

The effect of animal trampling and DCD on ammonia oxidisers, nitrification, and nitrate leaching under simulated winter forage grazing conditions

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Abstract

Purpose Dairy winter forage grazing on free-draining soils is a common practice within the Canterbury region of New Zealand. The high stocking rates involved and the associated deposition of urine onto wet soils during winter create a high risk of nitrate (NO_3^-) leaching from soil. The objective of this study was to determine the effect of animal trampling and the use of a nitrification inhibitor, dicyandiamide (DCD), on soil ammonia oxidisers, nitrification, and nitrate leaching under simulated winter forage grazing conditions.

Materials and methods A lysimeter trial was carried out using a Balmoral stony silt loam under a kale forage crop. Nitrate leaching losses, the effect of soil trampling, and the effect of DCD were measured. Soil nitrification rates and ammonia-oxidising community abundance and activity were measured in companion soil blocks under simulated dairy winter forage grazing conditions.

Results and discussion Animal trampling was found to reduce peak nitrate-N (NO_3^- -N) concentrations in drainage water from urine patch areas by 43 %. In addition, animal trampling reduced the total amount of NO_3^- -N leached from urine patches by 34 %. However, animal trampling did not affect the growth or activity of the ammonia oxidisers. The use of DCD was found to be highly effective in reducing the concentration and amount of NO_3^- -N leached from urine patches. Dicyandiamide applications reduced peak NO_3^- -N concentrations in drainage water by 66 %, and the total amount of NO_3^- -N leached was reduced by 61 % under the simulated dairy winter forage grazing conditions. Ammonia oxidising bacteria (AOB) were more

abundant than ammonia-oxidising archaea (AOA) and were responsible for mediating the nitrification process.

Conclusions These results suggest that both animal trampling and the use of DCD separately reduces soil nitrification rates and thus NO_3^- leaching.

Keywords Ammonia-oxidising archaea · Ammonia-oxidising bacteria · DCD · Nitrate leaching · Nitrification inhibitor · Nitrification rate · Trampling · Winter forage grazing

1 Introduction

Within New Zealand, livestock generally graze outdoors all year round. During the winter period when grass growth is slow, dairy cows in some regions of New Zealand are taken off pasture grazing to purposely grown winter forages. Within the Canterbury region, dairy winter forage grazing is a common practice as it prevents soil and pasture damage to the main farm during the wet winter period and increases pasture cover allowing high feed supplies when the cows return in spring. However, the winter grazing systems have the potential to cause NO_3^- leaching as they are often located on stony free-draining soils and stocking rates are high. During the winter period, NO_3^- leaching losses are generally greater than the remainder of the year due to low plant uptake of NO_3^- , low evapotranspiration, and high drainage (Cameron et al. 2013). Cow urine depositions in a localised area have significantly higher nitrogen (N) concentrations compared to the surrounding soil. For example, the localised N application rate in an individual urine patch may be equivalent to 400 kg N ha^{-1} from cows grazing kale. This has the potential to increase NO_3^- leaching losses from winter forage grazing areas. However, animal trampling of the soil during winter grazing

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can reduce the soil's air permeability and porosity (Drewry and Paton 2005; Ball et al. 2012) and may therefore reduce the rate of soil nitrification and NO_3^- leaching losses.

Increased NO_3^- leaching losses can lead to the degradation of surface water bodies through eutrophication. With potentially higher NO_3^- leaching losses under winter forage grazing, there is a need to develop technologies to help mitigate NO_3^- leaching losses. The nitrification inhibitor dicyandiamide (DCD) has been found to be a successful mitigation tool under grazed grassland conditions, reducing NO_3^- leaching losses by 36–76 % (e.g. Di and Cameron 2002, 2004; Shepherd et al. 2010). Dicyandiamide inhibits the first step of nitrification by deactivating the active site of the soil ammonia monooxygenase (AMO) enzyme produced by ammonia oxidisers (Di et al. 2009b). Thus, N remains in the soil as ammonium (NH_4^+) rather than being converted to NO_3^- .

In spite of the important role that nitrification plays in the loss of NO_3^- , it remains unclear whether ammonia-oxidising bacteria (AOB), ammonia-oxidising archaea (AOA), or both mediate the nitrification process in winter forage grazing systems. Previously, it has been determined that either AOB or AOA can mediate nitrification within agricultural soils (Di et al. 2009b; Offre et al. 2009; Zhang et al. 2010). However, it has been shown that AOB and AOA prefer different soil conditions for growth (Di et al. 2009b; He et al. 2007; Leininger et al. 2006; Shen et al. 2008; Valentine 2007; Xia et al. 2011). Leininger et al. (2006) found that AOA were more abundant than AOB in a number of European soils. However, Di et al. (2009b) showed that under high N loading soil conditions (e.g. dairy cow urine patches), AOB mediate nitrification, while AOA only play a minor role. In support of these findings, Di et al. (2009a) identified a significant relationship between AOB *amoA* gene abundance and NO_3^- leaching losses within high N-loaded grazed grassland soils, while no such relationship was found between AOA *amoA* gene abundance and NO_3^- leaching losses within the same soil. On the other hand, Valentine (2007) stated that AOA mediated nitrification under more extreme conditions including low soil fertility as well as extreme salinity and pH. In addition to this, a number of studies have shown the role of AOB and AOA under different soil pH (Baolan et al. 2014; He et al. 2007; Shen et al. 2008; Xia et al. 2011). Shen et al. (2008) and Xia et al. (2011) showed that when the soil pH conditions were alkaline or neutral, the application of N fertiliser provided a growth advantage to AOB. Conversely, He et al. (2007) showed that N fertilisation provided a growth advantage to AOA when the soil conditions were strongly acidic. However, to our knowledge, no studies have been reported on the role of AOB and AOA in nitrification under winter forage grazing conditions.

