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Review

Denitrification and N_2O:N_2 production in temperate grasslands: Processes, measurements, modelling and mitigating negative impacts

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HIGHLIGHTS

- We review denitrification, denitrifier populations and enzyme activities, nitrous oxide and dinitrogen production in soils.
- We describe molecular techniques to assess gene diversity and reveal enzymes that consume nitrous oxide.
- We consider a process-based approach to quantify the nitrous oxide:dinitrogen ratio and nitrous oxide emissions.
- We discuss the management strategies to enhance nitrous oxide reduction during denitrification.

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ABSTRACT

In this review we explore the biotic transformations of nitrogenous compounds that occur during denitrification, and the factors that influence denitrifier populations and enzyme activities, and hence, affect the production of nitrous oxide (N_2O) and dinitrogen (N_2) in soils. Characteristics of the genes related to denitrification are also presented. Denitrification is discussed with particular emphasis on nitrogen (N) inputs and dynamics within grasslands, and their impacts on the key soil variables and processes regulating denitrification and related gaseous N_2O and N_2 emissions. Factors affecting denitrification include soil N, carbon (C), pH, temperature, oxygen supply and water content. We understand that the N_2O:N_2 production ratio responds to the changes in these factors. Increased soil N supply, decreased soil pH, C availability and water content generally increase N_2O:N_2 ratio. The review also covers approaches to identify and quantify denitrification, including acetylene inhibition, ^{15}N tracer and direct N_2 quantification techniques. We also outline the importance of emerging molecular techniques to assess gene diversity and reveal enzymes that consume N_2O during denitrification and the factors affecting their activities and consider a process-based approach that can be used to quantify the N_2O:N_2 product ratio and N_2O emissions with known levels of uncertainty in soils. Finally, we explore strategies to reduce the N_2O:N_2 product ratio during denitrification to mitigate N_2O emissions. Future research needs to focus on evaluating the N_2O-reducing ability of the denitrifiers to accelerate the conversion of N_2O to N_2 and the reduction of N_2O:N_2 ratio during denitrification.

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1. Introduction

Grasslands cover about 25% of the earth's surface, occupy 117 million km^2 of vegetated land, and provide feed for over 1800 million livestock units (Saggar et al., 2009). Nitrogen (N) dynamics in temperate grasslands, occupying ca. 9 million km^2, have been extensively studied, particularly for managed grazed pasture systems (Haynes and Williams, 1993; Ledgard et al., 1999; Bolan et al., 2004; Saggar et al., 2009, 2011a). The direct effects of grazing grassland include consumption of aboveground biomass, removal of nutrients in animal products (milk, meat and wool), redistribution of nutrients through uneven deposition of excreta and gaseous and leaching/run-off losses of nutrients. Managed grasslands have a high demand for N to support plant growth, which is provided by symbiotic N fixation in association with legumes such as white clover (*Trifolium repens* L.) or/and by N fertiliser. The relative contributions of symbiotic N fixation and N fertiliser to the total N input to pastoral systems vary widely according to the intensity of the production system. Globally, about 14–15 Tg N [1 Tg = 10^{12} g = 1 million metric tonne] is applied as fertiliser in pastoral farms annually (Saggar et al., 2009), which is 14–15% of the total 103 Tg global fertiliser N use in 2010 (IFA, 2011).

Intensively managed pasture systems reach N saturation when plants, microorganisms and soils can no longer assimilate or retain excess N, which is lost from the system through leaching of nitrate (NO_3^-) and gaseous emissions (Saggar et al., 2011a). In grazed grassland soils, the N associated with animal excreta (both urine and faeces) is an additional important component and critical source contributing to and affecting the N cycle. In New Zealand grazing systems' daily excretal N ranges between 182 and 398 g N (mean 290 g N) for dairy cattle (Saggar et al., 2004a), and between 26 and 50 g N (mean 40 g N) for sheep (Saggar et al., 2007b). About 60–75% of the dietary N ingested is excreted in the urine consisting of over 70% urea, while the bulk of N in dung is in organic form. Nitrogen deposition ranges from 20 to 80 g m^{-2} in dung patches and 50 to 200 g m^{-2} in urine patches (Oenema et al., 1997; Bolan et al., 2004). The estimated amount of N excreted in animal manures on grazing grassland is as large as, or larger than, the current global annual N fertiliser consumption of 103 Tg N (IFA, 2011). For example, in New Zealand, animals void almost 5 times more N annually (1.5 Tg) than the N applied in fertilisers (0.31 Tg) (Saggar et al., 2005). Consequently, urine makes a much greater contribution to gaseous and leaching losses than N fertiliser. Although N is the major nutrient limiting pasture production, it is also a major contributor to environmental degradation where it occurs in excess of plant and microbial requirements in soil (Saggar et al., 2005). Accordingly, the challenge is to maximise the use of N for production in agricultural systems, while limiting the release of reactive N (biologically, chemically and radiatively active N) into the environment. Fig. 1 provides a schematic of N transformations in a leguminous pasture. A detailed description of the biotic and abiotic N transformations in grazed pastures is given by Bolan et al. (2004).

In the context of environmental pollution and global climate change, NO_3^- leaching attracts attention because of its potential effects on human and animal health, and on water pollution (Parliamentary Commissioner for the Environment, 2004). Gaseous N, such as ammonia (NH_3), nitrous oxide (N_2O) and nitric oxide (NO), cause concern because of their radiative or chemical effects on the atmosphere (Bolan et al., 2004). Denitrification completes the N-cycle by returning N_2 to the atmosphere (Fig. 1). Nitric oxide and N_2O are produced at intermediate steps during the denitrification process and may escape to the atmosphere before being reduced to N_2. While, N_2 is an environmentally benign gas, NO is a recognised pollutant that contributes to stratospheric ozone destruction and radiative forcing in the troposphere (Ravishankara et al., 2009). Nitrous oxide is also formed in soils during the microbial processes of nitrification. At low oxygen (O_2) concentrations, nitrifying bacteria may contribute to N_2O emissions through nitrifier-denitrification. However, biological denitrification is

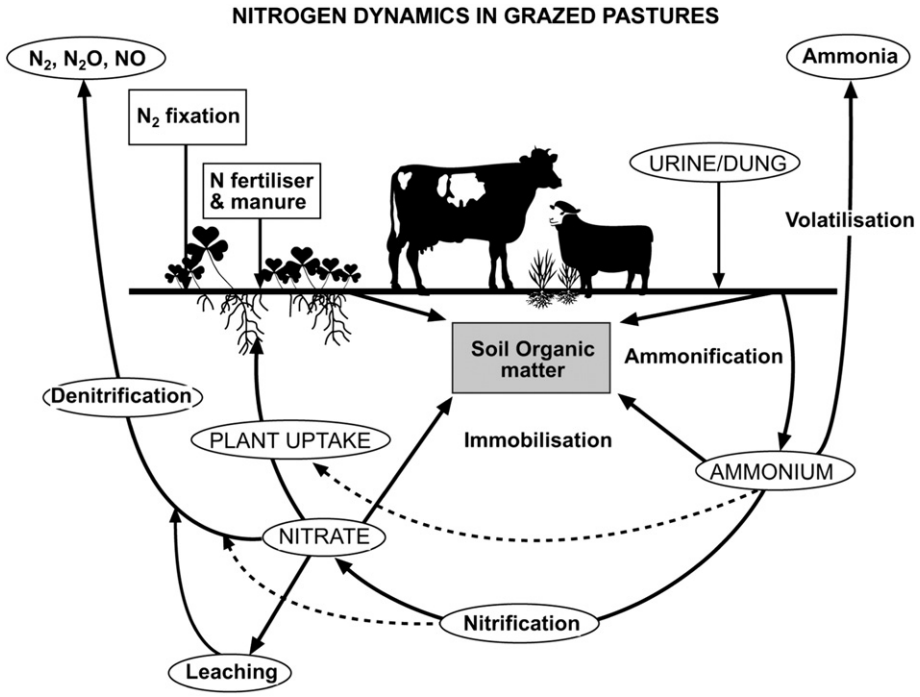

Fig. 1. Schematic representation of nitrogen transformations in legume-based pastures.

the primary process producing N_2O in temperate grassland soils (Saggar et al., 2009) and it accounts globally for about 60% of total N_2O emissions to the atmosphere (Mosier et al., 1998; Kroeze et al., 1999).

This review explores the biotic and abiotic transformations of nitrogenous compounds that occur during denitrification, and the factors that influence denitrifier populations and their enzyme activities, and hence affect the production of N_2O and N_2. We address denitrification with emphasis on temperate grasslands, and provide an overview of N inputs and dynamics and their impacts on the key soil processes regulating denitrification and related gaseous emissions. We also consider difficulties and limitations in measurement techniques, and describe a process-based approach that can be used to quantify N_2O emissions and the N_2O:N_2 product ratio with known levels of uncertainty. Finally, we discuss options for mitigating N_2O losses, identifying current gaps and limitations in our knowledge, and conclude by presenting the main research needs to develop future measurement, verification and mitigation strategies for N_2O emissions from managed temperate grasslands.

Only a brief description of N dynamics is given here, and the reader is referred to appropriate reviews that provide in-depth coverage of sources of N inputs, N transformations, gaseous and leaching losses, the environmental impacts of N losses, and their mitigation (Haynes and Williams, 1993; Ledgard et al., 1999; Bolan et al., 2004; de Klein and Eckard, 2008; Ledgard and Luo, 2008; Saggar et al., 2009, 2011a; Luo et al., 2010).

2. Overview of denitrification

Denitrification is considered as a much more potent source of N_2O than nitrification in grassland soils (Saggar et al., 2009). For the purposes of this review, denitrification is a microbially mediated process of dissimilatory reduction of one or both of the ionic nitrogen (N) oxides (NO_3^-, and nitrite NO_2^-) that may ultimately produce N_2 through a series of gaseous N oxide products (Eq. (1)).

$$\underset{\text{Nitrate}}{NO_3^-} \xrightarrow{\text{Nitrate reductase}} \underset{\text{Nitrite}}{NO_2^-} \xrightarrow{\text{Nitrite reductase}} \underset{\text{Nitric oxide}}{[NO]} \xrightarrow{NO^- \text{ reductase}} \underset{\text{Nitrous oxide}}{N_2O} \xrightarrow{N_2O^- \text{ reductase}} \underset{\text{Dinitrogen}}{N_2} \quad (1)$$

(1)

The general requirements for biological denitrification are:

- the presence of bacteria, fungi, other denitrifying eukaryote or archaea possessing metabolic activity
- suitable electron donors such as available organic C compounds
- anaerobic conditions or restricted supply of O_2
- availability of N oxides: NO_3^-, NO_2^-, NO, or N_2O as terminal electron acceptors.

The rate of denitrification, and the relative proportions of NO, N_2O, and N_2 produced, depend on the outcomes of complex interactions between soil properties, soil micro-organisms, climatic factors, and management practices. The availability of mineral N (both NH_4^+ and NO_3^-) and labile C, together with processes that affect reaction rates, such as temperature, pH and redox potential of soil microsites, controls the rate of denitrification in soils. These factors influence both the number of denitrifiers and the activity of denitrification enzymes (Luo et al., 1996; Cavigelli and Robertson, 2000; Wallenstein et al., 2006; Zaman et al., 2007, 2008a, 2008b; Liu et al., 2010).

The rates and percentage of N-input that was denitrified for 36 temperate grassland soils are shown in Table 1. This review of 241 datasets suggests a median daily loss of 0.017 kg N ha^{-1} through denitrification (Table 2) for a total mean loss of approximately 2.5% of available N. Thus globally each year, temperate grasslands could lose 5.6 Tg of N through denitrification.

Despite a qualitative understanding of the multiple factors that regulate the rates of denitrification in pastoral soils, we have only limited ability to integrate this knowledge to construct and validate robust and predictive process-based models of denitrification (Boyer et al., 2006). We lack knowledge of reliable quantitative denitrification rates for grazed pasture soils and how these rates and the N_2O:N_2 product ratios vary spatially and temporally. Issues that hinder this understanding include: difficulties in measuring denitrification rates (Groffman et al., 2006), the large spatial and temporal heterogeneity of denitrification products, and the large background atmospheric N_2 concentration (Davidson and Seitzinger, 2006). An understanding of the biological processes, and the soil and environmental factors that have the potential to reduce the production of N_2O during denitrification, is vital for the development of novel and effective N_2O mitigation technologies.

Table 1
Summary of denitrification studies in temperate grasslands.

