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Crop residue influence on denitrification, N_2O emissions and denitrifier community abundance in soil

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ABSTRACT

Bacterial denitrification plays an important role in the global nitrogen cycle and is a principal contributor of nitrous oxide (N_2O) to the atmosphere. The influence of simple (glucose) and complex (red clover and barley residue) carbon (C) sources on the amount of denitrification, N_2O molar ratio (N_2O:($N_2 + N_2O$)), and abundance of soil total bacterial and denitrifier communities was investigated using repacked soil cores. Quantitative PCR was used to determine the abundance of the total bacterial community (16S rRNA gene) and components of the denitrifier community, *cnorB*$_P$ (*Pseudomonas mandelii* and related species), *cnorB*$_B$ (*Bosea/Bradyrhizobium/Ensifer* spp.) and *nosZ* gene bearing communities. The relationship between the supply of, and demand for, terminal electron acceptors (TEAs), as determined by the relative availability of C and nitrate (NO_3^-), influenced the amount of denitrification and the N_2O molar ratio for both simple and complex C sources. Addition of glucose and red clover to the soil increased microbial activity, leading to NO_3^- depletion and an increased consumption of N_2O, whereas in soil amended with barley straw, there was not sufficient stimulation of microbial activity to create sufficient TEA demand to cause a measurable increase in emissions. This resulted in a higher N_2O molar ratio at the end of the incubation for the barley straw amended soil. A significant relationship ($R^2 = 0.83$) was found between respiration and cumulative denitrification, suggesting that the available C increased microbial activity and O_2 consumption, which led to conditions favorable for denitrification. The source of C did not significantly affect the total bacterial community or the *nosZ* copy numbers with an average of 4.9×10^7 16S rRNA gene copies g^{-1} dry soil and 4.6×10^6 *nosZ* gene copies g^{-1} dry soil, respectively. The addition of red clover plus NO_3^- significantly increased the *cnorB*$_P$ denitrifier community in comparison with the unamended control while the density of the *cnorB*$_P$ denitrifier community increased from 3.9×10^4 copies g^{-1} dry soil to a maximum of 8.7×10^5 copies g^{-1} dry soil following addition of glucose plus NO_3^- to soil. No significant correlations were found between the denitrifier community densities and cumulative denitrification or N_2O emissions, suggesting that the denitrification activity was decoupled from the denitrifier community abundance.

1. Introduction

Biological denitrification is central to the global nitrogen cycle. It is a dissimilatory process whereby nitrate (NO_3^-) and nitrite (NO_2^-), are used as alternative electron acceptors and reduced to gaseous nitric oxide (NO), nitrous oxide (N_2O) and molecular nitrogen (N_2). N_2O is an intermediate in the denitrification process, and consequently both the amount of denitrification and the N_2O molar ratio (N_2O:($N_2 + N_2O$)) are important in understanding and predicting N_2O emissions. The N_2O molar ratio is variable in space and time and this is of concern because N_2O is a greenhouse gas and a catalyst of stratospheric ozone degradation (Crutzen, 1981).

Both the rate of denitrification and the N_2O molar ratio in soil are regulated by various environmental factors including soil water content, temperature, soil pH, redox potential, nitrogen oxide concentrations and availability of carbon (C) (Firestone and Davidson, 1989; Hutchinson and Davidson, 1993). Denitrification is generally promoted under high soil moisture conditions where oxygen is limited, and NO_3^- and organic C are available for denitrifying microorganisms (Luo et al., 1999).

Carbon availability is one of the most important factors controlling denitrification rates (Beauchamp et al., 1989). It is of interest because it generally increases the amount of denitrification

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while either decreasing (Weier et al., 1993; Mathieu et al., 2006) or increasing (Dendooven et al., 1996; Mathieu et al., 2006) the N_2O molar ratio. This influence of C on denitrification is both through the provision of C directly to the denitrifiers, and/or stimulation of microbial metabolism, which increases the consumption of O_2 and creates conditions favorable for denitrification (Beauchamp et al., 1989). Laboratory studies have been conducted using simple C substrates (Weier et al., 1993; Jarvis and Hatch, 1994); however, fewer studies have used complex C sources that are common inputs in agricultural systems, such as crop residues (Dendooven et al., 1996). This is primarily due to the difficulty in assessing C availability in these more complex C sources (Beauchamp et al., 1989).

Microbial denitrification is believed to be a primary source of N_2O (Wrage et al., 2004); however, few studies have examined the denitrifier community and their influence on N_2O emissions. Denitrifier bacteria belong to a variety of physiological and taxonomic groups (Zumft, 1997) and have commonly been characterized by using most probable number (MPN) counts (Jacobson and Alexander, 1980; Lensi et al., 1995) and the denitrifier enzyme activity (DEA) assay (Martin et al., 1988). Using a modified DEA, Cavigelli and Robertson (2000) reported that under identical environmental conditions there was an increase in the N_2O molar ratio in soil from an agricultural field compared with a successional field, and suggested there was a significant functional role of the denitrifier community. More recently, molecular methods have been used to examine the denitrifying community composition and diversity (Rich and Myrold, 2004; Boyle et al., 2006), as well as the abundance of denitrifiers (Henry et al., 2004, 2006; Kandeler et al., 2006; Dandie et al., 2007a) by focusing on the amplification of functional genes involved in denitrification. The genes include nitrate reductase (*napA* and *narG*), nitrite reductase (*nirS* and *nirK*), nitric oxide reductase (*qnorB* and *cnorB)*, and nitrous oxide reductase (*nosZ*). However, not all denitrifying bacteria produce the complete suite of enzymes required to complete the denitrification process (Zumft, 1997).

Literature reviews have suggested that the composition and density of soil denitrifier communities may be factors affecting denitrification (Philippot and Hallin, 2005; Wallenstein et al., 2006), with studies reporting that the denitrifier community differs in response to environmental conditions that indirectly control the rate of denitrification (Cavigelli and Robertson, 2000; Holtan-Hartwig et al., 2000). Therefore, there is a need to understand the community dynamics of denitrifiers and the environmental factors influencing the abundance of the denitrifiers in soil to determine if the denitrifying community may play a role in controlling denitrification. For example, few studies have evaluated how C sources will affect the denitrifier community, and more specifically, the abundance of bacteria possessing these functional genes. Studies using quantitative real-time PCR revealed that soil amended with a mixed C substrate resulted in a four-fold increase in the number of *nirK* gene copies as compared with the same soil amended with water (Henry et al., 2004). Although quantification of soil denitrifier gene copy numbers has been reported previously in ecological studies, few studies have analyzed the response of denitrifier community abundance in agricultural soils to C amendment treatments, and none of these studies have evaluated the influence of crop residues on the denitrifier community abundance while comparing it with denitrification activity measured using biochemical assays. The objective of this study was to determine the influence of crop residue amendments on: (i) the amount and N_2O molar ratio of gaseous denitrification losses; and (ii) total bacterial and denitrifier community abundance. Bacterial abundance was measured using quantitative PCR and targeting the 16S rRNA gene for the total bacterial community, the *Pseudomonas mandelii* and related species $cnorB_P$, *Bosea*/*Bradyrhizobium*/*Ensifer* spp. $cnorB_B$ and *nosZ* functional genes for the denitrifier communities.

2. Materials and methods

2.1. Soil

In January 2006, frozen soil (0–15 cm) was collected from a field previously cropped to spring wheat (*Triticum aestivum* L.) in Fredericton, New Brunswick, Canada (45°52′ N, 66°31′ W). In order to maintain a low soil NO_3^- concentration, the soil was kept frozen at −20 °C. Three days prior to experimentation, the soil was thawed, air-dried to a gravimetric water content of 0.30 g g^{-1} dry weight, homogenized and passed though a 2 mm sieve and stored in the dark at 4 °C. Soil texture (pipette method with organic matter removal) was 406 g kg^{-1} sand, 475 g kg^{-1} silt and 119 g kg^{-1} clay. Soil total N and organic C concentrations (LECO CNS-1000) were 1.72 g kg^{-1} and 23.9 g kg^{-1}, respectively. Soil pH (1:1 water) was 6.2.