The objective of this study was therefore to determine the effect of animal trampling and DCD on soil ammonia oxidisers, nitrification, and NO_3^- leaching under simulated

winter forage grazing conditions. The free-draining soil studied here is typical of the soil type used for winter forage grazing in the South Island of New Zealand. However, findings from this study would also be relevant for other free-draining soils under similar management and climatic conditions elsewhere.

2 Materials and methods

2.1 Soil type

The soil used in this study was the Balmoral stony silt loam (acidic orthic brown soil in the New Zealand Soil Classification (Hewitt 1998) and Udic Haplustepts loamy skeletal in the USDA Soil Classification (Soil Survey Staff 1998)). This soil has developed from gravelly glacial outwash materials covered with varying depths of loess with key soil properties being the following: pH 5.9, Olsen P 24 mg L⁻¹, K⁺ 0.86 cmol_ckg⁻¹, Ca²⁺ 7.77 cmol_ckg⁻¹, Mg²⁺ 0.66 cmol_ckg⁻¹, Na⁺ 0.13 cmol_ckg⁻¹, CEC 17 cmol_ckg⁻¹, total C 42 g kg⁻¹, and total N 4.1 g kg⁻¹. Key features of this soil are that it is shallow (topsoil 0.18 m), stony, and well drained. The mean annual maximum and minimum temperatures in the region are 17 and 4 °C, respectively, with an average annual rainfall of about 650 mm.

2.2 Lysimeter collection and installation

Following well-established protocols and procedures (Cameron et al. 1992), undisturbed soil monolith lysimeters (50 cm diameter and 70 cm deep) were collected from the Lincoln University Ashley Dene Farm (43° 39' 2" S; 172° 19' 45" E). The collection of the lysimeters involved placing a metal cylinder casing on the soil surface and digging around the outside edge, so that it could be gradually pushed down around the soil column without disturbing the soil structure inside the casing. To avoid any negative impacts on microbial communities and their processes, the steel casings were not zinc coated and the space between the soil monolith and the casing was filled with Vaseline. Thus, there would be no effect of the casing on the soil. When large stones were encountered under the cutting edge of the lysimeter casing within the soil profile, they were removed and the casing pushed down. Once the casing reached the desired depth of 70 cm, the soil monolith was cut at the base of the casing with a cutting plate. The cutting plate was secured to the bottom of the lysimeter using long metal rods and the gap between the soil column and the metal casing was sealed using petroleum jelly to prevent edge flow.

After collection, the lysimeters were placed onto a specially designed trailer with air bag suspension which minimised the risk of soil disturbance within the lysimeters during

transportation. The lysimeters were then transported to a field trench lysimeter facility located at the Field Service Centre, Lincoln University, 20 km south of Christchurch (43° 38' 52" S; 172° 28' 7" E). The soil surface of each lysimeter was at the same level as that of the surrounding paddock, ensuring that plant growth occurred under near normal field conditions. The area outside each lysimeter was backfilled with soil to the same level as the surface of the lysimeters and the surrounding paddock.

2.3 Soil block installation

For each lysimeter, there was a corresponding soil block to enable periodic soil sampling to occur. Metal soil rings (50 cm diameter and 15 cm deep) were filled with Balmoral stony silt loam collected from the top 10 cm of the soil profile from Lincoln University Ashley Dene Farm. Any stones larger than 5 cm in diameter were removed from the soil during collection. These soil blocks were placed on a bed of sand (35 cm deep) to enable free drainage to occur.

The soil blocks were used for destructive soil sampling to determine the soil nitrification rates and ammonia oxidiser populations, since this could not be done on the lysimeters without adversely affecting the lysimeter properties.

2.4 Trial setup

During March 2012, kale (*Brassica oleracea* L.) seedlings of the 'Regal' cultivar were transplanted from the Lincoln University Ashley Dene Farm into each of the lysimeters and soil blocks. Three plants were transferred into each lysimeter and soil block representing the standard kale density on a winter feed system.

2.4.1 Treatment application

The following treatments were applied in replicates of four under both animal trampling and no animal trampling conditions: control, DCD 20 kg DCD ha⁻¹, urine 400 kg N ha⁻¹, and urine 400 kg N ha⁻¹ + DCD 20 kg DCD ha⁻¹ (Table 1). The treatments were applied in a random design between June 18 and 21, 2012. This period was selected to coincide with the start of winter forage grazing at the Ashley Dene field site. Animal grazing was simulated by cutting the kale off at ground level using handheld secateurs. After the simulated grazing, 20 ml of DCD (equivalent 20 kg DCD ha⁻¹) was applied to the relevant lysimeters and soil blocks using a handheld sprayer. The lysimeters and soil blocks were then wetted up to field capacity by applying 10 mm of simulated rainfall to represent winter conditions before the designated lysimeters and soil blocks were trampled using a handheld metal cow hoof device. The same number of trampling with the same weight was applied to each lysimeter and soil block.

