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Effect of seven years application of a nitrification inhibitor, dicyandiamide (DCD) on soil microbial biomass, protease and deaminase activities, and the abundance of bacteria and archaea in pasture soils

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Abstract

Purpose The nitrification inhibitor, dicyandiamide (DCD), has been shown to be highly effective in reducing nitrate (NO_3^-) leaching and nitrous oxide (N_2O) emissions, when used to treat grazed pasture soils. However, there have been few studies on possible effects of long-term DCD use on other soil enzyme activities or the abundance of the general soil microbial communities. The objective of this study was to determine possible effects of long-term DCD use on key soil enzyme activities involved in the nitrogen (N) cycle, and the abundance of bacteria and archaea in grazed pasture soils.

Materials and methods Three field sites used for this study had been treated with DCD for seven years in field plot experiments. The three pasture soils from three different regions across New Zealand were Pukemutu silt loam in Southland (SL) in the southern South Island, Horotiu silt loam in the Waikato (WK) in the central North Island, and Templeton silt loam (CT) in Canterbury in the central South Island. Control and DCD treated plots were sampled to analyse soil pH, microbial biomass C and N, protease and deaminase activity, and the abundance of bacteria and archaea.

Results and discussion The three soils varied significantly in the microbial biomass C (858 to 542 μg C g^{-1} soil) and biomass N (63 to 28 μg N g^{-1}), protease (361 to 694 μg tyrosine g^{-1} soil h^{-1}) and deaminase (4.3 to 5.6 μg NH_4^+ g^{-1} soil h^{-1}) activity, and bacteria (bacterial 16S rRNA gene copy number: 1.64 x 10^9 to 2.77 x 10^9 g^{-1} soil) and archaea (archaeal 16S rRNA gene copy number: 2.67x10^7 to 3.01x10^8 g^{-1} soil) abundance. However, seven years of DCD use did not significantly affect these microbial population abundance and enzymatic activities. Soil pH values were also not significantly affected by the long-term DCD use.

Conclusions These results support the hypothesis that DCD is a specific enzyme inhibitor for ammonia oxidation and does not affect other non-target microbial and enzyme activities. The DCD nitrification inhibitor technology therefore appears to be an effective mitigation technology for nitrate leaching and nitrous oxide emissions in grazed pasture soils with no adverse impacts on the abundance of bacteria and archaea and key enzyme activities.

Keywords Archaea • Bacteria • Dicyandiamide • Enzyme activity • Microbial abundance • Microbial biomass • Nitrification inhibitor

1 Introduction

In New Zealand, the major land-use is intensively grazed pasture farming where the animals graze pastures year round. In such production systems, animal urine patches have been identified as the major source of nitrate (NO_3^-) leaching and nitrous oxide (N_2O) emissions (Jarvis et al. 1995; Di and Cameron 2000, 2002a; De Klein et al. 2003). The N loading rate under a dairy cow urine patch may range between about 700 and 1200 kg Nha^{-1}, which is far greater than the N requirements of pastures (Haynes and Williams 1993; Jarvis et al. 1995). Thus, the excess N in the soil can lead to NO_3^--N leaching and N_2O emissions. These soil N losses represent both an inefficiency of N use in the agricultural system and also an environmental issue (Di and Cameron 2002a). Hence, major efforts have

been devoted to developing mitigation strategies to reduce N losses from the animal urine patches in grazed pastures.

One highly effective mitigation technology for reducing NO_3^- leaching and N_2O emissions from grazed pasture soil is the use of nitrification inhibitors such as dicyandiamide (DCD) (Di and Cameron 2002b, 2003, 2004, 2007; Di et al. 2007; Monaghan et al. 2009; de Klein et al. 2011). DCD works by deactivating the ammonia monooxygenase (AMO) enzyme responsible for the first step of the nitrification process, the conversion of ammonia to hydroxylamine (Amberger 1989; Di et al. 2009a). It is a non-toxic, non-volatile and water soluble compound and is degraded to CO_2, NH_4^+ and H_2O without leaving any long-term residues in the soil (Amberger 1989). More recent studies have shown that the use of DCD to treat grazed pasture soils did not adversely affect other soil agrobacteria and specific microbial communities such as methanotrophs (O'Callaghan et al. 2010; Di et al. 2011). However, possible long-term effects of repeated DCD use on other soil microbial enzyme activities and population abundance have not been widely studied. Such information is important for the long-term, sustainable use of DCD as an environmental mitigation tool.

