A lysimeter study of nitrate leaching from grazed grassland as affected by a nitrification inhibitor, dicyandiamide, and relationships with ammonia oxidizing bacteria and archaea

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Abstract

Nitrate (NO_3^-) can contribute to surface water eutrophication and is deemed harmful to human health if present at high concentrations in the drinking water. In grazed grassland, most of the NO_3^- -N leaching occurs from animal urine-N returns. The objective of this study was to determine the effectiveness of a nitrification inhibitor, dicyandiamide (DCD), in decreasing NO_3^- leaching in three different soils from different regions of New Zealand under two different rainfall conditions (1260 mm and 2145 mm p.a.), and explore the relationships between NO_3^- -N leaching loss and ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA). The DCD nitrification inhibitor was found to be highly effective in decreasing NO_3^- -N leaching losses from all three soils under both rainfall conditions. Total NO_3^- -N leaching losses from the urine patch areas were decreased from 67.7–457.0 kg NO_3^- -N/ha to 29.7–257.4 kg NO_3^- -N/ha by the DCD treatment, giving an average decrease of 59%. The total NO_3^- -N leaching losses were not significantly affected by the two different rainfall treatments. The total NO_3^- -N leaching loss was significantly related to the *amoA* gene copy numbers of the AOB DNA and to nitrification rate in the soil but not to that of the AOA. These results suggest that the DCD nitrification inhibitor is highly effective in decreasing NO_3^- leaching under these different soil and rainfall conditions and that the amount of NO_3^- -N leached is mainly related to the growth of the AOB population in the nitrogen rich urine patch soils of grazed grassland.

Keywords: Nitrate leaching, nitrification inhibitor, ammonia oxidizing bacteria, ammonia oxidizing archaea, grazed grassland

Introduction

Nitrate (NO_3^-) can contribute to surface water eutrophication and is deemed harmful to human health if present at high concentrations in drinking water (WHO, 1984). A major source for NO_3^- in water bodies around the world is that leached from agricultural land. In grazed grassland where animals graze outdoor pastures, most of the NO_3^- -N leached is from urine-nitrogen (N) deposited by the animal during grazing (Scholefield *et al.*, 1993; Ledgard *et al.*, 1999; Di & Cameron, 2002a). When animals graze pastures, between 70 and 90% of the N ingested is deposited on the pasture soil

in the dung and urine and about 70% of this N is in the urine (Jarvis *et al.*, 1995; Di & Cameron, 2002a). The N loading rate under a dairy cow urine patch can be as high as 1000 kg N/ha. Most of the N in the urine is urea which, upon deposition in the soil, is quickly hydrolysed to ammonium (NH_4^+) and then oxidized to NO_3^- . The large amount of NO_3^- produced under the animal urine patches is prone to leaching when there is drainage, particularly during the cold winter period when pasture growth and N uptake is minimal, evapotranspiration is low and drainage is high (Di & Cameron, 2002a).

A number of best management practices have been proposed to mitigate NO_3^- leaching from agricultural land (Addiscott, 1996; Di & Cameron, 2002a). However, the mitigation of NO_3^- leaching from animal urine patches in grazed

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pastures is a major challenge because animal urine is deposited randomly in the grazed field at different times of year after each grazing rotation. A major development in recent years has been the use of nitrification inhibitors to treat grazed pasture soils to mitigate NO_3^- leaching (Di & Cameron, 2002b, 2007). The nitrification inhibitor used in these studies, dicyandiamide (DCD), inhibits the first step of the nitrification process, that is the conversion from NH_4^+ to hydroxylamine. This keeps the N in the NH_4^+ form which can be held on the soil cation exchange complex and used for plant growth. DCD inhibits the nitrification process by deactivating the active site of the ammonia monooxygenase (AMO) enzyme in the ammonia oxidizing microbes. DCD is bacteriostatic and is not a biocide: it leaves no long-term residues in the soil (Amberger, 1989).

