The effect of soil pH and dicyandiamide (DCD) on N₂O emissions and ammonia oxidiser abundance in a stimulated grazed pasture soil

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Abstract

Purpose Nitrous oxide (N₂O) is a potent greenhouse gas which is mainly produced from agricultural soils through the processes of nitrification and denitrification. Although denitrification is usually the major process responsible for N₂O emissions, N₂O production from nitrification can increase under some soil conditions. Soil pH can affect N₂O emissions by altering N transformations and microbial communities. Bacterial (AOB) and archaeal (AOA) ammonia oxidisers are important for N₂O production as they carry out the rate-limiting step of the nitrification process.

Material and methods A field study was conducted to investigate the effect of soil pH changes on N₂O emissions, AOB and AOA community abundance, and the efficacy of a nitrification inhibitor, dicyandiamide (DCD), at reducing N₂O emissions from animal urine applied to soil. The effect of three pH treatments, namely alkaline treatment (CaO/NaOH), acid treatment (HCl) and native (water) and four urine and DCD treatments as control (no urine or DCD), urine-only, DCD-only and urine + DCD were assessed in terms of their effect on N₂O emissions and ammonia oxidiser community growth.

Results and discussion Results showed that total N₂O emissions were increased when the soil was acidified by the acid treatment. This was probably due to incomplete denitrification caused by the inhibition of the assembly of the N₂O reductase enzyme under acidic conditions. AOB population abundance

increased when the pH was increased in the alkaline treatment, particularly when animal urine was applied. In contrast, AOA grew in the acid treatment, once the initial inhibitory effect of the urine had subsided. The addition of DCD decreased total N₂O emissions significantly in the acid treatment and decreased peak N₂O emissions in all pH treatments. DCD also inhibited AOB growth in both the alkaline and native pH treatments and inhibited AOA growth in the acid treatment.

Conclusions These results show that N₂O emissions increase when soil pH decreases. AOB and AOA prefer different soil pH environments to grow: AOB growth is favoured in an alkaline pH and AOA growth favoured in more acidic soils. DCD was effective in inhibiting AOB and AOA when they were actively growing under the different soil pH conditions.

Keywords Acidification • Ammonia oxidising archaea • Ammonia oxidising bacteria • Denitrification • Nitrification • Nitrification inhibitor • Nitrogen cycle

1 Introduction

Nitrous oxide (N₂O) is a potent greenhouse gas and an ozone-depletion gas in the stratosphere (IPCC 2007). Global N₂O emissions have increased by 20 % in the last century (Thomson et al. 2012), with the main anthropogenic source being agricultural soils. The majority of N₂O is produced by microbial nitrification and denitrification as part of the soil nitrogen (N) cycle (Wrage et al. 2001). Various soil properties can increase N₂O emissions by causing incomplete denitrification or increased N₂O production from nitrifiers. Soil pH has been identified as an important variable for N₂O emissions as it can affect soil N transformations and microbial communities (Mørkved et al. 2007).

Denitrification is a microbial process whereby the nitrate (NO₃⁻) produced from nitrification is converted to di-nitrogen (N₂) through the obligatory intermediate N₂O in anaerobic

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conditions. However, in many cases, N_2 is not the end product of denitrification because some denitrifiers lack the appropriate enzymes. The pH of a soil affects denitrification by altering denitrification rates (Šimek and Cooper 2002) and disrupting the denitrification pathway (Wijler and Delwiche 1954). In acid soils, denitrification rates are reduced; however, N_2O production is favoured, as the synthesis of N_2O -reductase is inhibited at a low pH (Bergaust et al. 2010; Bakken et al. 2012). In contrast, at alkaline pH, N_2 production is favoured as functional N_2O reductase is produced, and complete denitrification occurs (Wijler and Delwiche 1954; Bergaust et al. 2010; Bakken et al. 2012).

A large proportion of N_2O is produced through denitrification; however, under some soil conditions, the nitrifiers' contribution to N_2O production can increase. Wrage et al. (2001) refers to this as nitrifier denitrification and states that mainly autotrophic ammonia oxidisers carry out the process. At an acidic pH, N_2O production from autotrophic nitrification can be enhanced (Martikainen and de Boer 1993), and it is expected that more nitrifiers may carry out nitrifier denitrification at a low pH due to an increase in energy gains (Wrage et al. 2001). Increased nitrification rates can also affect N_2O emissions indirectly through the enhancement of nitrate production, thus increasing the substrate for denitrification. This is referred to as coupled nitrification-denitrification and occurs in soils where the conditions are favourable for both denitrification and nitrification processes (Wrage et al. 2001).