The objective of this study was therefore to determine possible effects of long-term DCD use on key soil microbial enzyme activities involved in the nitrogen cycle, microbial biomass, and general soil bacteria and archaea population abundance in three different grazed pasture soils which had been treated with DCD for seven years.

2 Materials and methods

2.1 Soils

Three long-term field plot trials were established in 2004 to study the effect of DCD on nitrate leaching and/or pasture yield at three different locations across New Zealand: Southland (SL, southern South Island), Canterbury (CT, central South Island), and the Waikato (WK, central North Island) (see Table 1 for coordinates). The Southland site was located on a dairy farm at Tussock Creek about 20 km north of Invercargill (see Monaghan et al. 2009 for details). The SL soil is a poorly-drained Pukemutu silt loam (NZ classification: Argillic-mottled Fragic Pallic soil; USDA: Aeric Fragiaquept) (Monaghan et al. 2009). The pasture was dominated by a mixture of perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Twelve hydrologically-isolated plots (12 x 15 m) were established. Mole (installed at 450 mm depth) pipe (installed at 750 mm) drainage systems were installed in each plot to collect drainage water. Two treatments control and DCD, each with six replicates, were allocated to the plots in a randomized block design. DCD was applied at 10 kg ha^{-1} (active ingredient) to the DCD plots two or three times per year between autumn and early spring. The plots were grazed as per the normal grazing cycle of the dairy farm. The plots also received basal and N fertilizers as per normal farm practice (Monaghan et al. 2009).

The Canterbury site (CT) was located on the Lincoln University demonstration dairy farm, about 20 km south of Christchurch in the central South Island (see Table 1). The CT soil is a free draining Templeton silt loam (NZ classification: Immature Pallic Soil; USDA: Udic Haplustepts). Sixteen plots (20 x 7 m) were established and were fenced off from grazing. Treatments included control and DCD which were randomly allocated to the plots. DCD was applied to the DCD treatments at 10 kg ha^{-1} (active ingredient) twice per year, once in late autumn and once in early spring. The pasture, which was predominantly perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.), was cut at intervals similar to the grazing rotation of the dairy farm and was removed. The

plots received normal fertilizer applications as per the farm operations. The plots also received the same amount of irrigation water as the rest of the farm during the summer, typically about 500 mm per year. Soil samples were taken from Control plots where no animal urine was applied during the seven year period.

The Waikato site was located on a dairy farm in the central North Island. The soil is a Horotiu silt loam (NZ classification: Typic Orthic Allophanic Soil; USDA: Typic Udivitrand). This soil is a free-draining soil developed from volcanic parent material. The pasture was a mixture of perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Twelve field plots 24.5 x 29.5 m each were established on the Horotiu soil. Three treatments were applied to the plots: a control; DCD applied twice per year (10-15 kg DCD ha^{-1}, once in late autumn and once in mid-winter or early spring); and DCD applied three times per year (once in late autumn, once in mid-winter, and once in spring). The plots were grazed as per the normal grazing cycle of the farm. The plots also received the same N and other fertilizers as the rest of the farm. However, soil samples for this study were only collected from the control treatment and the plots where DCD was applied twice per year.

2.2 Soil sampling

Soil samples (0-0.1 m depth) were collected before DCD application in autumn (April) 2011 to determine the effect of DCD use since 2004. Twenty random samples were collected from each plot and bulked into a single sample. The samples were packed with ice blocks and kept in a cool room before being analysed.

2.3 Biochemical analysis

Soil pH was measured in de-ionised water (soil: water ratio 1:2.5). Soil microbial biomass C and N were determined by using the chloroform fumigation extraction method as described by Vance et al. (1987) and Brooks et al. (1985). Two 5 g samples of moist soil were taken from each replicate. Half of the samples were chloroform-fumigated for 24 hours and then extracted with 0.5 M K_2SO_4 by shaking at 120 rpm. The suspended samples were centrifuged and filtered. The other set of samples (controls) were unfumigated but were extracted in the same way. Total C and N in these filtrates were analysed using a Total Organic Carbon Analyser and a Flow Injection Analyser (FIA), respectively. Microbial biomass C and N were calculated as the difference between the amounts of C and N extracted from the fumigated and unfumigated samples.

