Soil Biology & Biochemistry 88 (2015) 344–353

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Impact of urine and the application of the nitrification inhibitor DCD on microbial communities in dairy-grazed pasture soils

Sergio E. Morales [a, *], Neha Jha [b, c], Surinder Saggar [b]

[a] Department of Microbiology and Immunology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand
[b] Ecosystems and Global Change Team, Landcare Research, Palmerston North 4442, New Zealand
[c] Earlier Soil & Earth Sciences Group, Institute of Agriculture and Environment, Massey University, Palmerston North 4442, New Zealand

ARTICLE INFO

Article history:
Received 26 November 2014
Received in revised form
12 May 2015
Accepted 6 June 2015
Available online 20 June 2015

Keywords:
Nitrous oxide
denitrification
Microbial functional genes
16S rRNA
Next generation sequencing
nitrification inhibitor

ABSTRACT

Tools to manage the emission of the greenhouse gas nitrous oxide (N_2O), an intermediate of both nitrification and denitrification, from soils are limited. To date, the nitrification inhibitor dicyandiamide (DCD) is one of the most effective tools available to livestock farmers for reducing N_2O emissions and minimizing leaching of nitrogen in response to increased urine deposition in grazed pasture systems. Despite its effectiveness in decreasing N losses from animal urine by inhibiting N processes in soils, the effect of DCD on the population structure of denitrifiers and overall bacterial community composition is still uncertain. Here we use three New Zealand dairy-grazed pasture soils to determine the effects of DCD application on microbial community richness and composition at both functional (genes involved in the denitrification process) and phylogenetic (overall bacterial community composition based on 16S rRNA profiling) levels. Results further confirm that the effects on microbial populations are minimal and transient in nature. The impact of DCD on microbial community structure was soil dependent, and a greater effect was attributed to intrinsic soil properties like soil texture, with community response to DCD in combination with urine being comparable to that under urine alone. Addition of DCD to cattle urine also reduced N_2O emission between 23 and 67%.

1. Introduction

New Zealand's pastoral based agricultural system is a cornerstone of its economy. In 2013 a total of 7.84 million hectares of land were dedicated to grasslands ("Statistics New Zealand," 2013), which support New Zealand's pasture based-livestock industries. It is estimated that approximately 42 million animals (30.79 million sheep, 6.48 million dairy, 3.70 million beef and 1.03 million deer) were supported in 2013 using a year-round grass fed outdoors approach ("Statistics New Zealand," 2013). As a result, animal excreta in the form of dung and urine compose a major part of New Zealand's N input into soils (de Klein et al., 2003; de Klein and Ledgard, 2005; van der Weerden et al., 2011). Nitrogen deposited in this form is normally in excess of what is assimilated by plants resulting in a superfluous N pool lost via three major mechanisms: volatilization, leaching, and denitrification (Ball et al., 1979; Monaghan et al., 2007).

Managing N losses linked to urine deposition is difficult due to the myriad of factors controlling N transformations at farm (Saggar et al., 2013) or larger (Morales et al., 2015) scales. This has resulted in the reliance on simple approaches including the use of nitrification inhibitors (Di and Cameron, 2006). In New Zealand, dicyandiamide (also known as cyanoguanidine, dicyanodiamide or DCD) applied at 10 kg ha^{-1} had been used as an effective management tool to block nitrification, thus reducing denitrification substrates while limiting N loss through leaching and nitrous oxide (N_2O) emission (Di et al., 2007; Zaman et al., 2007, 2009). Prior work suggests that DCD specifically inhibits bacterial ammonia oxidizer's by blocking the conversion of urine derived ammonia to hydroxylamine (Di et al., 2009). This inhibition is thought to be specific and to date no significant impacts on microbial communities have been observed (O'Callaghan et al., 2010; Wakelin et al., 2013). Once applied to soils, temperature dependent degradation of DCD occurs both *in situ* (Kelliher et al., 2008) and *in vitro* by common soil microorganisms (Hallinger et al., 1990). This degradation has been confirmed even after sustained application of the inhibitor over a 7 year period (Guo et al., 2013). However, previous methods used to assess the potential impacts of DCD on microbial

