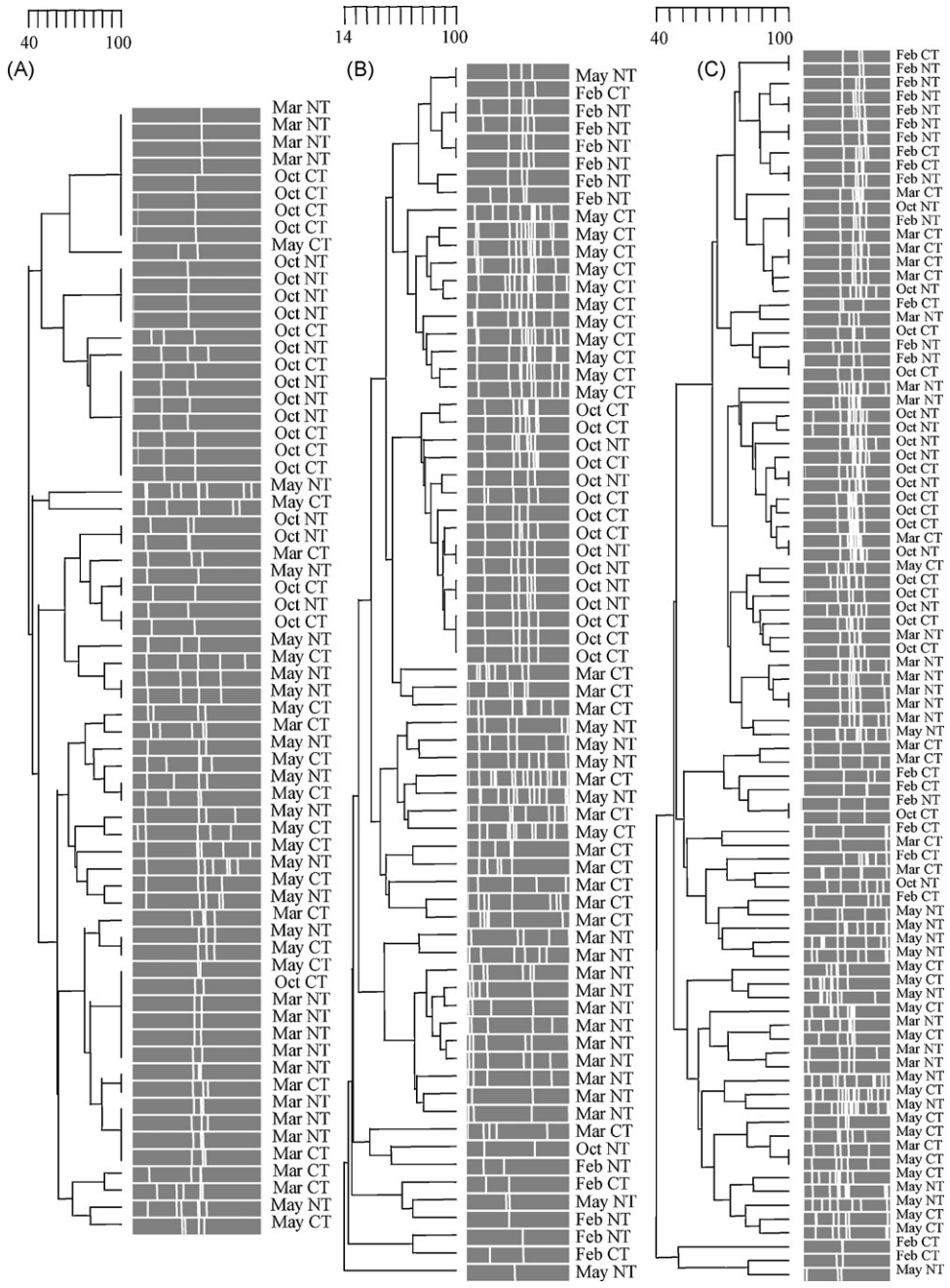

Fig. 2. UPGMA dendrograms of DGGE profiles of PCR-amplified *amoA* (A), and *nirS* (B) and *nirK* (C) gene fragments of bacterial communities from soils under CT and NT from October, February, March and May sampling dates. Each lane is a digitized DGGE profile produced by GeneTools software (Syngene). Scale bar shows percent similarity.

($P = 0.01$) for *nirS* and 0.25 ($P = 0.01$) for *nirK* indicating a significant change in denitrifying community structure during this time. No *amoA* could be detected in the February sampling. We assume that the abundance of this community was below the detection limit, however, by March, a community was again detected (Table 1).

