MEASURING BACTERIAL DENITRIFIER GENES DISTRIBUTION AND ABUNDANCE IN NEW ZEALAND DAIRY-GRAZED PASTURE SOILS

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Abstract

Knowledge of the abundance and distribution of soil denitrifiers as a function of soil physiochemical characteristics is pre-requisite to develop our understanding about the denitrification process and the factors enabling the conversion of N_2O to N_2. The objectives of this study were to determine the distribution and abundance of denitrifier genes (*nir*S, *nir*K and *nos*Z) in New Zealand pasture soils and to correlate gene abundance with measured soil physiochemical characteristics, N_2O emissions and denitrification rates. We collected 10 New Zealand dairy pasture soils with contrasting physiochemical characteristics and denitrification potentials. We determined the distribution and abundance of the total bacterial gene (*rpo*B) and denitrifier genes (*nir*S, *nir*K and *nos*Z) in field moist soils. *Nir*S, *nir*K and *nos*Z gene distributions were estimated using Terminal Restriction Fragment Length Polymorphism (T-RFLP) and their abundances were measured using quantitative Polymerase Chain Reaction (qPCR). Nitrous oxide emissions, denitrification rates (DR) and denitrification enzyme activities (DEA) were also measured.

The distribution and abundance of *nir*S, *nir*K, *nos*Z and *rpo*B genes varied among the soils. The average number of T-RFs for *nir*S, *nir*K and *nos*Z varied from 3-33 per sample. Similarly the gene copy numbers of *nir*S, *nir*K, *nos*Z and *rpo*B varied from 10^5 to 10^9 g^{-1} soil. The distribution and abundance of bacterial genes correlated significantly (P<0.05) with soil Olsen P, microbial biomass carbon (MBC), total C (TC), total N (TN) and mineral N (NO_3^- & NH_4^+). *Nos*Z, *nir*S and *nir*K gene copy numbers correlated positively with N_2O emissions. We found no clear relationship between *nos*Z gene copy numbers and N_2 emissions in our field moist soils. Since these soils had low moisture content, this suggests that the *nos*Z gene copy number does not predict N_2O and N_2 emissions from soils under aerobic conditions.

Introduction

Denitrification is an anaerobic microbial stepwise conversion of nitrate (NO_3^-) to dinitrogen (N_2, a harmless gas). Denitrification involves the sequential reduction of $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. The intermediate product nitrous oxide (N_2O) can have harmful environmental effects if lost to the atmosphere, however the final product, N_2, is benign. This process is mediated by four reductase enzymes; nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR) (Zumft, 1997). *Nir*S, and *nir*K are the bacterial genes encoding the NIR enzyme responsible for conversion of nitrite (NO_2^-) to nitric oxide (NO) and *nos*Z is the gene encoding the N_2OR enzyme for conversion of nitrous oxide (N_2O) to dinitrogen (N_2) during denitrification. The conversion of

NO_2^- to NO (the first intermediate gaseous product of denitrification) is a key step and distinguishes denitrifiers from nitrate respirers (Hallin & Lindgren, 1999). The gases produced in later stages of the denitrification process cannot be assimilated by nitrate respiring organisms (Zumft, 1997). The conversion of NO_2^- to NO is mediated by two distinct types of NIRs: one with heme *c* and d_1 (cd_1-Nir) prosthetic groups and another with a copper (cu-Nir) prosthetic group. These reductase enzymes are coded by *nir*S and *nir*K genes respectively. These two genes are structurally different while functionally and phylogenetically equivalent and generally do not co-occur in the same bacterial strain (Heylen *et al.*, 2006; Zumft, 1997). Since denitrifying bacteria harbour either of the genes, many studies tracing denitrification pathway have targeted the functional genes *nir*S and *nir*K to detect denitrifying bacteria in soil (Goregues *et al.*, 2005; Novinscak *et al.*, 2013).

Another key step in denitrification is conversion of N_2O to N_2 which is mediated by N_2OR. The N_2OR enzyme is encoded by the *nos*Z gene (Kloos *et al.*, 2001). This is the only enzyme to carry out the last step of denitrification (Bergaust *et al.* (2011), which makes the *nos*Z gene an important molecular marker to trace complete denitrification.

Denitrifying bacteria are taxonomically diverse and widely distributed in the environment (Philippot *et al.*, 2007). More than 60 genera of denitrifying bacteria have been identified so far (Chen *et al.*, 2012). The denitrifying bacteria may possess either all four enzymes or only some of them (Dandie *et al.*, 2008; Wallenstein *et al.*, 2006). *Paracoccus denitrificans* possesses genes encoding all four reductase enzymes, and thus is able to transform NO_3^- directly to N_2. However some denitrifying bacteria like *Agrobacterium tumefaciens* lack N_2OR and will emit N_2O as the end product of denitrification (Wood *et al.*, 2001). Dominance of this bacterium will lead to a high $N_2O/(N_2O+N_2)$ ratio (Bakken *et al.*, 2012). Thus, denitrifier community structure is an important factor determining the end product of denitrification in soil, and knowledge of the abundance of bacteria able to reduce N_2O is required to develop understanding of the key drivers of N_2O emissions from soil (Henry *et al.*, 2006).

Terminal restriction fragment length polymorphism (T-RFLP) is a PCR-based community profiling technique that has been used to study the distribution of denitrifying bacteria in environmental samples (Braker *et al.*, 2001; Castro-González *et al.*, 2005; Rich & Myrold, 2004; Zumft, 1992). The quantification of gene copy numbers during PCR provides measurements of gene abundances in soil (López-Gutiérrez *et al.*, 2004). Quantitative PCR (qPCR) allows for measurement of the number of copies of the target genes (Yoshida *et al.*, 2009). In order to assess the abundance of denitrifying bacteria in relation to the total bacterial community in the samples, quantification of a gene that is widespread among bacteria is carried out. Studies have reported use of 16S rRNA gene to quantify the total bacterial community (Case *et al.*, 2007). The occurrence of multiple copies of 16S rRNA genes in some bacteria poses a difficulty in accurately assessing the number of bacteria. The gene coding for the beta subunit of the RNA polymerase *rpo*B has been suggested as an alternative marker for the microbial community studies. This gene is described as possessing the same characteristics as 16S rRNA Dahllöf *et al.* (2000) and more importantly, the *rpo*B gene exists as a single copy in the bacterial genome (Mollet *et al.*, 1997). It therefore allows for accurate estimation of the abundance of bacteria in environmental samples. In order to avoid the bias due to the use of 16S rRNA genes to describe total bacterial abundance we have used *rpo*B genes abundance in this study.

Several biotic and abiotic factors such as competition, predation, O_2 content, pH, and availability of substrates affect the diversity of denitrifying bacteria in soils (Franklin & Mills, 2003; Ladd *et al.*, 1996) and ultimately the rate of denitrification. The abundance of denitrifiers can have a major impact on denitrification rates at a given location (Philippot & Hallin, 2005; Wallenstein *et al.*, 2006). Studies have shown that environmental variables affect microbial communities and their functions directly or indirectly. There are conflicting results in the literature about the effect of denitrifier community structure on denitrification rates. Some studies have found correlations between denitrification activity measured using the DEA assay and denitrifier community diversity (Wertz *et al.*, 2009) or abundance (Enwall *et al.*, 2010; Hallin *et al.*, 2009). However other studies have reported that denitrifier abundance ((Dandie *et al.*, 2008) or diversity (Attard *et al.*, 2011) are not related to variation in DEA.

