MICROBIAL PROCESSES AND NITRATE REMOVAL IN DENITRIFICATION BEDS

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Abstract

The anthropogenic abundance of reactive nitrogen (N) forms has increased in the last few decades, increasing food production, but also resulting in increased eutrophication, algae blooms, loss of biodiversity, and greenhouse gas (GHG) emissions, in aquatic and terrestrial ecosystems. Denitrification beds are one approach to return this reactive N back to the atmosphere. These beds are large containers filled with a carbon (C) substrate, often wood byproducts. This substrate acts as a C and energy source for denitrifiers to reduce nitrate (NO_3^-) from point source discharges into non-reactive dinitrogen (N_2) gas. This study investigated the biological mechanisms, controlling factors and adverse effects of NO_3^- removal in a woodchip denitrification bed (176 m x 5 m x 1.5 m) treating glasshouse effluent, and in barrels (0.2 m^3) testing alternative carbon substrates for use in denitrification beds (pine and eucalyptus woodchips, sawdust, green waste, maize cobs and wheat straw). Furthermore, different techniques for measuring denitrification rates were compared and an approach for determining reliable NO_3^- removal rates in denitrification beds was developed.

The NO_3^--N removal rates of the large denitrification bed averaged 7.6 g N m^{-3} bed volume d^{-1} and increased with increasing temperature (Q_{10} = 2.1). Microbial denitrification was the main NO_3^- removal mechanism in the denitrification bed and was always limited by C, rather than by NO_3^- availability. Dissimilatory nitrate reduction to ammonium (DNRA) and anammox were likely minor processes due to low ammonia (NH_4^+) and nitrite (NO_2^-) concentrations throughout the bed. Sulfate (SO_4^{2-}) reduction, and methanogenesis, could not compete with NO_3^- reduction for C due to continuously high NO_3^- concentrations

in the bed (>37 mg N L^{-1}). Aerobic processes dominated in the first few meters of the bed and close to the surface, but dissolved oxygen (DO) concentrations decreased rapidly along the bed from the inlet and remained low throughout most of the bed.

There were some adverse effects observed in the denitrification bed associated with NO_3^- removal. About 4.3% of NO_3^--N removed from the bed was released as nitrous oxide (N_2O), but methane (CH_4) emissions from the surface of the bed were very low. A total of 35.4 kg d^{-1} of carbon dioxide (CO_2) was released from the bed, but was not considered to contribute to a net increase in CO_2 concentrations of the atmosphere as the substrate (woodchips) used in the bed would likely decayed to CO_2 if used for other purposes. A net dissolved organic carbon (DOC) loss from the outlet was not detected. Longevity of the C substrate of the denitrification bed to support denitrification was about 39 years as calculated from the total C losses (CO_2 emissions and release of dissolved CO_2 and DOC from the bed).

In a barrel study of different carbon substrates, NO_3^- removal was predominantly limited by C availability and temperature (Q_{10} = 1.2) when NO_3^--N concentrations were above 1 mg L^{-1}. All C substrates showed high numbers of denitrification genes (nitrite reductase, *nirS* and *nirK*; nitrous oxide reductase, *nosZ*), providing further support that microbial denitrification was responsible for NO_3^- removal. Substrates incubated at 27.1 °C had greater ratio of *nir*/*nosZ* genes than substrates incubated at 16.8 °C, which was possibly a partial explanation for higher N_2O production in the warmer barrels. Wheat straw released 10% of NO_3^--N removed as dissolved N_2O, while all other carbon substrates released on average about

1.4% of the removed NO_3^--N as dissolved N_2O. Methane production occurred when NO_3^- concentrations were below 2 mg L^{-1} in the barrels. Maize cobs removed about 2.5 times more NO_3^- than woodchips, but released total organic carbon (TOC) in the outflow and a substantial portion of C was likely consumed by non-denitrifiers. Woodchips had low adverse effects and provided ideal conditions for denitrifiers determined by the relatively high ratio of denitrification gene copies/16S rRNA copies compared to the other C substrates examined.

Investigating different approaches to determine denitrification rates revealed that both the acetylene inhibition method and the copy number of nitrite reductase genes (*nirS*, *nirK*) were useful for comparative estimations of NO_3^- removal rates between different carbon substrates and temperatures. However, neither approach could be used to quantify actual rates of denitrification. The acetylene inhibition method overestimated the actual NO_3^- removal rate by five fold. An *in situ* push-pull test using enriched $^{15}NO_3^-$ was useful for determining denitrification rates at one specific point in a denitrification bed but would require multiple testing sites to obtain an average rate of NO_3^- removal for the bed. Comparing the ratio of the slopes of natural abundance ^{15}N-N_2 and ^{15}N- NO_3^- along the length of the bed determined the portion of NO_3^- removed by microbial denitrification, but not the denitrification rate. Measurements of dissolved N_2 concentration along the length of the bed were a useful approach to determine denitrification rates. This last approach was rapid and produced relatively accurate rates of NO_3^- removal compared to the other approaches conducted in this study.

In summary, denitrification beds are an efficient approach for removing NO_3^- from point source discharges, but the beds do produce some N_2O. Woodchips

could be combined with maize cobs to enhance NO_3^- removal rates while keeping adverse effects low in denitrification beds. Measurement of N_2 concentrations along the length and water flow of the bed was the most appropriate approach to determine denitrification rates of denitrifying bioreactors, and may also be useful in other ecosystems with high NO_3^- concentration and even flow.

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Chapter 1

General Introduction

1.1 Introduction

Nitrogen (N) is essential for life along with carbon (C), phosphorus (P), oxygen (O), sulphur (S) and a range of micro nutrients. The global mass of N is greater than the mass of these other four major elements (Mackenzie, 1998); however, most of this N is stored in its non reactive diatomic form (N_2) in the atmosphere. Natural processes that convert N_2 into reactive N forms are lightning and microbial N_2 fixation (Fig. 2.1). Reactive forms of N include ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), nitrous oxides (NO, N_2O), nitride and organically bound N. As a consequence of low availability in reactive forms, N often limits biological processes in a wide variety of ecosystems. During the last few decades, inputs of reactive N forms have rapidly increased on global and regional scales due to anthropogenic activities (Galloway et al. 2003; Galloway et al., 2004). Galloway et al. (2003) estimated that human-induced inputs of reactive N increased by 225% between 1970 and 2000, and Canfield et al. (2010) calculated that the anthropogenic production of reactive N contributed 45% of the total N fixation (conversion of N_2 into reactive N forms) on Earth in 2008, through the Haber Bosch process (30%; 135.8 Tg N $year^{-1}$), cultivation of N-fixing crops (10%; 46.2 Tg N $year^{-1}$) and combustion of fossil fuels (5%; 25.2 Tg N $year^{-1}$). The increase in reactive N production to support agriculture was necessary to feed the increasing human population in the 20^{th} century (Galloway et al., 2004). However, abundance of reactive N in ecosystems, especially terrestrial and coastal surface waters, has lasting adverse effects, such as eutrophication, acidification, hypoxia, algae blooms and loss of biodiversity (Vitousek et al., 1997; Howarth et al., 2000; Rabalais, 2002; Phoenix et al., 2006). A further important adverse effect

is the emission of nitrous oxide (N_2O) from land and water surfaces. Beaulieu et al. (2011) estimated that rivers emitted about 10% of the global anthropogenic N_2O. Nitrous oxides contribute to the greenhouse effect (IPCC, 2006), stratospheric ozone depletion (IPCC, 2006), production of tropospheric ozone (Crutzen, 1974), and increases in ozone concentrations in rural environments (Finlayson-Pitts and Pitts, 1986). These adverse effects have multiple environmental consequences (droughts, flooding, storms, etc.) (IPCC, 2006), affects human health (increases in rates of cancer, cardiac and respiratory diseases) (Wolfe and Patz, 2002), and harms plant growth (Mauzerall and Wang, 2001). Therefore, the need to reduce the production of reactive N and to develop strategies to convert unwanted reactive N in the environment back to N_2 is essential.

There are several approaches for reducing the N load into receiving aquatic environments. Sophisticated technological approaches for removing N, which are frequently used in wastewater treatment plants and septic tank systems, are often expensive and/or require ongoing maintenance (Oakley et al., 2010). Passive N removal technologies include constructed wetlands, riparian buffers, wastewater treatment ponds and denitrifying bioreactors (Schipper et al., 2010a). Riparian buffer zones and constructed wetlands are widespread approaches for removal of N from nonpoint source (e.g. runoff from agricultural areas and stormwater), and point source discharges (e.g. farm drains, commercial and domestic effluents)(Dinnes et al., 2002; Vymazal, 2006). Nitrate (NO_3^-) removal in riparian buffers occurs by dilution, plant uptake (assimilation) and denitrification (Dinnes et al., 2002). Constructed wetlands were first studied by Seidel (1953) in Germany (Campbell and Ogden, 1999). Sub-surface wetlands with horizontal flow showed

substantial nitrite (NO_2^-) and NO_3^- removal (Vymazal, 2006). However, a substantial portion of N is removed only temporarily by plant uptake and wetland vegetation may need continuous maintenance (Vymazal, 2006). Furthermore, Teiter and Mander (2005) measured relatively high N_2O and methane (CH_4) emissions from constructed wetlands and riparian buffers. Wastewater ponds generally have low NO_3^- removal rates (Lorch et al., 1992). Nevertheless, these systems are useful for decreasing N in waters, but other remediation systems need to be developed that have greater N removal and are easy to construct and maintain.

Recently, denitrification beds (Blowes et al., 1994; Robertson et al., 2005; Schipper et al., 2010b) have increasingly been used to remove NO_3^- from point source discharges due to relatively high rates of NO_3^- removal, low installation costs and relatively low maintenance requirements compared to other NO_3^- removal systems (Schipper et al., 2010a). Operational denitrification beds have been installed in New Zealand, Canada and USA (Table 2.1).

Fig. 1.1 Gas sampling by Sören at a large denitrification bed receiving effluent from a glasshouse in Karaka (NZ)

Denitrification beds are large containers filled with carbon substrate (predominantly wood byproducts) with an effluent inlet at one end and an outlet at the other end (Fig. 1.1). The effluent passes through the C substrate, which acts as an electron donor and C source for denitrifying bacteria to convert NO_3^- to N_2O and N_2 gases (Seitzinger et al., 2006). Different C substrates have been examined in laboratory studies to determine the most suitable material for large scale denitrification beds, which are currently operating predominantly with wood byproducts (Gibert et al., 2008; Cameron and Schipper, 2010). Wood byproducts generally have a high C/N ratio, high permeability, long persistence and are commercially available at low costs (Robertson and Anderson, 1999). Cameron and Schipper (2010) proposed that the NO_3^- removal rate does not depend on grain size and constitution (soft or hard) of the wood byproduct. There are many studies reporting NO_3^--N removal rates in denitrification beds (Robertson et al., 2005; Van Driel et al., 2006; Robertson and Merkley, 2009; Robertson et al., 2009; Schipper et al., 2010b; Robertson, 2010; Moorman et al., 2010; Elgood et al., 2010; Woli et al., 2010). Rates ranged from 1.8 g N m^{-3} d^{-1} (Robertson et al., 2005) to 9.7 g N m^{-3} d^{-1} (Schipper et al., 2010b; Table 2.1). These rates vary widely because of differences in effluent composition, temperature and specific site conditions. Additionally, measuring NO_3^- removal rates can be difficult and imprecise because of temporal variability in flow rates and inlet NO_3^- concentrations (Schipper et al., 2010a). Therefore, it is important to develop a reliable approach for measuring NO_3^- removal rates in denitrification beds.

Studies have generally assumed that NO_3^- removal in denitrification beds is mainly due to microbial denitrification, and that NO_3^- removal due to dissimilatory NO_3^- reduction to ammonium (DNRA), anammox and

microbial/plant immobilization are minor processes (Robertson et al., 2000; Greenan et al., 2006; Greenan et al., 2009; Robertson, 2010; Schipper et al., 2010a), but direct evidence of the relative importance of microbial denitrification is lacking.

In many ecosystems, the limiting factors of denitrification are temperature, C availability, pH, NO_3^-, and dissolved oxygen (DO) concentration (Seitzinger et al., 2006). These factors are also important controllers of N_2O emissions (Kampschreur et al., 2009). However, little is known about the importance of these factors for NO_3^- removal and N_2O emissions in denitrification beds. Some studies have reported increases in NO_3^- removal with increasing temperature in denitrification beds and also in column studies using wood byproducts (Robertson et al., 2008; Robertson et al., 2009; Elgood et al., 2010; Cameron and Schipper, 2010). Healy et al. (2006) observed that DO above 3.7 mg L^{-1} reduced the NO_3^- removal rate in laboratory experiments, and Elgood et al. (2010) reported that high DO concentrations increased the N_2O production. Competition by SO_4^{2-} reducers for C has also been reported (Robertson et al., 2009: Robertson, 2010; Elgood et al., 2010). It is important to determine all factors limiting NO_3^- removal in denitrification beds to enhance the NO_3^- removal of the beds and to reduce the adverse effects.

There have also been few studies examining potential adverse effects (GHG production, carbon release) arising from the use of denitrification beds. Moorman et al. (2010) and Elgood et al. (2010) measured dissolved N_2O in denitrification walls/beds, but did not measure fluxes of N_2O from the bed to the atmosphere. Dissolved methane was detected by Elgood et al. (2010) when NO_3^- concentration

was low in the bioreactor. Finally, the release of dissolved carbon was detected predominantly during the start up phases of C substrate column studies (Soares and Abeliovich, 1998; Greenan et al., 2009; Cameron and Schipper, 2010). Excess discharges of dissolved carbon to receiving waterways could potentially degrade these ecosystems by reducing dissolved oxygen (DO) concentrations (Cox et al., 2003). It is important to quantify all the potential adverse effects of operational denitrification beds to determine the overall benefits of these beds, and to develop strategies to reduce any unwanted effects.

1.2 Objectives of this thesis

The specific objectives of this thesis were to:

a. Determine the NO_3^- removal rate in a large denitrification bed over a 12 month period and determine whether denitrification was the main mechanism for NO_3^- removal;

b. Determine the environmental factors limiting NO_3^- removal and quantify any adverse effects in a large denitrification bed and for different carbon substrates;

c. Determine the most suitable natural C substrate for growth of denitrifying bacteria;

d. Estimate the lifetime of the C substrate in a denitrification bed, and the C use efficiency of the NO_3^- removal in a denitrification bed.

e. Develop a more reliable approach for measuring NO_3^- removal rates in operational denitrification beds;

1.3 Thesis outline

The six chapters of this PhD thesis are divided in Introduction (Chapter I), Literature Review (Chapter II), three research chapters (Chapter III to V) and a final overarching conclusion with recommendations for future work (Chapter VI). Research chapter III "Rates, controls and potential adverse effects of nitrate removal in a denitrification bed" addresses objectives a, c and e and was published in *Ecological Engineering* (2011) 37, 511-522. Chapter IV "Nitrate removal, communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds" was published in *Water Research* (2011) 45, 5463-5475 and addresses objectives c and d. Finally, chapter V "A comparison of different approaches for measuring denitrification rates in a nitrate removing bioreactor" addresses objectives a and b. This last research chapter was published in *Water Research* (2011) 45, 4141-4151.

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Chapter 2

A Review of Microbial Processes and Nitrate Removal in Denitrification Beds

2.1 General overview

In order to understand nitrate removal in denitrification beds within a broader context this literature review provides a general introduction to the nitrogen cycle (2.1.1.) and the denitrification process (2.1.2.). Then methods for quantifying denitrification are outlined (2.1.3.) before reviewing denitrification beds in greater detail (2.2.).

2.1.1 Nitrogen cycle

Nitrogen is a fundamental component of nucleic acids and proteins and thus essential for any form of life (Canfield et al., 2010). Nitrogen is composed of the stable isotopes ^{14}N and ^{15}N, with the majority of atmospheric N being ^{14}N (99.6337%) (Junk and Svec, 1958). The nitrogen cycle is dominated by microbial transformations of the N compounds (Fig. 2.1; Steinbüchle et al., 2003). Most of nitrogen is stored as N_2 gas in the atmosphere. This non-reactive form is unavailable to most organisms, but can be converted into reactive N via two natural processes; lightning and microbial N_2 fixation. Apart from N_2, all the other N compounds are considered reactive forms of N (Fig. 2.1; Galloway et al., 2003; Galloway et al., 2004). Nitrogen (N_2) fixation was first described by Sergej Winogradsky (1895) in the strain *Clostridium pasteurianum* which reduces N_2 to NH_4^+ using a nitrogenase enzyme system. Symbiotic and free living bacteria are capable of N_2 fixation under aerobic and anaerobic conditions (Steinbüchel et al., 2003). In 2008, the total natural N_2 fixation globally was about 250 Tg N $year^{-1}$ while the anthropogenic fixation of N_2 (Haber Bosch process, N fixing crops and combustion of fossil fuels) accounted for about 207 Tg N $year^{-1}$ (Canfield et al.,

2010). There are a wide range of microbial transformations of N. Organisms assimilate NH_4^+ to build up biomolecules and the mineralization of organic material releases NH_4^+. Some gram positive bacteria (*Nitrosomonas*- and *Nitrobacter* species) may oxidize NH_4^+ to NO_3^- under aerobic conditions (nitrification) (Fig. 2.1).

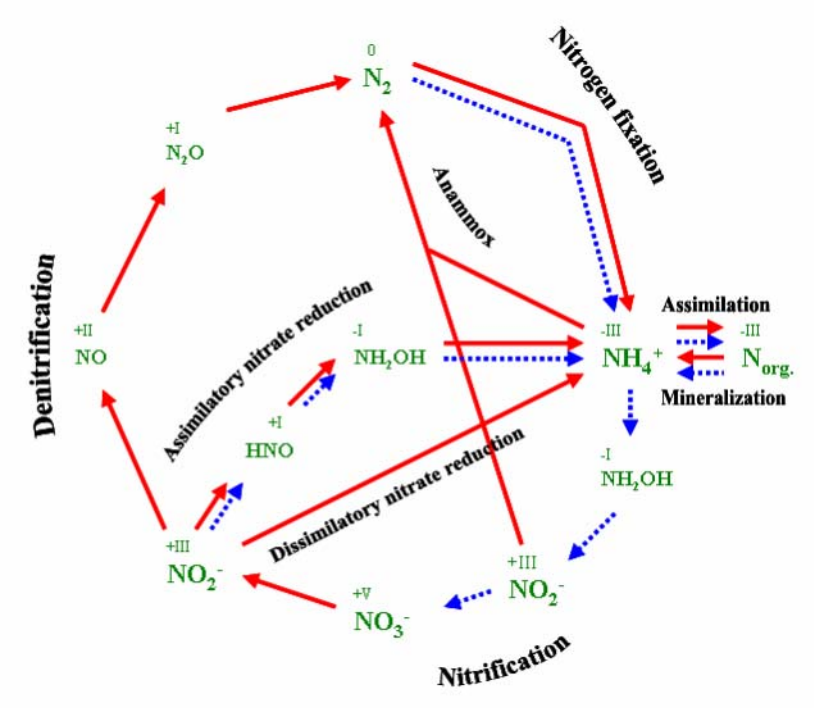

Fig. 2.1 Nitrogen cycle (modified after Steinbüchel et al., 2003). Blue broken arrows, aerobic process; red arrows, anaerobic process; Roman numerals, oxidation number of nitrogen in the compound.

The nitrification processes provide NO_3^-, which act as an electron acceptor for further reduction processes such as anammox, dissimilatory NO_3^- reduction to NH_4^+ (DNRA), assimilatory NO_3^- reduction and denitrification (Steinbüchel et al., 2003) (Fig. 2.1). Other organisms reduce NO_3^- to NH_4^+ (assimilatory NO_3^- reduction) for N assimilation and conversion to organic N compounds. This differs from the DNRA process, where facultative anaerobes reduce NO_3^- to NH_4^+ to oxidize their electron carrier (Tiedje, 1988). Anammox bacteria convert autotrophic NO_2^- and NH_4^+ into the non-reactive N_2 gas. There are three known

genera of anammox bacteria (*Candidatus* Brocadia, *Candidatus* Kuenenia and *Candidatus* Scalindua). Anammox bacteria produce at least 25 - 30% of total N_2 in the oceans, but their role in terrestrial ecosystems is not well understood (Brandes et al., 2007). Microbial denitrification is the other process, which converts NO_3^- to N_2 completing the N cycle (Tiedje, 1988). Many bacteria and some fungi can use NO_3^-, NO_2^- and N_2O as electron acceptors under anaerobic conditions for denitrification. There are also abiotic processes that convert N (organic and inorganic) into N gases (N_2, N_2O and NO) such as chemo-denitrification, but these are generally minor processes occurring containing acidic (pH < 5) or frozen soils with high concentrations of nitrite (Tiedje, 1988; Van Cleemput et al., 1998; Christianson and Cho, 1983).

2.1.2 The denitrification process

Microbial denitrification is the dominant process on Earth by which NO_3^- is converted to non-reactive N_2. In 2008, the denitrification rate in oceans and terrestrial systems was estimated to be about 240 Tg N $year^{-1}$ and 100 Tg N $year^{-1}$, respectively, which when combined was almost the same as the mass of N fixed by microorganisms (Canfield et al. 2010).

Denitrification is an anaerobic process, which has NO_2^-, nitrogen monoxide (NO) and nitrous oxide (N_2O) as intermediates (Fig. 2.2). Nitrogen monoxide is very reactive and reacts with oxygen to form N_2O (Tiedje, 1988).

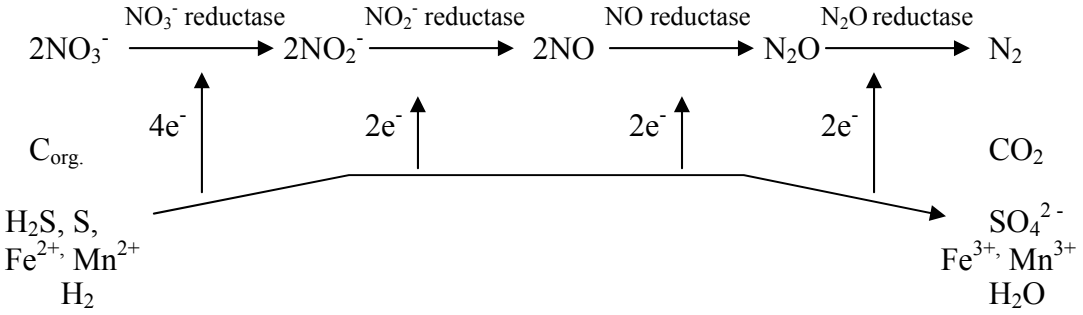

Fig. 2.2 Reduction of nitrate to non-reactive nitrogen gas (N_2) with the involved enzymes and the potential electron donors (modified after Steinbüchel et al, 2003).

The use of NO_3^- as an alternative to O_2 as terminal electron acceptor is common in bacteria, and between 1 – 10% of bacteria are capable of denitrification (Schmider and Ottow, 1984). Denitrifiers are widely distributed in bacteria including *Actinobacteria, Proteobacteria phyla, Bacteroides, Aquificae, Firmicutes,* and *Deinococcus- Thermus* (Zumft 1997). Malinowsky and Ottow (1985), Shoun et al. (1992) and Tanimoto et al. (1992) have also observed denitrification in some fungal species and denitrification was observed in Archaea by Philippot (2002).

The enzymes NO_3^- reductase, NO_2^- reductase, NO reductase and N_2O reductase catalyse the reduction of NO_3^- to N_2 (Fig. 2.2). The denitrification genes responsible for NO_3^- reduction (*nar*), NO_2^- reduction (*nir*), NO reduction (*nor*),and N_2O (*nos*) reduction are often assembled in clusters and are localised on different gene sets than the NO_3^- reduction genes of the assimilation process (Zumft et al., 1997). The NO_2^- reductase genes, *nirS* and *nirk* and the N_2O reductase gene *nosZ* have highly conservative regions. Therefore, these genes have been used as important markers for denitrification. The gene *nirS* expresses the cytochrome *cd*1-containing nitrite reductase, *nirK* expresses the cooper-

containing nitrite reductase and *nosZ* expresses the nitrous oxide reductase (Zumft et al., 1997; Braker et al., 1998).

Denitrifying bacteria have been identified in all three major physiological groups, which can use organic compounds (organotrophs), inorganic compounds (lithotrophs) and light (phototrophs) as electron donors (Fig. 2.2) (Tiedje, 1988) and occur in all ecosystems when the following requirements are met (Firestone and Davidson, 1989):

- presence of bacteria capable of nitrate reduction (denitrifiers are considered ubiquitous),
- availability of electron donor (e.g. microbially available carbon, H_2S, S, H_2 or light),
- low levels of oxygen, and
- supply of nitrate, nitrite or nitrous oxides which act as electron acceptors.

In ecosystems, the denitrification process can be limited by temperature, C availability, pH, NO_3^- and/or dissolved oxygen (DO) concentrations (Seitzinger et al., 2006). These factors may also contribute to incomplete denitrification, which increases the release of N_2O as an intermediate of denitrification (Kampschreur et al., 2009). For example, S^{2-} inhibits the N_2O reductase followed by N_2O release (Sörensen et al., 1980), high NO_3^- and low pH increase the N_2O/N_2 emission ratio (Blackmer and Bremner, 1978; Soto et al., 2006). Warmer temperatures (Johansson et al., 2003; Teiter and Mander, 2005) and low C/N ratios both enhance the N_2O emissions (Huang et al., 2004; Hunt et al., 2007). N_2O is of

concern because it is a potent greenhouse gas contributing to the greenhouse effect and stratospheric ozone depletion (IPCC, 2001).

2.1.3 Quantifying denitrification

It is important to quantify denitrification rates to determine the conversion of reactive N to non-reactive N_2 in a range of ecosystems and in technological approaches that enhance denitrification for water treatment (riparian buffers, constructed wetlands, denitrifying bioreactors etc.) to remove N from a range of different sources (groundwater, streams, drains, wastewater etc). However, the high background concentration of N_2 in the environment makes direct measurement of the main end product (N_2) of denitrification very difficult (Groffman et al. 2006).

