Glucose effects on denitrifier abundance, denitrification gene mrna levels, and denitrification activity in an anoxic soil microcosm

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Abstract
Organic carbon availability influences nitrous oxide (N_2O) emissions but its effect on denitrifier communities is not understood. Changes in denitrifier abundance, denitrification gene mRNA levels and denitrification activity were followed in anoxic soil microcosms in the presence and absence of glucose with non-limiting nitrate concentration for 48 h. *nosZ* and *nirS$_p$* (*Pseudomonas mandelii* and closely-related spp.) genes (qPCR) and mRNA levels (qRT-PCR) were quantified. Abundance of *nosZ* and *nirS$_p$* were unaffected by glucose addition and were stable over the duration of the incubation with average values of 4.3 x10^8 and 8.1 x10^4 gene number/g dry soil, respectively. *nirS$_p$* mRNA levels were increased by glucose addition. Glucose addition resulted in induction of *nirS$_p$* mRNA levels after 4 h, with a 2.5 fold increase in transcripts compared with 0 h, to 2.4 x10^4 transcripts /g dry soil. In contrast, *nosZ* mRNA levels were not affected by glucose addition and averaged 2.3 x10^6 transcripts /g dry soil. Glucose addition increased cumulative N_2O emissions, with final values of 4.9 and 0.9 mg N_2O-N /kg dry soil for the glucose amended and non-amended soils, respectively, at 48 hr. The increase in N_2O emissions resulting from glucose addition in this study were not clearly accompanied by significant changes in abundance or denitrification gene mRNA levels for the targeted bacterial communities.

Key Words
nosZ, nirS, quantitative PCR, gene expression, organic carbon, *Pseudomonas mandelii.*

Introduction
Denitrification, the dissimilatory reduction of nitrogen oxides, is a metabolic process performed by soil bacteria that produces the greenhouse gas nitrous oxide (N_2O) as an intermediate gaseous product. Denitrification is influenced by environmental factors including oxygen concentration and nitrate concentrations; however, carbon availability is likely one of the most important factors influencing denitrification (Miller *et al.* 2008). Organic carbon addition reduces soil oxygen supply by promoting microbial growth, favouring the denitrification process. Organic carbon is also used as an electron donor in denitrification (Zumft 1997). Glucose, a simple carbon source, was used in several denitrification studies (Dandie *et al.* 2007, Fischer *et al.* 2005, Miller *et al.* 2008, Murray *et al.* 2004) and was used in this study as a first step in a larger project to understand the relationships between denitrifier activity and organic carbon sources in agricultural soils. The objective of this study was to evaluate the effect of a simple carbon source, glucose, on denitrifier abundance, denitrification gene mRNA levels, and denitrification activity from an agricultural soil. We hypothesized that an increase in denitrification activity, after the addition of glucose, would be due to an increase in the abundance of denitrifiers and/or the abundance of denitrification gene transcripts.

Methods
Experimental Design
Treatments with or without glucose addition (0 or 500 mg glucose-C /kg dry soil) were applied to soil cores in a factorial arrangement (2 levels of glucose, 6 incubation times, 4 replicates) in a completely randomised design. Nitrate was added at 500 mg NO_3-N/g dry soil (as KNO_3) and all cores were incubated at 70% water-filled pore space in sealed jars. Two sets of jars were used to measure denitrification and N_2O emissions. Cumulative denitrification was measured in one set of jars by adding acetylene (C_2H_2) to the headspace to block N_2O reduction ($N_2O + N_2$). No C_2H_2 was added to a second set of jars to measure N_2O emissions. Headspace gas samples (20 mL) were taken using a syringe. Soils were destructively sampled at

0, 4, 8, 12, 24, and 48 h of incubation. For nucleic acid extractions, soil samples were flash frozen in liquid nitrogen immediately after sampling and stored at -80°C until processing.