Lastly, fresh kale-fed dairy cow urine was collected from non-lactating cows at the Ashley Dene field site and applied to the relevant lysimeters and soil blocks at 400 kg N ha⁻¹. In late August, a cereal crop of oats (*Avena sativa* L.) was planted into each lysimeter and soil block and was harvested in late November.

Simulated rainfall was used to supplement natural rainfall (Canterbury region average annual rainfall about 650 mm), generating a combined annual rainfall and irrigation input of approximately 1100 mm (the average water input on dairy farms in New Zealand). This simulated rainfall was applied using an electronically controlled metering system which delivered 10 mm of water to each individual lysimeter and soil block during irrigation events modelled using current rainfall totals and typical Canterbury farm irrigation patterns.

2.5 Leachate collection

Each lysimeter had a plastic collection container which sat within a metal box at the base of the lysimeter trench resulting in the leachate being kept cool and out of the sun before collection. Leachate collection began 4 weeks prior to treatment application to determine the background levels of NO₃⁻-N and ammonium-N (NH₄⁺-N) present in the soil and continued until early June the following year. Leachate was collected weekly or when the volume of drainage was greater than 200 ml. The volume of leachate was recorded and subsamples (100 ml) were taken for NO₃⁻ and NH₄⁺ analysis.

2.6 Soil block sampling and extractions

Three replicate 75-mm-deep soil cores were collected from each soil block on days 7, 21, 37, 50, 63, 83, 106, and 125 after treatment application. Each of the three replicates were bulked and thoroughly mixed before a subsample was removed and placed into a vial for molecular analysis. The vials were kept at -80 °C before molecular analysis occurred. Ammonium and NO₃⁻ were extracted from 5 g of soil using 25 ml of 2 M KCl (Blakemore et al. 1987). The samples were shaken for 60 min, centrifuged for 10 min before filtering using Advantec SC 110 mm filter papers, and then placed in a -20 °C freezer until analysis.

2.7 Chemical analysis

The leachate and soil extracts were analysed for NO₃⁻-N and NH₄⁺-N using a flow injector analyser (FIA; FOSS FIAstar 5000 triple channel analyser) with SoFIA software version 1.30 (FOSS Tecator AB, Sweden). Nitrate-N was analysed by the initial reduction of NO₃⁻-N to nitrite-N using a cadmium reduction coil, followed by the reaction of nitrite-N with sulphanilamide/NED to form an azo dye compound. The

Table 1 Treatments used in this study

Treatment	Urine (kg N ha ⁻¹)	DCD (kg DCD ha ⁻¹)	Trampling
Control trampled	0	0	Trampled
Control	0	0	Untrampled
DCD trampled	0	20	Trampled
DCD only	0	20	Untrampled
Urine trampled	400	0	Trampled
Urine only	400	0	Untrampled
Urine + DCD trampled	400	20	Trampled
Urine + DCD only	400	20	Untrampled

intensity of this compound is determined spectrophotometrically at 540 nm. Ammonium-N was determined using a gas diffusion membrane. Sodium hydroxide was added to increase the pH of the sample stream; any NH₄⁺-N ions present are converted into ammonia gas. The gas diffuses through the membrane into an indicator stream. The indicator stream changes colour (red to blue) with an increase at 590 nm. The extent of the colour change is proportional to the concentration of NH₄⁺-N ions present in the sample.

2.8 Soil AOB and AOA *amoA* gene abundance and gene expression

On days 7, 21, 37, 50, 63, 83, 106, and 125 after treatment application, DNA was extracted from the stored samples using a MO BIO™ PowerSoil® DNA Isolation Kit (MO BIO Laboratories, GeneWorks Pty Ltd, South Australia, Australia) following the manufacturer's instructions. On day 37, the AOB and AOA activity (*amoA* transcript abundance) was determined using the stored samples. Our previous work indicated that this was the time period when the treatments had the greatest effect on the population growth of AOB. RNA was extracted using the CTAB RNA extraction protocols (Griffiths et al. 2000), followed by DNase treatment using Turbo DNA-free Kit (Ambion®, Life Technologies, Auckland, New Zealand) and PCR inhibitor removal using Zymo-Spin IV-HRC clean-up columns (Zymo Research, Ngāio Diagnostics Ltd. Auckland, New Zealand). Complementary DNA (cDNA) was produced from total

RNA using SuperScript® III first-strand synthesis system (Life Technologies, Auckland, New Zealand) with a random hexamer primer following the manufacturer's recommendations. The AOB and AOA abundances and activity were determined by undertaking real-time quantitative PCR (qPCR).

