Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions

Hong J. Di1, Keith C. Cameron1, Ju-Pei Shen2, Chris S. Winefield3, Maureen O’Callaghan4, Saman Bowatte5 & Ji-Zheng He2

1Centre for Soil and Environmental Research, Lincoln University, Lincoln, Christchurch, New Zealand; 2Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China; 3Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln, Christchurch, New Zealand; 4AgResearch Ltd, Lincoln Research Centre, Christchurch, New Zealand; and 5AgResearch Ltd, Grasslands Research Centre, Palmerston North, New Zealand

Correspondence: Hong J. Di, Centre for Soil and Environmental Research, Lincoln University, PO Box 84, Lincoln 7647, Christchurch, New Zealand. Tel.: +64 3 325 2811; fax: +64 3 325 3607; e-mail: hong.di@lincoln.ac.nz

Received 11 November 2009; revised 15 February 2010; accepted 25 February 2010. Final version published online 29 March 2010.

DOI:10.1111/j.1574-6941.2010.00861.x

Editor: Christoph Tebbe

Keywords
ammonia-oxidizing bacteria; ammonia-oxidizing archaea; nitrification rate; nitrification inhibitor.

Abstract
Nitrification is a key process of the nitrogen (N) cycle in soil with major environmental implications. The recent discovery of ammonia-oxidizing archaea (AOA) questions the traditional assumption of the dominant role of ammonia-oxidizing bacteria (AOB) in nitrification. We investigated AOB and AOA growth and nitrification rate in two different layers of three grassland soils treated with animal urine substrate and a nitrification inhibitor [dicyandiamide (DCD)]. We show that AOB were more abundant in the topsoils than in the subsoils, whereas AOA were more abundant in one of the subsoils. AOB growth and the amoA gene transcription activity were significantly inhibited by DCD. Nitrification rates were much higher in the topsoils than in the subsoils and were significantly related to AOB abundance, but not to AOA abundance. These results suggest that AOB and AOA prefer different soil N conditions to grow: AOB under high ammonia (NH3) substrate and AOA under low NH3 substrate conditions.

Introduction
Nitrification, the microbial oxidation of ammonia (NH3) to nitrite (NO2), and then to nitrate (NO3), is a key process in the nitrogen (N) cycle in soil with major environmental and ecological consequences. It produces NO3, which is a water contaminant, and nitrous oxide (N2O), which is both a greenhouse gas and an ozone-depletion substance. The long-term global warming potential of N2O is 310 times that of carbon dioxide (IPCC, 1995). In addition, a recent study shows that N2O has become and will remain the greatest threat to the ozone layer in the 21st Century (Ravishankara et al., 2009).

The first and rate-limiting step of nitrification, the conversion of NH3 to hydroxylamine, is performed by the key ammonia monoxygenase (AMO) enzyme, which is encoded by the subunits of AMO genes. It has traditionally been assumed that this first step is carried out mainly by autotrophic ammonia-oxidizing bacteria (AOB) of the Beta- and Gamma-subgroups of Proteobacteria (Purkhold et al., 2000; Kowalchuk & Stephen, 2001; Prosser & Nicol, 2008). However, this view has recently been put into question by the discovery of the amoA gene in archaea populations, thus raising the prospect of the presence of ammonia-oxidizing archaea (AOA) in different ecosystems (Venter et al., 2004; Francis et al., 2005; Körneke et al., 2005; Wuchter et al., 2006; Prosser & Nicol, 2008). In fact, AOA populations were found to be more abundant than AOB in a range of soils, suggesting a potentially greater role for AOA than AOB in nitrification (Leininger et al., 2006; He et al., 2007; Chen et al., 2008; Prosser & Nicol, 2008). However, our collective knowledge and understanding of the relative role of AOB and AOA in nitrification is still very limited and conflicting. For example, NH3 oxidation by archaea has been shown to be important for N cycling in the ocean (Martens-Habbena et al., 2009) and in a soil (Offre et al., 2009). On the other hand, Di et al. (2009) and Jia & Conrad (2009) showed that AOB were functionally more important than...
AOA in NH₂ oxidation in some agricultural soils. It is therefore not clear what soil N environments are preferred by AOB and AOA for their growth. In addition, there is insufficient information on how the AOB and AOA growth may vary in different soil layers as the N and other nutrient levels change with soil depth.