2.2. Experimental design

Two experiments were conducted using repacked soil cores, assembled as previously described by Gillam et al. (2008). Briefly, the appropriate quantity of each C amendment was thoroughly mixed into the soil. The soil was hand packed to a bulk density of 1 Mg m^{-3} into Plexiglass cores. The cores were 5.5 cm in diameter by 6 cm high with a wall thickness of 0.5 cm, and had a series of 1 mm diameter holes to allow gas exchange. The soil cores were placed in 1 l canning jars and sealed with lids fitted with a perforable septum to allow for gas sampling. Each jar had either a 10% volume of acetylene (C_2H_2) or atmosphere added. The C_2H_2, generated from calcium carbide and water, was added to block N_2O reduction to N_2. Each treatment core was packed at 4 °C and remained at this temperature for 12 h after C_2H_2 injection to limit microbial activity while C_2H_2 diffused throughout the soil core. The jars were then incubated at 25 °C for 144 h. Time zero was the time at which the jars were placed in the incubation chamber.

2.2.1. Microcosm experiment 1

The objective of Experiment 1 was to determine the influence of C and NO_3^- availability on the amount and N_2O molar ratio of gaseous denitrification losses. The experiment used a factorial arrangement of treatments in a completely randomized design with twelve treatments replicated four times. The factors were three levels of C addition (0, 100 and 250 mg C kg^{-1} dry soil as glucose) and four levels of NO_3^- addition (0, 10, 25 and 50 mg N kg^{-1} dry soil as KNO_3). Each treatment was added in solution to the soil to attain a water-filled pore space (WFPS) of 70%. Each treatment was incubated with and without the addition of C_2H_2. At the end of the experiment (144 h), soil cores were sub-sampled for determination of NO_3^- and ammonium (NH_4^+) concentrations. Headspace gas samples (20 ml) were taken from each jar at 0, 8, 16, 24, 36, 48, 72, 96, 120 and 144 h and placed in an evacuated Exetainer vial (Labco, UK) containing 4 mg of magnesium perchlorate, a desiccant to remove water from the gas sample. An equal volume of air was injected into the jar immediately after sampling. Samples were analyzed for N_2O and CO_2 by gas chromatography.

2.2.2. Microcosm experiment 2

The objective of Experiment 2 was to determine the influence of crop residues on the amount and N_2O molar ratio of gaseous denitrification losses and the abundance of the denitrifier community. The experiment used a completely randomized design with six treatments replicated four times. Each treatment was added to achieve a WFPS of 70%. The treatments included a control with no C addition and with addition of 50 mg KNO_3-N kg^{-1} soil (G_0N_{50}), addition of 250 mg C kg^{-1} soil as glucose without or with addition of 50 mg KNO_3-N kg^{-1} soil ($G_{250}N_0$ and

$G_{250}N_{50}$), addition of 250 mg C kg^{-1} soil as red clover residue without or with addition of 50 mg KNO_3-N kg^{-1} soil ($RC_{250}N_0$ and $RC_{250}N_{50}$), and addition of 250 mg C kg^{-1} soil as barley straw with addition of 50 mg KNO_3-N kg^{-1} soil ($BS_{250}N_{50}$). A without NO_3^- addition treatment could not be conducted for the barley straw amendment due to limitations in the number of experimental units available. Red clover and barley straw were dried at 55 °C and ground to pass through a 2 mm sieve before being mixed into the soil. Total C concentrations were 449 and 452 g C kg^{-1} and total N concentrations were 33.5 and 10.1 g N kg^{-1} for red clover and barley straw, respectively. Headspace gas samples (20 ml) were taken from each jar at 0, 8, 16, 24, 36, 48, 72, 96, 120 and 144 h and analyzed for N_2O and CO_2 by gas chromatography. Soil was destructively sampled from additional replicates (four per treatment) at 0, 24, 72 and 144 h to allow for analysis of NO_3^-, NH_4^+, extractable organic C (EOC), DEA, DNA extraction and real-time PCR assays.

2.3. Gas sampling and analysis

Headspace gas samples were analyzed for N_2O and CO_2 by a Varian Star 3800 Gas Chromatograph (Varian, Walnut Creek, CA) fitted with an electron capture detector (ECD), thermal conductivity detector (TCD) and a Combi-PAL Autosampler (CTC Analytics, Zwingen, Switzerland). The ECD was operated at 300 °C, 90% Ar, 10% CH_4 carrier gas at 20 ml min^{-1}, Haysep N 80/100 pre-column (0.32 cm diameter × 50 cm length) and Haysep D 80/100 mesh analytical columns (0.32 cm diameter × 200 cm length) in a column oven operated at 70 °C. The pre-column was used in combination with a 4-port valve to remove water from samples. The TCD was operated at 130 °C, pre-purified helium (He) carrier gas at 30 ml min^{-1}, Haysep N 80/100 mesh (0.32 cm diameter × 50 cm length) pre-column followed by a Porapak QS 80/100 mesh (0.32 cm diameter × 200 cm length) analytical column maintained at 70 °C. N_2O was quantified based on ECD response for concentrations up to 50 μl N_2O l^{-1} and on TCD response for greater concentrations. Temperature and pressure changes in the jars were accounted for in all calculations. N_2O and CO_2 concentrations were also corrected for the gas dissolved in the water by using the Bunsen absorption coefficient (Moraghan and Buresh, 1977). Headspace N_2O concentration in the absence of C_2H_2 addition was considered cumulative N_2O emissions (N_2O) whereas headspace N_2O concentration in the presence of C_2H_2 addition was considered cumulative denitrification ($N_2O + N_2$) This ratio is used to represent the partitioning of gaseous losses from denitrification, assuming NO emissions to be negligible (Ryden et al., 1979). Headspace CO_2 concentration was considered to be soil respiration.

2.4. Denitrifying enzyme activity

Denitrifying enzyme activity was determined using a modified anaerobic slurry technique (Beauchamp and Bergstrom, 1993). Soil slurries were made by placing 25 g moist soil in a 125 ml flask with 25 ml of a solution containing 10 mM glucose, 10 mM KNO_3, 10 mM K_2HPO_4 and 0.100 g l^{-1} chloramphenicol, an antibiotic used to inhibit *de novo* synthesis of new denitrifying enzymes (Smith and Tiedje, 1979). Flasks were sealed with Suba stoppers and anaerobic conditions were created by evacuating to 2 torr and flushing the flasks with He four times. The internal pressure was equilibrated to atmospheric pressure. After addition of 20 ml of C_2H_2 to each flask, soil slurries were shaken continuously and sampled at 15, 30, 45, and 60 min. At each gas sampling, 20 ml of headspace was removed and placed in an evacuated Exetainer vial (Labco, UK) containing 4 mg of magnesium perchlorate. An equal volume of He was immediately injected into the flask. The N_2O concentration was measured as described above.

2.5. Soil sampling and analysis

Moisture content was determined gravimetrically at each sampling time. NO_3^-, NH_4^+ and EOC were extracted by shaking 25 g moist soil for 30 min with 50 ml 0.5 M K_2SO_4. Extracts were filtered and stored at −20 °C pending analysis. NO_3^-, NH_4^+ and EOC concentrations of the filtered aqueous extract were measured using a Technicon Auto Analyzer II system, following Technicon Industrial Method #100-70W, Technicon Industrial Method #98-70W and Technicon Industrial Method #455-76W/A, respectively. Blank solution samples were also filtered to estimate contamination.