Despite the importance of the nitrification process in N cycling and in influencing NO_3^- leaching, it remains unclear if this process is driven by the ammonia oxidizing bacteria (AOB) or ammonia oxidizing archaea (AOA) or both. Recently, AOA has been shown to be present in different environments, including the ocean (Francis *et al.*, 2005; Könneke *et al.*, 2005; Wuchter *et al.*, 2006), sediments (Schleper *et al.*, 1997) and soils (Leininger *et al.*, 2006; He *et al.*, 2007; Chen *et al.*, 2008; Shen *et al.*, 2008), suggesting a potential role for AOA in nitrification in these ecosystems (Nicol & Schleper, 2006; Prosser & Nicol, 2008). Our latest research has shown that in the high-N-loading urine patch areas of grazed grassland, the nitrification rate is more closely related to the dynamics of the AOB populations (Di *et al.*, 2009). However, the relationships between the dynamics of AOB and AOA and NO_3^- leaching losses are unknown. There is also insufficient information published on the effectiveness of

nitrification inhibitors, such as DCD, in decreasing NO_3^- leaching in different soil and environmental conditions, particularly under different rainfall conditions. It is not clear if different rainfall inputs may affect the transport and distribution of nitrogen and DCD in the soil thereby affecting the DCD effectiveness in decreasing NO_3^- leaching.

The objectives of this study were therefore to determine the effectiveness of the nitrification inhibitor, DCD, in reducing NO_3^- leaching in three grazed grassland soils under two rainfall conditions, and to explore relationships between NO_3^- leaching losses and AOB and AOA populations and nitrification rates.

Materials and methods

Soils and pasture

Three soils, from Canterbury (CT), the West Coast (WC) of the South Island, and Southland (SL) of New Zealand were used in this study (Table 1). The three soils differ significantly in their properties. The CT Lismore soil (NZ Classification: Pallic Orthic Brown Soil, Hewitt, 1998; USDA Soil Classification: Udic Haplustept loamy skeletal, Soil Survey Staff, 1998) is developed from gravelly glacial outwash covered with varying depths of loess. It has only a shallow layer of fine soil at the surface, below which the gravel content increases significantly, thus making this soil very free-draining. The WC Harihari soil (NZ classification: Mottled Fluvial Recent; USDA: Aquents) is a silt loam soil with a high silt content. The land at the site had been re-modelled to create 'humps' and 'hollows' to increase drainage, which is typical of local practise. The lysimeters were collected at the

Table 1 Properties of soils used for this study (0–0.1 m)

Location	Canterbury (CT) 43°45'54"S; 171°41'25"E	West Coast (WC) 42°42'12"S; 171°26'43"E	Southland (SL) 46°19'21"S; 168°16'02"E
Soil type	Lismore stony silt loam	Harihari recent silt loam	Mataura recent sandy loam
Particle size (%)			
Sand	10.5	4.2	69.1
Silt	66.0	93.6	29.0
Clay	23.5	2.2	1.9
pH (H ₂ O)	5.9	5.4	5.6
Organic C (g/kg)	36.5	68.8	62.6
Total N (g/kg)	3.5	5.6	5.7
Olsen P (mg/kg)	53.0	46.5	21.1
CEC (cmol _c /kg)	17.5	15.0	19.0
Exch. Ca ²⁺ (cmol _c /kg)	10.3	5.0	6.7
Exch. Mg ²⁺ (cmol _c /kg)	0.53	0.94	0.9
Exch. K ⁺ (cmol _c /kg)	0.71	0.25	0.24
Base saturation (%)	66.0	40.4	42.5

'hump' or shoulder places. The SL Matura soil is a recent sandy loam (NZ classification: Typic Fluvial Recent; USDA: Fluvents) developed in alluvium derived from schist and greywacke rock with a high sand content. The pasture on all three soils was a mixture of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) used for dairy grazing.