The following range of techniques has been used to measure denitrification rates in terrestrial and aquatic environments in the past.

a) Nitrate removal

Quantifying the denitrification rate by measuring the decrease in NO_3^--N concentration is often unsatisfactory because NO_3^- can be removed by various mechanisms such as DNRA, assimilation, anammox, precipitation and absorption. Furthermore, varying sources of NO_3^--N (groundwater, multiple surface water inlets) and varying NO_3^--N inlet concentrations over time can cause incorrect estimations of NO_3^--N removal data. When NO_3^--N removal is calculated as the

difference in NO_3^--N concentration at the inlet and outlet, the NO_3^- measured in examined water at the outlet likely represents the inlet water at some time before the sampling date (Schipper et al., 2010a). However, some studies have assumed that denitrification was the mechanism responsible for the NO_3^--N removal. Consequently these studies suggested that the NO_3^--N removal rate was identical with the denitrification rate (Robertson et al., 2000, Greenan et al., 2009; Moorman et al., 2010).

b) Changes in natural abundance of 15N ($\delta^{15}N$) in NO_3^- and N_2

Both biotic and abiotic mechanisms alter the natural $^{15}N/^{14}N$ ratio (expressed as $\delta^{15}N$) in ecosystems and different N conversion mechanisms result in distinct $\delta^{15}N$ signatures in these environments (Bedard-Haughn et al., 2003). Many studies have used these signatures to determine the source of N or to track N within an ecosystem (Kreitler and Browning, 1983; Wassenaar, 1995; Karr et al., 2001), even though the variability of $\delta^{15}N$ due to ongoing fractionation of $^{15}N/^{14}N$ make this kind of study difficult (Robinson, 2001). Denitrification increases $\delta^{15}N$-NO_3^- and decreases $\delta^{15}N$-N_2 due to discrimination against ^{15}N during denitrification (Mariotti et al., 1981). This natural fractionation of $^{15}N/^{14}N$ of the NO_3^- is described by a fractionation factor. After reviewing 15 different studies, Bedard-Haughn et al. (2003) calculated a median fractionation factor of 1.0185 for denitrification equivalent to an enrichment of ^{15}N-NO_3^- by 18.5 ‰. There was also the successful attempt from Erler and Eyre (2010) to estimate denitrification rates using the natural fractionation of ^{15}N-NO_3^- in a constructed wetland.

c) Acetylene block

The most commonly used technique to measure denitrification rates is the acetylene inhibition method (Groffman et al., 2006). Acetylene inhibits the reduction of the nitrous oxide during denitrification. Accumulation of N_2O can be easily measured via gas chromatography, because background concentration of N_2O is relatively low (Tiedje et al., 1989). However, there are a range of problems arising from the acetylene inhibition technique for quantifying denitrification rates. Acetylene can act as a carbon source for microorganisms (Kanner and Bartha, 1979; De Bont and Peck, 1980; Tam et al., 1983; Schink, 1985) and inhibits nitrifiers, which lead to a lack of NO_3^- production constraining NO_3^- supply to denitrifiers (Mosier et al., 1980; Walter et al., 1979). Furthermore, the acetylene-inhibition technique is generally performed in laboratory, which creates artifical conditions (e.g., constant temperature, shaking), that encourage denitrifiers and do not reflect the *in situ* denitrification rate. *In situ* measurements of denitrification rates using acetylene and soil chambers were evaluated by Ryden and Dawson (1982), but this approach is very labour intensive and is also subject to some problems described before. Therefore denitrification measurements via acetylene inhibition technique are not always accurate, but are most useful for comparing denitrification activity between different sites and seasons (Groffman et al., 1992; Groffman et al., 2006).

d) ^{15}N enrichment

Addition of ^{15}N is used to track N in ecosystems and to determine the rate of the N-cycle processes (Nadelhoffer and Fry, 1994). Hauck and Melsted (1956)

directly quantified denitrification in soils following addition of ^{15}N-labelled NO_3^- and measurement of the production of ^{15}N-N_2 and oxides of ^{15}N in a closed system. Hauck and Melsted (1956) recovered between 89 and 100% of N in the system using this ^{15}N enrichment technique. More recently, enrichment with ^{15}N has been used to determine denitrification rates in water-saturated environments using the push-pull method, where a solution of ^{15}N-labelled NO_3^- is injected into the area of interest and subsequently pulled out during an incubation period (Addy et al., 2002). The denitrification rate can then be determined by measuring the decrease of ^{15}N-NO_3^- or the increase of ^{15}N-N_2 relative to the conservative tracer (Hauck and Melsted, 1956; Addy et al., 2002; Baker and Vervier, 2004). Moorman et al. (2010) used this approach in a woodchip bioreactor and measured the decline of ^{15}N-NO_3^- concentration. The decline in ^{15}N-NO_3^- was attributed to denitrification, DNRA, anammox and/or biological uptake (immobilization). In general, the push-pull test is most appropriate in environments where NO_3^- is not limiting denitrification, otherwise adding NO_3^- may increase the denitrification rate (Groffman et al., 2006). When bromide is used as conservative tracer plant uptake has to be considered (Schnabel et al., 1995). For accurate measurements, the increase in denitrification products (^{15}N-N_2 and ^{15}N-N_2O) should be measured (Groffman et al., 2006).

e) Direct N_2 measurement

The direct measurement of N_2 emission would likely be the most accurate measurement approach, but is generally not possible due to the high background concentrations of N_2 in most environments. Butterbach-Bahl et al. (2002) successfully measured N_2 production from soils in N_2-free boxes, which was

extremely time consuming and was still prone to atmospheric N_2 contamination. In aquatic environments, denitrification rates can be calculated from the increase in dissolved N_2 concentrations (Blicher-Mathiesen et al., 1998) or the ratio of dissolved N_2/Argon (Laursen and Seitzinger, 2002). Blicher-Mathiesen et al. (1998) successfully measured the denitrification rate in the groundwater samples of a riparian wetland. They sampled groundwater along the groundwater-flow path in sealable vials and introduced helium gas headspaces, which were analysed for N2 after headspace equilibrium of the dissolved gases. Similarly, Laursen and Seitzinger (2002) estimated denitrification rates of three small rivers using membrane inlet mass spectrometry (MIMS) that measured increases in dissolved N_2/Argon of water down the length of the river.

This range of approaches for measuring denitrification should be tested in a denitrification bed to determine the denitrification rates of the bed and to compare the usefulness of each method for application in denitrification beds.

2.2 Denitrification beds

Excess NO_3^- in ecosystems can cause eutrophication, acidification, hypoxia, algae blooms, loss of biodiversity and N_2O emission (Vitousek et al., 1997; Howarth et al., 2000; Rabalais, 2002; Phoenix et al., 2006). Denitrification beds and walls along with a number of other passive approaches (constructed wetlands, riparian buffers, wastewater ponds) are simple low maintenance techniques for removing NO_3^- from water to reduce this wide range of impacts on terrestrial and aquatic environments. Denitrification beds were designed for treating surface point

discharges while denitrification walls were designed for treating ground water (Schipper et al., 2010a). Denitrification beds are containers predominantly filled with organic carbon-rich media, commonly wood byproducts such as woodchips and sawdust (Fig.1.1), which act as carbon source for denitrifying bacteria. The effluent passes through the wood byproducts of the bed from the inlet to the outlet (Schipper et al., 2010a). Wood by-products are used because of their high C/N ratio, commercial availability, low original costs, high permeability and long persistence (Robertson and Anderson 1999). Blowes et al. (1994) were the first to demonstrate NO_3^- removal from effluent in a denitrification bed. Blowes et al. (1994) treated successfully tile-drainage effluent using a 200 L denitrification bed. Today, denitrification beds have been constructed in Canada, USA and New Zealand to treat a wide range of point source discharges including streams, greenhouse wastewater, dairy farm wastewater, farmland groundwater, tile drainage, municipal effluent (Table 2.1). However, the mechanisms and factors controlling NO_3^- removal and potential adverse effects of these bioreactors are poorly examined.

Table 2.1 Global overview of the performance of denitrification beds

Location	Year built	Type of effluent	Bed volume (m^3)	NO_3^--N input ($g\ m^{-3}$)	Average NO_3^--N removal ($g\ N\ m^{-3}\ d^{-1}$)	Reference
New Zealand						
Kinloch	2003	municipal	600	5.5	0–11[a]	Schipper et al., 2010b
Bombay	2005	greenhouse	360	250	5-10	[b]NZ Hothouse Ltd.
Rotuehu	2005	stream	20	2.2	2[a]	[b]Landcare Res. (NZ)
Dargaville	2005	dairy farm	78	53	1.4[a]	Schipper et al., 2010b
Karaka	2006	greenhouse	1320	250	9.7	Schipper et al., 2010b
Motutere	2007	municipal	210	[c]No data	[c]No data	[b]Taupo Council (NZ)
Tikitere	2010	geothermal stream	200	[c]No data	[c]No data	[b]Rotorua Council (NZ)
Canada						
Waterloo	1994	drainage tile	1.9	5	10	Robertson et al., 2000
Ontario	1997	house effluent	9	17	1.8[a]	Robertson et al., 2005
Ontario	1998	trailer park effluent	108	38	2.4[a]	Robertson et al., 2005
Ontario	1999	motel effluent	360	14	5.1[a]	Robertson et al., 2005
Ontario	1999	municipal	120	35	2.5[a]	Robertson et al., 2005
Ontario	2001	drainage tile	17	10	3.4	Robertson et al., 2009
Woodstock	2002	farmland groundwater	0.7	9	2.1	van Driel et al., 2006
London	2003	farmland groundwater	0.2	13	3.7	van Driel et al., 2006
Stratford	2006	stream	40	5	3.2	Robertson and Merkley, 2009
Stratford	2006	stream	40	0.3 – 5.8	0.3 – 2.5 [a]	Elgood et al., 2010
USA						
DeLand	2007	drainage tile	77	2.8 – 18.9	6.4[a]	Woli et al., 2010

[a]Nitrate were depleted in these denitrifying bioreactors and NO_3^- removal rate was likely conservative;
[b]Data kindly provided by this institution;
[c]Data not available yet.

2.2.1 Nitrate removal and denitrification

2.2.1.1 Nitrate removal rates

The primary means for determining the usefulness of denitrification beds is the measurement of nitrate removal rates of different beds under a range of conditions. Nitrate removal rates, flow rate, NO_3^- concentration and bed longevity dictate the required size of denitrification beds and their cost for construction. Nitrate removal rates (g m^{-3} d^{-1}) in denitrification beds have often been measured using the difference of inlet and outlet NO_3^--N concentration (ΔNO_3^--N) multiplied with the flow rate (m^3 d^{-1}) and divided by the bed volume (m^3), expressed by following equation: NO_3^--N removal rate = ΔNO_3^--N x FR / V_{bed} (Schipper et al., 2010a). Average removal rates of operational denitrification beds have ranged between 1.8 g N m^{-3} d^{-1} (Robertson et al., 2005a) and 9.7 g N m^{-3} d^{-1} (Schipper et al., 2010b; Table 2.1). In some of these beds, NO_3^--N was completely removed (Schipper et al. 2010b; Robertson et al., 2005a; Woli et al., 2010; Elgood et al., 2010), which likely constrained the NO_3^- removal rates. Generally, when the average NO_3^- removal rates of denitrification beds (g m^{-3} d^{-1}) were converted to the units of NO_3^- removal rates reported for constructed wetlands (g m^{-2} d^{-1}), denitrification beds supported higher NO_3^- removal rates than wetlands which range between 0.68 g N m^{-2} d^{-1} and 2.52 g N m^{-2} d^{-1} (Vymazal, 2006).

The temporal variability in flow rates and inlet NO_3^- concentrations in real world field applications make calculating NO_3^--N removal rates difficult (Schipper et al., 2010a). In systems with fluctuating flows and loads the outlet concentration of NO_3^--N of a denitrification bed does not always correspond to the inlet

concentrations of NO_3^--N, when measured at the same time because of the travel time of the water from inlet to outlet. Hence, developing a relatively simple approach for measuring reliable NO_3^- removal rates (2.1.3) in denitrification beds is essential. It is also important to determine the factors controlling the NO_3^- removal (2.2.2).

2.2.1.2 Denitrification and other processes that remove nitrate

There are a range of processes that can remove NO_3^- from aquatic systems including denitrification, DNRA, anammox, absorption, biotic uptake and/or precipitation (Schipper et al., 2010a). Apart from denitrification, NO_3^- uptake by plants is an important mechanism for removal of NO_3^- in constructed wetlands, but is not necessarily a sustainable NO_3^- removal mechanism (unless plant biomass is harvested and removed), because the N can re-enter the wetland after plants drop litter and decomposition occurs (Vymazal, 2006). Many studies have assumed that microbial heterotrophic denitrification is the dominant NO_3^- removal mechanism in denitrification beds (Robertson et al., 2000; Greenan et al., 2006 and 2009; Robertson, 2010; Schipper et al., 2010a; Moorman et al., 2010).

There is some evidence to support this assumption. Robertson et al. (2000) and Robertson (2010) found that $\delta^{15}N\text{-}NO_3^-$ increased between inflow and outflow in two denitrification beds, and also in columns packed with woodchips from denitrification beds. They attributed this increase in $\delta^{15}N\text{-}NO_3^-$ to denitrification. This change in $\delta^{15}N\text{-}NO_3^-$ can not be due to anammox or DNRA, because of low NH_4^+ concentration (< 1 mg N L^{-1}) and also not due to immobilization, as Mariotti

et al. (1982) demonstrated that immobilization do not fractionate NO_3^- isotopes. Moorman et al. (2010) performed a push-pull test injecting a 20% ^{15}N-NO_3^- solution into a denitrification wall and measured a NO_3^- removal rate of 35.8 g N m^{-2} d^{-1}. These authors proposed that the decrease in ^{15}N-NO_3^- was primarily due to denitrification, because similar NO_3^- removal rates were measured using the acetylene inhibition method. Greenan et al. (2006) and Gibert et al. (2008) used ^{15}N labelled NO_3^- in woodchip column studies and showed that less than 5% of NO_3^--N were removed by DRNA. Furthermore, Greenan et al. (2009) showed that NO_3^--N immobilization accounted for 2 – 3.5% of NO_3^--N removed. Additionally, NH_4^+ concentrations in most operating beds are low further suggesting that DNRA is not a significant contributor to NO_3^- removal. These arguments and the fact that denitrification beds meet all the conditions for rapid denitrification proposed by Firestone and Davidson (1989) make it likely that denitrification is the main NO_3^- removal mechanism; however, direct measurements of denitrification end products (N_2 and N_2O) have not been made so far to confirm this hypothesis.

2.2.2 Factors controlling NO_3^- removal in denitrification beds

It is important to understand the factors controlling NO_3^- removal and other microbial processes that compete for C (2.2.3) in denitrification beds. In future knowledge of the factors controlling denitrification rates and processes competing for available organic C may allow manipulation of denitrification beds to enhance the NO_3^- removal rates, the carbon use efficiency and to reduce the potential adverse effects of the denitrification beds. In general, factors controlling microbial

denitrification are temperature, DO, pH, availability of C, number of denitrifying bacteria and concentrations of NO_3^-, NO_2^- and S^{2-} (Seitzinger et al., 2006).

Carbon is the electron donor for denitrification and usually the limiting factor for denitrification in anaerobic and NO_3^- rich environments (Knowles, 1982; Reddy et al., 1982) as present in denitrification beds. In column studies examining a range of substrates for use in denitrification beds, high NO_3^- removal rates corresponded with high dissolved TOC concentrations during start-up phases of the experiments (Soares and Abeliovich, 1998; Greenan et al., 2009, Cameron and Schipper, 2010). However, denitrifying activity measurements using the acetylene inhibition method in denitrification walls (woodchip bioreactors treating groundwater) by Schipper et al. (2005), and Moorman et al. (2010) showed no increase of denitrifying activity, when glucose alone was added. Schipper et al. (2005) suggested that the lack of response of denitrifying enzyme activity following glucose addition in woodchip samples of a denitrification wall was likely due to low NO_3^- concentration (5 – 15 mg NO_3^--N L^{-1} in the incoming groundwater (Schipper and Vojvodic-Vukovic, 2001)). It is necessary to determine whether C is limiting denitrification in denitrification beds.

Nitrate is the electron acceptor of the denitrification process (Tiedje, 1988). In woodchip column studies, Robertson et al. (2010) showed that NO_3^- removal followed zero-order kinetics, when NO_3^- concentrations were above 1 mg L^{-1}, which means that increasing NO_3^- concentration did not increase the rate of NO_3^- removal and consequently NO_3^- concentration was not limiting NO_3^- removal. The inlet NO_3^- concentrations in operating denitrification beds are often greater than the K_m values of denitrification measured in riparian soils (2.1 mg L^{-1} and < 0.1

mg L^{-1}) (Schipper et al., 1993; Ambus, 1993) and wetland soils (0.4 mg L^{-1} -1.3 mg L^{-1}) (Maag et al., 1997) (Table 2.1). Therefore nitrate removal in denitrification beds is probably not limited by NO_3^- concentration at the inlet end. However, it is likely that NO_3^- limits the NO_3^- removal process, when NO_3^- becomes depleted along the length of denitrification beds (Table 2.1). However, measurements of NO_3^- limitation of the NO_3^- removal process in operating denitrification beds are lacking.

The pH of many operating denitrification beds is within the range of the optimum for denitrifiers (7 – 8) (Bremner and Shaw, 1958; Knowles 1982). Decreases in pH have been measured in a number of denitrification beds (Robertson et al., 2005a; Van Driel et al., 2006; Robertson and Merkley, 2009), which is likely due to fermentative CO_2 and organic acid release. Nitrification would also decrease the pH producing hydronium ions (H_3O^+), which is unlikely due to low levels of oxygen in the beds. However, the denitrification reaction produces hydroxyl ions and should increase pH. DNRA also increase the pH consuming H_3O^+ ions. Therefore the pH depends on the dominating process in the beds.

In most natural environments, oxygen is the main inhibitor of the denitrification as denitrifiers will preferentially use O_2 rather than NO_3^- as terminal electron acceptor (Tiedje, 1988). The concentration of DO that inhibits denitrification varies between ecosystem studies. In a comparative study of denitrification in aquatic environments, Pina-Ochoa and Alvarez-Cobelas (2006) suggested a DO threshold for denitrification of about 0.5 mg L^{-1}. Laboratory studies showed that DO concentration of 2 mg L^{-1} reduced the denitrification rate by 85% in activated sludge material (Oh and Silverstein, 1999). In a denitrification bed study, Healy et

al. (2006) reported that denitrification was inhibited by DO concentration above 3.7 mg L^{-1} and Robertson et al. (2009) measured a sharp decline of DO in the first four meters of a denitrification bed and could not detect any DO inhibition of the NO_3^- removal process. The low DO sensitivity of the NO_3^- removal process in denitrification beds (Healy et al., 2006, Robertson et al., 2009) may due to the substrate of the beds. Aerobic microbial activity on the external surface of the woodchips could have consumed the oxygen dissolved in the effluent inside of the woodchips, providing internal anoxic microsites for denitrifiers, despite the oxygen present in the effluent between the woodchips. Gradients of oxygen concentrations in flocs of activated sludge were measured by Lens et al. (1995) and Schramm et al. (1999) and the resulting occurrence of denitrification within the flocs and nitrification outside of the flocs was observed by Satoh et al. (2003). Further measurements have to be performed to confirm the extent to which DO inhibits NO_3^- removal in denitrification beds.

Biological reactions generally increase with increasing temperature until an optimum temperature is reached. The optimum temperature for denitrification in soil ranges from 25 °C to 37 °C (Lensi and Chalamet, 1982; Saad and Conrad, 1993; Braker et al., 2010). Knowles et al. (1981) proposed a doubling of denitrification rate in soils, when the temperature increase is 10 °C between 10 °C and 35 °C. This is expressed as Q_{10} of 2, where Q_{10} is the factor of the reaction rate increase with every 10 °C rise in temperature. The temperature dependence of the NO_3^- removal has also been shown in denitrification beds (Robertson et al., 2008; Robertson et al., 2009; Elgood et al., 2010). Robertson et al. (2008) determined an extremely high Q_{10} of 10 in a column study using sawdust from a 15 year-old denitrification wall, whereas Elgood et al. (2010) and Cameron and

Schipper (2010) calculated a Q_{10} of 2 in an in-stream denitrification bed and a Q_{10} of 1.6 in a long-term woodchip barrel study, respectively. Furthermore, Cameron and Schipper (2010) reported that the temperature sensitivity of the NO_3^- removal process varied between different types of C substrates, which were likely due to different degradability of C substrates and the distinct bacterial communities of these substrates. Further studies of Q_{10} in operational denitrification beds and different substrates need to address their NO_3^- removal - temperature sensitivity. Better information on the relationship between denitrification, temperature and carbon substrate will ensure the optimum sizing of denitrification beds for specific temperature, C substrates and NO_3^- loads.

2.2.3 Microbial processes that compete for C in denitrification beds

Heterotrophic microorganisms in terrestrial and aquatic ecosystems compete with denitrifiers for C as electron donor (Madigan, 2003). Consequently, almost all processes that consume C (aerobic respiration, fermentation and anaerobic respiration) will reduce potential denitrification and NO_3^- removal in denitrification beds. However, some fermentative processes, which degrade complex carbon compounds may increase availability of carbon for denitrifiers.

When DO is present, aerobes will also compete with denitrifiers for available C and denitrification would be confined to anaerobic microsites in the carbonaceous media (Schipper et al., 2010a). This is only critical in a bioreactor with short retention times, but not in large denitrification beds with longer retention times due to the rapid consumption of DO near the inlet (Robertson, 2010, Robertson et

al., 2009). The retention time required to decrease DO concentration from 7 mg L^{-1} to 2 mg L^{-1} was 1 hour in a column study with woodchips of varying age (Robertson, 2010) and Robertson et al. (2009) measured a sharp decrease of DO in the first 4 meters of a denitrification bed.

Sulfate is present in many effluents and could support sulfate reduction in anaerobic environments, which compete with dentitrification for available C. Some studies have reported decreases in SO_4^{2-} concentration and an odour of H_2S which provided evidence of S^{2-} production when concentration of NO_3^- was very low (van Driel et al., 2006; Robertson and Merkley, 2009; Robertson, 2010; Elgood et al., 2010). Therefore it is likely that SO_4^{2-} reducers are present in denitrification beds and can compete for available C, when NO_3^- concentration is very low. Sulfate reduction will probably not occur, when NO_3^- concentration is high, because the energy yield of NO_3^- reduction for microorganisms is greater than of SO_4^{2-} reduction (Madigan, 2003).

DNRA is likely to be a minor process in denitrification beds. Rütting et al. (2011) proposed that DNRA is controlled by the C/NO_3^- ratio and the redox status of soils. Yin et al. (1998) showed that significant DNRA occurred only at a C/NO3− ratio above 12. In general DOC concentrations are below 10 mg/L in denitrification beds. Consequently, the NO_3^- concentration has to be below 1 mg L^{-1} to provide conditions for significant DNRA activity. The high NO_3^- concentration and the recalcitrant carbon source will likely lead to a low DNRA in denitrification beds. Scott et al. (2008) showed a DNRA activity only during summer below 5% of N removed in a freshwater wetland. Furthermore, DNRA contributed only about 5% to the NO_3^--N removal in column studies with different

carbon substrates being tested for use in denitrification beds (Greenan et al., 2006; Gibert et al., 2008).

The microbial processes occurring in denitrification beds have not been fully examined. In particular, the total carbon release of the bed has to be compared with total nitrate removed to estimate the quantity of carbon consumed by denitrification. The heterotrophic denitrification process releases theoretically about 5 CO_2-C per 4 NO_3^--N consumed (Robertson et al., 2000). This approache would allow an estimation of the proportion of carbon consumed by denitrifiers in the denitrification bed. This could be expressed as carbon consumption efficiency of a denitrification bed. Additionally, the ratio of denitrifying bacteria to total bacteria could be measured in future studies for rapid comparison of the carbon consumption efficiency.

2.2.4 Alternative carbon substrate for denitrification beds

The type of the C substrate used in denitrification beds is very important. The organic substrate acts as an electron donor for denitrification, provides bacteria with carbon for cell growth, and is apart from the treated effluent the main composition of the bacterial culture media. Therefore, a wide range of C substrates (wood, newspaper, wheat straw, green waste, corn stalks, corn cobs, rice husk, soil, cotton) have been tested in laboratory scale studies (Vogan, 1993; Healy et al., 2006; Greenan et al., 2006 and 2009; Gibert et al., 2008; Saliling et al., 2007; Robertson, 2010; Della Rocca et al., 2005; Soares and Abeliovich, 1998; Aslan and Türkman, 2004; Shao et al., 2008; Volokita et al., 1996 a and b;

Cameron and Schipper, 2010). Measured rates of NO_3^- removal have ranged 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). Wood material has been predominantly used in denitrification beds because it has a high C/N ratio, high permeability, long durability, low costs and ready availability (Robertson and Anderson, 1999). Some other C substrates (wheat straw, green waste, corn cobs, corn stalks, cotton, rice husk) have shown, on average, greater NO_3^- removal rates than wood byproducts (Cameron and Schipper, 2010; Della Rocca et al., 2005; Shao et al., 2008; Greenan et al., 2006; Saliling et al., 2007). However, the usefulness of some alternative C substrates for use in denitrification bed has often only been examined in short term laboratory studies. Typical start-up effects of these studies include high NO_3^- removal rates due to initially high release of organic carbon (Soares and Abeliovich, 1998 Greenan et al., 2009 and Cameron and Schipper, 2010) that do not prediction of the long term NO_3^- removal rates from these substrates. However, Cameron and Schipper (2010) found in a long term study (2 years) that corn cobs were able to remove 3 to 6.5 times more NO_3^- than woodchips without any substantial decrease in hydraulic conductivity. These NO_3^- removal and hydraulic conductivity measurements have been promising but adverse effects, carbon consumption efficiency and factors controlling NO_3^- removal of these C substrates have not been examined and should be determined before installation in denitrification beds.

2.2.5 Longevity of denitrification beds

Determining the longevity of denitrification beds is important for adequate maintenance and estimation of costs of these beds. The longevity of denitrification beds is determined by ongoing C supply to denitrifiers to sustain NO_3^- removal and degree of C substrate decay to maintain reasonable hydraulic conductivity.

Robertson et al. (2008) showed that a 15-year old denitrification wall still removed NO_3^-, but the removal rate had decreased from 7 g N m^{-3} d^{-1} to about 4 g N m^{-3} d^{-1}. Similarly, Long et al. (2011) showed that another denitrification wall removed almost all the inflowing NO_3^- from the groundwater. Long et al. (2011) estimated longevity of 66 years of the denitrification wall calculated from the observed C decay. Half lives of denitrification wall material (mixtures of wood material) taken from different depth were measured by Moorman et al. (2010). The estimated half lives of bioreactor material determined by weight loss over time ranged from 4.6 years near the surface to 36.6 years deeper in the wall. It was likely that aerobic microbial processes caused the relatively shorter life of the wood material near the surface of the denitrification wall. Mass balance calculations with woodchips of varying ages from a denitrification bed treating a stream also showed the potential for a long life time of denitrification beds. Denitrification has consumed only 10% of the carbon mass in the bioreactor after 7 years (Robertson, 2010). There is only one study of changes in hydraulic conductivity through time of different C substrates for denitrification beds (Cameron and Schipper, 2010). Cameron and Schipper (2010) suggested that the decrease of hydraulic conductivity in a 2 year study of different carbon substrates

was due to gas bubble formation beneath substrate particles rather than carbon substrate deterioration.

However, further research about ongoing carbon availability of denitrification beds and changes in hydraulic conductivity due to decay of wood material are needed to confirm the proposed long life time of denitrification beds.

2.2.6 Potential adverse effects

Potential adverse effects of denitrification beds include greenhouse gas production (N_2O and CH_4) and release of organic carbon to receiving waters. N_2O emission is the adverse effect of most concern, because it is likely to be produced as an obligate intermediate of the denitrification process (Fig. 2.2; Tiedje, 1988). N_2O contributes 6% to the anthropogenic greenhouse effect and has the longest atmospheric resident time (150 years) of all GHG (IPPC, 2001). Additionally, N_2O contributes 25% to ozone degradation in the stratosphere, which causes the ozone hole (Fabian, 1992). Methane is produced only by methanogenic archae bacteria (methanogenesis) under anaerobic conditions (Madigan, 2003). Methane contributes around 20% to the anthropogenic greenhouse effect. The atmospheric resident time of CH_4 is relatively low (9 – 15 Years), but has 25 times more global warming potential than CO_2 (IPPC, 2006). Carbon dioxide production is not an issue in denitrification beds because C substrate used in denitrification beds is C-neutral (C originally came from atmosphere) and would likely be degraded to CO_2 if used for other purposes.

A further potential adverse effect is the release of dissolved carbon from denitrification beds to receiving waterways. Excess dissolved carbon in aquatic environments can potentially harm these ecosystems by reducing the dissolved oxygen (DO) concentrations (Cox et al., 2003).

All these potential adverse effects have to be determined to evaluate the overall benefits and disbenefits of denitrification beds.

2.2.6.1 Nitrous oxide release

There are only three studies investigating potential N_2O production in denitrification beds. Greenan et al. (2009) measured dissolved N_2O concentration in the outflow of woodchip substrate columns, which on average was less than 0.033% of NO_3^--N removed. Elgood et al. (2010) and Moorman et al. (2010) determined about a magnitude higher dissolved N_2O production in their field studies. Elgood et al. (2010) measured dissolved N_2O in the outflow of a denitrification bed treating a stream. This bed removed 0.6% of NO_3^--N as N_2O-N while Moorman et al. (2010) measured that 0.84% of the NO_3^--N removed was released as dissolved N_2O-N from a denitrification wall treating agricultural drainage. The N_2O-N productions of these field bioreactors (Elgood et al., 2010, Moorman et al., 2010) are slightly greater than the N_2O-N production measured in wastewater treatment plants (0.5% of the total N removed was released as N_2O) and close to the IPCC emission factor (EF_5) for N_2O-N, which is 0.75% for N released in waterways (IPCC, 2006). Furthermore, Elgood et al. (2010) and Moorman et al. (2010) measured greater N_2O production in colder months than in warmer months. These studies suggested that colder temperatures lead to slow

reaction rates and less inlet DO consumption in the wood substrate, which may increase incomplete denitrification and N_2O production. Conversely, Teiter and Mander (2005) and Johansson et al. (2003) reported for constructed wetland greater N_2O production in summer months due to higher denitrifying activity. Further studies are needed to clarify N_2O production related to temperature in denitrification bed and column studies.

Sulfide is an important factor that can increase N_2O production. Sulfide inhibits the N_2O reductase, which leads to increased N_2O emission. Measurements of S^{2-} have not been conducted in denitrification beds, but an decrease of SO_4^{2-} and the odour of H_2S has been reported when NO_3^- was almost depleted (van Driel et al., 2006; Robertson and Merkley, 2009; Robertson, 2010; Elgood et al., 2010).

It is important to include total N_2O production from losses of both dissolved N_2O and N_2O emitted from the surface of the beds. Dissolved N_2O can still be consumed by downstream denitrification. Below a certain NO_3^- concentration, more N_2O will be consumed than produced by denitrification, which would lead to a reduction of dissolved N_2O concentration. Therefore, the release of N_2O to the atmosphere can vary between denitrification beds. Additionally, different surface atmosphere interactions will affect the N_2O emission of denitrification beds. Consequently, it is necessary to measure the N_2O emission of denitrification beds along with the dissolved N_2O release in receiving waters and determine potential factors controlling N_2O production.

2.2.6.2 Methane release

Heterotrophic methane producing bacteria compete with denitrifiers and sulfate reducing bacteria for C as energy source. However, it is likely that methanogenes will be suppressed by NO_3^- and/or SO_4^{2-} reducing bacteria in denitrification beds because of the low energetic yield of C reduction of the methanogensis (Madigan et al., 2003). Elgood et al. (2010) measured CH_4 in a stream bed bioreactor when NO_3^- was almost depleted and Tanner et al. (1997) detected relatively high CH_4 production in some constructed wetlands. Therefore, it is important to determine CH_4 production in denitrification beds.