Analyses and statistics

Extractable organic carbon (EOC) and NO_3^- concentrations were measured in K_2SO_4 extracts from soil samples using colorimetric analysis on a Technicon Auto Analyzer II system (Technicon Industrial Systems, Terrytown, MA, USA). Headspace gas was analyzed for N_2O and CO_2 concentrations using a Varian Star 3800 Gas Chromatograph (Varian, Walnut Creek, CA) fitted with an electron capture detector (to measure N_2O), thermal conductivity detector (to measure CO_2), and a Combi-PAL Autosampler (CTC Analytics, Zwingen, Switzerland) (Burton *et al.* 2008). Nucleic acids (DNA and RNA) were extracted from freeze-dried soil samples using methods adapted from Griffiths *et al.* (2000).

Denitrification genes and transcripts (*nosZ* and *nirS*$_p$) were quantified via qPCR and qRT-PCR using an Applied Biosystems (Streetsville, ON) ABI PRISM® 7000 thermal cycler and SYBR Green detection as described in Henderson *et al.* 2010. ANOVA was performed with treatment and time as fixed factors. Means comparisons were performed for significant main effects and interactions by performing post hoc Tukey HSD and Tukey adjusted LS means, respectively.

Results and discussion

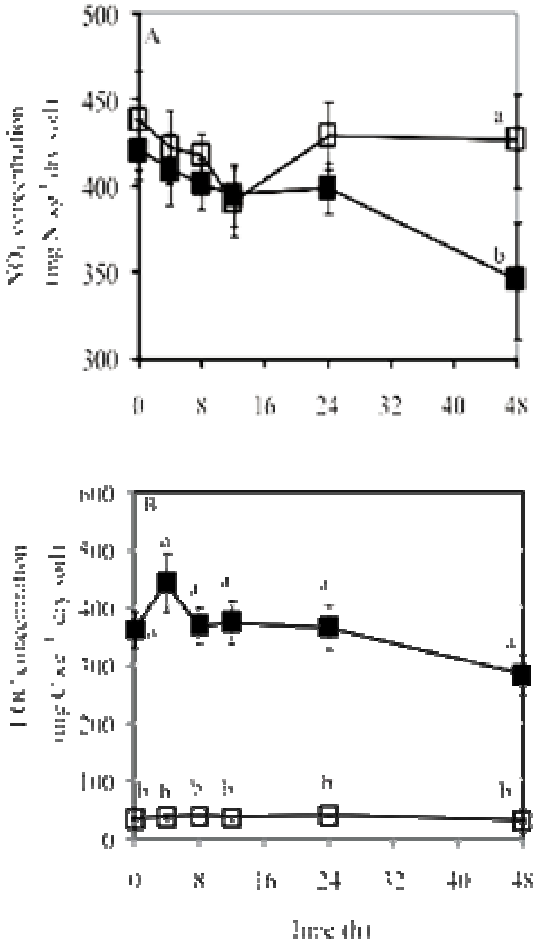

Figure 1. Soil concentrations of nitrate (NO_3^-) (A) and extractable organic carbon (EOC) (B) for soil incubated over 48 h following addition of glucose at 0 mg C-glucose /kg dry soil (G0) (□) or 500 mg C-glucose /kg dry soil (G500) (■). Values are means ± SEM (*n*=4).