Large undisturbed soil monolith lysimeters (0.5 m diameter and 0.7 m deep) were collected from each of the three sites. The collection of the lysimeters involved placing a metal cylinder casing on the soil surface, digging around the casing, and gradually pushing the casing down by small increments (Cameron *et al.*, 1992). Once the casing had reached the desired depth, the soil monolith was cut at the base with a cutting plate, secured on the lysimeter casing and lifted out of the collection site. The gap between the soil core and the casing was sealed using petroleum jelly to stop edge-flow effects. The lysimeters from the three sites were taken to and installed at a field lysimeter facility at Lincoln University which is about 20 km south of Christchurch on the east coast of the South Island. The lysimeters were installed on either side of a trench with the soil surface of the lysimeters at the same level as the surrounding field. The space outside the lysimeter casings was backfilled with soil to the same level as the surface of the lysimeters and the surrounding field. The lysimeters were thus exposed to the same climatic conditions as the soil and pasture in the surrounding field.

Treatments

For each soil type, there were two main treatments: urine; and urine plus soil treatment with the nitrification inhibitor DCD, and each treatment had four replicates. Fresh dairy cow urine was collected from lactating cows, analysed for total N concentrations and applied to the lysimeters at the equivalent rate of 1000 kg N/ha in May to simulate a typical dairy cow urine deposition (2 L of urine) (Di & Cameron, 2002a). The DCD was dissolved in water and applied to the lysimeters at 10 kg/ha after the urine application on the same day in May and again in August. The two DCD applications, one in the autumn and one in late winter/early spring followed standard recommendations in New Zealand to reduce nitrate leaching during this high drainage period. Urea fertilizer was applied to all the lysimeters at the annual rate of 200 kg N/ha, split into eight applications throughout the year, to simulate local practice of applying urea to the whole of grazed pasture including urine patch and non-urine patch areas.

Two rainfall conditions were created to test the influence of rainfall inputs on NO_3^- leaching. This was done by applying simulated rainfall to supplement natural rainfall to create 1100 mm and 2200 mm of annual rainfall. Simulated rainfall was applied using automated rainfall simulators. The amount of rainfall applied varied in different seasons with a greater amount applied during the summer to meet the higher water

demand by plants because of greater evapotranspiration. For the 1100 mm condition, total rainfall during the winter was to reach the 75th percentile of rainfall records during this period, and summer rainfall was to equal the total water input of rainfall plus irrigation normally applied in the Canterbury region where this research was conducted. The amount of water applied for the 2200 mm condition was simply to approximately double that of the 1100 mm water input in the different seasons to create two significantly different rainfall treatments. These two rainfall treatments would cover the range of rainfall conditions in the main dairy farming regions of New Zealand.

Pasture was cut to typical grazing heights and removed before the application of the treatments. Thereafter it and was cut at typical grazing intervals.

Leachate collection and analysis

Leachates from the lysimeters were collected as required (i.e. when the volume of drainage from the lysimeters was above 200 mL or weekly) for chemical analyses. The leachates were analysed for NO_3^- , nitrite (NO_2^-), and NH_4^+ concentrations by flow injection analysis (FIA) (Tecator Inc., Sweden).

AOB and AOA populations and nitrification rate

In order to explore the relationships between NO_3^- leaching, AOB and AOA populations and nitrification rate in the soil, a companion laboratory incubation study was conducted to quantify the dynamics of AOB and AOA in the soil and nitrification rate. Details of this study have been described by Di *et al.* (2009). Soils collected from the same areas as the lysimeters were treated with animal urine-N with and without DCD in a similar way to those described above and were incubated under simulated autumn-winter-spring conditions (12 °C) for about 3 months. Soil AOB and AOA populations were quantified using real-time PCR with primers targeting the *amoA* gene of the AOB and AOA DNA (Rotthauwe *et al.*, 1997; Francis *et al.*, 2005; Leininger *et al.*, 2006; He *et al.*, 2007). The *amoA* gene encodes a subunit of the AMO enzyme responsible for the first step of the nitrification process, the conversion from ammonia to hydroxylamine. Nitrification rate was quantified by determining the amount of NO_3^- -N produced during the incubation period by extracting soil samples with 2 M KCl followed by standard flow injection analysis of NO_3^- concentrations in the extracts.

Data analysis

Annual NO_3^- -N leaching losses were calculated on the basis of their concentrations in the leachate collected from each lysimeter and the volume of leachate. Average annual leaching losses were then calculated using values from the

four replicates. Analysis of variance (ANOVA) of the data was performed using Genstat (11th Edition, Lawes Agricultural Trust). Standard errors (SE) of the mean were calculated and presented with the mean values.