Many denitrification studies in New Zealand are based on relating N_2O emissions with soil and environmental parameters using the DEA assay (Luo *et al.*, 1994b, 1994a) and DR measurements using an acetylene (C_2H_2) inhibition (AI) technique (Ruz- Jerez *et al.*, 1994; Zaman & Nguyen, 2010; Zaman *et al.*, 2008). However, there is a lack of understanding of the key soil characteristics or environmental variables driving the abundance of denitrifying bacteria capable of reducing N_2O to N_2 and hence reducing N_2O emissions. Knowledge of the structure and abundance of denitrifier communities is needed to understand the dynamics of the denitrification process leading to N_2O or N_2 emissions, in order to develop mitigation tools to reduce N_2O emissions. Therefore, we measured the distributions and abundances of denitrifier genes in New Zealand dairy pasture soils with contrasting soil characteristics and denitrification potentials. In this study, we have used PCR-based molecular techniques to characterize and quantify the universal bacterial gene *rpo*B, and the denitrifier bacterial communities possessing *nirS, nirK* and *nos*Z genes present in New Zealand dairy pasture soils. Our aim was to understand the relationships of the distributions and abundances of denitrifier genes with the measured soil chemical attributes, DEA, DR and the N_2O and N_2 produced during denitrification. We hypothesised that the varying chemical characteristics of the selected soils would lead to diverse denitrifier community structures and varied denitrifier gene abundances.

Materials and Methods

Collection of soil samples

We collected soils from pasture sites on 10 New Zealand dairy farms (Appendix I). The soils had varying physical and chemical characteristics. Twenty five soil cores (25 mm diameter and 100 mm long) were collected using a steel corer from the 0-100 mm and 100-200 mm depths from six random locations (each with areas of 100 m^2) on each farm between August and December 2010 (10 sites × 6 replicates × 2 depths = a total of 120 samples). The 25 cores from each location were mixed together but the 6 replicates from each farm were stored separately. The field fresh soil cores were taken to the laboratory, sieved to 2 mm and stored at 4ºC in plastic bags. A sub-sample of each soil replicate was stored at -20^{o}C for molecular analysis. The following abbreviations are used to refer to soil texture: Fine Sandy Loam= FSL, Silt Loam = ZL and Stony Silt Loam is SZL. The two Manawatu soils were collected three weeks apart from two adjoining paddocks - one with no effluent irrigation and the other that had received dairy shed effluent irrigation at the rate of 10,000 l ha^{-1} every 2 months for the previous 4 years. The most recent effluent application was 2 weeks before the collection of soil samples. These soils are named as Manawatu FSL and Manawatu FSL effluent irrigated (EI). There were also two Paparua ZL soils collected from separate dairy farms in Springston and Lincoln.

Soil Characteristics

Soil samples were analyzed for soil water content, mineral N (NO_3^- and NH_4^+), total nitrogen (TN), total carbon (TC), pH, Olsen P, soluble C (K_2SO_4 extractable C from non-fumigated soils), and microbial biomass carbon (MBC), by following the protocols described in Jha *et al.* (2012). Denitrification enzyme activity (DEA) and denitrification rate (DR) were measured using the acetylene (C_2H_2) inhibition technique described in Jha *et al.* (2012). DEA is the total denitrification potential and the ambient reductase enzyme activity of soil measured as N_2O emitted when it is incubated with excess moisture, C and NO_3^-, together with chloramphenicol, in the presence of C_2H_2 under anaerobic conditions (N_2 atmosphere). The DR is the measure of total N_2O+N_2 emitted from soils unamended with water, C and NO_3^- in the presence of C_2H_2 during a 24 hour anaerobic incubation. The $N_2O/(N_2O+N_2)$ ratio was calculated from the N_2O emitted from soils incubated without and with C_2H_2.

Molecular Analysis

We employed two Polymerase Chain Reaction (PCR) based molecular techniques T-RFLP and Real-Time qPCR to characterize distribution and abundance of microbial populations that share same gene. Preliminary results indicated that DR and DEA were significantly lower in the subsurface samples (100-200 mm depth). We therefore performed molecular analyses only on the surface samples (0-100 mm depth). First, DNA was extracted from the soil samples using a PowerSoil™ DNA Isolation Kit following the manufacturer's instructions (MO Bio Laboratories Inc., Carlsbad, California USA). DNA was extracted from 0.25g soil samples (6 replicates, 10 soils). To reduce cost of analysis, the six replicate DNA extracts were arranged in three pairs and the extracts of each pair were pooled to yield 3 replicate DNA extracts per soil sample. The pooling was based on the DEA of the individual replicates. In each case, one sample with very high DEA and another with very low DEA were pooled together to obtain a combined sample with intermediate DEA. Thus the resultant three replicates all had comparable DEA.

In the current study we amplified *nir*S, *nir*K and *nos*Z for T-RFLP using methods described by Throbäck *et al.* (2004), Dandie *et al.* (2011) and Henry *et al.* (2006), respectively, with slight modifications described in Deslippe *et al.* (2013). We used real-time qPCR to quantify bacterial *nir*S, *nir*K, *nos*Z, and *rpo*B (Deslippe *et al.*, 2013). For *nir*S, *nir*K, *nos*Z we used the same primers as for the T-RFLP described above and for *rpo*B, we used the primers used by (Dahllöf *et al.*, 2000).

Statistical Analysis

The data for soil chemical characteristics, gaseous emissions and denitrifier community structure were analysed using Minitab 16 software. The normality of the distribution of the dataset was evaluated using the Shapiro-Wilk normality test (Shapiro & Wilk, 1965). As the assumptions of normality of data were violated, the data set was transformed using Box-Cox transformations and normality was tested again. Since the soil chemical characteristics, DEA and DR, were measured on 6 replicated samples, but pairs of the 6 replicates had been combined to give a total of 3 replicates for DNA analysis, the DEA and DR data were paired in the same way to give 3 replicates for statistical analysis. The differences in the means of soil characteristics such as pH, mineral N, TN, TC, Olsen P, MBC, soluble C, DEA, DR and molecular parameters (number of T-RFs and gene copy numbers) were assessed using a one-way analysis of variance (ANOVA) keeping soil characteristics as the response and soil origin as a factor. The Tukey's Studentized Range Test at alpha = 0.05 significance level was used *post hoc* to reveal significant differences among means. The relationships among soil chemical characteristics pH, mineral N, total N, total C, Olsen P, soluble C, MBC, DEA, DR and denitrifier gene distribution and abundance were determined using Pearson's correlation analysis.