All qPCR reactions were prepared using the CAS-1200 robotic liquid handling system (Corbett Life Science, Bio-Strategy, Auckland, New Zealand), and the real-time qPCR analysis was performed on a Rotor-Gene™ 6000 (Corbett Life Science, Bio-Strategy, Auckland, New Zealand). Both bacterial and archaeal *amoA* genes were quantified using the following PCR primer pairs: amoA1F/amoA2R primers (Rotthauwe et al. 1997) and Arch-amoAF/Arch-amoAR primers (Francis et al. 2005). The 16-μl reaction mixture consisted of 8.0 μl of 2× SYBR Premix Ex Taq™ (TaKaRa, Nori Biotech, Auckland, New Zealand), 0.4 μl of each primer at 10 μM concentration, and 1.5 μl of 1:10 diluted soil genomic DNA. A melting curve analysis was performed to confirm PCR product specificity after amplification by measuring fluorescence continuously as the temperature increased from 50 to 99 °C. Data analysis was then carried out using Rotor-Gene™ 6000 series software 1.7.

For each gene, a standard curve was produced as follows: bacterial and archaeal *amoA* genes were PCR amplified from extracted DNA with the primers amoA1F/amoA2R and Arch-amoAF/Arch-amoAR, respectively. Using the AxyPrep™ PCR Clean-up Kit (Axygen, Total Lab Systems, New Zealand), the PCR products were then purified and cloned into the pGEM-T

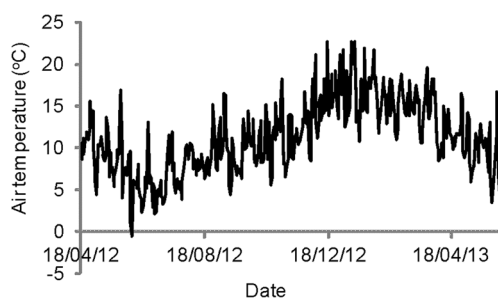
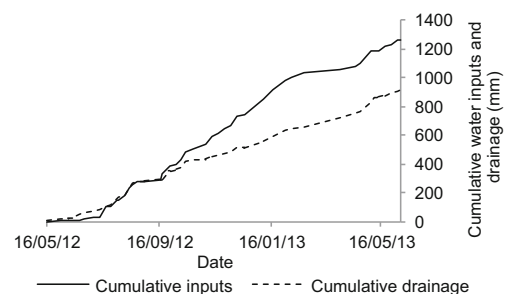
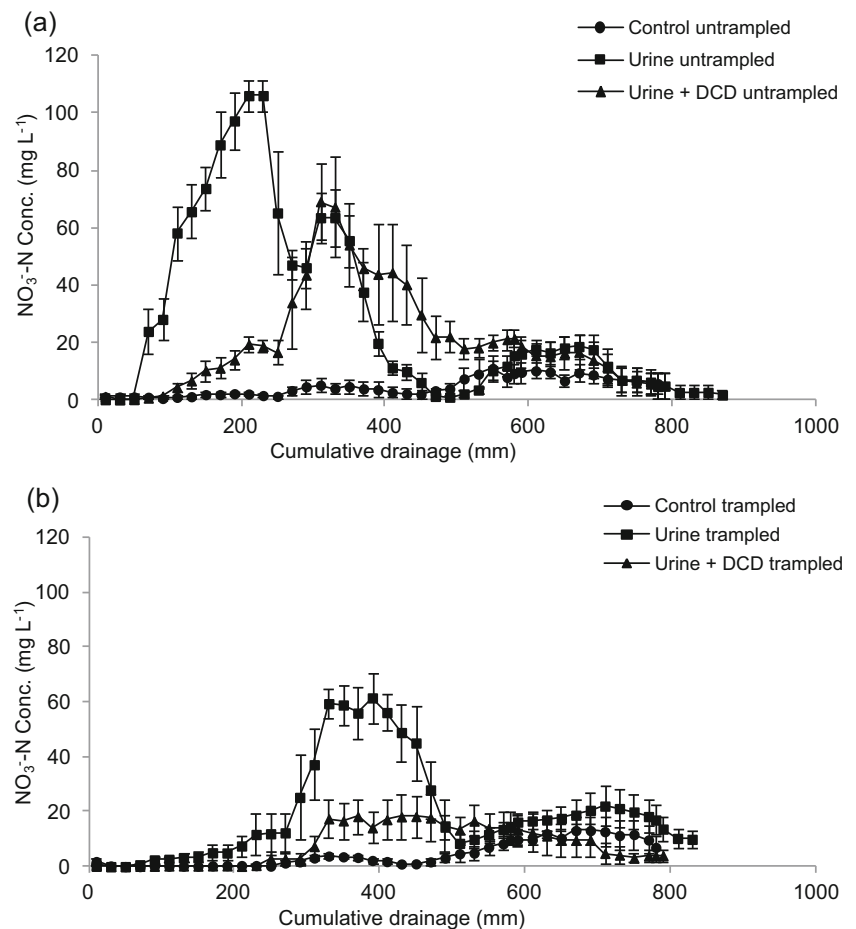
**Fig. 1** Daily average air temperatures for the trial period**Fig. 2** Cumulative water inputs and drainage for the trial period

Fig. 3 Nitrate-N concentrations in drainage water as affected by applications of cow urine-N, DCD, and trampling. **a** Untrampled. **b** Trampled. The error bars represent standard errors of the mean