2.2.6.3 TOC loss

The potential release of organic carbon from denitrification beds to receiving waters may cause impacts in aquatic ecosystems primarily by increasing microbial oxygen consumption, leading to DO depletion in the water column and sediments (Cox et al., 2003). Denitrifying woodchip bioreactors have been shown to release organic carbon particularly the first month of operation (Robertson and Cherry, 1995; Robertson et al., 2005b, Schipper et al., 2010a). This start-up effect has also been reported in laboratory scale studies testing carbon substrates for denitrifying bioreactors (Soares and Abeliovich, 1998; Greenan et al., 2009 and Cameron and Schipper, 2010). There are a range of approaches that might reduce the leakage of organic carbon in receiving waters during the start-up phases of denitrification beds (Schipper et al., 2010a). Apart from initial losses of carbon, Robertson (2010) observed escalating DOC releases from woodchips of denitrification beds in a column study, when NO_3^--N concentrations decreased below 1 mg L^{-1}. This

increasing DOC release was likely due to a decrease in carbon consumption by the denitrifiers, when they became NO_3^- limited. When alternative C substrates are examined for potential use in denitrification beds, organic carbon release should also be measured.

2.3 Conclusions

Denitrification beds seem to be very effective for removing NO_3^- from point source discharges. Denitrification beds filled with wood by-products have low installation and maintenance costs and have relatively high NO_3^- removal rates compared to other passive technologies. Additionally, it was shown that woodchip bioreactor can sustain NO_3^- removal for at least 14 years (Robertson et al., 2008, Long et al., 2011). However, NO_3^- removal rates have generally been measured as the difference between intlet and outlet concentrations of NO_3^-, but this approach may lead to either over- or underestimation of actual rates of NO_3^- removal. Many studies have suggested that microbial denitrification was responsible for NO_3^- removal in the beds, but direct evidence is lacking. Additionally, there are only few studies measuring adverse effects of denitrification beds, which are needed to evaluate the overall benefit of the beds and it is the first step for identifying mitigation approaches. Measurements of dissolved N_2O release of the beds have generally been within the range of the IPPC emission factor for N_2O-N release in waterways. However, these studies have not measured the N_2O emission from the bed surface. Methane emissions have been observed in only one denitrification bed study, when NO_3^- was almost depleted and further data is required. Similarly, dissolved organic carbon release was a concern during start up phases of the beds

and when NO_3^--N concentration was below 1mg L^{-1}. Controlling factors of NO_3^- removal and microbial processes that compete for available C have not been throughly examined in denitrification beds. Manipulation of factors controlling NO_3^- removal may enhance the NO_3^- removal of denitrification beds. There are many studies of alternative carbon substrates for denitrification beds. But measurement of potential adverse effects of alternative C sources has not been conducted.

The key aspects to evaluate the net benefit of denitrification beds and to develop strategies to enhance NO_3^- removal and reduce potential adverse effects of denitrification beds are listed below and are addressed in this thesis.

- It is important to develop an approach that measure reliable rates of NO_3^- removal and determine the mechanism for NO_3^- removal. The sustainability of the NO_3^- removal can be evaluated, when the mechanism for NO_3^- removal is established. Additionally, comparison of different techniques (2.1.3. a-e) for measuring denitrification rates help to use the appropriate techniques in ecosystems similar to denitrification beds.

- Measuring potential adverse effects including N_2O, CH_4 and TOC release of denitrification beds in comparison to other passive technologies designed for NO_3^- removal is important to evaluate the overall benefit of the bed and to minimize potential problems of this technology.

- The longevity of denitrification beds should be determined by measuring the net carbon consumption (CO_2, CH_4 and TC release) of the bed. Additionally, the quantity of C consumption by microbial processes other than denitrification should be determined to evaluate the efficiency of the bed. This knowledge could be used to develop strategies to enhance denitrifying processes and reduce unwanted carbon consuming processes of bacteria.

- Determining the factors controlling NO_3^- removal such as temperature, DO, pH, availability of C, number of denitrifying bacteria and concentrations of NO_3^-, NO_2^- and S^{2-} would allow development of strategies to enhance the NO_3^- removal of the bed.

- Alternative carbon substrates used in denitrification beds should be examined for adverse effects, longevity, carbon consumption efficiency and controlling factors before installations to evaluate the overall benefit of these substrates and to develop recommendations for bioreactor construction.

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Chapter 3

Rates, Controls and potential Adverse Effects of Nitrate Removal in a Denitrification Bed

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Rates, controls and potential adverse effects of nitrate removal in a denitrification bed

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ABSTRACT

Denitrification beds are a simple approach for removing nitrate (NO_3^-) from a range of point sources prior to discharge into receiving waters. These beds are large containers filled with woodchips that act as an energy source for microorganisms to convert NO_3^- to nitrogen (N) gases (N_2O, N_2) through denitrification. This study investigated the biological mechanism of NO_3^- removal, its controlling factors and its adverse effects in a large denitrification bed (176 m × 5 m × 1.5 m) receiving effluent with a high NO_3^- concentration (>100 g N m^{-3}) from a hydroponic glasshouse (Karaka, Auckland, New Zealand). Samples of woodchips and water were collected from 12 sites along the bed every two months for one year, along with measurements of gas fluxes from the bed surface. Denitrifying enzyme activity (DEA), factors limiting denitrification (availability of carbon, dissolved organic carbon (DOC), dissolved oxygen (DO), temperature, pH, and concentrations of NO_3^-, nitrite (NO_2^-) and sulfide (S^{2-})), greenhouse gas (GHG) production – as nitrous oxide (N_2O), methane (CH_4), carbon dioxide (CO_2) – and carbon (C) loss were determined. NO_3^--N concentration declined along the bed with total NO_3^--N removal rates of 10.1 kg N d^{-1} for the whole bed or 7.6 g N m^{-3} d^{-1}. NO_3^--N removal rates increased with temperature (Q_{10} = 2.0). In laboratory incubations, denitrification was always limited by C availability rather than by NO_3^-. DO levels were above 0.5 mg L^{-1} at the inlet but did not limit NO_3^--N removal. pH increased steadily from about 6 to 7 along the length of the bed. Dissolved inorganic carbon (C-CO_2) increased in average about 27.8 mg L^{-1}, whereas DOC decreased slightly by about 0.2 mg L^{-1} along the length of the bed. The bed surface emitted on average 78.58 μg m^{-2} min^{-1} N_2O-N (reflecting 1% of the removed NO_3^--N), 0.238 μg m^{-2} min^{-1} CH_4 and 12.6 mg m^{-2} min^{-1} CO_2. Dissolved N_2O-N increased along the length of the bed and the bed released on average 362 g dissolved N_2O-N per day coupled with N_2O emission at the surface about 4.3% of the removed NO_3^--N as N_2O. Mechanisms to reduce the production of this GHG need to be investigated if denitrification beds are commonly used. Dissolved CH_4 concentrations showed no trends along the length of the bed, ranging from 5.28 μg L^{-1} to 34.24 μg L^{-1}. Sulfate (SO_4^{2-}) concentrations declined along the length of the bed on three of six samplings; however, declines in SO_4^{2-} did not appear to be due to SO_4^{2-} reduction because S^{2-} concentrations were generally undetectable. Ammonium (NH_4^+) (range: <0.0007 mg L^{-1} to 2.12 mg L^{-1}) and NO_2^- concentrations (range: 0.0018 mg L^{-1} to 0.95 mg L^{-1}) were always very low suggesting that anammox was an unlikely mechanism for NO_3^- removal in the bed. C longevity was calculated from surface emission rates of CO_2 and release of dissolved carbon (DC) and suggested that there would be ample C available to support denitrification for up to 39 years.

This study showed that denitrification beds can be an efficient tool for reducing high NO_3^- concentrations in effluents but did produce some GHGs. Over the course of a year NO_3^- removal rates were always limited by C and temperature and not by NO_3^- or DO concentration.

1. Introduction

Reactive N at regional and global scales has rapidly increased during the last few decades due to anthropogenic activities (Galloway et al., 2003). Increased reactive N can have lasting adverse ecological effects contributing to eutrophication, hypoxia,

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toxic algal blooms, shifts in the food chain, loss of biodiversity, loss of fish stocks and habitat degradation in streams, lakes and coastal waters and elevated N_2O emissions (Howarth et al., 2000; Rabalais, 2002; Phoenix et al., 2006). Consequently, management practices to remove reactive N are needed to reduce adverse impacts on the receiving environment. There are several approaches to remove reactive N from effluents and waterways. These approaches attempt to enhance microbial denitrification, which reduces NO_3^- to unreactive N gas (N_2) (Schipper et al., 2010a) and anammox, which converts NO_3^- and NH_4^+ to N_2. For example, constructed wetlands are a widespread technology for N removal (Vymazal, 2006) and denitrification beds are increasingly being used to remove NO_3^- from point source discharge (Schipper et al., 2010c). Denitrification beds are large containers filled with wood by-products, such as woodchips or sawdust which act as a C source to denitrifying bacteria. Wood by-products are used because of their high C:N ratio, commercial availability, low original costs, high permeability and long persistence (Robertson and Anderson, 1999). A number of studies of NO_3^- removal in denitrification beds have been reported (Robertson et al., 2005, 2009; Van Driel et al., 2006; Robertson and Merkley, 2009; Schipper et al., 2010b; Robertson, 2010; Moorman et al., 2010; Elgood et al., 2010). Averages of NO_3^- removal rates ranged from 1.8 g N m^{-3} d^{-1} measured in a small bed in Canada (Robertson et al., 2005) to 9.7 g N m^{-3} d^{-1} measured in a large bed in New Zealand (Schipper et al., 2010b).

While NO_3^- removal has been demonstrated in denitrification beds, the microbial mechanisms responsible for NO_3^- removal, factors limiting NO_3^- removal and adverse effects such as N_2O emission, CH_4 emission and C loss in the effluent have not been fully examined. The factors that may limit denitrification in most ecosystems are temperature, C availability, pH, NO_3^- and dissolved oxygen (DO) concentrations (Seitzinger et al., 2006). These factors can also lead to incomplete denitrification and alter the production of the obligate denitrification intermediate, N_2O (Kampschreur et al., 2009). This potent GHG caused 6% of the anthropogenic glasshouse effect in 2001 and contributes to stratospheric ozone depletion (IPCC, 2001).

Previous studies have shown that NO_3^- removal increases with temperature in denitrification beds (Robertson et al., 2008, 2009; Elgood et al., 2010). Elgood et al. (2010) and Robertson et al. (2008) measured a Q_{10} of 2 and almost 10, respectively in denitrification beds.

Healy et al. (2006) reported that NO_3^- removal declined in laboratory scale experiments when DO concentration was greater than 3.7 mg L^{-1}, because oxygen (O_2) competes with NO_3^- as terminal electron acceptor. Elgood et al. (2010) reported that higher DO inlet concentrations led to incomplete denitrification which increased production of dissolved N_2O. However, the full extent to which DO concentration effects N_2O production and NO_3^- removal in larger denitrification beds has not been quantified. Denitrifying bacteria also face competition for available C from other anaerobic microorganisms, such as SO_4^{2-} reducers and methanogens. A few studies have reported declines of SO_4^{2-} concentrations and the odor of H_2S at the outlet of a denitrification bed when NO_3^- concentrations were low (<1 mg L^{-1}) (Robertson et al., 2009; Robertson, 2010; Elgood et al., 2010), but microbial SO_4^{2-} reduction has not been measured.

There have been few studies examining the adverse effects of denitrification beds, such as increased production of GHG and enhanced release of DOC from the bed. Dissolved N_2O-N production in woodchip bioreactors ranged from 0.6% to 0.85% of removed NO_3^--N (Moorman et al., 2010; Elgood et al., 2010); however, N_2O surface emissions were not measured. In a column experiment with woodchips, dissolved N_2O production was low ranging from 0.003% to 0.033% of removed NO_3^--N (Greenan et al., 2009). In a stream denitrification bed, Elgood et al. (2010) measured increases in dissolved CH_4 concentrations when NO_3^- concentrations were low during warmer periods. Some studies have measured DOC release from woodchips. Increased DOC concentrations were observed at the start up phases of woodchip bioreactors (Schipper et al., 2010c; Cameron and Schipper, 2010), and while NO_3^- concentrations were low (Robertson, 2010).

Our key objectives were to measure the rate of N removal along the length of a large bed for a year and to determine how NO_3^- removal was controlled by environmental factors such as respirable C, DO, DOC, temperature, and pH. Furthermore, we measured potential adverse effects of the bed including emitted and dissolved N_2O production, emitted and dissolved CH_4 production and DOC release. We evaluated the occurrence of competing anaerobic processes in the bed, such as NO_3^- reduction, SO_4^{2-} reduction, dissimilatory nitrate reduction to ammonium (DNRA) and anammox. Finally, the lifetime and efficiency of the denitrification bed was calculated.

2. Materials and methods

2.1. Study site

This study was conducted at a denitrification bed receiving effluent from a glasshouse at Karaka, New Zealand. The glasshouse predominantly grew tomatoes and cucumbers hydroponically. The bed (176 m × 5 m × 1.5 m) was constructed in 2006 and was filled with a mixture of woodchips and sawdust (pine wood) (Schipper et al., 2010b). The effluent from the glasshouse was pumped into one end of the denitrification bed through a PVC pipe 1 m below the surface of the woodchips. Final effluent leaving the bed was discharged into a drainage ditch. Twelve sampling sites were established along the length of the bed at 16 m intervals. Each sampling point included a fixed closed plastic chamber (22.5 cm high; Ø 25 cm) for gas (N_2O and CH_4) sampling on the surface of the bed, a soil collar (Ø 10.3 cm) for CO_2 surface emission rate measurements, and a fully screened PVC well (2 m long; Ø 5 cm) for water sampling. Flow rates of influent and effluent of the bed were measured with a mechanical water meter (LXLG-80, Bil, China) at the inflow and at the outflow.

2.2. Sampling and analysis

2.2.1. Field sampling

All sites along the bed were sampled every 2 months for 12 months in 2008/2009. Samples of water saturated woodchips were obtained from 0.2 m below the bed surface using a shovel and stored in plastic bags at 4 °C until laboratory analysis within 2 days for denitrification rate (DR) and factors that control denitrification. Water samples were taken from each well using a pump, stored in 50 mL plastic tubes on ice and subsequently analysed for pH in the laboratory within 8 h using a MP 220 pH meter (Mettler Toledo, Switzerland), and for NO_3^-, NO_2^-, NH_4^+, PO_4^{2-}, SO_4^{2}, S^{2-}, total carbon (TC), total organic carbon (TOC), total inorganic carbon (TIC), dissolved carbon (DC), DOC and dissolved inorganic carbon (DIC) concentrations (see methods below). Water samples were also collected in 3.7 mL exutainers (Labco, UK) prefilled with 0.2 mL H_2SO_4 (20%) as a preservative and analysed for dissolved gases (dissolved N_2O and dissolved CH_4) after 12 h headspace equilibrium at room temperature within 24 h. Temperature and DO of the bed water were measured in each well using an InLab 605 O_2-Sensor (Mettler Toledo, Switzerland). Gas samples (6 mL) were taken from the chambers using the closed-chamber technique described by

Table 1
Changes in NO_3^--N concentrations along the length of the bed and calculated removal rates for each month of sampling and average effluent temperature.

Month of sampling; temp. (°C)	NO_3^-N concentration (y); length (x)	R^2	p-value	NO_3^--N removal rate (g m^{-3} d^{-1})	NO_3^--N removed per day and bed (kg)
April; 16.7	$y = -0.35x + 144$	0.65	0.002	6.7	8.8
June; 15.5	$y = 0.15x + 92$	0.28	0.076	No removal	No removal
September 17.0	$y = -0.36x + 101$	0.77	<0.001	7.2	9.5
November; 19.3	$y = -0.37x + 139$	0.87	<0.001	7.7	10.1
December; 20.9	$y = -0.21x + 113$	0.90	<0.001	4.6	6.0
January; 23.7	$y = -0.53x + 146$	0.88	<0.001	11.2	14.8
March; 18.4	$y = -0.62x + 206$	0.72	0.001	8.4	11.1

Hutchison and Moiser (1981) at 30, 60, 90 and 120 min along the length of the bed and stored in 3.7 mL exutainers until analyses for N_2O and CH_4.

2.2.2. Chemical analysis

Water samples were filtered with disposable membrane filters (0.45 μm) prior to analysis for NO_3^-, NH_4^+, PO_4^{2-} and NO_2^- using a flow injection analyser (Lachat Instruments; Loveland, USA) (APHA, 1992). SO_4^{2-} concentrations were determined via ion chromatography (APHA, 2005). Filtered water samples were acid distilled into alkaline trapping solution using a Lachat Micro Dist system and analysed for S^{2-} concentrations via FIA (APHA, 2005). Unfiltered samples were analysed for TC, TOC and TIC using a Shimadzu TOC-5000 analyser, whereas DC, DOC and DIC were determined from filtered water samples.

2.2.3. GHG production

Collected gas samples and headspace equilibrium samples were analysed for N_2O and CH_4 concentration via gas chromatography using an electron capture detector (GC-ECD) and a flame ionization detector (GC-FIA), respectively (Varian; Palo Alto, USA). For N_2O analyses, the GC 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 and, the FID detector temperature was 150 °C and the flow rate of the N_2-carrier gas was 30 mL min^{-1}. Gas emission rates were calculated as the change of gas concentration with time. Dissolved N_2O and dissolved CH_4 gas concentrations along the length of the bed were calculated via headspace equilibrium using the Bunsen coefficient of the gases (Weiss and Price, 1980; Yamamoto et al., 1976).

CO_2 surface emission rates at each sampling point along the length of the bed were measured using a LI-8100 automated soil CO_2 surface emission rate system (LI-COR Inc.) with a soil collar area of 83.7 cm^2 and an observation length of 1.20 min.

2.2.4. Denitrification rates

Denitrification rates (DR) of the bed material were determined in the laboratory using a modification of the denitrifying enzyme activity method (DEA) of Tiedje et al. (1989). Woodchips (100 g fresh weight) and water (60 g) from each sampling site were placed into four airtight bottles (600 mL). To identify whether DR was C and/or NO_3^- limited, bottles were amended with one of four solutions (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). Headspace of the bottles was flushed with N_2 gas for 10 min and then 40 mL of acetylene gas was injected (10% of the headspace volume) to inhibit reduction of N_2O to N_2. Bottles were incubated at 27 °C on a shaker table at 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 exutainers (Labco, UK) until analysis for N_2O concentration within 7 days via GC-ECD (see above).

To determine how temperature controlled the DR under laboratory conditions, woodchip samples along the length of the bed collected in November 2008 were mixed thoroughly and DR measured as described above at 4, 8, 12, 16, 25 and 32 °C.

2.2.5. 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). Woodchips (100 g fresh weight) and effluent (60 g) from each sampling location was placed into airtight bottles (600 mL). Small beakers (30 mL) containing 10 mL of 0.5 M KOH were placed into the jars as a CO_2 trap. The jars were sealed and the headspace of bottles was flushed with N_2 gas for 10 min to remove oxygen and incubated at room temperature (22 °C) for 4 days. After incubation, 5 mL of the CO_2 trapping solution was transferred to flasks (100 mL) and 10% $BaCl_2$ (10 mL) was added along with phenolphthalein as a pH indicator. Solutions were back-titrated against the standard 0.1 M HCl to determine the amount of trapped CO_2 and expressed as CO_2-C g^{-1} woodchips (dry weight).

2.2.6. Data analyses

NO_3^- removal rates were calculated from the linear regression of the NO_3^--N concentrations along the length of the bed (ΔNO_3^-): NO_3^--N removal rate = ΔNO_3^--N × FR/V_{bed}, where FR was the flow rate of the effluent and V_{bed} was the volume of the bed. Statistical analyses were conducted using Systat (Version 10, SPSS Inc, Chicago, USA) to determine whether NO_3^- or C was the limiting factor of the denitrification. DR were log transformed to meet the normality assumptions of parametric tests, then analysed by two-way ANOVA to identify significant ($p < 0.05$) differences in denitrification rates among treatments of $+NO_3^-$ and +C (Tank and Dodds, 2003). This analysis is also able to determine co-limitation by both N and C based on the significance ($p < 0.05$) of the interaction term (N × C). Presented error bounds are standard errors.

3. Results

3.1. Solute concentrations

The average effluent flow rate of the bed was 145 m^3 d^{-1} ± 9 m^3 d^{-1} with a constant inlet flow.

There were significant linear declines in NO_3^--N concentrations along the length of the bed throughout the study, except for June (Table 1 and Fig. 1). NO_3^--N removal rates ranged from 4.6 (December) to 11.2 g N m^{-3} d^{-1} (January) with an average NO_3^--N removal rate of 7.6 g N m^{-3} d^{-1} which was equivalent to 10.1 kg NO_3^--N removed per day for the entire bed (Table 1). The bed temperature ranged from 15.5 °C to 23.7 °C on sampling dates and NO_3^--N removal rate measured in the bed generally increased with increasing temperature, excluding December and June (Fig. 2). An

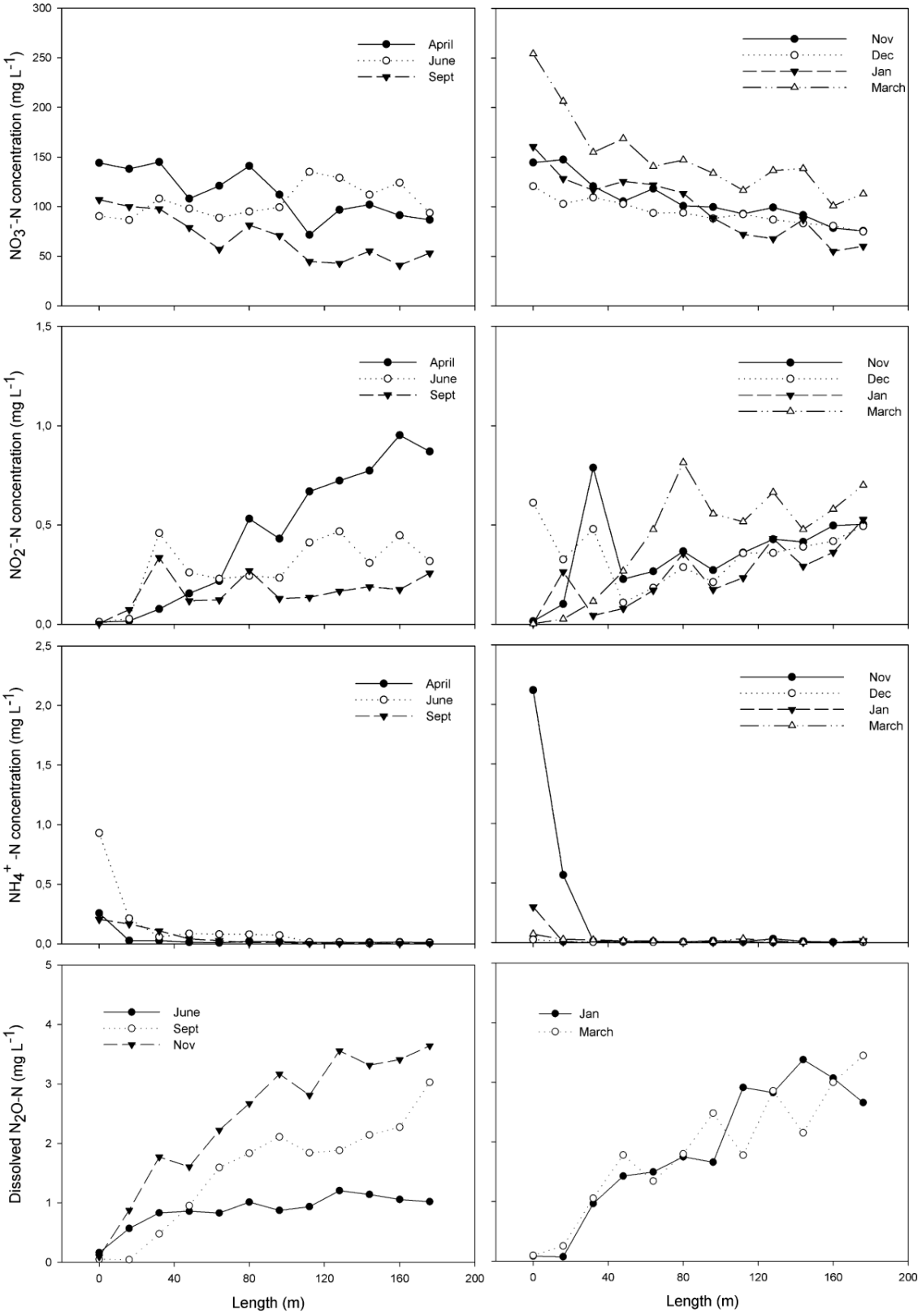

Fig. 1. Nitrogen key form concentrations along the length of the denitrification bed for every month of sampling.

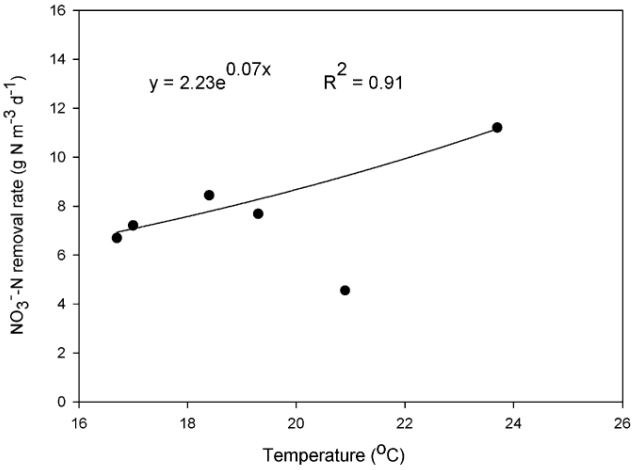

Fig. 2. *In situ* nitrate-N removal at different effluent temperatures for every month of sampling, except June. Exponential trend line is shown and does not include measurement in December at 20.9 °C.

exponential fit of NO_3^--N removal to temperature ($y = 2.23e^{0.07x}$; $R^2 = 0.91$, $p = 0.009$) gave a Q_{10} of 2.0 (Fig. 2). Q_{10} is the factor of the reaction rate increase with every 10 °C rise in temperature.

Ammonium-N concentrations from April to January were always less than 2.1 mg N L^{-1} at the inlet and rapidly decreased to less than 0.1 mg N L^{-1} in the first 50 m of the bed (Fig. 1). Nitrite-N concentrations increased along the length of the bed but were always very low (0.002 mg N L^{-1} to 0.95 mg N L^{-1}) (Fig. 1).

For three of six samplings, SO_4^{2-} concentrations decreased along the length of the bed (Fig. 3). Sulfate concentrations decreased from 370 mg L^{-1} to 290 mg L^{-1} in April, from 700 mg L^{-1} to 470 mg L^{-1} in September and from 570 mg L^{-1} to 410 mg L^{-1} in January. In June we observed an increase in SO_4^{2-} concentrations along the length of the bed (Fig. 3). Sulfide concentrations were almost always below the detection limit of 0.002 mg L^{-1}, except for April, when two sampling locations had S^{2-} concentrations of 0.0045 and 0.0039 mg L^{-1} (full data not shown). Phosphate-P concentrations in the bed were high ranging from 12.4 mg L^{-1} to 32 mg L^{-1} over the course of the study (Fig. 3) and decreased along the length of the bed on four occasions.

Dissolved oxygen concentrations in the inflow were relatively high, except for January (0.37 mg L^{-1}) ranging from 1.48 mg L^{-1} (September) to 2.84 mg L^{-1} (June) and decreased sharply in the first 60 m of the bed to less than 0.1 mg L^{-1} (Fig. 3). The pH increased along the length of the bed from about 6 to 7 on all sampling occasions (Fig. 3).

3.2. Denitrification and respirable C

Average DEAs (DR + C/N) ranged from 24.6 ± 3.9 μg N h^{-1} g^{-1} (September) to 38.3 ± 5.8 μg N h^{-1} g^{-1} (April) (Fig. 4). Unamended DRs ranged from 11.2 ± 1.6 μg N h^{-1} g^{-1} (November) to 20.3 ± 3.7 μg N h^{-1} g^{-1} (April). For all sampling times and locations, denitrification was limited by C rather than NO_3^- (2-way Anova, $p < 0.01$). Adding glucose enhanced the DR by about 85% while addition of NO_3^- did not increase enzyme activity in comparison to unamended controls (Fig. 4). Under laboratory conditions, DR of incubated woodchips increased with temperature and gave a Q_{10} of 2.1 ($y = 2.35e^{0.07x}$; R^2, 0.92; p, 0.0065) (full data not shown) similar to the Q_{10} of 2.0 of the NO_3^- removal field data measured over the course of one year (Fig. 2).

