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Nitrate removal, communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds

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Abstract

Denitrification beds are containers filled with wood by-products that serve as a carbon and energy source to denitrifiers, which reduce nitrate (NO_3^-) from point source discharges into non-reactive dinitrogen (N_2) gas. This study investigates a range of alternative carbon sources and determines rates, mechanisms and factors controlling NO_3^- removal, denitrifying bacterial community, and the adverse effects of these substrates. Experimental barrels (0.2 m^3) filled with either maize cobs, wheat straw, green waste, sawdust, pine woodchips or eucalyptus woodchips were incubated at 16.8 °C or 27.1 °C (outlet temperature), and received NO_3^- enriched water (14.38 mg N L^{-1} and 17.15 mg N L^{-1}). After 2.5 years of incubation measurements were made of NO_3^- – N removal rates, *in vitro* denitrification rates (DR), factors limiting denitrification (carbon and nitrate availability, dissolved oxygen, temperature, pH, and concentrations of NO_3^-, nitrite and ammonia), copy number of nitrite reductase (*nirS* and *nirK*) and nitrous oxide reductase (*nosZ*) genes, and greenhouse gas production (dissolved nitrous oxide (N_2O) and methane), and carbon (TOC) loss. Microbial denitrification was the main mechanism for NO_3^- – N removal. Nitrate–N removal rates ranged from 1.3 (pine woodchips) to 6.2 g N m^{-3} d^{-1} (maize cobs), and were predominantly limited by C availability and temperature (Q_{10} = 1.2) when NO_3^- – N outlet concentrations remained above 1 mg L^{-1}. The NO_3^- – N removal rate did not depend directly on substrate type, but on the quantity of microbially available carbon, which differed between carbon sources. The abundance of denitrifying genes (*nirS*, *nirK* and *nosZ*) was similar in replicate barrels under cold incubation, but varied substantially under warm incubation, and between substrates. Warm incubation enhanced growth of *nirS* containing bacteria and bacteria that lacked the *nosZ* gene, potentially explaining the greater N_2O emission in warmer environments. Maize cob substrate had the highest NO_3^- – N removal rate, but adverse effects include TOC release, dissolved N_2O release and substantial carbon consumption by non-denitrifiers. Wood-chips removed less than half of NO_3^- removed by maize cobs, but provided ideal conditions for denitrifying bacteria, and adverse effects were not observed. Therefore we recommend the combination of maize cobs and woodchips to enhance NO_3^- removal while minimizing adverse effects in denitrification beds.

Keywords

Denitrification; Controlling factors; Bioreactor; Denitrification genes; *nir*

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1. Introduction

Anthropogenic production of reactive nitrogen (N), through the Haber Bosch process, cultivation of N-fixing crops, and combustion of fossil fuels, contributes 45% of global N fixation (Canfield et al., 2010). This human impact on the nitrogen cycle leads to N enrichment of surface waters, with consequences including eutrophication, hypoxia, harmful algae blooms and habitat degradation in lakes, rivers and coastal zones, and an increase in N_2O emissions (Howarth et al., 2002; Rabalais, 2002; Phoenix et al., 2006). Denitrification beds are a promising approach to reduce reactive N release from point source discharges into waterways. These denitrifying bioreactors are containers filled with wood by-products, where the wood acts as carbon and energy source for denitrifying microorganisms (Schipper et al., 2010), which convert NO_3^- to unreactive N gas *via* microbial denitrification (Warneke et al., 2011b).

A wide range of carbon substrates have been trialled in column studies to find appropriate media for bioreactors (Volokita et al., 1996a,b; Soares and Abeliovich, 1998; Della Rocca et al., 2005, 2006; Saliling et al., 2007; Gibert et al., 2008; Cameron and Schipper, 2010). Nitrate removal rates in column studies range from 3 g N m^{-3} d^{-1} (woodchips; Cameron and Schipper, 2010) to 96 g N m^{-3} d^{-1} (rice husk; Shao et al., 2008). The exceptionally high NO_3^- removal rates of many carbon substrates (e.g., rice husks, wheat straw, cotton) were attributed to a large organic carbon release in the startup phase of the columns, and were not sustainable over a longer time period (Cameron and Schipper, 2010). In a long-term study, barrels filled with maize cobs removed 3–6.5 times more NO_3^- – N than wood substrate, but also had higher carbon leaching in the effluent (Cameron and Schipper, 2010). Greenan et al. (2006) also reported that maize stalks produced greater NO_3^- removal than woodchips. However, little is known about the mechanism responsible for NO_3^- removal, the controlling factors, denitrifying bacterial communities or adverse effects, such as greenhouse gas release, when using different carbon substrates than woodchips. Warneke et al. (2011a, b) demonstrated that the mechanism responsible for NO_3^- removal in a full-scale woodchip bioreactor was microbial denitrification, and the removal process was limited by microbially available carbon and temperature. Smaller-scale studies have also determined that microbial denitrification is the dominant N removal mechanism, rather than dissimilatory NO_3^- reduction to ammonium DNRA or NO_3^- immobilization (Robertson, 2010; Greenan et al., 2006, 2009; Gibert et al., 2008).

Greenhouse gas (GHG) production during denitrification is an important issue to address when studying denitrification beds. An *in field* woodchip bioreactor study by Warneke et al. (2011a) yielded total N_2O release of 4.3% of removed NO_3^- – N, whereas Greenan et al. (2009) reported negligible release of dissolved N_2O in a woodchip column study. However, there have been no studies examining GHG production in denitrification beds containing different carbon sources.

So far, the population of denitrifying bacteria has not been investigated in substrates for use in denitrification beds. The abundance of denitrifying communities can be estimated by quantifying the functional gene copy numbers for nitrite reductase, *nirS* and *nirK*, and nitrous-oxide reductase, *nosZ.* These denitrification genes express reductase enzymes involved in denitrification. *NirS* expresses the cytochrome *cd*1-containing nitrite reductase (which catalyses the reduction of nitrite to nitric-oxide), *nirK* expresses the copper containing nitrite reductase, and *nosZ* expresses nitrous oxide reductase (which catalyses the reduction of N_2O to N_2) (Zumft, 1997; Braker et al., 1998). The two different genes for nitrite reductase, *nirS* and *nirK*, have coevolved to produce two independent pathways and

no denitrifier is known to contain both pathways (Philippot, 2002). Interestingly many denitrifying organisms have been shown to reduce NO_3^- only to nitrous oxide (Cheneby et al., 1998, 2004) and some, such as *Agrobacterium tumerfaciens* C58 do not possess nitrous oxide reductase (*nosZ*) (Wood et al., 2001). Many studies have shown that differences in the diversity and abundance of denitrifying bacterial genes were correlated to a variety of physical and chemical conditions; organic carbon in glacier foreland (Kandeler et al., 2006), temperature in constructed wetlands (Chon et al., 2010), water logging in rice paddy soils (Yoshida et al., 2009), organic or conventional fertilizer in agricultural soils (Dambreville et al., 2006; Enwall et al., 2005), native and cultivated soils (Stres et al., 2004), soil pH in grassland soils (Cuhel et al., 2010), nitrous-oxide emissions (Philippot et al., 2009) and NO_3^- concentration in woodlands with different vegetation (Lindsay et al., 2010). However, the diversity and abundance of denitrifying bacteria under consistent environmental conditions (e.g., same temperature, NO_3^- concentration, DO concentration, flow rate), but with different carbon substrates are poorly known.

This study followed a 2.5-year trial by Cameron and Schipper (2010), where different C substrates were compared for their ability to remove NO_3^- from water at two temperatures. The main objectives of the present study were to determine the limiting factors and the microbial mechanisms of the NO_3^- removal for different C substrates such as woodchips (Pine and Eucalyptus), sawdust, green waste, maize cobs and wheat straw in these barrels. The abundance of the denitrification functional *genes nirS, nirK* and *nosZ* were compared across replicate barrels, different temperatures and substrates. The factors affecting denitrifying communities were examined and whether NO_3^- removal could be predicted from the copy number of denitrification genes. Adverse effects, including production of N_2O and methane (CH_4), and total organic carbon (TOC) release, were also determined to evaluate the benefit of the different C substrates. These findings can be used to help select the appropriate carbon substrate for denitrifying bioreactors (denitrification beds and walls) to optimise NO_3^- removal, reduce GHG production, and maximize the lifetime of the bioreactor.