Bacterial and archaeal ammonia oxidising communities, which convert ammonia to nitrate, can also be affected by soil pH. This is important for N_2O emissions as ammonia oxidation is the rate-limiting step, providing the substrate for denitrification. Furthermore, ammonia oxidisers can carry out nitrifier denitrification, further increasing N_2O emissions. Nicol et al. (2008) found in long term pH plots that bacterial *amoA* decreased with acidity, whereas archaeal *amoA* increased. However, the opposite occurs in high N soils, such as under urine patches in grazed grassland soils, with AOB being primarily responsible for ammonia oxidation (Di et al. 2009). Recent studies have hypothesised that ammonia oxidising archaea (AOA) may dominate ammonia oxidation in low-pH and low-nutrient soils due to their adaptation to unfavourable environments (Erguder et al. 2009; Di et al. 2010a).

The nitrification inhibitor, dicyandiamide (DCD), has been shown to effectively reduce N_2O emissions from agricultural soils (Di and Cameron 2002, 2005; Di et al. 2010b; de Klein et al. 2011). DCD works by inhibiting the ammonia monooxygenase enzyme (AMO) responsible for ammonia oxidation. Studies demonstrating DCD use have identified differences in DCD effectiveness with changing temperature (Amberger 1989; Di and Cameron 2004), application rate, soil type (Singh et al. 2008), drainage (Di and Cameron 2011; Shepherd et al. 2012), rainfall (Luo et al. 2010), organic matter content and liming (Puttanna et al. 1999). However, the

effect of short-term soil pH alteration on DCD effectiveness has not been determined, although Puttanna et al. (1999) state that increasing the soil pH will reduce DCD's efficiency.

Previous studies on the effects of soil pH on N_2O emissions and/or ammonia-oxidising communities have been conducted in the laboratory (Thomsen et al. 1994; Clough et al. 2004; Bakken et al. 2012) or on long term/natural soil pH plots (Weslien et al. 2009; Gubry-Rangin et al. 2010; Mahmood et al. 2011; Zhang et al. 2011). Few studies have determined the effect of soil pH change on N_2O emissions, ammonia oxidising communities and DCD effectiveness in the field. Soil pH changes may occur as a result of fertiliser applications or liming. Thus, the objectives of this study were to determine the effects of soil pH change on (1) N_2O emissions; (2) the growth of ammonia oxidising bacteria (AOB) and archaea (AOA) and (3) on the effectiveness of the nitrification inhibitor, DCD, in inhibiting ammonia oxidisers and in reducing N_2O emissions.

2 Materials and methods

2.1 Soil and treatments

A field trial was established at Lincoln University (43° 38' 55" S, 172° 28' 4" E) to determine the effect of soil pH on N_2O emissions, ammonia oxidiser abundance and DCD effectiveness. The soil at the site was a Temuka clay loam (Typic Orthic Gley Soil, New Zealand Soil Classification (Hewitt 1992), Mollic Endoaquept, USDA classification (Soil Survey Staff 1998) which had been under pasture for 8 years with regular fertiliser and irrigation additions.

Forty-eight experimental plots 1.5×1.3 m in size were established in August 2012. Three pH treatments were established by applying HCl (acid treatment) or CaO/NaOH (alkaline treatment) to alter the soil pH. The original (native) soil pH was 6, and was lowered to approximately 5 in the acid treatment, and raised to above 6.5 in the alkaline treatment. Initially, the soil pH was altered over the whole experimental plots (1.5×1.3 m), applying HCl or NaOH/CaO on the 4th, 7th and 9th of May 2012. For the alkaline pH treatment, 26.8 g of CaO was applied as a fine powder to the surface of the plots and watered in with 30 L of water in 10 L of aliquots using a watering can. For the acid pH treatment, 3.6 % HCl was applied in three 10-L aliquots to each acid plot. The same volume of water (30 L) was added to the control plots (native). The volume of liquid was determined on the basis of a pilot experiment to determine the best volume and concentration of acid/base to apply to the soil to alter the pH. The soil pH of the plots was monitored once a week for one month until the pH reached the target values.