Respirable C ranged from 59.6 ± 4.9 mg C g^{-1} d^{-1} (September) to 124.5 ± 7.1 mg C g^{-1} d^{-1} (January). Average respirable C was almost 1.5 times higher in January than in the other months of sampling and significantly different to the other months of sampling (Tukey's $p < 0.035$ for each month) (full data not shown).

3.3. Greenhouse gases and C budget

N_2O-N surface emission rates ranged from 42.8 ± 22.4 μg m^{-2} min^{-1} (November) to 110.3 ± 53.2 μg m^{-2} min^{-1} (January), with an average of almost 100 g d^{-1} bed^{-1} N_2O-N or 1% of NO_3^--N removed by the bed (Fig. 5). Dissolved N_2O-N concentrations increased along the length of the bed for every month of sampling, except for June when no N_2O surface emission and no nitrate removal was observed (Table 2 and Fig. 1). The net release of dissolved N_2O-N from the bed in the outflow was between 0.09 kg d^{-1} (June) and 0.51 kg d^{-1} (January, November) (Table 2), reflecting on average 3.3% of the removed NO_3^--N, coupled with the surface emitted N_2O-N the bed released 4.3% of the removed NO_3^--N as N_2O-N.

For the majority of sampling times and locations the bed was a net emitter of methane (Fig. 5). However, on occasions, methane concentrations in the chambers decreased suggesting methane oxidation in the surface of the bed (ranging from -1.02 ± 0.11 μg m^{-2} min^{-1} (November) to -2.9 ± 0.06 μg m^{-2} min^{-1} (March); full data not shown). Averages of CH_4 emission by sampling time and bed surface were always positive and ranged from 0.02 ± 0.08 μg m^{-2} min^{-1} (November) to 0.51 ± 0.24 μg m^{-2} min^{-1} (January) with great standard errors (Fig. 5). Over the year long sampling the CH_4 surface emission rate of the bed averaged 0.27 g d^{-1}. Dissolved methane concentrations within the bed ranged from 5.28 μg L^{-1} to 34.24 μg L^{-1} in the yearly sampling but there was no trend in CH_4 concentrations along the length of the bed (full data not shown).

CO_2 surface emissions ranged from 5.48 ± 0.72 mg m^{-2} min^{-1} (March) to 25.82 ± 5.27 mg m^{-2} min^{-1} (November) with an average CO_2-C emission of 4.4 kg bed^{-1} d^{-1} (Fig. 5). DIC (dissolved CO_2-C) increased along the length of the bed for every sampling, except for June. The net DIC outflow concentrations ranged from 17.58 mg L^{-1} (March) to 50.67 mg L^{-1} (January) (Fig. 6 and Table 2). The bed net released an average of 4.52 kg d^{-1} dissolved CO_2-C (Table 2). The increase of TC along the length of the bed was mainly due to the increase of DIC, and the bed net released on average 5.8 kg d^{-1} TC, except for June (Fig. 6). DOC concentrations were almost constant along the length of the bed (Fig. 6). The average DOC inflow concentration was 7.15 mg L^{-1} which was 1.2 mg L^{-1} greater than the DOC outflow concentration, reflecting an average DOC consumption of 0.2 kg d^{-1} of the bed. In June, DOC increased along the length of the bed and DIC was almost constant (Fig. 6).

4. Discussion

4.1. Nitrate removal and denitrification

Nitrate removal rates averaged 7.6 g N m^{-3} d^{-1} (ranging from 4.6 g N m^{-3} d^{-1} to 11.2 g N m^{-3} d^{-1}) and were at the higher end of NO_3^--N removal rates reported for denitrification bed studies (as reviewed by Schipper et al., 2010c). In many other reported bed studies, NO_3^- was often completely removed so that removal was likely limited by NO_3^- concentrations (Robertson et al., 2005; Schipper et al., 2010c). Generally, NO_3^--N removal rates of denitrification beds were higher than rates of constructed wetlands. Average rates of constructed wetlands ranged from 0.68 g N m^{-2} d^{-1} to 2.52 g N m^{-2} d^{-1} with different processes responsible for N removal (plant uptake, denitrification, annamox) (Vymazal, 2006). The low rates of NO_3^- removal measured in June

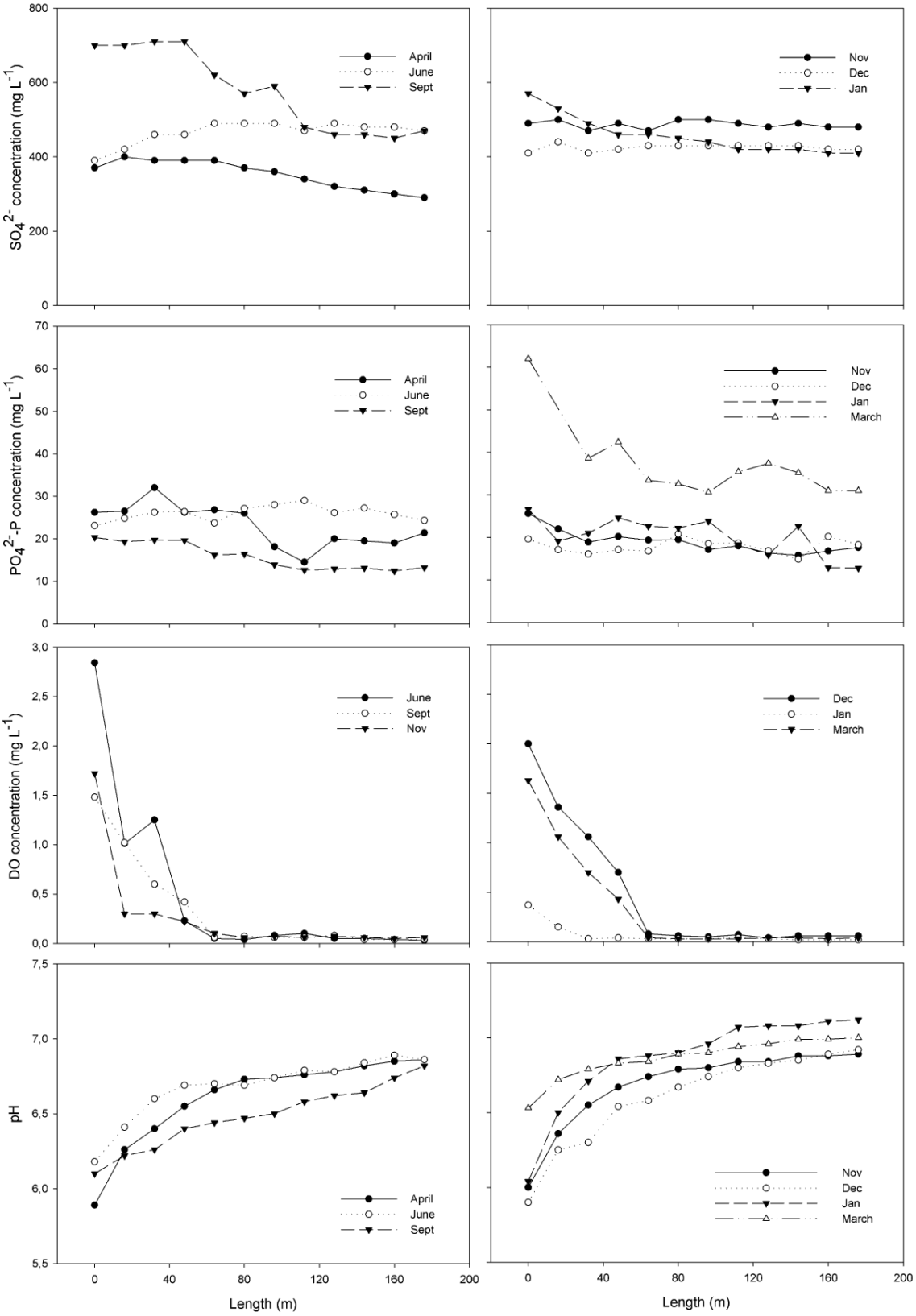

Fig. 3. Sulfate (SO_4^{2-})- (top), PO_4^{2-}P-, DO-concentrations and pH (bottom) along the length of the bed for every month of sampling.

Table 2
Changes in dissolved N_2O-N and DIC (dissolved CO_2) concentrations along the length of the bed and calculated release of dissolved N_2O-N and CO_2 in the outflow for each month of sampling.

Month of sampling	Dissolved N_2O-N conc. (y); length (x)	R^2	p-value	Dissolved N_2O-N released from the bed (kg d^{-1})
June	$y = 0.28x + 37.82$	0.65	0.002	0.09
September	$y = 1.2x + 12.05$	0.89	<0.001	0.41
November	$y = 1.32x + 57.1$	0.88	<0.001	0.51
January	$y = 1.30x + 18.15$	0.87	<0.001	0.51
March	$y = 1.2x + 25.86$	0.86	<0.001	0.30

Month of sampling	DIC conc. (y); length (x)	R^2	p-value	DIC released from the bed (kg d^{-1})
April	$y = 0.21x - 0.47$	0.96	<0.001	5.3
June	$y = 0.01x + 1.86$	0.73	<0.001	0.30
September	$y = 0.14x + 3.03$	0.90	<0.001	3.79
November	$y = 0.16x + 4.5$	0.88	<0.001	4.42
December	$y = 0.14x + 2.68$	0.96	<0.001	3.84
January	$y = 0.29x + 6.3$	0.86	<0.001	8.01
March	$y = 0.1x - 0.15$	0.92	<0.001	1.79

and December were attributed to antimicrobial treatment (composition unknown) applied in the glasshouse that leached into the bed and inhibited microbial activity. However, DR measured on June and December samples were similar to other sampling periods (Fig. 4) suggesting that any treatment lost its potency to inhibit microbial processes during 2 days of storing the woodchips prior to analysis. Further evidence of an antimicrobial treatment in June was a lack of N_2O production (emitted and dissolved), low CH_4 emission, increase of DOC and no increase of DIC along the length of the bed suggesting little *in situ* denitrification, methanogenesis, or microbial consumption of C.

Anaerobic microbial processes dominated in the bed as the DO concentration declined to less than 0.1 mg L^{-1} within the first 60 m. Initial degradation of woodchips was likely by fermentative bacteria which released C to other anaerobic microbes including denitrifiers. It is likely that denitrification was the main mechanism for NO_3^--N removal in the bed as exceptionally high DEAs were measured (average of 29.7 μg N h^{-1} g^{-1}) compared to other treatment systems e.g., the DEA in the bed were 20 to 1000 times higher than maximum DEAs of denitrification walls (0.030 μg N h^{-1} g^{-1} and 1.36 μg N h^{-1} g^{-1}) (Schipper et al., 2005; Moorman et al., 2010, respectively), a forested land based wastewater treatment system (0.24 μg N h^{-1} g^{-1}) (Barton et al., 2000), riparian forest sites (1.08 μg N h^{-1} g^{-1}) (Groffmann et al., 1992), a natural wetland (0.9 μg N h^{-1} g^{-1}) and a constructed wetland (0.36 μg N h^{-1} g^{-1})

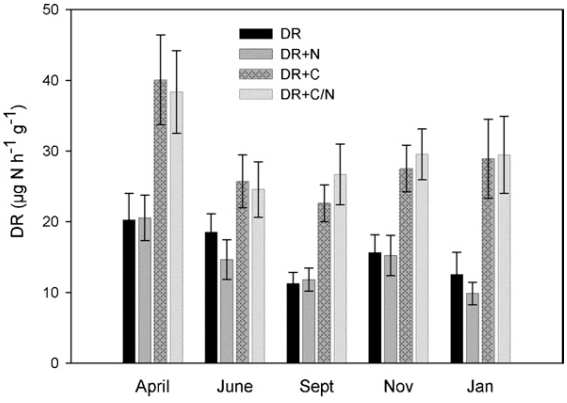

Fig. 4. Average DR at 27 °C for different months of sampling. *In vitro* DR assays – containing woodchips and effluent of the denitrification bed – were amended with glucose (DR + C), NO_3^- (DR + N) and glucose and NO_3^- (DR + C/N; DEA) and no amended (DR). Error bars are one standard error ($n = 12$). In all cases there were significant differences between samples amended with C (DR + C and DR + C/N) and samples without C amendment (DR and DR + N).

(Duncan and Groffmann, 1994). Using the measured bulk density of 210 kg m^{-3} of the woodchip material and an average bed temperature of 18.4 °C the DR measured in the lab can be converted to an *in situ* DR using a Q_{10} of 2.1 which gives an average *in situ* DR of 43.1 g N m^{-3} d^{-1}. This calculated DR at bed temperature was 6 times greater than measured NO_3^- removal rates in the bed suggesting that denitrification was more than sufficient to account for NO_3^- removal from the bed.

There was no evidence of other significant NO_3^- removal processes in the bed. Nitrate removal mechanisms such as anammox and DNRA were unlikely to be the major contributor to NO_3^--N removal in the bed as NH_4^+ concentrations were always too low to account for these removal rates, and N_2O an obligatory intermediate of the denitrification process was produced. Biotic uptake of NO_3^- can also be disregarded because there are no plants growing in the woodchips and uptake by microbes would occur only until reaching equilibrium shortly after the bed was first constructed. These findings agree with several other studies of denitrification beds indicating that microbial denitrification was the main NO_3^- removal process. *In situ* push pull test using ^{15}N-NO_3^- in a denitrifiction wall resulted in a NO_3^- removal of 35.76 mg N $g\,m^{-3}$ d^{-1} (Moorman et al., 2010), which was similar to the measured DR of 43.1 g N m^{-3} d^{-1} in this study. However, this is not proof of denitrification, because removal of ^{15}N-NO_3^- was measured, and not production of ^{15}N-N_2. Laboratory studies using ^{15}N-NO_3^- by Greenan et al. (2006) showed that less than 4% of total NO_3^- removed was due to DNRA in columns filled with woodchips. Furthermore, Robertson et al. (2000) and Robertson (2010) measured an increase of natural abundance ^{15}N in the residual NO_3^- in the outflow of denitrification beds and woodchip filled columns respectively. This isotopic enrichment was likely due to denitrification, because low NH_4^+ concentrations ruled out anammox and DNRA.

4.2. Factors controlling denitrification and C competing processes in the bed

There are a number of environmental factors that generally control denitrification including availability of C, DO, temperature, pH, number of denitrifying bacteria, and concentrations of NO_3^-, NO_2^- and S^{2-} (Firestone and Davidson, 1989; Seitzinger et al., 2006). In this study, denitrification appeared to be predominantly limited by C supply and temperature.

Laboratory testing of DR amended with NO_3^- and/or C clearly demonstrated that denitrification in the bed was limited by C but not by NO_3^- on all occasions. Carbon is usually the limiting factor for denitrification in anaerobic environment with high

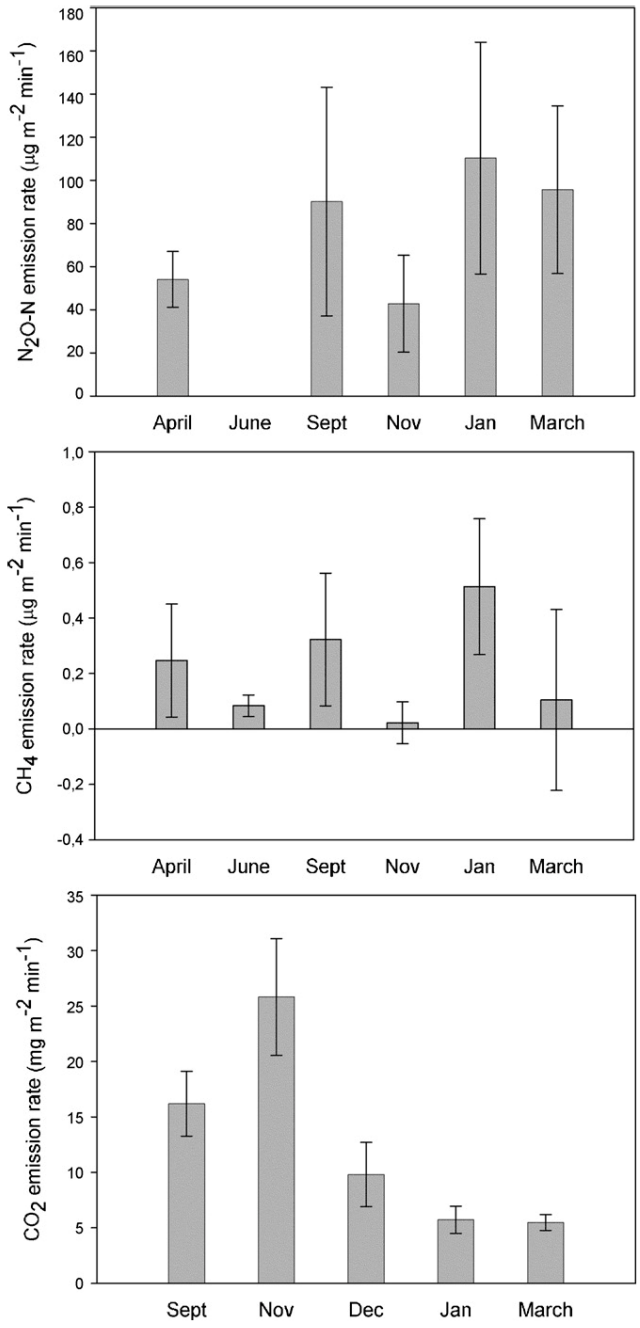

Fig. 5. GHG surface emission rates (N_2O-N emission rate, top; CH_4 emission rate, centre; CO_2 emission rate, bottom) for each month of study. Data of each plot were averaged across all 12 sampling sites from each month, and error bars are one standard error ($n = 12$).

NO_3^- loads (Knowles, 1982; Reddy et al., 1982). However, when compared to other ecosystems C availability, as assessed by measuring respirable C in the denitrification bed, was very high. The average C availability of our study (89 mg C d^{-1} g^{-1}) was at least 300 times higher than the maximum respiration rates of riparian forests (~0.28 mg C d^{-1} g^{-1}) (Groffmann et al., 1992), wetlands (0.15 mg C d^{-1} g^{-1}) (Duncan and Groffmann, 1994) and denitrification walls (0.055 mg C d^{-1} g^{-1}) (Schipper et al., 2005). We assume that the exceptionally high denitrifying activity in the bed consumed all the available C, so that C became the limiting resource for denitrification. The C limitation corresponds to the consistently low DOC concentrations along the length of the bed (Fig. 6).

Further evidence that NO_3^- concentration did not limit NO_3^- removal was the linear decline in NO_3^- concentration along the length of the bed. This decrease suggested that NO_3^- removal followed zero-order kinetics rather than first-order kinetics. NO_3^- removal rates were likely zero-order, because the NO_3^--N concentration of the outflow of the bed (>37 mg L^{-1}) was always two to three orders of magnitude greater than the apparent K_m values of denitrification in riparian soils (2.1 mg L^{-1} and < 0.1 mg L^{-1}) (Schipper et al., 1993; Ambus, 1993) and wetland soils (0.4 mg L^{-1}–1.3 mg L^{-1}) (Maag et al., 1997). Zero order kinetics of NO_3^- removal has also been measured in denitrification walls (Robertson et al., 2000; Schipper et al., 2005). Nitrate removal results of a woodchip media column study followed zero-order kinetics when NO_3^--N concentration exceeded 1 mg L^{-1}, otherwise first-order kinetics were observed (Robertson, 2010). However, in other aquatic ecosystems with much lower NO_3^- concentrations than in the denitrification bed, NO_3^- concentration and DO concentrations can explain the significant variability in denitrification rates (Pina-Ochoa and Alvarez-Cobelas, 2006).

While NO_3^- removal and denitrification rates were apparently limited by C, temperature also regulated the NO_3^- removal rate. Nitrate removal rates in the bed increased with increasing temperature with $Q_{10} = 2.0$ (Fig. 2), which was very similar to the Q_{10} (2.1) calculated from the laboratory measurements of DR at different temperatures and correspond to the Q_{10} of 2 measured by Elgood et al. (2010) in a streambed denitrifying bioreactor. Robertson et al. (2008) also measured increases in NO_3^- removal with increasing temperature in a denitrification bed study. In their study, NO_3^- removal rates ranged from 0.22 g N m^{-3} d^{-1} to 1.1 g N m^{-3} d^{-1} at 6 °C to 10 °C increasing to about 3.5 g N m^{-3} d^{-1} to 6 g N m^{-3} d^{-1} at 20 °C to 22 °C, implying a Q_{10} of almost 5 times greater than the present study. Higher temperatures are also likely to increase NO_3^- removal (in these C-limited systems) due to higher microbial activity, potentially providing the denitrifying microorganisms with more available C from the partial decomposition of wood material.

Nitrate removal did not appear to be constrained by pH in the bed, which was close to the optimum for denitrification (7–8) (Bremner and Shaw, 1958; Knowles, 1982) and increased along the bed, which is assumed to be due to the release of hydroxyl ions during denitrification. Other studies of denitrification beds observed a slight decrease of the pH at the outflow (Van Driel et al., 2006; Robertson et al., 2005; Robertson and Merkley, 2009).

We did not detect a decline in NO_3^- removal rates associated with higher DO concentration at the beginning of the bed even though DO concentrations averaged between 0.35 and 1.7 mg L^{-1} in the first 50 m of the bed. The threshold for DO inhibiting DR is not clear. Pina-Ochoa and Alvarez-Cobelas (2006) suggested a DO threshold for denitrification of 0.5 mg L^{-1} in their cross system study of factors controlling denitrification in aquatic environments. Oh and Silverstein (1999) reported an 85% decrease of the denitrification rate at 2 mg L^{-1} DO in a laboratory study, whereas Gomez et al. (2002) detected no inhibition below DO concentration of 4.5 mg L^{-1} and Healy et al. (2006) detected only inhibition above DO concentration of 3.7 mg L^{-1} in a denitrification bed. Anoxic micro sites present inside and at the surface of the woodchips would provide sufficiently low DO conditions for denitrification to occur in the first 60 m of the bed. After 60 m, DO concentrations were always below 0.1 mg L^{-1}, conditions suitable for denitrification. Robertson et al. (2009) also reported a sharp decrease of DO concentrations in the first 4 m of a woodchip bioreactor and also measured no inhibition of NO_3^- removal due to DO.

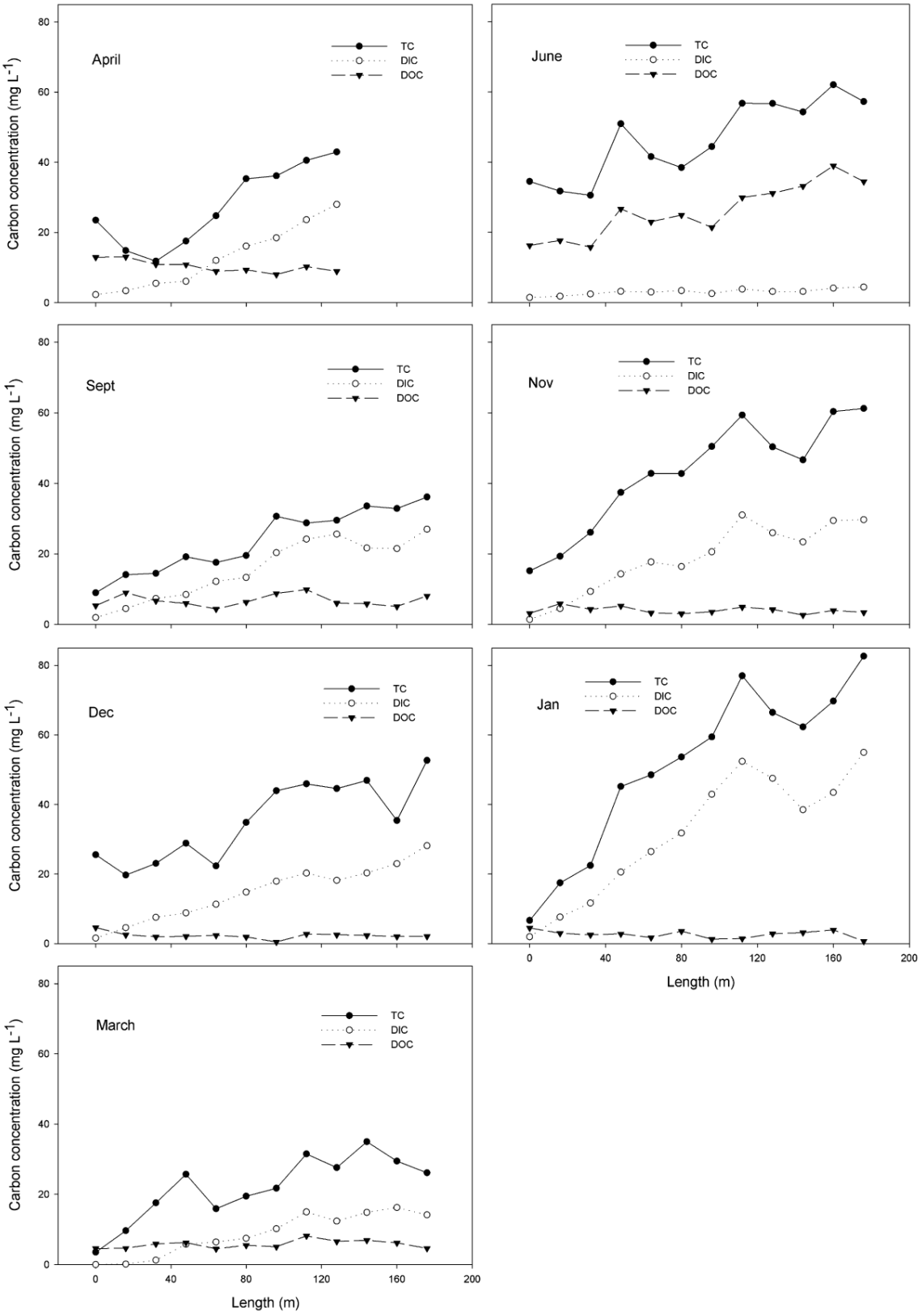

Fig. 6. Carbon species concentrations (TC, DIC and DOC) along the length of the denitrification bed for each month of sampling.

The small increase of NO_2^- concentration along the length of the bed suggested that there was little inhibition of NO_2^- reductase due to high NO_3^- concentrations (Kornaros et al., 1996), otherwise we would have expected to see a strong increase of NO_2^- concentrations along the length of the bed, because of the high NO_3^- concentration and the high denitrifying enzyme activity. Consequently, NO_2^- concentrations were always low.

Aerobic microbial activity may have competed for available C at the beginning of the bed, but thereafter, DO concentrations decreased sharply (see above). The decrease of NH_4^+ concentration along the length of the bed was possibly due to nitrification in the first meters of the bed when DO was still present, or uptake by microbes. Decreases in SO_4^{2-} concentration were measured along the length of the bed on three sampling occasions (Fig. 3). However, this decrease was unlikely to be due to microbial SO_4^{2-} reduction because S^{2-} concentrations were generally below detection limits. The pH in the bed was between 6 and 7 and at this pH around 50% of potential S^{2-} should be deprotonated (Kubli, 1946) and detectable. Furthermore we never noted the smell of hydrogen sulfide when collecting woodchip samples from the bed. It was likely that SO_4^{2-} reducers were out-competed by denitrifiers at the high NO_3^- concentrations in the bed. Robertson and Merkley (2009) suggested that SO_4^{2-} reduction initiated once NO_3^- concentrations were below 0.5–1 mg L^{-1}. Other studies on denitrification beds have shown that SO_4^{2-} reduction probably occurred (as evidenced by a decrease of SO_4^{2-} concentration and detectable H_2S odor), when NO_3^- was almost completely removed (Van Driel et al., 2006; Robertson and Merkley, 2009; Robertson, 2010; Elgood et al., 2010). The reasons for declines in SO_4^{2-} concentration in the Karaka bed were not clear but may have been due to variation in SO_4^{2-} concentrations of the inflow and/or SO_4^{2-} precipitation.

The DNRA process can also compete with denitrification for available C and NO_3^-, generally when the ratio of C to NO_3^- concentrations is high (Tiedje, 1988). DNRA appears to have been suppressed by denitrification because no increase in NH_4^+-N concentration was detected along the length of the bed. This was likely due to the low ratio of C to NO_3^- concentration in the denitrification bed. Greenan et al. (2006) also found that less than 4% of total NO_3^- removed was due to DNRA in a woodchip column study.

Methane emissions from the bed surface were generally low and it would appear that methanogens were not able to compete significantly with denitrifying bacteria for available C as the NO_3^- concentration was always very high in the denitrification bed (see below). On a few occasions we could also detect methane uptake, possibly indicating methanotrophic activity along the length of the bed.