2. Materials and methods

2.1. Study site and substrate

The design of the experimental setup was fully described in Cameron and Schipper (2010). In this study, 24 experimental barrels (0.2 m^3) filled with six different carbon substrates and placed in a 7 m long shipping container were continuously loaded with a self-prepared NO_3^- solution (in average about15.8 mg L^{-1}; Table 1). Barrels were divided between a cold treatment (16.8 °C average outlet temperature) and a warm treatment (27.1 °C average outlet temperature), and every carbon source had two replicate barrels at each temperature. The selected substrates were: woodchips of *Pinus radiata* (soft wood), woodchips of *Eucalyptus* “Red Duke” (hardwood), sawdust (*P. radiata*), maize cobs, wheat straw and green waste (shredded and chipped miscellaneous shrubbery leaves and stems). The barrels had been loaded with NO_3^- solution for 2.5 years before samples were taken for this study.

2.2. Solute concentrations and NO_3^- removal rate

Water was sampled from the inflow and outflow tubing of the cold and warm barrels. Samples were filtered through disposable membrane filters (0.45 μm) and analysed for NO_3^-, NH_4^+ and NO_2^- using a flow injection analyser (Lachat Instruments; Loveland, USA) (APHA, 1992). TOC was determined from unfiltered water samples using a Shimadzu TOC-5000 analyser (Shimadzu Corp.; Kyoto, Japan). Temperature and DO of the inlet and

outlet of the barrels were measured with an InLab 605 O_2-Sensor (Mettler Toledo, Switzerland).

Nitrate removal rate was calculated as follows:

$NO_3^- - N$ removal rate=$\Delta NO_3^- - N \times FR \times V^{-1}$, where $\Delta NO_3^- - N$ was the difference of inflow and outflow $NO_3^- - N$ concentration, FR was the flow rate of the NO_3^- solution, and *V* was the volume of the barrel.

2.3. Greenhouse gas production

Water from the inlet and outlet of the barrels was collected in 3.7 mL exetainers (Labco, UK) for analysis of dissolved N_2O and CH_4 concentrations. The exetainers contained 0.2 mL H_2SO_4 (20%) to prevent further bacterial activity. After 12 h head-space equilibrium at room temperature, headspace gas samples were analysed for N_2O and CH_4 concentration using a gas chromatograph equipped with an electron capture detector and flame ionisation detector, respectively (Varian; Palo Alto, USA). Dissolved N_2O and dissolved CH_4 gas concentrations were calculated after Weiss and Price (1980) and Yamamoto et al. (1976) using the Bunsen coefficients. For N_2O analyses, the gas chromatograph was fitted with a Hayesep D column (3.6 m × 1/8″ × 2.0 mm). The column oven temperature was 80 °C, the ECD detector temperature was 300 °C, and the flow rate of the carrier gas (10% methane in argon) was 40 mL min^{-1}. For CH_4 analyses, the GC was equipped with a Hayesep Q column (8′ × 1/8″ SS; Q 80–100). The column oven temperature was 90 °C, the FID detector temperature was 150 °C, and the flow rate of the N_2-carrier gas was 30 mL min^{-1}.

2.4. Denitrification rates

Denitrification rates (DR) of the different carbon substrates in the barrels were determined using a modification of the denitrifying enzyme activity (DEA) method of Tiedje et al. (1989). Carbon substrate (600 g wet weight) from each barrel was collected using a gloved hand from the centre of the barrel and stored in plastic bags at 4 °C. Rubber gloves were changed after each sampling. Water samples (500 mL) from the outlet of each barrel were stored in 1 L plastic bottles at 4 °C. In the laboratory, the substrate and water samples were equilibrated to room temperature in a water bath. Carbon substrate (100 g wet weight) and water (60 g) from each barrel were added to four airtight bottles (600 mL). The headspace of the bottles was flushed with N_2 gas for 10 min prior to injection of 40 mL of acetylene (10% of the headspace volume), to inhibit reduction of N_2O to N_2. Each assay was amended with one of four solutions (all 5 mL): i) glucose (8 g L^{-1}; DR + C), ii) potassium NO_3^- (4 g L^{-1}; DR + N), iii) glucose and KNO_3^- (8 g L^{-1} and 4 g L^{-1} respectively; DR + C/N), and iv) no amendment (DR), to identify whether DR was C and/or NO_3^- limited. After bottles were incubated at 27 °C on a shaker table (100 rpm), headspace gas samples were collected through a rubber septum after 30, 40, 50 and 60 min using a syringe. Gas samples were stored in 3.7 mL exetainers (Labco, UK) until analysis for N_2O concentration within 7 days *via* GC-ECD (see above).

2.5. DNA extraction

Carbon substrates (400 mL) were sampled from the centre of each barrel, sealed in 500 mL airtight plastic containers and stored at −24 °C until frozen samples were vacuum freeze dried. Several trial DNA extractions were performed on the 6 types of reactor bed material. It was determined that the corn cobs, green waste and sawdust, performed best with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH) whereas the bulkier samples, woodchips and wheat straw performed better with the Mo Bio Ultra Clean Mega Prep Soil DNA kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The criteria for selecting an extraction method was based on the amount of DNA extracted per amount of material extracted and the

total number of 16S rRNA genes per gram dry material as determined by quantitative PCR (data not shown). The corn cobs, woodchips and wheat straw were reduced in size with a sterile scalpel and or scissors, so that they could fit in the initial extraction tube. The FastDNA® SPIN Kit for Soil was used to extract 0.05–0.1 g of corn cobs, 0.13–0.22 g green waste and 0.1–0.14 g of sawdust as per manufacturer instructions. The Ultra Clean Mega Prep Soil DNA kit was used to extract 2.27–2.65 g of pine woodchips, 0.45–0.69 g of wheat straw and 3.32–4.04 g of Eucalyptus woodchips as per manufacturer instructions. All samples were extracted in duplicate. The quantity of DNA extracted was quantified with a Qubit fluorometer (Life Technologies, Carlsbad, CA).

2.6. Quantitative PCR

Thermal cycling, fluorescent data collection, and data analysis were performed on an ABI Prism 7300 sequence detection system (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions using SYBR-green based detection. Initially, the DNA extractions for each sample type were diluted from 20- to 1000-fold to determine the optimum DNA concentration for QPCR. It was determined that a 200-fold dilution was required for all samples to dilute past PCR inhibitors that were coextracted (data not shown). QPCR reactions for *nirK*, *nirS* and *nosZ* and 16S rRNA contain 5 uL of template DNA, 0.5 μM of each forward and reverse primer except *nosZ* which used 1.5 uM of primer, 12.5 μL of 2× SYBR GreenER QPCR Super Mix (Life Technologies, Carlsbad, CA), in a total volume of 25 μL. The primers (5′-3′) used to detect the *nirK*, *nirS* and *nosZ* and 16S rRNA genes are *nirK*876 (ATY GGC GGV AYG GCG A) and *nirK*1040 (GCC TCG ATC AGR TTR TGG TT) (Henry et al., 2005) *nirSCd3aF* (AAC GYS AAG GAR ACS GG) and *nirSR3cd* (GAS TTC GGR TGS GTC TTS AYG AA) (Kandeler et al., 2006), *nosZ2F* (CGC RAC GGC AAS AAG GTS MSS GT) and *nosZ2R* (CAK RTG CAK SGC RTG GCA GAA) (Henry et al., 2006), 341F (CCT ACG GGA GGC AGC AG) and 534R (ATT ACC GCG GCT GCT GGC A, also referred to as 515R) 16S rRNA primers (Lopez-Gutierrez et al., 2004) respectively. The conditions for *nirK* and *nirS* real-time PCR are 10 min at 95 °C for enzyme activation; afterwards six touchdown cycles are performed: 15 s at 95 °C for denaturation, 30 s at 63 °C for annealing, and 30 s at 72 °C for extension. The annealing temperature is progressively decreased by 1 °C down to 58 °C. Finally, a last cycle with an annealing temperature of 58 °C is repeated 40 times with the addition of a data acquisition step of 30 s at 80 °C after the extension phase. One last step of 95 °C for 15 s, 60 °C for 30 s and 95 °C for 30 s is added to obtain a specific denaturation curve. The thermal cycling conditions for *nosZ* are similar except for the annealing temperature, which is 65 °C for 30 s for the first 6 cycles and 60 °C for 15 s for the 40 cycles. 16S rRNA QPCR was performed with no touch-down cycle, just one annealing temperature at 60 °C for 30 s and only 35 cycles instead of 40. Purity of amplified products was checked by the observation of a single peak during the dissociation analysis. Copy Numbers were determined by using a standard curve obtained with serial plasmid dilutions of a known amount of plasmid DNA containing a fragment of the 16S rRNA gene, *nirK*, *nirS* and *nosZ* gene. Each DNA extraction was analyzed for each gene in triplicate along with three non-template controls. Denitrification gene copy numbers are reported as copies per gram dry material and also reported as normalized to 16S rRNA gene copies. The nitrite reductase to nitrous oxide reductase ratio (Σ*nir*/*nos*) was determined by summing the *nir* genes (*nirS* + *nirK*) and dividing the sum by the *nos* genes and was used as an indication of nitrous-oxide producing potential. To determine if each environment selected more for *nirS* or *nirK*, the ratio of *nirS/nirK* was also calculated. The authors acknowledge that novel bacterial sequences were likely missed by the 16S rRNA primers used in this study which might have resulted in an underestimation of the community size in our soil, which subsequently led to the calculation of higher relative abundances of *nirS* functional genes.