2.6. DNA extraction and quantitative PCR

Soil samples in 15 ml tubes were freeze-dried overnight until completely dry and stored at −80 °C prior to nucleic acid extraction. Soil DNA was extracted as previously reported by Griffiths et al. (2000) and modified by Dandie et al. (2007a). In brief, 0.25 g of freeze-dried soil was weighed into 2-ml screw-cap tubes containing 0.1 g each of washed and sterile 0.1-mm-diameter, 0.2- to 0.3-mm-diameter, and 0.7- to 1.2-mm-diameter-glass beads plus one 2.5-mm-diameter glass bead (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada). Modified hexadecyltrimethylammonium bromide extraction buffer (0.5 ml; equal volumes of 10% (wt/vol) hexadecyltrimethylammonium bromide in 0.7 M NaCl and 240 mM potassium phosphate buffer, pH 8.0, plus 2.5 mg ml^{-1} aurintricarboxylic acid), aluminum ammonium sulfate (50 μl of 200 mM filter-sterilized solution), and phenol–chloroform–isoamyl alcohol (0.5 ml; 25:24:1) were added to tubes and mixed thoroughly. Tubes were shaken at 20 strokes s^{-1} for 10 min (MO BIO 96-well plate shaker with tube adapter set) to lyse cells and then centrifuged (16,000 × *g*, 10 min, 4 °C) before removing the aqueous phase to a new tube. The aqueous phase was re-extracted with chloroform-isoamyl alcohol (24:1) and centrifuged as above, and the aqueous phase was removed to a fresh tube. Total nucleic acids were precipitated by the addition of 2 volumes 30% (wt/vol) polyethylene glycol 6000 (Fluka BioChemika)-1.6 M NaCl for 2 h at room temperature and then centrifuged (18,000 × *g*, 30 min, 4 °C). Pelleted nucleic acids were washed in ice-cold 70% (vol/vol) ethanol and air dried prior to resuspension in Tris–EDTA buffer (pH 8.0). DNA was quantified using the Picogreen assay (Invitrogen, Burlington, ON) and fluorescence was measured on a Fluoroskan Ascent fluorometer (Thermo Scientific, Waltham, MA, USA).

Amplification of quantitative PCR products was performed on an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) as previously described by Dandie et al. (2007a). Briefly, real-time PCR for *cnorB* was performed in 25 μl reaction mixtures containing 12.5 μl 2 × SYBR Green PCR Master Mix including Ampli*Taq* Gold DNA polymerase, uracil-*N*-glycosylase, deoxynucleotide triphosphates with UTP, passive reference dye and optimized buffer (Applied Biosystems); 900 nM of each primer, template DNA (5 μl of 100-fold dilutions of soil nucleic acid extracts and 5 μl of 10-fold dilutions of plasmid DNA for standard curves) and DNase-free water. T4 gene 32 protein (250 ng per reaction; New England Biolabs, Pickering, ON, Canada), which reduces the inhibitory effects of humic PCR inhibitors, was also included in the reactions containing soil DNA as template (Henry et al., 2006; Kandeler et al., 2006). Primers and thermal cycling conditions for amplification of the *cnorB* genes were as previously described by Dandie et al. (2007a). 16S rRNA amplifications were performed using 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of each primer, 300 nM of the TaqMan probe (Integrated DNA Technologies; Coralville, IA, USA) and template DNA as above. The primers and TaqMan probe used for 16S rRNA PCR was previously described by Suzuki et al. (2000) and thermal cycling conditions for the 16S rRNA were as described in Smith et al. (2006). Slight modifications

were made to the cycling conditions as described in Henry et al. (2006) for quantification of denitrifiers bearing the *nosZ* gene. Reactions were conducted in Applied Biosystems 2× Power SYBR Green Master Mix. Primers were optimized for use at 750 nM (nosZ1F) and 1000 nM (nosZ1R). Cycling conditions were as follows: 95 °C—15 min (1 cycle), 95 °C—15 s, touchdown 68–62 °C (−1 °C/cycle)—1 min, 81.5 °C—30 s (touchdown for 6 cycles), then the same cycling conditions with 62 °C annealing temperature for the remaining 35 cycles.

For each primer set, standard curves relating the C_T value and 16S rRNA or *cnorB* gene copy numbers were generated with three replicate 10-fold serial dilutions of a known quantity of plasmid DNA containing a fragment of the $cnorB_P$ or $cnorB_B$ gene (Dandie et al., 2007a). Similarly, primers nosZ1F and nosZ2R (Henry et al., 2006) were used to amplify a 700 bp fragment of the *nosZ* gene from *Pseudomonas brassicacearum* PD5 genomic DNA (Dandie et al., 2007b). The *nosZ* fragment was gel purified and ligated into pCR2.1-TOPO (Invitrogen) using the manufacturer's instructions. A positive clone was confirmed by sequencing to contain the correct fragment (data not presented). Plasmid DNA was prepared using Qiagen Midi Plasmid Extraction Kit and quantified using Picogreen. Plasmid DNA was restriction digested with SacI prior to use as a standard for *nosZ* quantitative PCR. Standard curves were used for each 96-well real-time PCR plate. Due to large sample numbers, multiple plates were used to analyze samples from the experiment and replicate calibrator samples were used on each plate.

2.7. Data analysis

All statistical analyses were conducted using the General Linear Model of SAS (SAS Institute Inc., Cary, NC, Version 8). All non-normal data was log transformed. Treatment means were calculated using the Uniform Minimum Variance Unbiased Estimators (Parkin and Robinson, 1994). Results were similar to the arithmetic means, therefore treatment means and standard errors presented in tables and figures are calculated from untransformed data. Means comparisons were performed using the LSmeans test, although if the interaction was not significant, treatment means were compared using a protected Least Significance Difference (LSD) test. Regression analyses were performed to assess the relationships between respiration, EOC and denitrification. Pearson correlation coefficients were determined between NO_3^-, EOC, respiration, cumulative denitrification or N_2O emissions and the density of the total bacteria and denitrifying communities. Significance was accepted at a level of probability of $P < 0.05$.

3. Results

3.1. Experiment 1

The objective was to determine the influence of C and NO_3^- availability on the amount and N_2O molar ratio of gaseous denitrification losses. The soil NO_3^- concentration prior to receiving NO_3^- and C additions was 3 mg NO_3^--N kg^{-1} soil. At the end of the incubation, soil NO_3^- concentrations decreased to <1 mg NO_3^--N kg^{-1} soil for all treatments except for the $G_{250}N_{50}$ treatment, and the three treatments which did not receive glucose but were amended with NO_3^- (Table 1). Soil NH_4^+ concentrations increased slightly during the incubation from 1.9 to 2.3 mg NH_4^+-N kg^{-1} soil and did not differ significantly among the treatments at the end of the incubation (data not presented).

When averaged across N rates, there was a significant increase in respiration as the rate of glucose addition, and hence C availability, was increased (Table 1). For soils not amended with glucose, all rates of NO_3^- addition significantly decreased respiration relative to the no NO_3^- addition treatment. In contrast, in soil amended with 250 mg glucose-C kg^{-1} soil, all rates of NO_3^- addition significantly increased respiration relative to the no NO_3^- addition treatment whereas in soil amended with 500 mg glucose-C kg^{-1} soil, there was no significant difference in respiration for addition of 0, 25 or 50 NO_3^--N kg^{-1} soil. Acetylene had no significant effect on respiration (data not presented).

Table 1
Influence of different levels of C (glucose) and NO_3^- (KNO_3) on soil NO_3^- concentration, cumulative CO_2 emissions, total denitrification and cumulative N_2O emissions at the end of a 144 h incubation

Treatment[a]	NO_3^- (mg N kg^{-1} soil)	Cumulative CO_2 emissions (mg CO_2-C kg^{-1} soil)	Denitrification ($N_2O + N_2$) (mg N_2O-N kg^{-1} soil)	Cumulative N_2O emissions (mg N_2O-N kg^{-1} soil)
G_0N_0	0.1 (0.01) D[b]	120 (4.6) D	2.2 (0.04) D	0.2 (0.002) G
G_0N_{10}	1.6 (0.69) CD	99 (16.2) EF	3.9 (0.78) CD	0.8 (0.168) E
G_0N_{25}	5.7 (1.13) B	102 (0.8) E	5.6 (0.69) BC	1.6 (0.160) BC
G_0N_{50}	12.8 (4.30)A	84 (7.5) F	5.1 (0.74) BC	2.6 (0.537) AB
$G_{250}N_0$	0.1 (0.01) D	61 (7.0) D	1.5 (0.01) D	0.04 (0.007) I
$G_{250}N_{10}$	0.1 (0.02) D	184 (2.8) C	2.8 (0.80) CD	0.4 (0.016) F
$G_{250}N_{25}$	0.1 (0.02) D	198 (1.7) C	6.0 (0.13) BC	1.1 (0.056) DE
$G_{250}N_{50}$	4.7 (1.12) BC	200 (3.9) C	13.3 (0.51) A	2.8 (0.101) A
$G_{500}N_0$	0.1 (0.01) D	304 (4.8) A	1.2 (0.02) D	0.02(0.006) I
$G_{500}N_{10}$	0.1 (0.02) D	284 (5.4) B	2.6 (0.12) CD	0.1 (0.008) H
$G_{500}N_{25}$	0.1 (0.03) D	291 (5.8) AB	5.1 (0.35) BC	0.2 (0.041) G
$G_{500}N_{50}$	0.2 (0.09) D	307 (10.2) A	8.9 (0.62) AB	1.2 (0.006) CD