4.3. Adverse effects, C loss and longevity of the bed

Adverse effects of concern when using denitrification beds are production of GHGs and the release of DOC in the effluent. The most likely GHG to be produced in a NO_3^- removing system is N_2O, as it is an obligatory intermediate of the denitrification process. Wastewater treatment plants release 0.5% of the total N content as N_2O, reflecting 1.3% of the total global N_2O emissions (IPCC, 2006, 2001). Constructed wetlands show a wide range of N_2O emission rates e.g., Teiter and Mander (2005) measured N_2O emission rates ranging from 1 μg N m^{-2} h^{-1} to 2600 μg N m^{-2} h^{-1}. Elgood et al. (2010) determined GHG production via dissolved gases in the outflow of a streambed woodchip bioreactor. Moorman et al. (2010) published dissolved N_2O-N gas concentrations for denitrification walls treating drainage water in the field and Greenan et al. (2009) determined dissolved N_2O-N gas concentrations for woodchips from bioreactors in a column study. To our knowledge, this is the first study measuring both surface emitted and dissolved GHGs from a denitrifying woodchip bioreactor. The annual average N_2O-N emission rate of 78.58 μg m^{-2} min^{-1} reflects an average N_2O-N surface emission of 1% of N removed by the bed. This is close to the reported results of dissolved N_2O-N by Elgood et al. (2010) and Moorman et al. (2010) (0.6% and 0.84%, respectively) but greater than the calculated factor from the column study by Greenan et al. (2009) (<0.033%). Furthermore the surface emission factor of our study was slightly greater than the IPCC N_2O-N emission factor (EF_5) of 0.75% for N released in waterways (IPCC, 2006). In our study an additional 3.3% of removed NO_3^--N was released as dissolved N_2O-N from the denitrification bed in the effluent, which is consistently about 4.5 times greater than the N_2O-N production rates in the studies of Elgood et al. (2010), Moorman et al. (2010), Greenan et al. (2009) and the EF_5 of the IPCC (2006). The denitrification bed released in total 4.3% of the removed NO_3^--N as emitted and dissolved N_2O. Dissolved N_2O-N is stored in the outflow water of the denitrification bed and can be still transformed by denitrification to N_2 gas when NO_3^- concentrations reach a level where N_2O production is lower than N_2O consumption by the denitrification process. Therefore it is critical to calculate the absolute N_2O production or emission from dissolved N_2O-N. In our study the dissolved N_2O will probably be released as N_2O gas to the atmosphere due to the high NO_3^- concentration in the outflow and the probably decreasing denitrifying activity in the ditch water. Consequently denitrification beds should be constructed to reduce the NO_3^- concentration under a threshold, where mainly dissolved N_2O-N will be used and converted in N_2 gas by the denitrification process.

We detected the highest N_2O emission in summer (highest temperature) when NO_3^- removal and dissolved N_2O concentrations were greatest. These results correspond with findings in constructed wetlands, where in warmer months N_2O production was greater than in colder months (Teiter and Mander, 2005; Johansson et al., 2003). Conversely, Elgood et al. (2010) and Moorman et al. (2010) reported greater N_2O production in colder months due to slower reaction rates and higher inlet DO concentrations leading to incomplete denitrification.

We detected low CH_4 emission and no dissolved CH_4 production at our study site probably due to high NO_3^- concentrations in the system so that methanogenes were outcompeted by denitrifiers for C supply. A constructed wetland in similar climatic conditions (New Zealand) showed at least 140 times greater CH_4 emission than measured in this denitrification bed (Tanner et al., 1997). Dissolved CH_4 production measured by Elgood et al. (2010) increased when NO_3^- concentrations were almost depleted, a scenario which we could not observe due to the continually high NO_3^- concentrations in the bed.

The average yearly CO_2 emisson of 15.97 kg d^{-1} from the surface of the bed and the average yearly dissolved CO_2 loss from the bed of 19.44 kg d^{-1} did not contribute to a net increase in CO_2 concentrations in the atmosphere as wood used for other purposes would decay to CO_2 in any case.

Robertson (2010) observed an increase of DOC in the effluent of woodchip columns, when NO_3^- concentrations were low (<1 mg N L^{-1}). We did not observe losses of organic C in the effluent from the bed, probably due to the high NO_3^- concentrations throughout the bed. The stoichiometric ratio of denitrification (5 CO_2-C will be released per 4 NO_3^--N removed) was close to the actual measured ratio of the average CO_2-C emission and dissolved CO_2-C release per NO_3^--N removed (4.1 CO_2-C were released per 4 NO_3^--N removed), suggesting that denitrification was the main mechanism in the bed and that microbial carbon consumption was mainly due to denitrification.

We calculated that the wood material in the bed will last for 39 years, using the measured daily total C loss (combining the CO_2 surface emission and dissolved net C losses at the outflow) of the bed, the measured woodchip bulk density of 210 kg m^{-3} and

an assumed C content of 50% for the woodchips. Moorman et al. (2010) measured the weight loss of woodchips over time at different depths to estimate half lives of wood in a denitrification wall, with half lives ranging from 4.6 years near the surface to 36.6 years deeper in the wall.

5. Conclusions

This study suggests that denitrification was the main mechanism for NO_3^- removal in the denitrification bed and ranged from 4.6 to 11.2 g N m^{-3} d^{-1}. Nitrate removal and denitrification activity were generally limited by availability of C and temperature rather than NO_3^- concentration, which was always very high relative to the known K_m of denitrifiers (Barton et al., 1999). Concentrations of DO (<3 mg L^{-1}) at the inlet of the bed and SO_4^{2-} (>290 mg L^{-1}) did not inhibit NO_3^- removal in the denitrification bed.

Significant adverse effects were measured with a total N_2O-N release of 4.3% of NO_3^--N removed. However the measured N_2O-N emission (1%) was slightly greater than the IPCC emission factor (0.75%). CH_4 emissions from the bed were very low in comparison to other treatment systems and no additional DOC was released by the bed in the connected waterway.

Role of the funding source

WaikatoLink Ltd. (New Zealand) and Hans-Sauer-Foundation (Germany) funded this study. They were not involved in project design, sampling, data analysis/interpretation and writing the manuscript.

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Chapter 4

Nitrate Removal, Communities of Denitrifiers and Adverse Effects in Different Carbon Substrates for Use in Denitrification Beds

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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. Woodchips 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

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recommend the combination of maize cobs and woodchips to enhance NO_3^- removal while minimizing adverse effects in denitrification beds.

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 start-up 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 = ΔNO_3^-–N × FR × V^{-1}, where ΔNO_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 headspace 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;

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).

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 touchdown 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

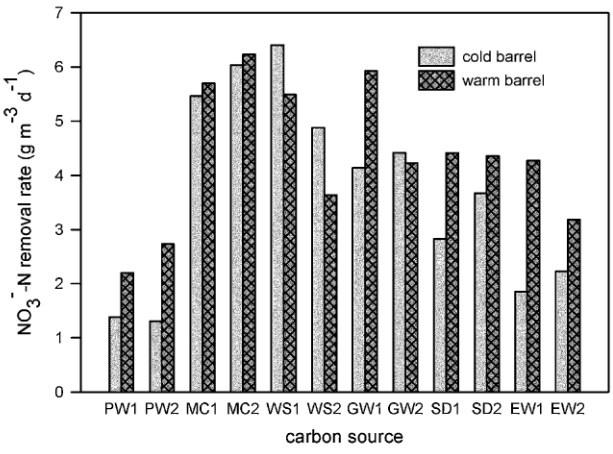

Fig. 1 – Nitrate removal rates for different carbon substrates in cold (16.8 °C) and warm (27.1 °C) barrels. PW1 and PW2, soft woodchips (pine), replicates; MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, hard woodchips (eucalyptus).

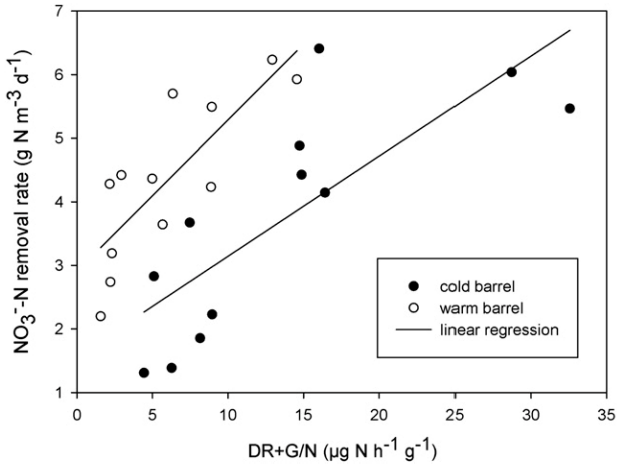

Fig. 2 – Nitrate–N removal rate as a function of *in vitro* DR amended with glucose and nitrate (DR + G/N) for cold and warm incubated substrate. Linear regression statistics are reported in text.

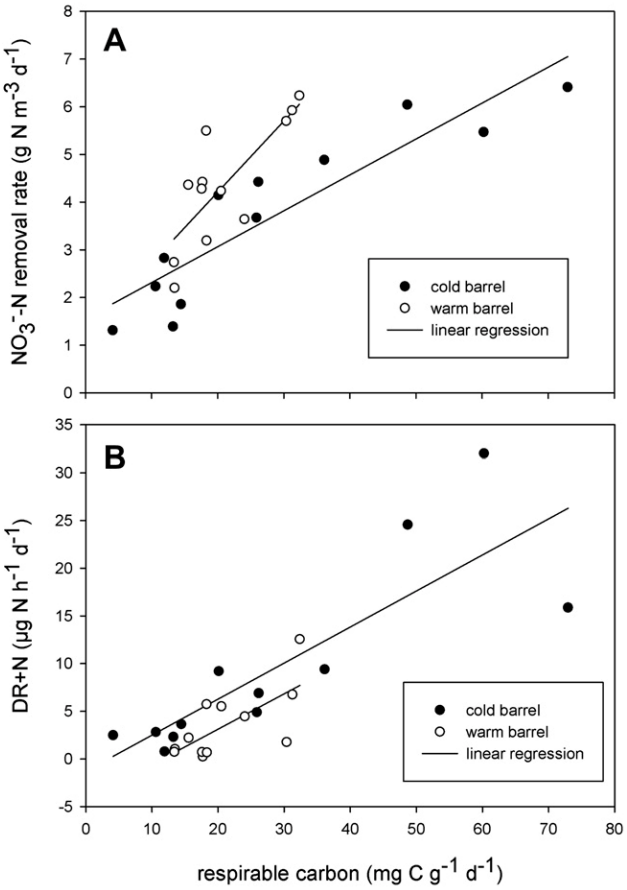

Fig. 3 – Nitrate–N removal rate (A) and *in vitro* DR amended with nitrate (DR + N) (B) as a function of respirable carbon for cold and warm incubated substrate. Linear regression statistics are reported in text.

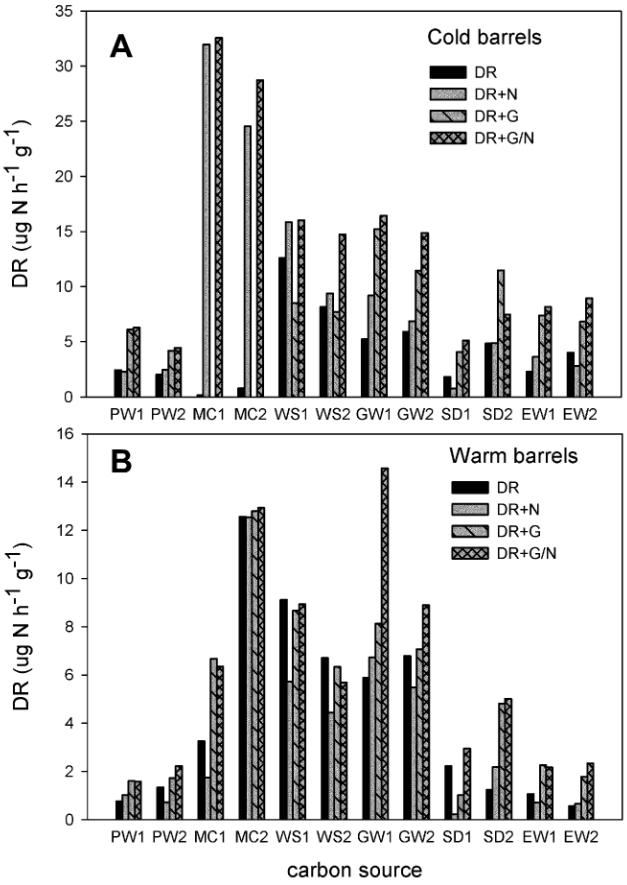

Fig. 4 – In vitro denitrification rates (DR) at 27 °C for different carbon substrates in cold (A) and warm (B) barrels. DR assays were amended with glucose (DR + C), NO_3^- (DR + N), glucose and NO_3^- (DR + C/N), and none amended (DR). PW1 and PW2, soft woodchips (pine); MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, hard woodchips (eucalyptus).

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

Table 2 – Average copy number (×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).

$1.5 \pm 0.07 \times 10^{10}$ (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.

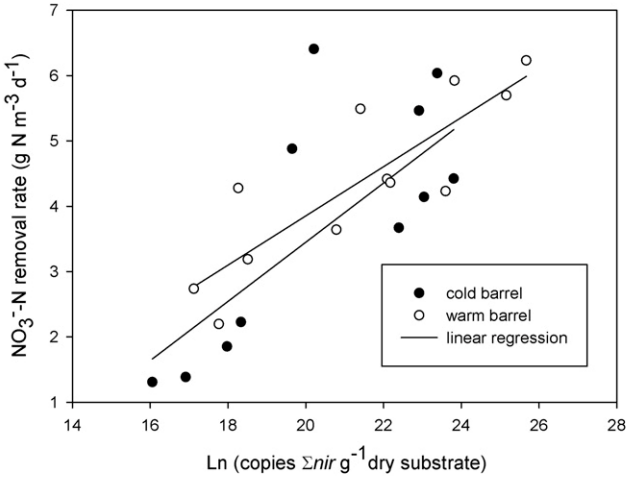

Fig. 5 – Nitrate–N removal rate as a function of total nitrite reductase gene (Σ*nir*) copies for cold and warm incubated substrates. Linear regression statistics are reported in text.

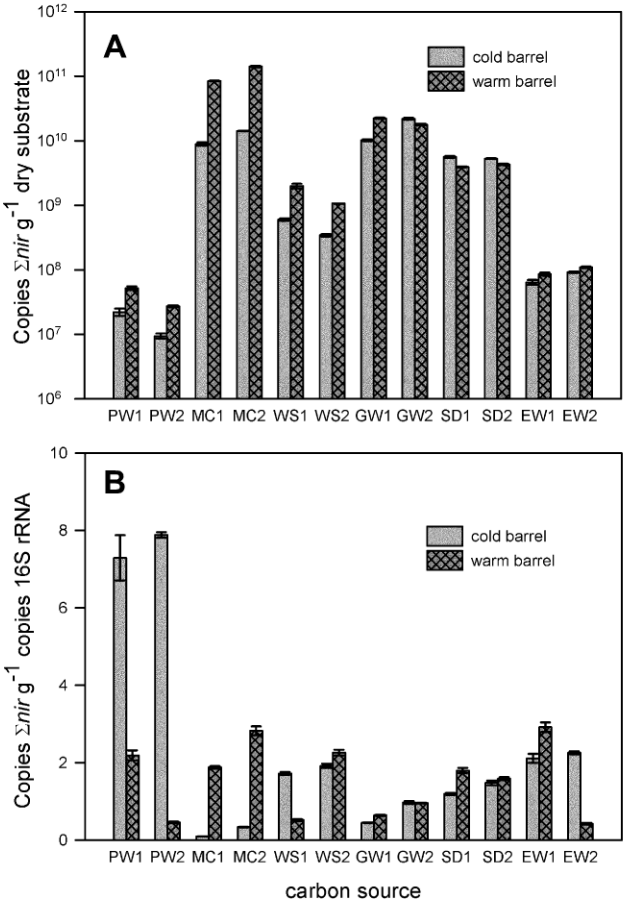

Fig. 6 – Total number of nitrite reductase genes (Σ*nir*) normalized per gram carbon substrate (A) and normalized to total bacteria (16S rRNA) (B) of the different carbon substrates used in the barrels under cold and warm incubation. PW1 and PW2, pine woodchips; MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, eucalyptus woodchips. Error bars are one standard error (*n* = 3).

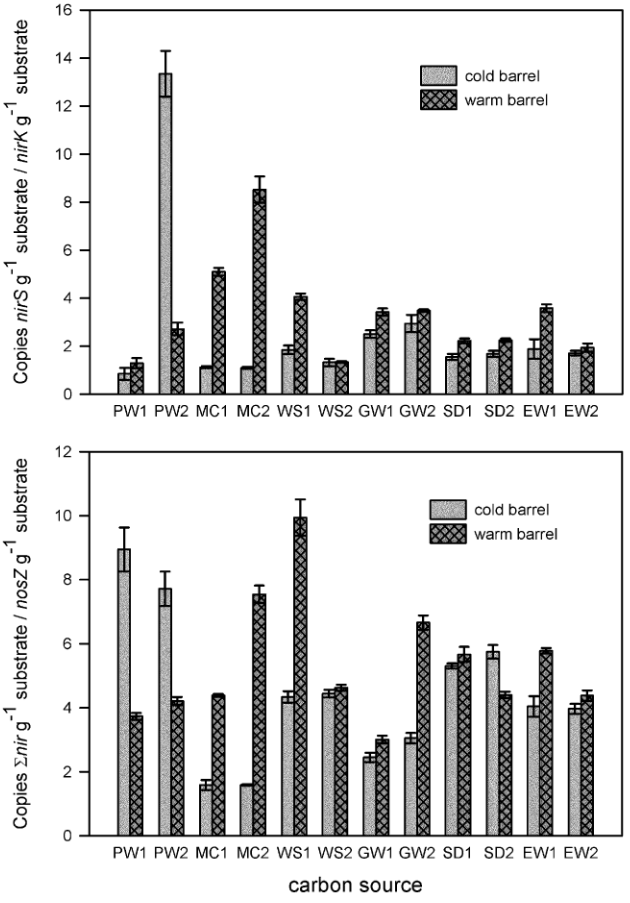

Fig. 7 – Ratios of gene copies of *nirS*/*nirK* (A) and total nitrite reductase (Σ*nir*) to nitrous oxide reductase (*nosZ*) (B). PW1 and PW2, pine woodchips; MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, eucalyptus woodchips. Error bars are one standard error (*n* = 3).

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).

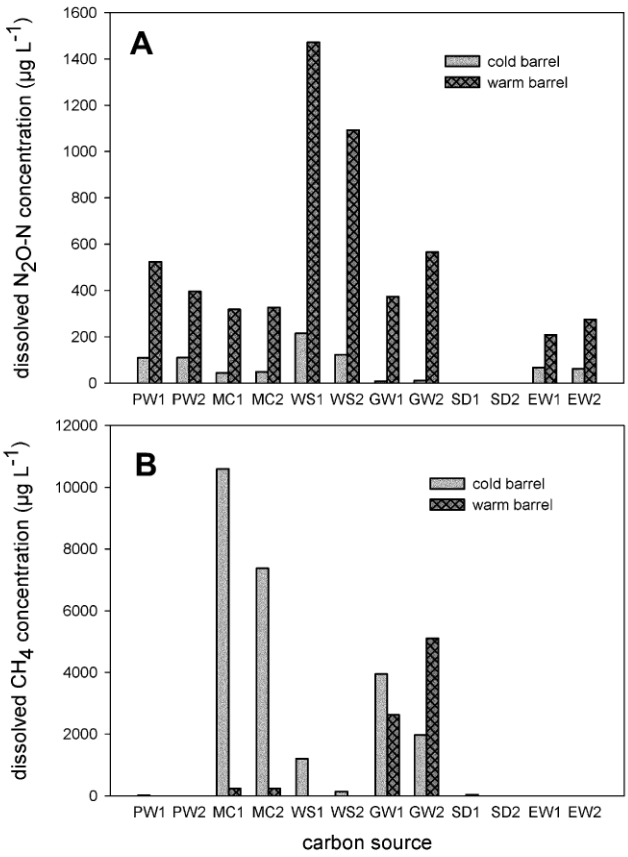

Fig. 8 – Dissolved nitrous oxide (A) and methane (B) concentrations in the outlet water of different carbon substrates in cold and warm barrels. PW1 and PW2, soft woodchips (pine); MC1 and MC2, maize cobs; WS1 and WS2, wheat straw; GW1 and GW2, green waste; SD1 and SD2, sawdust; EW1 and EW2, hard woodchips (eucalyptus).

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 woodchips 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 (∑*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 good approaches 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 ∑*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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Chapter 5

A Comparison of different Approaches for Measuring Denitrification Rates in a Nitrate Removing Bioreactor

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A comparison of different approaches for measuring denitrification rates in a nitrate removing bioreactor

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ABSTRACT

Denitrifying woodchip bioreactors (denitrification beds) are increasingly used to remove excess nitrate (NO_3^-) from point-sources such as wastewater effluent or subsurface drains from agricultural fields. NO_3^- removal in these beds is assumed to be due to microbial denitrification but direct measurements of denitrification are lacking. Our objective was to test four different approaches for measuring denitrification rates in a denitrification bed that treated effluent discharged from a glasshouse. We compared these denitrification rates with the rate of NO_3^- removal along the length of the bed. The NO_3^- removal rate was 8.73 ± 1.45 g m^{-3} d^{-1}. *In vitro* acetylene inhibition assays resulted in highly variable denitrification rates (DR_{AI}) along the length of the bed and generally 5 times greater than the measured (NO_3^-—N removal rate. An *in situ* push–pull test, where enriched ^{15}N—NO_3^- was injected into 2 locations along the bed, resulted in rates of 23.2 ± 1.43 g N m^{-3} d^{-1} and 8.06 ± 1.64 g N m^{-3} d^{-1}. The denitrification rate calculated from the increase in dissolved N_2 and N_2O concentrations (DR_{N2}) along the length of the denitrification bed was 6.7 ± 1.61 g N m^{-3} d^{-1}. Lastly, denitrification rates calculated from changes in natural abundance measurements of $\delta^{15}N$–N_2 and $\delta^{15}N$—NO_3^- along the length of the bed yielded a denitrification rate (DR_{NA}) of 6.39 ± 2.07 g m^{-3} d^{-1}. Based on our experience, DR_{N_2} measurements were the easiest and most efficient approach for determining the denitrification rate and N_2O production of a denitrification bed. However, the other approaches were useful for testing other hypotheses such as factors limiting denitrification or may be applied to determine denitrification rates in environmental systems different to our study site. DR_{N_2} does require very careful sampling to avoid atmospheric N_2 contamination but could be used to rapidly determine denitrification rates in a variety of aquatic systems with high N_2 production and even water flows. These measurements demonstrated that the majority of NO_3^- removal was due to heterotrophic denitrification.

1. Introduction

The global production of anthropogenic nitrogen (N) is increasing due to food and energy production (Vitousek et al., 1997; Canfield et al., 2010). This N also has lasting adverse effects on the environment, including increased greenhouse gas emissions, stratospheric ozone depletion, pollution of drinking water, and eutrophication of streams, lakes and coastal waters (Galloway et al., 2004, 2008; Canfield et al., 2010). There are a range of strategies to reduce the N load to

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aquatic ecosystems from agricultural practices, such as the construction or preservation of wetlands and riparian buffers, and installation of denitrification beds or walls (Dinnes et al., 2002; Vymazal et al., 2006; Schipper et al., 2010). Denitrification beds are large containers filled with wood by-products that act as a carbon source to support heterotrophic denitrification, which converts nitrate (NO_3) to nitrous oxide (N_2O) and N_2 gases (Seitzinger et al., 2006). These beds are increasingly being installed to remove NO_3 from point-source discharges such as effluent streams and drainage systems (Schipper et al., 2010).

It is generally presumed that microbial denitrification is predominantly responsible for the NO_3—N removal in these beds (Schipper et al., 2010) and that other NO_3 removal processes such as dissimilatory NO_3 reduction to ammonium (DNRA), anammox and microbial/plant uptake are relatively low. For example, Greenan et al. (2006, 2009) showed that less than 4% of NO_3—N removal in woodchip columns was due to DNRA and that microbial uptake only accounted for 2–3.5% of NO_3—N removed. Isotopic enrichment of natural abundance of ^{15}N in NO_3 was measured in the outflow of a denitrification bed and in a column study while NH_4^+ concentrations were low, was also suggestive of microbial denitrification (Robertson et al., 2000; Robertson, 2010). However, there are various processes beside heterotrophic denitrification that can account for ^{15}N—NO_3 increase in natural systems (Bedard-Haughn et al., 2001). Therefore, measurement of the products of denitrification (N_2, N_2O), is critical to establish that denitrification is responsible for NO_3 removal. Our previous work suggested that denitrification is the primary pathway for NO_3 removal in denitrification beds because we measured very high potential rates of denitrification using the acetylene inhibition method. Anammox and DNRA were likely negligible due to low NH_4^+ concentrations and the lack of plant/algae growth on the denitrification bed ruled out biotic uptake of NO_3 (Warneke et al., 2011). However, there are no direct measurements of denitrification rates in operating denitrification beds to demonstrate that denitrification dominates other NO_3 removal processes. Developing a method to directly measure denitrification rates would also allow reliable determination of NO_3 removal rates in denitrification beds and potentially in other similar aquatic systems because determining NO_3 removal via measurement of inflow and outflow NO_3 concentrations is difficult in many of these systems due to high temporal variability in NO_3 concentrations and flow rates at inflow and outflow (Schipper et al., 2010).

A number of different techniques may be used to measure denitrification rates in terrestrial and aquatic environments (Groffman et al., 2006). The acetylene inhibition method has probably been the most commonly used approach for measuring denitrification (Groffman et al., 2006). Acetylene inhibits the reduction of N_2O to N_2 and accumulated N_2O can be measured using gas chromatography. However, the acetylene block technique can lead to inaccurate measurements of denitrification rates because acetylene has a number of other unwanted effects on microbial populations e.g., acting as an inhibitor of nitrifiers or as a carbon source (Groffman et al., 2006). Denitrification rates measured in soils using acetylene inhibition technique are generally an underestimate of actual rates (Groffman et al., 2006).

Denitrification rates in water-saturated environments (e.g., groundwater or wetlands) can also be estimated using the push–pull method (Addy et al., 2002) where a slug of ^{15}N-labelled NO_3 is added into the denitrifying environment and the accumulation of ^{15}N–N_2 and ^{15}N–N_2O is measured with time (Hauck and Melsted, 1956; Addy et al., 2002; Baker and Vervier, 2004).

Direct quantification of denitrification by measuring N_2 emissions from soils has also been attempted, although it is technically challenging due to the high atmospheric background concentration of N_2 (Butterbach-Bahl et al., 2002). Similarly, in aquatic environments, increases in dissolved N_2 can be measured but are also confounded by high background levels of dissolved N_2 concentrations derived from the atmosphere (Groffman et al., 2006). However, conditions for measuring denitrification in rivers via dissolved N_2 concentrations described by Laursen and Seitzinger (2005) suggested that it may be possible to directly measure increases in dissolved N_2 concentrations along the length of denitrification beds due to their turbulent-free water flow and their potentially high production of N_2 through denitrification.

A final approach that could be used to demonstrate denitrification as the main mechanism for NO_3 removal in denitrification beds is the measurement of changes in the $^{15}N/^{14}N$ natural abundance of NO_3 and nitrogen gases along the length of the bed. If denitrification was the main mechanism of NO_3 removal then there should be increases in natural abundance $^{15}N/^{14}N$ in NO_3, observed as $\delta^{15}N$–NO_3, due to the strong discrimination against ^{15}N during denitrification (Mariotti et al., 1981) and a negative congruent decrease in the $^{15}N/^{14}N$ of N_2 gas produced, reported as $\delta^{15}N$–N_2.

The main objectives of this study were to determine whether denitrification rates were high enough to account for the observed NO_3 removal in an operational denitrification bed and to compare different methods for measuring denitrification rates in denitrification beds. A range of the techniques were trialled for accuracy, ease, and expense of measurement, including measuring changes in the dissolved nitrogen gases and natural abundance stable isotope (^{15}N–N_2 and ^{15}N—NO_3) along the length of the bed, acetylene inhibition assays, and accumulation of ^{15}N-labelled N_2 and N_2O following introduction of an ^{15}N-labelled NO_3 spike.

2. Materials and methods

2.1. Study site

This study was performed at a large denitrification bed (176 m × 5 m × 1.5 m) constructed in 2006 and filled with a mixture of woodchips and sawdust (Warneke et al., 2011). The bed treated effluent from a glasshouse, which grew hydroponic cucumbers, tomatoes and capsicums at Karaka, New Zealand. The effluent from the glasshouse was pumped into one end of the denitrification bed through a PVC pipe 1 m below the surface of the woodchips and was discharged from the other end of the bed into a drainage ditch. Twelve fully screened PVC wells (2 m long; diameter 0.05 m) were installed along the length of the bed at 16 m intervals for effluent sampling. Mechanical water metres (LXLG-80, Bil, China) at

the inflow and at the outflow of the bed measured the flow rate of the incoming and outcoming effluent of the bed.