2.7. Respirable C

Respirable C was measured, as an index of the availability of C to microorganisms, using a modified alkali trap method of Cheng and Coleman (1989). Carbon substrates (100 g wet weight) and effluent (60 g) from each barrel were added to airtight bottles (600 mL). Small beakers (30 mL) filled with 10 mL of 0.5 M KOH were placed into the jars to trap CO_2. After sealing the bottles, the headspaces were flushed with N_2 gas for 10 min and incubated at room temperature (22 °C) for 4 days. After incubation, 5 mL of the CO_2 trapping solution were removed from the bottles and mixed with 10% $BaCl_2$ solution (10 mL) and phenolphthalein (pH indicator) in 100 mL flasks. After back-titration of these solutions against the standard 0.1 M HCl to determine the amount of trapped CO_2, respirable carbon was expressed as CO_2–C g^{-1} carbon substrate (dry weight).

2.8. Statistical analysis

Similarities and differences of nitrite reductase gene copies (Σ*nir*) per gram carbon substrate, *nirS*/*nirK* and *nir*/*nosZ* were evaluated calculating the Wald confidence interval (95%) of these gene copy numbers for each barrel (data not shown). Associated errors of the results are reported as ±standard errors.

3. Results

3.1. Solute concentrations

The average temperature of the cold and warm incubation outlet was 16.8 °C and 27.1 °C respectively, and used as calculation basis for determining the Q_{10}. Q_{10} is the factor of the reaction rate increase with every 10 °C rise in temperature. The inlet NO_3^- – N concentration of the cold barrels was 14.4 ± 0.6 mg L^{-1}, and for the warm barrels was 17.2 ± 1 mg L^{-1}. The average flow rates of the cold and warm barrels were 48.3 ± 2.0 ml min^{-1} and 58.5 ± 2.3 ml min^{-1}, respectively. Nitrite–N concentrations in the outflow were always below 0.2 mg L^{-1} for cold barrels and ranged from 0.08 to 0.82 mg L^{-1} for warm barrels (Table 1). In the cold incubations wheat straw, green waste and sawdust, and in the warm incubations all the carbon substrates, except green waste, released NH_4^+ ranging from 0.03 to 0.79 mg L^{-1} (Table 1). All the barrels showed a slight decrease in pH at the outflow (Table 1). DO decreased from 7.1 mg L^{-1} (inlet concentration) to below 1.9 mg L^{-1} (outlet concentration) in cold barrels, and from 5.9 mg L^{-1} to below 1.3 mg L^{-1} in warm barrels (Table 1). TOC was released in high concentrations from the cold incubated maize cobs (70.2 and 76.8 mg L^{-1}) and wheat straw (16.5 and 11 mg L^{-1}), and in the warm incubated maize cobs (9.5 and 9.7 mg L^{-1}). However other carbon substrate barrels released either low concentrations, or consumed, TOC (Table 1).

3.2. Nitrate removal and controlling factors of denitrification

Nitrate–N removal rates ranged from 1.3 (soft woodchip barrel 2) to 6.2 g N m^{-3} d^{-1} (maize cobs barrel 2), and were dependent on temperature with a Q_{10} of 1.2 ± 0.13 (Fig. 1). Maize cobs, wheat straw and green waste showed the highest NO_3^- – N removal rates, ranging from 4.3 g N m^{-3} d^{-1} (green waste) to 5.7 g N m^{-3} d^{-1} (maize cobs) in cold barrels, and from 4.5 g N m^{-3} d^{-1} (wheat straw) to 6.0 g N m^{-3} d^{-1} (maize cobs) in warm barrels (Fig. 1).

Nitrate–N removal increased linearly with the *in vitro* denitrification rate DR + C/N for cold and warm incubation ($y = 0.16x + 1.6$; $R^2 = 0.63$; $p = 0.002$ and $y = 0.24x + 2.9$; $R^2 = 0.65$; $p = 0.001$ respectively; where y=NO_3^- – N removal rate in g N m^{-3} d^{-1} and x = DR + C/N in μg N h^{-1} g^{-1}) (Fig. 2). Furthermore, the NO_3^- – N removal rate depended on the available carbon content as shown in three ways. Nitrate–N removal rate was linearly correlated with respirable carbon for both cold and warm incubated carbon substrates ($y = 0.08x + 1.6$; R^2 =

0.82; $p < 0.001$ and $y = 0.15x + 1.2$; $R^2 = 0.62$; $p = 0.002$ respectively; where y=NO_3^- – N removal rate in g N m^{-3} d^{-1} and x = respirable carbon in mg C g^{-1} d^{-1}) (Fig. 3). *In vitro* measured DR could be enhanced with a glucose amendment for all carbon substrates, except for maize cobs and wheat straw in cold and warm barrels. DR in cold incubated maize cobs and wheat straw were NO_3^- limited (NO_3^- – N concentration <1 mg L^{-1}) (Fig. 4; Table 1) and DR in warm incubated maize cobs and wheat straw were not limited by glucose or NO_3^-, except for one warm barrel of maize cobs (MC1), which was also limited by glucose (Fig. 4). Nitrate amended DR (DR + N) was also significantly correlated with respirable carbon for cold and warm incubations ($y = 0.38x - 1.3$; $R^2 = 0.70$; $p < 0.001$ and $y = 0.37x - 4.3$; $R^2 = 0.48$; $p = 0.013$ respectively; where y = DR + N in μg N h^{-1} g^{-1} and x = respirable carbon in mg C g^{-1} d^{-1}) (Fig. 3B).

3.3. Copies of denitrification genes (*nirS*, *nirK* and *nosZ*)

The abundance of *nirS*, *nirK* and *nosZ* ranged from $8.7 \pm 0.8 \times 10^6$ (pine woodchips) to $1.6 \pm 0.01 \times 10^{10}$ (green waste) copies of *nirS* g^{-1} dry substrate, $0.7 \pm 0.1 \times 10^6$ (pine woodchips) to $6.8 \pm 0.1 \times 10^9$ (maize cobs) copies of *nirK* g^{-1} dry substrate, and $1.2 \pm 0.1 \times 10^6$ (pine woodchips) to $9.0 \pm 0.2 \times 10^9$ (maize cobs) copies of *nosZ* g^{-1} dry substrate for cold incubations (Table 2). Abundance of *nirS*, *nirK* and *nosZ* in warm incubated substrate ranged from $2.0 \pm 0.1 \times 10^7$ (pine woodchips) to $1.3 \pm 0.04 \times 10^{11}$ (maize cobs) copies of *nirS* g^{-1} dry substrate, $7.4 \pm 0.4 \times 10^6$ (pine woodchips) to $1.5 \pm 0.07 \times 10^{10}$ (maize cobs) copies of *nirK* g^{-1} dry substrate, and $6.5 \pm 0.3 \times 10^6$ (pine woodchips) to $1.9 \pm 0.02 \times 10^{10}$ (maize cobs) copies of *nosZ* g^{-1} dry substrate (Table 2). The NO_3^- removal rate increases exponentially with the total copy number of nitrite reductase genes (Σ*nir*) per gram substrate and was significantly linearly correlated with the ln (Σ*nir* g^{-1} substrate) in cold and warm barrels ($y = 0.45x - 5.62$; $R^2 = 0.48$; $p = 0.012$ and $y = 0.38x - 3.68$; $R^2 = 0.73$; $p < 0.001$ respectively; where y=NO_3^- – N removal rate in g N m^{-3} d^{-1} and x = ln (copies Σ*nir* g^{-1} substrate) (Fig. 5). Generally, the copies of Σ*nir* were greater in warm than in cold barrels, except for sawdust. A temperature increase of 10 °C yielded 4-fold increases in Σ*nir* (Fig. 6A).

The carbon substrates maize cobs and green waste had the greatest bacterial population ranging from$18592.3 \pm 919.1 \times 10^6$ copies of 16S rRNA g^{-1} dry substrate (warm incubated green waste) to $96761.9 \pm 2649.3 \times 10^6$ copies of 16S rRNA g^{-1} dry substrate (cold incubated maize cobs), and the greatest Σ*nir* per gram carbon substrate (Table 2). In contrast, nitrite reductase gene copies (Σ*nir*) normalized to total bacteria (16S rRNA genes) of these substrates (maize cobs and green waste) were at the lower end of the data generated in this study, ranging from 0.1 ± 0.00 (cold incubated maize cobs) to 2.82 ± 0.11 copies Σ*nir* copies^{-1} 16S rRNA g^{-1} dry substrate (warm incubated maize cobs) (Fig. 6B). Cold incubated pine woodchips had the highest Σ*nir* copy number normalized to total bacteria (7.29 ± 0.58 and 7.89 ± 0.07 copies Σ*nir* copies^{-1} 16S rRNA g^{-1} dry substrate), followed by eucalyptus woodchips for cold incubations (Fig. 6B).