Location	Texture	Treatments	Denitrification method	Denitrification (kg N ha^{-1} d^{-1})	N loss by denitrification (% of total N input)	Reference
				mean (min.–max.)	mean (min.–max.)	
Australia	[a]	AN + U (200 kg N ha^{-1})	Soil core	0.040 (0.018–0.061)	0.73 (0.32–1.11)	Eckard et al. (2003)
Belgium	Loamy, sandy loam	NK (200 kg N ha^{-1})	Soil core	0.038 (0.024–0.052)	7.52 (3.7–11.31)	Vermoesen et al. (1996)
Belgium	Sandy loam	NK (200 kg N ha^{-1})	Chamber	0.329 (0.008–0.654)	3.06 (1.2–5.7)	Vermoesen et al. (1997)
Belgium	Sandy loam	AN (295 kg N ha^{-1})	Soil core	0.240	22.8	Van Cleemput et al. (1994)
Belgium	Sandy loam	AN (215 kg N ha^{-1})	Soil core	0.059	4.9	Kaiser et al. (1996)
China	Sandy loam	Grazing intensity	Soil core	0.0001 (0.0000076–0.00013)	[a]	Xu et al. (2008)
Denmark	Loamy sand	U, ammonium solution (529 kg N ha^{-1})	Soil core	0.0140	[a]	Carter (2007)
France	Sandy	Grazing intensity	Soil Slurry	1.4 (1.2–1.8)	[a]	Patra et al. (2005)
Germany	Sand	CAN (78 kg N ha^{-1})	Chamber	[a]	2.3	Mogge et al. (1999)
Germany	Loam	AN, PS, CS (450 kg N ha^{-1})	Chamber	0.003 (0.001–0.008)	0.32 (0.1–0.7)	Schwarz et al. (1994)
New Zealand	Silt loam	U (500 kg N ha^{-1})	N balance	1.414 (0.863–1.974)	27.0 (16.4–37.5)	Clough et al. (1996)
New Zealand	Silt loam	Natural rainfalls (26 mm)	Soil core	0.020 (0.003–0.247)	[a]	Luo et al. (2000)
New Zealand	Fine sandy loam	U (500 kg N ha^{-1})	Soil core	0.014 (0.005–0.031)	3.61 (2.6–4.8)	Ruz-Jerez et al. (1994)
New Zealand	Silt loam	KN (200 kg N ha-1), Lime, Zeolite	Sieved soil	1.48 (0.80–2.33)	24.7 (20.0–32.0)	Zaman et al. (2008a)
New Zealand	Silt loam	U, KN (400 kg N ha^{-1}), Lime, Zeolite	Soil core	0.0145 (0.0009–0.03)	0.591 (0.39–0.84)	Zaman and Nguyen (2010)
Netherlands	Sand	CAN, AS, U, KN, CA, O (kg N ha^{-1})	Soil core	0.152 (0.006–0.382)	4.99 (0.2–22.8)	Velthof et al. (1996)
Netherlands	Sand, loam, peat	Irrigation (0, 10 mm), temperature (10, 20 °C), KN (40, 80 kg N ha^{-1})	Soil core	1.413 (0.03–3.6)	[a]	de Klein and Van Logtestijn (1996)
Netherlands	Heavy clay	CS, KN, deposition (33–264 kg N ha^{-1})	Soil core	0.276	20	van der Salm et al. (2007)
Switzerland	Sandy loam	AN (85 kg N ha^{-1})	Soil core	0.078 (0.065–0.091)	18.1 (14.6–21.6)	Rudaz et al. (1999)
USA	Sandy loam to loamy sand	*	Chamber	0.015 (0.07–0.024)	[a]	Hixson et al. (1990)
USA	[a]	U + AS (240 kg N ha^{-1})	Soil core	0.142 (0.011–0.280)	10.6 (10.4–10.8)	Horwath et al. (1998)
USA	Sandy loam, loam, silt loam	Poultry litter (103–254 kg N ha^{-1})	Soil core	0.016 (0.002–0.042)	1.85 (0.4–4.8)	Marshall et al. (1999)
USA	[a]	KN (300 kg N ha^{-1})	N balance	5.67 (4.49–6.84)	56.7 (44.9–68.4)	Rolston et al. (1976)
USA	Sandy loam to loamy sand	Dry/wet meadow	Chamber	0.026	[a]	Walker et al. (1992)
USA	[a]	PS, AN (112 kg N ha^{-1})	Soil core	0.0003 (0.000072–0.00048)	0.03 (0.007–0.052)	Sullivan et al. (2005)
USA	[a]	U (510 kg N ha^{-1})	Soil core	0.0008 (0.00025–0.0015)	0.002 (0.0058–0.002)	Frank and Groffman (1998)
UK	Clay, clay loam	CAN, U (100, 200, 300 kg N ha^{-1})	Soil core	0.071 (0.004–0.216)	11.4 (3.3–26.3)	Jordan (1989)
UK	Clay	AN (210 kg N ha^{-1})	Chamber	0.009	1.0	Colbourn et al. (1984)
UK	Sandy loam over clay loam	CS, KN (700 kg N ha^{-1})	Chamber	0.071 (0.018–0.174)	5.04 (1.0–9.1)	Egginton and Smith (1986)
UK	[a]	AN (200 kg N ha^{-1})	Soil core	0.168 (0.080–0.24)	44.7 (41.3–48.1)	Elliott et al. (1991)
UK	Silty clay loam	CS (45 kg N ha^{-1}), AN (60 kg N ha^{-1})	Soil core	0.117 (0.06–0.151)	1.41 (0.9–1.7)	Ellis et al. (1998)
UK	Loam, silty clay loam	CS + (DCD, acid, nitrapyrin) (200–296 kg N ha^{-1})	Soil core	0.065 (0.003–0.237)	4.12 (0.1–15.6)	Pain et al. (1990)
UK	Loam over clay	AN (250, 500 kg N ha^{-1})	Chamber	0.038 (0.004–0.080)	5.13 (4.4–5.8)	Ryden (1983)
UK	Sandy loam	CS (44–72 kg N ha^{-1}), PS (223–278 kg N ha^{-1})	Soil core	0.059 (0.002–0.101)	5.59 (0.6–14.0)	Misselbrook et al. (1996)
UK	Coarse sandy loam	NK (280 kg N ha^{-1})	Soil core	0.645 (0.02–0.37)	[a]	Jarvis et al. (2001)
UK	Sandy loam	Clover, grass	Soil core	0.141 (0.024–0.464)	[a]	Šimek et al. (2004)

NK: Not known fertiliser, AN: ammonium nitrate, CAN: calcium ammonium nitrate, PS: Pig slurry, CS: Cattle slurry, U: urea, AS: ammonium sulphate, KN: potassium nitrate.
[a] No data available.

Table 2
Summarised values for denitrification rate and percent N loss for temperate grassland soils.

Site	Number study	Min	Max	Median	Mean ± s.e.	95% confidence interval of mean
Denitrification rate (kg N ha^{-1} d^{-1})	241	0.0001	6.84	0.0171	0.258 ± 0.050	0.098
Nitrogen loss (% of total N input)	236	0.002	68.4	2.45	7.97 ± 1.26	2.51

Mitigation approaches should focus on ways to reduce the production of N_2O during denitrification and enhancing the reduction of N_2O to N_2 thus lowering the N_2O:N_2 product ratio.

2.1. Denitrification reactions

In grazed pastures, autotrophic nitrification of various ammonium (NH_4^+) inputs, including those released from animal excreta, fertiliser, and soil organic matter, leads to continuous formation of NO_3^-–N, which is either taken up by plants, lost from soils as leachate or denitrified. Microbially mediated denitrification is an energy-yielding process catalysed by a range of intracellular enzymes. During denitrification, N-oxides are reduced through the addition of two electrons per N-atom. The product of each reduction forms the substrate for the subsequent step in the process, as follows:

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \quad (2)$$

$$NO_2^- + 2e^- + 4H^+ \rightarrow NO + H_2O + 2OH^- \quad (3)$$

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad (4)$$

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad (5)$$

These reactions differ with regard to the amounts of energy they yield. The relative redox potential (E′o) of the reduction of NO_3^- to NO_2^- is +420 mV; of NO_2^- to NO is +375 mV; of NO to N_2O is +1175 mV; and of NO to N_2 gas is +1355 mV (Zannoni, 2004).

2.2. Denitrifiers

Organisms capable of all reaction steps 2–5, as described above, are facultative-anaerobes that utilise NO_3^- (dissimilative reduction) when O_2 becomes limiting in the environment. Denitrifiers utilise N oxides as the terminal electron acceptor in the generation of ATP and are phylogenetically diverse, spanning three kingdoms: bacteria, archaea, and eukarya. While ubiquitous in soil (Payne, 1981; Firestone, 1982; Tiedje, 1988) denitrifiers are not numerically dominant, typically representing only 0.5–5% of the total bacterial community (Tiedje, 1988). More than 60 genera of bacteria and archaea, as well as many saprobic fungi, and protozoans, are capable of denitrifying N oxides. Most prokaryotic, and all eukaryotic denitrifiers are heterotrophs, and many can grow by fermenting organic compounds. Chemolithotrophic denitrifying bacteria and archaea, which couple the reduction of N-oxides to the oxidation of inorganic compounds, are also known (Van Cleemput and Samater, 1996; Zumft, 1997; Fernández et al., 2008). All these microorganisms can use NO_2^- instead of NO_3^- for denitrification.

Many denitrifiers lack one or more enzymes to catalyse reduction steps 2–5, and are thus often said to be 'incomplete'. In soil, complete denitrification of NO_3^- to N_2 predominantly occurs through the synergistic activities of microbial consortia (Zumft, 1997; Wallenstein et al., 2006). The denitrification systems of most fungi (Kobayashi et al., 1996, and references therein) and approximately one third of sequenced bacterial denitrifiers (Philippot et al., 2011) lack N_2O reductase, and consequently evolve N_2O as the final denitrification product. In fact the composition of microbial communities is an important factor in determining the N_2O:N_2 product ratio emitted from temperate pasture soils. Indeed, increased N_2O emissions that were reported resulted from the addition of bacteria lacking N_2O reductase to soils (Philippot et al., 2011) and also from fungi dominated temperate grassland soil (Laughlin and Stevens, 2002) where the mole fraction of N_2O was 0.7 of total $N_2O + N_2$-N.

2.3. Denitrification enzymes

Denitrification is catalysed by the action of four, independent intracellular enzymes: Nitrate reductase (Nar), Nitrite reductase (Nir), Nitric-oxide reductase (Nor) and Nitrous oxide reductase (Nos) (Eq. (1)). Denitrification genes are named for their enzyme products. To distinguish the enzyme from the gene, it is conventional to use an italic font so that, for example, the respiratory Nar is encoded by the *Nar* genes (Fig. 2). The denitrification proteome (Nar, Nir, Nor and Nos plus several other proteins) is synthesised in response to oxygen depletion, and is blocked by high oxygen concentrations (both transcriptional and post-transcriptional control) (Van Spanning et al., 2007). With the onset of anaerobic conditions and addition of C substrate, these enzymes are usually sequentially induced (Holtan-Hartwig et al., 2000; Tavares et al., 2006). Nar is activated within 2–3 h, Nir between 2 and 12 h, and Nor between 24 and 48 h, which explains the sequence of production of these enzymes with developing anaerobic conditions in soil (Holtan-Hartwig et al., 2000).

Under anaerobic growth conditions, a NO_3^- sensor protein controls the expression of the membrane-bound respiratory Nar. The Nar enzymes are typically composed of three subunits. The large alpha subunit, which varies in mass from 112 to 145 kDa (kilodalton), carries the molybdenum cofactor, the active site of the reductase (Hille, 1996). The smaller, beta subunit of the enzyme binds four Fe–S clusters, which are important for maintaining enzyme structure and the retention of the cofactor. The catalytic site of Nar is oriented inwards, and its action releases NO_2^- into the cell cytoplasm (Fig. 2). During denitrification, NO_2^- must cross the cell membrane before reduction by Nir, which is localized in the periplasmic space (Zumft, 1997). Where Nar and Nap co-occur within an organism, it is likely that Nar catalyses the first step of anaerobic denitrification since this process is energetically more favourable (Richardson et al., 2001).

Denitrifying bacteria contain one of two forms of Nir: a cytochrome cd_1 form or a Cu-containing form. The cytochrome cd_1 form is a homodimer composed of two 60 kDa subunits (Nurizzo et al., 1998). Each of the subunits contains a heme C and a heme D_1 prosthetic group. Heme D_1 is the catalytic site of the enzyme, and both the substrate NO_2^- and the product NO, bind via their N-atoms to the Fe^{2+} in heme D_1 (Nurizzo et al., 1998). At the catalytic site, NO_2^- is protonated before water is cleaved to yield NO. The Cu-containing form of Nar in bacteria is similar to the fungal Nar. The Cu-Nir in bacteria and fungi are trimeric enzymes. The three 40 kDa subunits are tightly associated around a three-fold axis with a central channel. At the intersection of the subunits are six copper atoms, two at each junction. Copper is believed to bind the oxygen atom in NO_2^-, and subsequent hydrolysis of the Cu-ONO intermediate, yields NO. Both forms of bacterial Nir are periplasmic and, analogous with fungi, Cu-Nir is located in the intermembrane space of the mitochondria (Kobayashi et al., 1996; Zumft, 1997).