2.2. *Nitrate-N removal rate*

Effluent samples for NO_3 concentration were taken from each well along the length of the bed using a simple pump and stored in plastic tubes (50 mL) before being frozen at −16 °C until analysis. After filtering (0.45 μm disposable membrane filters), NO_3—N concentrations of effluent samples were analysed with a flow injection analyser (Lachat Instruments; Loveland, Colorado, USA; APHA, 1992). NO_3—N expresses the nitrogen (N) of the nitrate (NO_3). NO_3 removal rates (g N m^{-3} bed material d^{-1}) were calculated from the linear decline of the NO_3—N concentrations (g m^{-3}) along the length of the bed (ΔNO_3): NO_3—N removal rate = ΔNO_3—N × FR/V_{bed}, where FR was the flow rate of the effluent (m^3 d^{-1}) and V_{bed} was the volume of the bed (m^3).

2.3. *Denitrification rates*

2.3.1. *In vitro denitrification measurement using acetylene inhibition technique − DR_{AI}*

A modified version of the acetylene inhibition technique (Tiedje et al., 1989) was used to measure *in vitro* denitrification rates (DR_{AI}) (see Warneke et al., 2011). Woodchips (effluent-saturated bed material) were sampled along the bed from 0.2 m below the bed surface using a shovel and stored in plastic bags at 4 °C until analysis within 2 days. Woodchips (100 g) and effluent (60 g) from each sampling location (12) was placed into airtight glass jars (600 ml). After addition of acetylene (10% of the headspace volume) the increase in N_2O concentration was measured using a gas chromatograph (Varian; Palo Alto, USA) equipped with an electron capture detector, and 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 argon/methane-carrier gas was 40 mL min^{-1}. The DR_{AI} was calculated from linear increase in N_2O concentration with time. This rate was adjusted for the difference in temperature between the bed at the time of sampling (19 °C)and laboratory incubation temperature (27 °C) using a Q_{10} of 2.1 (Warneke et al., 2011). Q_{10} is the factor of the reaction rate increase with every 10 °C rise in temperature. The temperature of the bed effluent was measured in each well using an InLab 605 O_2-Sensor (Mettler Toledo, Switzerland).

2.3.2. *In situ denitrification measurement using push–pull technique − DR_{PP}*

In situ denitrification rates (DR_{PP}) were measured using the push–pull ^{15}N—NO_3 technique (Addy et al., 2002; Baker and Vervier, 2004) at two locations (location A at 48 m bed length; location B at 128 m bed length) in the denitrification bed within 2 days. ^{15}N—NO_3 expresses the nitrogen isotope ^{15}N of the nitrate (NO_3). Effluent flow in the bed was stopped at the inlet 6 days before sampling to reduce movement of the introduced tracers away from the sampling locations. Effluent from 1.2 m depth from the bed (20 L) was pulled (400 mL min^{-1}) using a peristaltic pump and a stainless steel pipe fitted with a screen at the base which was connected to Tygon®tubing (internal diameter 4 mm) directly into a Tedlar gas bag (25 L) to avoid contact with the atmosphere and to maintain anoxic water conditions (Fig. 1). The extracted effluent was amended with ^{15}N—NO_3 (95.0% $^{15}NO_3$) to about 20% above background NO_3 concentration. Sodium bromide (NaBr) was also added (10 times background Br^- concentration) to allow calculation of the dilution of added ^{15}N—NO_3 and ^{15}N gas during subsequent sampling. Then the amended effluent was pumped back into the bed through the same pipe. Prior to amendment, background effluent and gas samples were collected to determine background concentrations of NO_3—N, ^{15}N–N_2O and ^{15}N–N_2 (t_{-1}). ^{15}N–N_2O and ^{15}N–N_2 express the nitrogen isotope ^{15}N of the nitrous oxide (N_2O) and nitrogen (N_2) gas respectively

Immediately after the enriched effluent was pumped back in the bed, effluent samples were taken at time 0 (t_0) and subsequently every 30 min for 3 h (t_1–t_6). For each sampling, effluent was pumped out of the stainless steel pipe with the first litre of the effluent being discarded. Subsequent effluent samples were stored in 50 ml PP tubes (Greiner Bio-one, Germany) at 4 °C until analysis of Br^-, NO_3, and ^{15}N—NO_3 concentrations.

To obtain samples for analysis of dissolved ^{15}N–N_2O and ^{15}N–N_2, the effluent was pumped through the pipe and tubing into a 50-ml plastic syringe fitted with a 3 way stopcock. This sampling procedure was performed in a transparent plastic container (20 L) filled water to avoid atmospheric contact (Fig. 1). The first outlet of the stopcock was connected to the syringe, the second outlet of the stopcock was connected to the tubing of the ultra-high purity helium tank (99.999% purity, BOC Ltd., New Zealand) and the third outlet was connected after the helium purging step (see below) to the tubing of the stainless steel pipe (effluent) or later on to a needle (Fig. 2a–g). Before collecting the effluent with the syringe, the stopcock and the syringe was generously purged (five times) with helium to remove traces of background N_2 gas (Fig. 2a–b). The effluent tube from the pipe was connected to the stopcock while continuously pumping out effluent from the bed. Exactly 35 ml of effluent was pumped into the syringe (Fig. 2c).

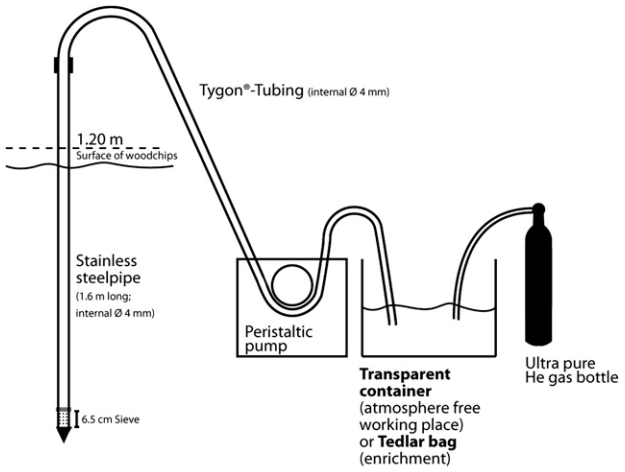

Fig. 1 − Overview of experimental setup to sample effluent and their dissolved gases (gas headspaces) from 1.20 m depth of the denitrification bed without atmosphere contact.

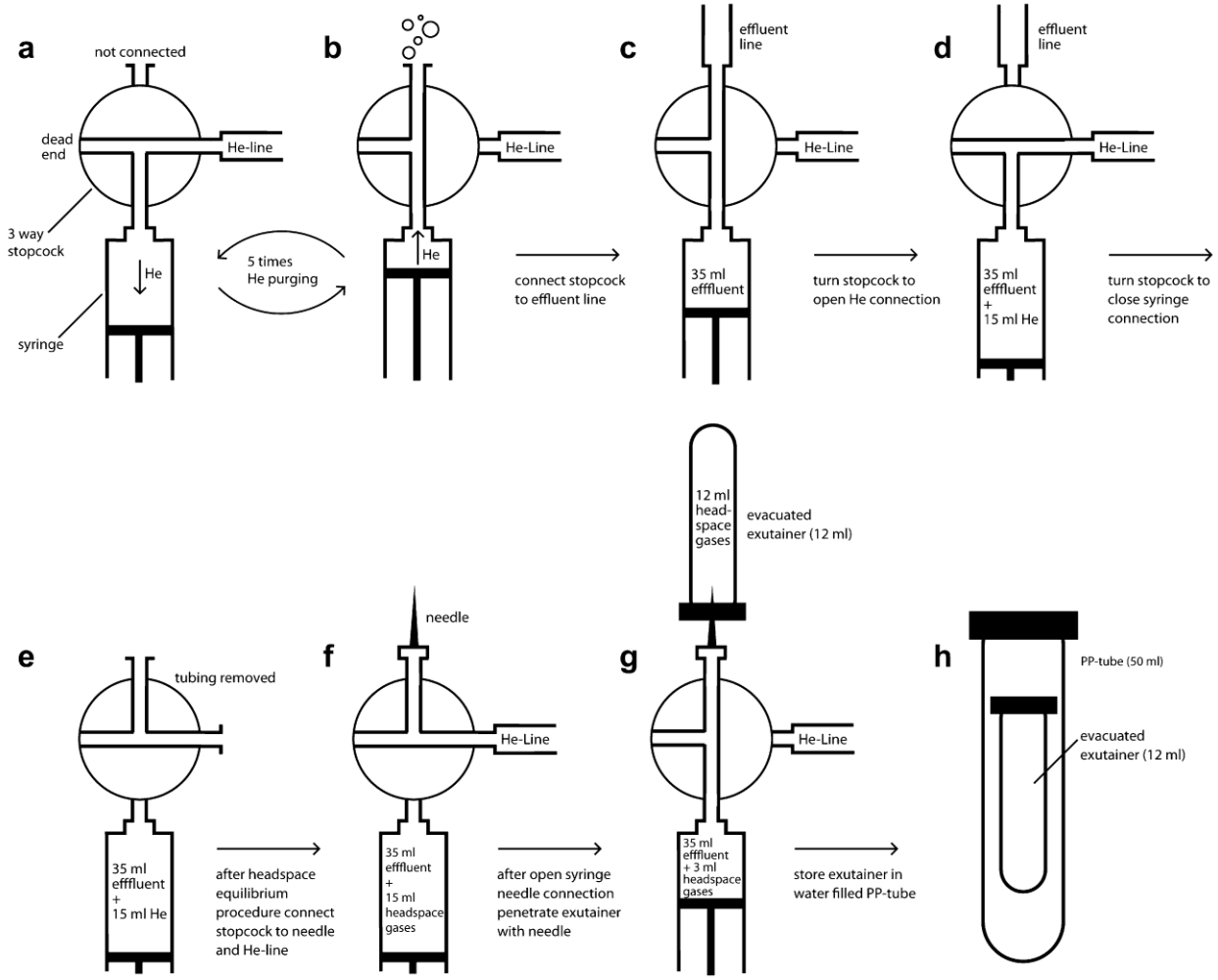

Fig. 2 – Detailed view on the sampling steps under water in the transparent container of Fig. 1 to obtain headspace gas samples from the effluent for analysis of dissolved N_2 and N_2O. a) and b) He purging; c) effluent sampling; d) Filling with He; e) Transport to headspace equilibrium procedure; f) Purging needle with He; g) Transfer of headspace gases into evacuated exetainer; h) Storage of exetainer in water filled PP tube. See further explanation in text.

The stopcock was switched to the helium connection to add 15 mL of helium into the syringe (Fig. 2d). The stopcock was turned to close the syringe connection and the He and effluent tubing were removed (Fig. 2e) before starting the headspace equilibrium procedure (see below). For each sampling, duplicate syringes were filled with effluent. To achieve equilibrium between dissolved gases with the helium headspace, the two syringes of the transparent water filled container were placed into a small water filled container which was closed carefully and was placed onto a shaker table. The syringes were shaken for 10 min at 450 rpm to equilibrate gases dissolved in the effluent with the He headspace. After equilibration, the container with the syringes was returned to the large transparent container filled with water (Fig. 1). The syringes were removed from the small container under water. A needle and the helium tube were connected to the 3 way stopcock valve (Fig. 2f). After purging the needle with helium, the headspace gas in the syringe (~12 ml of 15 ml) was transferred to a evacuated exetainer (12 mL Labco, UK) (Fig. 2g). The exetainers were then stored in water filled PP tubes (50 ml) (Fig. 2h). These samples were analysed for $^{15}N_2O$ and $^{15}N_2$ at the stable isotope facility at the University of California, Davis, USA using a SerCon Cryoprep trace gas concentration system interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Gas samples were analysed within 3 weeks of collection. Prior to sample collection, the exetainers were evacuated with an Edwards RV3 roughing pump and stored in helium-sparged water. The DR_{pp} (R; µg l^{-1} min^{-1}) from this experiment was determined using the calculations after Clilverd et al. (2008) as followed:

$$DR_{pp} = (({}^{15}N_{gas(t)} - \beta {}^{15}N_{gas(t_0)})\ ({}^{15}NO_3^- \%\beta)^{-1})\ t^{-1},$$

$^{15}N_{gas(t)}$ was the concentration of dissolved $^{15}N{-}N_2$ and dissolved $^{15}N{-}N_2O$ at each sampling time (t). $^{15}N_{gas(t_0)}$ was the concentration of dissolved $^{15}N{-}N_2$ and dissolved $^{15}N{-}N_2O$ at time 0 of each assay. To calculate the dissolved $^{15}N{-}N_2$ and $^{15}N{-}N_2O$ concentrations, total dissolved $N_2{-}N$ and $N_2O{-}N$ concentrations were multiplied by the respective atom % ^{15}N. The respective atom % ^{15}N expresses the fraction of ^{15}N in %

(^{15}N ($^{15}N + {}^{14}N)^{-1}$) of the N gas (N_2 or N_2O). Dissolved N_2–N and N_2O–N concentrations were calculated using the Bunsen solubility coefficient from Weiss (1970) and Weiss and Price (1980). β was the dilution factor for each sampling. Dilution was caused by water movement and diffusion. β was calculated by dividing Br^- concentration at time (t) by the Br^- concentration at time 0. $^{15}NO_3$ % was the percent $^{15}NO_3$ of total NO_3 added to the well at the start of the push–pull test (20%) and t was sampling time in minutes. Lastly, denitrification rates were converted to bed volume by multiplying the effluent volume with the determined tracer porosity of 7 years old woodchips (60%) by Robertson (2010).

2.3.3. *In situ denitrification measurement using N_2 and N_2O concentrations – DR_{N_2}*

In situ denitrification rates were measured by collecting effluent samples along the length of the bed using the equipment described above and analysing collected effluent for total dissolved N_2–N and N_2O–N concentrations (DR_{N_2}) (Figs. 1 and 2). The headspaces of these effluent samples (duplicates) were collected from 8 locations along the bed (0 m, 16 m, 32 m, 48 m, 64 m, 96 m, 128 m, 160 m) and sent to the stable isotope facility at the University of California, Davis, USA for analysis of N_2–N and N_2O–N concentrations using a SerCon Cryoprep trace gas concentration system interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The denitrification rate (mg N L^{-1} effluent m^{-1} bed length) was calculated from the slope of a linear regression of the dissolved N_2–N and N_2O–N along the length of the bed. The denitrification rate per unit time and bed volume (g N m^{-3} bed volume d^{-1}) was calculated by multiplication of the effluent flow velocity through the bed and a wood chip porosity of 60% (Robertson, 2010). The theoretical flow velocity of the effluent through the bed was determined using Darcy's law (1856) as followed:

$$v = (V \times (A \times p)^{-1})^{-1}$$

where v was the effluent velocity (min m^{-1}) through the bioreactor, V was the inlet volume (m^3 min^{-1}) of the effluent, A(m^2) was the cross-sectional area of the bioreactor and p is the tracer porosity of the bed material. The inlet volume of 170 m^3 d^{-1}, the cross-sectional area of 7.5 m^2 and a tracer porosity of 60% (Robertson, 2010) yielded an effluent velocity of 38.1 min m^{-1}.

2.3.4. *Comparison of in situ $\delta^{15}N$—NO_3 increase to $\delta^{15}N$–N_2 decline – DR_{NA}*

We compared the natural enrichment of ^{15}N in NO_3 with the depletion of ^{15}N in dissolved N_2 in the effluent along the length of the bed. We hypothesised that if denitrification was the main mechanism for NO_3 removal that the rate of change in natural abundance ^{15}N along the length of the bed for NO_3 and N_2 would be of similar value but with opposite sign.

The natural abundance ^{15}N–N_2, expressed as $\delta^{15}N$–N_2 was determined by collecting equilibrated headspace samples at eight locations along the length of the bed using the equipment described above (Figs. 1 and 2). $\delta^{15}N$ describes the difference between the $^{15}N/^{14}N$ ratio of the sample (R_{sample}) and the $^{15}N/^{14}N$ ratio ($R_{standard}$) of the natural abundance standard (air) in ‰ ((R_{sample}–$R_{standard}$) $R_{standard}^{-1}$) to allow the detections of small differences in natural abundance of ^{15}N in dissolved gases ($\delta^{15}N$–N_2; $\delta^{15}N$–N_2O) and nitrate ($\delta^{15}N$—NO_3). The equilibrated headspace samples of duplicate were analysed at the stable isotope facility at the University of California, Davis, USA using a SerCon Cryoprep trace gas concentration system interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Additionally, $\delta^{15}N$–N_2 was measured at 12 locations along the length of the bed via vacuum extraction of the dissolved gases. This measurement was performed during a different season with a similar NO_3—N removal rate of the bed (7.2 g N m^{-3} bed material d^{-1}) than in the time when $\delta^{15}N$—NO_3 was measured (8.7 g N m^{-3} bed material d^{-1}). Samples of water from 1.20 m depth of the bed were withdrawn through a tube into a 50 mL plastic syringe taking care to keep all air out of the sample. A two way tap was placed on the outlet of the syringe and excess water sample purged through the outlet to ensure all parts were filled with the water sample. With the tap turned off, the plunger of the syringe was pulled down to create a vacuum in the syringe and the syringe was shaken vigorously. The created vacuum extracted dissolved gases from the water and these gases formed a bubble in the syringe. Tests with air-saturated water and with isotopically-labelled gases dissolved in water showed that we could obtain at least 90% of the theoretical amount of dissolved gases by this technique and the recovered gases contained close to appropriate gas ratios of air (data not shown). The bubble was displaced into a 1 mL, zero dead space, insulin syringe fitted to the two way tap and previously purged with sample water to eliminate any air contamination. The 1 mL syringe while attached to the apparatus was tipped up so that the small amount of water in it formed a seal and it was withdrawn, the water expelled and needle of syringe pushed into a rubber bung for storage until analysis. This gas sample, normally of 0.3–0.4 mL, was injected into a port in the continuous flow mass spectrometer (PDZ Europa 20-20) and isotopic abundance of N measured.

Furthermore, $\delta^{15}N$—NO_3 of water samples was determined using chemical reduction via cadmium and azide to convert NO_3 to NO_2 and then N_2O (McIlvin and Altabet, 2005) and measured on an IsoPrime isotope spectrometer at New Zealand's National Isotope Centre. This measurement includes nitrite, typically in negligible amounts. The ratio of the rate of increase of $\delta^{15}N$—NO_3 and the decrease of $\delta^{15}N$–N_2 multiplied by the measured NO_3—N removal gives the denitrification rate – DR_{NA}. The negative congruence of the slopes shows the proportion of NO_3—N removed by microbial denitrification.

The fractionation factor associated with denitrification was estimated using the Rayleigh equation by plotting $\delta^{15}N$—NO_3 versus the natural logarithm of the ratio of NO_3—N concentrations along (C_t) and at the inlet of the bed (C_0) (Fig. 3). The slope of the linear regression curve through the data points expressed the isotope fractionation factor in ‰.

2.4. *Statistical analysis*

Standard errors (SE) for each DR approach, nitrate removal and fractionation factor were calculated with linear regressions analysis using SAS software version 8.2 (SAS Institute Inc., Cary, USA). Results are shown as DR ± SE and as SE of the

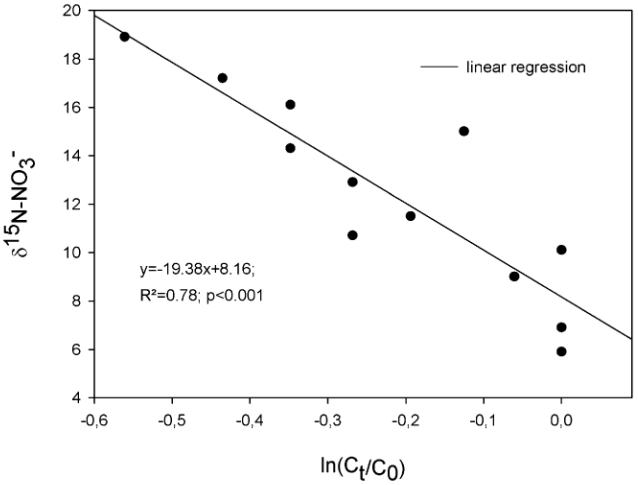

Fig. 3 – $\delta^{15}N$—NO_3^- measured along the length of the bed versus $\ln(C_t/C_0)$, where C_t is the NO_3^-—N concentration along the length and C_0 at the inlet of the bed). Linear regression line indicates isotopic fractionation factor of −19‰.

slope in equations. The mean DR_{AI} and the belonging SE of the DR_{AI} approach were calculated including DR_{AI} estimates resulting of $n = 12$ measuring points along the length of the bed. Furthermore we compared the different DR approaches by calculating the 95% Wald confidence intervals with the SAS software for each approach as shown in Fig. 6. Bounds express the range of the confidence intervals in Fig. 6.

The experimental errors of DR_{N_2}, DR_{PP} and $\delta^{15}N$–N_2 measurements during sampling, transport and analysis processes are expressed as experimental standard errors (SE_{exp}) and are shown for the sampling points in the Figs. 7–9.

3. Results

3.1. Nitrate removal rate

There was a significant linear decline ($y = -0.39 \times +173$; $R^2 = 0.78$; $p < 0.001$; SE = 0.064; $y = NO_3$—N concentration in mgL^{-1}; x = length of the bed in m) in NO_3—N concentration along the length of the bed (Fig. 4). This yielded a NO_3—N removal rate of 8.73 ± 1.45 g N m^{-3} bed material d^{-1} or 11.5 ± 1.9 kg N bed^{-1} d^{-1}.

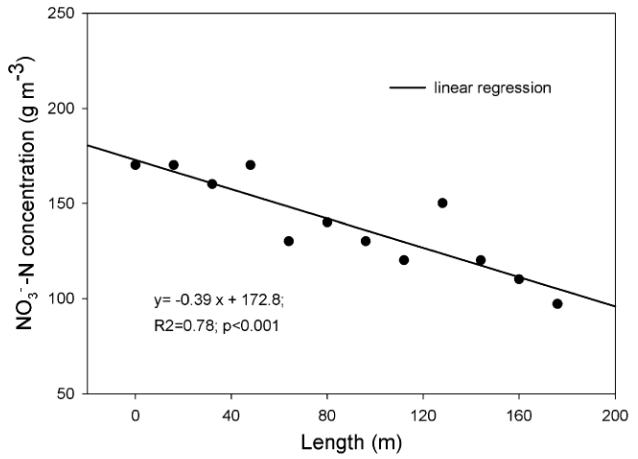

Fig. 4 – Concentrations of NO_3^-—N along the length of the denitrification bed (April). Linear regression line was fitted.

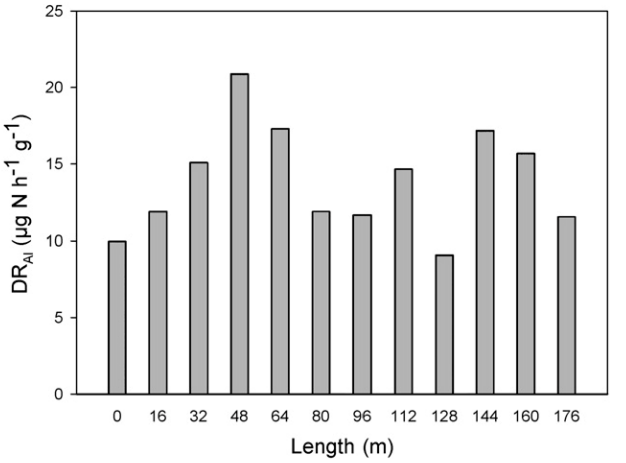

Fig. 5 – *In vitro* via acetylene inhibition method measured DR_{AI} at 27 °C along the length of the bed.

3.2. Denitrification rates

DR_{AI} ranged from 9.07 µg N h^{-1} g^{-1} dried media of the bed to 20.88 µg N h^{-1} g^{-1} (Fig. 5) averaging 13.9 ± 1 µg N h^{-1} g^{-1}. These analysis were conducted at 27 °C and when corrected to the bed temperature at the time of sampling (19 °C) using a Q_{10} of 2.1, resulted in a DR_{AI} of 7.94 ± 0.58 µg N h^{-1} g^{-1} (13.9 ± 1 divided by the Q_{10} associated factor 1.75) or 40.02 ± 2.9 g N m^{-3} d^{-1} based on a measured bulk density of 210 kg dried media m^{-3} (volume of bed) (Fig. 6).

The *in situ* push–pull technique with enriched ^{15}N—NO_3 measured concentrations of dissolved ^{15}N gas (^{15}N–N_2 and ^{15}N–N_2O) at locations A (48 m) and B (128 m). There were linear increases with time (^{15}N–N_2-gas concentration at

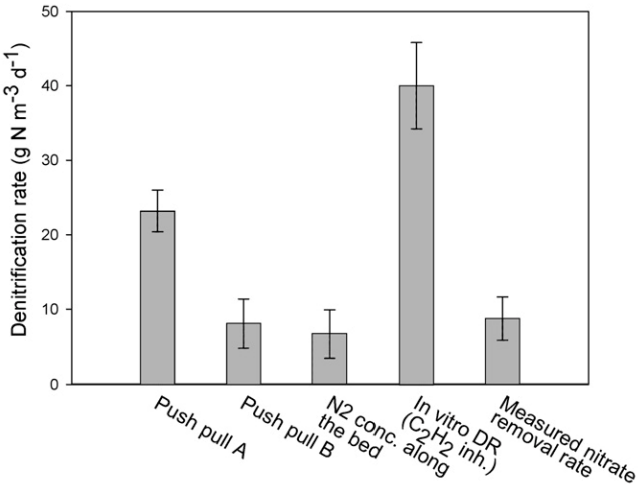

Fig. 6 – Denitrification rates (bars) of different approaches compared with the measured NO_3^-—N removal rate. Bounds express the range of the Wald confidence interval (95%).

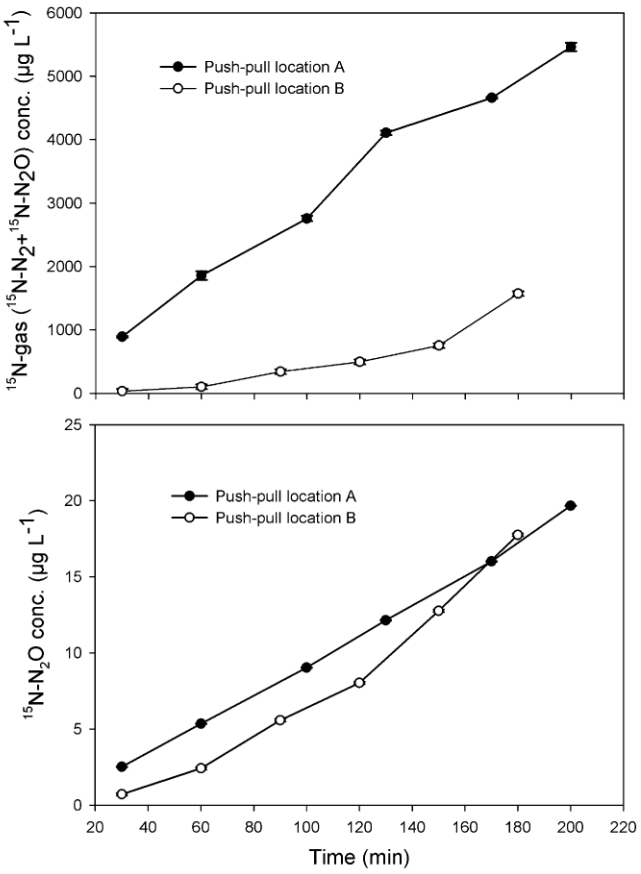

Fig. 7 – ^{15}N-Nitrogen gas concentration (top) and ^{15}N–N_2O concentration (bottom) at different times determined via push–pull technique at location A and B. Error bar is one experimental standard error (n = 2).

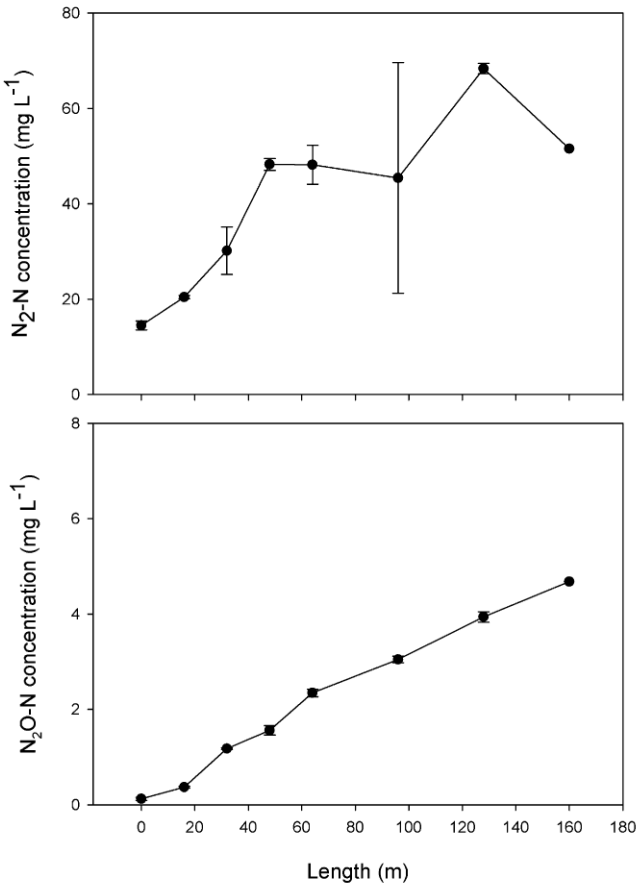

Fig. 8 – Dissolved N_2 gas concentration (top) and dissolved N_2O–N gas concentration along the length of the denitrification bed. Error bar is one experimental standard error (n = 2).

location A and B: $y = 26.9x + 201$; $R^2 = 0.99$; $p < 0.001$; SE = 1.65 and $y = 9.3x - 429$; $R^2 = 0.86$; $p = 0.008$; SE = 1.90 respectively; where $y = {}^{15}N-N_2$-gas concentration in µg L^{-1} and x = time in min) ($^{15}N-N_2O$ at location A and B: $y = 0.1x - 0.7$; $R^2 = 0.99$; $p < 0.001$; SE = 0.002 and $y = 0.11x - 4$; $R^2 = 0.97$; $p < 0.001$; SE = 0.01 respectively; $y = {}^{15}N-N_2O$-gas concentration in µg L^{-1}; x = time in min) (Fig. 7). From these increases, DR_{pp} were calculated as 26.8 ± 1.65 µg N L^{-1} min^{-1} at location A and 9.3 ± 1.9 µg N L^{-1} min^{-1} at location B. Assuming a bed material porosity of 60% (Robertson, 2010) the DRs were converted to 23.2 ± 1.43 g N m^{-3} d^{-1} for site A and 8.06 ± 1.64 g N m^{-3} d^{-1} for site B (Fig. 6). The dissolved N_2O–N release was 0.4% and 1.55% respectively of the potentially removed NO_3—N (Fig. 7).