In grazed grassland where animals graze outdoor pastures, the sites that have the highest concentrations of NH₃ substrate for nitrification are the animal urine patch areas. These are also the locations where most of the NO₃ leaching and N₂O emissions occur in grazed pastures (Di & Cameron, 2002a). As animals graze pastures in the field, a large proportion (70–90%) of the ingested N is returned to the soil in the animal excreta, particularly in the urine. The N loading rate under a dairy cow urine patch in intensively grazed dairy grassland can be as high as 1000 kg N ha⁻¹ s⁻¹ (Di & Cameron, 2002a). Most of the N in the urine is urea, which, upon hydrolysis in the soil, produces ammonium, and is subsequently subject to nitrification. Recent estimates show that animal manure is a major source of N₂O emissions globally (Davidson, 2009). A mitigation technology that has been shown to be effective in reducing N₂O emissions and NO₃ leaching from animal urine patches in grazed grassland is the use of nitrification inhibitors [e.g. dicyandiamide (DCD)] to treat the soil (Di & Cameron, 2002b; Di et al., 2007). However, questions remain regarding how the NH₃-oxidizer populations, AOB and AOA, would respond to the urine-derived NH₃, particularly in different soil layers (depths) with contrasting soil fertility and how effective nitrification inhibitors are in inhibiting their growth in different soil layers. There is a need for an improved understanding of the population dynamics of AOB and AOA in different soil layers with contrasting soil N fertility in order to better manage and mitigate N₂O emissions and NO₃ leaching in grazed grasslands.

The objectives of this study were to determine: (1) AOB and AOA growth as affected by contrasting soil N conditions in the topsoil (0–0.2 m) and subsoil (0.4–0.6 m) of three grazed grassland soils in New Zealand and (2) the effects of animal urine and a nitrification inhibitor (DCD) on the growth of AOB and AOA in these different soil layers.

Materials and methods

Soils

Soil samples were collected from intensively grazed dairy pasture soils from three different regions across New Zealand: the Waikato region in the northern part of the North Island; the Canterbury region in the Central South Island; and Southland in southern South Island (Table 1). The soil in Waikato is a Horotiu silt loam (NZ classification: Typic Orthic Allophanic Soil, Hewitt, 1998; USDA: Typic Udident, Soil Survey Staff, 1998). The Canterbury soil is Templeton silt loam (NZ classification: Immature Pallic Soil; USDA: Udic Haplustepts). The Southland soil is Mataura recent sandy loam (NZ classification: Typic Fluvial Recent; USDA: Fluvents). The Waikato Horotiu soil is developed from volcanic parent material, whereas the Canterbury soil is developed from glacial outwash covered with varying depths of loess, and the Southland soil is developed in alluvium derived from schist and greywacke rock.

Soil samples were collected from two different depths, 0–0.2 and 0.4–0.6 m, from 10 different random locations. For each site, the samples were bulked into a single sample for each layer, packed with ice packs to keep it cool and transported to the laboratory to be stored in the fridge before being used for the incubation study below. A subsample was taken from each soil to determine the soil water retention capacity (at −10 kPa) on a tension table.