[a] G: C addition as glucose where numeric values represent mg C kg^{-1}; N: NO_3^--N addition as KNO_3 where numeric values represent mg N kg^{-1} soil.
[b] Values followed by the same letters within a column are not significantly different ($P > 0.05$) based on a protected LSD test. Values are means ($n = 4$).

3.1.1. Denitrification and N_2O emissions

Addition of 25 or 50 mg NO_3^--N kg^{-1} soil increased denitrification as compared with the no NO_3^- addition treatment, regardless of rate of glucose addition (Table 1). Rate of glucose addition had no significant effect on denitrification for treatments with NO_3^- addition, whereas addition of 250 mg glucose-C kg^{-1} soil increased denitrification compared with 0 mg glucose-C kg^{-1} soil for treatments with addition of 50 mg NO_3^--N kg^{-1} soil.

Cumulative N_2O emissions increased in response to NO_3^- addition within each rate of glucose addition. Except for the 50 mg NO_3^--N kg^{-1} soil addition treatments, addition of glucose decreased cumulative N_2O emissions compared with 0 mg glucose-C kg^{-1} soil treatment within each rate of NO_3^- addition. Amendment of soil with both glucose and NO_3^- increased denitrification following addition of ≥25 mg NO_3^--N kg^{-1} soil as compared to the G_0N_0 treatment. In contrast, cumulative N_2O emissions decreased compared with soil amended with NO_3^- alone, except in the $G_{250}N_{50}$ treatment.

As the level of NO_3^- amendment increased within each rate of glucose addition, the N_2O molar ratio also increased (Fig. 1). This resulted in a greater proportion of gaseous emissions as N_2O. In comparison, for the same rate of NO_3^-addition, the N_2O molar ratio decreased in response to increasing glucose addition. At the end of the incubation, the G_0N_{50} treatment had the highest N_2O molar ratio, 0.49, whereas the lowest ratio of 0.02 was measured in the $G_{500}N_0$ treatment (Fig. 1).

3.2. Experiment 2

3.2.1. NO_3^-, NH_4^+ and EOC concentrations

The objective of Experiment 2 was to determine the influence of crop residues on the amount and N_2O molar ratio of gaseous denitrification losses and the abundance of the total bacterial and denitrifier communities. The soil NO_3^- concentration prior to receiving NO_3^- and C additions was 3 mg NO_3^--N kg^{-1} soil. Treatments not amended with NO_3^- decreased to <0.5 mg NO_3^--N kg^{-1} soil by the

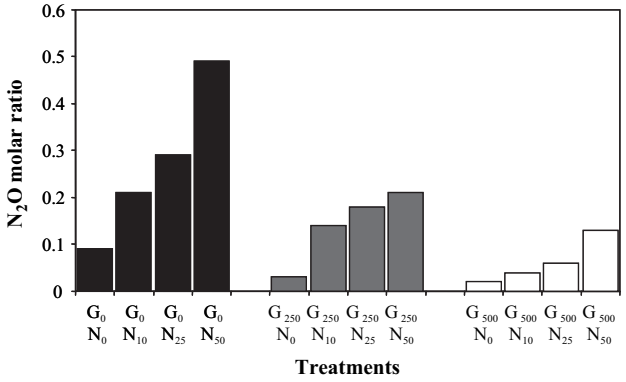

Fig. 1. N_2O molar ratio (N_2O:($N_2O + N_2$)) at the end of a 144 h incubation for different glucose (G) and NO_3^- (N) addition treatments, where numeric values represent mg C kg^{-1} soil and mg N kg^{-1} soil, respectively. N_2O molar ratio was calculated using treatment means ($n = 4$).

end of the incubation (Fig. 2A). Soil NO_3^- concentrations significantly decreased over time in all treatments which received 50 mg NO_3^--N kg^{-1} soil. Soil NO_3^- concentration in the $G_{250}N_{50}$ treatment decreased to <1 mg NO_3^--N kg^{-1} soil within 24 h, and was not significantly different from the $G_{250}N_0$ treatment at 144 h. Soil NO_3^- concentrations decreased by 50, 66 and 86% in the G_0N_{50}, $BS_{250}N_{50}$ and $RC_{250}N_{50}$ treatments, respectively, by the end of the incubation.

The soil NH_4^+ concentration significantly increased in all treatments during the incubation (Fig. 2B). The G_0N_{50} and $BS_{250}N_{50}$ treatments had the greatest increase, from 3.0 mg NH_4^+-N kg^{-1} soil at the beginning of the incubation to 9.6 mg NH_4^+-N kg^{-1} soil at 144 h. The soil NH_4^+ concentration increased from 5.0 and 1.2 mg NH_4^+-N kg^{-1} soil to 9.3 and 4.1 mg NH_4^+-N kg^{-1} soil in the $RC_{250}N_{50}$ red and $G_{250}N_{50}$ treatments, respectively.

The $K_2SO_4^-$ extractable organic C (EOC) content at the start of the incubation averaged 208 mg C kg^{-1} soil in glucose amended soil, 45 mg C kg^{-1} soil in the red clover amended soil, and 37 mg C kg^{-1} soil for the barley straw amended and unamended soils (Fig. 2C). Soil EOC concentrations significantly decreased during the incubation in all treatments except the G_0N_{50} treatment, which did not change. At the end of the incubation, EOC averaged 28 mg C kg^{-1} soil and did not differ significantly among treatments.

3.2.2. Respiration

The application of different sources of organic C at 250 mg C kg^{-1} soil resulted in a significant increase in respiration (i.e., cumulative CO_2 emissions) (Fig. 3). Maximum respiration of 177 mg CO_2-C kg^{-1} soil was measured in the $G_{250}N_{50}$ treatment. Increases in respiration over time in the $RC_{250}N_{50}$ and $G_{250}N_0$ treatments were comparable and significantly higher than the G_0N_{50} treatment, which had the lowest respiration. The respiration induced by C amendment, estimated as respiration for the amended treatment less respiration for the G_0N_{50} treatment, accounted for 40%, 21% and 10% of the C added in the glucose, red clover, and barley straw, respectively. NO_3^- addition significantly increased respiration in soil amended with glucose, but there was no effect of NO_3^- addition on respiration from red clover amended treatments. There was no significant effect of C_2H_2 addition on respiration (data not presented).