* Corresponding author. Tel.: +64 3 479 3140; fax: +64 3 479 8540.
E-mail address: sergio.morales@otago.ac.nz (S.E. Morales).

http://dx.doi.org/10.1016/j.soilbio.2015.06.009
0038-0717/

community structure have relied on low-resolution tools (e.g. DGGE) or focused solely on specific functional groups (e.g. ammonia oxidizers) (Di et al., 2014). The identification of vulnerable microbial populations is critical to maintaining soil health, and is particularly important given the recent detection of DCD in surface waters (Smith and Schallenberg, 2013).

In this study we use soil microcosms, gas chromatography, soil chemical analyses, high throughput 16S rRNA gene amplicon sequencing, terminal restriction fragment length polymorphism (T-RFLP) and quantitative PCR (qPCR) on three denitrifier functional genes (*nirS*, *nirK* and *nosZ*) to assess the impact of DCD on target denitrifier communities, as well as the overall microbial community by means of 16S rRNA gene analysis. The objectives were to determine: (1) the impact of urine and DCD application on total bacterial community composition and diversity, (2) the impact of urine and DCD application on specific target populations within the denitrifier guild (by assessing community abundance and diversity), (3) if microbial community response to DCD was influenced by soil type.

2. Materials and methods

2.1. Study site and sample collection

Soils were collected from three different farms (Tokomaru [TM] silt loam from Massey University No.4 dairy farm in Palmerston North [40°22′58.26″S, 175°36′31.01″E], Manawatu [MW] fine sandy loam from a Longburn dairy farm [40°22′56.99″S, 175°32′24.49″E] and an Otorohonga [OH] silt loam from an AgResearch Ruakura dairy farm in Hamilton [38°11′19.70″S, 175°12′35.67″E]) that have been characterized previously and shown to exhibit contrasting denitrification enzyme activities and N_2O emissions (Jha unpublished) (Table 1, Fig. S1). At each farm 25 soil cores (25 mm diameter and 100 mm long) were collected from each of the four randomly selected areas (100 m^2 each). This resulted in 4 biological replicates per site, with each replicate consisting of 25 composited field cores. During sampling, areas around paddock entrances, water troughs and obvious urine or dung patches were avoided. The collected soil from each randomly selected area resulted in 4 field replicates of the soil on each farm. Soil samples for each replicate were sieved separately to 2 mm and immediately stored in plastic bags at 4 °C for chemical analysis. Subsamples from each of the plastic bags were stored at −20 °C for molecular analysis.

2.2. Microcosms

Four microcosms were established for each farm using four field replicates to address the variability in soil characteristics identified across the grazed farm. Each microcosm consisted of 50 g (dry weight equivalent) subsamples of each soil placed in plastic containers (r = 2.25 cm, h = 7.4 cm, vol = 117.63 cm^3) with 1 mm holes (15 in number) on the walls of the container to allow for the acetylene (C_2H_2) gas to penetrate the soil and N_2O to be released from the soil. Treatments were applied by bringing soils to saturation by gradually adding either deionized water in control treatments, urine (700 mg N kg^{-1} dry soil) or urine (700 mg N kg^{-1} dry soil) + DCD (10 mg DCD kg^{-1} dry soil). In urine and urine + DCD treatments the same amounts of deionized water, minus the volumes of bovine urine and DCD, were applied to respective containers to increase the soil water contents. The DCD application rate was based on prior work conducted in New Zealand (Singh et al., 2008; Kim et al., 2012; Zaman and Nguyen, 2012). To reduce N losses from grazed pasture 10 kg ha^{-1} of DCD in 800 L water using a fine particle suspension twice per year in late autumn and late winter is recommended (Di and Cameron, 2005). Single application of DCD was decided based on the results of (de Klein et al., 2011) suggesting repeated use of DCD does not impact its effectiveness as compared to single use. Urine used in the experiment was collected fresh from cows during milking (avoiding contamination from dung), and stored in tightly sealed plastic bottles at 4 °C to avoid urea hydrolysis. Total C and N content in urine were determined and the amount of urine required for application to achieve desired N application was calculated based on this. Microcosms were set up in the presence and absence of acetylene (C_2H_2) (10% headspace volume of the glass jars i.e. 100 ml) resulting in 72 total microcosms (3 soils × 3 treatments × 4 replicates × 2 ± C_2H_2). A separate set of four replicated soil samples (250 g each) for each treatment was prepared and incubated in glass jars for periodic measurements of changes in mineral-N, soil pH and microbial community structure. Once established, all microcosms were incubated at 25 °C for 28 days. Gas sampling, chemical and molecular analysis were conducted on days 1, 3, 7, 15 and 28 days of application of treatments.

