CHANGES IN DENITRIFICATION RATE, BACTERIAL DENITRIFIER COMMUNITY STRUCTURE AND ABUNDANCE IN DAIRY-GRAZED PASTURE SOILS TREATED WITH CATTLE URINE AND DCD

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Abstract

Urine excreted by cattle can produce very high concentrations of available N in relatively small volumes of soil and lead to high nitrous oxide (N_2O) emissions. Application of the nitrification inhibitor dicyandiamide (DCD) can inhibit nitrification. DCD application results in lower nitrate (NO_3^-) concentrations and N_2O emissions from denitrification in urine affected soils. However, the effect of urine and DCD on denitrification may vary depending on the soil's inherent capacity to denitrify. We assessed the effect of DCD additions on N_2O emissions, denitrifier community structure of *nir*S, *nir*K and *nos*Z genes and their abundance in urine affected soils in order to gain insight into how DCD affects the denitrification process within soils to which urine had been applied.

We used surface samples from 3 New Zealand dairy pasture soils with contrasting denitrification enzyme activities (DEA) (Manawatu Fine Sandy Loam, Tokomaru Silt Loam and Otorohonga Silt Loam) with the highest DEA in Manawatu and the lowest in Otorohonga soil. The treatments applied were; cattle urine (700 mg N kg^{-1} soil), cattle urine + DCD (10 mg DCD kg^{-1} soil) and control (deionised water). Soils were saturated with water and incubated at 25^{o}C for four weeks. Gas samples and soil extracts collected during the incubation were analyzed to determine denitrification rate (DR), N_2O and N_2 emissions, pH, and mineral N contents in soils. We also determined the denitrifier community structure of *nir*S, *nir*K and *nos*Z genes and their abundance using molecular techniques. We observed increased DR and denitrifier genes abundances after 24 hours of incubation in soils that had urine applied. The DCD was ineffective in controlling denitrification after 24 hours of incubation. The results of the longer incubation time are under analysis.

Introduction

Pastoral agriculture is the dominant source of greenhouse gas emissions in New Zealand with a 23% increase in N_2O emissions from pastures since 1992 (MFE, 2012). Apart from fertilizer and dairy waste effluent, a major proportion of N input to pasture is in the form of animal excretion (Di *et al.*, 2002). In a grazed pasture 60-90% of the N ingested by animals is returned to soil in the form of urine and dung and more than 70% of this N is returned as urine (Haynes & Williams, 1993; Jarvis *et al.*, 1995). Urine patches are among the highest sources of N_2O emissions from agricultural soils (Van Groenigen *et al.*, 2005). Urine patches provide high concentrations of readily available N and C in relatively small volumes of soil, which then become a source of high N_2O emissions (Yamulki *et al.*, 2000).

N_2O is produced through the action of both nitrifying and denitrifying organisms and despite producing N_2O, denitrifiers are the only organisms that can reduce N_2O to N_2 (Conrad, 1996). Using a ^{15}N labelling technique, Di & Cameron (2008) reported that denitrification contributes 60% of the total N_2O emitted from a urine patch. Emissions of N_2O generally increase immediately after urine application/deposition (Yamulki *et al.*, 1998). The exact mechanism that leads to higher N_2O emissions just after urine application is not well understood and the amount of emission might be affected by soil and environmental factors such as soil type, moisture content, soil pH, microbial activity in the soil, temperature and the amount of urine-N deposited.

There are various ways of mitigating N_2O emissions from pasture soils. These include improved soil structure, optimum mineral N fertilizer application, use of nitrification inhibitors, and improved animal and pasture management (Luo *et al.*, 2008; Saggar *et al.*, 2009; Saggar *et al.*, 2011). Nitrification inhibitors restrict the conversion of ammonium (NH_4^+) to NO_3^-. One of the commonly used inhibitors is DCD (Amberger, 1989). Di & Cameron (2008) reported that DCD can achieve a 72% reduction in N_2O emissions from urine patches through both nitrification and denitrification.

Most of the N_2O mitigation research in New Zealand dairy pasture soils is related to the ability of DCD to inhibit nitrification (Di & Cameron, 2006, 2008; Luo *et al.*, 2010) and thus N_2O emissions in urine applied soils. The effect of DCD application on denitrifier community structure and its abundance is not very well understood in New Zealand dairy pasture. This information is vital to develop mitigation strategies to reduce denitrification and thus N_2O emissions from dairy pasture soils. Therefore, we planned an experiment with the following objectives: (1) to estimate the variation in DR and emissions of N_2O and N_2 with urine and urine + DCD applications on three dairy pasture soils contrasting in DEA, (2) to determine the effectiveness of DCD in reducing the production of NO_3^- and thereby reducing the DR and (3) to elucidate the changes in denitrifier genes abundance with the application of urine and urine + DCD. The experiment was designed to test the following hypotheses: (1) application of cattle urine to the soils will increase the NO_3^- content in soils which will increase denitrification rate and also emissions of N_2O and N_2, (2) application of DCD with urine will restrict the supply of NO_3^- and decrease the DR and (3) application of DCD will reduce the abundance of denitrifier genes in soils.

Material and methods

Soil collection

The three soils with contrasting DEA (Jha et al 2013) that were used for this study were: Tokomaru Silt Loam from Massey University No.4 dairy farm in Palmerston North, Manawatu Fine Sandy Loam from a Longburn dairy farm and Otorohonga Silt Loam from an AgResearch Ruakura dairy farm in Hamilton. About 25 soil cores (25 mm diameter and 100 mm long) were collected from four randomly selected areas (100 m^2 each) in each farm. During sampling, areas around paddock entrances, water troughs and obvious urine or dung patches were avoided. The 25 soil cores collected from each randomly selected area were bulked together resulting in 4 field replicates of the soil on each farm. Replicate soil samples were sieved to 2 mm and immediately stored in plastic bags at 4^oC for chemical analysis. Subsamples from each of the plastic bags were stored at -20^oC for molecular analysis.

Application of treatments and incubation of soils

Fresh cattle urine was collected from cows during milking, (avoiding contamination from dung), and stored in tightly sealed plastic bottles at 4^oC to avoid urea hydrolysis. Total C and N contents of the urine were determined and the amount of urine required for application was calculated.
The treatments were:

1. Control (C) (deionised water)
2. Urine (700 mg N kg^{-1} dry soil) (U)
3. Urine (700 mg N kg^{-1} dry soil) + DCD (10 mg DCD kg^{-1} dry soil) (UI)

Incubation: For each treatment, four replicate 50g (dry weight equivalent) subsamples of each soil were placed in plastic containers with 1 mm holes to allow for the exchange of gases and incubated at 25^oC for 24 hours for measurements of DR (3 soils × 3 treatments × 4 replicates × 2 $\pm C_2H_2$= 72 total). Soils were brought to saturation by gradually adding only deionized water in control treatments. In urine and urine + DCD treatments the same amounts of deionised water, minus the volumes of bovine urine and DCD, were applied to respective containers to increase the soil water contents. Finally, the pre-calculated amounts of urine and DCD were added to saturate the soils. Another set of four replicated soil samples (250g each) for each treatment were also amended with urine and urine + DCD and incubated in glass jars at saturated soil water content for collection of subsamples for chemical and molecular analysis.