Results

Temperature and water inputs and outputs

Daily average air temperature varied from a low of 0.8 °C in June 2008 to a high of 34 °C in January 2009 (with an average 13.9 °C for the study period) (Figure 1a). Total annual water input, including rainfall and supplementary irrigation, reached 1260 mm for the lower rainfall target, slightly above the 1100 mm target because of a relatively wet winter (Figure 1b). Total water input in the higher rainfall regime reached 2145 mm, just slightly under the target rainfall of

Figure 1 (a) Daily average air temperature; (b) daily and cumulative water inputs in the 1260 mm annual water input treatment; and (c) daily and cumulative water inputs in the 2145 mm annual water input treatment.

2200 mm (Figure 1c). An average of 335 mm of drainage water was collected from the 1260 mm lysimeters and this is equivalent to about 27% of the annual water input. Under the 2145 mm rainfall treatment, an average of 683 mm of drainage was collected, equivalent to about 32% of the water input.

Nitrate concentrations in the drainage water

Under the 1260 mm rainfall treatment, nitrate-N concentration in the drainage water in the CT Lismore soil reached a peak of 263 mg NO_3^- -N/L in the urine treatment, and this was significantly ($P < 0.05$) reduced to 84 mg NO_3^- -N/L when DCD was applied (Figure 2a). In the SL Matura soil, the NO_3^- -N concentration reached a peak of 240 mg NO_3^- -N/L in the urine treatment. This was significantly

Figure 2 Nitrate-N concentrations ($\pm$ SE) in the drainage water from: (a) CT Lismore soil; (b) SL Matura soil; and (c) WC Harihari soil lysimeters under 1260 mm of water input.

($P < 0.05$) decreased to 80 mg NO_3^- -N/L with the application of DCD (Figure 2b). In the WC Harihari soil, the NO_3^- -N concentrations were considerably lower than those in the CT Lismore and SL Matura soils, reaching a peak of 72 and 27 mg NO_3^- -N/L for urine and urine + DCD treatments, respectively (Figure 2c).

Under the 2145 mm rainfall treatment, the NO_3^- -N concentration in the drainage water in the SL Matura soil reached a peak of 239 mg NO_3^- -N/L in the urine treatment (Figure 3a). This was significantly reduced to 93 mg NO_3^- -N/L when DCD was applied. In the WC Harihari soil, the NO_3^- -N concentrations in both the urine and urine + DCD treatments were significantly lower, reaching a peak of 52 and 29 mg NO_3^- -N/L in the two treatments, respectively (Figure 3b). There was a blockage in some of the lysimeters in the CT Lismore lysimeters under the higher rainfall treatment which made the results unreliable (data not reported).

Total nitrate-N leaching losses

Under the 1260 mm rainfall treatment, total NO_3^- -N leaching losses in the urine treatment ranged from a low of 122.9 kg NO_3^- -N/ha in the WC Harihari soil to a high of 435.8 kg NO_3^- -N/ha in the SL Matura soil (Table 2). These were significantly decreased to between 35.8 and 176.5 kg NO_3^- -N/ha when DCD was applied ($P < 0.05$). These reductions in NO_3^- leaching losses by DCD were equivalent to 56–71%.

Under the 2145 mm rainfall treatment, the total NO_3^- -N leaching losses in the urine treatment varied from 67.7 kg NO_3^- -N/ha in the WC Harihari soil to 457.0 kg NO_3^- -N/ha in the SL Matura soil (Table 2). These were decreased to 29.7 and 257.4 kg NO_3^- -N/ha with the application of DCD. These reductions in NO_3^- leaching because of DCD were equivalent to 44–56%. The difference in the amount of

Figure 3 Nitrate-N concentration in the drainage water from: (a) SL Matura soil; and (b) WC Harihari soil lysimeters under 2145 mm of annual water input.

NO_3^- -N leached between the two rainfall treatments was not statistically significant ($P > 0.05$). The average reduction in NO_3^- -N leaching loss by the DCD treatment under both rainfall treatments was 59%.