3.2.3. Denitrification and N_2O emissions

Minimal cumulative denitrification (<2.5 mg N_2O-N kg^{-1} soil) and N_2O emissions (<1 mg N_2O-N kg^{-1} soil) were measured in treatments without NO_3^- addition (Fig. 4). Cumulative denitrification and N_2O emissions were greater in the $G_{250}N_{50}$ treatment relative to the G_0N_{50} treatment after 24 h. The majority of cumulative denitrification occurred within 36 h in the $G_{250}N_{50}$ treatment and within

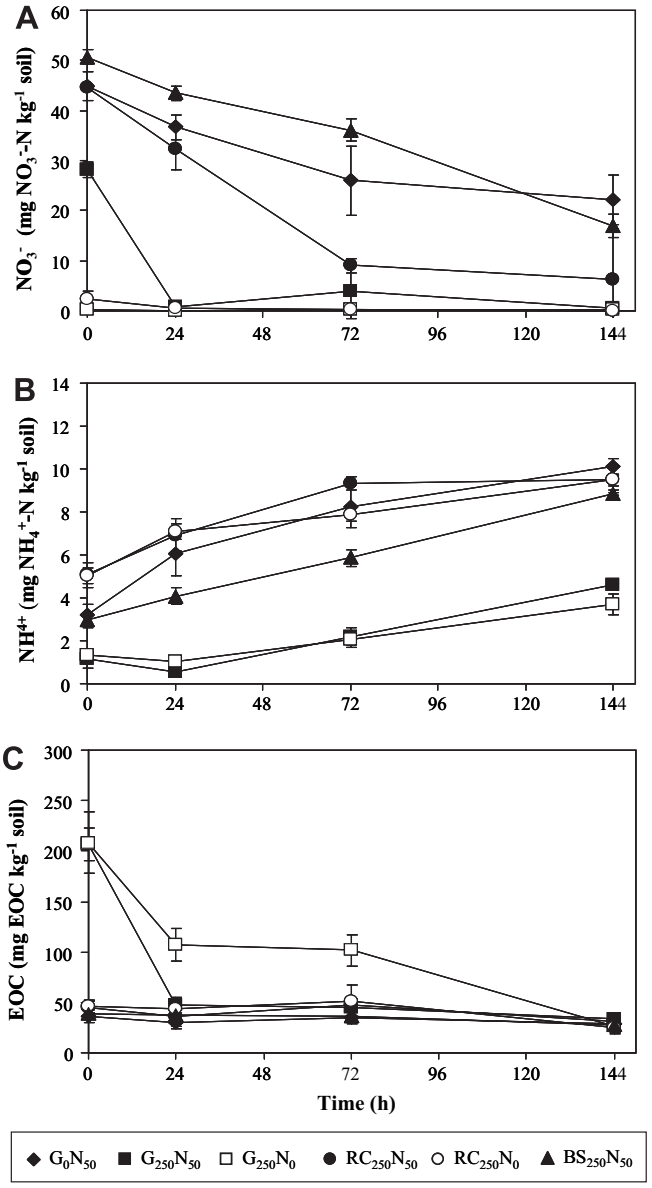

Fig. 2. Soil concentrations of (A) NO_3^-, (B) NH_4^+ and (C) EOC during a 144 h incubation in response to different C and NO_3^- addition treatments. Carbon additions as glucose (G), red clover (RC) and barley straw (BS) with numeric values indicating rate of addition in units of mg C kg^{-1} soil; NO_3^- additions as KNO_3-N (N) with numeric values indicating rate of addition in units of mg N kg^{-1} soil. Values represent means ($n = 4$) ± standard errors.

72 h in the $RC_{250}N_{50}$ treatment (Fig. 4A). In contrast, cumulative denitrification in the $BS_{250}N_{50}$ treatment, similar to the G_0N_{50} treatment, increased at a relatively constant rate over the entire incubation. A significant relationship was found between cumulative denitrification ($N_2O + N_2$) and respiration in treatments amended with both C and NO_3^- over all sampling times:

$$\text{Denitrification} = 0.152^{*}\text{respiration} + 0.184;$$
$$R^2 = 0.83; P\text{-value} = < 0.001$$

where denitrification is cumulative denitrification (mg N_2O-N kg^{-1} soil) and respiration was measured as cumulative CO_2 emissions (mg CO_2-C kg^{-1} soil).

The maximum value of cumulative N_2O emissions in treatments not amended with C_2H_2 occurred within 24 h in the $G_{250}N_{50}$ and $RC_{250}N_{50}$ treatments (Fig. 4B). The highest cumulative N_2O

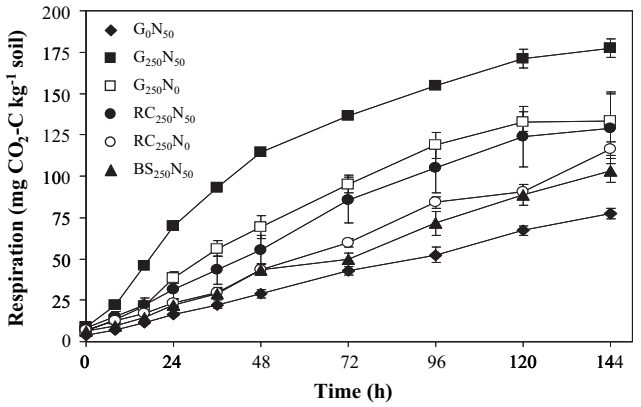

Fig. 3. Respiration, measured as cumulative CO_2 emissions, in soil during a 144 h incubation in response to different C and NO_3^- addition treatments. Carbon additions as glucose (G), red clover (RC) and barley straw (BS) with numeric values indicating rate of addition in units of mg C kg^{-1} soil; NO_3^- additions as KNO_3-N (N) with numeric values indicating rate of addition in units of mg N kg^{-1} soil. Values represent means ($n = 4$) ± standard errors.

emissions, 4.6 mg N_2O-N kg^{-1} soil, were measured in the $G_{250}N_{50}$ treatment. The highest cumulative N_2O emissions in the $BS_{250}N_{50}$ and G_0N_{50} treatments were measured at 120 h with average values of 2.4 and 2.9 mg N_2O-N kg^{-1} soil, respectively.

The N_2O molar ratio changed with time during the incubation for all treatments, initially increasing from 0–8 h and subsequently decreasing for the remainder of the incubation (Fig. 5). For the $G_{250}N_{50}$ treatment, the cumulative N_2O emissions began to decrease after 24 h resulting in a decrease in the N_2O molar ratio, and suggesting that N_2O was being consumed by the soil denitrifiers (Fig. 4B). This decrease in cumulative N_2O emissions corresponded to the time at which NO_3^- decreased to a low (<1 mg NO_3^--N kg^{-1}) concentration in the soil (Fig. 2A). Similar cumulative N_2O emissions were measured in the $RC_{250}N_{50}$ and $BS_{250}N_{50}$ treatments, however the addition of barley straw resulted in a higher N_2O molar ratio at the end of the incubation. At the end of the incubation the N_2O molar ratio ranged from 0.04 to 0.09 in the $G_{250}N_0$, $G_{250}N_{50}$ and $RC_{250}N_{50}$ treatments and from 0.16 to 0.18 in the G_0N_{50}, $RC_{250}N_0$ and $BS_{250}N_{50}$ treatments.

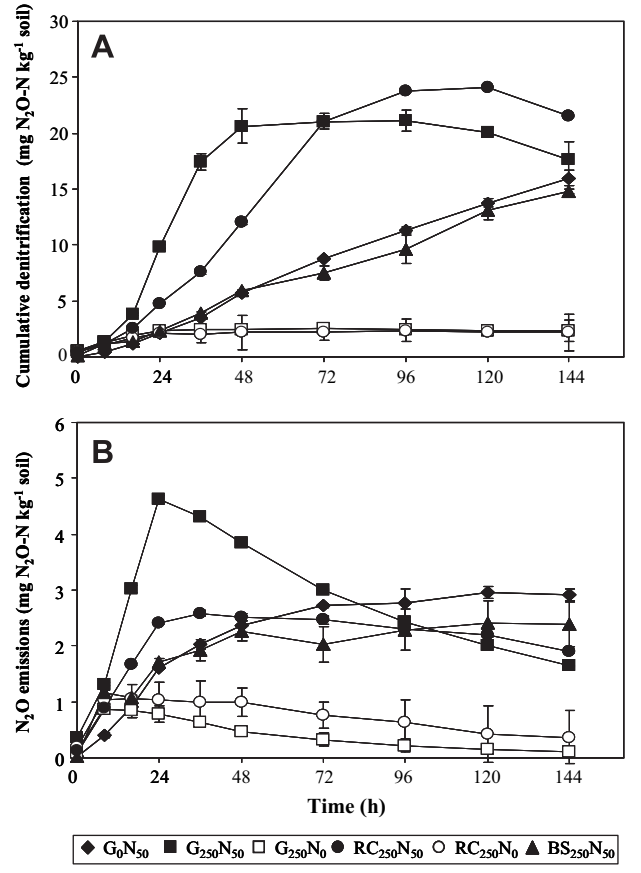

Fig. 4. Cumulative N_2O emissions in soil over a 144 h incubation in response to different C and NO_3^- addition treatments: (A) cumulative denitrification ($N_2O + N_2$); (B) N_2O emissions. Carbon additions as glucose (G), red clover (RC) and barley straw (BS) with numeric values indicating rate of addition in units of mg C kg^{-1} soil; NO_3^- additions as KNO_3-N (N) with numeric values indicating rate of addition in units of mg N kg^{-1} soil. Values represent means ($n = 4$) ± standard errors.