Measurements of soil chemical characteristics and denitrification

Gravimetric soil water content, pH, microbial biomass carbon (MBC), mineral N (NO_3^- and NH_4^+), total C (TC), total N (TN) and Olsen P contents were measured in soil samples before incubation. Subsamples collected from incubated soils after 24 hours were also analysed for mineral N and soil pH by following the standard protocols. We measured DR and DEA in the 3 soils at the original field moist condition and DR after 24 hours of incubation, using the methods reported in (Jha *et al.*, 2012).

Molecular analysis

Approximately 0.25 g subsamples of the freshly collected soils and incubated soils stored at -20^{o}C were used for DNA extraction. Quantification of the distributions and abundances of denitrifier genes encoding *nos*Z, *nir*S and *nir*K was accomplished using T-RFLP and qPCR as described in (Jha *et al.*, 2013). Both *nir*S and *nir*K encode for same reductase enzyme (nitrite reductase) and these do not co-occur in same bacteria. Therefore for simplification we summed the numbers of gene phylotypes (T-RFs) and gene copy numbers of these two genes in each soil and the added values are reported in this paper.

Data 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 some of the data sets were violated, those data sets were transformed to normal based on Box-Cox transformations. The transformation normalised the dataset. The effect of saturation on soil characteristics (pH, mineral N, DR, numbers of gene T-RFs and gene copy numbers) was determined using 2 samples T-tests. The effects of soil type and treatments on the means of soil characteristics (pH, mineral N, TN, TC, Olsen P, MBC, DEA, DR) and molecular parameters (numbers of T-RFs and gene copies) were assessed using two way analysis of variance (ANOVA). The differences in the means of soil characteristics (pH, mineral N, TN, TC, Olsen P, MBC, DEA, DR), and molecular parameters (numbers of T-RFs and gene copies) were assessed using one-way ANOVA with soil characteristics as response variables and soil treatments as the predictive factor. Tukey's Studentized Range Test, at alpha = 0.05 significance level, was used *post hoc* to reveal significant differences among the means. The relationship among denitrification rates, N_2O and N_2 emissions with soil characteristics such as NH_4-N content and molecular parameters such as numbers of T-RFs and gene copies were determined using Pearson's correlation analysis.

Results

Soil Characteristics

The pH did not differ among the three original field moist soils (mean=6.0; range= 5.9 to 6.3; Table 1). The NO_3-N contents of the soils varied from 12 to 55 mg kg^{-1} soil, and were significantly higher in the Manawatu soil. The NH_4-N content was similar in the three soils and ranged from 70.8 to 85.0 mg kg^{-1} soil. The TC and TN contents of the three soils ranged from 28 to 80.6 mg kg^{-1} soil and 2.7 to 8.7 mg kg^{-1} soil, respectively, and were the highest in Otorohonga and the lowest in Tokomaru soil. The Olsen P and MBC contents ranged between 25 and 110 mg kg^{-1} soil and from 1.17 to 1.75 mg g^{-1} soil respectively and were the lowest in the Tokomaru soil.

Changes in soil pH and mineral N after 24 hours of incubation

After 24 hours of incubation we found a decrease in soil pH with saturation only in Tokomaru soil. In Manawatu and Otorohonga soils there was no change in soil pH with saturation. The soil NO_3-N and NH_4-N contents in control soils after 24 hours of incubation were lower than in the original field moist soil (Table 2).

In the incubated treatments the pH was higher in the urine and urine + DCD treatments than in the controls (Table 3). The pH in the urine + DCD treatment was significantly higher than control in all the three soils. The NO_3-N content in Manawatu and Tokomaru soils was higher in the urine and urine + DCD treatments than in the controls. In Otorohonga soil the NO_3-N content was higher than control in the urine-only treatment. The NH_4-N content in urine-treated soils was similar to but higher than control in Manawatu and Tokomaru soils. In Otorohonga soil the NH_4-N content was highest in the urine + DCD treatment followed by urine-only and control. There was nearly a 10 fold increase in NH_4-N contents in soils with urine application. However, when compared to the amount of N applied in the urine treatments, the recovered soil NO_3-N and NH_4-N contents were lower in all the three soils. The recovered N varied from 44 to 79% of the total applied N as cattle urine in these soils.

Table 1: Soil chemical characteristics before the start of incubation

Soil	pH	NO_3-N (mg kg^{-1} soil)	NH_4-N (mg kg^{-1} soil)	Total C (mg kg^{-1} soil)	Total N (mg kg^{-1} soil)	Olsen P (mg kg^{-1} soil)	MBC (g kg^{-1} soil)	DR (µg N_2O-N kg^{-1} soil hr^{-1})	DEA (µg N_2O-N kg^{-1} soil hr^{-1})
Manawatu (MW)	6.3 ± 0.07^a	55.3 ± 2.2^a	70.8 ± 2.9^a	46.7 ± 2.29^b	5.3 ± 0.23^b	110.7 ± 22.0^a	1.61 ± 0.08^a	19.09 ± 0.83^a	938.82 ± 183.05^a
Tokomaru (TM)	5.9 ± 0.05^a	12.0 ± 1.0^c	85.0 ± 6.8^a	28.02 ± 0.79^c	2.7 ± 0.073^c	24.8 ± 2.0^b	1.17 ± 0.04^b	10.39 ± 0.88^b	471.44 ± 139.92^b
Otorohonga (OH)	6.0 ± 0.23^a	43.1 ± 3.9^b	74.5 ± 5.3^a	80.62 ± 3.0^a	8.7 ± 0.29^a	82.2 ± 5.4^a	1.45 ± 0.12^a	5.9 ± 0.69^c	149.93 ± 39.85^c

(n=4) 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 2: Soil characteristics in field moist and saturated control soils after 24 hours of incubation

Soil Characteristics	**Field Moist**	**Control**	***P* value**	**Field Moist**	**Control**	***P* value**	**Field Moist**	**Control**	***P* value**
	MW			**TM**			**OH**		
pH	6.3 ± 0.07	6.05 ± 0.38	0.623	5.9 ± 0.05	6.50 ± 0.09	**0.001**	6.0 ± 0.23	6.39 ± 0.18	0.283
NO_3-N (mg kg^{-1} soil)	55.3 ± 2.2	4.4 ± 0.7	**0.001**	12.0 ± 1.0	1.6 ± 0.1	**0.002**	43.1 ± 3.9	7.9 ± 1.4	**0.005**
NH_4-N (mg kg^{-1} soil)	70.8 ± 2.9	34.6 ± 4.5	**0.001**	85.0 ± 6.8	52.9 ± 3.7	**0.014**	74.5 ± 5.3	33.8 ± 4.4	**0.002**
DR (µg N_2O-N kg^{-1} soil hr^{-1})	19.09 ± 0.83	143.30 ± 16.26	**0.0001**	10.39 ± 0.88	107.36 ± 11.54	**0.0001**	5.9 ± 0.69	49.25 ± 7.84	**0.0001**
***Nir*S+*nir*K gene T-RFs #**	21.50 ± 3.0	24.25 ± 0.63	0.430	17.0 ± 2.6	5.75 ± 1.2	0.171	16.00 ± 0.58	8.25 ± 1.6	**0.020**
***Nos*Z gene T-RFs #**	10.25 ± 0.75	6.75 ± 0.25	0.210	7.25 ± 1.0	5.25± 12	0.383	5.75 ± 0.75	4.50± 0.50	0.224
***Nir*S+*nir*K gene copies g^{-1} soil**	$7.6\times10^{9}\pm1.1\times10^{9}$	$9.0\times10^{9}\pm1.4\times10^{9}$	0.472	$2.7\times10^{9}\pm1.4\times10^{9}$	$7.3\times10^{9}\pm1.4\times10^{9}$	0.071	$4.3\times10^{7}\pm1.1\times10^{7}$	$3.2\times10^{9}\pm3.1\times10^{8}$	**0.002**
***Nos*Z gene copies g^{-1} soil**	$2.5\times10^{8}\pm2.5\times10^{7}$	$3.9\times10^{8}\pm5.1\times10^{7}$	0.579	$6.2\times10^{7}\pm4.4\times10^{7}$	$2.0\times10^{8}\pm3.6\times10^{7}$	0.054	$7.2\times10^{6}\pm4.7\times10^{6}$	$2.7\times10^{7}\pm4.4\times10^{6}$	**0.029**

(n=4) All means are reported ± standard error of the mean (S.E.M.). *P* values suggest significance of 2 samples T-test.