Soil protease activity was assayed using the method of Ladd and Butler (1972), as modified by Burton and McGill (1992). Four grams of moist soil was weighed into a 50 ml centrifuge tube and 10 mL of sodium caseinate (10 mg mL^{-1} in 0.1 M Tris (hydroxymethyl) aminomethane (TRIS) buffer (pH = 8.1) added. The mixture was shaken at 120 oscillations min^{-1} on a reciprocating shaker bath at 50°C. After one hour the reaction was stopped with four mL of 17.5% trichloroacetic acid (TCA), and then the tubes were centrifuged at 2000 x g for 20 minutes. Two mL of supernatant was removed and added to 3.0 mL of 1.4 M Na_2CO_3 and 1 mL of threefold diluted Folin & Ciocalteu's phenol reagent (BDH). For soil controls, 0.1 M TRIS buffer was substituted for the caseinate-buffer solution. Reagent controls included all reagents but no soil. Absorbance at 700 nm was measured after 10 min using a solution spectrophotometer, and then compared to tyrosine standards.

Soil deaminase activity was measured using the procedure as described by Alef and Kleiner (1987). Five grams of moist soil was incubated at 37°C for three hours after adding two mL of 11.5 mM arginine solution. A blank was similarly prepared, but immediately frozen at -20°C. After incubation, 18 mL of 2 M KCl solution was added to the soil, shaken for 30 minutes and filtered. Three mL KCl solution, two mL of 0.12 M sodium phenolate solution, one

mL of 0.17 mM sodium nitroprusside and one mL sodium hypochlorite solution were added to one mL of the filtrate. After 30 min, the color formed was measured spectrophotometrically against the blank at 630 nm.

2.4 Determination of bacterial and archaeal abundance

Bacterial and archaeal 16S rRNA gene abundance was quantified using real-time quantitative PCR (qPCR). DNA was extracted from 0.4 g soil (fresh weight) using MoBio Powersoil™ DNA isolation kits (MoBio Laboratories, GeneWorks Pty Ltd, SA, Australia) according to the manufacturer's instructions. Four replicated DNA extractions were prepared for each treatment at each location. Concentration and quality of the extracted DNA was estimated using Qubit fluorometer (Invitrogen, Auckland, New Zealand) and NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, USA). All DNA extractions were diluted 1:10 to reduce potential PCR inhibition. PCR primer pair 1369F and 1492R (Suzuki et al. 2000), and primer pair A364aF and A934bR (Burggraff et al. 1997; Großkopf et al. 1998) were used to amplify regions of the bacterial and archaeal 16S rRNA genes respectively (Table 2). All reactions were set up using the CAS-1200 Robotic liquid handling system (Corbett Life Science, BioStrategy, Auckland, New Zealand), and real-time qPCR was performed with SYBR® Premix Ex Taq™ master mix (TaKaRa, Norrie Biotech, Auckland, New Zealand) on a Rotor-Gene™ 6000 real-time rotary analyser (Corbett Life Science,BioStrategy, Auckland, New Zealand). A typical 16 µL reaction consisted of 8.0 µL of SYBR®Premix Ex Taq™, 0.5 µL each primer (10 µM) for bacteria or 0.2 µL each primer (10 µM) for archaea, 1.5 µL of template DNA and sterile water to the final volume. The PCR cycling conditions are detailed in Table 2.