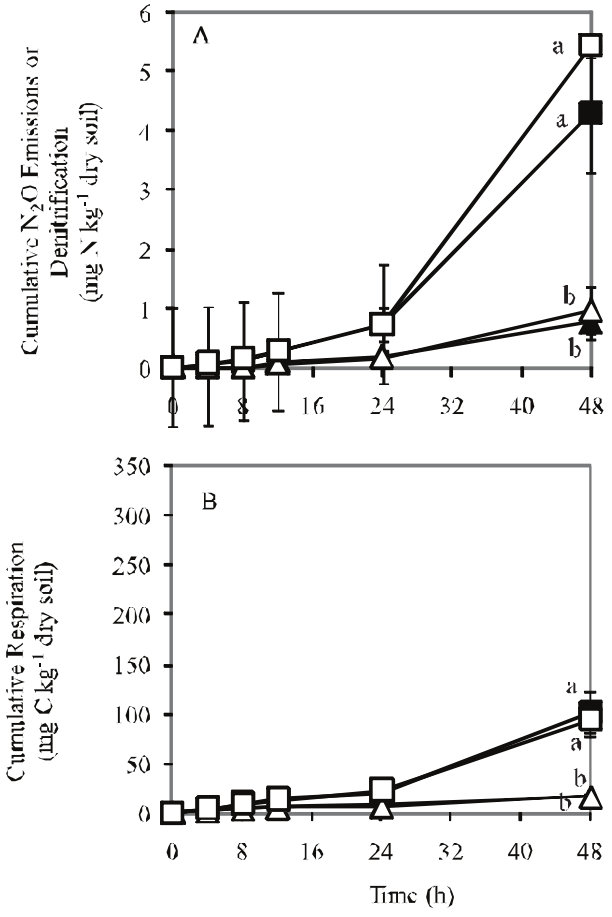

Figure 2. Cumulative emissions of nitrous oxide (N_2O) and denitrification (i.e. $N_2O + N_2$) (A) and carbon dioxide (CO_2) (respiration) (B) from soil incubated over 48 h following addition of glucose at 0 mg C-glucose/kg dry soil (G0) with (▲) or without (△) C_2H_2, or addition of 500 mg C-glucose/kg dry soil (G500) with (■) or without (□) C_2H_2. Values are means ± SEM (*n*=4).

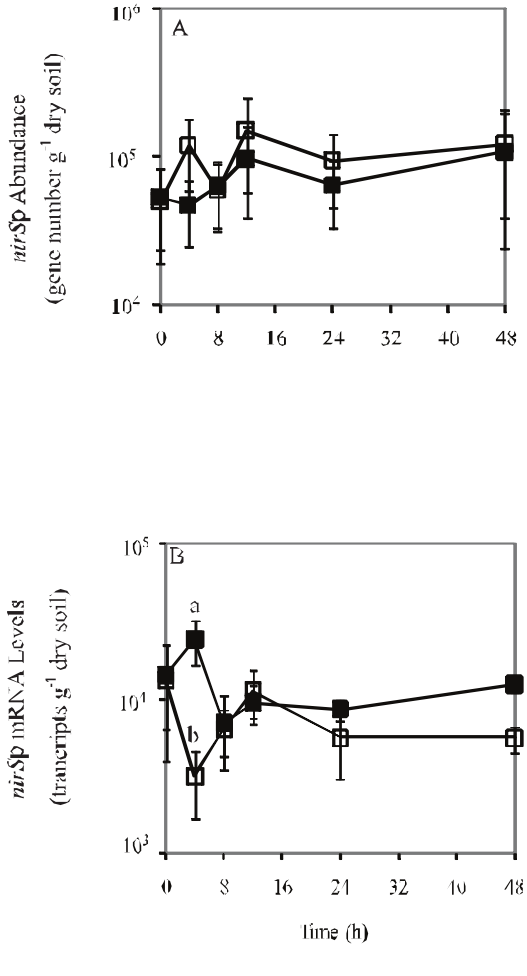

Figure 3. Quantification of *nirS$_p$* gene abundance using qPCR (A) and *nirS$_p$* mRNA levels using qRT-PCR (B) in soil incubated over 48 h following addition of glucose at 0 (G0) (□) or 500 mg C-glucose /kg dry soil (G500) (■). Standard curve descriptors of quantification of *nirS$_p$* gene numbers and detection levels: y = -3.39x + 36.0, R^2 = 0.998, E = 97.1%, no template control (NTC) = undetected. Standard curve descriptors of quantification of *nirS$_p$* transcripts and detection levels: y = -3.43x + 36.3, R^2 = 0.994, E = 95.3%, NTC = undetected. Values are means ± SEM (*n*=4).