Table 3: Soil characteristics after 24 hours of incubation following addition of treatments

	pH	NO_3-N (mg kg^{-1} soil)	NH_4-N (mg kg^{-1} soil)
MW (Control)	6.05 ± 0.38^{b}	4.4 ± 0.7^{c}	34.6 ± 4.5^{b}
MW (Urine)	6.74 ± 0.33^{ab}	29.3 ± 1.2^{a}	393.9 ± 118.3^{a}
MW (Urine +DCD)	7.37 ± 0.25^{a}	11.8 ± 2.3^{b}	456.5 ± 39.4^{a}
TM (Control)	6.50 ± 0.09^{b}	1.6 ± 0.1^{c}	52.9 ± 3.7^{b}
TM (Urine)	6.95 ± 0.06^{a}	28.4 ± 6.5^{a}	434.1 ± 82.3^{a}
TM (Urine +DCD)	7.05 ± 0.06^{a}	7.4 ± 0.7^{b}	418.0 ± 29.8^{a}
OH (Control)	6.39 ± 0.18^{b}	7.9 ± 1.4^{b}	33.8 ± 4.4^{c}
OH (Urine)	6.88 ± 0.13^{ab}	47.8 ± 8.5^{a}	258.6 ± 105.9^{b}
OH (Urine +DCD)	6.98 ± 0.14^{a}	6.3 ± 1.7^{b}	529.0 ± 45.7^{a}

(n=4) All means are reported ± standard error of the mean (S.E.M). Letters indicate differences in the means of treatments within each soil. Means sharing same letter are not significantly different. The letters indicate the differences in the values only within the block they are presented in.

DR and N_2O/ (N_2O+N_2) ratio before and after incubation

DR and DEA measured at time zero in the original field moist soils varied from 5.9 to 19.1µg N_2O-N kg^{-1} soil hr^{-1} and from 149.9 to 938.8µg N_2O-N kg^{-1} soil hr^{-1} respectively (Table 1). Both DR and DEA were highest in the Manawatu soil and lowest in the Otorohonga soil. After 24 hours of incubation a significant increase in DR in the control soil compared to the original field moist soil was observed. DR in the incubated control soil ranged from 46.9 to 143.3µg N_2O-N kg^{-1} soil hr^{-1}. These DRs in the saturated soils were 7-10 times greater than in the field moist soils (Table 2).

DR in the urine and urine + DCD treatments was also significantly higher than in the control treatments (Figure 1). Application of DCD with urine slightly reduced DR in soils compared to urine-only, but this reduction was not statistically significant in any of the soils. The N_2O/ (N_2O+N_2) ratio ranged from 0.07 to 0.32 in incubated soils (Table 4). There was higher N_2 production in urine and urine + DCD treatments in Manawatu and Tokomaru soils and no significant increase in N_2O emission with urine application compared to the control treatment. Consequently the N_2O/(N_2O+N_2) ratio was significantly lower in urine and urine + DCD treatments in Manawatu and Tokomaru soils. We did not observe any difference in the N_2O/ (N_2O+N_2) ratio among the three treatments in the incubated Otorohonga soil. Therefore either the N_2O emission with respect to total denitrification product was similar or there was similar increase in both N_2O and N_2 emissions in all the three treatments in the Otorohonga soil.

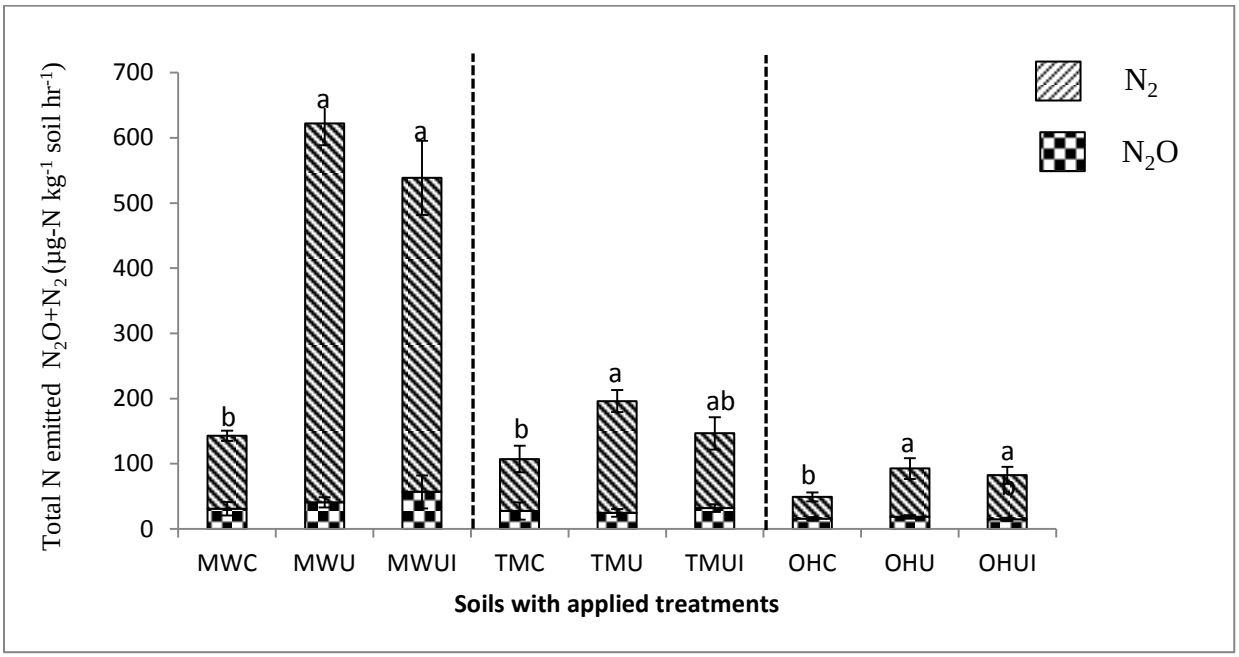


Figure 1: Denitrification rates of soils after incubation for 24 hours following application of treatments. Error bars denote S.E.M. Bars with the same letter are not significantly different. The letters indicate the differences in the means only within the section they are present in.