Results

Soil chemical characteristics, DEA and DR

The soils had a range of physiochemical characteristics, DEA, DR and $N_2O/(N_2O+N_2)$ ratio (Tables 1 to 4). The gravimetric SWC of field moist soils ranged from 23 to 53% and their WFPS varied from 26 to 64% in surface and sub-surface soils. The SWC and WFPS values presented here correspond to the moisture levels in the sieved soils at the time of DR measurements. The NO_3-N content ranged from 1.7 to 58.7 mg kg^{-1} soil. Tokomaru ZL sub-surface soil contained the least NO_3^- and the Manawatu FSL (EI) soil had the highest NO_3-N content. The NH_4-N content in the soils varied from 0.7 to 13.4 mg kg^{-1} soil. The Manawatu FSL (EI) sub-surface soil had the least amount of NH_4-N. The amount of Olsen P in soils ranged from 18.3 to 122.5 µg g^{-1} soil with the highest P content in the Manawatu FSL (EI) soil. MBC ranged from 0.20 to 0.97 mg kg^{-1} soil and was highest for both Manawatu FSL soils. The soluble C ranged from 0.05 to 0.26 mg kg^{-1} soil. The DEA varied from 1.37 to 3738 µg N_2O-N kg^{-1} soil hr^{-1} and was lowest in the Otorohanga ZL sub-surface soil and highest in the Paparua ZL (Springston) soil. Among these dairy pasture soils DR ranged between 1.68 and 21.8µg N_2O-N kg^{-1} soil hr^{-1} with the lowest in the Lismore SZL sub-surface soil and the highest in the Te Kowhai soil. The $N_2O/(N_2O+N_2)$ ratio was the lowest in both Paparua ZL surface soils and highest in both Manawatu ZL surface and sub-surface soils. The Manawatu FSL soil that had received effluent applications had higher WFPS, NO_3-N, TN, TC and Olsen P contents and lower soil pH than the non-irrigated soil. This difference between the two Manawatu soils was apparent at both depths.

Table 1: Chemical characteristics of soils (0-100 mm depth) used in the experiment

Soil Name	Gravimetric SWC (%)	WFPS (%)	pH	Nitrate-N (mg kgsoil^{-1})	Ammo-N (mg kgsoil^{-1})	Total N (mg kg soil^{-1})	Total C (mg kg soil^{-1})	Olsen P (mg kgsoil^{-1})	MBC (mg gsoil^{-1})	Soluble C (mg gsoil^{-1})
Manawatu FSL (EI) (MWEI)	52.8 ± 1.0^{a}	52.1±1.2^{s}	5.9 ± 0.05^{bc}	58.7 ± 3.5^{a}	0.9 ± 0.1^{e}	5.2 ± 0.2^{c}	51.0 ± 2.0^{c}	122.5 ± 10.9^{a}	0.97 ± 0.07^{a}	0.05 ± 0.013^{e}
Manawatu FSL (MW)	36.1 ± 1.4^{bc}	45.8±2.3^{b}	6.3 ± 0.15^{a}	21.2 ± 4.0^{c}	3.8 ± 0.3^{d}	4.4 ± 0.1^{d}	44.6 ± 1.2^{d}	95.6 ± 10.6^{b}	0.84 ± 0.04^{a}	0.08 ± 0.007^{cd}
Tokomaru ZL (TM)	39.1 ± 3.4^{b}	64.2±1.2^{a}	5.7 ± 0.08^{cd}	5.4 ± 0.3^{e}	5.6 ± 1.4^{bcd}	2.7 ± 0.1^{f}	36.2 ± 2.0^{e}	85.4 ± 12.2^{b}	0.64 ± 0.03^{b}	0.07 ± 0.005^{de}
Te Kowhai ZL (TeK)	39.9 ± 0.6^{b}	55.8±1.1^{ab}	5.6 ± 0.01^{d}	13.5 ± 0.9^{d}	13.4 ± 1.8^{a}	2.7 ± 0.1^{f}	25.87 ± 1.5^{f}	39.8 ± 2.20^{c}	0.55 ± 0.04^{bcd}	0.10 ± 0.004^{c}
Otorohonga ZL (OH)	54.5 ± 0.7^{a}	31.3±0.98^{d}	5.6 ± 0.02^{d}	12.6 ± 0.9^{d}	11.6 ± 0.3^{a}	8.4 ± 0.4^{a}	82.6 ± 1.1^{a}	49.6 ± 1.8^{c}	0.5 ± 0.05^{cd}	0.26 ± 0.010^{a}
Horotiu ZL (HR)	54.3 ± 1.3^{a}	60.3±2.3^{a}	5.8 ± 0.02^{bcd}	10.5 ± 1.6^{d}	12.8 ± 1.0^{a}	6.4 ± 0.1^{b}	62.7 ± 0.8^{b}	53.0 ± 2.1^{c}	0.54 ± 0.02^{bcd}	0.19 ± 0.006^{b}
Paparua ZL (Springston) (PSP)	33.5 ± 1.3^{cd}	43.7±1.4^{c}	6.0 ± 0.06^{b}	32.8 ± 3.1^{b}	4.5 ± 0.4^{cd}	3.5 ± 0.1^{e}	38.6 ± 0.6^{e}	55.8 ± 8.15^{c}	0.59 ± 0.06^{b}	0.10 ± 0.017^{c}
Lismore SZL (LM)	31.7 ± 1.6^{d}	34.7±1.2^{c}	5.7 ± 0.08^{cd}	10.6 ± 1.5^{d}	12.9 ± 0.9^{a}	3.7 ± 0.1^{e}	38.2 ± 0.7^{e}	55.8 ± 8.15^{c}	0.66 ± 0.05^{b}	0.08 ± 0.004^{cd}
Mayfield ZL (MF)	24.3 ± 0.6^{e}	29.6±2.2d	4.8 ± 0.08^{e}	8.1 ± 0.8^{d}	8.1 ± 0.1^{b}	4.4 ± 0.2^{d}	43.6 ± 1.8^{d}	41.4 ± 2.51^{c}	0.44 ± 0.07^{d}	0.21 ± 0.012^{b}
Paparua ZL (Lincoln) (PL)	30.5 ± 1.3^{d}	42.8±2.1^{c}	6.4 ± 0.04^{a}	34.4 ± 4.0^{b}	6.7 ± 0.3^{bc}	3.0 ± 0.5^{f}	37.0 ± 1.1^{e}	41.4 ± 2.5^{c}	0.59 ± 0.02^{bc}	0.08 ± 0.003^{cd}

n=6, all means are reported ± standard error of the mean (S.E.M). Values sharing same letter are not significantly different. The letters indicate the differences in

Table 2: DEA, DR and $N_2O/(N_2O+N_2)$ molar ratios of soils (0-100 mm depth) used in the experiment

Soil Name	DEA (ugN_2O-N $kgsoil^{-1}hr^{-1}$)	DR (ugN_2O-N $kgsoil^{-1}hr^{-1}$)	$N_2O/(N_2O+N_2)$
MWEI	2533.3 ± 378.9^{ab}	7.3 ± 0.3^{c}	0.9 ± 0.05^{a}
MW	1130.2 ± 243.9^{c}	5.3 ± 0.31^{cde}	0.9 ± 0.05^{a}
TM	608.4 ± 105.1^{cd}	19.1 ± 1.8^{ab}	0.3 ± 0.02^{c}
TeK	180.35 ± 67.1^{d}	21.8 ± 1.5^{a}	0.5 ± 0.10^{b}
OH	173.3 ± 27.1^{d}	6.8 ± 0.8^{cd}	0.4 ± 0.08^{bc}
HR	924.1 ± 137.4^{c}	17.4 ± 3.3^{b}	0.6 ± 0.09^{b}
PSP	3738.2 ± 277.8^{a}	4.7 ± 0.04^{cde}	0.002 ± 0.0004^{d}
LM	469.9 ± 100.6^{cd}	2.9 ± 0.43^{e}	0.5 ± 0.05^{b}
MF	1026.4 ± 119.8^{c}	4.6 ± 0.41^{cde}	0.4 ± 0.09^{b}
PL	1930.3 ± 119.5^{b}	3.3 ± 0.5^{de}	0.003 ± 0.0002^{d}

n=6, all means are reported ± standard error of the mean (S.E.M). Values sharing same letter are not significantly different. The letters indicate the differences in the values