For each pH, the following treatments were applied: control (no urine or DCD), urine (700-kg urine-N ha⁻¹), DCD (10-kg DCD ha⁻¹) and urine plus DCD (700-kg urine-N ha⁻¹+10-kg DCD ha⁻¹). These treatments are referred to as “control”, “DCD-only”, “urine-only”, and “urine + DCD”, respectively. All treatments were replicated four times.

2.2 Installation of N₂O measurement frames

Following soil pH alteration, stainless steel base frames 500 mm in diameter by 150-mm deep were inserted 700-mm deep into the soil in each of the 48 plots for N₂O sampling. The frames allowed a modified closed chamber method (Hutchinson and Mosier 1981) to be employed for N₂O sample collection. On top of the base frames was a trough filled with water which provided an airtight seal when the gas chamber was placed on top. The frames were left permanently in the field for the duration of the trial, while the top chambers were placed on top of the base frames only when N₂O samples were collected. Corresponding paired frames were installed beside each of the gas sampling frames to allow destructive soil sampling to determine soil pH, mineral N, AOB and AOA abundance.

2.3 Application of treatments

Following the installation of the N₂O measurement frames, urine and DCD treatments were applied to the appropriate plots. Synthetic urine was used due to the large volume required (total urine requirements for the trial; 14-g L⁻¹ urea, 3.5-g L⁻¹ glycine, 16-g L⁻¹ potassium bicarbonate, 3-g L⁻¹ potassium chloride and 2-g L⁻¹ potassium sulphate (Fraser et al. 1994). DCD solution of 15 mL (0.13 g/mL) was applied to the appropriate plots. Herbage was cut by hand fortnightly or when required for ease of soil and gas sampling and to stimulate a grazed pasture environment. The soil pH was measured at soil sampling days to ensure the pH was maintained at the desired level and adjustments were made if necessary by applying NaOH or HCl solutions as appropriate to the soil and gas sampling rings.

2.4 Soil sampling and analysis

Soil samples were taken weekly for the first month then fortnightly until the end of the trial. For each soil sampling, two soil cores (52 cm³) were taken to a depth of 7.5 cm from each soil sampling ring. These were homogenised, and sub-samples were taken to determine moisture content, soil pH (H₂O), NO₃⁻ concentration, DCD concentration and for microbial assays (see below).

Soil NO₃⁻ was extracted using 5 g of soil with 20 mL of 2 M KCl. Samples were shaken for 1 h, centrifuged for 10 min and filtered through the Whatman 41 filter paper. Nitrate

concentration was analysed using a flow injection analyser (FIA) (FOSS FIA star 5,000 triple channel analyser).

DCD was extracted from 5 g of soil using 25 mL of deionised water. Samples were shaken for 1 h, centrifuged for 20 min and filtered using the Whatman 41 filter paper. DCD concentration was analysed on a Shimadzu high-performance liquid chromatography (Di and Cameron 2004).

2.5 Nitrous oxide flux measurements

N₂O fluxes were measured twice per week for the duration of the trial using methods described in Hutchinson and Mosier (1981). Samples were collected early afternoon at time zero, 20 min and 40 min using a syringe through a rubber septum on the gas chamber (Di et al. 2010b). The samples were injected into 6-mL exetainers for later determination of N₂O

Fig. 1 Soil pH after urine and DCD treatment application (day 0); **a** native, **b** acid treatment and **c** alkaline treatment. Arrow indicates re-application of pH treatments (18th September 2012) after the application of urine and DCD treatments. Error bars show a standard error of the mean (SEM)

concentration by gas chromatography (SRO8610 linked to a Filson 222XL autosampler and an electron capture detector (ECD) (SRI Instruments, USA)).

2.6 AOB and AOA assays

Subsamples of soil were taken at days 1, 7, 14, 30, 45 and 90 after the application of treatments to determine the abundance of the *amoA* gene for AOB and AOA. Soil samples were stored at -80°C prior to extraction.