4.3. Tillage impacts on community composition of nitrifiers and denitrifiers

Multivariate analysis showed a significant difference in community structure of nitrifiers associated with tillage over all sampling dates ($R = 0.121$, $P = 0.04$). The most obvious variation in diversity and community structure due to tillage for the *amoA* gene was in March where the ANOSIM value was 0.66 ($P = 0.01$) (Fig. 4B). The diversity (H) was also significantly different between the two treatments at this point (Table 1).

We also found a significant effect of tillage on the *nirS* denitrifier community structure over all sampling dates ($R = 0.38$; $P = 0.01$). When examining sampling dates individually we found significant variation in the *nirS* community due to tillage treatment in October (Fig. 4D), March (Fig. 4F), and May (Fig. 4G) but not February (Fig. 4E). Ecological indices revealed a significant difference in richness (d) due to tillage in May ($P \leq 0.05$) (Table 2), but no other differences between communities.

Tillage also had a significant impact on the structure of the *nirK* denitrifier community ($R = 0.19$, $P = 0.01$). Although the R-value was significant, it was low, likely an indication of substantial overlap in the individual denitrifying community between the conventional tilled and no tilled communities, as seen in the

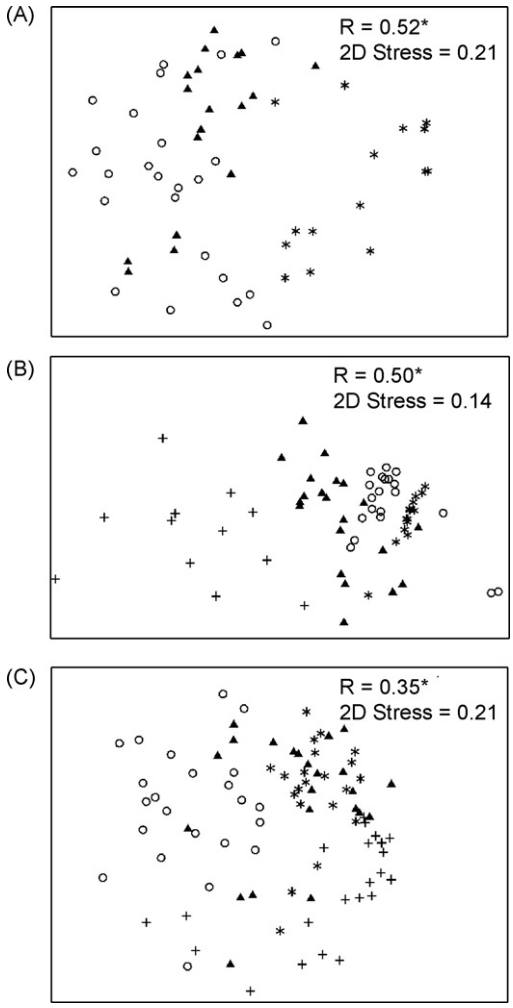

Fig. 3. Multidimensional scaling plots of all time periods for *amoA* (A), *nirS* (B) and *nirK* (C). Each point on the plot represents the community structure at one of the sample locations (CT and NT) from either October (*), February (+), March (▲) or May (○). The 2D stress values and the ANOSIM values (*R*) are indicated. *The *R*-value is significant ($P < 0.01$).

dendrogram presented in Fig. 2. Multivariate analysis indicated that all time periods for denitrifiers showed some degree of variation of community structure due to tillage treatment with *R*-values ranging from 0.35 in May to 0.53 in October (Fig. 4H–K). However, ecological indices of the communities did not differ due to tillage (Table 2).

Table 2
Means of indices for diversity (H'), richness (d) and evenness (J) for the denitrifying community on sample dates from October 2005 to May 2006 for conventional tillage (CT) and no-tillage (NT) treatments.

Gene	Date	Trt	H'	d	J
nirS	October	CT	1.66 *ab*	2.01 *ab*	2.17 *a*
nirS	February	CT	0.75 *d*	0.58 *c*	2.20 *a*
nirS	March	CT	1.54 *abcd*	1.75 *bc*	1.99 *a*
nirS	May	CT	2.08 *a*	2.98 *a*	2.12 *a*
nirS	October	NT	1.43 *abcd*	1.72 *bc*	2.07 *a*
nirS	February	NT	0.96 *cd*	0.95 *c*	1.60 *a*
nirS	March	NT	1.45 *bcd*	1.48 *bc*	1.89 *a*
nirS	May	NT	1.66 *abc*	1.86 *bc*	2.23 *a*
nirK	October	CT	1.47 *ab*	1.73 *abcd*	2.08 *a*
nirK	February	CT	1.07 *b*	1.21 *d*	2.12 *a*
nirK	March	CT	1.44 *ab*	1.68 *bcd*	2.18 *a*
nirK	May	CT	1.59 *ab*	2.23 *abc*	2.04 *a*
nirK	October	NT	1.78 *a*	2.35 *ab*	2.15 *a*
nirK	February	NT	1.14 *b*	1.18 *cd*	2.06 *a*
nirK	March	NT	1.55 *ab*	1.95 *abcd*	2.08 *a*
nirK	May	NT	1.77 *a*	2.58 *a*	2.07 *a*

