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# Effect of the nitrification inhibitor dicyandiamide (DCD) on microbial communities in a pasture soil amended with bovine urine

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ABSTRACT

Nitrification inhibitors, such as dicyandiamide (DCD), have been shown to decrease leaching from urea- and ammonium-based fertilisers and from urine patches in grazed pastures. To date there have been few studies on effects of nitrification inhibitors on non-target soil microbiota. This pot trial examined the short-term effects of DCD on the activity and diversity of both target (ammonium-oxidising bacteria and archaea) and non-target soil microbial populations. Bovine urine at a rate equivalent to 600 kg urine-N $ha^{-1}$ with or without DCD at 30 kg $ha^{-1}$ was applied to pots planted with perennial ryegrass. This rate of DCD was typical of the amount applied to pasture in New Zealand, although this annual rate may be spread over several applications carried out over 2–3 months. The single high rate application was used to provide a "worst case scenario" to assist detection of potential impacts of DCD application to non-target soil microflora. Treatments also included DCD alone and untreated control pots. Soil used was a Horotiu sandy loam and pots were maintained at 80% WHC in a controlled-environment room at 12 °C/16 h (day) and 8 °C/8 h (night). Soil mineral N, hot water extractable C and N concentrations, soil pH, microbial biomass C and N, and DCD persistence were measured at regular intervals. Diversity and composition of the overall soil bacterial community were analysed by serial analysis of ribosomal sequence tags (SARST). Effects on ammonium-oxidising bacterial and archaeal communities were monitored more closely by determining the size of these populations using real-time PCR and their transcriptional activity by comparing RNA-denaturing gradient gel electrophoresis (DGGE) profiles following RT-PCR of the *amoA* gene. Changes in soil pH and mineral N following application of urine in the pot trial reflected patterns typically demonstrated in the field. Application of DCD to soil did not change the diversity of the soil bacterial community, with the four predominant phyla (Proteobacteria, Actinobacteria, Acidobacteria and Firmicutes) remaining in proportions that were similar to control soils. In contrast, urine application to soil resulted in a significant increase in members of Firmicutes, some of which are relatively stress tolerant. In line with the SARST results, shifts in the structure of the active component of the general soil bacterial community were detected in the urine and urine + DCD treatments only, further suggesting DCD had little impact on the overall soil bacterial activity. In contrast the microbes targeted by DCD, the ammonium-oxidising bacteria, were significantly affected by DCD with reductions in population size and altered activity. Ammonium-oxidising archaea, however, showed no response to application of DCD to soil, and were only minimally affected by application of urine. The results suggest that application of DCD to pasture is a relatively benign intervention that has an important role to play in mitigating the environmental hazards imposed by ongoing land use intensification.



## 1. Introduction

Intensification of New Zealand's farming systems has resulted in an increase in fertiliser inputs and stocking rates, with a five-fold increase in nitrogenous fertiliser use being recorded between 1990 and 2004 (Parliamentary Commissioner for the Environment, 2004). Intensively grazed dairy pastures in New Zealand routinely receive

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large inputs of nitrogen (N) through deposition of urine by grazing animals, with additional input through application of N fertiliser (urea). Several studies have indicated that, at least in New Zealand, animal urine patches are the major source of N leached from grazed pastures (Ledgard et al., 1999; Davidson and Mosier, 2004; Zaman and Blennerhassett 2010). The N loading in soil under a bovine urine patch can range between 700 and 1200 kg N $ha^{-1}$ which greatly exceeds the ability of pasture plants to absorb N (Haynes and Williams, 1993; Jarvis et al., 1995). The urea in urine is rapidly converted to ammonium and then to nitrate ($NO_3^-$), a process known as nitrification, by the activities of soil microorganisms. Excess $NO_3^-$-N in the soil can lead to nitrous oxide ($N_2O$) emissions through denitrification. In addition, leaching of nitrate from soil into aquifers, rivers and lakes causes adverse environmental impacts, such as eutrophication, and is a source of increasing public health concern; under some circumstances nitrate-N concentrations are close to or exceed currently acceptable guidelines for drinking water (11.3 mg $L^{-1}$ of $NO_3^-$-N; New Zealand Ministry for the Environment, 2007). Hence, much research has been aimed at mitigating the effects of increasing N inputs on pastoral land.

One of the technologies showing great promise for reducing N losses from soil and increasing N use efficiency is the application of nitrification inhibitors, and these are being used increasingly in New Zealand pastures. Nitrification inhibitors have been shown to decrease leaching and denitrification from urea- and ammonium-based fertilisers and from urine patches in grazed pastures (Di et al., 2007; Monaghan et al., 2009) and, under some conditions, can lead to improved pasture productivity (Moir et al., 2007; Sprosen et al., 2009; Zaman and Blennerhassett, 2010).