Easy Vector (Promega, In Vitro Technologies, Auckland, New Zealand). Following the manufacturer's instructions, the resulting clones were transformed in *Escherichia coli* JM109 competent cells (Promega, In Vitro Technologies, Auckland, New Zealand). The transformed *E. coli* cells were grown on solid lysogeny broth (LB) plates at 37 °C overnight. Ten to 15

bacterial colonies from the plate were then individually inoculated into a 3-ml LB broth medium and incubated overnight in an orbital incubator shaker at 37 °C and 250 rpm. The plasmids carrying correct *amoA* gene inserts were then extracted from bacterial cultures using QIAprep Spin Miniprep Kit (Qiagen, Bio-Strategy, Auckland, New Zealand) and sent for sequencing. The plasmid DNA concentration was determined on a QubitTM fluorometer using the Quant-iTTM dsDNA BR Assay Kit (Life Technologies, Auckland, New Zealand). The copy numbers of the target genes were then calculated directly from the concentration of the extracted plasmid DNA. Tenfold serial dilutions of a known copy number of the plasmid DNA were included in each real-time qPCR run. Triplicates were used to generate a

Table 2 Total NO₃⁻-N leaching losses

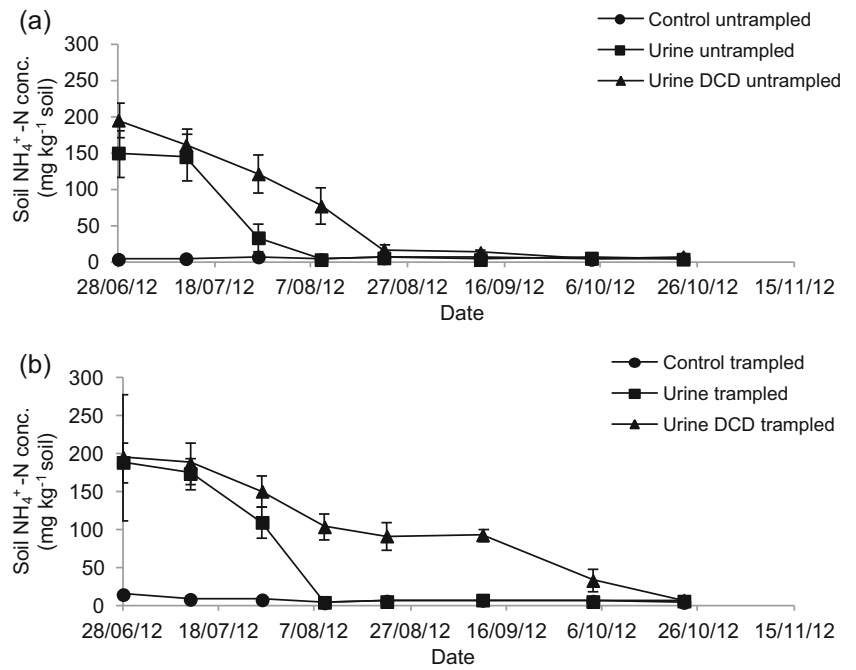
	Total NO ₃ ⁻ -N leached (±SE) (kg NO ₃ ⁻ -N ha ⁻¹)	% Reduction	
		DCD	Trampled
Control trampled	39 (5.3)	— ^a	—
Control	40 (5.5)	—	—
DCD trampled	29 (4.3)	—	—
DCD only	47 (3.0)	—	—
Urine only	253 (10.0)	—	—
Urine trampled	168 (26.3)	—	34 %
Urine + DCD only	167 (7.4)	40 %	—
Urine + DCD trampled	66 (23.3)	61 %	61 %
LSD	58	—	—

^a Not applicable

Table 3 ANOVA analysis summary for nitrate leaching losses

	Peak NO ₃ ⁻			Total NO ₃ ⁻		
	<i>p</i>	<i>F</i>	<i>DF</i>	<i>p</i>	<i>F</i>	<i>DF</i>
DCD effect trampled	0.05	10.49	1	0.05	8.60	1
DCD effect untrampled	NS	2.84	1	<0.001	39.11	1
Trampling effect DCD	0.05	6.04	1	0.01	18.32	1
Trampling effect no DCD	0.01	17.46	1	0.05	8.32	1

Fig. 4 Ammonium-N concentrations in the soil as affected by applications of cow urine-N, DCD, and trampling. **a** Untrampled. **b** Trampled. The error bars represent standard errors of the mean



standard curve and estimate amplification efficiency for each assay.

2.9 Statistical analysis

Annual NO₃⁻ leaching losses were calculated using the NO₃⁻ concentrations in, and volume of, the leachate collected from each lysimeter. Average annual NO₃⁻ leaching losses were then calculated using the four replicates. Mean values and standard errors of the means for the NO₃⁻ leaching losses, soil ammonium concentration, and ammonia oxidiser populations were calculated based on the four replicates for each treatment using Microsoft Excel 2010 (Microsoft Corporation, USA). A basic 1×1 factorial design was used for both the leachate and soil block data. *p* and *F* values were calculated by undertaking a general analysis of variance (ANOVA) using GenStat[®] (Version 15.1, VSN International Ltd, UK) with the comparison being between the following treatments: urine only and urine + DCD, urine trampled and urine + DCD trampled, urine only and urine trampled, and urine + DCD and urine + DCD trampled.