Means followed by the same letter within one column for both treatments together are not significantly different according to Tukey's, $P < 0.05$. $n = 12$ maximum.

5. Discussion

Seasonal variation has been shown to affect microbial communities. Mergel et al. (2001b) found peaks of denitrifying bacteria numbers in the autumn and winter/early spring and cited freezing/thawing as a contributing factor. Specifically, Wolsing and Priemé (2004) used T-RLFP to reveal that *nirS* and *nirK* denitrifiers showed a significant seasonal shift from spring to fall in an agricultural soil and attributed this to the combined effect of abiotic seasonal changes. Multiple factors such as soil moisture and temperature differed between our sampling dates, and are known to affect community diversity. Avrahami and Bohannan (2007) found that the abundance of *amoA* ammonia oxidizers increased with increasing soil moisture (30–60%) and decreased with increasing temperature (20–30 °C). In a laboratory study, Avrahami et al. (2003) and Avrahami and Conrad (2005) used DGGE of *amoA* ammonia oxidizers to find that the lowest diversity occurred at high (30 °C) and low (4 °C) temperatures. Nutrient availability in spring and fall was related with an increase in total bacterial community diversity at these times and a decrease in diversity in July was seen due to warm, dry and nutrient limited conditions (Smit et al., 2001).

In our study we saw changes in nitrifying bacteria as well as changes in nutrient levels. The byproduct of organic matter mineralization is ammonium, however, in general very little ammonium builds up in an agricultural soils because it is quickly converted to NO_2^- and NO_3^- by the nitrification process. However, it has been observed that the conversion of soil organic N to ammonium (NH_4^+-N) occurs more readily than subsequent nitrification at soil temperatures below 10 °C (Campbell and Biederbeck, 1972; Cookson et al., 2002; Emmer and Tietema, 1990), therefore NH_4^+-N often builds up in the soil over winter. Soil N analysis showed that NH_4^+-N, remained near zero during the October and May sampling points when soil temperatures were above 10 °C, but were detectable at low levels in the soil in the February and March sampling dates, when temperatures were colder (Fig. 1). We were not able to amplify the *amoA* gene from our February sampling date, suggesting that the community of nitrifiers was sufficiently low during this period that there was not enough DNA template for PCR amplification to work effectively (Table 1). The higher levels of ammonium in the winter sampling points may be partially associated with the low levels of nitrifying bacteria detectable at this time.

It has been suggested that nitrifying bacteria gradually acclimatize to fluctuating soil temperatures in late fall, an initial kill of cells occurs with sub-zero temperatures, followed by population growth of adapted microorganisms (Cookson et al., 2002). Subsequently, as they adapt to new conditions, changes in the composition and function of the microbial population may occur. Fatty acid biomarker analysis indicated that total community composition did change over a period of decreasing temperatures (25–5 °C) (Zogg et al., 1997). Our data showed that the community composition of nitrifiers changed significantly over the winter (Fig. 3A). Similarly, indices for diversity, evenness and richness all decreased over winter between October and February, and then increased again between the March and May samplings (Table 2).

Once acclimatized, the community of nitrifiers may be active at soil temperature as low as 0 °C (Savard et al., 2007). In an earlier ^{15}N labelled fertilizer tracer study at our field site, Jayasundara et al. (2007) showed that nitrification progressed slowly over the winter, producing NO_3^- that is almost immediately lost from the