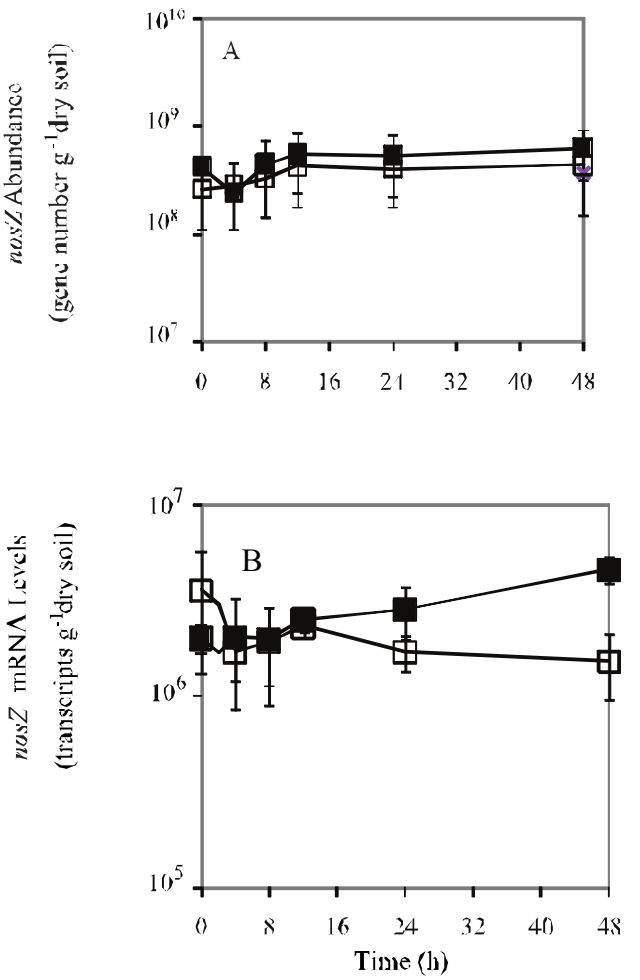

Figure 4. Quantification of *nosZ* gene abundance using qPCR (A) and *nosZ* mRNA levels using qRT-PCR (B) in soil incubated over 48 h following addition of glucose at 0 (G0) (□) or 500 mg C-glucose /kg dry soil (G500) (■). Standard curve descriptors of quantification of *nosZ* gene numbers and detection levels: y = -3.35x+ 43.0, R^2 = 0.990, E = 99.0%, no template control (NTC) = undetected. Standard curve descriptors of quantification of *nosZ* transcripts and detection levels: y= -3.38x + 38.4, R^2 = 0.994, E = 97.4%, NTC = undetected. Values are means ± SEM (*n*=4).

Effect of glucose on analytical measurements

Glucose addition resulted in a significant (p = 0.048) decrease in soil NO_3^- concentration, with average values of 425 and 373 mg NO_3^--N /kg dry soil in the G0 and G500 treatments, respectively (Figure 1A). The EOC concentration significantly ($p < 0.001$) increased following glucose addition with average values of 37 and 367 mg C /kg dry soil in the G0 and G500 treatments, respectively (Figure 1B). Reduction in EOC concentration over time indicated that glucose was metabolized.

Over the 48h incubation period, there was no significant difference between cumulative N_2O production from soil incubated without C_2H_2 (i.e. N_2O emissions) or with C_2H_2 (i.e. total denitrification) (Figure 2A), indicating that gaseous emissions from denitrification occurred primarily as N_2O. Cumulative denitrification was significantly ($p = 0.002$) increased by glucose addition (Figure 2A). Addition of glucose significantly increased respiration (cumulative CO_2 emissions) (Figure 2B). Similarly, soil amendment with glucose and other sources of organic carbon such as plant residues or manure have previously been shown to increase denitrification activity, and the increase in denitrification was commonly related to the increase in respiration (Dandie *et al.* 2007; deCatanzaro *et al.* 1985; Miller *et al.* 2008; Miller *et al.* 2009).