Table 1. Properties of the soils used

<table>
<thead>
<tr>
<th>Region and location</th>
<th>Waikato 38°46′38″S; 175°18′26″E</th>
<th>Canterbury 43°38′11″S; 172°26′18″E</th>
<th>Southland 46°19′21″S; 168°16′02″E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil type</td>
<td>Horotiu silt loam</td>
<td>Templeton silt loam</td>
<td>Mataura recent sandy loam</td>
</tr>
<tr>
<td>Soil layer and depth (m)</td>
<td>Topsoil 0–0.2</td>
<td>Subsoil 0.4–0.6</td>
<td>Topsoil 0–0.2</td>
</tr>
<tr>
<td>Particle size (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sand</td>
<td>37.1</td>
<td>24.9</td>
<td>44.2</td>
</tr>
<tr>
<td>Silt</td>
<td>60.2</td>
<td>73.8</td>
<td>53.7</td>
</tr>
<tr>
<td>Clay</td>
<td>2.7</td>
<td>1.3</td>
<td>2.1</td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td>5.7</td>
<td>6.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Organic C (g·kg⁻¹)</td>
<td>53.1</td>
<td>7.6</td>
<td>25.5</td>
</tr>
<tr>
<td>Total N (g·kg⁻¹)</td>
<td>5.0</td>
<td>0.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Olsen P (mg·kg⁻¹)</td>
<td>9.8</td>
<td>4.7</td>
<td>19.4</td>
</tr>
<tr>
<td>CEC (cmol·kg⁻¹)</td>
<td>16.0</td>
<td>7.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Exchange Ca²⁺ (cmol·kg⁻¹)</td>
<td>4.9</td>
<td>5.2</td>
<td>5.8</td>
</tr>
<tr>
<td>Exchange Mg²⁺ (cmol·kg⁻¹)</td>
<td>0.5</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Exchange K⁺ (cmol·kg⁻¹)</td>
<td>0.2</td>
<td>0.08</td>
<td>0.1</td>
</tr>
<tr>
<td>Base saturation (%)</td>
<td>36.7</td>
<td>79.0</td>
<td>51.9</td>
</tr>
</tbody>
</table>

Published by Blackwell Publishing Ltd. All rights reserved
Design of the incubation study

A laboratory incubation study was carried out to determine: (1) AOB and AOA abundance in the topsoil (0–0.2 m) and subsoil (0.4–0.6 m) of the three soils (Waikato, Canterbury and Southland); (2) growth responses of AOB and AOA to animal urine substrate (to simulate animal urine deposition during grazing); and (3) the inhibitory effect of a nitrification inhibitor, DCD, on AOB and AOA growth.

The experiment used the six soil samples (two different depths of the three soils) and each soil had the following treatments: A, Control; B, 1000 kg urine-N ha⁻¹; and C, 1000 kg urine-N + 10 kg DCD ha⁻¹. Each treatment had four replicates. Treatment B was to simulate a urine patch in a grazed pasture soil where the N input from the animal urine was 1000 kg N ha⁻¹. DCD is a nitrification inhibitor used here to determine its effect on AOB and AOA growth and on the nitrification rate.

For each replicate, a 1 kg soil sample (dry weight basis) was weighed into an incubation vessel (polypropylene plastic containers) and placed in an incubator at 12 °C for 1 week for preincubation equilibration. A temperature of 12 °C was used to represent the temperature in the autumn in many parts of New Zealand when nitrification inhibitors are recommended for use to reduce NO₃ leaching and N₂O emissions in grazed pastures. Before the application of the treatments, soil samples were taken for AOB and AOA assays.

Fresh dairy cow urine was collected and analyzed for its total N concentration before being applied. DCD was applied in a liquid form. Following the application of urine and DCD, the soil moisture was adjusted to 80% of the water-holding capacity for each soil, and the soil sample within each incubation vessel was thoroughly mixed. A lid with two aeration holes (each with 0.5 cm diameter) was placed on top of each soil container. These incubation vessels were then placed randomly inside the incubator with a constant temperature at 12 °C. The soil moisture was maintained at 80% water-holding capacity by adjusting the weight of each vessel with deionized water. The soil samples were incubated for about 3 months, during which time subsamples were taken to determine AOB, AOA and the nitrification rate.

AOB and AOA assays

Subsamples were collected 6, 27, 69 and 97 days after the application of the treatments and were extracted to determine the amoA gene copy numbers of AOB and AOA. Fresh soil samples were stored in a freezer at − 80 °C before DNA extraction. A subsoil sample was used to determine the soil moisture content.

Frozen soil (0.4 g) was extracted for nucleic acid using MoBio Powersoil™ DNA Isolation Kits (San Diego, CA) according to the manufacturer’s instructions. DNA was eluted with 100 µL of solution C6 (MoBio Laboratories, CAT. No. 12888–100) and stored at − 80 °C before being analyzed.