Total soil genomic DNA was extracted from frozen soil (0.25 g) using NucleoSpin® Soil kit (Macherey-Nagel, Norrie Biotech, Auckland, New Zealand) according to the

manufacturer's instructions. DNA was eluted with 100 μL of buffer SE and stored at -20°C before analysis.

AOB and AOA *amoA* gene abundance was estimated using quantitative real-time polymerase chain reaction (qPCR). All PCR reactions were set up using a CAS1200 Robotic liquid handling system (Corbett Life Science, BioStrategy, Auckland, New Zealand), and real-time qPCR analysis was performed on a Rotor-Gene™ 6000 (Corbett Life Science, BioStrategy, Auckland, New Zealand). Tenfold diluted soil genomic DNA was used as a template for the qPCR. Bacterial and archaeal *amoA* genes were quantified using the primer pairs *amoA1F/amoA2R* (Rotthauwe et al. 1997) and

Fig. 2 N_2O flux for the native, acid and alkaline treatments; **a** control, **b** DCD-only, **c** urine-only and **d** urine + DCD during field trial. Error bars show SEM

Fig. 3 Total N₂O emitted during the trial period for the native, acid and alkaline treatments. Standard errors show SEM. LSD_{0.05} demonstrates the least significant difference ($p < 0.05$)

Fig. 4 Nitrate concentrations for **a** control, **b** DCD-only, **c** urine-only and **d** urine + DCD. Error bars indicate SEM

Arch-amoAF/Arch-amoAR (Francis et al. 2005), respectively, with SYBR® Premix Ex Taq™ (TaKaRa, Japan) using the thermal profiles as described in Di et al. (2009). The 16-μL reaction mixture contained 8 μL of SYBR® Premix Ex Taq™ (2× concentrated), 0.4 μL of each primer (final concentration 0.25 μM), 1.5 μL of template DNA and 5.7 μL of nuclease-free deionised water. To confirm PCR product specificity, a melting curve analysis was carried out at the end of each qPCR run, by measuring fluorescence continuously as the temperature was increased from 50 to 99 °C. Standard curves for real-time qPCR assays were

developed using the same method as described in Di et al. (2009). Data analysis was carried out using Rotor-Gene™ 6000 series software 1.7. The PCR amplification efficiencies were 96–98 % and 92–94 % for AOB and AOA reactions, respectively, ($R^2 > 0.995$).

2.7 Statistical analysis

Mean values and standard errors of the means for N_2O emissions, NO_3^- concentrations and ammonia oxidiser abundances were calculated based on the four replicates for each

treatment using Microsoft Excel 2010 (Microsoft Corporation, USA). Total N_2O emissions were calculated by calculating the area under the curve. Average AOA and AOB abundance per day was calculated by dividing the area under the curve of AOB or AOA abundance by the number of sampling days. Least significant differences and p values were calculated using general analysis of variance for each sampling date as required in Genstat® (version 15.1, VSN International Ltd, UK). N_2O values were log-transformed before analysis to achieve a normal distribution of the data.

Fig. 5 AOB *amoA* gene abundance for the native, acid and alkaline treatments **a** control, **b** DCD-only, **c** urine-only and **d** urine + DCD. Error bars indicate SEM

3 Results

3.1 Soil pH

During the trial period, the soil pH ranged from 6.07–7.13 in the native pH (Fig. 1a). In the acid treatments, the pH ranged from 4.33–6.41, and in the alkaline treatments, the pH ranged from 6.84–7.76 (Fig. 1b, c). The addition of urine caused an increase in soil pH in all pH treatments (Fig. 1b). DCD application did not significantly affect the soil pH.

3.2 N₂O emissions

3.2.1 Daily N₂O emissions

The highest emissions were recorded in the urine-only treatments, where emissions peaked at day 7, reaching 0.64 mg of N₂O-N m⁻² h⁻¹ in the acid treatment and 0.26 mg of N₂O-N m⁻² h⁻¹ in the alkaline treatment (Fig. 2c). In the native pH treatment, N₂O emissions peaked on day 13, reaching 0.34 mg of N₂O-N m⁻² h⁻¹. Compared with the urine-only treatment, N₂O emission peaks in the urine + DCD treatments were 71 % lower in the acid treatment, 54 % lower in the alkaline treatment and 50 % lower in the native pH treatment (Fig. 2d).