3.2.4. Quantification of 16S rRNA, nosZ and cnorB genes

The total bacterial community, measured as the 16S rRNA gene copy number, was not significantly different among the treatments, averaging 4.9×10^7 16S rRNA gene copies g^{-1} dry soil (Fig. 6). The density of the *nosZ* gene bearing community was also not significantly different among the treatments and averaged 4.6×10^6 *nosZ* gene copies g^{-1} dry soil (Fig. 6). Both the 16S rRNA and *nosZ* gene bearing communities did not significantly change over time.

The *cnorB*$_B$ (*Bosea*/*Bradyrhizobium*/*Ensifer*) gene copy numbers did not change significantly over time. The *cnorB*$_B$ gene copy numbers for the G_0N_{50} and $G_{250}N_{50}$ treatments, average of 7.5×10^4 *cnorB*$_B$ gene copies g^{-1} dry soil, were significantly higher than all other treatments, average of 3.0×10^4 *cnorB*$_B$ gene copies g^{-1} dry soil (Fig. 6).

In the absence of an additional C source, the density of *cnorB*$_P$ (*P. mandelii*-and related species) did not change significantly over time (Fig. 7). Likewise, there were no significant changes over time in the *cnorB*$_P$ gene copy numbers in the $RC_{250}N_{50}$ and $BS_{250}N_{50}$ treatments. Although, the density of the *cnorB*$_P$ gene copy numbers in the G_0N_{50} and $RC_{250}N_{50}$ treatments did not change significantly over time, the *cnorB*$_P$ gene bearing community was significantly greater in the $RC_{250}N_{50}$ treatment, average of 8.1×10^4 *cnorB*$_P$ gene copies g^{-1} dry soil, compared with the G_0N_{50} treatment, average of 3.7×10^4 *cnorB*$_P$ gene copies g^{-1} dry soil. In the $G_{250}N_{50}$ treatment, the *cnorB*$_P$ gene copy numbers significantly increased from a starting population of 3.9×10^4 *cnorB*$_P$ gene copies g^{-1} dry soil to a maximum of 8.7×10^5 *cnorB*$_P$ gene copies g^{-1} dry soil at 24 h, then subsequently declined over the remainder of the incubation to 1.5×10^5 *cnorB*$_P$ gene copies g^{-1} dry soil at 144 h. The *cnorB*$_P$ density also significantly increased between 0 and 24 h in the $G_{250}N_0$ treatment, but was significantly lower than was measured in the $G_{250}N_{50}$ treatment at this time. No significant correlations were found between the denitrifier community densities and the

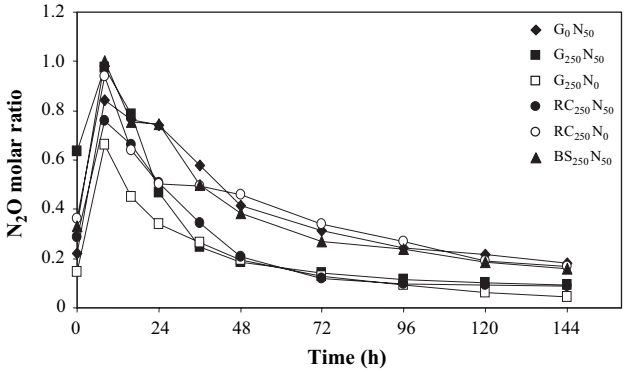

Fig. 5. N_2O molar ratio (N_2O:($N_2O + N_2$)) in soil over a 144 h incubation in response to different C and NO_3^- addition treatments, calculated using treatment means ($n = 4$). Carbon additions as glucose (G), red clover (RC) and barley straw (BS) with numeric values indicating rate of addition in units of mg C kg^{-1} soil; NO_3^- additions as KNO_3-N (N) with numeric values indicating rate of addition in units of mg N kg^{-1} soil.

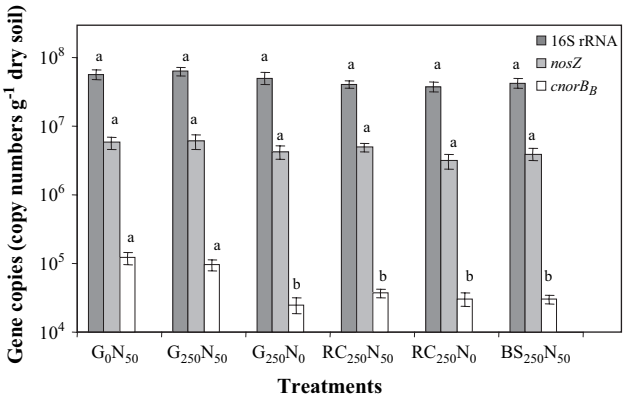

Fig. 6. Quantification of 16S rRNA, *nosZ* and *cnorB*$_B$ gene copy numbers in response to different C and NO_3^- addition treatments averaged over time. Carbon additions as glucose (G), red clover (RC) and barley straw (BS) with numeric values indicating rate of addition in units of mg C kg^{-1} soil; NO_3^- additions as KNO_3-N (N) with numeric values indicating rate of addition in units of mg N kg^{-1} soil. Treatment means with the same letter are not significantly different based on protected LSD test ($P < 0.05$). Values represent means ($n = 16$) ± standard errors. Standard curve descriptors and detection levels: 16S rRNA: $R^2 = 0.999$, y intercept = 39.2, E (amplification efficiency) = 94.9%, NTC = undetected. *cnorB*$_B$: $R^2 = 0.999$, y intercept = 35.7, $E = 100\%$, NTC = undetected. *nosZ*: $R^2 = 0.998$, y intercept = 37.2, $E = 88.9\%$, NTC = undetected.

measured soil parameters (respiration, EOC, NO_3^-, DEA, cumulative denitrification or N_2O emissions) within individual sampling times.

3.2.5. Denitrifying enzyme activity

At 24 h, DEA for all treatments, excluding the $BS_{250}N_{50}$ and the G_0N_{50} treatments, had significantly increased compared with time zero (Fig. 8). DEA in the $G_{250}N_{50}$ and $RC_{250}N_{50}$ treatments continued to significantly increase between 24 and 72 h. At 72 h, DEA in the $BS_{250}N_{50}$ and G_0N_{50} treatments had also increased from time zero. Maximum DEA values of 1.3, 1.9 and 1.4 mg N kg^{-1} soil h^{-1} were measured at 72 h in the $G_{250}N_{50}$, $RC_{250}N_{50}$, and $BS_{250}N_{50}$ treatments, respectively.

4. Discussion

4.1. Denitrification and N_2O emissions

Microbial denitrification and nitrification are responsible for the majority of N_2O emissions in many soil environments (Firestone and Davidson, 1989). N_2O resulting from denitrification generally occurs in soil when the WFPS is >60% (Davidson, 1991). In the present study, decreasing soil NO_3^- concentrations were accompanied by an increase in N_2O emissions in the presence of C_2H_2 in soil cores with a WFPS of 70%. This suggests that the N_2O emissions were primarily a result of denitrification. Similarly, Bateman and Baggs (2005) reported that denitrification accounted for all the N_2O produced when soil WFPS was 70% or higher. Preliminary research revealed that under similar soil core conditions, N_2O emissions were not significantly different in soil cores incubated with or without the nitrification inhibitor nitrapyrin (unpublished data), indicating that nitrification did not significantly contribute to N_2O emissions in this experimental system soil.