Field moist soil samples were analyzed for soil pH, gravimetric water content, mineral N (NO_3^- and NH_4^+), total nitrogen (TN), total carbon (TC), Olsen P, soluble C (K_2SO_4 extractable C from non-fumigated soils), microbial biomass carbon (MBC), denitrification enzyme activity (DEA), and denitrification rate (DR) before application of treatments. Periodic measurements of soil pH, MBC, soluble C, and mineral N contents were conducted following the application of the treatments during the entire incubation.

Soil pH was measured in a 1:2.5 (w/w) soil to water mixture stirred vigorously then left to stand overnight before measurements using a PHM 83 Autocal pH meter (Blakemore, 1987). Soil water content (SWC) was determined gravimetrically by first weighing the wet soil samples, oven drying at 105 °C for 24 h and re-weighing the dried soil. Soil NO_3^-–N and NH_4^+–N were determined by 1 h soil extraction with 2 M KCl solution at soil extract ratio of 1:5, and subsequent analysis of the filtrate colorimetrically using an automatic analyzer method (Downes, 1978). TN and TC were determined by combustion using a LECO CNS-1000 (Bremner, 1996; Nelson and Sommers, 1996). Olsen P was determined in 0.5 M $NaHCO_3$ soil extracts (Olsen et al., 1954), by the phosphomolybdate method (Murphy and Riley, 1962) using a Spectrophotometer PU 8625 UV/VIS at 712 nm. Microbial biomass carbon (MBC) was determined using the chloroform fumigation-extraction technique (Vance et al., 1987). Fumigated and non-fumigated soils were extracted with 0.5 M K_2SO_4 for 30 min (1:5 soil:extractant ratio), filtered and an aliquot was analyzed for organic C by acid-dichromate oxidation (Jenkinson and Powlson, 1976) in which an aliquot of soil extract was added to a mixture of sulphuric acid and orthophosphoric acid and boiled under refluxing condition for 30 min. Excess dichromate was titrated with ferrous ammonium sulfate. The additional oxidisable C obtained from the fumigated soils was taken to represent the microbial-C flush and converted to microbial-biomass C using the relationship: microbial C=C flush/0.41.

Denitrification enzyme activity (DEA) was determined using the slightly modified method described by Luo et al. (2010). Soil samples (10 g equivalent dry weight) were placed in 125 ml flasks. Slurries were prepared by adding 25 ml of a solution containing 2.2 mg NO_3^- (35 μmoles NO_3^-) as KNO_3^-, 2.5 mg C (208 μmol C) as D-Glucose and 250 μg chloramphenicol. The flasks were sealed using Suba-Seal® septa (Sigma–Aldrich) with air flushed from the flasks using N_2 gas to create anaerobic conditions. Ten percent of the headspace volume (approx. 10 ml) of the flasks was replaced with purified (acetone-free) acetylene (C_2H_2). Gas samples (5 ml) for time 0 (T0) were taken immediately and replaced with an equal quantity of N_2. The flasks were then placed on an orbital shaker (set at 125 rpm) and incubated at 25 °C for 6 h. A 5 ml gas sample was