In order to estimate how the abundance of the different genes in the denitrifying pathway changed with respect to the other steps in denitrification, the ratios of copies of *nirS*/*nirK*, and Σ*nir*/*nosZ* (nitrous oxide reductase) were determined (Fig. 7). Increasing temperature increased the ratio of *nirS*/*nirK*, and Σ*nir*/*nosZ*, except for pine woodchips.

For cold incubations the ratios of *nirS*/*nirK* within the same carbon substrate (replicates) were not different from each other applying the Wald confidence interval (95%), except for pine wood. The same was observed for the ratios of Σ*nir*/*nosZ* within the same carbon substrate in cold barrels, whereas in warm barrels differences in ratios of *nirS*/*nirK*, or Σ*nir*/

nosZ were shown for each carbon source, except for *nirS*/*nirK* ratios of warm incubated green waste and sawdust barrels (Fig. 7).

3.4. Greenhouse gases

The inlet concentrations of dissolved N_2O–N were below the detection limit (<1.1 μg L^{-1}). Therefore the measured dissolved N_2O–N and CH_4 concentrations in the outlet water of the barrels are the net dissolved N_2O–N release from the barrels in the outlet water. The dissolved N_2O–N release from the cold barrels in the outlet ranged from below detection limit (sawdust) to 214.5 μg L^{-1} (wheat straw) and from the warm barrels from below detection limit (sawdust) to 1472.5 μg L^{-1} (wheat straw). Wheat straw was the largest source of N_2O for both cold and warm incubations, followed by green waste in warm incubations. Warm wheat straw barrels released almost 10% of the removed NO_3^- – N as dissolved N_2O–N in the outlet water. All substrates at the warmer temperature released on average about seven times more dissolved N_2O–N in the outlet than cold barrels (Fig. 8).

The inlet concentration of dissolved CH_4 was 5.4 μg CH_4 L^{-1} for cold and 16.8 μg CH_4 L^{-1} for warm barrels. There was little net dissolved CH_4 release in the outlet of woodchips (hard and soft wood) and sawdust (<40 μg L^{-1}) detected. Wheat straw released some dissolved CH_4 in the outlet water at cold incubations (139 μg L^{-1} and 1201 μg L^{-1}) and maize cobs released large amounts of dissolved CH_4 at cold incubations (10,600 μg L^{-1} and 7375 μg L^{-1}) in the outlet of the barrels, but less dissolved CH_4 at warm incubation. Barrels of green waste released dissolved CH_4 in the outlet from cold and warm barrels, with an average of 2970 μg L^{-1} and 3870 μg L^{-1}, respectively (Fig. 8).

4. Discussion

In this study, several different carbon substrates (maize cobs, wheat straw, green waste, sawdust, hardwood and softwood) receiving NO_3^- from a simulated household effluent (inlet NO_3^- concentration between 14 and 18 mg L^{-1}) were examined to determine factors controlling NO_3^- removal and the extent of possible adverse effects. The denitrifying bacterial communities in the different barrels were also examined to determine whether microbial community structure could account for differences in activity (NO_3^- removal, dissolved GHG concentrations). The experimental barrels had been operating for 2.5 years prior to these measurements, thereby eliminating short term study effects (i.e., high TOC release coupled with high NO_3^- removal rates), as have been described in other column and barrel studies (Greenan et al., 2009; Cameron and Schipper, 2010; Soares and Abeliovich, 1998). In our study a single sampling was taken. However, we consider that steady state had been reached in the microbial community, which allow comparisons between substrates; e.g., Warneke et al. (2011a) found only very small differences in dissolved N_2O and CH_4 concentrations along the length of a field-scale woodchip bioreactor during a sampling period of one year.

4.1. Nitrate removal and microbial processes

The mean NO_3^- – N removal rates of the experimental barrels were less than the NO_3^- – N removal rates reported by Cameron and Schipper (2010) in the same experimental barrels for the previous 2.5 years, and less than the reported rates of most other column studies with alternative carbon substrates (Gibert et al., 2008; Saliling et al., 2007; Greenan et al., 2006; Della Rocca et al., 2005; Shao et al., 2008; Soares and Abeliovich, 1998). These lower NO_3^- removal rates were most likely due to the age of the carbon material (>2.5 years in use) and the 10-fold lower NO_3^- – N inlet concentration than used by Cameron and Schipper (2010). For example, in this study, NO_3^- – N removal rates of cold incubated maize cobs and wheat

straw were clearly limited by NO_3^- – N concentrations (NO_3^- – N outlet concentrations <1 mg L^{-1}; Table 1). Nitrate removal rates of pine and eucalyptus woodchip and sawdust ranged from 1.3 to 4.4 g N m^{-3} d^{-1} and were at the lower end of removal rates determined for woodchip bioreactors in the field (Schipper et al., 2010). Maize cobs, followed by wheat straw and green waste, exhibited a higher NO_3^- removal rate than wood substrates in this study, as also reported by Cameron and Schipper (2010) for the same experimental system. However, the NO_3^- – N removal rates for wood substrates in this study were in the same range as the NO_3^- – N removal rates (3.9 g N m^{-3} d^{-1}) measured by Greenan et al. (2009) in a column study. Other column studies with wood-chips showed NO_3^- – N removal rates 2–10 times higher than this study (Robertson, 2010; Saliling et al., 2007).

As expected, there was good evidence that the mechanism for NO_3^- – N removal in the substrates was most likely microbial denitrification, because the measured *in vitro* DR + C/N of each experimental barrel were higher than many other NO_3^- reducing ecosystems e.g., denitrification walls (Schipper et al., 2005; Moorman et al., 2010), forested land-based wastewater treatment system (Barton et al., 2000), riparian forest sites (Groffmann et al., 1992), a natural wetland and a constructed wetland (Duncan and Groffmann, 1994). Additionally, nitrite reductase genes (*nirS* and *nirK*), which are responsible for the second step of denitrification, were on average more abundant in this study (Table 2) than in constructed wetlands (Chon et al., 2010), or rice fields (Yoshida et al., 2009). Furthermore, the significant linear relationship of the increase of NO_3^- removal, and the increase of measured DR + C/N, indicated that microbial denitrification was responsible for the NO_3^- – N removal, regardless of the carbon substrate in the experimental barrels and showed that the acetylene inhibition method was a good measure for comparative NO_3^- removal estimations between C substrates (Fig. 2).

Although seven of the 12 cold barrels, and eight of the warm barrels produced small amounts of NH_4^+, neither anammox or DRNA appeared to be significant contributors to NO_3^- removal, because of the low NH_4^+ – N concentration (<0.8 mg L^{-1}) at the outlet. Both Gibert et al. (2008) and Greenan et al. (2006) also suggested that DNRA is a minor process involved in NO_3^- removal (less than 5%).

As NO_3^- was depleted in the cold incubated maize cobs and wheat straw barrels, methanogenic bacteria were able to compete successfully with denitrifiers for carbon as suggested by the high dissolved CH_4 production of cold incubated maize cobs and wheat straw barrels. Although NO_3^- – N concentrations were above 2 mg L^{-1} in the outlet of cold green waste barrels and warm maize cobs and green waste barrels, we observed dissolved CH_4 production (Table 1, Fig. 8), which suggests that methanogenes may occur even at relatively moderate NO_3^- concentrations. It is likely that once the microbial consumption of NO_3^- exceeded diffusion of NO_3^- within the carbon substrate, methanogenes could develop in the interior of the substrate.

4.2. Factors controlling NO_3^- removal

In general, denitrification is primarily controlled by carbon availability, NO_3^-, NO_2^-, sulphide, temperature, DO, and the number of denitrifiers (Firestone and Davidson, 1989; Seitzinger et al., 2006). In this study, carbon availability and temperature were identified as the main factors limiting nitrate removal in the experimental barrel systems, when NO_3^- concentrations were more than 1 mg L^{-1}; below this concentration NO_3^- limited denitrification.

The warm barrels removed more NO_3^- than the cold barrels, with a Q_{10} factor of 1.2 ± 0.13 (Fig. 1). Cameron and Schipper (2010) found a greater temperature dependence of NO_3^- removal (Q_{10} = 1.6) in the same experimental system, but these measurements were made with 10 times higher NO_3^- inlet concentrations, whereas in the present study NO_3^- limited in some barrels the NO_3^- removal. Studies of woodchip bioreactors by Robertson et al. (2008), Elgood et al. (2010) and Warneke et al. (2011a), also determined higher Q_{10}s than in the present study.