Standards used in the qPCR analysis were prepared as follows. The archaeal and bacterial 16S rRNA gene fragments from the extracted DNA using Premix Ex Taq™ master mix (TaKaRa, Norrie Biotech, Auckland, New Zealand) with primers and conditions listed in Table 2. The PCR products were then gel purified using AxyPrep DNA Gel Extraction Kit (Axygen, Total Laboratory Systems, Auckland, New Zealand), cloned into the pGEM-T Vector (Promega, In Vitro Technologies, Auckland, New Zealand) and transformed into JM109 High Efficiency Competent Cells (Promega, In Vitro Technologies, Auckland, New Zealand) according to the manufacturer's recommendations. The transformed cells were then grown in 3 mL of Lurea Bertani (LB) medium overnight and plasmids containing correct inserts were extracted and purified using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, Auckland, New Zealand). The concentration and quality of the extracted plasmid DNA were estimated as described earlier. Standard curves used for each gene quantification were generated using a series of 1:10 dilutions of plasmid DNA, giving a concentration range from 10^2 to 10^8 copies·µL^{-1}. Amplification efficiencies of 95-101% ($R^2 > 0.99$) and 92-99% ($R^2 > 0.99$) were observed for the bacterial and archaeal standards respectively.

A typical qPCR run included soil DNA samples, corresponding standards and no-template control (NTC) reactions. Data analysis was carried out using Rotor-Gene™ 6000 series software 1.7 (Corbett Life Science, BioStrategy, Auckland, New Zealand).

2.5 Data analysis

Mean values and standard errors of all data were calculated based on the replicates of each treatment. Analysis of variance was performed using Genstat Version 14 (Lawes Agricultural Trust) to test the effect of DCD use on the soil properties.

3 Results

3.1 Soil pH

The pH in the three soils varied slightly from 5.9 in the SL soil to 6.3 in the CT soil (Fig. 1). However, there was no significant difference in soil pH between the control and DCD treatment in all the three soils.

3.2 Microbial biomass C and N

Microbial biomass carbon varied from 858 µg C g^{-1} soil in the SL soil to 542 µg C g^{-1} soil in the CT soil (Fig. 2). The application of DCD did not significantly affect the soil microbial biomass carbon in the three soils. Soil microbial biomass N varied from 63 µg N g^{-1} soil in the SL soil to 28 µg N g^{-1} soil in the CT soil (Fig. 3). Similarly, the application of DCD over seven years did not result in significant changes in microbial biomass N in the three soils.

3.3 Soil protease and deaminase activity

Soil protease activity varied from 361 µg tyrosine g^{-1} soil h^{-1} in the SL soil to 694 µg tyrosine g^{-1} soil h^{-1} in the CT soil (Fig. 4). The use of DCD did not significantly change the soil protease activity. The soil deaminase varied from 4.3 µg NH_4^+ g^{-1} soil h^{-1} in the WK soil to 5.6 µg NH_4^+ g^{-1} soil h^{-1} in the CT soil (Fig. 5). The DCD treatment did not result in a significant difference in deaminase activity in the three soils.

3.4 Abundance of bacteria and archaea

The bacteria DNA gene copy numbers varied from 1.64 x 10^9 g^{-1} soil in the WK control soil to 2.77 x 10^9 g^{-1} in the SL control soil (Fig. 6). After seven years of DCD treatment, there was no significant difference in the bacteria gene copy numbers between the control and DCD treatment. The archaea gene copy numbers varied significantly between the three soils but were more than 10 fold lower than the bacteria gene copy numbers (Fig. 7). Overall, the use of DCD did not significantly affect the archaea gene copy numbers. The archaea gene copy numbers in the DCD treatment were slightly higher than in the control treatment for the CT soil. Further research is required to determine the significance of this difference.

4 Discussion

The nitrification inhibitor DCD is increasingly used in New Zealand as a means of mitigating NO_3^- leaching and N_2O emissions in grazed pastures. One of the questions arising from this practice is whether the long-term use of DCD has an impact on soil microbial communities and key N cycling processes (e.g., N mineralization). Results from this study clearly show that after seven years of DCD use on three different grazed dairy pasture soils across different parts of New Zealand, no significant effect was observed on soil microbial C and N levels, protease and deaminase activities, and the abundance of bacteria and archaea in the three soils. These results would support the hypothesis that DCD is a specific inhibitor for ammonia oxidizers which carry the ammonia monooxygenase gene, and does not affect the abundance of bacteria and archaea and the key enzyme activities which are important for the N cycle.