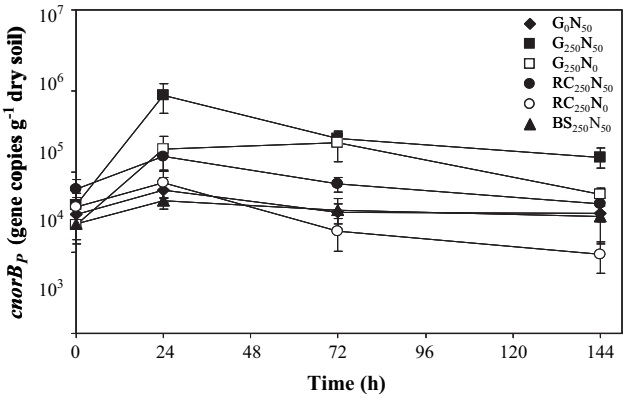

Fig. 7. Real-time PCR quantification of *cnorB*$_P$ gene copy numbers over 144 h incubation in response to different C and NO_3^- addition treatments. Carbon additions as glucose (G), red clover (RC) and barley straw (BS) with numeric values indicating rate of addition in units of mg C kg^{-1} soil; NO_3^- additions as KNO_3-N (N) with numeric values indicating rate of addition in units of mg N kg^{-1} soil. Values represent means ($n = 4$) ± standard errors. Standard curve descriptors and detection levels: *cnorB*$_P$: $R^2 = 0.999$, y intercept = 37.9, $E = 94.5\%$, NTC = undetected.

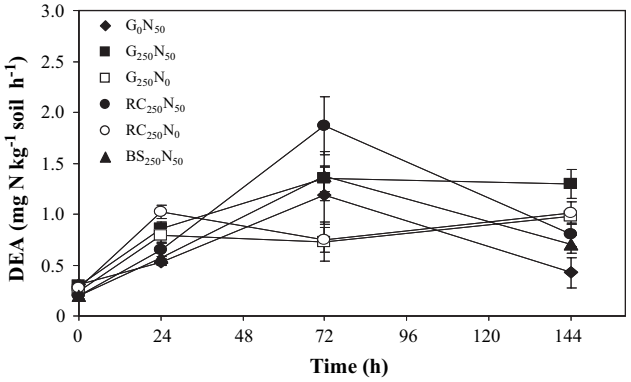

Fig. 8. Denitrifying enzyme activity in soil over 144 h in response to different C and NO_3^- addition treatments. Carbon additions as glucose (G), red clover (RC) and barley straw (BS) with numeric values indicating rate of addition in units of mg C kg^{-1} soil; NO_3^- additions as KNO_3-N (N) with numeric values indicating rate of addition in units of mg N kg^{-1} soil. Values represent means ($n = 4$) ± standard errors.

Use of the C_2H_2 blockage method has recently been criticized due to reports that an oxidation reaction may occur between NO and O_2 in the presence of C_2H_2 which could lead to an underestimation of denitrification (McKenney et al., 1997; Bollmann and Conrad, 1997). More recently, Murray and Knowles (2003) found that scavenging of NO by the C_2H_2-catalyzed NO oxidation reaction in the presence of trace amounts of O_2 would not cause a serious underestimate of long-term measurements of denitrification in anaerobic soils containing sufficient C and NO_3^-. Similar to this study, Murray and Knowles (2003) measured higher rates of denitrification as compared with previous work where the water content was lower (60% WHC) and not supplemented with NO_3^- (Bollmann and Conrad 1997). In the current study, the N_2O molar ratio did not increase above the theoretical maximum value of 1, suggesting that this oxidation reaction was limited under the soil incubation conditions. There was also no evidence of C_2H_2 inhibition break-down as characterized by a rapid decrease in cumulative N_2O emissions (Simarmata et al., 1993). The lack of an effect of C_2H_2 addition on soil respiration in both experiments also confirmed that C_2H_2 was negligible as a C source.

In Experiment 1, the amount of denitrification and N_2O molar ratio were influenced by the relative availability of oxidant (N oxides) and reductant (C). This was previously postulated by Hutchinson and Davidson (1993). Using a simple C source (i.e., glucose), Experiment 1 demonstrated that as the NO_3^- concentration increased the N_2O molar ratio also increased. Previous studies have shown that increasing soil NO_3^- concentrations can inhibit N_2O reductase activity (Blackmer and Bremner, 1978; Weier et al., 1993). NO_3^- is preferred over N_2O as a terminal electron acceptor (TEA) and N_2O can accumulate whenever NO_3^- supply is greater than the reducing demand of the denitrifiers (Swerts et al., 1996). A simultaneous increase in glucose decreased the N_2O molar ratio by supplying electron donors to the denitrifiers and/or by increasing

soil microbial metabolism, thereby increasing consumption of O_2 and the demand for alternative TEAs (Beauchamp et al., 1989).

An examination of more complex carbon sources (i.e., red clover and barley straw) in Experiment 2 found that a similar relationship existed in that the amount of denitrification and N_2O molar ratio were again influenced by the availability of N-oxides and C. Soil which received the $RC_{250}N_{50}$ treatment became NO_3^- limited after 72 h of incubation, resulting in increased consumption of N_2O. In comparison, C availability in the soil receiving the $BS_{250}N_{50}$ treatment was not sufficient to deplete the soil NO_3^-, and consequently increased consumption of N_2O did not occur. Ryden (1983) suggested that N_2O emissions are limited by NO_3^- when concentrations are lower than 5–10 mg N kg^{-1} soil. Soil NO_3^- decreased to <10 mg NO_3^--N kg^{-1} in the soil amended with $RC_{250}N_{50}$. This relationship resulted in lower N_2O molar ratios in the $G_{250}N_{50}$ and $RC_{250}N_{50}$ treatments at the end of the incubation. In comparison, more N_2O was produced as a proportion of cumulative denitrification in the $BS_{250}N_{50}$ and G_0N_{50} treatments.

Previous studies have reported on the variability of the N_2O molar ratio in response to C addition. Comparisons among studies can be difficult because various soils, methods and environmental parameters are evaluated over varying time periods. However, in agreement with this study, Stevens et al. (1998), using ^{15}N-gas flux measurements, found that the N_2O molar ratio declined from 0.8 to 0.05 over 80 h of incubation when soil was amended with glucose and NO_3^-. Similarly, additions of glucose and starch increased the amount of N_2 produced whereas addition of cellulose, a more complex C source, increased N_2O emissions (Murray et al., 2004). Gillam et al. (2008) used the C_2H_2 blockage method to examine the N_2O molar ratio and found that it was generally higher than 0.8 in soil amended with glucose, red clover and barley straw. However, in contrast to this study, soil NO_3^- was non-limiting which may have decreased N_2O consumption by denitrifiers because of their preference to use either O_2 or NO_3^- over N_2O as a TEA. Also, the soil WFPS was maintained at 60% by Gillam et al. (2008), and consequently the N_2O molar ratio may have been overestimated somewhat due to N_2O emissions arising from nitrification.

Decreased respiration demonstrated that the $BS_{250}N_{50}$ treatment had significantly less available C compared with the $G_{250}N_{50}$ and $RC_{250}N_{50}$ treatments. Barley straw had a higher C:N ratio (45:1) and is composed of a greater proportion of highly resistant lignin and cellulose, whereas red clover had a lower C:N ratio (13:1), and is composed of more labile C compounds. Plant residues with low C:N ratios, such as red clover, would promote mineralization and creation of anaerobic microsites whereas incorporation of residues with high C:N ratios may cause immobilization of soil N (Baggs et al., 2000). The increased respiration in the $G_{250}N_{50}$ and $RC_{250}N_{50}$ treatments as compared with the G_0N_{50} treatment would result in increased O_2 consumption, creating conditions favoring denitrification (Aulakh et al., 2000; Azam et al., 2002). The G_0N_{50} treatment resulted in similar denitrification, N_2O emissions and respiration as compared with the $BS_{250}N_{50}$ treatment. This suggests that in the barley straw amended soil, there was not enough additional available C incorporated to create sufficient TEA demand to cause a measurable increase in N_2O emissions.