In most of the other experimental barrels, carbon amendment (glucose) increased the denitrification activity (Fig. 4), as reported by Warneke et al. (2011a) for a field-scale woodchip bioreactor. Furthermore, NO_3^- removal and the denitrification rate (DR + N; removing NO_3^- limitation) were found to increase linearly with the availability of carbon (measured as respirable carbon, Fig. 3). Therefore, nitrate removal in the experimental barrels was most likely limited by carbon availability, except for cold maize cobs and cold wheat straw barrels. Nitrate removal in cold maize cobs and cold wheat straw barrels was limited by NO_3^- likely due to low NO_3^- – N outlet concentrations below 1 mg L^{-1} (Fig. 4; Table 1). These findings confirm that in anaerobic, NO_3^- rich environments, carbon limits microbial denitrification (Knowles, 1982; Reddy et al., 1982). This study shows that respirable carbon measurements could also be used to make comparative estimations of NO_3^- removal in carbon limited systems (Fig. 3).

In this study, the pH decreased slightly from inlet to outlet as found in other studies (Van Driel et al., 2006; Robertson et al., 2005; Robertson and Merkley, 2009), but was still in the optimal range for denitrifiers (Bremner and Shaw, 1958; Knowles, 1982). In contrast Warneke et al. (2011a) reported an increase in pH along the length of a field-scale woodchip bioreactor.

DO concentrations decreased from above 6 mg L^{-1} at the inlet, to below 2 mg L^{-1} at the outlet. Robertson (2010) also measured a similar decrease in DO in a woodchip column study and that a substantial portion of microbially available carbon was consumed by aerobic respiration. However, Gibert et al. (2008) measured declines in DO from 4 to 1.2 mg L^{-1} in the first 10 cm of a 90 cm long woodchip column. This fine-scale work suggested that most of the substrate close to the inlet served to provide anaerobic conditions for denitrifiers.

The NO_3^- removal rate was significantly correlated to the copy number of nitrite reductase genes (*nirS* and *nirK*) (Fig. 5). Furthermore the average nitrite reductase gene copies per gram dry substrate increased 4-fold with a temperature increase of 10 °C (Fig. 6A), but the NO_3^- – N removal rate increased 1.2 times. This temperature dependence of denitrification genes corresponds with seasonal measurements of nitrite reductase gene copies in wetlands (Chon et al., 2010). The copies of 16S rRNA genes also increased with temperature, with the exception of the sawdust barrel (Table 2), so the greater copy number of denitrification genes in the substrate at higher temperature was also partially due to an increase in bacterial biomass.

4.3. Denitrifying bacterial communities

Abundance of *nirS*, *nirK* and *nosZ* genes in maize cob, green waste, sawdust and wheat straw ranged from 10^7 to 10^{11} copies g^{-1} dry substrate (Table 1, Fig. 6A), and these values were on average greater than those measured in constructed wetlands or rice fields (Chon et al., 2010; Yoshida et al., 2009). However, the abundance of denitrification genes in pine and eucalyptus woodchips were slightly lower, but in the same range as the wetland and rice

field studies (Chon et al., 2010; Yoshida et al., 2009). But woodchips, especially those from cold incubations, showed the greatest abundance of nitrite reductase genes as a proportion of total bacterial DNA (16S rRNA), coupled with low 16S rRNA gene copies (Table 2, Fig. 6B). Green waste and maize cobs, particularly cold incubated maize cobs, had a low copy number of denitrification genes as a proportion of total bacteria, and gave high 16S rRNA gene copies (Table 2, Fig. 6B). Therefore, the bacterial community in green waste and maize cob barrels had a low ratio of denitrifying genes per copy number of 16S rRNA genes even though green waste and maize cobs had on average more denitrifiers per gram substrate than woodchips (Table 2, Fig. 6). Consequently, a substantial proportion of carbon in green waste and maize cob barrels was likely consumed by non-denitrifying bacteria, fungi and/or yeasts, whereas a greater proportion of C released from woodchips appeared to be consumed by denitrifiers.

The ratios of *nirS*/*nirK*, and Σ*nir*/*nosZ*, were similar between replicate barrels in cold incubations, except for pine wood barrels (Fig. 7). In warm incubations, there was much greater variation in replicates, and the ratios of *nirS*/*nirK*, and *nir*/*nosZ*, varied significantly among carbon substrates (Fig. 7). Therefore we assume that it was likely that the composition of denitrifying bacteria in replicate barrels under cold incubation was very similar, but in warm barrels the denitrifying population varied greatly between replicates. Furthermore it is likely that the composition of denitrifier was also very distinct in different carbon substrates, in both warm and cold barrels.

At warm temperatures, the *nirS*/*nirK* ratio increased (except in one pine woodchip barrel), suggesting that higher temperature enhanced growth of *nirS* containing bacteria, or did not encourage the growth of *nirK* containing bacteria (Fig. 7). The *nirS/nirK* has been shown to be greater in unfertilized soils, compared to those that were fertilized (Hallin et al., 2009). The ratio also decreased with the presence of cattle and increased with increasing nitrate, pH and soil moisture (Philippot et al., 2009). Similar temperature dependence was observed with the nitrite reductase/nitrous oxide reductase gene ratio (Σ*nir/nosZ*). The Σ*nir/nosZ* was significantly higher in warm barrels than in cold barrels (Fig. 7). This finding corresponded with the higher N_2O concentrations in warm barrels compared to cold barrels, and the observed increase in N_2O emission at higher temperatures in previous studies (Warneke et al., 2011a; Teiter and Mander, 2005; Johansson et al., 2003). High N_2O fluxes have been shown to correlate with a low ratio of *nosZ/narG*, where *narG* is the gene responsible for nitrate reduction the first step in the denitrification pathway (Philippot et al., 2009). Similarly, a high ratio of $N_2O/N_2O + N_2$ has also been shown to correlate with the Σ*nir/nosZ* ratio (Cuhel et al., 2010).

4.4. Evaluation of the different carbon substrates

Maize cobs, wheat straw and green waste barrels removed more NO_3^- than wood substrates. The dissolved N_2O–N production of maize cobs, green waste and wood-filled barrels was moderate and the dissolved N_2O–N outlet concentrations ranged from 7 to 110 μg L^{-1} for cold barrels, and from 207 to 566 μg L^{-1} for warm barrels. Wheat straw produced on average about three times more dissolved N_2O (Fig. 8) than other carbon substrates. This corresponded with the relatively high ratio of nitrite reductase gene copies to nitrous oxide reductase gene copies (Σ*nir*/*nosZ*) in the wheat straw barrels (Fig. 7), which lead likely to more N_2O production than N_2O consumption. The N_2O–N release from wheat straw in the effluent was almost 10% of the removed NO_3^- – N, which is also about three times greater than the dissolved N_2O–N release of a field-scale wood chip denitrification bed (Warneke et al., 2011a). Only sawdust showed no N_2O release.

Maize cobs had the highest NO_3^- removal rate and were additionally limited by NO_3^- concentration. Therefore, a higher NO_3^- removal rate could be expected for maize cobs if it was loaded with more NO_3^- as shown by Cameron and Schipper (2010). However, maize cobs also released high concentrations of TOC and dissolved CH_4. It would be expected that CH_4 release from maize cobs in the outlet water would decrease with a higher NO_3^- concentration in inlet water because denitrification would outcompete methanogenesis. Additionally maize cobs had a low denitrifier/bacteria ratio, which would probably yield substantial carbon loss due to carbon consumption by non-denitrifiers, whereas woodchips seemed to be an ideal substrate for denitrifying bacteria. Furthermore, wood substrate showed moderate NO_3^- removal rates, with almost no adverse effects. As demonstrated in previous studies (Warneke et al., 2011a; Schipper et al., 2010; Robertson, 2010; Long et al., 2011) woodchips provide sustained NO_3^- removal due to slow decomposition of wood in the bioreactor.

5. Conclusions

This study suggested that microbial denitrification was the main mechanism for nitrate removal for all carbon sources tested, due to the high *in vitro* DR, the linear relationship between NO_3^- removal and *in vitro* DR + C/N, high abundance of nitrite reductase genes, and uniformly low NH_4^+ concentrations.

The denitrification process in the experimental barrels was limited by carbon availability and temperature, except when NO_3^- – N outlet concentrations were below 1 mg L^{-1}, when NO_3^- – N limitation occurred. The NO_3^- – N removal rate was dependent on the quantity of microbially available carbon, which varied between carbon sources. Both the acetylene inhibition method for measuring denitrification activity, and the quantification of denitrification genes were goodapproaches for determining comparative NO_3^- removal in carbon limited systems (Figs. 3 and 5). It would be useful to determine and compare the slope of the linear regressions between NO_3^- removal and Ln (Σ*nir* g^{-1} substrate) in different ecosystems to estimate the nitrate removal rates only by the copy number of nitrite reductase genes in similar ecosystems (Fig. 5).