In situ measured dissolved N_2–N and N_2O–N concentrations increased significantly linear along the length of the denitrification bed ($y_1 = 0.27x + 22.74$; $R^2 = 0.7$; $p = 0.01$; SE = 0.072 and $y_2 = 0.03x + 0.16$; $R^2 = 0.99$; $p < 0.001$; SE = 0.001 respectively; $y_1 = N_2$-N gas concentration in mg L^{-1}; x = length of bed in m; $y_2 = N_2O$–N gas concentration in mg L^{-1}; x = length of bed in m) (Fig. 8). A DR_{N_2} of 295 ± 71 µg N L^{-1} m^{-1} or 6.7 ± 1.61 g N m^{-3} d^{-1} was calculated based on a theoretical flow velocity of 38.1 min m^{-1} and a woodchip porosity of 60% (Fig. 6).

The natural abundance of $\delta^{15}N—NO_3$ of the remaining NO_3 increased linearly along the length of the bed ($y = 0.07x + 6.18$; $R^2 = 0.99$; $p < 0.001$; SE = 0.002; $y = \delta^{15}N—NO_3$; x = length in m).

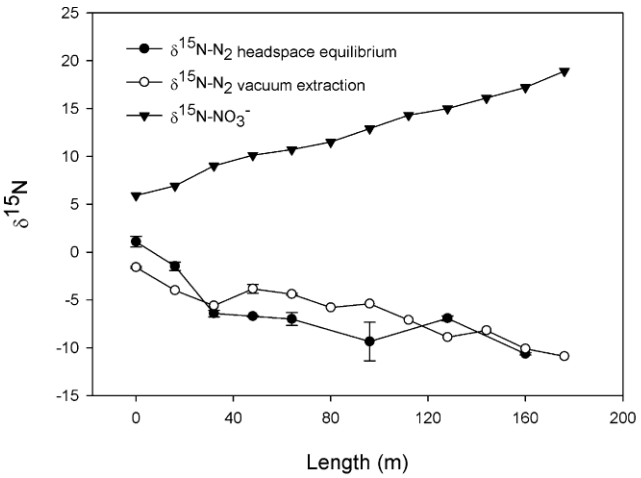

Fig. 9 – $\delta^{15}N—NO_3^-$, $\delta^{15}N–N_2$ measured via headspace equilibrium and $\delta^{15}N–N_2$ measured via vacuum extraction along the length of the denitrification bed. Error bar is one experimental standard error (n = 2).

As expected the $\delta^{15}N{-}N_2$ decreased along the length of the bed for both the vacuum extracted $^{15}N{-}N_2$ ($y = -0.05x{-}2.31$; $R^2 = 0.89$; $p < 0.001$; SE = 0.005; $y = \delta^{15}N{-}N_2$; x = length in m) and the via headspace equilibration method gained $^{15}N{-}N_2$ ($y = -0.06x{-}2.02$; $R^2 = 0.69$; $p = 0.011$; SE = 0.016; $y = \delta^{15}N{-}N_2$; x = length in m) (Fig. 9). A comparison of the slopes of $\delta^{15}N{-}NO_3$ and the average of the slopes of $\delta^{15}N{-}N_2$ indicated that about 73.2 ± 23.8% of removed NO_3—N was converted via denitrification to N_2 gas.

$\delta^{15}N{-}NO_3$ measured along the length of the bed decreased linearly ($y = -19.4 \times +8.2$; $R^2 = 0.78$; $p < 0.001$; SE = 3.2; $y = \delta^{15}N{-}NO_3$; $x = \ln(C_t/C_0)$) with the natural logarithm of the ratio of NO_3—N concentrations along (C_t) and at the inlet of the bed (C_0). The slope of this linear regression indicated an isotopic fractionation of natural abunded $^{15}N{-}NO_3$ of −19.4 ± 3.2‰ (Fig. 3).

4. Discussion

The NO_3 removal rate (g N m^{-3} bed material d^{-1}) of this study was similar to NO_3 removal previously measured in this denitrification bed (Warneke et al., 2011), and in the range of NO_3—N removal of other denitrification beds (Schipper et al., 2010). The majority of NO_3—N removal rates measured in denitrification beds have been calculated from the difference in total NO_3—N inputs (concentration multiplied by inlet flow rates) and total NO_3—N at the outlet divided by bed volume. It is likely that these measured NO_3—N removal rates are imprecise estimates of the average NO_3—N removal due to variability in NO_3 inlet concentrations and time taken for effluent to move to the outlet. Consequently, the measured outflow NO_3—N concentration may not reflect the NO_3—N removal of the measured inlet NO_3—N concentration. Therefore, it was necessary to develop a method to obtain reliable rates of NO_3 removal and to determine whether this NO_3—N removal was due to denitrification or other removal processes, such as DNRA, precipitation, absorption and/or biotic uptake. These other processes lead to temporary removal of NO_3 from the effluent and can be subsequently released whereas denitrification represents a permanent N sink. A substantial advantage of this study was that we could obtain reliable results for the NO_3—N removal rate due to only one effluent inlet, an almost constant inlet flow rate (149 ± 8 m^3 d^{-1}; 7 measurements $year^{-1}$) and constant inlet solute concentration (146 ± 13 mg NO_3—N L^{-1}; 7 measurements $year^{-1}$), the construction of the bioreactor (only open to the top) and the number of NO_3—N concentration measurements along the length of the bed ($n = 12$). Therefore we were able to compare the rate of NO_3—N removal with the measured denitrification rates using different techniques.

4.1. *Microbial denitrification was the main NO_3—N removing process in the bed*

There have been measurements of NO_3 removal in denitrification beds, and it has often been assumed that microbial denitrification was the main mechanism for NO_3—N removal (Greenan et al., 2006, 2009; Robertson et al., 2000; Robertson, 2010; Schipper et al., 2010; Moorman et al., 2010; Warneke et al., 2011), but none of these studies have directly measured denitrification rates via products of the denitrification process (N_2 and N_2O).

Using four different approaches for assessing the importance of denitrification for NO_3 removal we found persuasive evidence that NO_3 removal was largely due to denitrification. Both *in situ* measurements of dissolved $^{15}N{-}N_2$ via push–pull test (DR_{PP}) and the direct measurement of dissolved N_2 gas concentrations DR_{N_2} along the length of the bed resulted in denitrification rates that were sufficient to account for the actual NO_3—N removal of the bed (Fig. 6). Furthermore, the increase of dissolved $\delta^{15}N{-}NO_3$ and the decline of dissolved $\delta^{15}N{-}N_2$ along the length of the bed were almost negatively congruent (Fig. 9) suggesting that at least 73% of NO_3—N removal was due to production of dissolved N_2. This was likely an underestimate of the proportion of NO_3 removal due to denitrification because N_2O production rates could be not included in this calculation. Anammox could be eliminated as a significant NO_3—N removal process as NH_4^+ concentrations were always low (Warneke et al., 2011).

The fractionation factors of $^{15}NO_3$ caused by denitrification (enrichment of $^{15}N{-}NO_3$ in the system) are highly variable and depend on various conditions in a denitrifying system e.g., NO_3 concentration, concentration and microbial availability of carbon (electron donor), temperature, denitrification rate, species of the denitrifying bacterium (Mariotti et al., 1982; Bryan et al., 1983; Macko et al., 1987).

The calculated fractionation factors of $^{15}N{-}NO_3$ along the length of our study site was −19.4 ± 3.2‰, similar to the fractionation factors measured in laboratory studies under optimal denitrifying conditions by Blackmer and Bremner (1977) (−17‰) and by Mariotti et al. (1981) (−29.4‰). Our fractionation factor is greater than the fractionation factors calculated by Robertson et al. (2000) for two denitrification bed (−7.8 and −5.7‰) and calculated by Robertson (2010) in a woodchip column study (−13‰). Furthermore, fractionation factors determined in other field studies were also lower due to nitrogen transformations other than denitrification, e.g., Spalding and Parrott (1994) determined a fractionation factor of −9.6‰ in a groundwater study and Lund et al. (2000) a fractionation factor of −2.5‰ in a large constructed wetland. This is further evidence that microbial denitrification was responsible for the NO_3 removal in our study. The higher fractionation factor in our study may be due available carbon limitation of the denitrification process (electron donor) and the consistently high concentration of electron acceptor (NO_3) (Mariotti et al., 1981).

4.2. *Evaluation of different approaches to determine denitrification rates of the bed*

Our second objective was to determine the advantages and disadvantages of these different approaches for measuring denitrification rates in denitrification beds. This is the first study that has compared denitrification rates measured using different techniques with known rate of NO_3 removal in a denitrification bed.

The DR_{N_2} measurement technique resulted in a denitrification rate close to the measured NO_3—N removal rate in the denitrification bed, whereas the DR_{PP} at location A and the

DR_{AI} approach were significantly higher than the measured NO_3—N removal rate (Fig. 6). The DR_{N_2} method is based on measuring the increase of dissolved N_2 and N_2O along the length of the bed and required the assumption that the N_2 emission was constant along the length of the bed to calculate a reliable denitrification rate. N_2O gas emissions measured in chambers along the length of the bed showed no trend and consequently support this assumption (Warneke et al., 2011). The results suggest that the DR_{N_2} method was accurate, inexpensive and easy to use; however, the water sampling must be done with conservable care to avoid N_2 contamination from the atmosphere. Furthermore this method allows determination of dissolved N_2O production, which was about 2 times greater than the annual average total N_2O release from this denitrification bed (Warneke et al., 2011) and could be in the range of N_2O variations over months.

The temperature adjusted DR_{AI} was highly variable along the length of the bed (ranging from 26.1 to 60.1 g N m^{-3} bed material d^{-1}) similar to previous measurements at this site (Warneke et al., 2011) and generally 5 times greater than the calculated NO_3 removal rate (8.7 g N m^{-3} bed material d^{-1}; Fig. 6). This corresponds with the findings of Bernot et al. (2003) who measured significantly lower denitrification rates using the membrane inlet mass spectrometry technique than using the acetylene-block technique without adding chloramphenicol in aquatic systems. Although the acetylene inhibition technique is simple to perform, allows for a high degree of replication, and is not influenced by atmospheric N_2 contamination, it is likely to overestimate denitrification due to favourable incubation conditions (constant shaking of samples, constant temperature) and the addition of acetylene could act as a carbon source (Kanner and Bartha, 1979; De Bont and Peck, 1980; Tam et al., 1983; Schink, 1985). Furthermore, this method does not distinguish between N_2 and N_2O production. Consequently DR_{AI} has limited utility for estimating denitrification rates in denitrification beds, but can be used to determine whether C or N is limiting denitrification (e.g., Warneke et al., 2011) and it is also useful as a comparative index of denitrification activity across different sites or seasons (Groffman et al., 1992, 2006).

The push–pull technique was a reasonable method for determining the denitrification rate at a specific location within the bed; however, there was considerable variability of DR_{PP} between the two sampling locations. DR_{PP} was almost 3 times greater at location A than at the location B. This magnitude of variability between location A and B was similar to the variability of DR_{AI} at the same measurement locations (Fig. 5), but average DR_{AI} was more than 2 times greater than DR_{PP}. In contrast, Moorman et al. (2010) measured DR_{PP} in a denitrifcation wall in the same range as measurements of DR_{AI}. The Wald confidence interval (95%) of average DR_{PP} of the bed ranged from 13.5 g N m^{-3} to 17.8 g N m^{-3} and was significantly greater than the measured NO_3 removal rate. Multiple push–pull tests along the bed would be required to obtain an average denitrification rate of the entire bed. This approach required substantial effort and resources to minimize N_2 contamination risk and to obtain sufficient samples of dissolved gases at each location (minimum 16 per site), which can be costly. Furthermore, the effluent flow through the bed had to be stopped during each experiment, which may have altered the denitrifying conditions of the bed. Denitrification in the bed was previously shown to be C limited (Warneke et al., 2011), so the injection of additional ^{15}N—NO_3 was unlikely to alter the denitrification process in our experiment. However, addition of NO_3 would be an important consideration in systems where NO_3 concentrations were much lower and potentially limiting. Dissolved N_2O production also could be determined via push–pull technique and was in the range of the measured N_2O emission via chambers by Warneke et al. (2011) at the denitrification bed (around 1% of the removed NO_3—N). Summing up, the DR_{PP} method yielded reliable rates, but was a time consuming and expensive approach for measuring denitrification rates in denitrification beds particularly in comparison to the DR_{N_2} approach.

Measurements of natural abundance $\delta^{15}N$-gas and $\delta^{15}N$—NO_3 along the length of the bed measured a DR_{NA} that accounted for 73.2 ± 23.8% of NO_3—N removal. This method was also susceptible to contamination by atmospheric N_2 due to very small increases in dissolved ^{15}N–N_2-gas. Additionally, the NO_3—N removal rate based on inflow and outflow NO_3 concentration was needed to calculate DR_{NA} using this method. However, this approach was useful for determining whether the main pathway of NO_3—N removal was microbial denitrification.

5. Conclusions

All four approaches for measuring denitrification rates supported the hypothesis that microbial denitrification was the main mechanism for NO_3—N removal in the denitrification bed.

Our results suggest that the DR_{N_2} approach was useful for obtaining reliable NO_3 removal rates and was superior to the other investigated techniques for determining denitrification rates. This method may allow rapid measurement of denitrification rates for a variety of beds constructed in different locations with different carbon substrates and NO_3 loadings and for similar aquatic systems, so long as care is taken to avoid atmospheric contamination of samples. *In situ* push–pull experiments with enriched ^{15}N—NO_3 (DR_{PP}) were more intensive and only useful for measuring denitrification rates at specific locations when NO_3 was non-limiting. Denitrification rates determined via changes in natural abundance of $\delta^{15}N$—NO_3 and $\delta^{15}N$–N_2 (DR_{NA}) required data on NO_3—N removal rates to determine the proportion of NO_3 removed by denitrification and was prone to atmospheric N_2 contamination. The DR_{AI} method largely overestimated the denitrification rate of our system, but was useful for determining whether denitrifcation was C or N limited in the bioreactor.

Future denitrification rate measurements can also identify where the highest DR activity is in a bed (depth and length of the bed), and may lead to recommendations about the construction size of the bed.

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Chapter 6

Conclusion and Outlook

6.1 Objectives, conclusions and future work

As denitrification beds continue to be used to remove excess NO_3^- from point sources before discharge into sensitive aquatic ecosystems, it is critical to determine the mechanism of NO_3^- removal to evaluate the sustainability of denitrification beds. The controlling factors of NO_3^- removal were examined for future development of strategies to enhance the rates of denitrification, and to minimize the potential adverse effects of denitrification beds. Adverse effects such as GHG production and carbon loss were determined to evaluate the net benefit of this NO_3^- removing technology. Furthermore, it was necessary to develop a methodology for measuring denitrification rates in denitrification beds rather than calculating estimates of NO_3^- removal using changes in NO_3^- concentrations between inlets and outlets. Additionally, this study examined NO_3^- removal and microbial properties of 6 different carbon substrates (pine and eucalyptus woodchips, sawdust, green waste, maize cobs and wheat straw) in barrels (0.2 m^3) prior to their use in full scale denitrification beds.

The objectives and the conclusions achieved in this study are presented as follows. Resulting ideas of future work are also listed.

Objective a) Determine the NO_3^- removal rate in a large denitrification bed over a 12 month period and determine whether denitrification was the main mechanism for NO_3^- removal

Monitoring of a large denitrification bed over a 12 month period measured a NO_3^--N removal rate of 7.6 g N m^{-3} d^{-1}. In most cases, denitrification beds remove more NO_3^- from water discharges than other passive wastewater treatment technologies such as constructed wetlands (Vymazal, 2006). This thesis showed that NO_3^- removal by denitrification beds was predominantly due to denitrification rather than N immobilization or DRNA, because denitrification rates were determined from measurements of the end products (N_2 and N_2O) of the denitrification process and were sufficiently high to explain the measured NO_3^--N removal rate. The measured reduction from NO_3^- to non reactive N_2 demonstrated that denitrification beds are a sustainable technology to remove NO_3^- from point source discharges.

Objective b) Determine the environmental factors limiting NO_3^- removal and quantify any adverse effects in a large denitrification bed and for different carbon substrates

Controlling factors

Controlling factors of denitrification, including availability of C, DO, temperature, pH, number of denitrifying bacteria, and concentrations of NO_3^-, NO_2^- and S^{2-} (Firestone and Davidson, 1989; Seitzinger et al., 2006) were measured in a denitrification bed, and in barrels (0.2 m^3) filled with different carbon sources and incubated at two different temperatures.

Generally, denitrification was controlled by temperature and C supply. I calculated a temperature dependence of NO_3^- removal with a Q_{10} of 2 and 1.2 for the denitrification bed and the barrel study, respectively. NO_3^- removal followed zero-order kinetics and the availability of NO_3^- did not limit NO_3^- removal in the bed, probably because NO_3^- concentrations were above the known K_m of denitrifiers (Barton et al., 1999). However, NO_3^- limitation was observed in the barrel study, when NO_3^--N outlet concentrations were below 1 mg L^{-1}.

Dissolved oxygen was rapidly consumed in the denitrification bed and in the barrels and did not appear to inhibit denitrification.

Furthermore, S^{2-} as a potential inhibitor of the N_2O reductase was almost always below the detection limit in the denitrification bed. However, previous studies of denitrification beds have reported a decrease of SO_4^{2-} concentration and the odour of H_2S, which suggested active SO_4^{2-} reduction and the formation of S^{2-} (Van Driel et al., 2006; Robertson and Merkley, 2009; Robertson, 2010; Elgood et al., 2010). Therefore, S^{2-} should be measured and managed in further denitrification bed studies to prevent high N_2O emissions.

As C availability limited NO_3^- removal, future studies should focus on increasing the microbially available C concentration in the denitrification beds. One approach could be the addition of a labile carbon substrate (e.g., maize cobs) to the woodchips of a denitrification bed.

Adverse effects

Potential adverse effects of NO_3^- removal in denitrification beds include production and release of N_2O, CH_4 and DOC to the atmosphere and receiving waters.

The N_2O emission (1% of NO_3^--N removed) from the surface of the monitored denitrification bed was slightly greater than the IPCC emission factor for N released in waterways (EF_5 = 0.75%; IPCC, 2006). But when the N_2O emissions from the surface of the bed were combined with the release of N_2O dissolved in the outflow of the bed, the total N_2O release was about 4.3% of the removed NO_3^--N. Future work should investigate approaches to decrease the N_2O release of denitrification beds. One approach to reduce N_2O release would be to optimise the size of the bed. The bed should be large enough to decrease the NO_3^- concentrations below a threshold where the accumulated dissolved N_2O will be used as an electron acceptor by denitrifiers and reduced to N_2 gas. This unknown threshold of NO_3^- concentration should be determined in future studies.

Furthermore, I observed that N_2O release from the denitrification bed increased during warmer seasons. Similarly in the barrel study, warm incubated barrels (27.1 °C) released on average seven times more dissolved N_2O than cold incubated barrels (16.8°C). Additionally, the ratio of nitrite reductase gene (*nir*)/nitrous oxide reductase gene (*nosZ*) copies increased with increasing temperature in the substrates examined. Therefore, it was likely that the production of N_2O increased (from reduction of nitrite) more than the reduction of N_2O to N_2 with increasing temperature, which possibly explaining the higher N_2O release from warm barrels than cold barrels. Further studies should investigate the

correlation between ratio of *nir*/*nosZ* and temperature in different environments to understand observations of increasing N_2O emissions with increasing temperature (Teiter and Mander, 2005 and Johansson et al., 2003).

Methane release from the denitrification bed was very low, probably due to the high NO_3^- concentrations throughout the bed. However, methanogens competed successfully with denitrifiers for available carbon in the barrel study, when NO_3^--N concentrations were below 2 mg L^{-1}.

Further studies on the ideal outlet NO_3^- concentration from denitrification beds to limit GHG production should be conducted and construction sizes of denitrification beds may be adapted to achieve the desired NO_3^- concentrations. NO_3^- concentration close to the outlet should be sufficiently low to reduce dissolved N_2O to N_2 during denitrification, but sufficiently high to prevent CH_4 emissions.

The denitrification bed filled with woodchips had no net release of DOC, but the barrel study showed that some carbon substrates such as maize cobs released high concentrations of carbon. Dissolved organic carbon release has to be considered when carbon substrates other than wood by-products are used in denitrification beds.

Studies should be performed that test the combination of different C substrates in denitrification beds to provide the denitrifiers with sufficient available C to maximise NO_3^- removal rates, while ensuring that all dissolved C is consumed by microorganisms before the water leaves the denitrification bed.

Objectives c) and d) Determine the most suitable natural C substrate for growth of denitrifying bacteria. **Estimate the lifetime of the C substrate in a denitrification bed, and the C use efficiency of the NO_3^- removal in a denitrification bed**

I evaluated different carbon substrates in the barrel study for possible implementation in denitrification beds. Wheat straw removed more NO_3^- than woodchips, but released 10% of the removed NO_3^--N as N_2O. Maize cobs removed more NO_3^- than all other carbon substrates examined, but released TOC in the outflow and a substantial amount of carbon consumption by non-denitrifiers was likely as there was a relatively low ratio of denitrification gene copies/16S rRNA copies. This barrel study showed that wood substrates had moderate NO_3^- removal rates, and were an ideal medium for denitrifiers as there was a relatively high ratio of denitrification gene copies/16S rRNA copies suggesting a large proportion of the microbial population was dominated by denitrifiers.

Furthermore, longevity of the denitrification bed in my study was about 39 years as calculated from the total C losses including CO_2 emissions and release of dissolved CO_2 and DOC from the bed and support the estimated long life times of woodchip bioreactors (Moorman et al., 2010; Long et al., 2011). Additionally, the C consumption in the woodchip substrate of the denitrification bed was mainly due to microbial denitrification.

Summarizing, woodchips supported a relatively high ratio of denitrification gene copies to 16S rRNA copies, a long life time, relatively low adverse effects and a moderate NO_3^- removal rate. These findings suggest that wood by-products should

be used as the base substrate in denitrification beds. But addition of other carbon substrates such as maize cobs could enhance TOC availability and consequently increase NO_3^- removal rates without necessarily increasing adverse effects substantially in the denitrification bed. Future studies are needed to confirm the benefit of maize cob addition to woodchips in a denitrification bed.

Objective e) Develop a more reliable approach for measuring NO_3^- removal rates in operational denitrification beds

Finally, this thesis compared four different methods to determine denitrification rates in denitrification beds. The DR_{N2} method, which measured dissolved N_2 and dissolved N_2O concentrations along the length of the denitrification bed allowed calculation of a denitrification rate which was close to the measured NO_3^- removal rate. This method was also relatively quick to implement. This approach will be useful for determination of denitrification rates and NO_3^- removal rates of denitrification beds and possibly for aquatic environments with high denitrification activity and even flow. Future application of this technique may allow for areas of higher denitrifying activity to be localized, which may lead to changes in bed construction (depth and length of the bed).

The DR_{PP} approach, which measured production of $^{15}N_2$ and $^{15}N_2O$ after enrichment with $^{15}NO_3^-$ at a defined location in the denitrification bed, using push-pull technique (Addy et al., 2002), was time consuming and expensive. It would be an enormous effort to use the push-pull technique to obtain a reliable average of denitrification rates along the length of a denitrification bed.

Nevertheless, this approach might be useful to determine denitrification rates at specific locations in NO_3^- rich environments (e.g., Addy et al., 2002).

I also determined denitrification rates (DR_{NA}) by comparing the slopes of the increase of $\delta^{15}N\text{-}NO_3^-$ and decrease of dissolved $\delta^{15}N\text{-}N_2$ along the length of the bed. However, the calculation of DR_{NA} required knowledge of the NO_3^--N removal rate to determine the proportion of NO_3^- removed by denitrification. Therefore this method is less useful for determining denitrification rates, but could be used to determine the extent of denitrification in a nitrate removing system.

The most widely applied approach to measure denitrification rates are *in lab* acetylene inhibition measurements (DR_{AI}) (Groffman et al., 2006). These measurements overestimated NO_3^- removal in the denitrification bed, but were useful to determining whether denitrification was C or N limited (Tiedje et al., 1989). Furthermore, I showed that the acetylene inhibition method and the copy number of Σ(*nirS*; *nirK*) were good approaches to compare NO_3^- removal activity in different denitrifying systems.

In summary, results of this thesis suggest that denitrification beds were a useful passive approach for removing NO_3^- from point source discharges, with a great potential for further optimization. However, ways must be found to reduce the N_2O production of these beds.

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Appendix

Nitrate Removal from three different effluents using large-scale denitrification beds

My contribution to the following paper was provision of data and review of paper.

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Nitrate removal from three different effluents using large-scale denitrification beds

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ABSTRACT

Simple technologies that remove nitrate from effluents and other point discharges need to be developed to reduce pollution of receiving waters. Denitrification beds are lined containers filled with organic carbon (typically wood chip or coarse sawdust) and are a technology that is proving promising. Water containing NO_3^- (treated effluent or agricultural drainage) is passed through the bed and the wood chips act as an energy source for denitrifying bacteria that convert NO_3^- to N gases. There are few data on the efficiency of NO_3 removal in large-scale beds. We report here NO_3^- removal results from three large denitrification beds with volumes of 83, 294, and 1320 m^3 treating dairy shed effluent, treated domestic effluent and glasshouse effluent, respectively. Nitrate was nearly completely removed from the dairy shed effluent (annual load of 31 kg N) and domestic effluent (annual load 365 kg N). In these beds, NO_3^- removal, presumably by denitrification, was limited by NO_3^- concentration. However, the bed treating glasshouse effluent was overwhelmed by very high NO_3^- concentration (about 250 g $N\,m^{-3}$) and high flow rates (about 150 $m^3\,d^{-1}$) but still reduced NO_3^- concentration to about 150 g $N\,m^{-3}$. For this bed, long-term NO_3^- removal was between 5 and 10 g $N\,m^{-3}$ of bed material when NO_3^- was non-limiting and was similar to rates reported for other smaller denitrification beds. As expected, organic N, ammonium and phosphorus were not removed from any of the effluents following passage through the beds. Our results suggest that denitrification beds are a relatively inexpensive system to construct and operate, and are suitable for final treatment of a range of NO_3^--laden effluents.

1. Introduction

Excess nitrogen entering and polluting aquatic environments continues to grow as a global problem (Galloway et al., 2003). This N is predominantly derived from use of fertilizers or through enhanced N fixation, both of which are aimed at increasing food and fiber production. Not all of the applied N is assimilated by crops and animals and once nitrified, excess NO_3^- can leach to groundwater and then to surface waters as a non-point source of pollution. Where agricultural land is drained, N can enter surface waters as point discharges through sub-surface drains. N that is captured in food ultimately ends up in effluent treatment systems designed to remove nutrients. Generally, these treatment systems are not 100% efficient, resulting in discharges of some N and other nutrients to water bodies.

There is a need to develop simple, low-cost treatment systems (both in terms of construction and ongoing maintenance) to remove NO_3^- from effluents and from point source discharges from drained agricultural land. To complete the N cycle, NO_3^- can be converted to dinitrogen gas via denitrification. Nitrate removal of non-point source discharges can occur in wetlands and riparian buffers when these ecosystems contain significant organic matter, which provide an energy source to denitrifying bacteria (Hill, 1996; Dinnes et al., 2002). With increasing land-use intensity and urbanization, the N removal capacity of the remaining, often highly modified, wetlands and riparian buffers can be overwhelmed or bypassed by tile drainage systems (Dinnes et al., 2002). Constructed and natural wetlands have also been used in many parts of the world to remove nutrients from a range of effluents, but denitrification in these systems can be limited by carbon availability to denitrifying bacteria (Kadlec, 2005).