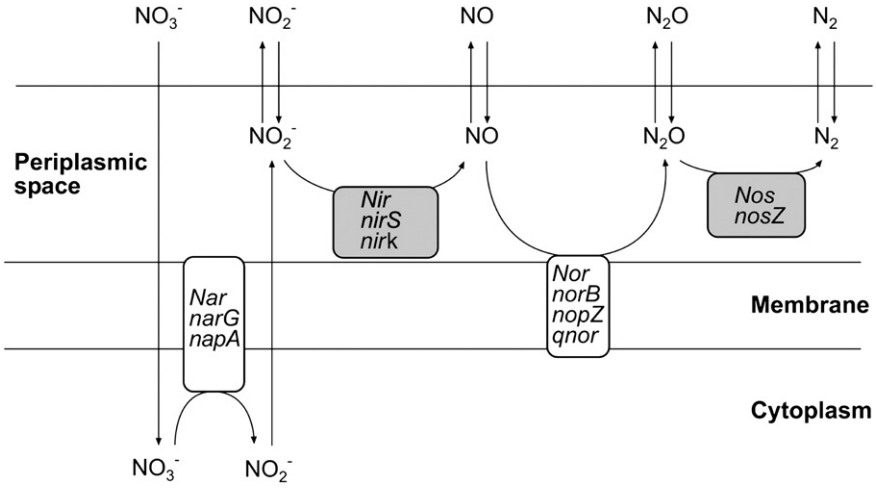

Fig. 2. Sequential reductive pathway of denitrification showing the location of enzymes relative to the cytoplasmic membrane. The enzymes are nitrate reductase Nar (encoding genes narG, napA), nitrite reductase Nir (encoding genes *nir*K, *nir*S), nitric oxide reductase Nor (encoding genes *nor*B, *nor*Z, *q*Nor), and nitrous oxide reductase Nos (encoding gene *nosZ*). Only one gene is required to carry out each step. The genes frequently targeted by molecular approaches are shaded in grey.
Adapted from Ye et al. (1994) and Wallenstein et al. (2006).

Nitric oxide is membrane-labile and highly toxic to most bacteria and fungi including those that denitrify. As a result, all denitrifiers synthesize Nor to rid themselves of the potentially lethal product of Nir (de Boer et al., 1996; Butland et al., 2001; Richardson et al., 2009). During steady-state denitrification, extracellular NO is in the low (1–30) nanomolar range, and NO toxicity occurs at concentrations exceeding 1 mM. The Nor enzyme is virtually identical among bacterial denitrifiers that use either the Cu-Nir or the cytochrome cd_1 form of Nir. Bacterial Nor are complexes of at least two subunits of 17 and 53 kDa. The large subunit NorB is a hydrophobic cytochrome b, located within the cell membrane. The small subunit, NorC, is a c-type cytochrome containing a large heme-binding domain that protrudes into the periplasm. Nor reduces NO to N_2O and requires two electrons to catalyse the formation of the N–N bond. These electrons are transferred from a periplasmic electron donor to the heme group of NorC, and subsequently to the catalytic site of NorB, thought to be a binuclear heme B- and non-heme Fe^{2+} site, which would allow for the close proximity of two NO molecules (Zumft, 1997). The reaction mechanism of $P450_{nor}$, the fungal N_2O reductase, differs from that in bacteria. In fungi, a 44 kDa monoprotoheme cytochrome exists in the ferric-state. NO-binding induces a conformational change in the enzyme, allowing it to accept electrons directly from NADH, to fuel the production of N_2O by reaction with a second NO molecule (Zumft, 1997). The activity of isolated $P450_{nor}$, is more than five times higher than the activity of bacterial NO reductases (reviewed in Morozkina and Kurakov, 2007).

The reduction of N_2O to N_2 is a respiratory process in its own right, and several bacteria have been identified that can use N_2O as a sole electron acceptor to fuel heterotrophic growth. Conversely, not all complete denitrifiers will reduce exogenous N_2O. Nos is similar in all the heterotrophic, phototrophic and chemolithotrophic bacteria where it has been studied, but is often absent in fungi. Located in the periplasm, it is a dimer of identical 66 kDa subunits. Each subunit contains up to four Cu atoms, which have been proposed as the catalytic sites of the enzyme (Zumft, 1997). Persistence of Nos was reported to be lower under aerated conditions in pastoral soil microcosms, and once anaerobic conditions were induced, N_2O reduction occurred only after 16 h (Dendooven and Anderson, 1994), suggesting that the activity of this enzyme may be a strong short-term control on the N_2O:N_2 ratio.

2.4. Denitrification genes

Due to the phylogenetic diversity of denitrifiers, studies that seek to identify these organisms in natural habitats often target genes that encode denitrification enzymes. The target gene usually codes for a key structural component of the enzyme of interest. For example, the *narG* or *napA* genes, which encode the large, catalytic subunit of Nar and periplasmic Nar, respectively, have been targeted for quantification in soils (Bru et al., 2007). However, because these genes can also be present in dissimilatory reducers of NO_3^- to NH_3, *narG* and *napA* have not been widely used to characterise denitrifying bacterial communities (Philippot et al., 2002; Chèneby et al., 2003, 2004).

Molecular characterization of the organisms that drive the denitrification process often begins at the second step of denitrification, with the genes that encode Nir. *Nir*K encodes the Cu-containing form of Nir, while *nir*S encodes the cytochrome cd_1 form, and both genes have been widely used to characterise bacterial denitrifiers in soil (Sharma et al., 2005; Kandeler et al., 2006; Wertz et al., 2006, 2007; Bremer et al., 2007; Yoshida et al., 2009; Liu et al., 2010; Smith, 2010).

While polymerase chain reaction (PCR) primers to target two types of *nor*B genes, which code for the large subunit of nitric oxide reductase, are available (Braker and Tiedje, 2003), relatively few studies have examined the presence of these genes as a marker for denitrifying bacteria in soils. Like the *narG* and *napA* genes, the *qnorB*-type genes have been discovered in a variety of non-denitrifying organisms and may be involved in the detoxification of exogenous NO (Richardson, 2000). Consistent with its radically different product, the fungal $P450_{nor}$ gene shows marked contrast to the bacterial *nor* genes (Kobayashi et al., 1996; Zhang et al., 2001). As yet, studies that target fungal $P450_{nor}$ genes have been limited to cultured organisms (Zhang et al., 2001, 2002; Kaya et al., 2004), and their role in denitrification in pasture soils requires further study.

NosZ encodes N_2O reductase, and is unique in being the only known gene whose product catalyses the final step of denitrification (Burger and Matiasek, 2009). Like the *nir* genes, it is widely used to describe denitrifier communities (Chèneby et al., 1998; Rich and Myrold, 2004; Henry et al., 2006; Philippot et al., 2009, 2011; Čuhel et al., 2010). Primers for the amplification of *nosZ* from soils have improved over time. In an early study Chèneby et al. (1998) found that *nosZ* was unamplified from 35% of the N_2O-reducing bacteria isolated from an agricultural soil. Later, updated primers allowed for the detection of *nosZ* in all but one N_2O-reducing organism in a riparian soil (Rich and Myrold, 2004). The addition of serial dilutions of a bacterium lacking *nosZ* to experimental microcosms recently proved the principle that the genetic composition of the denitrifying community can influence the nature of the denitrification end products (Philippot et al., 2011), a finding that is likely to stimulate research in this area.

Denitrification genes are typically held in clusters within genomic DNA. Adjacent to genes that encode the structural gene products are genes that encode associated proteins such as those involved in co-factor synthesis, electron donation and protein assembly processes (Zumft, 1997; Demanèche et al., 2009). An interesting feature of *Nir* and *Nos* gene clusters is the occurrence of individual putative binding sites for cyclic AMP receptor proteins (Crp) and fumarate/nitrate reductase (Fnr) regulatory proteins (Demanèche et al., 2009). These proteins are thought to suppress the transcription of *Nir* and *Nos* in the presence of oxygen and NO (Vollack et al., 1999) and suggest that the production of enzymes to catalyse each step in the denitrification process may be under independent environmental control in some organisms. Conversely, in other organisms, gene clusters coding for denitrification enzymes may be linked to one another, forming denitrification "gene islands". For example, *Nir*, *Nor* and *Nos* genes are closely associated in *Brucella melitensis* and share a single *Nnr*A binding site for Crp/Fnr regulators (Haine et al., 2006), suggesting that the production of these three enzymes is under a single regulatory control.

In bacteria, the organization of denitrification gene clusters is often variable (Demanèche et al., 2009), and denitrification genes may be held in multiple copies within a single organism (Cramm et al., 1997; Etchebehere and Tiedje, 2005; Jones et al., 2008; Demanèche et al., 2009). Where denitrification genes occur in multiple copies, one or more copies are often plasmid borne (Cramm et al., 1997; Schwintner et al., 1998). These observations, together with the wide distribution of denitrification genes among phylogenetically unrelated bacteria, suggest that horizontal gene transfer is an important mechanism for the acquisition of the ability to denitrify (Philippot, 2002; Braker and Tiedje, 2003; Demanèche et al., 2009). Accordingly, affiliating denitrification gene sequences to bacterial taxa is difficult because of poor congruence between denitrification gene trees and the 16S rRNA gene phylogeny (Jones et al., 2008; Demanèche et al., 2009). In a recent review, Bakken et al. (2012) established that denitrification phenotypes differ strongly in their tendency to emit N_2O and suggested that characterisation of community structure and diversity of denitrifier genes have a limited value in revealing the potential N_2O production. These authors proposed to identify regulatory gene sequences that characterise various denitrification regulatory phenotype (DRP) across phyla.

3. Factors affecting denitrification

Environmental factors that can affect denitrification have been categorised as proximal and distal regulators (Tiedje, 1988; Groffman and Tiedje, 1989; Groffman, 1991; de Klein et al., 2001; Wallenstein et al., 2006) (Fig. 3). Proximal regulators of denitrification are those that immediately affect denitrifying communities leading to instantaneous changes in denitrification rates. These include factors such as NO_3^- concentrations, C availability, O_2 concentration, and temperature (Beauchamp et al., 1980; Knowles, 1982; Weier et al., 1993; Thomas et al., 1994; de Klein et al., 2001, 2010; Mosier et al., 2002; Dalal et al., 2003; Bolan et al., 2004; Wallenstein et al., 2006; Drury et al., 2008; Saggar et al., 2009). Distal regulators control the composition and diversity of denitrifying communities over larger spatial and temporal scales for a longer term and on a larger scale than proximal regulators (Tiedje, 1988; Groffman and Tiedje, 1989; Groffman, 1991; de Klein et al., 2001; Wallenstein et al., 2006). These regulators include factors such as plant growth, management practices (i.e. cultivation and animal grazing), soil

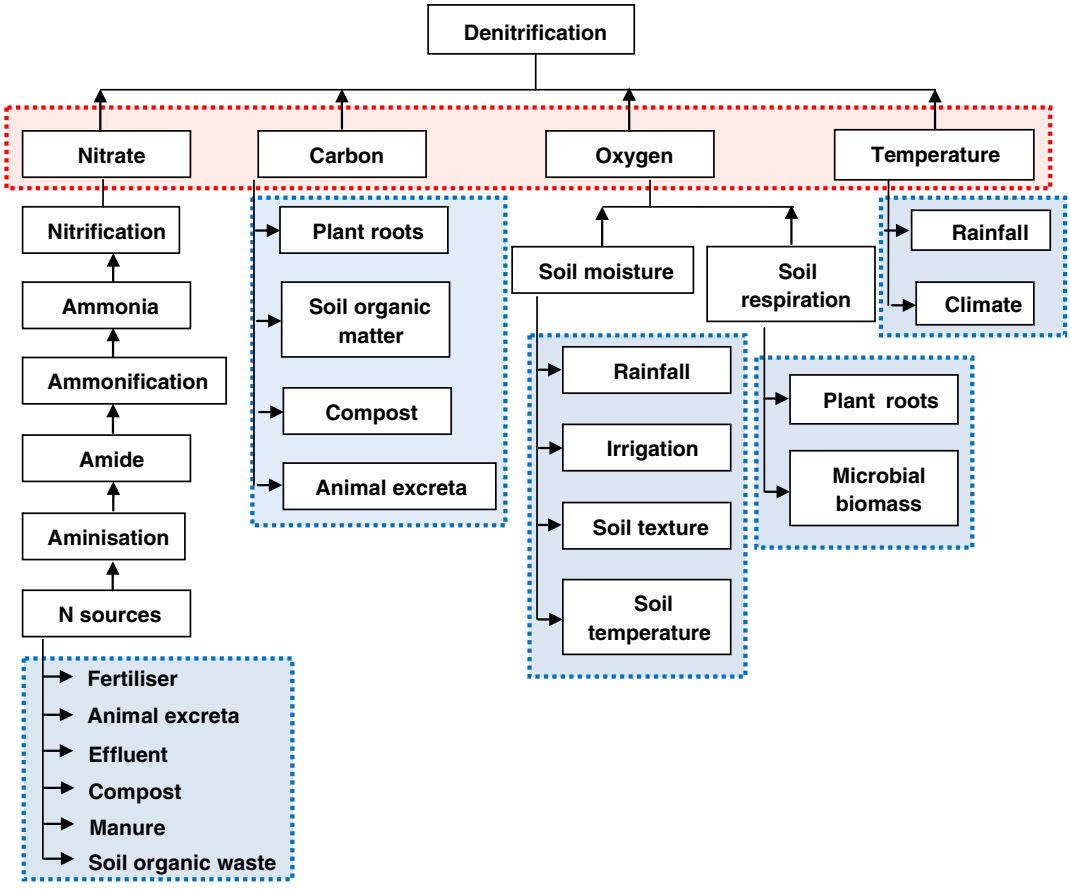

Fig. 3. Factors affecting denitrification in soils (red shaded: Proximal factors; blue shaded: Distal factors). Adapted from Tiedje (1988); Groffman (1991); and de Klein et al. (2001).

texture, soil pH, and water availability. In general, complete denitrification is promoted by high soil water content, neutral to slightly basic soil pH, high soil temperature, low rates of O_2 diffusion and the presence of labile C.