Soil microbial biomass is a relatively small but important component of the soil organic matter, and is often used as a sensitive indicator for soil quality change. The lack of a significant effect following seven years of DCD

use would suggest that DCD appears to be benign to the bulk of the soil microbial communities, while specifically inhibiting the growth of ammonia oxidizers (Di et al. 2009a). This conclusion is further supported by the lack of a significant impact on bacteria and archaea in the three soils. Similarly, O'Callaghan et al. (2010) also found that DCD was relatively benign and did not affect the soil microbial communities. Di et al. (2011) studied the effect of DCD on a specific group of the soil microbial community, methanotrophs, and also found no significant effect. Other studies (e.g., Di and Cameron 2004; Zaman et al. 2009) also found no effect of DCD on soil microbial biomass. The significance of the slightly higher archaea gene copy numbers in the DCD treatment in the CT soil is unclear and will require further investigation.

The two soil enzymes, protease and deaminase, chosen for this study are important for N cycling in the soil, responsible for the sequential mineralization of soil organic N. Soil enzyme activities and microbial biomass are closely related, and are sometimes also used as indices of soil quality. The lack of a significant effect after seven years of DCD use on the activity of the two enzymes would suggest that DCD can specifically inhibit one step of the nitrogen cycle, namely the oxidation of ammonia, whilst not affecting other key soil N cycling processes.

5 Conclusions

These results and those reported earlier would support the conclusion that DCD is a relatively benign nitrification inhibitor which specifically targets the inhibition of the ammonia oxidation process, and which has little or no long-term effect on the abundance of bacteria and archaea and key soil enzyme activities that are responsible for N cycling in the soil.

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Captions for tables and figures

Table 1 Sampling locations and properties of the Southland (SL), Waikato (WK) and Canterbury (CT) soils used in the study

Table 2 Primer sets used in the real-time PCR to quantify bacterial and archaeal 16S rRNA gene copies in the soils (Suzuki et al. 2000; Burggraaf et al. 1997; Grosskopf et al. 1998)

Fig. 1 pH of SL, WK and CT soils with and without DCD. The error bars indicate the standard error of mean (SEM)

Fig. 2 Microbial biomass C for SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

Fig. 3 Microbial biomass N for SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

Fig. 4 Protease activity in SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

Fig. 5 Soil deaminase activity in SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

Fig. 6 Bacterial 16S rRNA gene abundance in SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

Fig. 7 Archaeal 16S rRNA gene abundance for SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

Table 1 Sampling locations and properties of the Southland (SL), Waikato (WK) and Canterbury (CT) soils used in the study

Soil region and location		Southland (SL) 48°14'43" S; 168°20'43" E	Waikato (WK) 38°46'38" S; 175°18'26" E	Canterbury (CT) 43°38'11" S; 172°26'18" E
Annual mean temperature (°C)		10.3	13.8	11.6
Annual total rainfall (mm)		1080	1150	650
Soil type		Pukemutu silt loam	Horotiu silt loam	Templeton silt loam
Particle size distribution (%)	Sand	44.8	55.3	59.1
	Silt	40.7	33.6	29.4
	Clay	14.5	11.1	11.5
pH (H_2O)		5.8	5.8	5.9
Organic matter (%)		8.1	11.4	6.4
Total N (%)		0.44	0.65	0.34
Total C (%)		4.71	6.59	3.73
C/N (ratio)		11	10	11
Olsen P ($\mu g\ g^{-1}$)		25	39	39
CEC ($cmol_C\ kg^{-1}$)		13.0	18.0	13.0
Calcium ($cmol_C\ kg^{-1}$)		7.5	8.9	8.1
Magnesium ($cmol_C\ kg^{-1}$)		0.93	1.48	1.49
Potassium ($cmol_C\ kg^{-1}$)		0.35	0.59	0.50
Sodium ($cmol_C\ kg^{-1}$)		0.22	0.40	0.22
Sulphate S ($mg\ kg^{-1}$)		10	113	21
Base sat. (%)		66.5	63.5	77.4

Table 2 Primer sets used in the real-time PCR to quantify bacterial and archaeal 16S rRNA gene copies in the soils (Suzuki et al. 2000; Burggraaf et al. 1997; Grosskopf et al. 1998)