Table 3: Chemical characteristics of soils (100-200 mm depth) used in the experiment

Soil Name	SWC (%)	WFPS (%)	pH	Nitrate-N (mg kgsoil^{-1})	Ammo-N (mg kgsoil^{-1})	Olsen P (mg kgsoil^{-1})	Total N (mg kg soil^{-1})	Total C (mg kg soil^{-1})	MBC (mg gsoil^{-1})	Soluble C (mg gsoil^{-1})
MWEI	38.5 ± 0.7^{b}	43.4 ± 1.52^{b}	6.0 ± 0.03^{ab}	26.2 ± 2.3^{a}	0.7 ± 0.0^{e}	33.9 ± 2.2^{bc}	2.6 ± 0.13^{c}	24.8 ± 1.09^{f}	0.47 ± 0.05^{ab}	0.04 ± 0.010^{ef}
MW	24.1 ± 0.8^{d}	40.2 ± 1.13^{c}	6.2 ± 0.12^{a}	7.7 ± 1.6^{c}	2.5 ± 1.7^{de}	41.7 ± 6.1^{b}	2.1 ± 0.17^{a}	20.4 ± 1.30^{g}	0.20 ± 0.04^{c}	0.07 ± 0.002^{de}
TM	36.3 ± 2.3^{b}	52.8 ± 1.23^{a}	6.0 ± 0.08^{ab}	1.7 ± 0.2^{d}	6.6 ± 0.8^{bc}	70.5 ± 9.1^{a}	2.6 ± 0.10^{e}	31.6 ± 1.60^{c}	0.51 ± 0.03^{a}	0.06 ± 0.005^{bc}
TeK	26.2 ± 1.9^{cd}	36.5 ± 1.25^{d}	5.7 ± 0.03^{c}	7.7 ± 0.4^{c}	10.1 ± 1.1^{a}	18.3 ± 1.0^{d}	2.0 ± 0.02^{e}	16.5 ± 0.14^{h}	0.26 ± 0.02^{cd}	0.09 ± 0.007^{cd}
OH	54.6 ± 0.8^{a}	29.1 ± 0.92^{de}	6.0 ± 0.02^{ab}	7.9 ± 0.8^{c}	8.0 ± 0.8^{ab}	40.4 ± 2.4^{b}	6.2 ± 0.10^{a}	59.4 ± 1.05^{a}	0.25 ± 0.02^{cd}	0.25 ± 0.012^{a}
HR	49.7 ± 2.3^{a}	56.7 ± 2.10^{a}	5.7 ± 0.14^{c}	5.0 ± 0.4^{cd}	9.6 ± 1.4^{ab}	41.0 ± 1.4^{b}	4.3 ± 0.13^{b}	40.3 ± 1.2^{b}	0.21 ± 0.03^{d}	0.15 ± 0.008^{a}
PSP	29.3 ± 0.6^{c}	42.3 ± 1.01^{b}	5.8 ± 0.08^{bc}	23.9 ± 4.1^{a}	4.0 ± 0.4^{cd}	35.0 ± 3.3^{bc}	2.3 ± 0.31^{cd}	29.2 ± 0.49^{cd}	0.41 ± 0.02^{b}	0.07 ± 0.003^{fg}
LM	23.9 ± 0.9^{d}	34.2 ± 1.34^{d}	5.8 ± 0.06^{bc}	5.0 ± 0.4^{cd}	9.6 ± 1.4^{ab}	35.0 ± 3.3^{bc}	2.2 ± 0.08^{cde}	19.4 ± 0.58^{g}	0.32 ± 0.03^{c}	0.05 ± 0.003^{g}
MF	22.9 ± 0.5^{d}	25.6 ± 1.3^{e}	4.6 ± 0.09^{d}	2.0 ± 0.3^{d}	7.9 ± 1.1^{ab}	25.6 ± 1.8^{cd}	2.5 ± 0.09^{cd}	25.3 ± 1.29^{ef}	0.20 ± 0.04^{d}	0.13 ± 0.010^{b}
PL	25.4 ± 2.5^{cd}	34.8 ± 2.01^{d}	6.1 ± 0.06^{a}	18.3 ± 1.6^{b}	4.0 ± 0.4^{cd}	25.6 ± 1.8^{cd}	2.4 ± 0.07^{cd}	27.8 ± 0.65^{de}	0.43 ± 0.03^{ab}	0.05 ± 0.005^{g}

n=6. All means are reported ± standard error of the mean (S.E.M). Values sharing same letter are not significantly different. The letters indicate the differences in the values only within the column they are presented in.

Table 4: DEA, DR and $N_2O/(N_2O+N_2)$ molar ratios of soils (100-200 mm depth) used in the experiment

Soil Name	DEA (ugN_2O-N $kgsoil^{-1}hr^{-1}$)	DR (ugN_2O-N $kgsoil^{-1}hr^{-1}$)	$N_2O/(N_2O+N_2)$
MWEI	48.71 ± 10.93^{c}	5.50 ± 0.11^{a}	0.97 ± 0.01^{a}
MW	10.54 ± 2.57^{c}	3.82 ± 0.19^{b}	0.87 ± 0.05^{ab}
TM	7.72 ± 1.98^{c}	2.51 ± 0.92^{bcde}	0.69 ± 0.08^{bc}
TeK	33.13 ± 12.08^{c}	1.08 ± 0.13^{e}	0.28 ± 0.04^{e}
OH	1.37 ± 0.32^{c}	1.96 ± 0.26^{cde}	0.47 ± 0.10^{d}
HR	170.93 ± 42.18^{b}	2.76 ± 1.11^{bcd}	0.64 ± 0.09^{cd}
PSP	435.10 ± 85.11^{a}	3.31 ± 0.36^{bc}	0.008 ± 0.0005^{f}
LM	38.44 ± 7.49^{c}	1.68 ± 0.43^{de}	0.62 ± 0.11^{cd}
MF	40.50 ± 8.41^{c}	3.26 ± 0.29^{bc}	0.58 ± 0.03^{cd}
PL	236.05 ± 65.84^{b}	2.23 ± 0.31^{cde}	0.05 ± 0.004^{f}

Distributions and abundances of denitrifier genes in NZ dairy grazed pasture soils
Genes encoding the initial steps of denitrification (*nir*S+*nir*K) were more abundant than those encoding the final step (*nos*Z). Since both *nir*S and *nir*K genes encode for the same reductase enzyme (nitrite reductase) and these do not co-occur in same bacteria (Heylen *et al.*, 2006), we summed the distributions and abundances of *nir*S and *nir*K in each sample.