3 Results

3.1 Temperature and cumulative water inputs and drainage

The average daily air temperature varied from -0.6 °C in June 2012 to 22.7 °C in January 2013, with an average of 11.3 °C for the study period (Fig. 1). The total annual water input over the study period from both rainfall and supplementary irrigation reached 1264 mm (Fig. 2). An average of 913 mm drainage water was collected from the lysimeters which accounted for 72 % of the total annual inputs.

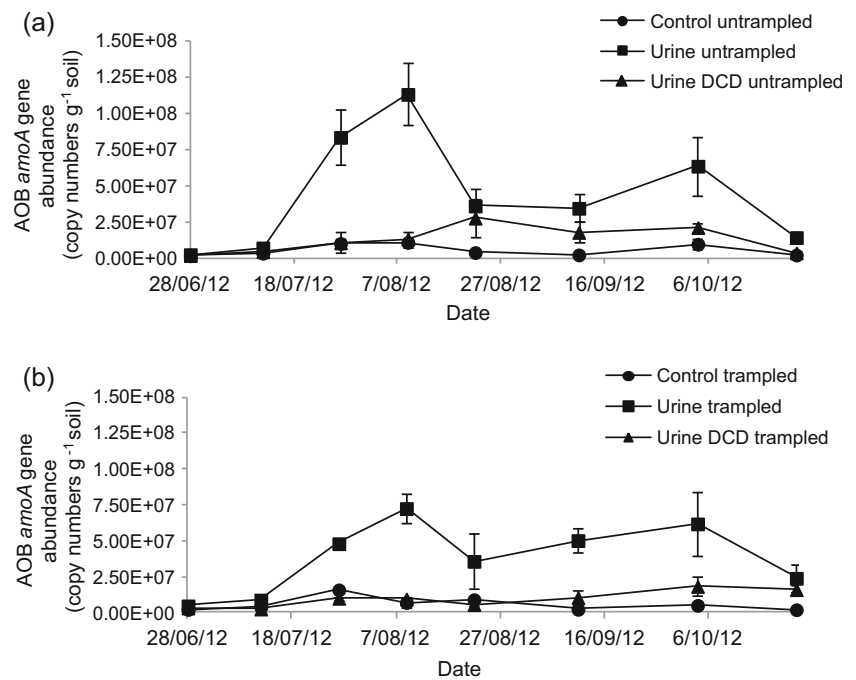
3.2 Nitrate concentrations in the drainage water

In the urine only treatment, the NO₃⁻-N concentration in the drainage water peaked at 106 mg NO₃⁻-N L⁻¹ (Fig. 3a), and this was significantly (*p*<0.01) reduced to 61 mg NO₃⁻-N L⁻¹ when the soil was trampled (Fig. 3b and Table 3). In the urine + DCD treatment, the NO₃⁻-N concentration in the drainage water peaked at 72 mg NO₃⁻-N L⁻¹, and this was significantly (*p*<0.05) reduced to 21 mg NO₃⁻-N L⁻¹ when the soil was

Table 4 ANOVA analysis summary for ammonium concentration and ammonia oxidisers from the soil blocks

	NH ₄ ⁺ -N			AOB abundance			AOA abundance			AOB activity			AOA activity		
	<i>p</i>	<i>F</i>	<i>DF</i>	<i>p</i>	<i>F</i>	<i>DF</i>	<i>p</i>	<i>F</i>	<i>DF</i>	<i>p</i>	<i>F</i>	<i>DF</i>	<i>p</i>	<i>F</i>	<i>DF</i>
DCD effect trampled	0.01	20.58	1	0.01	25.73	1	0.05	12.13	1	0.01	27.42	1	NS	0.219	1
DCD effect untrampled	NS	4.799	1	<0.001	64.97	1	NS	0.316	1	0.01	18.47	1	NS	1.161	1
Trampling effect DCD	0.05	6.562	1	NS	2.134	1	NS	0.359	1	NS	0.013	1	NS	1.125	1
Trampling effect no DCD	NS	3.172	1	NS	0.804	1	NS	3.843	1	NS	0.003	1	NS	2.046	1

Fig. 5 AOB gene copy *amoA* abundance in the soil as affected by applications of cow urine-N, DCD, and trampling. **a** Untrampled. **b** Trampled. The error bars represent standard errors of the mean

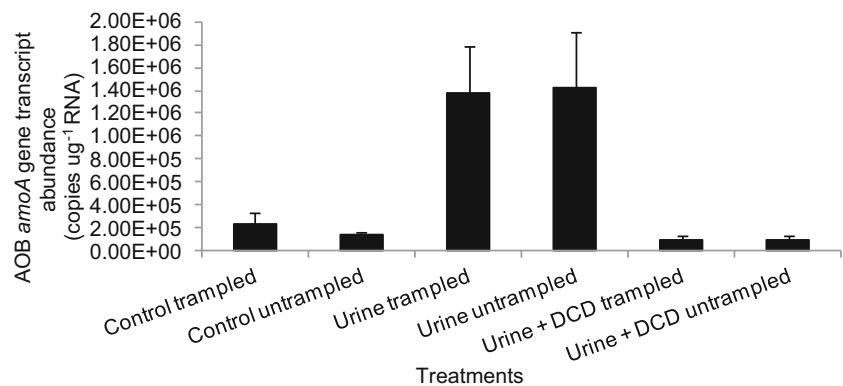


trampled. The application of DCD to the trampled soil significantly ($p < 0.05$) reduced the peak NO_3^- -N concentration. No significant peaks were observed for the control and DCD treatments for both trampled and untrampled soils.