Dynamics of AOB and AOA populations

Detailed results of the AOB and AOA populations are reported by Di *et al.* (2009). Briefly, both AOA and AOB

Soil	Rainfall (mm p.a.)	Treatment	NO_3^- -N leached ($\pm$ SE) (kg/ha)	% Reduction of NO_3^- -N leaching
CT	1260	Urine	399.0 (27.3)	— ^a
CT	1260	Urine + DCD	176.5 (15.5)	56
SL	1260	Urine	435.8 (13.7)	—
SL	1260	Urine + DCD	142.2 (0.9)	67
WC	1260	Urine	122.9 (59.1)	—
WC	1260	Urine + DCD	35.8 (17.4)	71
SL	2145	Urine	457.0 (53.4)	—
SL	2145	Urine + DCD	257.4 (22.3)	44
WC	2145	Urine	67.7 (25.1)	—
WC	2145	Urine + DCD	29.7 (11.5)	56
SED for treatment			20.0	
Average reduction of NO_3^- -N leaching by DCD				59

^aNot applicable.

Table 2 Total NO_3^- -N leaching losses

were detected in these soils in large numbers. The AOA population size was similar in the three soils, with *amoA* gene copy numbers varying from 3.85×10^6 in the SL Mataura soil to 4.65×10^6 in the WC Harihari soil. However, the AOB population size varied significantly from 1.07×10^6 in the WC Harihari soil to 2.0×10^7 in the SL Mataura soil. The AOA to AOB ratio varied from 0.2 in the SL Mataura soil to 4.4 in the WC Harihari soil.

Following the application of dairy cow urine-N to the soils, the AOB population size grew 10.4-, 4.7- and 5.4-fold compared with the controls in the CT, WC and SL soils, respectively. The AOB growth was significantly inhibited by the application of the nitrification inhibitor, DCD. A significant relationship was found between the nitrification rate and the AOB population size. The AOA population, on the other hand, did not grow with the application of animal urine-N, and was not related to the nitrification rate. The *amoA* gene copy numbers of the AOB and AOA DNA were used here to determine the relationship between NO_3^- -N leaching losses and the abundance of AOB and AOA.

Relationships between nitrate leaching and AOB and AOA populations

The following significant relationship was found between total NO_3^- -N leaching loss from both rainfall treatments of the three soils and the AOB *amoA* gene copy numbers (Figure 4a):

$$y = 57.2 + 2.93x$$

$$R^2 = 0.51; P < 0.01 \quad (1)$$

where y is the total NO_3^- -N leaching loss (kg NO_3^- -N/ha), and x is the AOB *amoA* gene copy numbers (million copies per gram of soil). However, no relationship was found between the total NO_3^- -N leaching loss and the AOA populations (Figure 4b).

A significant relationship was also found between the total NO_3^- -N leaching loss from both rainfall treatments of the three soils and the nitrification rate as indicated by the amount of NO_3^- -N produced (Figure 4c):

$$y = -44.8 + 0.592x$$

$$R^2 = 0.67; P < 0.01 \quad (2)$$

where y is the total NO_3^- -N leaching loss (kg NO_3^- -N/ha) and x is the nitrate-N concentration in the soil (mg NO_3^- -N per kg soil) after 49 days of incubation when the AOB population reached a peak (Di *et al.*, 2009).

Discussion

Results from this study show that the nitrification inhibitor, DCD, was highly effective in mitigating NO_3^- leaching from all three soils under the two different rainfall treatments. The

Figure 4 Relationships between total nitrate-N leaching losses from both rainfall treatments of the three soils and: (a) soil AOB DNA *amoA* gene copy numbers; (b) soil AOA DNA *amoA* gene copy numbers; and (c) amount of nitrate-N in the soil after 49 days when AOB populations peaked in soil in the companion incubation study.

59% average reduction of NO_3^- -N leaching loss by DCD achieved in the three soils here are of a similar magnitude to those reported previously (e.g. Di & Cameron, 2002b, 2007). The lower rainfall treatment (1260 mm) used in the present study is similar to the annual water inputs in the major dairy farming regions in the Waikato in the northern North Island, Southland in the southern South Island, and in Canterbury (rainfall + irrigation) in mid South Island of New Zealand. The higher rainfall treatment (2145 mm) is similar to those in the regions of Rotorua Lakes in Central North Island, and parts of the west coast of both Islands. These rainfalls are thus relevant to the major dairy farming regions across New Zealand.