The average number of T-RFs of the *nos*Z gene ranged from 12 to 27, the *nir*S 4 to 33, the *nir*K 3 to 31, and the number of *nir*S+*nir*K gene T-RFs varied from 9 to 49. The average numbers of *nir*S+*nir*K gene T-RFs were higher than *nos*Z gene T-RFs in these soils (Figures 1 and 2). We found that the numbers of *nir*S+*nir*K T-RFs ranged from 9 to 49 and were lowest in Paparua ZL (Lincoln) and highest in both Manawatu FSL soils. The numbers of *nos*Z T-RFs varied from 12 in the Paparua ZL (Lincoln) to 27 in the Manawatu FSL (EI). Overall, the Paparua ZL (Lincoln) soil had significantly ($P<0.05$) lower numbers of denitrifier gene T-RFs than any other soil and the Manawatu FSL (EI) had the highest.

The evenness of *nir*S and *nir*K T-RFs was calculated as the Pielou's coefficient of evenness (J) using Shannon diversity index (H') and was the measure of the evenness of distribution of T-RFs of denitrifier gene relative to the total number of T-RFs per sample. It showed that the evenness of the distributions of *nir*S gene T-RFs (Table 5) in the Paparua ZL (Lincoln) was the lowest (0.28) and was the highest in Manawatu FSL (EI) and Paparua ZL (Springston) (0.94-0.96). The *nos*Z and *nir*K genes evenness were lowest in Horotiu ZL (0.21, 0.68) and highest in Manawatu FSL (EI) (0.98, 0.98) respectively.

The abundances of total bacteria and denitrifying bacteria were measured as the gene copy numbers of the respective bacterial genes in soils (Figures 3 and 4). The average gene copy numbers of the total bacterial gene *rpo*B ranged from 3.5×10^8 to 1.6×10^9 g^{-1} soil, the *nos*Z gene from 9.9×10^5 to 4.8×10^8 g^{-1} soil, the *nir*S gene from 2.5×10^7 to 4.6×10^8 g^{-1} soil, the *nir*K gene from 1.5×10^8 to 5.9×10^8 g^{-1} soil, and the *nir*S+*nir*K gene copy numbers varied from 2.6×10^8 to 7.5×10^8 g^{-1} soil (Figures 3 and 4).

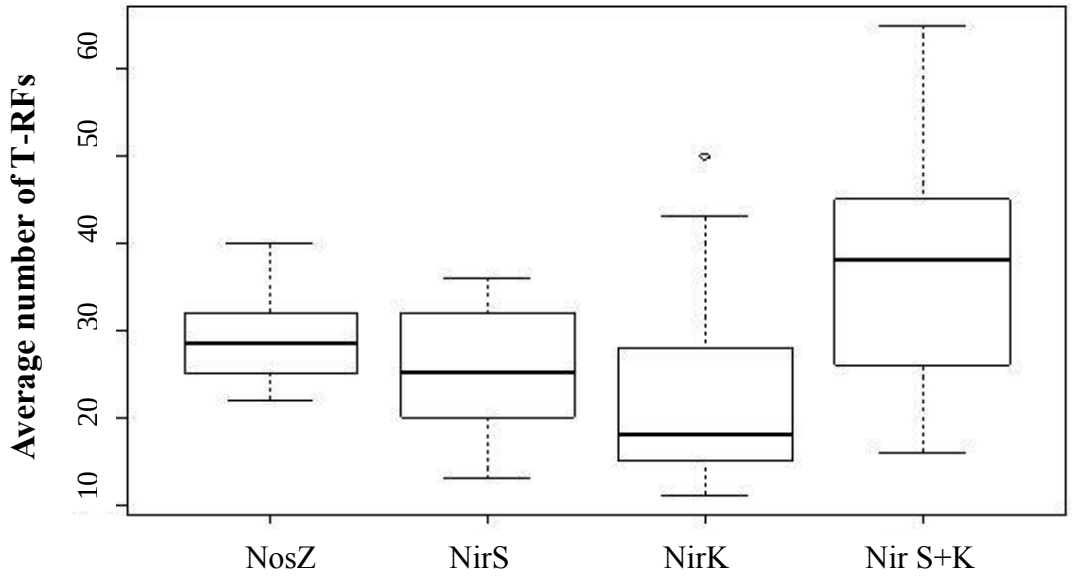

Figure 1: Distribution of denitrifier genes T-RFs in soils

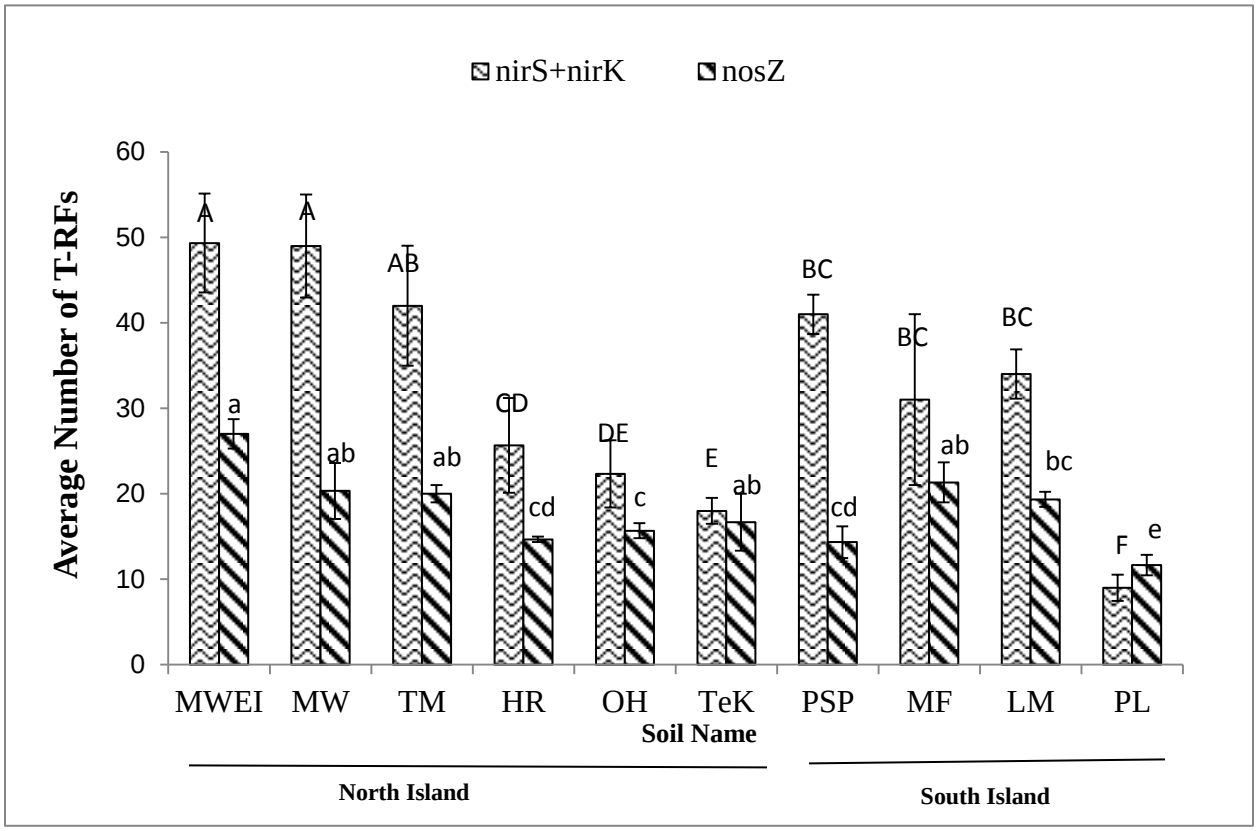

Figure 2: Average denitrifier gene phylotype numbers in soils, bars denote standard error of mean (S.E.M). Bars with same letter values are not significantly different.