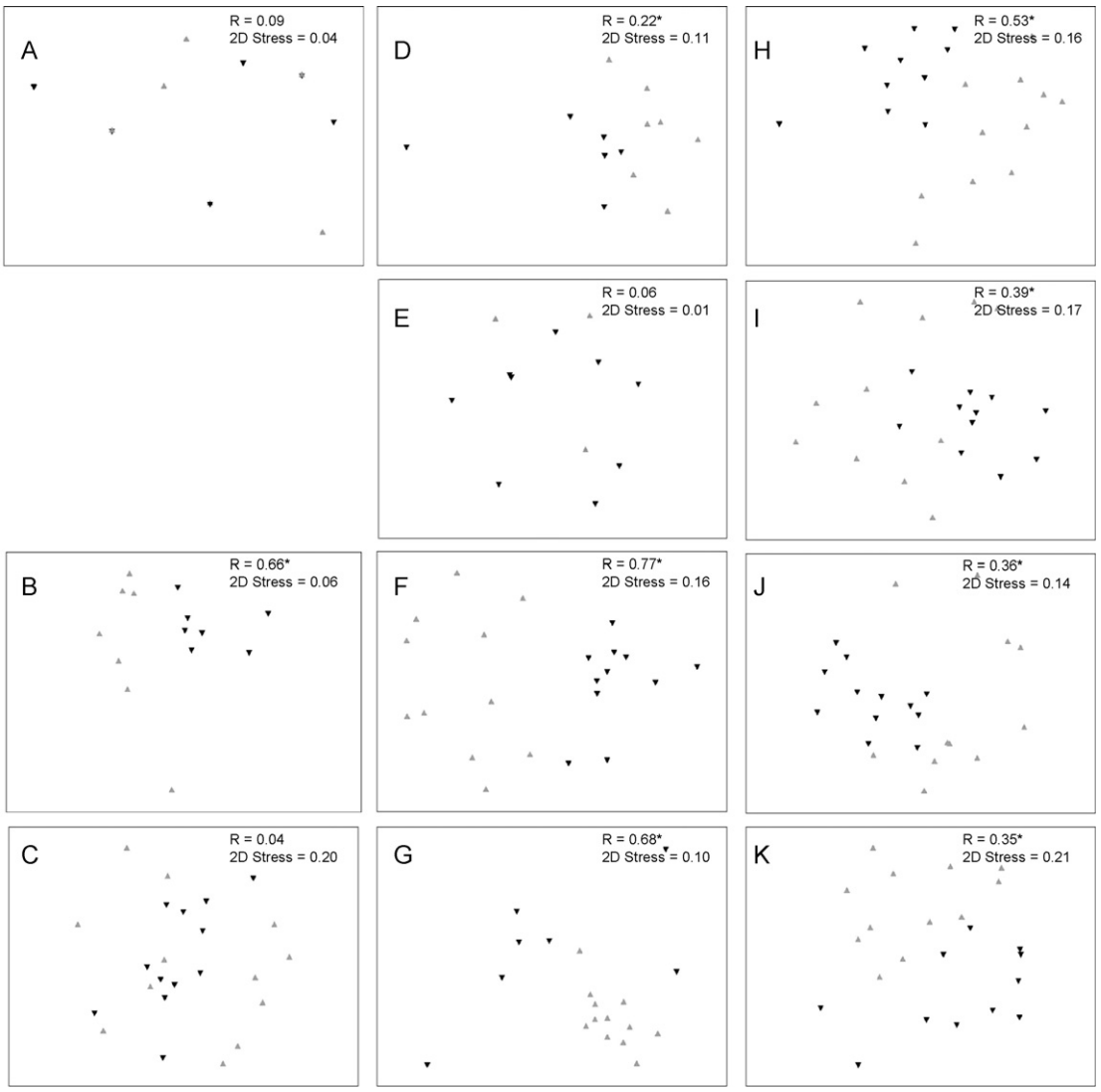

Fig. 4. Multidimensional scaling plots of October (A, D, H), February (E, I), March (B, F, J), and May (C, G, K) time periods for *amoA* (A–C), *nirS* (D–G) and *nirK* (H–K). Each point on the plot represent the community structure at either a CT (▼) or NT (Δ) sampling location. The 2D stress values and the ANOSIM values (*R*) are indicated. *The *R*-value is significant ($P < 0.05$).

soil profile, by close coupling of nitrification with denitrification. In the current field study we also found very little build up of NO_3^- over the winter months (February and March), associated with significant N_2O fluxes during this time period (Fig. 1A), suggesting that denitrification is coupled with any production of NO_3^-.