3.3 Total nitrate-N leaching losses

In the urine only treatment, the total NO_3^- -N leaching was 253 kg NO_3^- -N ha⁻¹, and this was significantly ($p < 0.05$) reduced by 34 % to 168 kg N ha⁻¹ when the soil was trampled (Tables 2 and 3). In the urine + DCD treatment, the total NO_3^- -N leaching losses was 167 kg NO_3^- -N ha⁻¹, and this was significantly ($p < 0.01$) reduced by 61 % to 66 kg NO_3^- -N ha⁻¹ when the soil was trampled. The application of DCD to the soil significantly ($p < 0.05$) reduced total NO_3^- -N leaching losses by 40–61 %.

Fig. 6 AOB *amoA* transcript abundance in the soil as affected by the application of cow urine-N, DCD, and soil trampling at day 37. The error bars represent standard errors of the mean



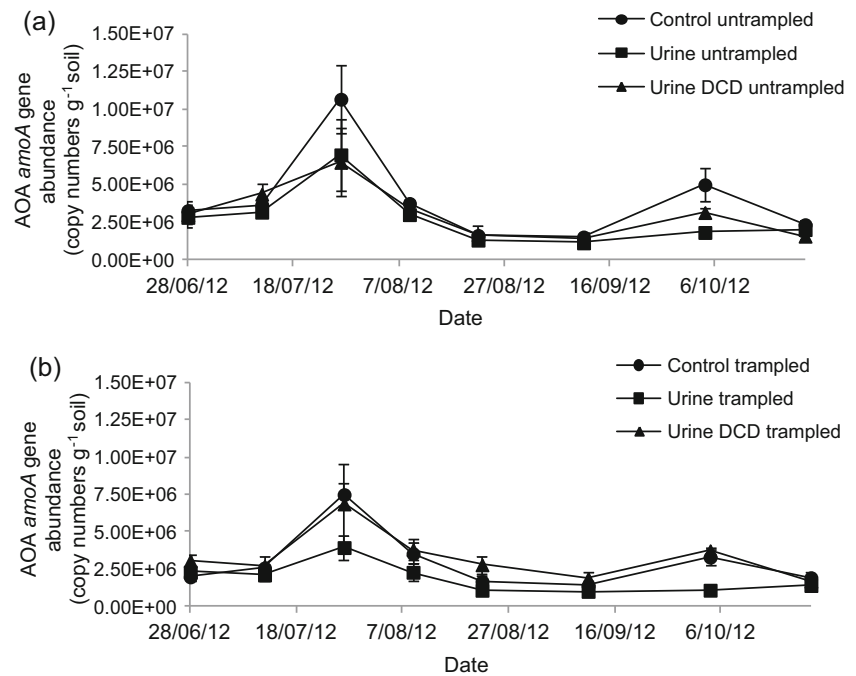
3.4 Soil ammonium concentrations

In the urine only treatment, the average soil NH_4^+ -N concentration was 31 mg NH_4^+ -N kg⁻¹ dry soil (Fig. 4a). With trampling, the NH_4^+ -N concentration was 50 mg NH_4^+ -N kg⁻¹ dry soil (Fig. 4b and Table 4) which was not statistically different. In the urine + DCD treatment, the average soil NH_4^+ -N concentration was 59 mg NH_4^+ -N kg⁻¹ dry soil. This significantly ($p < 0.05$) increased when the soil was trampled (98 mg NH_4^+ -N kg⁻¹ dry soil). The application of DCD to the trampled soil resulted in a significantly ($p < 0.01$) greater average soil NH_4^+ -N concentration.

3.5 Dynamics of AOB and AOA populations

The total number of AOB *amoA* gene copies present in the soil was significantly ($p < 0.001$) greater than the number of AOA

Fig. 7 AOA *amoA* abundance in the soil as affected by applications of cow urine-N, DCD, and trampling. **a** Untrampled. **b** Trampled. The error bars represent standard errors of the mean



amoA gene copies. Following the application of dairy cow urine-N to the soil, the AOB *amoA* gene abundance and AOB *amoA* transcript abundance significantly ($p < 0.01$) increased (Figs. 5 and 6, Table 4). However, trampling the soil did not impact the peak or average AOB *amoA* gene abundance or the AOB *amoA* transcript abundance. The AOB growth and activity was significantly ($p < 0.01$) inhibited by the application of DCD. The AOB *amoA* gene abundance was significantly ($p < 0.01$) reduced by 78 % in the trampled soil and by 70 % in the untrampled soil when DCD was applied. In addition, the AOB *amoA* transcript abundance was significantly ($p < 0.01$) reduced by 93 % in both trampled and untrampled soils when DCD was applied (Fig. 6).