Table 5: **Pielou's coefficient of T-RF Evenness** ***of denitrifier phylotype distribution in soils***

Soil	MWEI	MW	TM	TeK	OH	HR	PSP	LM	MF	PL
***nir*S**	0.94^{a}	0.96^{a}	0.59^{e}	0.50^{f}	0.68^{d}	0.72^{c}	0.96^{a}	0.59^{e}	0.87^{b}	0.27^{g}
***nir*K**	0.98^{a}	0.71^{c}	0.87^{b}	0.36^{e}	0.27^{fg}	0.21^{g}	0.41^{d}	0.47^{d}	0.28^{fg}	0.32^{ef}
***nos*Z**	0.98^{a}	0.96^{ab}	0.98^{a}	0.80^{c}	0.72^{d}	0.68^{e}	0.95^{b}	0.74^{d}	0.80^{c}	0.73^{d}

n=3. All means are reported ± standard error of the mean (S.E.M). Values sharing same letter are not significantly different. The letters indicate the differences in the values only within the column they are presented in.

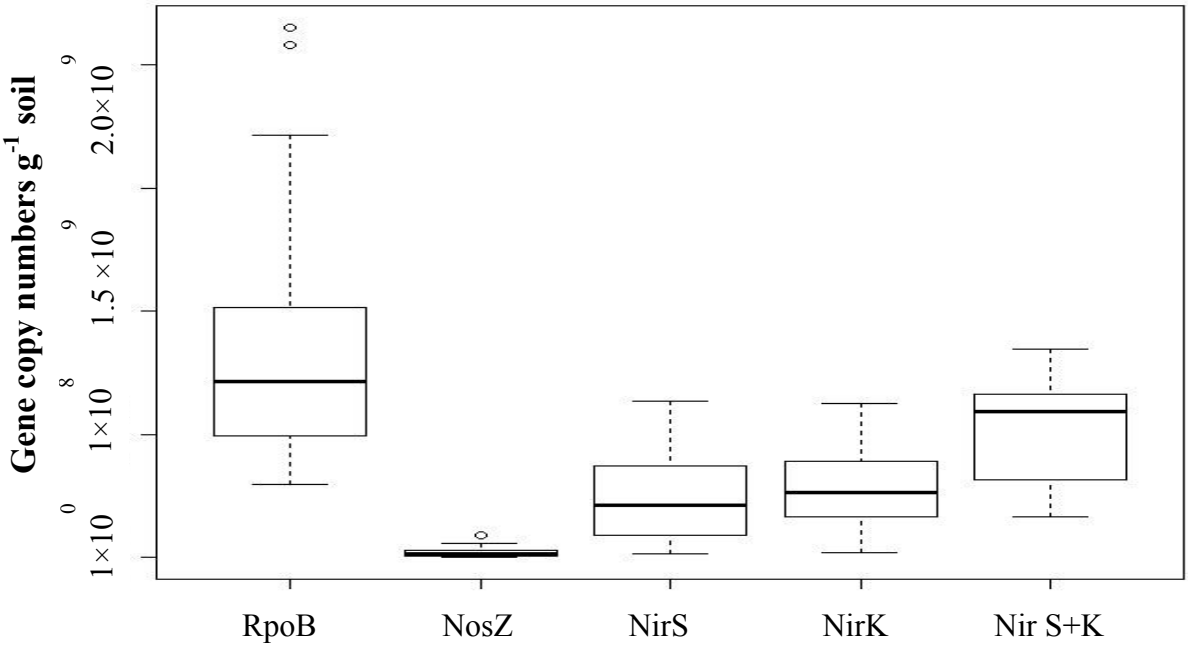

Figure 3: Abundance of universal bacterial gene copy numbers in soils

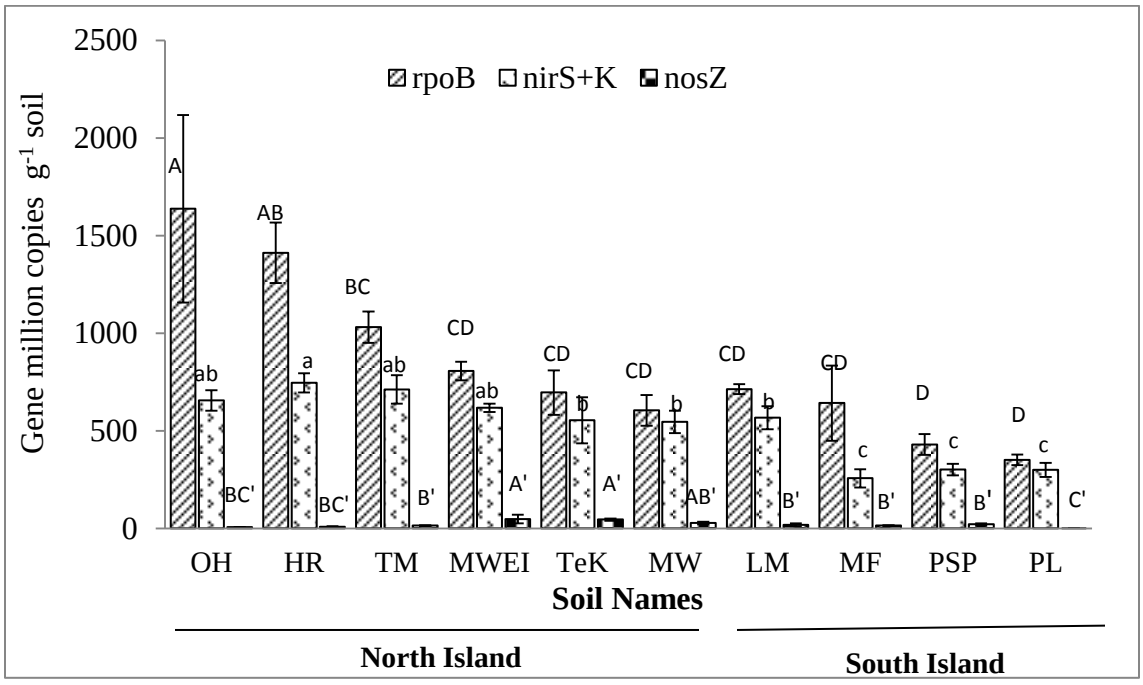

Figure 4: Average universal bacterial (rpoB) and denitrifier gene copy numbers in soils, error bars denote S.E.M. Bars with same letter values are not significantly different. Letter values with same case or symbol denotes one test.