Table 4: N_2O/ (N_2O+N_2) ratio in soils after 24 hours of incubation following addition of treatments

Soil	Treatments	$N_2O/(N_2O+N_2)$
MW	Control	0.21 ± 0.04^a
	Urine	0.07 ± 0.01^b
	Urine + DCD	0.10 ± 0.05^b
TM	Control	0.26 ± 0.13^a
	Urine	0.12 ± 0.04^b
	Urine + DCD	0.22 ± 0.08^b
OH	Control	0.32 ± 0.05^a
	Urine	0.20 ± 0.03^a
	Urine + DCD	0.18 ± 0.05^a

(n=4) All means are reported ± standard error of the mean (S.E.M). Letters indicate differences in the means of treatments within each soil. Means sharing same letter are not significantly different. The letters indicate the differences in the values only within the block they are presented in.

Denitrifier community structure and abundance before and after incubation

Overall, in all the soils the numbers of *nir*S+*nir*K gene T-RFs were higher than the numbers of *nos*Z gene T-RFs. The numbers of *nir*S+*nir*K T-RFs varied from 16.0 to 21.5 and *nos*Z from 5.7 to 10.5 in field moist soils. In control saturated soils numbers of *nir*S+*nir*K gene T-RFs varied from 5.7 to 24.2 and *nos*Z T-RFs from 4.5 to 6.7. In incubated treatments the numbers of *nir*S+K and *nos*Z gene T-RFs varied from 4.7 to 24.5 and from 4.5 to 6.7 respectively in three soils (Figures 2a, b). The *nir*S+*nir*K gene T-RFs were higher in Manawatu and Tokomaru soils than in Otorohonga soil. In all the three soils the number of *nir*S+*nir*K gene copies varied from 4.3×10^{7} to 7.6×10^{9} g^{-1} soil in field moist soils and from 3.2×10^{9} to 9.0×10^{9} g^{-1} soil in saturated control soils. The *nir*S+*nir*K gene copies were higher than the *nos*Z gene copies which varied from 7.2×10^{6} to 2.5×10^{8} g^{-1} soil in field moist soils and from 2.7×10^{7} to 3.9×10^{8} g^{-1} in saturated control soils (Figures 3a, b &c). We observed lower numbers of *nir*S+*nir*K gene T-RFs in the control treatment than the field moist soil only in the Otorohonga soil. Similarly, we observed higher *nir*S+*nir*K and *nos*Z gene copies in the control than in the field moist treatment only in Otorohonga soil. In the other two soils we observed no difference in either numbers of denitrifier genes T-RFs or gene copies with saturation. In saturated soils with applied treatments the *nir*S+*nir*K gene copies varied form 3.2×10^{9} to 2.0×10^{10} g^{-1} soil. Similarly the *nos*Z gene copies varied from 2.7×10^{7} to 3.9×10^{8} g^{-1} soil with the lowest number in Otorohonga control soil and highest in Manawatu urine+DCD.