Transcription of the amoA gene was studied by determining the RNA copy numbers in the Canterbury topsoil samples. One gram of fresh soil was collected 69 days after the start of the incubation and was extracted using the MoBio RNA Power Soil™ Total RNA Isolation Kit, following the manufacturer’s instructions. Other soil samples were not extracted and analyzed for RNA due to resource constraints. To generate cDNA, the extracted RNA was treated using the Turbo DNA-free Kit (ABI) to remove the DNA. cDNA was produced using Superscript III reverse transcriptase (Invitrogen NZ, Auckland). Random primers (Invitrogen NZ) were used at a concentration of 2.5 nmol per reaction. Two negative controls were performed with all reactions. The first control contained soil RNA template and all DNase/RT reagents, except for the final addition of the RT enzyme. A second control contained no template (water only) to ensure that all reagents were free from possible contaminants.

All the PCRs were set up using the CAS-1200 Robotic liquid handling system (Corbett Life Science, Australia), and real-time PCR was performed on a Rotor-Gene™ 6000 (Corbett Life Science). Possible inhibition of the real-time PCR was assessed by running a series of 10-fold dilutions of the extracted DNA and determining the amplification efficiency of each diluted sample. Inhibition was observed without dilution, but high amplification efficiencies of 92–99% were obtained for both the AOB and the AOA quantifications after a 10-fold dilution. A 10-fold dilution of each sample was thus used for the final analysis.

Bacterial and archaeal amoA genes were quantified using the primers amoA1F/amoA2R (Rotthauwe et al., 1997) and Arch-amoAF/Arch-amoAR (Francis et al., 2005) with SYBR® Premix Ex Taq™ (TaKaRa, Japan) using the thermal profiles as described in Di et al. (2009). The 20-µL reaction mixture consisted of 10.0 µL of SYBR® Premix Ex Taq™, plus primers and 1.5 µL of template 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 carried out using Rotor-Gene™ 6000 series software 1.7.

Standard curves for real-time PCR assays were developed as follows: briefly, the bacterial and archaeal amoA genes were PCR amplified from extracted DNA with the primers amoA1F/amoA2R and Arch-amoAF/Arch-amoAR, respectively. The PCR products were purified using a PCR cleanup kit (Axygen) and cloned into the pGEM-T Easy Vector (Promega, Madison, WI). The resulting ligation mix was transformed in Escherichia coli JM109 competent cells (Promega) following the manufacturer’s instructions. Plasmids used as standards for quantitative analyses were...
extracted from the correct insert clones of each target gene and sent for sequencing. The plasmid DNA concentration was determined on a Qubit™ fluorometer (Invitrogen NZ) and the copy numbers of target genes were calculated directly from the concentration of the extracted plasmid DNA. Tenfold serial dilutions of a known copy number of the plasmid DNA were subjected to a real-time PCR assay in triplicate to generate an external standard curve and to check the amplification efficiency. High efficiencies of 88–102% were obtained for AOB amoA amplification, with the R value ranging between 0.997 and 0.999, and efficiencies of 86–98% were obtained for AOA amoA amplification, with the R value ranging between 0.994 and 0.999.

Nitrification rate
Subsamples were taken at 1, 6, 13, 20, 27, 41, 55, 69, 83 and 96 days after the application of the treatments and extracted with 2 M KCl to determine the NO3/C0 concentrations on a flow injection analyzer (Tecator Inc., Sweden).

Results

AOB and AOA abundance in different soil layers
The AOA amoA gene copy numbers were greater than those of the AOB in all three soils in both the topsoil and the subsoil (Fig. 1a and b). The amoA gene copy numbers of AOB varied from $7.7 \times 10^3$ in the Waikato subsoil to $7.7 \times 10^4$ in the Southland topsoil, whereas that of AOA varied from $1.2 \times 10^5$ in the Waikato subsoil to $2.0 \times 10^7$ in the Southland subsoil. The ratios of the AOA to AOB amoA gene copy numbers were 21.9, 5.7 and 7.4 for the Waikato, Canterbury and Southland topsoils, respectively, and 3.0, 53.5 and 154.3 for the Waikato, Canterbury and Southland subsoils, respectively.

The AOB community was more abundant in the topsoil than in the subsoil for all three soils (Fig. 1a). The ratio of the AOB amoA gene copy numbers in the topsoil to that in the subsoil was 5.9, 36.5 and 6.0 in the Waikato, Canterbury and Southland soils, respectively. However, the AOA amoA gene copy numbers were only greater in the topsoil than in the subsoil in the Waikato and Canterbury soils, and were much smaller in the topsoil than in the subsoil for the Southland soil (Fig. 1b). The ratios of the AOA amoA gene copy numbers in the topsoil to that in the subsoil were 42.7, 3.9 and 0.3 for the Waikato, Canterbury and Southland soils, respectively.