Nitrification inhibitors are not a new technology; one of the most widely used inhibitors, dicyandiamide (DCD, $C_2H_4N_4$), was shown to affect plant growth in the 1920s (McGuinn, 1924). DCD is bacteriostatic rather than bacteriocidal and impairs the activity of ammonium-oxidising bacteria by restricting the uptake or utilization of ammonium (Zacherl and Amberger, 1990), thus reducing the production of $NO_2^-$. Various formulations of DCD have been developed and their efficacy has been measured under a range of environmental and soil conditions (Di et al., 2007; Monaghan et al., 2009; Kelliher et al., 2008). Because it is readily water soluble, has little or no losses through volatility and is reasonably priced, DCD is the most used nitrification inhibitor in New Zealand (Zaman et al., 2009). While the literature about the mechanisms and benefits of nitrification inhibitors is extensive, there have been few studies on the non-target effects of these now widely used agricultural compounds. Cuttle (2008) concluded that there appeared to be no evidence of widespread environmental impacts arising from the use of these products but after reviewing the available literature, Edmeades (2004) stated that research was needed to quantify the long- and short-term impacts of these chemicals, and their repeated use, on soil quality, which includes soil microbial function. In the face of growing evidence that changes in microbial community structure may lead to changes in soil microbial function, the effects of nitrification inhibitors on the soil microbial community must be considered.

The objective of this study was to determine the short-term effects of DCD on the activity and diversity of the soil bacterial and archaeal communities. The functional group most likely to be impacted by application of DCD are the ammonium-oxidising bacteria and archaea. As ammonium-oxidising bacteria play a key role in terrestrial N cycling, specific molecular primers have been developed to allow molecular characterisation of these populations in soil (Rotthauwe et al., 1997). Archaea were included in the analysis as it was not known whether DCD was active against these organisms, which have only recently been recognised as being widespread in soil ecosystems (Leininger et al., 2006), including New Zealand pastoral soils (Bowatte et al., 2009; Di et al., 2009). Several recent studies have suggested there is potential for archaea to play a dominant role in soil N cycling, given that archaeal *amoA* genes may be present at levels between 2 and 3000 times greater than their bacterial counterparts (Schauss et al., 2009), depending on soil type. Evidence for ammonium oxidation by bacteria and archaea in soil has largely been based on abundance of ammonia monooxygenase (amo) genes, rather than activity. This study reports on the treatment effect of DCD and bovine urine, applied alone and in combination, on some soil biochemical characteristics as well the activity of both target (ammonium-oxidising bacteria and archaea) and non-target soil microbial populations by analysis of the transcriptional activity of 16S rRNA genes and a key functional gene, *amoA*. Changes in abundance of *amoA* genes in response to amendment of soil with bovine urine and DCD were also measured.

## 2. Materials and methods

### 2.1. Soil, experimental treatments and sampling

Horotiu sandy loam soil (a Vitric Hapludand, Horotiu sandy loam) (Hewitt, 1998) was obtained from white clover (*Trifolium repens*)/perennial ryegrass (*Lolium perenne*) pasture to a depth of 15 cm, near Hamilton, New Zealand (37° 47′S, 175° 20′ E). This soil had the following characteristics: organic carbon, 4.1%; total nitrogen, 0.4%; Olsen P 29 μg $g^{-1}$ soil; soil pH (water) 5.6. Approximately 1 kg of field-moist soil (oven-dry equivalent was 658 g) was placed in each of 120 pots (14 cm diameter × 10 cm high). Four ryegrass seedlings (cv. 'Samson AR1') were planted in each pot and pots were maintained in a controlled-environment (CE) room operating at 12 °C/16 h (day) and 8 °C/8 h (night). These conditions were used throughout the experiment.

Pots containing soil were allocated into four groups of 30, with each group being allocated to one of four treatments: control; urine only; urine + DCD; DCD only. Bovine urine was collected from a dairy farm near Hamilton, New Zealand. Three urine samples were collected and bulked. The bulked sample (21.4 L) had a concentration of 4.14 g N and 9.2 g C $L^{-1}$. Two sub-samples of urine, 8 L each, were put in 2 containers. One container received 100 ml of DCD containing 1.6 g DCD (urine + DCD) and the other container received 100 ml of distilled water (urine only treatment). A third container containing 8 L of distilled water also received 100 ml of DCD solution containing 1.6 g DCD (DCD only treatment). All the treatments were applied at a rate of 230 ml of liquid per pot. Control pots received 230 ml of distilled water. Applications were made over a 5 day period to minimise drainage from each pot (any leachate was collected in a saucer and reabsorbed into soil within 12 hours). Amounts of DCD and urine applications were selected to represent 600 kg $ha^{-1}$ of urine-N and 30 kg $ha^{-1}$ of DCD. The rates were calculated on the surface area of soil in each pot. The rate of urine-N is within the range of N concentration typically found under urine patches, while the rate of DCD applied is typical of the amount applied to pasture in New Zealand, although this annual rate may be spread over several applications carried out over 2–3 months (Zaman et al., 2009). This one time high application was selected to investigate adverse impacts (if any) of DCD application to soil microflora. Also, Sprosen et al. (2009) reported that high rate of DCD is more effective at retaining inorganic N in top layers of soil than lower rates. Pots were maintained in a CE room as described above and were regularly watered to a fixed weight to maintain a soil water holding capacity of approximately 80%.

Before treatment application, four pots were destructively sampled to obtain baseline data. At each sampling, four replicate pots from each treatment were destructively sampled; soil from individual pots was mixed thoroughly and a bulk sample from each pot