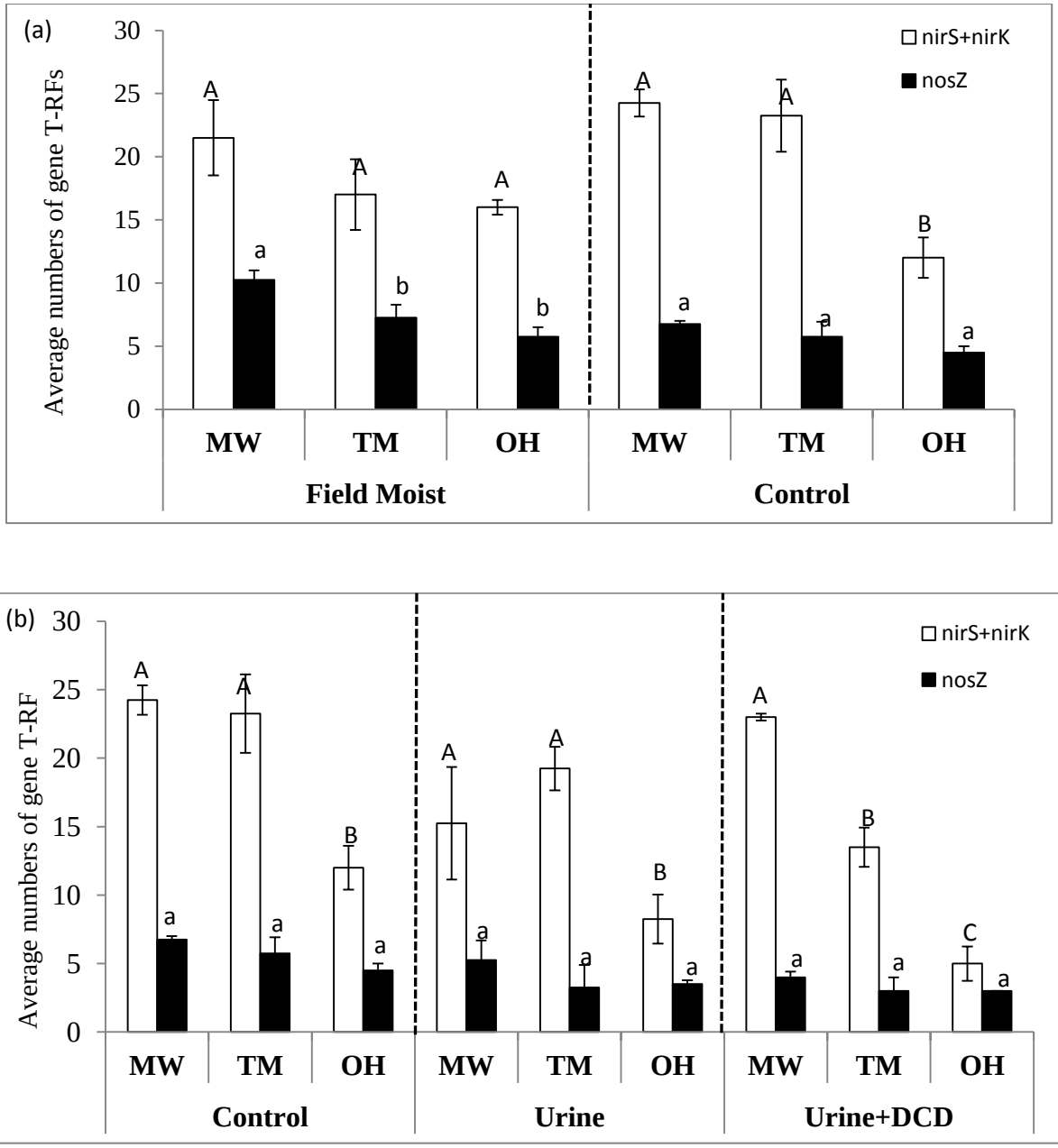
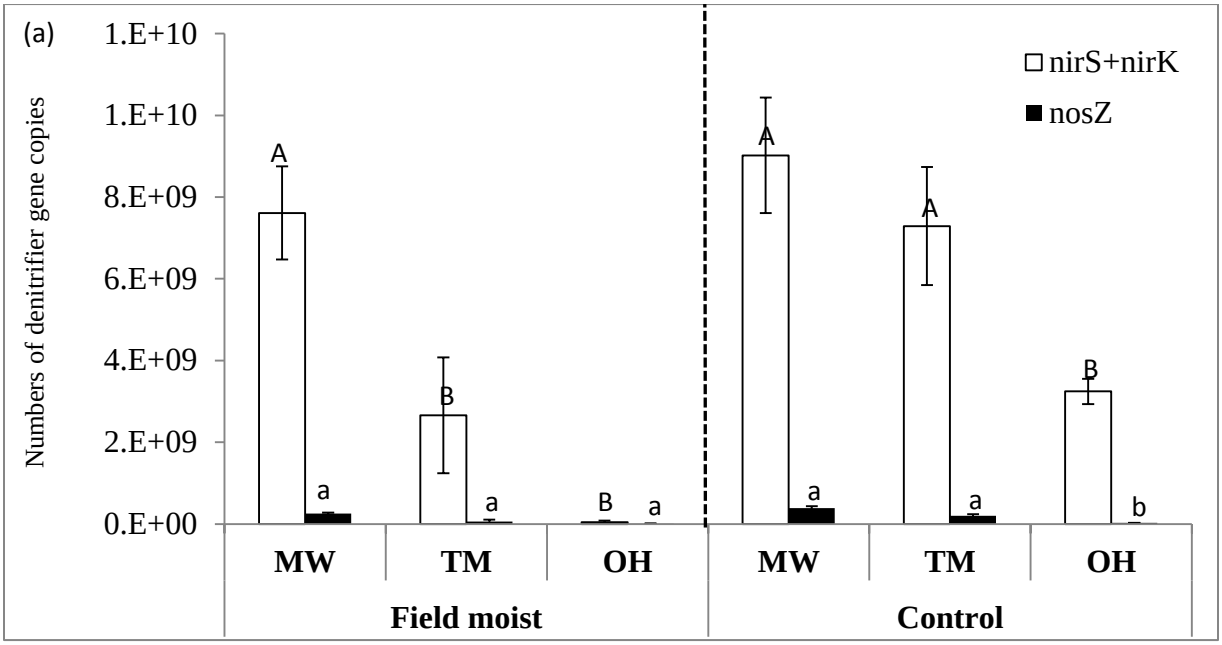
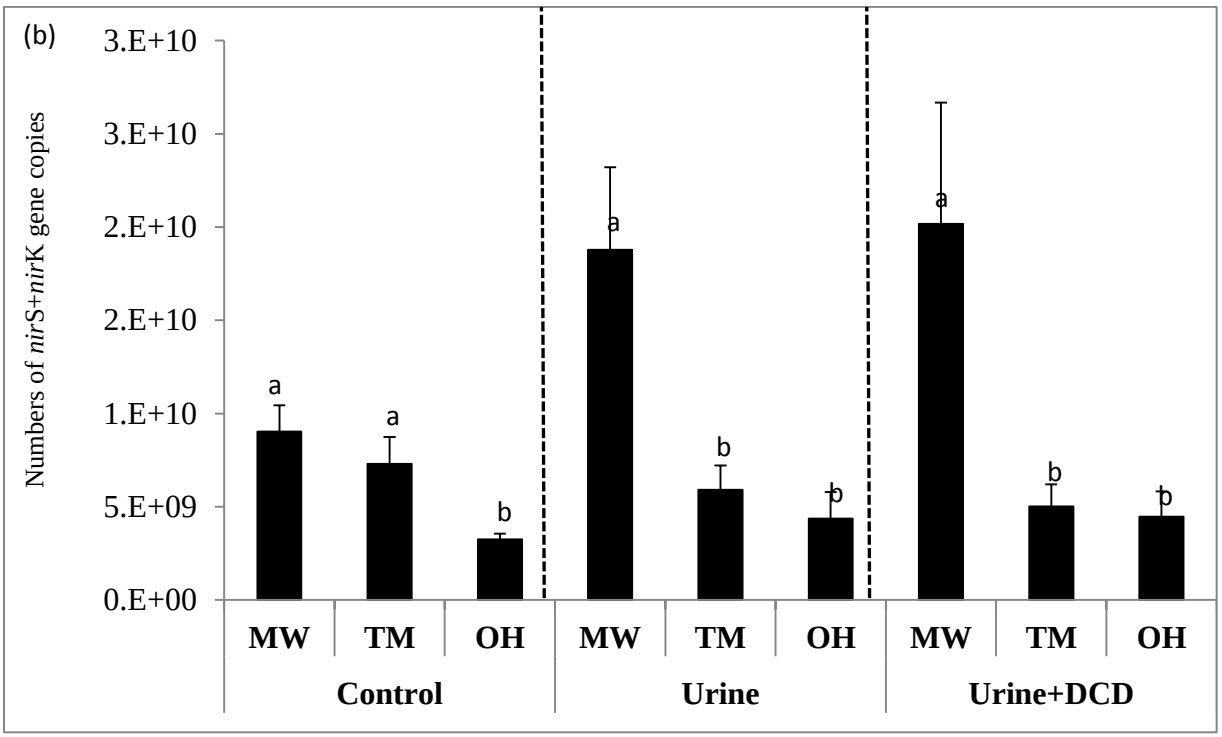


Figure 2: Numbers of denitrifier gene T-RF in soils (a) original field moist and control treatment (b) three saturated treatments. Error bars denote S.E.M. Bars with same letter values are not significantly different. Letter values indicate differences in means only in the section they are displayed in. Letters with same font represent one test.


(a)
Numbers of denitrifier gene copies
1.E+10
1.E+10
8.E+09
6.E+09
4.E+09
2.E+09
0.E+00
nirS+nirK
nosZ
A
a
B
a
B
a
A
a
A
a
B
b
MW
TM
OH
MW
TM
OH
Field moist
Control


(b)
Numbers of nirS+nirK gene copies
3.E+10
3.E+10
2.E+10
2.E+10
1.E+10
5.E+09
0.E+00
a
a
b
a
b
b
a
b
b
MW
TM
OH
MW
TM
OH
MW
TM
OH
Control
Urine
Urine+DCD