Target group	Primer	Sequence (5'-3')	Length of amplicon (bp)	Conc. (nM)	Thermal profile
Bacterial	1369F	CGGTGAATACGTTCYCGG	100	0.312	2 min at 94°C, followed by 40 cycles of 10 s at 94°C, 30s at 56°C
	1492R	GGWTACCTTGTTACGACTT		0.312	
Archaeal	A364aF	CGGGGYGCASCAGGCGCGAA	548	0.125	2 min at 94°C, followed by 40 cycles of 20 s at 94°C, 30s at 58°C and 20 s at 72°C
	A934bR	GTGCTCCCCCGCCAATTCCT		0.125	

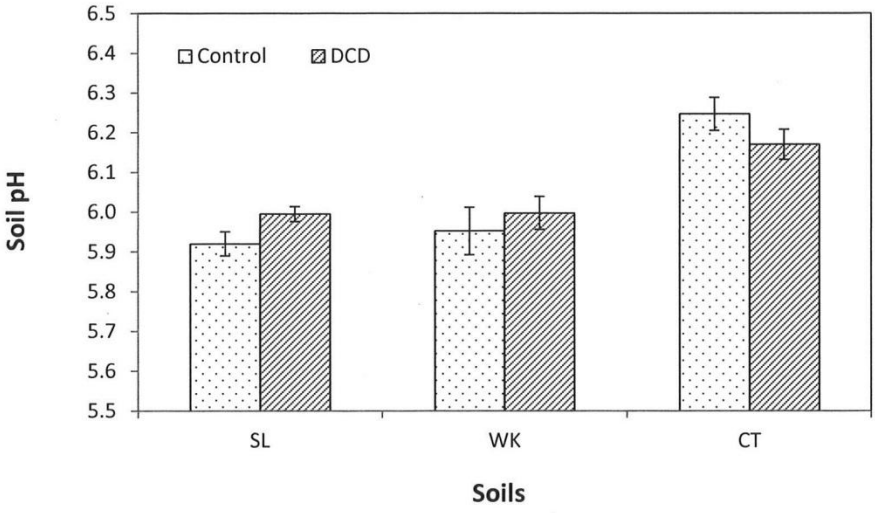

Fig. 1 pH of SL, WK and CT soils with and without DCD. The error bars indicate the standard error of mean (SEM)

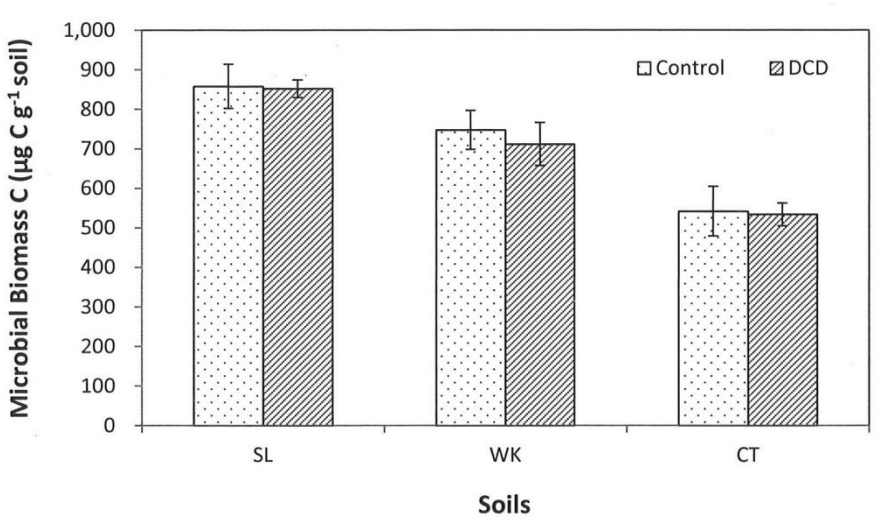

Fig. 2 Microbial biomass C in SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