Daily N₂O emissions from the control and DCD-only treatments remained very low throughout the experimental period (Fig. 2a, b).

3.2.2 Total N₂O emissions

The control plots, on average, emitted a total of 300 g of N₂O-N ha⁻¹ over the study period. With the addition of urine, total N₂O emissions increased significantly to 1445 g of N₂O-N ha⁻¹ in the alkaline treatment, 1291 g of N₂O-N ha⁻¹ in the native pH treatment and 2,450 g ha⁻¹ in the acid treatment (Fig. 3). The addition

of DCD to the urine treatment significantly ($p < 0.05$) reduced total N₂O emissions by 64 % in the acid treatment, but the reduction in the native and alkaline treatments were not statistically significant (Fig. 3). The addition of DCD in the absence of urine did not significantly affect the total amount of N₂O emitted.

3.2.3 Soil NO₃⁻ concentration

The addition of urine caused a transient accumulation of NO₃⁻ in all soil pH treatments, with peak concentrations increasing with soil pH (Fig. 4c). Transient accumulation of NO₃⁻ was also seen in the urine + DCD treatments; however, the peak concentrations were lower compared to the urine-only treatments (Fig. 4d). In the treatments without the addition of urine, the NO₃⁻ concentrations remained low throughout the trial period (Fig. 4a, b).

In the acid treatment, the NO₃⁻ concentrations remained low throughout the trial period compared to the native pH treatment. However, in the alkaline treatment, the NO₃⁻ concentration was similar to that in the native pH treatment.

When comparing the urine-only and urine + DCD treatments, there was an average decrease in NO₃⁻ concentration of 65 % in the acid treatment, 28 % in the native pH treatment and 42 % in the alkaline treatment.

3.3 Ammonia oxidiser abundance

3.3.1 Ammonia oxidising bacteria

The AOB *amoA* gene abundance was increased by the application of urine, and this was particularly evident in the native pH treatment and the alkaline treatment (Figs. 5 and 6).

Fig. 6 Weighted average for AOB *amoA* gene abundance (*amoA* copy numbers g⁻¹ soil) per day. Error bars indicate SEM and *LSD* indicates least significant difference ($p = 0.05$)

Fig. 7 AOA *amoA* gene abundance for the native, acid and alkaline treatments; **a** control, **b** DCD-only, **c** urine-only and **d** urine + DCD. Error bars indicate SEM

With the application of DCD to the urine treatment, AOB *amoA* gene copy numbers significantly ($p < 0.05$) decreased in the alkaline treatment and the native pH treatment (Fig. 6), but the DCD effect in the acid treatment was not statistically significant.

3.3.2 *Ammonia oxidising archaea*

There were no obvious trends of growth of the AOA communities as judged by the AOA *amoA* gene copy numbers (Fig. 7). However, there was a slight decrease in AOA *amoA* gene abundance from day 1 until day 28

following the addition of urine in the acid treatment (Fig. 7c). The AOA *amoA* gene abundance then gradually recovered from day 28 until the end of the trial (Fig. 7c). Thus, at day 90, the AOA *amoA* gene abundance in the urine-only treatment was significantly ($p < 0.05$) higher in the acid treatment than in the alkaline treatment.

In the acid treatment, the AOA *amoA* gene abundance was significantly lower in the urine + DCD treatment compared to the urine-only treatment (Fig. 8). However, such differences were not found in the native pH treatment and the alkaline treatment.

Fig. 8 Weighted average for AOA *amoA* gene abundance (*amoA* copy numbers g^{-1} soil) per day. Error bars indicate SEM and *LSD* indicates least significant difference ($p=0.05$)

3.4 DCD concentration

The concentration of DCD decreased exponentially in all treatments over the trial period (Fig. 9). There was no clear treatment effect on the reduction of DCD concentration in the soil over time.

4 Discussion

4.1 N_2O emissions

This study showed that pH had a significant effect on total N_2O emissions with the amount emitted from the urine-only treatment being significantly ($p<0.05$) higher in the acid treatment than in the alkaline treatment (Fig. 2). This was

likely to have been caused by the interruption of the denitrification pathway under acid conditions (Wijler and Delwiche, 1954). At a low pH, the assembly of the N_2O reductase enzyme, responsible for the conversion of N_2O to N_2 , can become inhibited (Bergaust et al. 2010; Bakken et al. 2012). This results in an ineffective enzyme, thus incomplete denitrification and a higher proportion of N_2O (Knowles 1981). This has implications for soil pH management in terms of potential impacts on N_2O emissions.