The composition of the denitrifier communities also changed significantly over the winter and spring (Fig. 3B and C). This was expected since other studies have shown seasonal variation in soil bacterial communities including denitrifiers (Bossio et al., 1998; Bremer et al., 2007; Mergel et al., 2001b; Schutter et al., 2001; Smit et al., 2001; Wolsing and Priemé, 2004). Multiple factors such as soil moisture and temperature differed between our sampling dates, and are known to affect community diversity (Avrahami and Bohannan, 2007; Avrahami and Conrad, 2005; Avrahami et al., 2003; Drenovsky et al., 2004; Schutter et al., 2001; Smit et al., 2001; Zhou et al., 2002). Significant flushes of N_2O associated with spring thaw occurred overwinter. These events occurred in conjunction with increased soil temperatures and volumetric water content (VWC), and were associated with a unique denitrifier community (Figs. 1 and 2). At this experimental site, total non-growing season (November–April) emissions of N_2O were mostly due to an increased N_2O flux at spring thaw (Wagner-Riddle et al., 2007). In addition, ^{15}N tracer studies of our soil indicated that the large flux of N_2O during thaw was due primarily to denitrification, and the production of N_2O in the surface soil layer (Wagner-Riddle et al., 2008). Molecular analysis of the denitrifying community indicates that there is a rapid change in the diversity and richness of the community during spring, with the lowest diversity occurring in mid-winter February and increasing until May. Importantly, we were able to amplify *nirS* and *nirK* from all of our soil sampling periods, suggesting that the community of denitrifiers is not as affected by cold temperatures as the nitrifiers. At spring thaw a rapid increase in temperature and liquid water in both treatments, likely created a flush of nutrients leading to increased microbial activity, promoting changes in diversity and structure of the communities of interest, and in turn an N_2O flux.

Diversity indices were analyzed against soil temperature and VWC separately and no significant correlations were revealed. This suggests that the seasonal variation that caused significant changes in the diversity and community structure of nitrifying and denitrifying bacteria is caused by a combination of seasonal factors including soil temperature, VWC, nutrient levels and other potential factors.

During this experiment, lower N_2O emissions were observed from soils under NT, and this was primarily due to lower emissions in

NT during a spring thaw event (Fig. 1A). This confirms that prior studies by Wagner-Riddle et al. (2007) showing lower N_2O emissions in BMP fields at our site were associated with physical processes related to the NT treatment, such as reduced soil freezing due to the insulating effects of snow cover plus crop residue, rather than solely to the decreased fertilizer application in the earlier study. In addition, earlier studies have found that new production of N_2O through denitrification in surface soil layers is the major source of N_2O during this time period (Ruser et al., 2006). Denitrifiers have been shown to easily metabolize the organic compounds released from microbes killed as a result of freezing (Christensen and Tiedje, 1990; Koponen and Martikainen, 2004; Sehy et al., 2004) and from disintegrated aggregates (Christensen and Christensen, 1991, van Bochove et al., 2000) during subsequent thawing. Therefore, the lower nutrient availability in the NT plots due to the lower degree of soil freezing, results in lower N_2O production. Our study further indicates that there are significant differences in the composition of the denitrifier and nitrifier communities in the CT and NT plots during the March sampling period, immediately after a spring thaw event (Fig. 4B, F and J). In this study we did not specifically measure microbial activity, hence, the assumption is that for the community structure and diversity to change drastically, the organisms must be dividing and active.

Cavigelli and Robertson (2000) found that denitrification and communities containing *nosZ* were different between a successional field as compared to a tilled agricultural field, and suggested that the difference in community composition of these two fields was responsible for a difference in N_2O emissions (Cavigelli and Robertson, 2000). It was confirmed in a later study that the taxonomic diversity of the two fields was functionally significant by measuring sensitivity of denitrification to oxygen of isolates from these two soils (Cavigelli and Robertson, 2001). In our study we could not confirm that taxonomic diversity in these communities is functionally significant, however, we showed that sampling periods associated with high N_2O emissions were also associated with changes in the composition of these communities. A study using RNA analysis and isotope probing may be necessary to confirm relations between diversity and function. Furthermore, we did not assess archaeal ammonium oxidizers in our study and observing changes in this community may be needed to complete this assessment, as archaea are abundant in soils and are affected by temperature (Leininger et al., 2006; Tourna et al., 2008).

6. Conclusions

In a field-based study we have combined field-scale micrometeorological measurements of N_2O flux with molecular characterization of the structure of nitrous oxide producing bacterial communities in our field plots. Over the non-growing season (October–April), we observed important functional shifts in the nitrogen cycle, including soil NH_4^+-N build up over winter, and a large N_2O flux at spring thaw, as well as alterations in the community structure of *amoA*, *nirS* and *nirK* containing bacteria. We also observed that NT plots had significantly lower cumulative N_2O emissions compared to CT plots, due primarily to a lower spring flux of N_2O. We identified significant differences in the microbial community structure associated with each management system, and these differences were most obvious in the March sampling date, immediately after the spring thaw event. Our study demonstrates that the structure of the nitrifier and denitrifier communities varies both with season and with tillage practice.

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