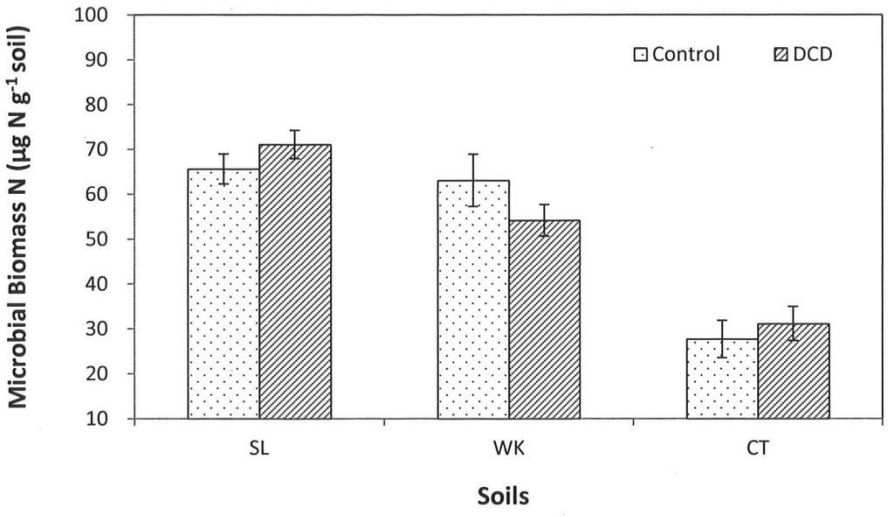

Fig. 3 Microbial biomass N for SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

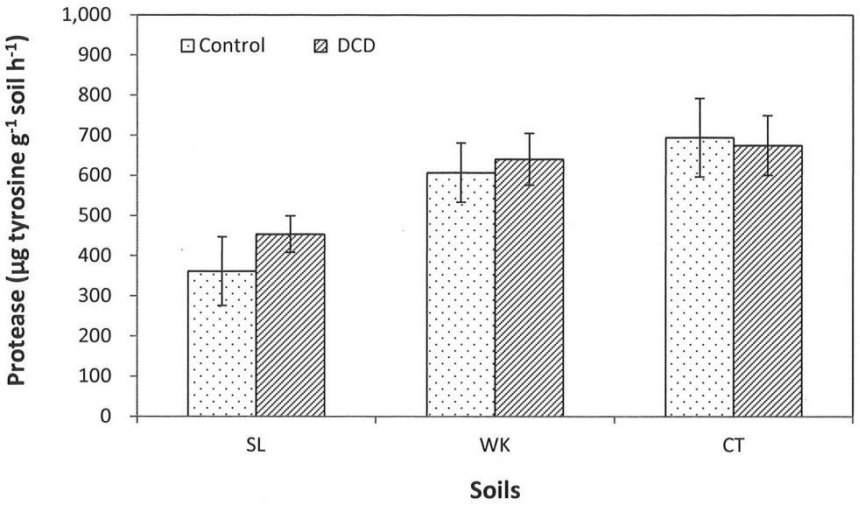

Fig. 4 Protease activity in SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

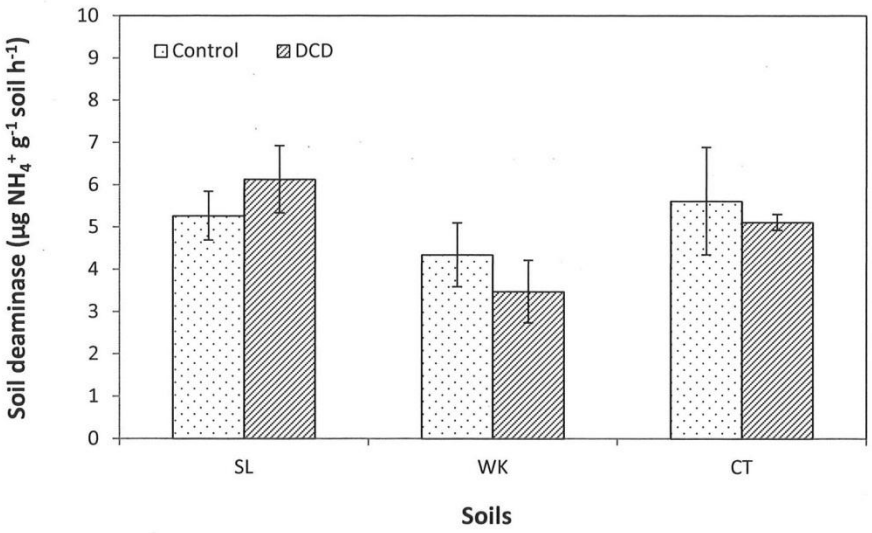

Fig. 5 Soil deaminase activity in SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