Greatest dissolved N_2O release in the outlet water was detected for wheat straw and was about 10% of the removed NO_3^- – N, which was much greater than reported in previous studies for wood substrates. Methanogenesis could compete with denitrification when NO_3^- – N concentrations were below 2 mg L^{-1} and Σ*nir*/*nosZ* ratio was high.

Maize cobs had the highest NO_3^- – N removal rate, but released elevated amounts of TOC, and substantial carbon consumption by non-denitrifiers was likely. Wood substrates exhibited moderate and sustained NO_3^- removal, and appeared to be ideal for denitrifiers under anaerobic, high NO_3^- conditions. Therefore it may be useful to combine maize cobs with woodchips, to enhance C availability and increase the denitrifying activity in the woodchip material. This approach would possibly generate higher NO_3^- – N removal rates than woodchips alone, with only moderate adverse effects. Furthermore, findings in this study suggest that increased temperatures enhance the growth of *nirS*-containing and *nosZ*-lacking bacteria, but further research is needed to understand this effect.

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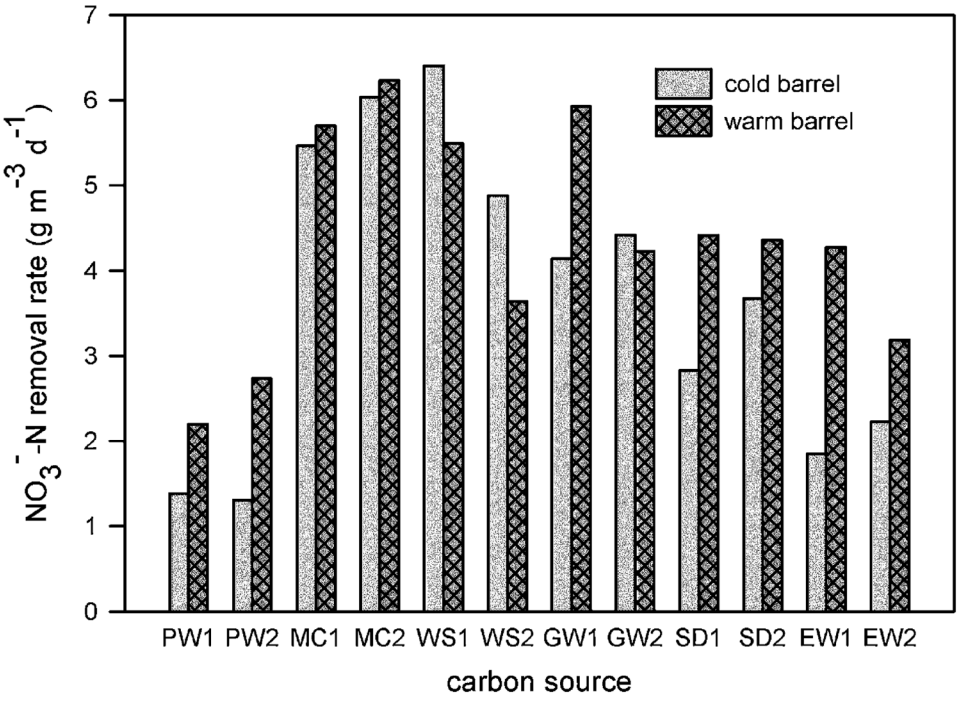

Fig. 1.

Nitrate removal rates for different carbon substrates in cold (16.8 °C) and warm (27.1 °C) barrels. PW1 and PW2, soft woodchips (pine), replicates; MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, hard woodchips (eucalyptus).

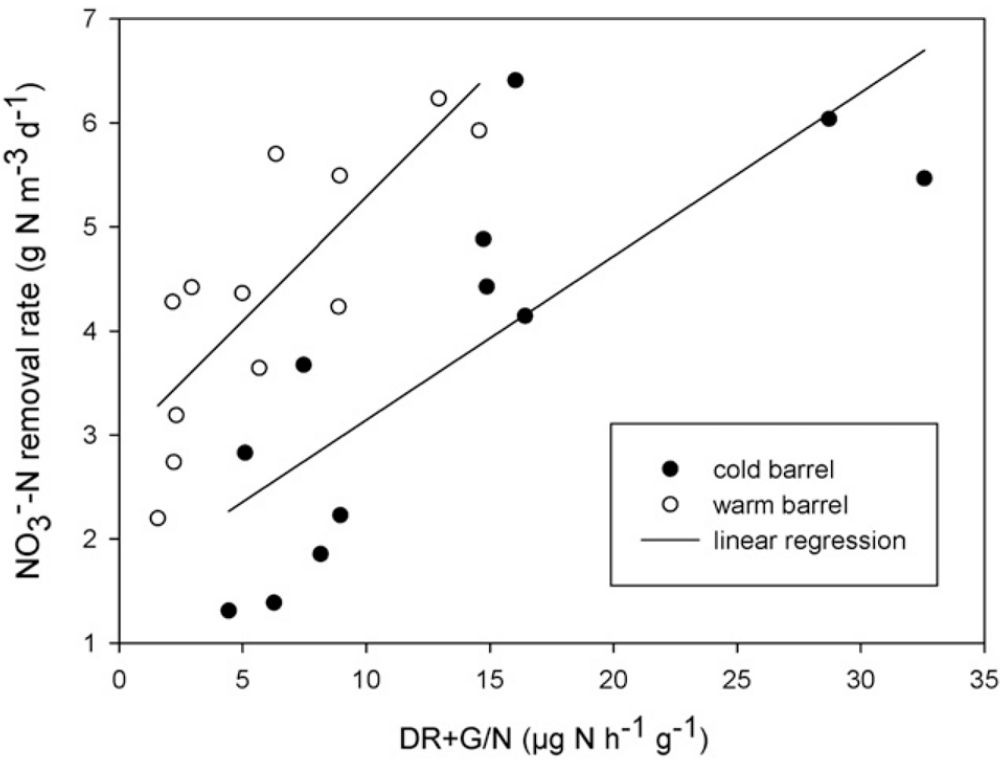

Fig. 2.

Nitrate–N removal rate as a function of *in vitro* DR amended with glucose and nitrate (DR + G/N) for cold and warm incubated substrate. Linear regression statistics are reported in text.

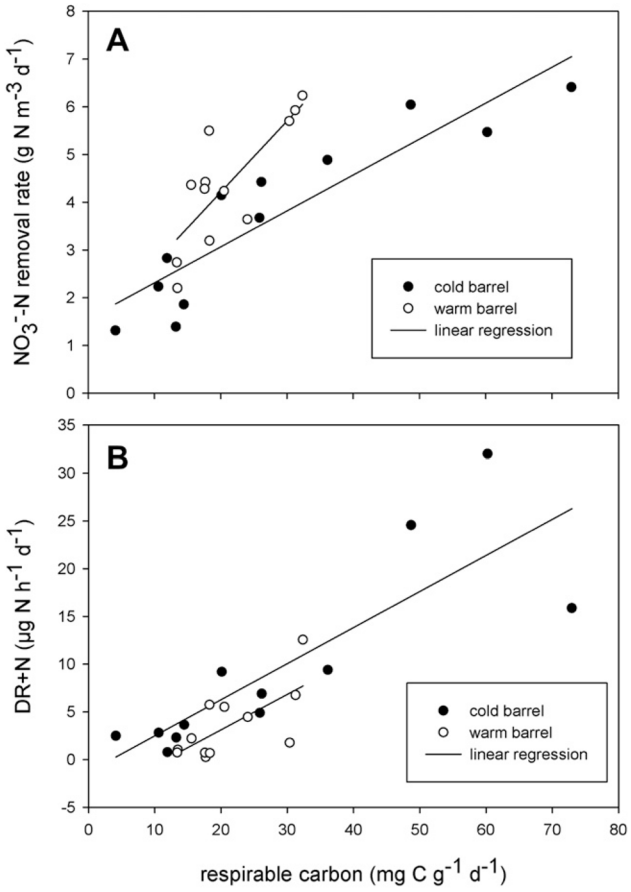

Fig. 3.

Nitrate–N removal rate (A) and *in vitro* DR amended with nitrate (DR + N) (B) as a function of respirable carbon for cold and warm incubated substrate. Linear regression statistics are reported in text.