AOB and AOA growth
The application of the NH3 substrate in the form of animal urine stimulated substantial AOB growth in all three topsoils (Fig. 2a, c, e). The peak amoA gene copy numbers in the urine treatments were 38.0, 62.8 and 28.4 times those in the respective Control treatments in the Waikato, Canterbury and Southland topsoils, respectively.

The application of the DCD nitrification inhibitor to the urine treatments significantly inhibited the AOB growth in the three topsoils ($P < 0.05$) (Fig. 2a, c, e). The inhibition was particularly effective in the Waikato and Southland topsoils, but less effective in the Southland topsoil.

There was no AOB growth in the Waikato subsoil, and only slow AOB growth in the Canterbury and Southland subsoils (Fig. 2b, d, f).

The AOA growth was in total contrast to that of the AOB (Fig. 3). AOA growth was recorded in the Control treatments of the Canterbury and Southland topsoils (Fig. 3b and c) where no NH3 substrate was applied. The application of urine in the Canterbury and Southland subsoils (Fig. 3d and f) decreased the AOA amoA gene copy numbers below those in the Controls, which had high copy numbers of the amoA gene AOA at the start of the incubation study.

AOB and AOA amoA gene transcription
The transcription activity of the AOB amoA gene, as measured by the RNA copy numbers, in the Canterbury
topsoil increased 64 times with the application of the urine substrate compared with that in the Control after 69 days of incubation (Fig. 4a). When DCD was applied with the urine, the AOB RNA copy numbers remained the same as that in the Control, showing the inhibition effect of DCD on the AOB activity.

However, the expression of the AOA amoA gene, as indicated by the RNA copy numbers, was greater in the Control than in the urine treatment (Fig. 4b). In the Urine treatment, the RNA copy numbers of the AOB were 414 times greater than that of the AOA. Even in the Control treatment, the RNA copy number of the AOB was 1.4 times that of the AOA despite the AOA amoA gene copy numbers being 15.5 times greater than those of the AOB after 69 days of incubation.

**Nitrification rate**

The application of the urine substrate significantly increased the nitrification rate in the three topsoils, resulting in higher NO₃⁻-N concentrations ($P < 0.05$) (Fig. 5a, c, e). The application of the DCD nitrification inhibitor to the urine treatments inhibited the nitrification rate, restricting the NO₃⁻-N concentration to below those in the urine treatments. The nitrification rate was negligible in the Waikato subsoil and was slow in the Canterbury and Southland subsoils (Fig. 5b, d, f). These patterns were similar to the patterns of AOB growth as shown in Fig. 2.

**Relationships between the nitrification rate and AOB and AOA**

Regression analysis between the NO₃⁻-N concentration and amoA gene copy numbers of AOB and AOA after 69 days of incubation (when the treatment effects on the AOB and AOA growth were at a maximum for a majority of the soils) showed the following significant relationship between the nitrification rate and the AOB abundance:

$$y = 76.3 + 15.0x$$

where $y$ is the NO₃⁻-N concentration (mg NO₃⁻-N kg⁻¹ soil) and $x$ is the AOB amoA gene copy numbers.
However, no relationship was found between the NO$_3$-N concentration and the AOA amoA gene copy numbers (Fig. 6b).

**Discussion**

Despite the discovery of large archaea populations in a diverse range of environments, including the ocean, sediments and soils (Francis *et al.*, 2005; Könneke *et al.*, 2005; Leininger *et al.*, 2006; Wuchter *et al.*, 2006; He *et al.*, 2007), the drivers of archaea vs. bacteria growth are poorly understood. The results from this study clearly show that AOB and AOA prefer different N conditions for their growth. While AOB abundance and activity grew substantially when supplied with a high dose of urine substrate in the topsoils, the AOA community only grew in the Controls, where no urine substrate was applied. In fact, the AOA community seemed to be inhibited by the high dose of urine substrate in two of the subsoils (Fig. 3d and e). This differential trend of AOB and AOA growth is supported by the transcription data of the amoA gene (RNA copy numbers) in the Canterbury topsoil (Fig. 4b). These findings provide further evidence to support and extend those of Di *et al.* (2009). In the previous study (Di *et al.*, 2009), only the fertile surface soil (0–0.1 m) was used and there was insufficient evidence to show a preferred environment for AOA to grow. The present study, by including both high-fertility topsoils and low-fertility subsoils, provides clear evidence of contrasting patterns of AOB and AOA growth under different N conditions.