In the alkaline treatment, NO_3^- concentrations and AOB abundances in the urine treatment were higher and N_2O emissions were lower than in the native pH treatment and acid treatment. This was probably because of complete denitrification leading to higher proportions of N_2 emitted rather than N_2O in the higher pH treatments (Thomsen et al. 1994; Stevens et al. 1998; Clough et al. 2004). Similar findings have been reported by Weslien et al. (2009) who found a strong negative correlation between N_2O emissions and increasing soil pH. However, N_2 was not measured in the present study, and further studies are required to test this hypothesis.

4.2 Ammonia oxidising communities

Soil pH affected the ammonia oxidising communities, with the abundance of AOB being lower in the acid treatment following the addition of urine. This was likely due to lower NH_3 availability in the lower pH soil since it is NH_3 rather than NH_4^+ that is the substrate used for AOB growth (Nicol et al. 2008). As a result, NO_3^- concentration remained low throughout the trial period in the acid treatment. Soil pH is, therefore, an important factor determining the growth and function of AOB in the soil.

AOA abundance showed a different pattern to that of AOB. When urine was applied, there was an initial decline in the AOA abundance from day 1 to day 28. This was probably the result of an inhibitive effect by the high rates of urine N applied (Di et al. 2010a). However, as the urine was hydrolysed, and the NH_4^+ nitrified, the AOA abundance increased

Fig. 9 Concentration of DCD (mg DCD/kg of soil) in the native, acid treatment and alkaline treatment; **a** DCD-only and **b** urine + DCD. Error bars indicate SEM.

in the acid treatment. This led to a significantly ($p < 0.05$) higher AOA abundance at day 90 compared to the alkaline treatment. Therefore, while AOB growth was inhibited under strongly acid conditions, AOA grew once the inhibitive effects of the NH_4^+ was dissipated. These results clearly indicate that AOB and AOA prefer different soil environments to grow, and soil N status and pH are two of the important parameters affecting their growth and function in the soil ecosystem (Nicol et al. 2008; Di et al. 2009, 2010a, b; Gubry-Rangin et al. 2010; Zhang et al. 2011).

4.3 DCD effectiveness

DCD was highly effective at significantly reducing total N_2O emissions in the acidic treatment; and had lower effectiveness in the native and alkaline treatments (Fig. 2). However, DCD did reduce peak N_2O emissions in all pH treatments (Fig. 1c, d).

DCD was highly effective in inhibiting the growth of AOB in the alkaline and native pH treatments following the application of the urine substrate. In the acid treatment, DCD inhibition of AOB was less effective. This however was not caused by reduced DCD concentration in the acid treatment (Fig. 9). This was probably because of the limited growth of AOB, already inhibited by the strong acidity of the soil in the acid treatment.

AOA abundances were not significantly decreased in the alkaline and native treatments by the addition of DCD (Fig. 8). This was probably because AOA growth was initially inhibited by the high concentrations of NH_4^+ in the urine-applied treatments (Di et al. 2009). However, when the AOA did grow in the acid treatment (after day 40), the application of DCD did inhibit their abundance. These results demonstrate that DCD is effective in inhibiting AOB or AOA when these communities are actively growing. If these communities are not actively growing, due to other inhibitions, then DCD effectiveness declines. This hypothesis is also supported by earlier results (Di et al. 2009, 2010a, b).

5 Conclusions

Results from this study showed that soil pH is an important parameter regulating N_2O emissions from urine-treated soils, with higher total N_2O emissions in the acid treatment than the alkaline treatment. When the urine substrate was applied, AOB growth was favoured in the alkaline treatment, whereas AOA growth was favoured in the acid treatment once the urine was hydrolysed (after day 28). DCD was effective in inhibiting the growing communities of AOB and AOA following the application of a urine substrate. These inhibitions significantly decreased the rate of nitrate production and the emissions of N_2O from the urine N.

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