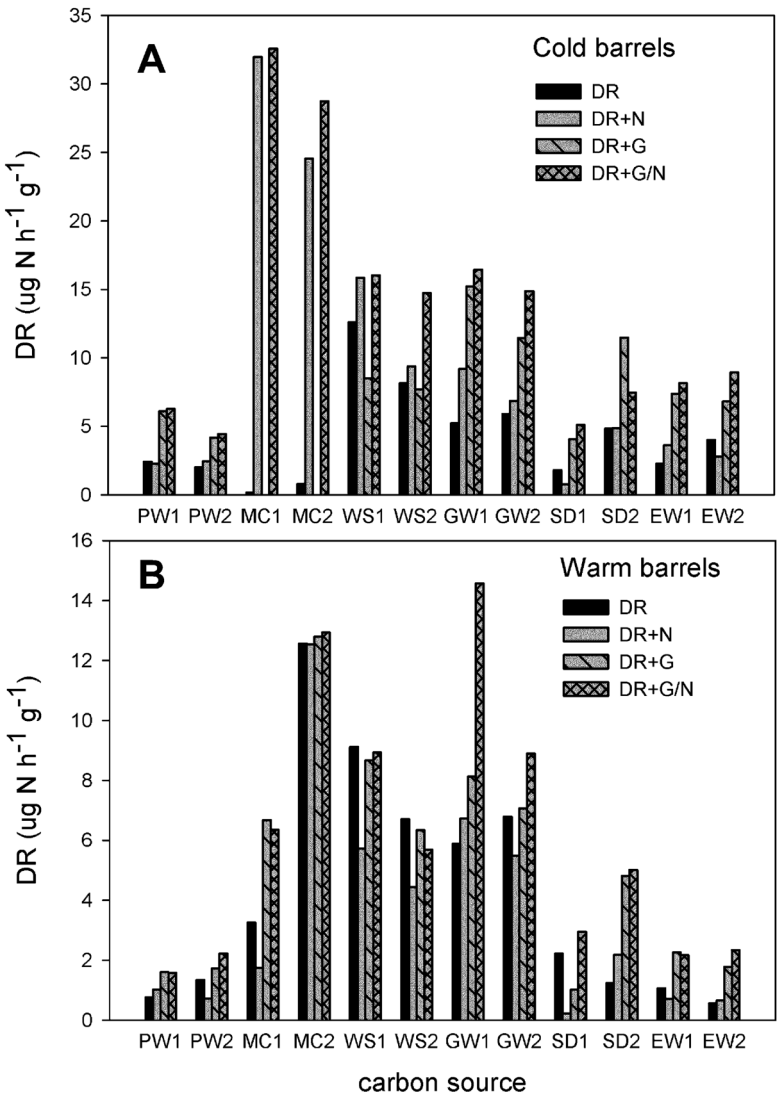

Fig. 4.

In vitro denitrification rates (DR) at 27 °C for different carbon substrates in cold (A) and warm (B) barrels. DR assays were amended with glucose (DR + C), NO_3^-, glucose and NO_3^- (DR+C/N), and none amended (DR). PW1 and PW2, soft woodchips (pine); MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, hard woodchips (eucalyptus).

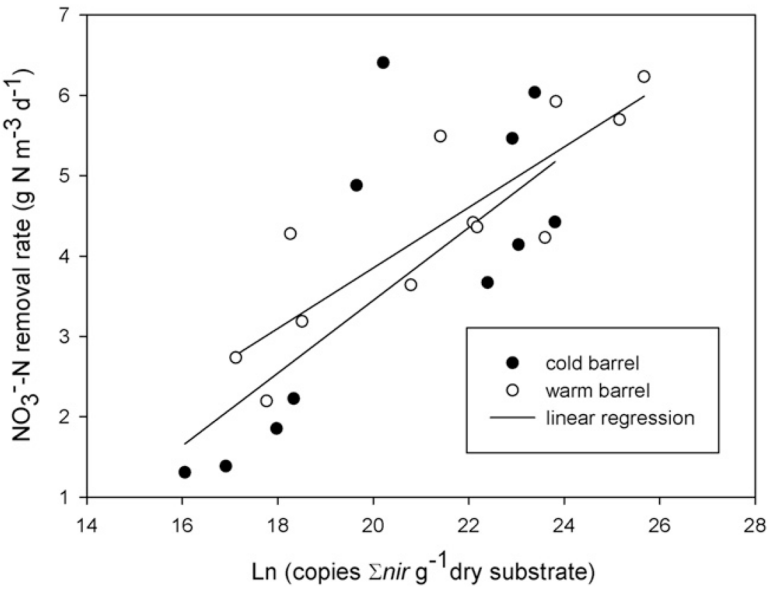

Fig. 5.

Nitrate–N removal rate as a function of total nitrite reductase gene (Σ*nir*) copies for cold and warm incubated substrates. Linear regression statistics are reported in text.

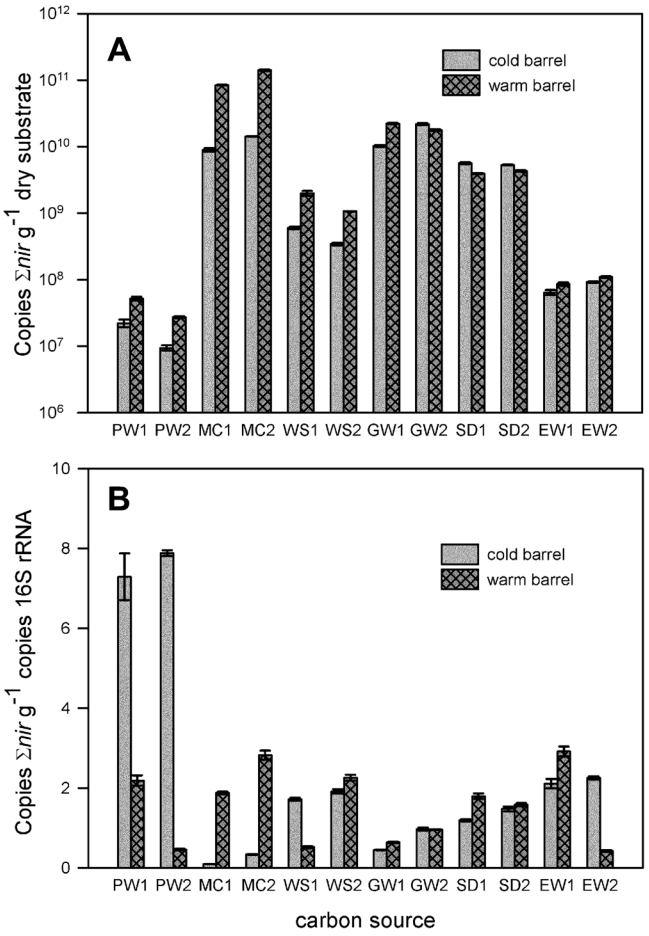

Fig. 6.

Total number of nitrite reductase genes (Σ*nir*) normalized per gram carbon substrate (A) and normalized to total bacteria (16S rRNA) (B) of the different carbon substrates used in the barrels under cold and warm incubation. PW1 and PW2, pine woodchips; MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, eucalyptus woodchips. Error bars are one standard error ($n = 3$).

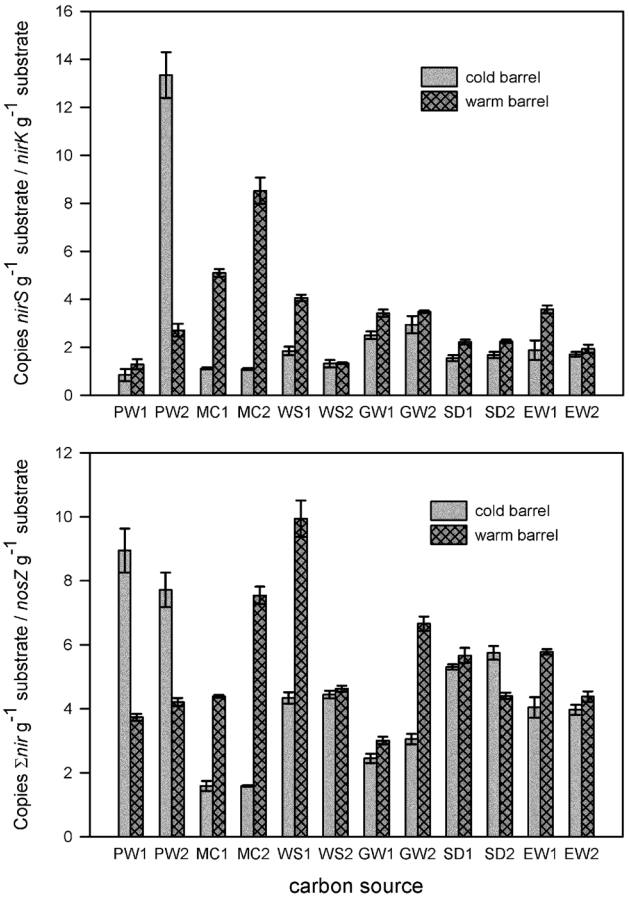

Fig. 7.