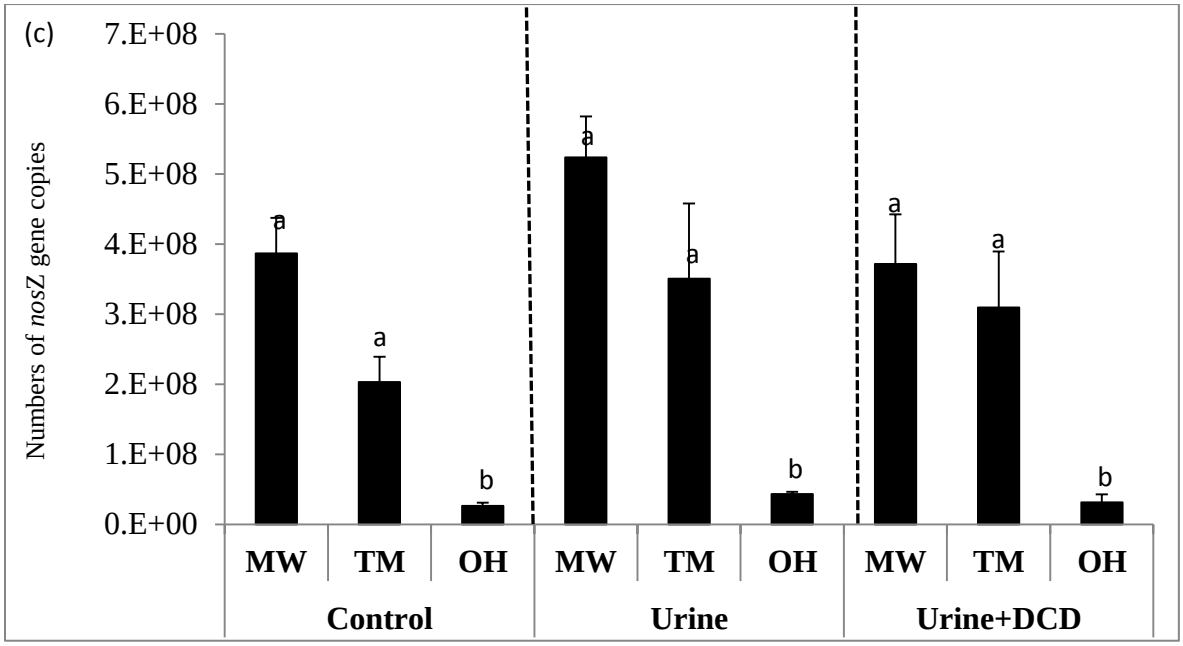


Figure 3: Abundance of denitrifier gene copies in soils after incubation for 24 hours after application of treatments (a) in field moist and control soils (b) nirS+nirK gene copy numbers in treated soils (c) nosZ gene copy numbers in treated soils. Error bars denote S.E.M. Bars with same letter values are not significantly different. Letter values indicate differences in means only in each section they are displayed in.

Table 5: Two way analysis of variance p-values of soil characteristics in different soil types receiving different treatments:

Source	**Soil pH**	**NO_3^-N**	**NH_4-N**	**DR**	***Nos*Z gene copies**	***Nir*S+K gene copies**	***Nos*Z gene T-RFs**	***Nir*S+K gene T-RFs**
Soil Type	0.797	0.001	0.856	0.0001	0.0001	0.0001	0.069	0.0001
Treatments	0.0001	0.0001	0.0001	0.0001	0.008	0.285	0.012	0.004
Interaction	0.386	0.0001	0.272	0.0001	0.180	0.174	0.084	0.893

Effect of soil type and treatments on DR, soil chemical characteristics and molecular parameters after 24 hours of incubation with applied treatments

The influence of soil, applied treatments and the interaction of soil and treatments varied among the soil parameters measured (Table 5). The analysis suggested that the soils, treatments and their interactions had significant effects on the DR and NO_3-N contents in these soils. This implied that both DR and NO_3-N contents responded to the applied treatments (control, urine and urine+DCD) differently in the three soils. The DR in Manawatu and Tokomaru soils was significantly higher than in Otorohonga soil. Also the DR in urine and urine + DCD treatments was higher than in the control treatments in all the soils.

The soil and treatments effects, but not their interactions, were significant for the numbers of *nos*Z gene copies and *nir*S+*nir*K T-RFs. The numbers of *nos*Z gene copies and *nir*S+*nir*K T-RFs exhibited similar trends in all the three soils, with the applications of the various treatments. The numbers of *nos*Z gene copies and *nir*S+*nir*K T-RFs were higher in Manawatu and Tokomaru soils than in Otorohonga soil. Also these numbers were higher in urine treatments than the control. The *nir*S+*nir*K gene copy numbers varied among the three soils with higher numbers of gene copies in Manawatu soil than in Tokomaru and Otorohonga soils. The effect of treatments, or the interaction of treatments with soils, was not significant for *nir*S+*nir*K gene copy numbers. The treatments (but not the soils or the interaction between soils and treatments) had a significant effect on soil pH, NH_4-N content and the number of *nos*Z gene T-RFs. Soil pH and NH_4-N contents were higher in urine-applied soils. The numbers of *nos*Z gene T-RFs were lower in urine + DCD applied soils than the control treatments in all the three soils.

Effect of soil characteristics on DR, N_2O and N_2 emissions and $N_2O/(N_2O+N_2)$ ratio after 24 hours of incubation of soils with applied treatments.

The correlation analysis (Table 6) illustrated the relationships between the soil characteristics (NH_4-N content, denitrifier gene distribution and abundance) DR, N_2O and N_2 emissions. Denitrfication rate following 24 hour incubation of treated soils was significantly and positively correlated to *nir*K gene copy numbers, *nir*S+*nir*K gene copy numbers, *nos*Z gene copy numbers and soil NH_4-N content. The N_2 emitted during denitrification was positively correlated to *nir*S+*nir*K, *nos*Z and *nir*K gene copy numbers. The N_2O emitted during denitrification was positively correlated with numbers of *nir*S+*nir*K gene copies and *nir*S+*nir*K T-RFs present in the incubated soils. The N_2O molar ratio [$N_2O/(N_2O+N_2)$] was positively correlated to (*nir*S+*nir*K)/ total denitrifier gene copies measured in the incubated soils and negatively correlated to *nos*Z gene copies. The numbers of nosZ gene T-RFs were negatively correlated to soil NH_4-N content.

Table 6: Significant Pearson's correlation coefficients between soil characteristics and denitrifier gene distribution and abundance in soils after incubation for 24 hours after application of treatments

	Variable	Correlation coefficient (r)	*p*
DR	*nir*K gene copy numbers	0.804	0.0001
	*nir*S+*nir*K gene copy numbers	0.800	0.0001
	*nos*Z gene copy numbers	0.683	0.0001
	Soil NH_4^+ content	0.357	0.0320
N_2 emissions	*nir*S+*nir*K gene copy numbers	0.774	0.0001
	*nos*Z gene copy numbers	0.665	0.0001
	*nir*K gene copy numbers	0.776	0.0001
N_2O emissions	*nir*S+*nir*K gene copy numbers	0.548	0.0010
	*nir*S+*nir*K T-RF numbers	0.547	0.0001
N_2O/N_2O+N_2	*(nir*S+*nir*K) / *(nir*S+*nir*K+*nos*Z) gene copy numbers	0.316	0.0050
	*nos*Z gene copy numbers	-0.331	0.0490
*nos*Z T-RF numbers	Soil NH_4-N content	-0.460	0.005

Discussion

Recovery of Applied N

The three soils in this study were selected to have different soil chemical characteristics (NO_3-N, TC, TN, Olsen P, MBC, DEA and DR) (Table 2). The effect of cattle urine and the hydrolysis of urea in the applied urine in increasing NO_3-N and NH_4-N, compared to the incubated control treatment were significant in all the three soils. Similar effects of application of cattle urine to soil have been suggested by Haynes & Williams (1993) and Lovell & Jarvis (1996). The amount of N in urine applied soils, (measured as NO_3-N and NH_4-N contents) was lower (306-535 mg N kg^{-1} dry soil) as compared to the amount of N (700 mg N kg^{-1} dry soil) applied as cattle urine at the start of the incubation and was similar in all the three soils. The low recovery of N as NO_3-N and NH_4-N in urine treated soils could be because the urea present in the cattle urine was not completely hydrolyzed in 24 hours. The hydrolysis of urea might have been slow during the beginning of the incubation, as suggested by Hongprayoon *et al.* (1991), and might increase with incubation time. The reduced oxygen content under saturated conditions might also retard urea hydrolysis (Sahrawat, 1984; Savant *et al.*, 1985).