Influence of soil characteristics on denitrifier gene distributions and abundances

The relationships between soil properties (SWC, WFPS, pH, MBC, NO_3-N, NH_4-N,Olsen P, TC, TN, DR, DEA, N_2O, N_2 emission, $N_2O/(N_2O+N_2)$ ratio) and the gene distributions and abundances of *nos*Z, *nir*S, *nir*K, *nir*S+*nir*K, and *rpo*B were assessed using Pearson's correlation coefficients. The analysis suggested that not all the soil characteristics shown in Tables 1&2 had significant relationships with the denitrifier gene distribution and abundance and therefore, only significant correlations are displayed in Tables 6 & 7. The numbers of T-RFs of *nos*Z, *nir*S, *nir*K, and *nir*S+*nir*K genes were significantly positively correlated with MBC and Olsen P and negatively correlated with the NH_4-N content in the soils. Also, the numbers of T-RFs of denitrifier genes were positively correlated to their respective gene copy numbers. In the case of gene copy numbers, *nos*Z gene abundance was positively correlated with MBC, Olsen P and soil NO_3-N content. N*ir*S gene copies were positively related to NH_4-N and negatively related to NO_3-N contents of soils. On the other hand, *nir*K gene copies were positively correlated with MBC and NO_3-N content of the soils. The *nir*S+*nir*K gene copy numbers were positively correlated with MBC. The *rpo*B gene copy numbers were positively correlated with MBC, TN and TC.

When the relationship between gaseous emissions, gene distribution and abundances was considered (Table 7), the N_2O emission during denitrification was positively correlated with *nos*Z, *nir*S, *nir*K, *nir*S+*nir*K, and *rpo*B gene copy numbers however, overall there was no significant correlation observed between denitrifier gene copies and N_2 emissions in these soils. The DR was positively correlated only to *nir*S+*nir*K gene copy numbers. The proportion of N_2O emitted with respect to total denitrification was positively correlated to the number of *nos*Z gene T-RFs and gene copies. It was negatively correlated with *nir*K/*nos*Z gene copies and *nir*S+*nir*K/ *nos*Z gene copies. The N_2O/N_2 emission ratio during denitrification was positively correlated with the numbers of *nir*K/*nos*Z gene T-RFs. The DEA was positively correlated with numbers of *nir*S/*nos*Z and *nir*S+*nir*K/*nos*Z gene T-RFs.

Table 6: **Significant** ***Pearson's correlation coefficients between soil characteristics denitrifier gene distribution and abundance***

	Variable	**Correlation Coefficient (r)**	**p**
Number of T-RFs			
***Nir*K**	MBC	0.709	0.0001
	Olsen P	0.585	0.0001
	NH_4-N	-0.607	0.0001
	NO_3-N	0.414	0.0230
	*nir*K gene copy numbers	0.512	0.0040
***Nir*S+*Nir*K**	MBC	0.578	0.0010
	Olsen P	0.591	0.0190
	NH_4-N	-0.481	0.0070
	*nir*S+*nir*K gene copy numbers	0.420	0.0321
***Nos*Z**	MBC	0.500	0.0001
	Olsen P	0.729	0.0050
	NH_4-N	-0.319	0.0050
	*nos*Z gene copy numbers	0.600	0.0042
Gene Copy Numbers			
***Nir*K**	MBC	0.532	0.0020
	NO_3-N	0.364	0.0480
***Nir*S**	NH_4-N	0.506	0.0040
	NO_3-N	-0.546	0.0020
***Nir*S +*Nir*K**	MBC	0.332	0.0050
***Nos*Z**	MBC	0.483	0.0070
	Olsen P	0.344	0.0430
	NO_3-N	0.393	0.0320
	Soluble C	-0.436	0.0160
***Rpo*B**	MBC	0.445	0.0140
	TN	0.669	0.0000
	TC	0.537	0.0020
	Soluble C	0.563	0.0001

Table 7: Significant Pearson's correlation coefficients between soil N_2O emissions, DR, $N_2O/(N_2O+N_2)$, N_2O/N_2, and denitrifier gene distribution and abundance.

	Variable	**Correlation Coefficient (r)**	**p**
N_2O	*nir*K gene copy numbers	0.375	0.0410
	*nir*S+*nir*K gene copy numbers	0.554	0.0020
	*nos*Z gene copy numbers	0.356	0.0500
	*rpo*B gene copy numbers	0.356	0.0540
DR(N_2O+N_2)	*nir*S+*nir*K gene copy numbers	0.497	0.0050
$N_2O/(N_2O+N_2)$	*nos*Z gene copy numbers	0.423	0.0020
	*nos*Z T-RFs	0.613	0.0001
	*nir*K gene copy numbers	0.414	0.0220
	*nir*K gene T-RFs	0.424	0.0200
	*nir*S+*nir*K gene copy numbers	0.417	0.0220
	*nir*S+*nir*K T-RFs	0.487	0.0070
	*nir*K / *nos*Z gene copy numbers	-0.432	0.0170
	*nir*S+*nir*K / *nos*Z gene copy numbers	-0.438	0.0160
N_2O/N_2	*nir*K/*nos*Z gene T-RFs	0.451	0.0120
	*nos*Z gene abundance	0.465	0.0100

Discussion

The soil characteristics (SWC, WFPS, mineral N, TC, TN, MBC, Olsen P), DR and DEA were significantly higher in surface soils than in sub-surface soils, which reflected the higher root mass, and substrate availability in surface soils. Soil water content is a key factor for controlling DR in pasture soils (de Klein & van Logtestijn, 1994; Jarvis *et al.*, 1991). Higher soil water content, or lower oxygen content, activates N_2O reductase and reduces diffusion of N_2O from the site of production (Petersen & Andersen, 1996). This creates a greater opportunity for reduction of N_2O to N_2 and thereby reduces the proportion of N_2O in the total denitrification product (Weier *et al.*, 1993). Most of the soils we studied had low SWC and consequently we observed low DRs in these soils. The soils with higher WFPS (> 60%), such as Te Kowhai ZL, Otorohonga ZL and Tokomaru ZL had comparatively higher DRs than the rest of the soils with lower WFPS. Drury et al. (2003) reported similar findings and suggested that DR reaches its maximum value at 90% WFPS.

The DEA of a soil is its maximum potential to denitrify under ideal conditions and reflects the variability of soil's microbial enzyme activities. Despite lower DEA in soils such as Te Kowhai ZL, Otorohonga ZL and Tokomaru ZL, these soils displayed higher DRs due to their higher

WFPS. This confirms that WFPS probably played a big role in determining the relative rates of denitrification in these soils.

In this study the distributions and abundances of denitrifying bacteria and total bacteria in a range of New Zealand dairy grazed pasture soils were assessed. As expected, in all the 10 soils the universal bacterial genes were the most abundant gene followed by *nir*S +*nir*K, *nir*S, *nir*K and the least abundant was the *nos*Z gene. This has also been confirmed in other studies measuring denitrifier abundance in environmental samples (Chon *et al.*, 2011). The total number of denitrifying bacteria ranged from 10^5 to 10^9 bacteria g^{-1} soil. The higher abundance of *nir*S+*nir*K genes than the *nos*Z gene is explained by the fact that some bacteria like *Agrobacterium tumefaciens* lack *nos*Z gene.