Ratios of gene copies of *nirS*/*nirK* (A) and total nitrite reductase (Σ*nir*) to nitrous oxide reductase (*nosZ*) (B). PW1 and PW2, pine woodchips; MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, eucalyptus woodchips. Error bars are one standard error (n = 3).

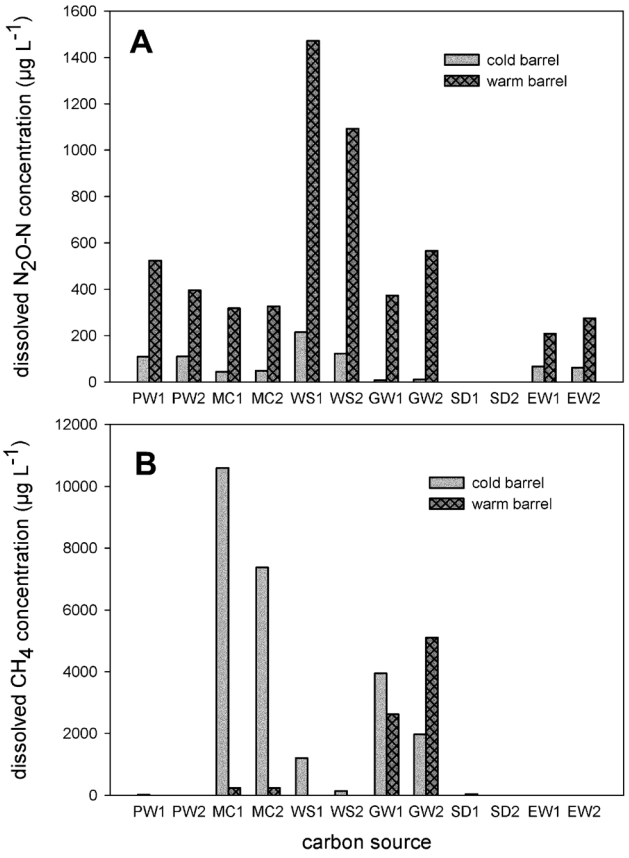

Fig. 8.

Dissolved nitrous oxide (A) and methane (B) concentrations in the outlet water of different carbon substrates in cold and warm barrels. PW1 and PW2, soft woodchips (pine); MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, hard woodchips (eucalyptus).

Table 1

Solute concentrations and temperature at the inlet and outlet of barrels filled with different carbon substrates under warm and cold incubation.

Barrel[a] (cold line)	$NO_3^- - N$ (mg L^{-1})	$NO_2^- - N$ (mg L^{-1})	$NH_4^+ - N$ (mg L^{-1})	pH	Temp (°C)	DO (mg L^{-1})	TC (mg L^{-1})	TOC (mg L^{-1})
Inlet	14.4	0.023	<0.001	7.7	19.2	7.1	13.5	5.4
Outlet PW1	10	0.080	<0.001	6.9	18	1.9	18.3	6.8
Outlet PW2	10.5	0.179	<0.001	6.9	17.2	1.3	26.6	9.1
Outlet MC1	0.4	0.033	<0.001	6.2	17.2	1.1	100.4	70.2
Outlet MC2	0.1	0.003	<0.001	5.9	16.2	0.5	86.8	76.8
Outlet WS1	0.5	0.060	0.134	6.9	17.1	0.7	56.1	16.5
Outlet WS2	1	0.025	0.065	6.9	16.6	0.5	49.3	11
Outlet GW1	6.2	0.153	0.178	6.8	16.6	0.9	25.1	7
Outlet GW2	2.2	0.024	0.785	6.6	16.3	0.5	60	14.7
Outlet SD1	8.3	0.164	0.364	6.8	16.9	0.4	23.2	5.9
Outlet SD2	5.7	0.020	0.344	7.1	16.7	0.3	14.8	4.6
Outlet EW1	9.7	0.082	0.033	7.0	16.6	0.6	21.6	4.7
Outlet EW2	9.6	0.424	<0.001	7.0	16.3	0.5	29	7.4
Inlet	17.2	0.007	<0.001	8.3	36	5.9	14	6.0
Outlet PW1	12	0.234	0.027	7.6	26	1	19.5	5.8
Outlet PW2	11.2	0.171	<0.001	7.5	27.3	0.6	18.2	6.2
Outlet MC1	3.7	0.079	0.081	7.6	26.3	1	51.4	9.5
Outlet MC2	6.1	0.094	0.364	7.7	29	1	53.7	9.7
Outlet WS1	8.5	0.410	0.259	7.6	29.1	0.4	33.71	7.8
Outlet WS2	9.3	0.562	0.194	7.6	27	1.1	10.9	0.6
Outlet GW1	4.3	0.088	<0.001	7.5	27.4	0.9	52.1	8.3
Outlet GW2	7.2	0.234	<0.001	7.6	25	1.1	46.4	7.9
Outlet SD1	8.5	0.444	0.307	7.6	27.2	1.3	23.8	5.8
Outlet SD2	8.6	0.494	0.084	7.6	28.4	0.6	13.7	3.8
Outlet EW1	9.6	0.816	0.024	7.6	27.7	0.4	30	5.8
Outlet EW2	10.9	0.516	0.123	7.6	25.3	1.1	20.9	5.1

[a] PW1 and PW2, soft woodchips (pine); MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, hard woodchips (eucalyptus).

Table 2

Average copy number ($\times 10^6$) of denitrification genes (*nirS*, *nirK* and *nosZ*) and 16S rRNA isolated from different carbon substrates used in denitrifying barrels under cold and warm incubations.

Treatment	Carbon substrate[a]	*nirS* copies g^{-1} dry substrate	*nirK* copies g^{-1} dry substrate	*nosZ* copies g^{-1} dry substrate	16S rRNA copies g^{-1} dry substrate
Cold	PW1	10.1 ± 2.9	12 ± 0.2	2.5 ± 0.1	3.1 ± 0.1
	PW2	8.7 ± 0.8	0.7 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
	MC1	4718 ± 299.2	4217 ± 301.8	5760.1 ± 783.8	96761.9 ± 2649.3
	MC2	7474.5 ± 160.2	6815.9 ± 147	8990.3 ± 180.4	43138.8 ± 2076.5
	WS1	388.6 ± 29.4	211.3 ± 4.8	138.3 ± 0.2	351.1 ± 28.1
	WS2	195 ± 17.9	148.4 ± 3.3	77.2 ± 1.6	1.2 ± 0.1
	GW1	7296.5 ± 368.3	2919 ± 72.4	4183.6 ± 109.3	22755.1 ± 596.1
	GW2	16163.2 ± 137.3	5664.1 ± 667.7	7168.5 ± 143.8	22660.2 ± 1595.2
	SD1	3412.4 ± 43.5	2227.5 ± 185.9	1064.2 ± 50.4	4745.9 ± 253.5
	SD2	3319.6 ± 53.3	1998.5 ± 126.2	927 ± 25.2	3648.9 ± 338.6
	EW1	41.5 ± 6.3	22.7 ± 1.7	15.9 ± 0.5	30.3 ± 1.0
	EW2	57.9 ± 1.3	34 ± 1.6	23.3 ± 1.3	40.8 ± 1.6
Warm	PW1	29.2 ± 4	22.8 ± 0.6	14 ± 1.1	23.9 ± 1.2
	PW2	19.9 ± 1.1	7.4 ± 0.4	6.5 ± 0.3	59.6 ± 1.2
	MC1	70533.5 ± 481.5	13860 ± 504.3	19230.2 ± 171.3	45035.9 ± 1781.8
	MC2	126400 ± 3695.2	14926.2 ± 719.7	18745.9 ± 365	50774.6 ± 4753.5
	WS1	1596.6 ± 135.9	393.6 ± 33.5	200 ± 9.9	3870.4 ± 19.1
	WS2	610.8 ± 3.9	456.3	230.9 ± 5	476.7 ± 33.4
	GW1	17237.9 ± 488.2	5044.3 ± 88.3	7415.2 ± 182.7	35076.9 ± 1550.5
	GW2	13763.3 ± 385.2	3957.4 ± 158.3	2670.5 ± 157.7	18592.3 ± 919.1
	SD1	2716.2 ± 35.8	1224.2 ± 48.2	696.8 ± 18.5	2217.6 ± 187.1
	SD2	2967.5 ± 98.4	1321.6 ± 25.4	974.1 ± 7.7	2710.5 ± 61.5
	EW1	67.1 ± 4.4	18.7 ± 0.4	14.8 ± 0.6	29.6 ± 0.8
	EW2	72.1 ± 3	37.5 ± 2.4	25.1 ± 0.8	263.8 ± 18.8

[a]PW1 and PW2, soft woodchips (pine); MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, hard woodchips (eucalyptus).