A significant relationship was found between respiration and denitrification ($N_2O + N_2$) in treatments amended with both C and NO_3^-. This is consistent with previous reports (Burford and Bremner, 1975; De Wever et al., 2002). Correlation between denitrification and other measurements of C such as water soluble organic carbon have also been reported (Burford and Bremner, 1975; de Catanzaro and Beauchamp, 1985). In the present study, no significant relationship was found between EOC measured at the beginning of the incubation and cumulative denitrification or N_2O emissions measured at the end of the incubation. Respiration appeared to be a better estimate of available C for denitrification than measured EOC. EOC likely measures the instantaneous size of the available pool of labile C, whereas respiration represents mineralized organic C.

4.2. Total bacteria and denitrifier community abundance

The total bacterial community, measured as the 16S rRNA gene copy number, was not significantly different among treatments. This suggests that the C additions were not sufficient to cause a measurable increase in the total bacterial community. Similarly, He et al. (2007), using real-time PCR, found no effect on the abundance of the total bacterial community between treatments receiving both mineral fertilizer and organic manure in comparison to soil receiving no amendments for 16 years in a wheat–maize rotation system.

Components of the indigenous soil denitrifier community were assessed using the N_2O reductase (*nosZ*) and NO reductase gene (*cnorB*) as molecular markers. Neither the rate nor source of C amendment used in this study induced a measurable change in the abundance of the *nosZ* gene bearing community. The $cnorB_B$ (*Bosea/Bradyrhizobium/Ensifer*) gene copy numbers were higher for unamended and glucose amended soil than for plant residue amended soil, however changes in copy numbers over time were not significant. The reason for this finding is unclear, but it suggests that the $cnorB_B$ community was less competitive in the presence of plant residues. Similarly, Dandie et al. (2007a) found that the $cnorB_B$ gene bearing community did not change over time in response to increasing additions of glucose (0–500 mg C kg^1).

The abundance of the $cnorB_P$ (*Pseudomonas mandelii* and related species) denitrifier community increased in response to glucose-C addition and reached a maximum within 24 h and then subsequently declined over the remainder of the incubation. This decrease in abundance coincided with depletion of soil NO_3^- and induction of N_2O consumption. Using the denitrifier MPN technique, Jacobson and Alexander (1980) found that in soil amended with glucose, the denitrifier community increased over 48 h and then decreased as the rate of NO_3^- reduction also decreased. Microbial communities can be altered by addition of labile organic material by selecting for populations that are most competitive in terms of growth rates and their ability to absorb nutrients (Drenovsky et al., 2004). This component of the $cnorB_P$ gene bearing denitrifier community may be able to more effectively compete for the available glucose-C under the induced denitrification conditions. *Pseudomonas* species have commonly been found in soil (Cheneby et al., 2000; Dandie et al., 2007b) and this is presumably because of their versatility and ability to compete for C substrates (Tiedje, 1988).

Studies using molecular techniques to examine the influence of C on soil denitrifiers are limited. Kandeler et al. (2006) used real-time PCR to quantify denitrification genes in a receding glacier foreland and found that the highest correlation existed between the quantity of soil organic C and the abundance of eubacteria and denitrifiers as measured by *nirK* and *nosZ* genes. Also using real-time PCR, Henry et al. (2004) found a significant four-fold increase in the number of *nirK* gene copies found in soil amended with a mixed C substrate as compared with the same soil amended with water. However, few details are provided regarding the type of C substrate utilized and if denitrification was induced. In the present study, the amount of denitrification and the N_2O molar ratio were influenced by the addition of red clover and barley straw residues; however, the density of the $cnorB_P$ and $cnorB_B$ communities did not significantly change in response to the barley straw addition. In contrast, an increase in the abundance of the $cnorB_P$ gene copy numbers was measured in the $RC_{250}N_{50}$ treatment in comparison with the G_0N_{50} treatment.

No significant correlations were found between the denitrifier community densities and denitrification or N_2O emissions for

individual sampling times, suggesting that the denitrification activity was decoupled from denitrifier community abundance. Likewise, no significant correlations were found between the denitrifier community densities and EOC or respiration for individual sampling times. The availability of C substrates is determined by their decomposability and capacity to support microbial growth (Bremner and Shaw, 1958) and the quantity of the denitrifiers may have increased in response to higher inputs of crop residue. Plant residues are only one source of C in agricultural systems and other organic C sources such as manure should be examined for their influence on the denitrifier community.

4.3. Denitrifying enzyme activity

Denitrifying enzyme activity was measured to assess existing denitrifier enzyme content in the soil at the time of sampling. Using a short assay incubation time (60 min), and chloramphenicol to inhibit protein synthesis, ensured that the activity measured was due to pre-existing enzymes and not to enzymes synthesized during incubation (Smith and Tiedje, 1979). The increase in DEA over time in specific treatments, including the G_0N_{50} treatment, reflected an increase in the denitrifying enzyme content. This suggested that the denitrifiers were active within these treatments and may reflect soil warming which occurred once the soils were thawed and incubated at 25 °C. The DEA was significantly increased in the $G_{250}N_{50}$ and $RC_{250}N_{50}$ treatments compared with the G_0N_{50} treatment. This increase in DEA indicates that the C amendments increased denitrification activity. The increase in DEA coincided with an increase in abundance of the *cnorB*$_P$ gene bearing community but not in the *cnorB*$_B$ and/or *nosZ* gene bearing communities, in the $G_{250}N_{50}$ or $RC_{250}N_{50}$ treatments. It has been hypothesized that the activation-inactivation of existing enzymes is influenced by O_2 to a greater extent than the growth of denitrifiers (Smith and Parsons, 1985). DEA represents pre-existing enzymes in the soil and may better correlate with the expression (mRNA) of the denitrification genes since detection of mRNA suggests that the organism was actively expressing the gene and producing the associated enzyme (Wallenstein et al., 2006).

4.4. Conclusions

The relative availability of C and NO_3^- was found to influence both the amount of denitrification and the N_2O molar ratio, and it was demonstrated that these relationships exist for both simple and complex C sources. Increases in available C led to significant increases in microbial respiration in treatments amended with glucose and red clover in comparison with the unamended control. A significant relationship ($R^2 = 0.83$) was found between respiration and cumulative denitrification. This suggests that the available C increased microbial activity and O_2 consumption, which led to conditions favorable for denitrification.

The source of C did not significantly affect the abundance of the total bacterial community or the *nosZ* gene copy numbers; however, the addition of glucose or red clover plus NO_3^- significantly increased the denitrifier *cnorB*$_P$ gene copy numbers, suggesting that there was an increase in the number of *cnorB*$_P$ gene bearing denitrifiers in the soil with the capability to denitrify (Wallenstein et al., 2006). Whether this increase in the *cnorB*$_P$ gene bearing community coincided with an increase in expression of the *cnorB* gene or other genes in the denitrification pathway remains unknown. DEA increased in the $G_{250}N_{50}$ and $RC_{250}N_{50}$ treatments relative to the G_0N_{50} treatment, indicating that these C amendments increased denitrification activity and that denitrifiers were active within the C amended treatments.

Quantitative measurements of functional genes in soil using real-time PCR is becoming more important as a tool to better understand the role of microorganisms in nutrient cycling, recognizing however that it has the same limitations as conventional PCR regarding extraction efficiency and PCR inhibition (Sharma et al., 2007). This experiment assessed components of the soil denitrifier community bearing the *nosZ* and *cnorB* genes. Further research should focus on the quantification of denitrifiers bearing both these and other denitrification genes in order to capture larger components of the denitrifier community. There is a need to determine the abundance and composition of the soil denitrifier community, and also the environmental factors which affect the specific activity of the enzymes involved in the denitrification process. This information will provide greater insights into the regulation of denitrification, as well as N_2O and N_2 emissions.

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Appendix A. Supplemental material

Supplementary information for this manuscript can be downloaded at doi: 10.1016/j.soilbio.2008.06.024.

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