While considerable experimental work has led to the generalizations about the effects of distal and proximal factors on denitrification and on the N_2O:N_2 ratio (Knowles, 1982; Weier et al., 1993; de Klein and Van Logtestijn, 1994; Thomas et al., 1994; Mosier et al., 2002; Dalal et al., 2003; Bolan et al., 2004; Wallenstein et al., 2006; Zaman et al., 2007; 2008a, 2008b; Saggar et al., 2009; Zaman and Nguyen, 2010), the interactions among these factors are likely to control denitrification and the N_2O:N_2 ratio under field conditions, and these interactions remain poorly understood. We understand the general trends of change in the N_2O:N_2 ratio with changes in some of the individual factors. But how these factors interact to affect denitrification and N_2O:N_2 ratios under various edaphic and environmental conditions remains poorly understood.

3.1. Soil and plant factors

3.1.1. Soil mineral nitrogen

The availability of N, especially in the form of NO_3^-, for denitrifying bacteria is the primary requirement for denitrification and therefore the NO_3^- concentration in the soil solution can be one of the principal factors limiting denitrification. Soil NO_3^- concentrations depend on mineralisation and nitrification rates, plant N uptake, microbial immobilisation and NO_3^- movement by leaching and diffusion (Tiedje, 1988; Zaman et al., 2007). Diffusion of NO_3^--N to active microsites is the primary limiting factor for denitrification in grazed pasture, especially in dry soils (Luo et al., 1999b). This positive relationship between denitrification rate and NO_3^- concentration has been reported when the NO_3^--$^-$N concentration is <1 mmol (Ogilvie et al., 1997; Zhong et al., 2010). Luo et al. (1996) found that the increase in NO_3^--N concentration beyond 50 μg g^{-1}soil is detrimental for denitrification activity. This negative or 'toxic' effect of high NO_3^- concentration on denitrification has also been identified in a recent study by Senbayram et al. (2012) who found a significant decline in denitrification rate with increasing soil NO_3^- concentration from 2 to 20 mM.

The NO_3^- concentration is one of the key factors that influence the N_2O:N_2 ratio of denitrification, with higher NO_3^- concentrations usually resulting in a higher N_2O:N_2 ratio (Bremner and Blackmer, 1978; Mosier et al., 1983; Arah and Smith, 1990; Weier et al., 1993; Zaman et al., 2007). In diary grazed pastures, NO_3^- concentrations under urine patches are usually high (up to 418 mg N kg^{-1} soil) compared with non-urine areas (1 to 11 mg N kg^{-1} soil) (Saggar et al., 2004a). A higher level of NO_3^- in soil is also known to result in incomplete denitrification and thus higher N_2O:N_2 due to suppression of Nos activity, the enzyme responsible for the conversion of N_2O to N_2 (Blackmer and Bremner, 1978; Sahrawat and Keeney, 1986; Cho et al., 1997; Scholefield et al., 1997; Stevens and Laughlin, 1998). Because denitrifiers obtain more energy by reducing NO_3^- than by reducing N_2O, high NO_3^- concentration under the urine patches may favour N_2O production. This is supported by the results of Weier et al. (1993), who measured both N_2O and N_2 emissions from four soils treated with three different rates of applied NO_3^- and found that high NO_3^- concentrations inhibited the conversion of N_2O to N_2 and resulted in a higher ratio of N_2O:N_2.

The relatively few simultaneous measurements of N_2O and N_2 emissions currently limit our ability to quantify changes in the N_2O:N_2 product ratio in response to changes in NO_3^- concentration. Cai et al. (2002) measured both N_2O and N_2 emissions and found an increase in N_2O:N_2 ratio with increasing NO_3^- concentration, but the actual emission of N_2 did not change. As NO_3^- concentration decreases, a greater proportion of the N is emitted as N_2 due to the stimulation of Nos, where N_2O acts as a major electron acceptor (Swerts et al., 1996; Dendooven et al., 1997). Senbayram et al. (2009) observed low N_2O emissions following a large input of labile C, without significant NO_3^- input in soils amended with organic matter and suggested that most of the N_2O could have been converted to N_2 — although they did not measure N_2 emissions. Difficulties in accurately measuring N_2 production under an N_2-rich atmosphere is the bottle-neck in further understanding the influence of NO_3^- on N_2O:N_2 ratio during denitrification research. Recent developments (Molstad et al., 2007) in simultaneous quantification procedure for N_2O and N_2 measurements are likely to improve understanding and estimation of gaseous losses of N during denitrification.

3.1.2. Carbon availability

Denitrifiers require a readily available C before reduction of added NO_3^- can occur (Burford and Bremner, 1975; Delwiche, 1981).

$$5C_6H_{12}O_6 + 24NO_3^- = 12N_2 + 30CO_2 + 18H_2O + 24OH^- \quad (6)$$

The heterotrophic denitrification as described in Eq. (6) shows that 5 mol of glucose (30 mol of C) is required for the denitrification of 24 mol of NO_3^-. The theoretical ratios of COD (chemical oxygen demand) during denitrification are 2.86 for NO_3^- and 1.71 for NO_2^- and Akunna et al. (1993) found higher experimental ratios 3.7–4.1 for NO_3^- and 2.0–2.8 for NO_2^- in acetic and lactic acid media. The difference between theoretical and experimental ratios is due to the assimilation of C for cell synthesis. Heterotrophic denitrification is, therefore, often limited by labile C in agricultural soils, and any process that influences the rate of C mineralisation in soils (e.g., temperature, drying–wetting, tillage, liming, incorporation of crop residues, organic or inorganic fertiliser application, root exudates) can have a major impact on the denitrification rate.

Permanent pasture soils develop surface layers rich in organic material with high potential for denitrification when fertilised or when urine and dung are deposited during grazing (Ryden, 1986). The amendment of soils with farm effluents, slurries and organic manures containing readily available C as an energy source for denitrifiers creates anaerobic conditions through enhanced respiration and thereby trigger denitrification under anoxic conditions (Burford and Bremner, 1975; Beauchamp et al., 1989; Bhandral et al., 2007a; Nishina et al., 2009; Cayuela et al., 2010). Application of farm effluents such as dairy and piggery effluents has been shown to enhance N_2O emissions by increasing soil C availability (Bhandral et al., 2007a), and increased N_2O emissions following the application of animal waste slurries are often attributed to the increased labile C availability (Barton et al., 1999; Chadwick et al., 2000). Likewise, increased rates of denitrification following application of urea fertilisers compared with ammonium-based fertilisers (e.g., DAP) have often been attributed to the direct supply of dissolved organic C (DOC) from the urea and the solubilisation of soil C resulting from an increase in soil pH caused by urea hydrolysis (Barton et al., 1999). Interestingly, applying urea fertiliser with a C source (wheat straw and green manure) reduced N_2O emissions substantially compared with applying urea fertiliser alone (Aulakh et al., 2001). This is possibly due to microbial immobilisation of some of the added N (Tiedje, 1988) or dissimilatory nitrate reduction to ammonium (DNRA) (Matheson et al., 2002).

High denitrification rates are found in soils where the plants have been cut or damaged, and this has been attributed to the leaking of available organic C into the soil from the roots remaining in the soil (Beck and Christensen, 1987; Robertson et al., 1987). Thus the increases in rhizosphere available C following a winter grazing event may enhance denitrification when the soil moisture levels are high.

The accessibility of available C to microorganisms is, therefore, an important factor in controlling denitrification, especially under field conditions. The rate of C supply is likely to be faster in the case of DOC when compared with other more stable C sources such as lignin and cellulose in soils (Myrold and Tiedje, 1985). However, the presence of low molecular weight DOC fractions rather than total DOC

concentrations is an important factor controlling N_2O release in upland and peat soils after disturbance (Beauchamp et al., 1980; Drury et al., 2008; Saari et al., 2009). Indeed, Vallejo et al. (2006) noticed a decrease in N_2O:N_2 with increasing concentration of DOC in various sources of effluents. Organic C and DOC concentrations decrease with depth in most mineral soils. Thus, although leaching of NO_3^--N into lower horizons is a common phenomenon, the availability of C is usually limiting denitrification activity in subsoils (Parkin and Meisinger, 1989; Luo et al., 1996; Brye et al., 2001; Barton and Schipper, 2001). However, the drainage conditions that favour DOC losses may enhance denitrification in subsoil.

It is generally considered that increasing C availability decreases the ratio of N_2O:N_2 (Smith and Tiedje, 1979; Arah and Smith, 1990; Dendooven et al., 1998). However, the effect of available C on the absolute and relative amounts of N_2O and N_2 produced and emitted from soils also varies with NO_3^- concentration and aeration status (Water-filled pore-space; WFPS). It has been suggested that in anaerobic zones of fertilised soils, NO_3^- concentration may control the denitrification product ratio (N_2O:N_2), while labile C concentration controls the denitrification rate (Tiedje, 1988; Weier et al., 1993). The change in the N_2O:N_2 product ratio from denitrification due to the changing labile C:NO_3^- ratio could be explained by changes in enzyme status, and/or the diffusion rate of NO_3^- into denitrifying microsites (Swerts et al., 1996; Weymann et al., 2010). The N_2O:N_2 ratio is generally lower in the rhizosphere, which provides more available C in the form of root exudates and root debris, has generally low NO_3^- concentrations due to plant N uptake, and is characterised by low partial pressures of O_2 (due to O_2 consumption by plant roots) (Casella et al., 1984; Henry et al., 2008). On the other hand, a high NO_3^- concentration immediately after fertiliser application or animal urine deposition may increase the N_2O:N_2 ratio in the rhizosphere (Gift et al., 2010).

In addition to its effect on denitrification and the N_2O:N_2 ratio, the nature and availability of soil C affect the denitrification enzyme activity (DEA) and microbial community composition. In comparison with glucose, addition of liquid manure to soil increases the abundance of *Nos*Z. This in turn enhances denitrification (Paul and Beauchamp, 1989; Van Kessel and Reeves, 2000) due to the ability of the manure to provide both labile and complex forms of C for the soil organisms.

3.1.3. Oxygen supply and water content

As denitrification is an anaerobic reaction, O_2 availability is one of the most important factors inhibiting this process in soil (Knowles, 1982; Lloyd, 1993). The soil water content, together with the rate of O_2 consumption by plant roots and microorganisms (respiration), determines the O_2 availability in the root zone (Tiedje, 1988). Field studies have shown an increase in denitrification rate under restricted soil aeration associated with high soil water content (e.g., de Klein and van Logtestijn, 1994; Ledgard et al., 1999; Luo et al., 2000). Studies (e.g., de Klein and Van Logtestijn, 1994; Hefting et al., 2003) have shown the existence of a soil water threshold above which denitrification rates increased sharply with increasing soil water content. Below this critical soil water threshold value, denitrification rates appeared to be unrelated to soil water content. The soil water threshold value differs according to soil type and is expressed as water-filler pore space (WFPS). de Klein and van Logtestijn (1996) suggested that the critical WFPS for denitrification in many soils is equivalent to field capacity or above. Although the presence of small quantities of O_2 (0.02 atm) can cause large decreases in DEA (Firestone et al., 1979), denitrification and DEA have also been reported during an oxic phase following an anoxic phase (Robertson et al., 1989; Baumann et al., 1996; Morley et al., 2008). Among N-reductase enzymes, Nos was more severely inhibited by oxygen than Nar, Nir and Nor indicating denitrification may continue for some time during drainage following flooding even if the O_2 supply is partly or fully restored (Morley et al., 2008). However, because of higher inhibition of Nos these anoxic/oxic phases may result in higher production of N_2O than N_2.

Oxygen supply is also affected by soil compaction. In grazed pastures compaction can be caused by animal treading especially with high stocking rates (Bilotta et al., 2007; Drewry et al., 2008). Soil compaction restricts O_2 diffusion within the soil, thereby increasing the rate of denitrification (Rosswall et al., 1989; Luo et al., 1999a). McTaggart et al. (1997) and Hansen et al. (1993) observed that soil compaction increased N_2O emission by 100% from an imperfectly drained clay loam and by 36% from a sandy loam soil. Soil compaction also affects the nitrification process and thereby influences the supply of NO_3^- for denitrification. For example, Bhandral et al. (2007b) monitored N_2O emissions from various N sources in a pasture soil affected by compaction. While N_2O emissions from a NO_3^- source (KNO_3) were 14 times more in the compacted than the uncompacted soils, N_2O emission from ammonium and urea sources was only 3–4 times higher in compacted than uncompacted soil. The different N_2O emission rates were attributed to different transformations of the added N substrates and to the consequently different soil NO_3^- supplies. These findings highlight an important interaction among N-source and compaction that acts to control in situ N_2O emission rates.