Although different amounts of drainage (335 mm vs. 683 mm) were collected under the two rainfall treatments, there was no statistically significant difference between the

amounts of NO_3^- -N leached from the two rainfall treatments. This was partly because the 1260 mm water input was sufficient to leach most of the NO_3^- out of the soil profile, any additional water input would not result in significantly more NO_3^- being leached. This is shown by the very low NO_3^- -N concentrations in the drainage water at the later part of the graph in Figure 3a under the high rainfall condition. In addition, the wetter conditions of the higher rainfall treatment may have created an environment more conducive for denitrification, thus resulting in greater losses of nitrogen gases.

It is interesting that despite the large urine-N input at 1000 kg N/ha, the amount of NO_3^- -N leached from the WC Harihari was small, particularly in relation to the large NO_3^- -N leaching losses from the other two soils. This was probably related to the very high silt content (93.6%) of the WC Harihari soil and thus slower drainage rate which may have resulted in the conversion of a large amount of the NO_3^- into nitrogen gases by denitrification. This may also be why the NO_3^- -N leaching loss under the higher rainfall treatment was less than that under the lower rainfall treatment in this soil; the higher rainfall treatment would have created a more favourable condition for denitrification. Further research is required to ascertain the mechanisms responsible for the small NO_3^- leaching loss from the WC Harihari soil.

Despite the critical importance of nitrification in the nitrogen cycle, our understanding of the role of the different ammonia oxidizers (e.g. AOB and AOA) in nitrification in different soil and environmental conditions and in affecting NO_3^- leaching is still limited. The recent discovery of the *amoA* gene in the domain archaea and the detection of putative AOA in different ecosystems point to a potentially large role by AOA in nitrification (Francis *et al.*, 2005; Könneke *et al.*, 2005; Leininger *et al.*, 2006; He *et al.*, 2007; Prosser & Nicol, 2008). Results from this study, however, show that the total NO_3^- -N leaching loss was significantly related to the abundance of the AOB population but not to the AOA population. This would support our finding reported separately that the nitrification rate was mainly related to the growth of AOB population rather than the AOA population in the urine patch soils with high nitrogen loads (Di *et al.*, 2009). AOA may be particularly adapted to unfavourable or oligotrophic environments (Mahmood & Prosser, 2006; Valentine, 2007; Shen *et al.*, 2008; Di *et al.*, 2009; Schauss *et al.*, 2009). The high-N loading environment in the urine-treated soil therefore stimulated the growth of *Nitrosopira* species of the AOB but may be inhibitive for the AOA populations, thus limiting AOA growth and its role in nitrification and NO_3^- leaching at a low level (Di *et al.*, 2009). The highly significant relationship between the total NO_3^- -N leaching loss and the nitrification rate (Figure 4c) and that between the nitrification rate and the AOB populations (Di *et al.*, 2009) also provides a strong indirect link between the AOB population and NO_3^- leaching in grazed grassland with high

nitrogen inputs. However, it must be noted that the abundance of AOB was only able to account for about 51% of the variation in NO_3^- -N leaching losses (as shown by the R^2 value). Other microbes and variables therefore also contribute to the variations in NO_3^- -N leaching, a topic that requires further research.

Conclusions

Results from this study demonstrate that the DCD nitrification inhibitor technology was highly effective in decreasing NO_3^- -N leaching in three different soils under two contrasting rainfall treatments. The total NO_3^- -N leaching losses were not significantly affected by the amount of rainfall applied, partly because of the effect of rainfall on nitrogen losses from denitrification and partly because the amount of NO_3^- -N leached may have been limited by the amount of NO_3^- available for leaching rather than the amount of drainage. The significant relationship between the total NO_3^- -N leaching loss and the abundance of the AOB population indicates the important role that the AOB population plays in nitrification and nitrogen cycling in the nitrogen rich urine patch soils of grazed grassland.

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