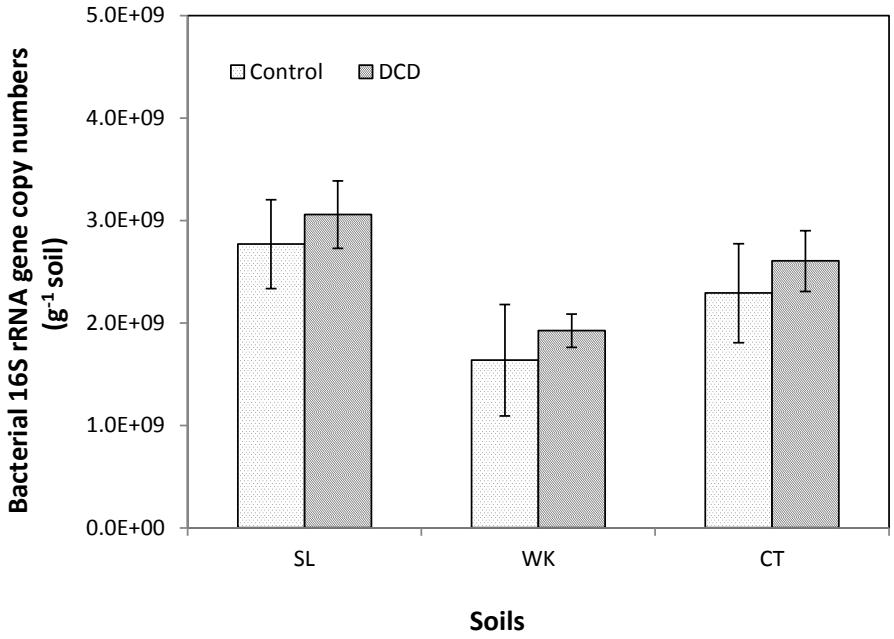

Fig. 6 Bacterial 16S rRNA gene abundance in SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)

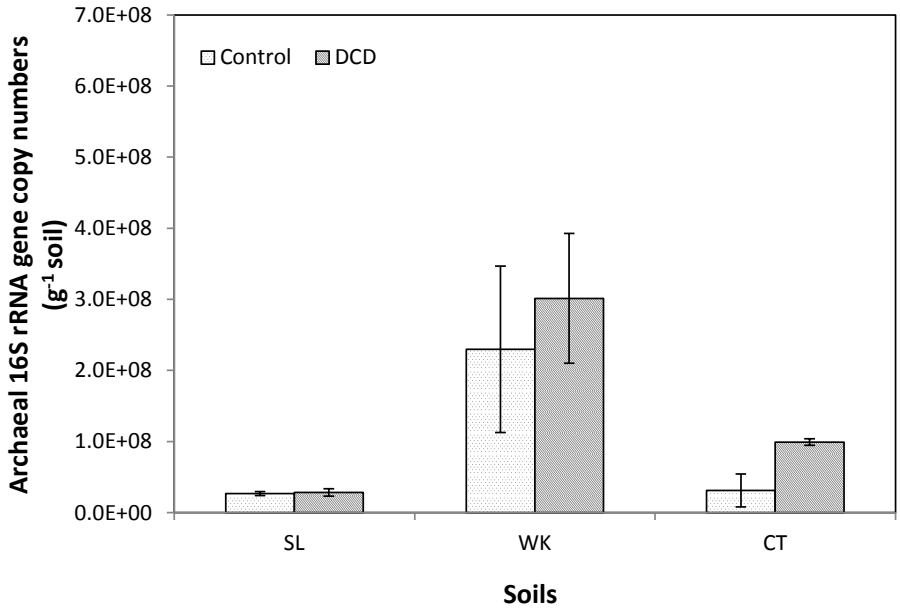

Fig. 7 Archaeal 16S rRNA gene abundance for SL, WK and CT soils, with and without DCD. The error bars indicate the standard error of mean (SEM)