Effect of saturation on soil characteristics, DR, denitrifier community structure and size

The decrease in mineral N contents in these soils with saturation and incubation suggested both nitrification and denitrification were occurring with the increase in soil water content. The increase in DR with increasing soil water content was consistent with the work done by Grundmann & Rolston (1987) and Ruz- Jerez *et al.* (1994) who have suggested that soil water is the major factor influencing the rate of denitrification. Similarly, higher DRs in saturated soils were also reported in our earlier study (Jha *et al.*, 2012). The higher soil water content with anaerobic conditions might have activated the N_2O reductase enzymes facilitating reduction of N_2O to N_2, thus increasing total denitrification in saturated soils. We observed higher numbers of copies of *nir*S+*nir*K and *nos*Z genes in the control treatment than in the field moist Otorohonga soil. The higher carbon content in this soil compared to the other two soils might be influencing the denitrifier community at saturation. Miller *et al.* (2008) also reported higher denitrifier abundances in soils with higher carbon contents.

The effect of applied DCD on nitrification

Application of DCD appeared to have restricted the conversion of NH_4^+ to NO_3^- during incubation, thus restricting the amounts of NO_3^-N in soils to which DCD had been applied. The lower NO_3-N contents in the control incubated soils (as compared to the original field moist soils) suggest NO_3^- was denitrified in the saturated conditions. The higher NO_3-N contents in urine treatments may have resulted from the nitrification of ammonium produced from hydrolysis of urea in the urine and the lower NO_3-N contents in urine + DCD treatments could be due to inhibition of nitrification by DCD in all the three soils. This was consistent with our hypothesis that DCD application would restrict the NO_3-N content in urine applied soils. When the individual soils were considered, the DCD application resulted in significantly higher NH_4-N contents in the urine + DCD treatment than in the urine-only treatment in Otorohonga soil (Table 3). This implies that nitrification occurring in Otorohonga soil was inhibited by DCD. In contrast, nitrification rates in the urine-only treatments in Manawatu and Tokomaru soils were already much lower than in the Otorohonga soil and therefore in these soils there was no significant difference in NH_4-N content between urine and urine + DCD treatments. Di *et al.* (2007) have reported significant decreases in nitrification, and consequent retention of NH_4-N in soils applied with DCD.

The effect of urine and DCD addition on denitrification

We found a significant effect of urine addition on DR. Within 24 hours a 2-6 fold increase in DR compared to the controls (water only) was observed. The rate magnitude of this increase varied among the soils. This result is in agreement with the increase in denitrification activity with addition of artificial urine treatments reported by Carter *et al*. (2007). Urine application provided additional C, N, an increase in pH and together with the existing saturated soil water content created ideal anaerobic conditions for an increase in denitrification rates in these soils. de Klein & van Logtestijn, (1994) have reported that denitrification may increase by over 0.6g N m^{-2} d^{-1} following urine deposition.

We hypothesized that DCD application would reduce DR in soils however, our hypothesis did not hold true and the DCD application was not effective in restricting denitrification after 24 hours of incubation following treatment application. It could be possible that DCD might not be effective instantaneously and we might observe an increased effectiveness of DCD in controlling DR in subsequent measurements during incubations.

The sources of N_2O and N_2 emissions

We have noticed differences among the three soils in the relative production of N_2O and N_2 during denitrification in the incubated treatments (Figure 1). Studies have reported higher N_2O emissions with urine application to soil (Luo *et al.*, 2008) and reductions 60-85% in N_2O emissions with DCD application to urine applied soils (de Klein & Eckard, 2008; Di *et al.*, 2007). The higher N_2O emissions reported in studies with urine application is mostly due to nitrification occurring under aerobic conditions (Uchida *et al.*, 2012) as a result of the higher NH_4^+ availability (Bremner & Blackmer, 1978). In contrast to these previous results we found no significant increase in N_2O emission with urine application or reduction in N_2O emission with DCD application to urine treated soils. We found higher N_2 emissions than N_2O which is due to activation of reductase enzymes in the anaerobic conditions and subsequent reduction of more N_2O to N_2 in incubated soils. Monaghan & Barraclough (1993) also detected immediate large emissions of N_2 with application of urine to soil. Using a ^{15}N technique Panek *et al.* (2000) reported that after application of either ammonium ^{15}N or nitrate ^{15}N, N_2O production was mainly derived from denitrification, immediately after irrigation and mainly derived from nitrification as the soil drained.

High N_2O emissions after urine applications have been recorded by Van Groenigen *et al.* (2005) only in soils with 60-70% WFPS and low emissions have been observed at both higher and lower WFPS. The soil/treatments in our experiment were at saturation soil water content and therefore there was no opportunity for N_2O production through nitrification and at the same time most of the N_2O formed during denitrification might have been reduced to N_2. As a result, little increase in N_2O emissions with urine application and no reduction in N_2O emissions due to DCD application to soils were observed. The measurements taken in our experiment were only after 24 hours of incubation. We might expect an increase in N_2O emissions in urine-only treatments with increasing incubation time. Monaghan & Barraclough (1993) also observed higher emissions of N_2 than N_2O from soil with applied urine one day after the urine application and from then on the N_2 emissions decreased and N_2O emissions increased over the 30-day incubation period - probably due to nitrification of the higher available NH_4-N contents in the soil and the onset of aerobic conditions.

The soil characteristics affected by application of urine and DCD

The application of treatments had a variable influence on soil characteristics. In the urine + DCD treatment in Manawatu soil, we have found higher pH values than in the urine + DCD treatments in other two soils. In the Manawatu soil the number of *nir*S+*nir*K T-RFs were similar in the control and urine + DCD treatments. However, the numbers of gene copies of *nir*S+*nir*K were higher in the urine + DCD treatment than in the control. The *nir*S+*nir*K gene copy numbers were

either same or lower in urine and urine + DCD treatments than in control in other two soils. These differences in Manawatu from Tokomaru and Otorohonga might have led to observed variations in DR under similar conditions (Figure1).

With higher NH_4-N contents in urine and urine + DCD treatments - especially in Otorohonga soil - the numbers of denitrifier gene (*nir*S+*nir*K and *nos*Z) T-RFs were lower than control soil. In our previous study Jha *et al.* (2013) we reported a significant negative correlation between numbers of denitrifier genes (*nir*S+*nir*K and *nos*Z) T-RFs and soil NH_4-N contents in NZ dairy pasture soils. With increase in soil NH_4^+ content, ammonia oxidizing bacteria (AOB) and archea (AOA) might have increased in number. The increasing numbers of AOB and AOA might have generated some competition for the bacterial denitrifier communities and thus later became sensitive to soil NH_4-N content. This suggests there could be many denitrifying bacteria present in these soils before the start of incubation and with the onset of anaerobic condition only the dominant phylotypes that were resistant to quick biochemical changes increased in number and were observed. The abundance of total gene copies of denitrifier genes was significantly higher in saturated and incubated soils than in the field moist soils. The higher soil pH and NH_4-N contents in anaerobic incubated soils seem to relate to the higher denitrifier gene copies (*nir*S+*nir*K and *nos*Z) found in incubated soils with applied urine and urine + DCD.