Molecular analysis of denitrifier abundance and mRNA levels

Despite the increases in respiration and denitrification in response to glucose addition, there were no measurable changes in the $nirS_p$ (Figure 3A) and *nosZ* (Figure 4A) abundance in soil over the 48 h incubation period. Previous studies also found that the *nosZ*-bearing denitrifier community did not increase in abundance after the addition of glucose to anoxic soil microcosms in 6 day incubations (Miller *et al.* 2008). In contrast, glucose addition at 250 mg-C /kg dry soil increased abundance of *P. mandelii* and closely related species quantified using *cnorB* primers ($cnorB_p$) in anoxic soil microcosms (Dandie *et al.* 2007, Miller *et al.* 2008). In soil amended with glucose, $nirS_p$ gene transcript abundance was increased compared with unamended soil only at 4 h ($p = 0.009$) (Figure 3B). The increase in $nirS_p$ transcripts occurred without a measurable increase in $nirS_p$ abundance, suggesting this increase was through increased mRNA levels per cell. Surprisingly, *nosZ* mRNA levels were not significantly affected by glucose addition and did not change significantly over time during the 48 h incubation ($p = 0.320$) (Figure 4B). Glucose is commonly thought to induce denitrification gene expression through oxygen depletion resulting from increased microbial respiration, however in this experiment where soil anoxic conditions were implemented by changing the headspace gas composition, such a response would not occur.

We hypothesized that the measured denitrification increase in glucose amended soil was due to an increase in denitrifier abundance and/or denitrification gene mRNA levels. Soil amendment with glucose increased microbial respiration and denitrification without a significant increase in abundance of *nosZ* and $nirS_p$ denitrifier communities and with a measurable and transient increase in transcripts for $nirS_p$ only. Therefore, under the experimental conditions used, the increase in denitrification activity was not well linked to denitrifier gene copy and mRNA suggesting that enzyme activity might be important in understanding the control of N_2O emissions in soil systems.

References

Burton DL, Zebarth BJ, Gillam KM, MacLeod JA (2008) Effect of rate and time of fertilizer nitrogen application on N_2O emissions from potato. *Canadian Journal of Soil Science* **88,** 229-239.

Dandie CE, Miller MN, Burton DL, Zebarth BJ, Trevors JT Goyer C (2007) Nitric oxide reductase-targeted real-time PCR quantification of denitrifier populations in soil. *Applied and Environmental Microbiology* **73,** 4250-4358.

deCatanzaro JB, Beauchamp EG (1985) The effect of some carbon substances on denitrification rates and carbon utilization in soil. *Biology and Fertility of Soils* **1,** 183-187.

Fischer EN, Whalen SC (2005) Rates and control of denitrification in an agricultural soil fertilized with lagoonal swine waste. *Nutrient Cycling in Agroecosystems* **71,** 271-287.

Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environmental Microbiology* **66,** 5488-5491.

Henderson SL, Dandie CE, Patten C, Zebarth BJ, Burton DL, Trevors JT, Goyer C (2010) Changes in denitrifier abundance, denitrification gene mRNA levels, nitrous oxide emissions and denitrification in anoxic soil microcosms amended with glucose and plant residues. *Appl. Environ. Microbiol.* doi:10.1128/AEM.02993-09

Miller MN, Zebarth BJ, Dandie CE, Burton DL, Goyer C, Trevor JT (2008) Crop residue influence on denitrification, N_2O emissions and denitrifier community abundance in soil. *Soil Biology and Biochemistry* **40,** 2553–2562.

Murray PJ, Hatch DJ, Dixon ER, Stevens RJ, Laughlin RJ, Jarvis SC (2004) Denitrification potential in a grassland subsoil: Effect of carbon substrates. *Soil Biology and Biochemistry* **36,** 545-547.

Zumft WG (1997) Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* **61,** 522-616.