The N_2O:N_2 ratio has often been found to decrease with increasing soil water content (Colbourn and Dowdell, 1984; Davidson, 1992; Rudaz et al., 1999), particularly when the soil water content exceeds 75% WFPS (Davidson, 1992; Weier et al., 1993). Likewise, the measured N_2O:N_2 ratio was highest (≥ 1) under dry conditions during summer and early autumn when denitrification was relatively inactive (Ruz-Jerez et al., 1994). Changes in N_2O:N_2 ratio with increased WFPS will also depend on the ability of denitrifiers to produce N_2 or N_2O as an end product (Sigman et al., 2001; Wood et al., 2001; Casciotti et al., 2002; Morley et al., 2008).

3.1.4. Soil pH

Soil pH is another key regulator of the microbiological processes that affect N_2O and N_2 production. It is generally agreed that denitrification is slower in acid conditions (Bremner and Shaw, 1958; Bryan, 1981; Nägele and Conrad, 1990; Fageria and Baligar, 2008), but denitrification can still occur at pH values as low as 3.5 and can account for significant N losses in naturally acid soils (Parkin et al., 1985; Weier and Gilliam, 1986). Šimek et al. (2000) found no significant relationship between soil pH and denitrifying enzyme activity, although denitrification potential was significantly correlated with soil pH. This and other work led Šimek and Cooper (2002) to conclude that the expression "optimum pH for denitrification has little or no meaning" unless it is qualified by specifying the particular aspect of denitrification that is being considered.

It is also generally accepted that the level of soil acidity influences the N_2O:N_2 product ratio of denitrification. At lower soil pH the N_2O:N_2 ratio is increased (Koskinen and Keeney, 1982; Nägele and Conrad, 1990; Struwe and Kjøller, 1994; Liu et al., 2010), with N_2O frequently appearing as the dominant product in acid soil (Christensen, 1985; Parkin et al., 1985). Several laboratory experiments using pH modifying treatments, and field experiments where acidifying fertilisers were applied, have shown that at lower soil pH, denitrification produces more N_2O and the ratio N_2O to N_2 is increased (Koskinen and Keeney, 1982; Struwe and Kjøller, 1994; Šimek and Cooper, 2002; Wolf and Brumme, 2003; Venterea, 2007; Zaman et al., 2007, 2008a; Zaman and Nguyen, 2010; Čuhel and Šimek, 2011). Rochester (2003) derived a negative exponential function between N_2O:N_2 ratio and soil pH from a number of laboratory and field studies across a range of soil types. Approximately equivalent amounts of each gas were emitted from the soil at pH 6.0 but for the alkaline grey clays (pH 8.3–8.5) the N_2O:N_2 ratio was 0.02.

The mechanism of pH control of the N_2O:N_2 ratio of denitrification is not fully understood. The increased N_2O:N_2 product ratio in acidic soils may reflect an enhanced post-transcriptional sensitivity of bacterial Nos to low pH, relative to the other denitrification enzymes (Liu et al., 2010). The lower activity of Nos in acidic soils (Knowles,

1982; Thomsen et al., 1994) may also reflect an increased dominance of fungi relative to bacteria in these systems, as fungi generally lack Nos. Acidic soils may also favour N_2O production by autotrophic and heterotrophic nitrifiers (Martikainen and de Boer, 1993). It has been observed that in *Pseudomonas mandelii* expression of *nirS* and *cnorB* was unchanged over the pH range of 6–8; however, it reduced substantially with a decrease in pH to 5 (Saleh-Lakha et al., 2009a). Furthermore, the presence of increasing amounts of NO_2^- at lower pH levels due to enhancement of abiotic transformations of NO_3^- may contribute to the increased proportion of N_2O produced (Koskinen and Keeney, 1982).

Increasing soil pH above 6.0 may offer a mechanism to mitigate N_2O emissions by shifting the balance between N_2O and N_2 (Stevens and Laughlin, 1998; Šimek and Cooper, 2002; Zaman et al., 2007, 2008a; Liu et al., 2010; Zaman and Nguyen, 2010). While there is a little doubt that the N_2O:N_2 product ratio decreases with increasing pH, reduction may be partially offset by the tendency toward increased total denitrification rates with increasing pH.

3.2. Environmental factors

3.2.1. Soil temperature

Denitrification can occur over a wide range of soil temperatures ranging from sub-zero to 75 °C (Knowles, 1982). The minimum temperature for N_2O emissions during denitrification is generally associated with the occurrence of free water, while the maximum temperature is approximately that value limiting biological activity and NO_3^- availability in the soil. Within normal physiological range for the cell, DEA follows Michaelis–Menten kinetics with a typical Q_{10} value of approximately 2 in soils (Stanford et al., 1975) and with optimal measured denitrification rates converging at 30 °C. Dobbie and Smith (2001) observed that the temperature response of N_2O emissions was greater over a lower range of temperature than over a higher range, with Q_{10} values of 3.7 and 2.3 for 5–12 °C and 12–18 °C, respectively. Temperature is one of the main factors causing temporal fluctuations in denitrification (Ryden, 1983).

In addition to the direct effects of temperature on denitrifier activity, indirect effects include changes in gas solubility, and gas diffusion (Craswell, 1978), as well as temperature limitation of substrate supply through direct effects on N-mineralisation, C, NO_3^- availability and nitrification. Several researchers have found that N-losses through denitrification during the winter period were limited by soil temperature (Ryden, 1986; Jarvis et al., 1991; de Klein and Van Logtestijn, 1996), although rapid losses of N through denitrification were also reported after the addition of mineral fertilisers and organic manure at relatively low soil temperatures (Egginton and Smith, 1986; Thompson et al., 1987; Schwarz et al., 1994).

In temperate countries, generally winter application of fertilisers to grasslands is limited/prohibited. However, denitrification loss could be higher in winter (soil temperature below 10 °C) (Ruz-Jerez et al., 1994; Luo et al., 2000; Saggar et al., 2004a). The high winter N_2O fluxes and denitrification loss have been attributed to a combination of WFPS exceeding 0.60, a high soil NO_3^-N content and low plant uptake of N.

Denitrifying bacteria acclimate and denitrifying communities adapt to the historic range of soil temperatures (Schimel and Gulledge, 1998), resulting in different temperature optimums for denitrification in different regions (Powlson et al., 1988; Malhi et al., 1990). Dorland and Beauchamp (1991) reported that C amendment lowered the temperature threshold at which denitrification can occur in soil. Temperature changes affect the substrate availability and thus control the expression of specific enzymes involved in transformation, however, abundances and community structure of denitrifiers remain unaffected (Stres et al., 2008). However, Saleh-Lakha et al. (2009a) observed a negative effect of low temperature (below 10 °C) on denitrification activity and the sensitivity of *nirS* and *cnorB* expression which increased positively with increasing the temperature from 10 to 30 °C.

Because the activation energy of N_2O reduction is higher than the activation energy of N_2O production (Holtan-Hartwig et al., 2002), it has been suggested that more N_2O is produced at low temperatures and the N_2O:N_2 product ratio is increased (Keeney et al., 1979; Avalakki et al., 1995). While some studies have reported a decrease in the N_2O:N_2 ratio with increasing soil temperature (Bailey, 1976; McKenney et al., 1984; Maag and Vinther, 1996), no relationship between denitrification rate and soil temperature could be detected in others (Focht, 1974; Rudaz et al., 1999). The variable effects of temperature observed in these studies likely reflect the many interacting ways that temperature may affect denitrification rates; many of these have not been fully elucidated.

3.2.2. Rainfall

Enhanced denitrification rates, denitrification activity and N_2O emissions following rainfall commonly reported for agro-ecosystems have been generally attributed to the dual effects of bringing applied substrates into contact with soil micro-organisms and a reduction in soil O_2 levels (Ellis et al., 1998; Van Kessel et al., 1993; Luo et al., 2000). Peak N_2O emissions or denitrification rates in temperate grassland soils generally occur following rainfall or irrigation events (Velthof et al., 1996; Luo et al., 2000; Saggar et al., 2004a). The emissions are much shorter lived in coarse-textured soils than in fine-textured soils (e.g., 12 h in a sandy loam soil and 48 h in a clay loam soil) (Sexston et al., 1985).

Local topography also plays an important role in influencing the effect of rainfall on denitrification and N_2O emission rates. Corré et al. (1990) observed a better correlation between rainfall levels and emission rates at the foot of a slope ($R^2 = 0.73$) than from a flat area ($R^2 = 0.57$). The time taken for emissions to return to background levels was also longer (48 h) from the foot of the slope than from the flat area (24 h). This likely resulted from differences in the rate of soil drying between these two areas. Luo et al. (2000) also found differing denitrification rates on various topographic areas in a dairy grazed pasture, possibly due to differences in soil moisture contents.

3.2.3. Soil drying–rewetting and freezing–thawing

Plant water uptake followed by rapid rewetting during precipitation generally creates drying–wetting cycles in the rooting zone. For up to several days following the wetting of dry soil, organic matter mineralisation is enhanced by i) growth and turnover of microorganisms, ii) rhizosphere release of osmotically active substances such as sugar, and iii) the disruption of bonds that physically protect soil organic matter (Adu and Oades, 1978; Lundquist et al., 1999; Fierer and Schimel, 2002; Muhr et al., 2008). For example, increased soil denitrification rates and N_2O emissions following wetting of dry soil by rainfall or irrigation have been reported for various agricultural systems (Kessavalou et al., 1998; Kim et al., 2009; Nobre et al., 2001) including grazed pastures (Luo et al., 1998; Garcia-Montiel et al., 2003; Saggar et al., 2004a; Kim et al., 2010). Increased soil N_2O emissions resulting from denitrification following thawing of frozen soils have also been observed in various agricultural systems including grasslands (Flessa et al., 1995; Kammann et al., 1998; van Bochove et al., 2000; Priemé and Christensen, 2001; Ludwig et al., 2004; Öquist et al., 2004; Mørkved et al., 2006; Holst et al., 2008; Wagner-Riddle et al., 2008; Wolf et al., 2010; Virkajärvi et al., 2010).

The mechanisms considered responsible for increased denitrification and N_2O emission following rewetting and thawing are:

- increased availability and accessibility of the substrate (Kieft et al., 1987; Edwards and Cresser, 1992; Davidson et al., 1993; Schimel and Clein, 1996; van Bochove et al., 2000; Priemé and Christensen, 2001; Pesaro et al., 2003; Grogan et al., 2004; Saetre and Stark, 2005; Yergeau and Kowalchuk, 2008)

- creation of anaerobic conditions (Nyborg et al., 1997; Li et al., 2000; de Bruijn et al., 2009).
- release of trapped N_2O and N_2 (Burton and Beauchamp, 1994; Koponen et al., 2006; de Bruijn et al., 2009; Goldberg et al., 2010; Virkajärvi et al., 2010).

3.2.4. Availability of trace metals

Denitrification enzymes require several metal ions as cofactors, including iron, copper and molybdenum (Ferguson, 1998). Thus soils that for reasons of parent material composition, or biological, or chemical immobilisation, lack these metals may have reduced rates of denitrification. For example, molybdate ion (Mo^{6+}) acts as a component of Mo-cofactor for Nar and ferric ion (Fe^{3+}) is essential for the cytochrome subunits of both Nar and Nir. The addition of Mo^{6+} and Fe^{3+} usually affects the growth and denitrification rates of microbial communities by accelerated NO_3^- utilisation and NO_2^- accumulation (Labbe et al., 2003; Zhou et al., 2007; Pintathong et al., 2009). For other copper-dependent enzymes like Nar or Nir, there is one more non-copper dependent variant available, but Nos is the only enzyme with no alternative non-copper type available to carry out reduction of N_2O in the absence of Cu. Richardson et al. (2009) consider that the failure of the reduction of N_2O to N_2 in bacterial denitrification process could be attributed to incomplete assembly of *NosZ* due to lack of sufficient Cu to provide full complement of Cu co-factors. However, the Cu limitation will directly impact only on release of *NosZ*. The limitation of other trace metals such as Fe or Mo that form co-factors in Nar and Nir would lead to inhibition of NO_2^- and NO fluxes so that N_2O may not be formed (Richardson et al., 2009).

4. Approaches to identify and quantify denitrification and N_2o and N_2 emissions

Accurately measuring N_2 and N_2O emissions, identifying denitrifiers, estimating the N_2O:N_2 ratio, and predicting denitrification rates from grazed pastures are always problematic. This is because of:

i) natural, and livestock induced spatial and temporal variability (uneven excretal-N deposition and animal treading) where denitrification occurs and also in the factors driving the process, and the complex way in which they interact (Dandie et al., 2008)
ii) methodological limitations such as associated change in substrate concentration, disturbances to the physical environment, lack of sensitivity, and high costs
iii) difficultly in accurately measuring N_2, the final product of denitrification due to the high background concentration in the atmosphere.