Denitrifier gene abundance (*nir*S+*nir*K and *nos*Z) was related to total denitrification and emissions of N_2O and N_2 during denitrification in soils with applied treatments. The significant correlation of N_2O emissions with denitrifier gene abundances suggested that in these soils either both *nir*S+*nir*K and *nos*Z genes were present on the same bacterial community or the two types of bacteria coexist in similar conditions to complete the denitrification process. There could be a strong possibility that the N_2O produced by NO_2^- reducing bacteria might have been taken up by N_2O reducing bacteria. In a long-term experiment Chen *et al.* (2012) showed a considerable effect of mineral fertiliser application with and without rice straw on denitrifier gene (*nar*G, *qnor*B, *nir*S, *nir*K and *nos*Z) abundance in paddy soil. This was attributed to higher substrate availability through fertilisation encouraging denitrifiers to flourish and thus increased denitrifier abundance. They also found that potential denitrification activity significantly correlated positively with denitrifier gene abundance (*nar*G, *nir*K, *nir*S and *nos*Z) in fertilized plots which suggests the anaerobic conditions in paddy soils are favourable for increasing denitrifier genes and their activities. Similarly, Miller *et al.* (2009) have reported higher *nos*Z gene abundance in soils treated with liquid manure than in the untreated soils.

Conclusions

Application of cattle urine and bringing the soils to saturation water content increased the overall DR and decreased the $N_2O/(N_2O+N_2)$ ratio after 24 hours of application of treatments. The increase in total denitrification was influenced by the higher soil mineral-N contents (NO_3^- and NH_4^+) with urine application. The urine application influenced the bacterial denitrifier community structure and the gene copy numbers of dominant species increased in urine applied soils and thus affected the DR. The denitrifier gene abundance was higher in urine applied soils and correlated with the DRs, N_2O and N_2 emissions. The application of DCD did not significantly affect DR in soils after 24 hours of application of treatments however it did

influence N_2O and N_2 emission during denitrification in soils. This paper only reports the changes in soil mineral-N, pH, N_2O emissions, and denitrification and denitrifier gene abundance within 24 hours of soil saturation and addition of urine or urine + DCD. There might not have been complete urea hydrolysis during the first 24 hours. There could be ongoing N transformations in the three soils with longer incubations and these might influence soil conditions accordingly. The changes in denitrifier gene abundance, denitrification rates and N_2O emissions associated with amended soils may vary with incubation time. Measurements after longer times of incubation are currently underway. Therefore the results reported in this paper should be regarded as preliminary, and interpreted with caution.

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References

Bremner, J. M., & Blackmer, A. M. (1978). Nitrous oxide emission from soils during nitrification of fertilizer nitrogen. *Science. USA., 199*, 295-296.

de Klein, C. A. M., & Eckard, R. J. (2008). Targeted technologies for nitrous oxide abatement from animal agriculture. *Australian Journal of Experimental Agriculture, 48*(1-2), 14-20.

Di, H. J., & Cameron, K. C. (2006). Nitrous oxide emissions from two dairy pasture soils as affected by different rates of a fine particle suspension nitrification inhibitor, dicyandiamide. *Biology and Fertility of Soils, 42*(6), 472-480.

Di, H. J., & Cameron, K. C. (2008). Sources of nitrous oxide from ^{15}N-labelled animal urine and urea fertiliser with and without a nitrification inhibitor, dicyandiamide (DCD). *Australian Journal of Soil Research, 46*, 76-82.

Di, H. J., Cameron, K. C., & Sherlock, R. R. (2007). Comparison of the effectiveness of a nitrification inhibitor, dicyandiamide, in reducing nitrous oxide emissions in four different soils under different climatic and management conditions. *Soil Use and Management, 23*(1), 1-9. doi: 10.1111/j.1475-2743.2006.00057.x

Grundmann, G. L., & Rolston, D. E. (1987). A water function approximation to degree of anaerobiosis associated with denitrification. *Soil Science, 144*(6), 437-441.

Haynes, R. J., & Williams, P. H. (1993). Nutrient cycling and soil fertility in grazed pastue ecosystem. *Adv. in Agronomy, 49*, 119-199.

Hongprayoon, C., Lindau, C. W., Patrick, W. H., Jr., Bouldin, D. R., & Reddy, K. R. (1991). Urea transformations in flooded soil columns. I. Experimental results. *Soil Science Society of America Journal, 55*(4), 1130-1134.

Jha, N., Deslippe, J., Saggar, S., Tillman, R., & Giltrap, D. (2013). Measuring bacterial denitrifier genes distribution and abundance in New Zealand dairy-grazed pasture soils. In L. D. Currie & C. L. Christensen (Eds.), *Accurate and efficient use of nutrients on farms*. Occasional Report No. 26. Fertilizer and Lime Research Centre http://flrc.massey.ac.nz/publications.html.Massey University, Palmerston North, New Zealand.

Jha, N., Saggar, S., Tillman, R. W., & Giltrap, D. (2012). Changes in denitrification rate and N_2O/N_2 ratio with varying soil moisture conditions of New Zealand Pasture soils. In L. D. Currie & C. L. Christensen (Eds.), *Advanced nutrient management: Gains from the past-Goals for the future. Fertilizer and Lime Research Centre Occasional Report No. 25, Massey University Palmerston, North New Zealand*.

Lovell, R. D., & Jarvis, S. C. (1996). Effects of urine on soil microbial biomass, methanogenesis, nitrification and denitrification in grassland soils. *Plant and Soil, 186*(2), 265-273.

Luo, J., de Klein, C. A., Shepherd, M. A., & Ledgard, S. (2010). *Effect of nitrification inhibitor on nitrous oxide emissions in pasture soils.* Paper presented at the 19th World Congress of Soil Science, Soil Solutions for a Changing World, 1-6 August 2010, Brisbane, Australia.

Luo, J., Lindsey, S. B., & Ledgard, S. F. (2008). Nitrous oxide emissions from animal urine application on a New Zealand pasture. *Biology and Fertility of Soils, 44*(3), 463-470. doi: 10.1007/s00374-007-0228-4

Miller, M. N., Zebarth, B. J., Dandie, C. E., Burton, D. L., Goyer, C., & Trevors, J. T. (2008). Crop residue influence on denitrification, N_2O emissions and denitrifier community abundance in soil. *Soil Biology and Biochemistry, 40*(10), 2553-2562.

Monaghan, R. M., & Barraclough, D. (1993). Nitrous oxide and dinitrogen emissions from urine-affected soil under controlled conditions. *Plant and Soil, 151*(1), 127-138.

Panek, J. A., Matson, P. A., Ortíz-Monasterio, I., & Brooks, P. (2000). Distinguishing nitrification and denitrification sources of N_2O in a Mexican wheat system using ^{15}N. *Ecological Applications, 10*(2), 506-514.

Ruz- Jerez, B. E., White, R. E., & Ball, P. R. (1994). Long term measurement of denitrification in three contrasting pastures grazed by sheep. *Soil Biology and Biochemistry, 26*, 29-39.

Sahrawat, K. L. (1984). Effects of temperature and moisture on urease activity in semi-arid tropical soils. *Plant and Soil, 78*, 401-408.

Savant, N. K., James, A. F., & McClellan, G. H. (1985). Effect of Soil Submergence on Urea Hydrolysis. *Soil Science, 140*(2), 81-88.

Uchida, Y., von Rein, I., Akiyama, H., & Yagi, K. (2012). Contribution of nitrification and denitrification to nitrous oxide emissions in Andosol and from Fluvisol after coated urea application. *Soil Science and Plant Nutrition, 59*(1), 46-55. doi: 10.1080/00380768.2012.708646

Van Groenigen, J. W., Velthof, G. L., Van Der Bolt, F. J. E., Vos, A., & Kuikman, P. J. (2005). Seasonal variation in N_2O emissions from urine patches: Effects of urine concentration, soil compaction and dung. *Plant and Soil, 273*, 15-27.