One relatively inexpensive approach to address the issue of carbon limitation of denitrification is to add a commonly available carbon source into the path of the NO_3^- discharge. This approach was trialed by Robertson and Cherry (1995) who added wood chip material into the flow path of groundwater (termed a denitrification wall) and also intercepting a tile drain (denitrification bed). Both these systems passively removed considerable NO_3^- for at

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least 15 years (Robertson et al., 2008). Similarly, Schipper and Vojvodic-Vukovic (1998) and Schipper et al. (2005) demonstrated the ability of sawdust to remove NO_3^- from groundwater for more than 7 years. A major advantage of these systems was the relatively low construction cost and no maintenance for many years. The denitrification beds tested by Robertson et al. (2005) ranged in size from 9 to 360 m^3 treating septic tank effluent volumes of 1–73 $m^3\ d^{-1}$. Often effluent flows can be much greater than these volumes and there is a need to determine whether denitrification beds may also be useful for treating variety of other effluent types with greater influent flows and masses of NO_3^-.

Key questions remain when considering scaling up of denitrification beds and utility for treating different effluents. The main uncertainty is what the upper limit for NO_3^- removal would be for very large denitrification beds where hydraulic flow properties may differ from smaller systems. It is also unclear whether other forms of N (ammonium and organic N) might be removed by denitrification beds via microbial immobilization. This information is critical in order to design future denitrification beds for other applications. However, obtaining such performance information from large denitrification beds can constrain the opportunity for replication at a specific site and effluent type. Here, we report rates of NO_3^- removal in three medium- to large-scale denitrification beds treating effluents derived from a hydroponic glasshouse, wash-water from a farm dairy milking shed and feedlot, and a sewage treatment plant. Even without on-site replication, rates of nitrate removal for a range of systems is valuable for design purposes.

2. Material and methods

We measured the performance of denitrification beds that received effluent from three different treatment systems. Effluent composition, flow rates, and operator objectives of these treatment systems differed and as a consequence the beds were all of different dimensions (Table 1). Bed temperatures were occasionally measured at all beds, but not generally at the same time as water sampling, and therefore only temperature ranges are presented here (Table 1).

2.1. Glasshouse effluent – Karaka

To avoid accumulation of salts and decrease risk of plant diseases some hydroponic glasshouse systems discharge a proportion of their nutrient solutions, which can have quite high N concentrations predominantly as NO_3^- (200–300 g N m^{-3}). In November 2006, a denitrification bed was constructed at Underglass Hothouses in Karaka just south of Auckland, New Zealand. This bed was 136 m long by 1.5 m deep and 7 m wide at the ground level tapering to 3 m wide at the bottom of the bed. For construction, a pit was dug into the soil and lined with plastic silage wrap and then the pit was backfilled with coarse sawdust and wood chip derived from softwood *Pinus radiata* (about a 50:50 mix). In April 2007, the bed was extended a further 40 m. Effluent entered through a single horizontal pipe (150 mm diameter) which discharged onto the top of one end of the bed. The outlet pipes consisted of four (150 mm diameter) vertical pipes 1 m apart linked via t-junctions to a single horizontal pipe at the other end of the bed. Final discharge of effluent was to a small stream on the property or irrigated onto nearby pasture land.

Inlet and outlet water samples were taken periodically by glasshouse operators, stored at 4 °C, couriered to a commercial analytical firm (Hills Laboratories, Hamilton, New Zealand) for analysis for total Kjeldahl N, NO_3^-, NO_2^-, and NH_4^+ using standard methods (APHA, 2005). Effluent flow rates were measured using a water meter (LXLG-80, Water Supply Products Ltd., New Zealand) that was read at the same time as sample collection.

2.2. Domestic effluent – Kinloch

A second bed was installed at Kinloch subdivision on the northern shore of Lake Taupo. This subdivision has large seasonal variations in populations because it is a popular vacation destination. The effluent from the subdivision was reticulated to a sequencing batch reactor (SBR) for secondary treatment and then into the denitrification bed. Two beds were constructed, each being 49 m long by 4 m wide and 1.5 m deep. Carbon material was a 50:50 woodchip (predominantly 10–30 mm diameter) and sawdust (predominantly 1–4 mm diameter) mix derived from *P. radiata*. Effluent from the treatment plant was diverted into both beds by a concrete diversion chamber that had two PVC pipes exiting from the base of the chamber. Final effluent exited the bed via two PVC pipes that were combined and then injected into shallow groundwater.

Inlet and outlet water samples were collected about every 2 weeks by the staff of the Taupo District Council who are responsible for the operation of the effluent treatment plant and analysed in their laboratories for NO_3^- and NH_4^+, total Kjeldahl N, total P and BOD (APHA, 2005). Flow was determined from an inline sonic flow meter (Wastemaster V, ABB, New Zealand).

2.3. Dairy farm effluent – Dargaville

The third bed (13 m long by 4 m wide by 1.5 m deep) was constructed on a dairy farm near Dargaville, Northland. The bed was filled with a 50:50 woodchip (predominantly 10–30 mm diameter) and sawdust (predominantly 1–4 mm diameter) mix derived from *P. radiata*. The bed was installed down stream of a membrane biological reactor (MBR) which received wash-down water from the dairy shed and feed lot. Effluent was pumped into the MBR plant from a storage pond that provided some reduction in suspended sediment load. The MBR plant converted the majority of the effluent N to NO_3^-. Effluent from the denitrification bed was discharged into a surface water drain.

Inlet and outlet samples from the bed were taken weekly for the first 16 months of operation and then at monthly intervals for the remaining 6 months. Total flow into the MBR plant for the sampling interval was measured by an impellor water meter. Flow data were only available for the latter part of the trial, which constrained calculation of N removal rates. Water samples were analysed for NO_3^- and NH_4^+, total Kjeldahl N, and total P using Hach test kits (Hach Company, Colorado).

Table 1
Properties of denitrification beds including size (length by width by depth), effluent type, bed temperature range, typical effluent NO_3^- concentrations and flow rates.

Denitrification bed	Bed dimensions (m)	Effluent type	Temperature (min–max) (°C)	NO_3 concentration (g N m^{-3})	Flow rate ($m^3\ d^{-1}$)
Karaka	176 × 5 × 1.5[a]	Glasshouse	14–25	250	150
Kinloch	49 × 4 × 1.5	2° treated domestic	14–22	5.5	182
Dargaville	13 × 4 × 1.6	2° treated dairy shed	15–26	53	1.6

[a] Surface width is 7 m tapering down to 3 m at bottom of bed.

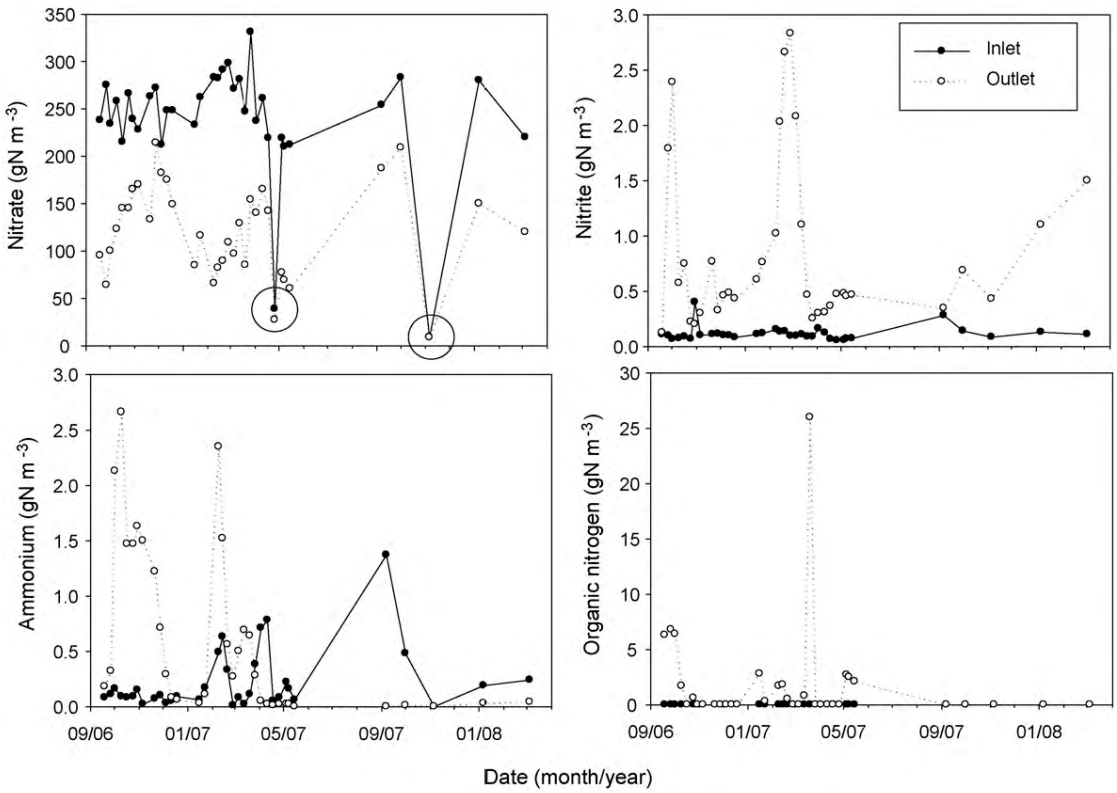

Fig. 1. Karaka denitrification bed: changes in N species as hothouse effluent passed through the denitrification bed. Note two circled points in NO_3^- graph were responsible for the low NO_3^- removal rates in Fig. 4.

3. Results and discussion

3.1. Nitrogen species in inflows and outflows

Total N loads and composition of effluent varied greatly between the three sites and with time. At the Karaka hothouse site, total N concentrations were very high (up to 350 g N m^{-3}) and was predominantly NO_3^- with little organic N or ammonium (Fig. 1). The bed receiving domestic effluent from Kinloch township received lower total N concentrations (up to 20 g N m^{-3}) dominated by NO_3^- with lesser amounts of NH_4^+ and organic N (Fig. 2). The Dargaville site which received effluent from dairy wash-down water had greatly fluctuating total N concentrations (up to 250 g N m^{-3}) that consisted of both NO_3^- and NH_4^+ (Fig. 3).

Nitrate was the major form of N removed from the effluents passing through the beds. At both Kinloch and Dargaville, nearly all the NO_3^- was removed; however, at the Karaka site, the bed was overwhelmed and NO_3^- concentration exiting the bed were often greater than 100 g N m^{-3} (Fig. 1). Nitrite was consistently analysed for at the Karaka bed (Fig. 1) but was generally low (generally less than 3 g N m^{-3}) relative to NO_3^- concentrations (>100 g N m^{-3}). Nitrite was only occasionally measured in the Dargaville bed (data not shown) but was always less than 1 g N m^{-3}. The Dargaville bed was the only bed that received substantial amounts of NH_4^+ and organic N in the inflow and did not display any consistent reduction in concentration of these N species (Fig. 3). Similarly, Robertson et al. (2005) did not measure any reduction in NH_4^+ concentrations through the beds they studied.

3.2. Removal mechanism

Denitrification was the most likely pathway for the removal of N as effluent passed through the beds because NO_3^- was consistently the main form of N removed. Alternative mechanisms for NO_3^- removal would include: immobilization into microbial biomass, dissimilatory NO_3^- reduction to NH_4^+ (DNRA), and anaerobic NH_4^+ oxidation (ANAMMOX).

When beds were initially flooded with effluent, N immobilization into an establishing microbial population might have been important until the biomass reached a steady-state when presumably immobilization would be matched by mineralization with no further net accumulation of N. If immobilization was a major long-term sink for N, then decreases in NH_4^+ and organic N concentrations through the beds would also be expected and these were not observed. DNRA is the anaerobic reduction of NO_3^- to NH_4^+ using a carbon source as an electron donor (Tiedje, 1988). It seems unlikely the DNRA was a major sink for NO_3^- in the denitrification beds as no accumulation of NH_4^+ was observed. The lack of significant NO_3^- removal by immobilization or DNRA was supported by a laboratory study conducted by Greenan et al. (2006). They incubated different carbon sources (including wood chips) with ^{15}N-labelled NO_3 under anaerobic conditions and found less than 4% of removed NO_3^- could be attributed to DNRA and immobilization.

ANAMMOX is the microbial conversion of NO_3^- and NH_4^+ to N_2 gas via an intermediate NO_2^- (Kunen, 2008). There was no consistent decrease in NH_4^+ through the Dargaville and Kinloch beds (where inflow contained ammonium) and so it would seem unlikely that ANAMMOX was a major process for N removal. However, the role of ANAMMOX should be explored further. If ANAMMOX could be enhanced in denitrification beds this would be beneficial because the beds would also reduce incoming NH_4^+. Kunen (2008) argued that the slow growth of ANAMMOX microbes means long start-up periods (more than 2 years in effluent treatment plants) to establish an active population of these organisms. Consequently, it may be that populations of

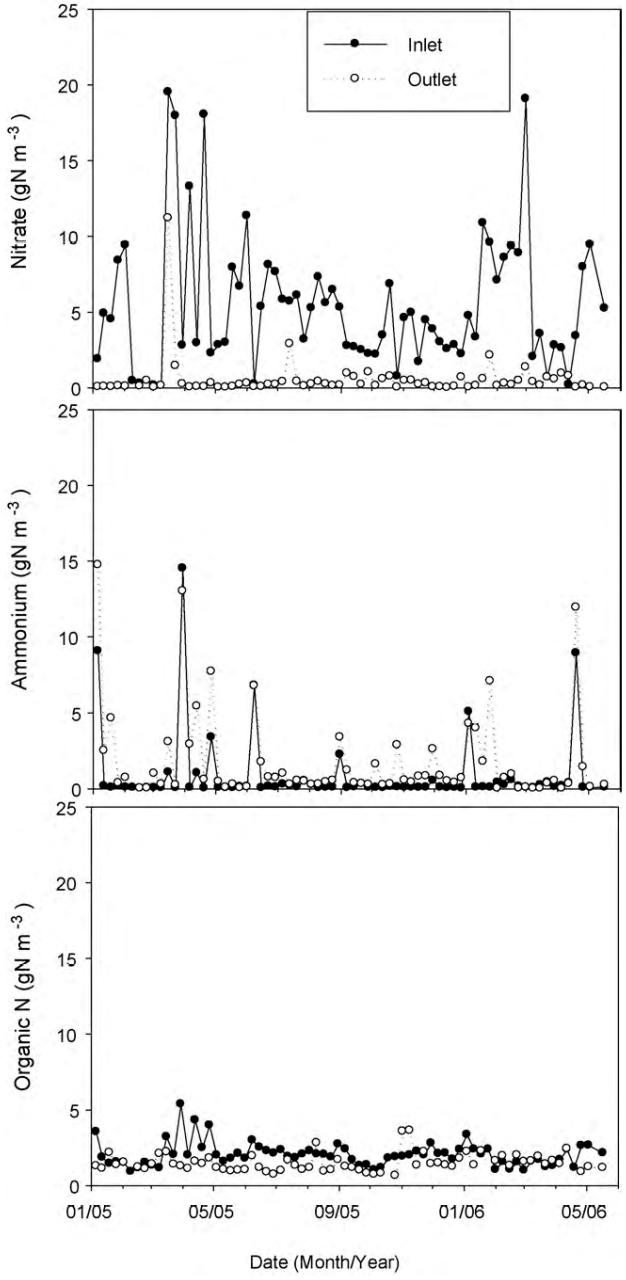

Fig. 2. Kinloch denitrification bed: changes in N species as treated domestic effluent passed through the denitrification bed.

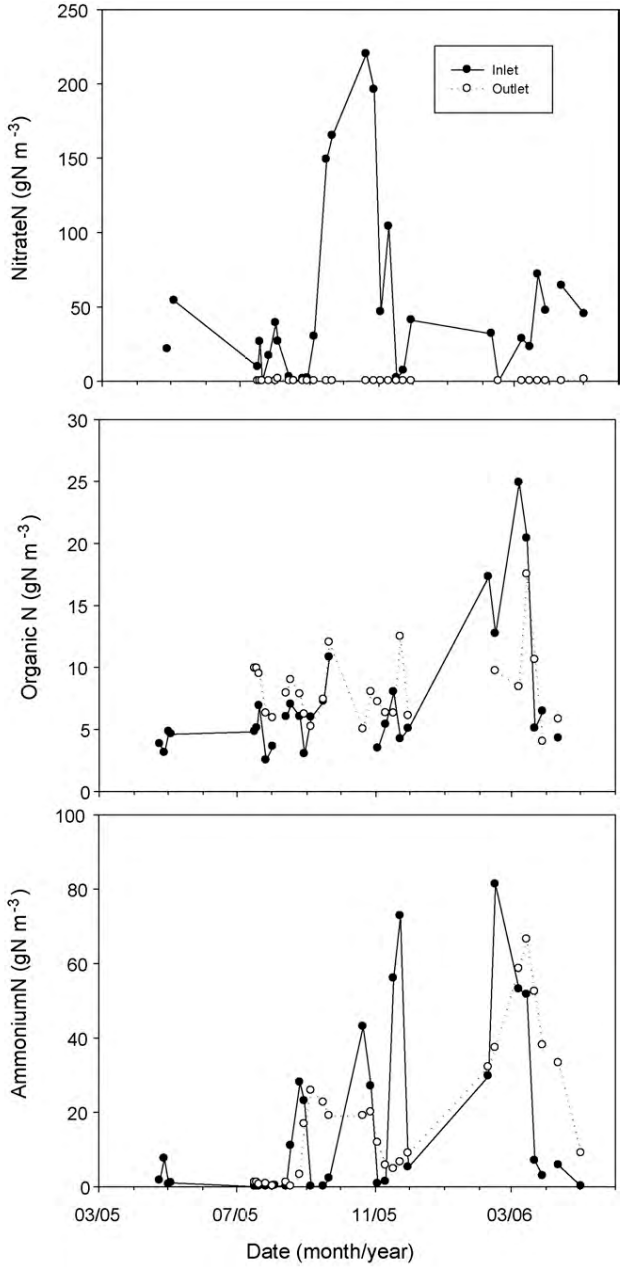

Fig. 3. Dargaville denitrification bed: changes in N species as treated dairy wash-down water passed through the bed.

ANAMMOX bacteria have not yet established in the beds studied here.

3.3. Nitrate removal rates

Nitrate removal rates were calculated as the difference between the mass of NO_3^- in the inlet and outlet (concentration in g N m^{-3} multiplied by flow in m^3) divided by the volume of the bed (Table 1). Nitrate removal rates (Fig. 4) ranged from around 11 g N m^{-3} d^{-1} down to near 0 g N m^{-3} d^{-1} in the Kinloch bed while NO_3^- removal in the Dargaville bed was generally lower (average 1.4 g N m^{-3} d^{-1}). However, the low rates of NO_3^- removal in both Dargaville and Kinloch occurred when there was complete removal of NO_3^- and so denitrification was likely limited by availability of NO_3^-. The same order of magnitude of NO_3^- removal was recorded at Karaka, where rates were initially more than 10 g N m^{-3} d^{-1} but with time decreased to between 5 and 10 g N m^{-3} d^{-1}. On two occasions, there was no apparent NO_3^- removal in the Karaka bed coinciding with large decrease in the concentrations of NO_3^- in the inlet. As with the other beds, low inputs of NO_3^- would result in denitrification being NO_3^--limited.

A few other studies have examined NO_3^- removal in generally smaller denitrification beds. Robertson et al. (2005) measured NO_3^- removal in denitrification beds ranging in size between 9 and 360 m^3, van Driel et al. (2006) tested beds between 0.2 and 0.7 m^3 and finally Robertson and Merkley (2009) investigated a bed 40 m^3 in volume. Despite these large differences in size between these beds and the beds we studied (up to 1320 m^3 at Karaka), rates of

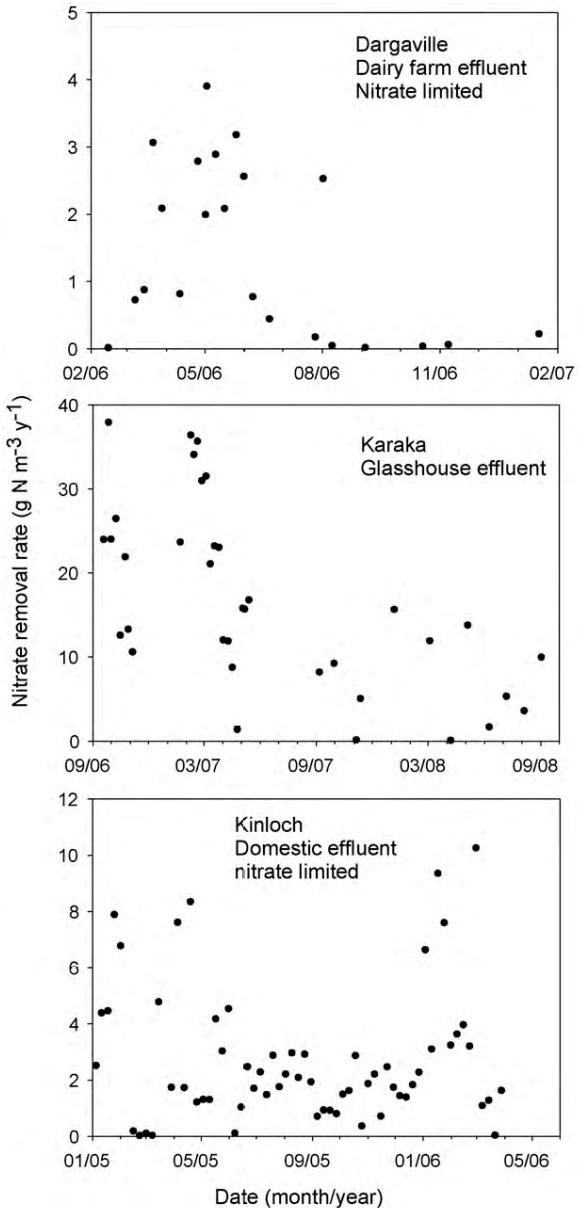

Fig. 4. Nitrate removal rate of all three beds. On many occasions at the Kinloch and Dargaville sites, NO_3^- was completely removed before exiting the bed and denitrification was likely limited by NO_3^- concentration; consequently NO_3^- removal rates were low, see text for full discussion. Note differences in scales of axis.

NO_3^- removal were similar across all beds when not limited by NO_3^- concentration. The previous reports by Robertson and colleagues have presented NO_3^- removal rates on the basis of mobile fluid volume of the beds (cf., effective porosity) rather than on a woodchip volume (Robertson, W.D., pers. comm.). To convert rates of NO_3^- removal based on effective porosity (NR_{ep}) to the rate of NO_3^- removal based on bed volume (NR_{bv}) requires the following adjustment: $NR_{bv} = NR_{ep} \times$ effective porosity, where effective porosity is the ratio of the pore space through which water flow occurs to the total volume of the bed. Effective porosity is generally determined by tracer studies. Applying this adjustment to reported measurements, Schipper et al. (this issue) recalculated NO_3^- removal rates of these studies. Average removal rates in the beds reported by van Driel et al. (2006) ranged between 2.1 and 3.7 g N m^{-3} d^{-1} in upflow denitrification beds installed adjacent to a stream treating groundwater. Adjusted rates of NO_3^- removal reported by Robertson et al. (2005) ranged from 1.8 to 5.1 g N m^{-3} d^{-1} and for the study of Robertson and Merkley (2009) average NO_3^- removal was 3.2 g N m^{-3} d^{-1}. As with our studies at Kinloch and Dargaville, NO_3^- was often completely removed in the beds studied by Robertson and colleagues suggesting that the maximal removal rates of NO_3^- had not always been reached. The rates of NO_3^- removal measured in the denitrification beds were greater than removal rates measured in denitrification walls of about 1 g N m^{-3} d^{-1} (Robertson et al., 2008; Schipper and Vojvodic-Vukovic, 2000; Schipper et al., 2005; Jaynes et al., 2008). The lower removal rates in denitrification walls are most likely because denitrification walls have lower proportion of wood chips or sawdust material.

In summary, across these studies NO_3^- removal rates for wood chips and sawdust are generally less than 10 g N m^{-3} d^{-1}. Removal rates can initially be greater (such as Karaka) most likely due to initial release of soluble carbon which is more biologically degradable. Other solid substrates (e.g., corn stalks) may be able to support greater removal rates (Greenan et al., 2006; Cameron and Schipper, this issue) but have yet to be tested at field scales.

3.4. Total P and BOD changes

Total P was measured in the inlet and outlets of the Kinloch and Dargaville beds and there were no consistent changes in phosphorus concentration as effluent passed through either bed

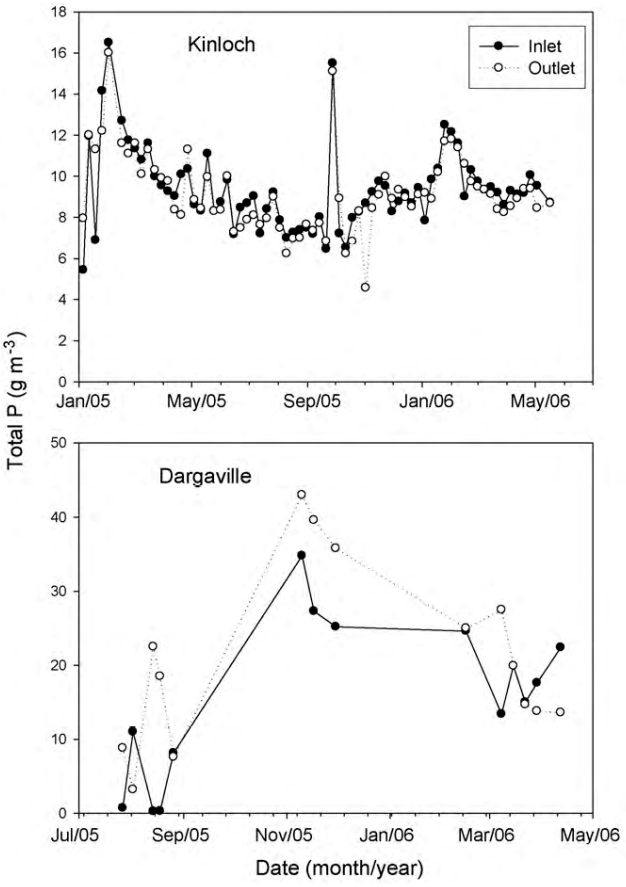

Fig. 5. Changes in total P in inlet and outlet of Kinloch and Dargaville denitrification beds. Note change in axis scales.

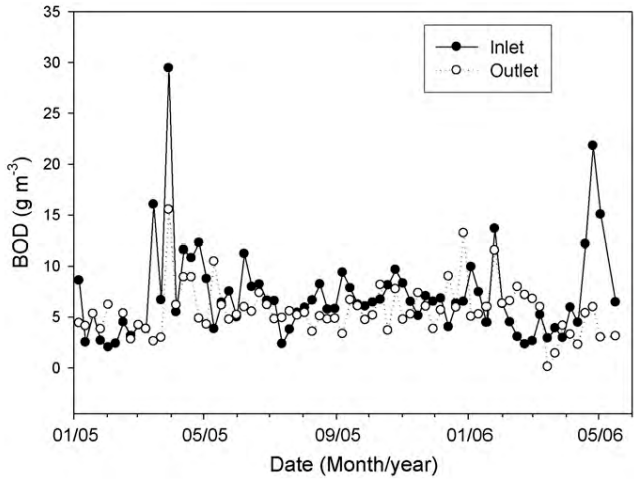

Fig. 6. Changes in BOD between inlet and outlet of the Kinloch bed.

(Fig. 5). Other approaches for removing P have been tested, such as the use of iron compounds (e.g., Robertson, 2000; McDowell et al., 2008) that could be easily incorporated in denitrification beds.

As beds are filled with woodchips there is the potential for the release of soluble carbon compounds through the outlet, which might enhance undesired oxygen consumption in receiving waters. BOD was only continuously measured in the Kinloch bed and generally ranged between 0 and 15 g m^{-3} and there was no obvious difference between concentrations in inlet and outlet (Fig. 6). Further measurements of BOD need to be made during the start-up period of beds to ensure that BOD loss is not a short-term concern (Robertson et al., 2005).

4. Conclusions

Denitrification beds are a relatively simple technology for removing NO_3^- from a variety of effluent streams. We demonstrated that NO_3^- removal rates of large denitrification beds receiving large effluent volumes with high NO_3^- concentrations are similar to smaller beds (van Driel et al., 2006; Robertson et al., 2005). Nitrate removal rates were between 5 and 10 g N m^{-3} d^{-1} when the beds were not NO_3^- limited and denitrification was the most likely mechanism for NO_3^- removal. Despite good NO_3^- removal, there was no evidence of removal of NH_4^+, organic N, BOD or total P as effluent passed through the bed. For denitrification beds to be effective, effluent needs to be filtered and oxidized to convert N to NO_3^- and remove organic matter; otherwise, N removal is likely to be limited and clogging of the bed might occur.

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