The abundance of *nos*Z, *nir*S, *nir*K and *nir*S+*nir*K genes relative to *rpo*B genes varied from 0.28 to 6.5%, 3.22 to 55.3%, 17 to 74% and 46 to 91% respectively. This shows a very large proportion of the total bacterial population in these pasture soils have the ability to denitrify. However, only a small proportion of total bacteria are capable of reducing N_2O to N_2 and thus completing the denitrification process in these soils. Wu *et al.* (2012) have found the abundance of *nir*S, *nir*K genes relative to the total bacterial 16S rDNA gene to vary from 2.5 to 22% and from 6.25 to 50% respectively. The abundances of denitrifying genes (*nos*Z) relative to the total bacterial genes in various environmental samples have been reported to vary between 0.1% and 5.0% (Jones *et al.*, 2013). The studies by (Chen *et al.*, 2012; Henry *et al.*, 2006; Kandeler *et al.*, 2006) have reported lower (<10%) proportions of abundances of denitrifier genes to total bacterial genes than reported in current study. There was a significant negative correlation (r =-0.372, *P*= 0.043) between the individual abundances of *nir*S and *nir*K genes in these soils. This supports the fact that denitrifying bacteria harbour either of the *nir*S and *nir*K genes (Goregues *et al.*, 2005) and the soils used in the current experiment might possess bacterial denitrifiers possessing either of the genes.

The distribution of denitrifier genes in New Zealand pasture soils also suggested that the denitrifier communities in these soils are dominated by NO_2^- reducers compared to N_2O reducers. The Pielou's coefficient of evenness (J) for denitrifying bacteria illustrated that the number of T-RFs of the N_2O reducing bacterial community is more equally present than the NO_2^- reducing community in the pasture soils tested. This suggests that some genotypes of NO_2^- reducers are dominant in these soils and a few are rarely present. It might also mean that the differences in DR in these soils could be driven more by variation in the NO_2^- reducing communities than the N_2O reducers.

A correlation analysis was performed to relate soil chemical characteristics to denitrifier community structure, denitrification rates and N_2O emissions measured in New Zealand pasture soils. We found key soil factors such as Olsen P, MBC, NO_3-N and NH_4-N contents were correlated with the bacterial denitrifier gene distribution and abundance in New Zealand pasture soils. The soils with higher MBC and Olsen P, like Manawatu FSL (EI), had higher abundances of denitrifying bacteria than other soils. The Paparua ZL (Lincoln) had lower abundances of denitrifier genes and the associated lower MBC, Olsen P also agreed with the lower *rpo*B copies in this soil - suggesting a small bacterial population. Also, the higher NH_4-N content of this soil likely favoured nitrifying or ammonia oxidising bacteria over denitrifying bacteria. Previous

studies have also shown that soil chemical characteristics such as pH, EOC, TN, TC, NO_3^-, MBC, and MBN (Liu *et al.*, 2013) influence the denitrifier gene abundances a soil.

Soils of similar pH and textures to those used in our study have yielded conflicting results with regard to the relationship among denitrifier community structure and denitrification activity or N_2O emissions (Enwall *et al.*, 2005; Peralta *et al.*, 2010; Rich & Myrold, 2004). In our study, the sums of *nir*S and *nir*K abundances were significantly correlated to our DR measurements. This finding is similar to other studies that have shown an influence of denitrifier community structure on N_2O emissions during denitrification (Cavigelli & Robertson, 2000; Cavigelli & Robertson, 2001; Holtan-Hartwig *et al.*, 2000). We found no correlation between *nos*Z gene abundance with N_2 emission or any of the denitrifier gene abundances with DEA. This suggests that differences in denitrifier gene abundance were independent of denitrifier activity and were driven by changes in soil chemical characteristics. However, we observed that total bacterial gene abundance and denitrifier gene abundance were positively related to N_2O emissions during denitrification. This is a similar finding to other studies that have shown an influence of denitrifier community size on N_2O emissions during denitrification. The variation in size and the ability of the denitrifier community to denitrify may result in variable N_2O emission during denitrification.

Since *nos*Z controls the reduction of N_2O to N_2, it was hypothesised that *nos*Z abundance would be negatively correlated to N_2O/N_2 or N_2O/DR ratio. Contrary to this expectation, the relative emission of N_2O with respect to N_2 emission during denitrification or total DR was positively correlated to *nos*Z gene abundance. However, in this experiment most of the soils had low SWC and WFPS. In both the Manawatu soils that had high *nos*Z gene abundance, the comparatively lower WFPS meant that there was only limited reduction of N_2O to N_2. There are very few studies relating N_2O/DR ratio with denitrifier gene abundance. (Miller *et al.*, 2008, 2009) reported no correlation between N_2O molar ratio and *nos*Z gene abundance in soils amended with crop residues and animal manure. Cavigelli &Robertson (2000), Cavigelli &Robertson (2001) and Holtan-Hartwig *et al.* (2000) have suggested that the N_2O emissions during denitrification are regulated by the denitrifier community structure due to their physiological differences in the soil. They have emphasised that the ability of denitrifiers to either produce or reduce N_2O under certain soil or climatic condition should be given emphasis when making models to develop mitigation techniques for N_2O emissions from soil.

Conclusions

Denitrification is a critical component of the nitrogen cycle in agricultural pastures and is a multistep process. This is one of the few studies in New Zealand that contributes to our understanding of the microbial community associated with two key steps in the denitrification process, and their environmental regulation. The soils used had varying physiochemical characteristics and DEA. We found that NO_2^- reducers are more abundant in these soils than N_2O reducing bacteria and also that soils show wide variation in denitrifier community structure. The correlation analysis suggested the denitrifier gene distribution and abundance is related to characteristics such as MBC, Olsen P, and mineral N contents. *Nos*Z, *nir*S and *nir*K gene copy numbers correlated positively with N_2O emissions. We found no clear relationship between *nos*Z gene copy numbers and N_2 emissions in our field moist soils having gravimetric SWC between 23 to 54% or WFPS between 26 to 64%. For many of the soils, these water contents were below

field capacity, and it may be that the *nos*Z copy number may only predict N_2 emissions under anaerobic conditions. The results of this study suggest that *nos*Z genes were present but were not being transcribed and not actively participating in the N transformations. The next step could be to look into the denitrifier gene abundance in soils with increased soil water content.

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Appendix I

Soil Name	Geographical Location	Location of the dairy farm
Te Kowhai Silt Loam (TeK)	37°44'57.55"S 175°10'27.06"E	AgResearch Ruakura Waikato
Otorohonga Silt Loam (OH)	38°11'19.70"S 175°12'35.67"E	Tokanui Waikato
Horotiu Silt Loam(HR)	37°46'30.80"S 175°18'23.27"E	AgResearch Ruakura Waikato
Tokomaru Silt Loam(TM)	40°22'58.26"S 175°36'31.01"E	Massey University, Palmerston north
Manawatu Fine Sandy Loam (MW)	40°22'56.99"S 175°32'24.49"E	Longburn, Palmerston North
Manawatu Effluent irrigated Fine Sandy Loam (MWEI)	40°22'58.26"S 175°32'21.65"E	Longburn, Palmerston North
Paparua Silt Loam (Springston) (PSP)	43°38'15.97"S 172°28'13.81"E	Springston, Christchurch
Paparua Silt Loam (Lincoln) (PL)	43°38'43.91"S 172°25'21.86"E	Lincoln, Christchurch
Lismore Stony Silt Loam (LM)	43°53'17.44"S 171°38'28.43"E	Ashburton, Canterbury
Mayfield Silt Loam (MF)	43°38'30.12"S 171°43'47.28"E	Methven, Canterbury

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