The two most commonly used approaches for determining denitrification rate from measurements of N_2 and N_2O production include a technique based on the acetylene (C_2H_2) inhibition (AI) of N_2O reduction (Balderston et al., 1976; Yoshinari and Knowles, 1976; Tiedje, 1988) and an isotopic method using substrates enriched in ^{15}N that allows subsequent ^{15}N gases to be determined by isotope-ratio mass spectrometry (Mosier and Klemedtsson, 1994). More recently, direct quantification of N_2 has been attempted using airtight systems containing He or Ar either with continuous flow of $He+O_2$ (Butterbach-Bahl et al., 2002) or a closed system with periodic headspace sampling and replacing the sampled volume with He. Only a brief description of these three methods is given here, and the reader is referred to the recent review of Groffman et al. (2006) on the methods available to measure and calculate denitrification in terrestrial systems. Here we also discuss the potential of using molecular techniques to improve our understanding of the denitrification processes. All the techniques, together with their advantages and disadvantages, have been summarised in Table 3.

4.1. Acetylene inhibition (AI) technique

The AI method has been widely used for more than 40 years and is still the most commonly used method to study denitrification rates and denitrification enzyme activities in both aquatic and terrestrial systems. The limitations include the use of C_2H_2 as a microbial substrate (Yeomans and Beauchamp, 1982; Terry and Duxbury, 1995), insufficient blockage of the Nos and incomplete inhibition of reduction of N_2O to N_2 (Yeomans and Beauchamp, 1978; Oremland et al., 1984; Simarmata et al., 1993; Yu et al., 2010; Qin et al., 2011), inhibition of NO_3^- production through nitrification (Seitzinger et al., 1993), interactions in the presence of sulphide (Soerensen et al., 1978; Dalsgaard and Bak, 1992; Jones and Knowles, 1992), or inhibition of denitrification that is tightly coupled to nitrification (Rysgaard et al., 1993; Nielsen and Revsbech, 1994) and

Table 3
Summary of denitrification measurement techniques, their advantages and disadvantages.

Denitrification measurement method	Advantage	Disadvantage
Acetylene inhibition (AI) technique Approaches: ▪ Calcium carbide (CaC_2) granules. ▪ In situ chambers in field. ▪ Static cores	• Simple to conduct • Can run large number of samples at a time • Removes the spatial and temporal variability of denitrification rate • Useful in studying the effect of soil and environmental factors on denitrification and denitrification enzyme activity assay	• Can only be used in NO_3-dominated systems • Inhibits nitrification; can underestimate denitrification • Slow diffusion of C_2H_2 into soil or sediments limits blockage of Nor • Rapid decomposition of C_2H_2 by microbes • Contamination of C_2H_2 with other gases can affect denitrification • Scavenging of NO leading to underestimation of denitrification
^{15}N tracer technique	• Considered better than AR technique • Gives reliable estimates of denitrification	• Laborious process • Requires costly instruments • Addition of ^{15}N to the N limiting condition results in overestimation of denitrification
Direct N_2 quantification	• No labelled N or inhibitor is added • Highly sensitive method can even detect low denitrification changes • Can be used to study temperature and moisture effect	• Can only be used in enclosed incubation experiments • Complex and difficult system • Not suitable for longer period. • Challenge to measure N_2 against high atmospheric N_2 concentration
Molecular approaches ▪ Polymerase chain reaction ▪ DNA microarray technique ▪ Immunological techniques	• Give reliable and realistic qualitative indication of enzymes and encoding genes involved in denitrification • Can be used both in aquatic and terrestrial	• Require very expensive equipment • Require technical expertise to extract, analyse and interpret enzymes and encoding genes • Useful to study microbial diversity

the C_2H_2-catalysed decomposition of NO (Bollmann and Conrad, 1997) that may result in under- or over-estimation of the denitrification rate under some conditions (Table 3). Murray and Knowles (2003) reported that C_2H_2 does not scavenge for NO during denitrification and hence does not lead to underestimation of soil denitrification potential. In a recent study, Qin et al. (2011) observed incomplete inhibition of the reduction of N_2O to N_2 by C_2H_2 but little changes in soil denitrification potential, and proposed the use of a correction factor to account for the incomplete inhibition.

The technique has also been applied in studies that have led to better understanding of the spatial and temporal variability of denitrification (Groffman et al., 1999). Recently, Seo and DeLaune (2010) used AI in combination with selective antibiotics to quantify the contributions from denitrifying bacteria and fungi in a swamp forest. They reported that fungi were the main contributor to total denitrification (34% compared with 1% for denitrifying bacteria) under aerobic to weakly reducing conditions (Eh +250 and Eh +400 mV), whereas bacteria were the main contributor (65% compared with 17%) under strongly reducing conditions (Eh<−100 mV).

4.2. ^{15}N tracer technique

The ^{15}N tracer technique is used for direct measurements of the ^{15}N labelled gases ($^{15}N_2O$ and $^{15}N_2$) produced following the addition of either $^{15}NO_3^-$ alone or with $^{15}NH_4^+$ to the soils, sediments, ground, or surface water (Hauck and Melsted, 1956; Hauck et al., 1958; Nishio et al., 1983; Aulakh et al., 1991; Stevens and Laughlin, 2001). Although small additions of labelled NH_4^+ and NO_3^- do not alter the coupled nitrification/denitrification processes in fertilised agricultural soils, the ^{15}N method may cause over estimation of denitrification in N-limited agricultural systems. The analysis of ^{15}N in soils and $^{15}N_2O$ and $^{15}N_2$ gases produced during denitrification by isotope-ratio mass spectrometry (IRMS) can be fully automated (Stevens et al., 1993). Thus the fluxes of N_2 and N_2O can be measured simultaneously in laboratory and field experiments by using the ^{15}N stable isotope labelling method. When compared with the AI method, the ^{15}N labelling method is considered to be better for direct quantification of denitrification. However, its application is often limited because of the high cost of ^{15}N-substrates, ^{15}N analyses, the expensive IRMS required, and the laborious procedures involved in sample preparation. The limitations associated with the use of the ^{15}N technique are listed in Table 3. These include the difficulties in homogenous mixing and effects of denitrification, nitrification and plant N uptake on the uniform distribution of the labelled N in denitrifying zone during the experiment. However, despite reports of significant under- (Seitzinger et al., 1993) or over-estimation (Groffman et al., 2006) of denitrification by the ^{15}N technique, it is still favoured for generating reliable estimates of denitrification rates in a range of environments, compared with the AI method. Use of this technique in conjunction with the AI method can provide improved estimates of denitrification rates in soils.

4.3. Direct N_2 quantification

In this method intact soil cores or sediments are kept in air-tight systems with the air inside replaced by an inert gas like He or Ar, and the background N_2 concentration is decreased from 79% N_2 to approximately 1% N_2 (Stefanson and Greenland, 1970; Wickramasinghe et al., 1978; Seitzinger et al., 1980). The N_2 produced during denitrification within the air-tight container is then analysed by gas chromatography. In general, two types of incubation systems are used for direct N_2 measurements: i) continuous air flow with $He + O_2$ but no N_2, with changes in N_2 gas concentration at the outlet used for calculating N_2 emission or N_2/Ar ratio (Butterbach-Bahl et al., 2002; Cardenas et al., 2003) and ii) a closed system with the changes in the composition of gases in the headspace measured by periodically sampling small amounts of gases and replacing the sampled volume with He (Liu et al., 2010). In both systems it is necessary to remove all N_2 present in the test soil aggregates or cores before the measurements commence. The continuous flow incubation system has been improved by the development of a technique to measure N_2 and N_2O emissions simultaneously (Scholefield et al., 1997; Butterbach-Bahl et al., 2002; Cardenas et al., 2003; Groffman et al., 2006; Khalil and Richards, 2011). An improvement in this technique is the use of Membrane Inlet Mass Spectrometer (MIMS) capable of measuring a large number of dissolved trace gases without any interference of high atmospheric background of N_2. This method can measure N_2 at a precision of <0.05% for the N_2/Ar where Ar is considered as a tracer to minimise variations in N_2 production. This technique is only suitable for short-term in-situ qualitative and quantitative denitrification measurements. For determining N_2O:N_2 ratios N_2O measurements have to be conducted separately. Thus further studies are needed to test the suitability of MIMS technique.

A major advantage of this method is that no addition of substrate (labelled or otherwise) or inhibitor is required. Thus, there is no interference with soil microbial processes, including those involved in denitrification, during the measurements. However, the incubation system used is complex. As it is not possible to measure very small quantities of N_2 gas, this method is not able to detect small changes in denitrification products, and the method appears to be only useful for measuring denitrification in highly fertilised agricultural and grassland soils (Scholefield et al., 1997). However, in spite of its limitations this method is increasingly being used for quantification of N_2 emissions in both aquatic and terrestrial ecosystems (Butterbach-Bahl et al., 2002) and further improvements and automations are made using robotized sampling and analysing system to overcome some of the limitations.

4.4. Molecular techniques

Recently, molecular techniques based on the identification and quantifications of the genes encoding enzymes which catalyse key denitrification processes (*NirS*, *Nirk*, *Nosz*) have been used (Braker et al., 2000; Philippot, 2002; Henry et al., 2004). Many commercial kits are available for nucleic acid (DNA or RNA) extraction from soils. These and published protocols (e.g. Griffiths et al., 2000) typically involve physical cell lysis and the inactivation of nucleases, nucleic acid extraction from the soil matrix and purification followed either by targeted polymerase chain reaction (PCR)-based analyses or direct characterisation.

Molecular characterization of the micro-organisms that drive the denitrification process often begins at the second step of denitrification, with the genes that encode NIR. *Nir*K encodes the copper-containing form of NIR while *nir*S encodes the cytochrome cd_1 form, and both genes have been widely used to characterise bacterial denitrifiers in soil (Sharma et al., 2005; Kandeler et al., 2006; Wertz et al., 2006, 2007; Bremer et al., 2007; Yoshida et al., 2009; Liu et al., 2010; Smith et al., 2010). The desire to characterise only those organisms that are actively denitrifying, and thus potentially contributing to N_2O emissions or consumption, has motivated the use of reverse transcription polymerase chain reaction (RT-PCR) in studies of denitrifiers. In addition to studies of pure cultures of denitrifiers (Saleh-lakha et al., 2009a, 2009b) RT-PCR has now been successfully applied to the study of denitrifier activity in sediments (Nogales et al., 2002; Smith et al., 2007) and more recently also in soils (Dandie et al., 2011; Shannon et al., 2011). Once the gene of interest is amplified the 'community profile' or 'fingerprint' of denitrifying organisms is obtained using terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) or its variant temperature gradient gel electrophoresis (TGGE), and single stranded conformation polymorphism (SSCP) (e.g. Braker et al., 2001; Rich and Myrold, 2004; Rösch and Bothe, 2005; Bremer et al., 2007). Quantitative real-time PCR (qRT-PCR) is an evolution of PCR that allows for the detection and quantification of DNA template copy number in "real-time" as the PCR proceeds. It has been used to quantify several denitrification genes in soils including *nirK*, *nirS*, *narG* and *nosZ* (Grüntzig et al., 2001; López-Gutiérrez et al.,

2004; Henry et al., 2004; Kandeler et al., 2006; Siciliano et al., 2009; Levy-Booth and Winder, 2010; Chen et al., 2011). Gene copy number is calculated based on the rate of product accumulation in the exponential phase of the PCR. A critical issue in qRT-PCR is the construction of adequate controls so that samples and standards are carefully matched.

All of the molecular approaches for the characterization of denitrifiers in soils described above share a common dependence on PCR, which has several inherent biases, such as:

- PCR primer design, based on the sequences of known denitrifiers, represents only a small portion of all potential denitrifiers in soils and may not represent the gene's abundance (Acinas et al., 2005; Cardon and Gage, 2006)
- amplification errors may result in the production of sequence artefacts
- many community profiling methods saturate very quickly with regard to the complexity of the community that can be resolved and underestimate the diversity of the community
- it is not possible to link the characterization of a single phylotype of a functional gene to a species.

For direct characterisation of denitrifiers in soils immunological techniques, based on the identification of specific translated proteins, are used (Metz et al., 2003). This is accomplished by direct hybridization of a labelled antibody that can be detected fluorometrically, chemically or radioactively. While the efficacy of immunological techniques is well established in complex environments like soil (Metz et al., 2003; Maron et al., 2004) concerns exist about the sensitivity of the methods to very low concentrations of the target protein (Philippot and Hallin, 2005). Similarly, functional gene arrays, which consist of probes for a variety of functional genes from a range of species have been applied to quantify denitrification gene expression in soils with a varying degree of success (Chèneby et al., 1998; Henry et al., 2006; Babic et al., 2008; Hallin et al., 2009). Currently, while metagenomics, the culture-independent analysis of the metagenome, and the collective genomes of all organisms present in an environmental sample (Handelsman et al., 1998; Rondon et al., 2000; Gillespie et al., 2005), allow for the very rapid accumulation of microbial gene and genome data, an understanding of the functional and ecological relevance of these is evolving more slowly (Suenaga, 2012).