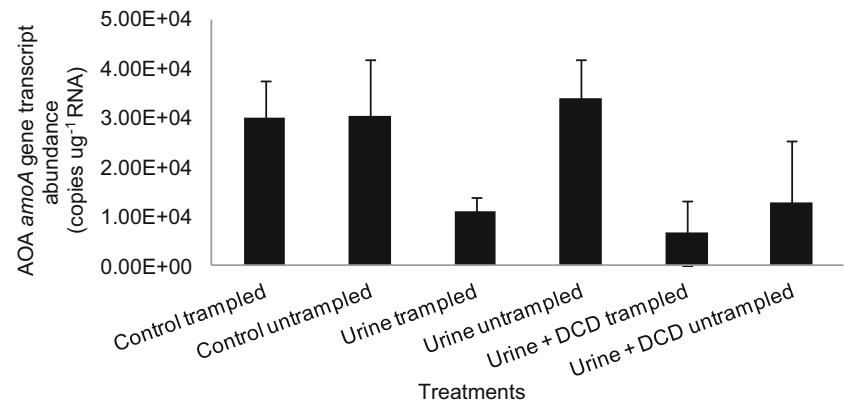
The application of urine onto the soil significantly ($p < 0.05$) decreased the AOA *amoA* gene abundance (Fig. 7 and Table 4). Trampling the soil did not impact the AOA *amoA* gene abundance or the AOA *amoA* transcript abundance (Figs. 7 and 8). When DCD was applied to the urine trampled treatment, the

AOA *amoA* gene abundance was significantly ($p < 0.05$) greater by 81 %. In all other treatments, DCD had no significant effect.

4 Discussion

Animal trampling significantly ($p < 0.05$) reduced peak NO_3^- -N concentrations in drainage water and total NO_3^- -N leaching losses from the lysimeters by 34–71 %. The reduction in NO_3^- -N leaching resulted from changes in soil physical properties, including air permeability and soil porosity (Drewry and Paton 2005; Ball et al. 2012), consequently decreasing nitrification rates and potentially increasing denitrification rates. Optimum conditions for nitrification were removed due to a reduction in airflow through the soil, thus decreasing soil aeration and increasing water-filled pore space. These anaerobic soil conditions are less favourable to ammonia oxidisers which could

Fig. 8 AOA *amoA* transcript abundance in the soil as affected by the application of cow urine-N, DCD, and soil trampling on day 37. The error bars represent standard errors of the mean



have decreased the rate of nitrification, reduced the amount of NO_3^- in the soil solution, and decreased the amount of NO_3^- leached. However, these soil conditions are favourable for denitrifying microbes and denitrification (Cameron et al. 2013; de Klein and Eckard 2008; Jetten 2008); therefore, in addition to the reduction of nitrification, denitrification might have been enhanced following trampling. Consequently, a reduction in the peak NO_3^- -N concentrations in drainage water and total NO_3^- -N leaching losses was observed.

The lysimeters demonstrated that DCD can reduce NO_3^- leaching under wet, cold, and draining soil conditions due to a reduction in soil nitrification rates. DCD significantly ($p < 0.05$) reduced peak NO_3^- -N concentrations and total NO_3^- -N leaching losses by 40–66 %, results similar to previous work on NO_3^- leaching under several agricultural systems (Di and Cameron 2002, 2004; Di et al. 2009a; Moir et al. 2010; Williamson et al. 1996). The companion soil blocks showed that the AOB growth and activity was significantly inhibited by the application of DCD, indicating that its application significantly inhibited the nitrification process, thus reducing the amount of NO_3^- available for leaching.

There was a clear and significant response by the AOB population to the addition of cow urine with an increase in AOB *amoA* gene abundance by 7-fold when the soil was trampled and by 9-fold when the soil was untrampled. A significant increase in AOB *amoA* transcript abundance was also observed following the addition of cow urine. The dominant AOB population led to high nitrification rates (Di et al. 2010; Kowalchuk and Stephen 2001), thus leading to higher amounts of NO_3^- leached in the urine treatments. In contrast, the role of AOA in nitrification was minor under dairy winter forage grazing conditions with no positive response for the AOA *amoA* gene abundance or transcript abundance to the application of cow urine. In fact, the application of cow urine significantly ($p < 0.05$) reduced the AOA population abundance suggesting that AOA does not grow in response to the addition of ammonia from urine. It has previously been hypothesised that AOA prefer different growing conditions compared to AOB (Di et al. 2010). The current findings are similar to those of Di et al. (2009b), Di et al. (2010), and Parfitt et al. (2012).

In addition to AOB mediating the nitrification process under dairy winter forage grazing conditions, this current study identified a significantly ($p < 0.001$) greater AOB abundance compared to the AOA abundance. This finding is in contrast to those of Leininger et al. (2006) and He et al. (2007) who found that AOA was the numerically dominant ammonia oxidiser in a soil. This difference in ammonia oxidisers' abundance was most likely due to the high N loading in the current study which is the preferred growing conditions for AOB rather than AOA.

5 Conclusions

Severe trampling of the wet soil decreased NO_3^- leaching losses. The use of the nitrification inhibitor DCD even under the severe trampling conditions was effective in reducing NO_3^- leaching, soil nitrification rates, and AOB abundance and activity. AOB was more abundant than AOA and was responsible for mediating the nitrification process.

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