The above review suggests the application of a common methodology and molecular techniques for the identification and quantification of genes and characterisation of denitrifiers is a pre-requisite for comparisons across land use and land management systems under variable soil conditions (mineralogy, SOC levels, pH).

5. Modelling the denitrification process and emissions of N_2O and N_2

As a major source of N_2O production from agricultural soils, the denitrification process must be an important part of any process-based soil N_2O model. As discussed in the previous section, separate measurements of N_2O and N_2 emissions from denitrification can be carried out at the field scale. However, emissions of both N_2O and N_2 during denitrification are too variable, both spatially and temporarily, to be practically measured simultaneously at the field scale. Moreover, denitrification does not occur in isolation, and the success of a process-based denitrification model also depends upon how well the model simulates the other processes affecting the soil environment.

5.1. Modelling approaches

Simple empirical models can be used to estimate emissions, but these are limited to the types of soils and management practices used in the model parameterisation. More detailed, process-based models can be used to create emission scenarios and to examine the potential impacts of novel mitigation strategies. A recent review by Heinen (2006) identified three types of denitrification models: i) microbial growth models; ii) soil structural models; and iii) simplified process models that represent the denitrification rate in terms of easily measurable parameters such as soil moisture, temperature and NO_3^- concentration.

The microbial growth models consider the dynamics of microorganisms involved in N cycling processes. Grant (1991) used the maximum O_2 requirements for the growth and maintenance of microorganisms to calculate the maximum potential respiration and denitrification rates. But his model did not consider the relative production of N_2O and N_2. In an earlier attempt, Leffelaar and Wessel (1988) developed a denitrification and respiration model considering three pools of denitrifiers that respectively used NO_3^-, NO_2^-, and N_2O as electron acceptors. This scheme has been used by more detailed process-based models such as DNDC (Denitrification–Decomposition) (Li et al., 1992) and NLOSS (N Loss) (Riley and Matson, 2000). DNDC also includes temperature and pH rate modifying factors in the denitrifier growth and electron acceptor consumption equations.

Soil structural models consider gaseous diffusion into and out of soil aggregates with denitrification only occurring in anoxic parts of soil aggregates. Arah and Smith (1989) considered steady-state denitrification in a spherical soil aggregate only occurring within the anaerobic part of the aggregate. In these models denitrification is calculated from the estimates of NO_3^- diffusion into the aggregate using Michaelis–Menten kinetics with respect to the local NO_3^- concentration at radius r within the pore. The model was then scaled up to an assembly of aggregates by assuming log-normal distribution of aggregate radii and oxygen reduction potentials. Vinten et al. (1996) subsequently used an approximation of the anaerobic volume fraction (Φ) based on the mean aggregate radius, the mean oxygen reduction potential, the O_2 concentration in the inter-aggregate air-filled pore space, the water content, the water content of saturated aggregates, the diffusion coefficients of O_2 in air and water, and the volume fraction of soil occupied by aggregates.

A simpler "anaerobic balloon" (i.e. volumetric fraction of anaerobic microsites) concept is included in the DNDC (denitrification–decomposition) model to account for the fact that both nitrification and denitrification could occur simultaneously within the soil (Li et al., 2000), and is based on the relative partial pressures of O_2 in the liquid and the air. Substrates such as soil N and DOC are partitioned between the aerobic and anaerobic soil fractions, with nitrification occurring in the aerobic fraction and denitrification occurring within the anaerobic fraction.

The simplified process-based models represent denitrification as a base rate multiplied by factors accounting for the effect of soil nitrate (f_N) water content (f_S),temperature (f_T) and pH (f_{pH}) (Heinen, 2006). This type of equation assumes that the effects of all the rate modifying influences are independent of each other. If the denitrification rate is independent of the reactant concentration then the relationship is called a zero-order process, while a first-order process is one where the denitrification rate is directly proportional to the reactant concentration. A Michaelis–Menten or Monod type relationship (Eq. (6)) can also be used to simulate both reactant-limited and non-reactant limited cases:

$$f_N \propto \frac{N}{N+K} \tag{6}$$

where N is the concentration of NO_3^- and K is a constant. Eq. (6) becomes approximately constant (zero order) for high values of N and approximately linear (first order) for low values of N. Table 4 lists the form of f_N used in a selection of denitrification models.

Denitrification processes are highly sensitive to the O_2 status of the soil, as the denitrifying bacteria are anaerobic. Many models use some form of soil moisture measurement (such as WFPS) as a proxy for O_2 availability. As described earlier, there is a threshold WFPS

Table 4
Form of f_N used in some denitrification models.

Model Name	Form of f_N	Reference
ANIMO	Zero-order	Rijtema and Kroes (1991)
FASSET	Michaelis–Menten	Chatskikh et al. (2005)
InfoCrop	First-order	Aggarwal et al. (2006)
NEMIS	Michealis–Menten	Hénault and Germon (2000)
NOE	Michealis–Menten	Hénault et al. (2005)
WNMM	First-order	Li et al. (2007)

level beneath which denitrification does not occur, which varies depending on soil type (de Klein and van Logtestijn, 1996). Most denitrification models therefore use $f_S = 0$ below a threshold WFPS, although the level of this threshold varies between models. For WFPS above the threshold, f_S is commonly (Heinen, 2006) expressed as:

$$fs = \begin{cases} \left(\frac{WFPS - WFPS_{th}}{WFPS_{max} - WFPS_{th}}\right)^{w} & , WFPS_{th} \le WFPS_{max} \\ 1 & , WFPS > WFPS_{max} \end{cases} \quad (7)$$

where $WFPS_{th}$ is the denitrification threshold WFPS, $WFPS_{max}$ is the WFPS above which $f_S = 1$, and w is an empirical parameter.

At higher WFPS values a greater proportion of the denitrified N appears as N_2 rather than as N_2O. In most models D_a represents the total $N_2O + N_2$ produced by denitrification but some models, such as InfoCrop (Aggarwal et al., 2006), calculate only N_2O. Models primarily aimed at N-availability for plant growth or water contamination risk usually do not disaggregate the separate N_2O and N_2 emission rates. However, for greenhouse gas calculations the proportion of denitrified N released as N_2O is critical. The ratio of N_2O:N_2 can be calculated in numerous ways; some models assume a constant N_2O:N_2 ratio, while others use functions based on various soil properties. Table 5 shows the factors considered in calculating the N_2O:N_2 ratio in a number of models.

In general, microbial activity and denitrification will increase with increasing temperature, although at very high temperatures microbes will begin to die off, reducing the denitrification rate. Most models assume this will not occur at temperatures usually found in field conditions. Some models, such as NOE (Hénault et al., 2005) and NEMIS (Hénault and Germon, 2000), use stepwise functions for f_T, representing different microbial populations that dominate in different temperature regimes.

There are many different relationships used to account for the temperature effect, but one commonly used is a Q_{10} response of the form:

$$f_T = Q_{10}^{(T - T_b)/10} \quad (8)$$

where Q_{10} represents the factor by which the reaction is increased given an increase of 10 °C from some base temperature T_b.

As discussed earlier, denitrification rates are lower in acidic soils than neutral or slightly alkaline soils, and low pH also influences the proportions of N_2O and N_2 produced. However, many models neglect the pH factor as soil pH does not change rapidly, except around events such as liming, and urine or urea application and deposition. The DNDC model (Li et al., 1992) uses three different pH reduction factors for the different stages of denitrification ($NO_3^- \rightarrow NO_2^-$; $NO_2^- \rightarrow N_2O$; $N_2O \rightarrow N_2$).

In simplified process models, the microbial activity or population status of the soil in the denitrification rate calculation is usually incorporated into the base denitrification rate. Some models use soil organic C as an indicator of the relative size of the microbial pool or explicitly track the size of microbial pools, and use soil respiration as an indicator of microbial activity. Other models use site-specific empirical parameters that implicitly incorporate microbial activity. The approaches used to account for microbial activity in different models are summarised in Table 6.

Table 5
Calculation of N_2O:N_2 ratio in some denitrification models.

Model name	Variables used in N_2O:N_2 ratio function	Reference
DayCent	NO_3^-/CO_2 ratio, WFPS	Stehfest and Müller (2004)
FASSET	Temperature, WFPS, clay, depth	Chatskikh et al. (2005)
NGAS	Soil NO_3^-, respiration and WFPS	Parton et al. (1996)
NOE	Empirical site-based parameter	Hénault et al. (2005)
WNMM	WFPS	Li et al. (2007)

5.2. Model validation

Testing denitrification models is complicated by the fact that under field conditions there are many interacting processes that will affect denitrification rates. e.g., heat and water transport through soil and other N transformation processes. Therefore, the success of a process-based denitrification model depends not only on the quality of the denitrification component but also on its ability to simulate soil conditions accurately. For example, denitrification rates are highly dependent on soil moisture and improvements to the soil water-modelling component have been found to improve predictions of N_2O emission (Saggar et al., 2007a; Norman et al., 2008). Therefore, there would be little point in improving the denitrification process in a model if the poor performance of some other component was the limiting factor. In grazed grasslands the spatial variability of N_2O emissions (due to the non-uniform nature of excretal N inputs) is an additional challenge to model validation (Giltrap et al., 2011).

The denitrification model of Leffelaar and Wessel (1988) (based on the growth, maintenance and death rates of denitrifier populations) was tested against laboratory incubation experiments where the model produced reasonable simulations of the progression $NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$. The availability of appropriate datasets to fully parameterise the model was noted as a key limitation. However, most models are tested under field conditions. This means that the denitrification component itself is not explicitly tested, but rather the combined effect of all the model processes is tested. Models are frequently validated against N_2O measurements. This is not necessarily a good test of the denitrification model as N_2O is a product of both nitrification and denitrification, while denitrification also produces N_2. So it is possible for a model to predict the N_2O emissions correctly while incorrectly simulating the overall denitrification rate. Frolking et al. (1998) found in a comparison of four different process-based models (DNDC, DayCent, CASA, and Expert-N) that even when the models produced similar N_2O fluxes they often produced very different estimates of gaseous losses as NO, N_2 and NH_3 indicating differences in the simulated N-transformation processes. Therefore, in order to have confidence that a model is simulating denitrification well it is necessary to ensure that other processes such as NO_3^--leaching, NH_3 volatilisation, and plant uptake are also well simulated. Even though it is not usually feasible to measure all the possible forms of N (e.g. N_2O, N_2, NH_3, soil NO_3^- and NH_4^+)

Table 6
Approach used to account for microbial activity for a number of denitrification models.

Model name	Indicator of microbial activity	Reference
DayCent	CO_2 concentration	Stehfest and Müller (2004)
DNDC	Microbial biomass	Li et al. (1992)
FASSET	Mineralisation rate	Chatskikh et al. (2005)
InfoCrop	Microbial biomass	Aggarwal et al. (2006)
NEMIS/NOE	Site-specific constant	Hénault and Germon (2000), Hénault et al. (2005)
NGAS	Respiration rate	Parton et al. (1996)
PaSim	Decomposition rate	Schmid et al. (2001)
WNMM	Soil organic carbon	Li et al. (2007)

in a given experiment, the more that are measured the more valuable the data set will be for model validation.

5.3. Future requirements

All process-based models contain empirical approximations at some level, either from limitations of understanding the underlying processes or from practical difficulties in measuring all the required parameters. The different approaches to denitrification modelling discussed above differ in the point at which empirical approximations begin. Simplified process models can often give good results for situations for which they have been well-parameterised (e.g., Hénault et al., 2005) and may be more suitable for some applications. However, these simplifications limit the model's ability to only providing insight into the simplified processes. For example, a model that uses a fixed $N_2O:N_2$ ratio for denitrification products is not capable of determining situations where the $N_2O:N_2$ ratio changes.

Improved understanding of the underlying processes can lead to improvements in the models. However, as N_2O production is the result of many interacting processes, improving the simulated denitrification process alone will not necessarily improve the model's performance if other processes are poorly simulated. Another limitation to improving models is the availability of appropriate parameter values.

Many models are validated at field scale simply by comparing measured N_2O emissions with the modelled results. However, this does not indicate which processes are well modelled and where the model is performing poorly. Further modelling work that examines more closely the performance of the different component processes leading to changes in $N_2O:N_2$ ratio is needed to improve model predictability.

6. Management practices to control denitrification and N_2O and N_2 emissions

From an agricultural perspective denitrification results in the loss of a valuable plant nutrient and should therefore be minimised. From an environmental perspective denitrification presents both a threat and an opportunity. Where denitrification uses NO_3^- that would otherwise be leached into rivers or lakes this may be an environmental benefit. If, however, denitrification results in emission of N_2O, this poses an environmental threat. Management of agricultural systems should therefore minimise the accumulation of NO_3^- in soil and thereby restrict both leaching and denitrification. But where denitrification is inevitable, or even desirable to protect water quality, management should ensure that as much of the denitrified NO_3^- as possible is emitted as N_2 rather than N_2O.

A range of on-farm management options to control denitrification by either emitting less N_2O or by shifting the balance between harmful N_2O and the non-greenhouse gas N_2 in grazed pastures are summarised in Table 7. These management options include increasing C inputs, minimising N inputs from animal excreta and chemical fertilisers, reducing animal feed crude protein, decreasing soil NO_3^- concentrations, and improving soil aeration and soil pH.

6.1. Soil C availability

Soil C availability has been shown to regulate denitrification rate in grassland soils (Section 3). Shepherd et al. (2010) found that sucrose addition immobilised urine N and decreased N leaching by up to 66%. This mitigation option could also be used to potentially reduce denitrification.

When NO_3^- N has leached below the root zone of pastures, there may still be opportunities to remove it by denitrification. Two options are wetlands and denitrification walls where C, such as in sawdust, is added in a trench (e.g., Schipper and Vojvodic-Vukovic, 1998), both having the effect of intercepting the hydrological flow of drainage water to surface waterways. Both options have shown potential to remove NO_3^- by denitrification. However, they are highly dependent on the fit to the landscape and the ability to intercept a significant component of the drainage water flows. Increasing soil C through effluent irrigation and (in future) biochar application may also be a management option to lower the $N_2O:N_2$ ratio. However, not all the biochar created is equal and contradictory findings on the effect of biochar in reducing N_2O emissions or affecting $N_2O:N_2$ ratios from denitrification are limiting its use (Clough et al., 2010; Saggar et al, 2011b). Aspects of biochar on soil denitrification community, enzyme activity and $N_2O:N_2$ ratios need to be investigated.

6.2. N availability

The simplest approach for controlling denitrification in managed pasture systems is to reduce the NO_3^- concentration in the soil. This both reduces N_2O emissions and shifts the balance between N_2O and N_2. As discussed in Section 2.1, the major sources of NO_3^- in grazed pasture system are animal excreta, effluent, and chemical fertilisers. Soil generally remains wet during late-autumn/winter and early spring in the temperate region due to excessive precipitation, and during this time soil compaction by animal treading, low pasture N uptake, and low N immobilisation as a result of low soil temperature are likely to accelerate denitrification (Saggar et al., 2004b; de Klein et al., 2006; Hyde et al., 2006; Bhandral et al., 2007b;). The cut and carry systems that avoid direct excretal N input of grazing animals tend to be more N efficient than the grazed pasture systems (Ball and Ryden, 1984; Grignani and Laidlaw, 2002) during this high risk period of N losses. Minimising N inputs to the soil during these critical months can therefore potentially reduce N losses by denitrification. This can be achieved by:

- minimising the time animals spend grazing in the field (Ledgard et al., 2006; Luo et al., 2006) during the critical period. This practice enables farmers to have a better control over animal excreta which are collected in feed pads or animal houses and can be later

Table 7
Summary of management practices to control denitrification in temperate grassland.

Control factor	Goal	Impact	Management practices
Carbon availability	Increase	Lower $N_2O:N_2$	Effluent irrigation and biochar application
Nitrogen availability	Decrease	Lower denitrification and $N_2O:N_2$	Reducing excreta deposition during late-autumn/winter and early spring (use of stand-off/feed pads or animal houses)
			Reducing the concentration and amount of N in urine (increase urine volume and low protein feed)
			Increasing hippuric acid concentration by manipulating animals diet
			Nitrification and urease inhibitors
			Avoiding fertilisation of urine or dung patches
Oxygen supply	Increase	Lower denitrification	Preventing soil compaction
			Improving drainage
			Avoiding excessive amount of irrigation
Soil pH	Increase	Lower $N_2O:N_2$	Liming
Microbial enzymes	Increase Nos	Increased N_2O reduction	Supply of Cu as a cofactor for release of *NosZ*

applied to pastures when the risk of N losses is minimal (van der Meer, 2008). In several farmlet studies in New Zealand, N_2O emissions were reported to be reduced by up to 60% when animals were on stand-off/feed pads or in animal houses for 3–4 months during late-autumn/winter compared with year-round grazing (de Klein et al., 2006; Ledgard et al., 2006; Luo et al., 2008).

- adding salts to animal feed (to increase urine volume and spread) and feeding animals with low protein feed such as maize silage when on stand-off/feed pads or in animal houses, can be effective management tools for reducing the concentration and amount of N in urine (Mulligan et al., 2004; Ledgard et al., 2007). However, increasing urine volume through salt addition can only decrease N concentration in urine but may not change the total N in urine. Reduction of pasture N content through the use of controlled N release fertiliser or decreasing fertiliser application rate to match pasture N requirements can also be an effective option to reduce N contrition in urine thereby reducing denitrification rate and potentially N_2O:N_2 product ratios from denitrification (Luo et al., 2010).
- increasing hippuric acid concentration in urine by manipulating animals' diet may also reduce denitrification and N_2O emission (Kool et al., 2006; Bertram et al., 2009). However, a New Zealand field study (Clough et al., 2009) did not observe any effect of the increased hippuric or benzoic acid concentrations in cow urine on N_2O emissions.
- applying nitrification and/or urease inhibitors to retain N in the NH_4^+ form and/or reduce the hydrolysis of urea-N (Di et al., 2007; Saggar et al., 2008; Majumdar, 2009; Zaman et al., 2009; Parkin and Hatfield, 2010; Zaman and Blennerhassett., 2010). The inhibitors, by controlling hydrolysis and nitrification processes, can reduce the amount of NO_3^- available for denitrification and may potentially reduce the N_2O:N_2 product ratio. Sun et al. (2008) found that the use of NI, dicyandiamide (DCD), reduced denitrification N losses by 22% in a grazed pasture soil.
- including deep rooted pasture species may reduce the amount of NO_3^- in the subsurface soil horizons where there is a greater potential for denitrification. Dear et al. (2009) found that incorporating perennial pasture species in swards reduced NO_3^- leaching and prevented a decline in surface soil pH.

6.3. Plant composition

Plants with specific constituents, such as tannins or saponins, can increase N utilisation by animals and increase N excretion in the low-risk dung N forms relative to urine N (e.g., Miller et al., 2001). Feeding animals with grass silage containing elevated concentrations of water-soluble carbohydrates increased the N use efficiency for microbial growth in the rumen from 46 to 68% (Merry et al., 2003). Similarly, Miller et al. (2001) found that dairy cows on a "high sugar" variety of perennial ryegrass excreted 18% less N in total and 29% less urine N. Thus, manipulation of plant composition offers a potential to reduce N excretion in the urine, thereby reducing subsequent denitrification and N_2O losses. Plants also have the potential to alter soil N cycling via altering the quality of their residues or releasing compounds which have nitrification inhibition characteristics. Ledgard et al. (1998) measured a 10-fold difference in gross N immobilisation rate between non-N-fertilised grassland dominated by *Agrostis* and *Holcus* spp. compared with ryegrass with a regular N fertiliser history. Such differences in immobilisation potential may be important controllers of denitrification and N_2O losses from N sources such as animal urine, N fertilisers, and effluent/manure.

6.4. Oxygen supply

As discussed earlier, denitrification becomes a major source of N_2O and N_2 production at low O_2 partial pressure (<0.5 vol.%) and high WFPS (>60%) (Section 3.1.3). Improving soil drainage conditions, avoiding soil compaction and pugging in wet soil conditions, and avoiding over-irrigation can all reduce the amount of denitrification N losses. In addition, biochar application to soils could reduce the amount of denitrification N losses by decreasing bulk density and improving aeration status of the soil (Lehmann and Joseph, 2009; Sohi et al., 2010). Biochar application to soils also increases soil organic matter contents which could subsequently reduce the product ration of N_2O and N_2 through denitrification.

6.5. Soil pH

Liming has also been mooted as a mitigation option for lowering soil N_2O emissions. For example, Clough et al. (2004) showed that while liming is likely to decrease N_2O emission from urine patches where soils are at field capacity, the increased NO_3^- concentration from lime-induced nitrification would be susceptible to denitrification if the soil moisture subsequently exceeds the field capacity. However, Zaman and Nguyen (2010) recently noticed that lime had no effect on N_2O emission in either urine- or KNO_3^--treated soils, but increased N_2 emissions and lowered N_2O:N_2 ratios in a short-term field experiment. In soils without an added C source, transcription of denitrification genes was below detectable levels but the addition of glutamate showed sharp peaks in the transcription of denitrifying genes with the transcription of nosZ exceeding that of nirS at lower pH. This led Liu et al. (2010) to conclude that the increased N_2O:N_2 product ratio at low pH reflects a post transcriptional phenomenon, whereby Nos assembly is more sensitive to low pH relative to other denitrification enzymes. As discussed, adding biochar to soil generally raises the pH of acid soils and may consequently reduce N_2O emissions. Technologies for applying biochar to perennial grasslands need to be developed to ensure that the biochar is fully mixed with the surface soil for maximising effects on N_2O reduction. Application of biochar during the cultivation of perennial grasslands for pasture renewal could be an approach.

6.6. Microbial enzymes

Denitrification engages several proteins that require metal ions as a cofactor (Ferguson, 1998). For example, addition of Mo^{6+} and Fe^{3+} usually affects the growth and denitrification rates of microbial communities by accelerated NO_3^- utilisation and NO_2^- accumulation (Labbe et al., 2003; Zhou et al., 2007; Pintathong et al., 2009). The accumulation of NO_2^- results from the higher increase in the membrane-bound Nar activity and consumption of NO_3^- compared with the smaller increase in periplasmic Nir activity and utilisation of NO_2^-. Nos is the only enzyme with no alternative non-copper type available to carry our reduction of N_2O in the absence of Cu. Application of supplements/fertilisers rich in Cu will be helpful to boost the activity of *NosZ* and thus converting more N_2O to N_2 (Richardson et al., 2009).

7. Conclusions and future research needs

Denitrification is the primary source of N_2O emissions and a key process in temperate pastoral soils that could annually denitrify 5.6 Tg of reactive N. The need both for removal of excess NO_3^- from soil for protection of ground and surface waters, and for reductions in N_2O emissions produced during denitrification to mitigate global warming and ozone depletion, is now urgent. The last three decades have witnessed a resurgence of interest in denitrification in soils. Molecular techniques involving PCR-based amplification of DNA extracted directly from soil have advanced our understanding of the composition and abundance of the microbial community involved in the denitrification process. However, the DNA only indicates the presence of the denitrifier from which it was extracted, and not its quantitative functional contribution towards denitrification. Therefore, the extrapolation of DNA gene copy abundance to nitrification and

denitrification rates must be confirmed by next generation sequencing (NGS) technologies. As understanding of the denitrification process improves, this knowledge can be used to develop effective mitigation technologies.

It is clear from research on ecology of denitrification that while the ability to denitrify is widely distributed among a taxonomic and phylogenetically diverse group of microorganisms, not all can reduce N_2O to N_2. Therefore, the amount of N_2O produced can vary from almost 0 to over 90% of the total N-gases produced by denitrification. The research suggests that a significant number (~1/3) of denitrifying organisms do not have the capacity (*nosZ*) to reduce N_2O. This may provide an opportunity to manipulate soil denitrifying organisms to enhance *nosZ* activity. It could be done through altering the interaction between mineral N (NH_4^+ and NO_3^-) or Cu supply, organic C, soil aeration and pH to stimulate the activity of Nos, which will help result in emissions of more N_2 than N_2O.

Simultaneous information on soil and environmental parameters and bacterial processes is needed to identify and assess the mechanisms regulating the production and reduction of N_2O. The multiplicity of soil and environmental conditions that could influence the ability of denitrifiers to complete denitrification and result in reducing N_2O emissions may explain why to date no clear picture has emerged as to the efficacy of soil management practices in reducing N_2O emissions. Understanding those mechanisms and the denitrifiers that have the potential to reduce the production of N_2O during denitrification is critical to the development of novel and effective N_2O mitigation strategies. Thus the key research questions are:

- What is the relationship between soil, microbial and environmental parameters and processes and N_2O/N_2 ratio of denitrification?
- Is the number of high capacity denitrifiers (*nosZ*) in soils sufficient to reduce the N_2O/N_2 during denitrification?
- How can the denitrifier community be manipulated and the *nosZ* enhanced?
- What is the effectiveness of soil manipulations for reducing N_2O production during denitrification?
- What are the optimum soil and environmental conditions required for maximum N_2O mitigation using the most effective soil amendment?

There are substantial knowledge gaps that require both laboratory and field research to determine the best soil type and optimal environmental conditions to encourage the activity of Nos during denitrification. Future research, therefore, needs to focus on evaluating the N_2O-reducing ability of the denitrifiers to accelerate the conversion of N_2O to N_2 and the reduction of N_2O:N_2 ratio during denitrification. More research is also required to measure N_2O and N_2 emissions at field scale and quantitatively determine the emissions under different land-use and land management systems and to improve model approaches to quantify the N_2O:N_2 ratio and N_2 emissions from denitrification with greater certainty.

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