It has been suggested that AOB growth is favored by fertile soil conditions, whereas AOA have a preference for low-fertility or oligotrophic environments (Valentine, 2007; Erguder *et al.*, 2009). The significant growth of AOB in the urine-treated soils, the lack of AOA growth when supplied with a high dose of urine substrate and the substantial AOA growth in the Control soils would support such a hypothesis. Archaeal growth and NH$_3$ oxidation activity were also observed in a Scottish study (Offre *et al.*, 2009) where no N fertilizer treatment was applied (equivalent to the Controls of the present study). Although the expression of the archaean amoA gene has been detected previously (Könneke

(million copies g$^{-1}$ soil) after 69 days of incubation (Fig. 6a).
et al., 2005; Leininger et al., 2006; Di et al., 2009) and in the present study, AOA may not use NH$_3$ oxidation as their sole or main energy source and may grow heterotrophically or mixotrophically (Leininger et al., 2006; Nicol & Schleper, 2006; Jia & Conrad, 2009). Additional evidence of the different environments for bacterial and archaeal growth may also be seen in their population distribution at the two different soil layers. The AOB abundance was much greater in the high-fertility topsoils (0–0.2 m) than in the low-fertility subsoils (0.4–0.6 m) (Fig. 1 and Table 1), whereas the AOA abundance was much greater in the Southland subsoil than in the Southland topsoil (Fig. 1). The average ratio of the AOA to AOB amoA gene copy numbers of the three soils from a previous study was 3.9 for the 0–0.1 m depths (Di et al., 2009), and that from the present study was 11.7 for the 0–0.2 m depths and 70.3 for the 0.4–0.6 m depths, showing a clear increasing trend with soil depth. Leininger et al. (2006) also reported declining AOB abundance with soil depth whereas little vertical variation was observed with AOA, thus resulting in a greater AOA to AOB ratio with soil depth. It is not clear what is responsible for the differential growth of AOB and AOA at different soil depths. Soil pH has been suggested as a possible factor for archaea populations in different soils in other studies (He et al., 2007; Nicol et al., 2008; Lehtovirta et al., 2009), but was
not found to be significant in this study where the soil pH was in a narrow range. We hypothesize that the greater AOB populations in the topsoils are mainly related to the higher fertility and higher N inputs to these topsoils (fertilizer N, animal excreta-N and N fixation in legume-based pastures), which stimulated the growth of the predominantly Nitrospirapira species (Di et al., 2009), whereas the larger AOA community in the subsoil is related to the low-fertility environment, demonstrating archaea’s adaptation to a less fertile environment. However, further research is required to study the role of AOA in nutrient cycling in different soils.

The rate of nitrification corresponded to the growth of AOB rather than AOA in the different soils. Indeed, there was a significant quantitative relationship between the nitrification rate and the AOB amoA gene copy numbers and no such relationship was found with AOA. These findings provide further evidence to support those by Di et al. (2009) and Jia & Conrad (2009) that AOB are functionally more important in nitrification in high-N agricultural soils despite the greater abundance of AOA in many soils (Leininger et al., 2006; He et al., 2007; Shen et al., 2008). AOA clearly have a different requirement for NH₃ substrate for their growth from the AOB populations. The contribution of AOA to nitrification may be more significant in low-fertility soils.

The substantially greater AOB abundance in the topsoils than in the subsoils and the close relationship between the nitrification and the AOB amoA gene copy numbers would indicate that most of the nitrification in the grazed grassland soils takes place in the surface soil layer and the nitrification activity is low deeper in the soil profile. The initial AOB population was so low in the subsoils that even when supplied with a high dose of urine substrate, the growth was very slow, resulting in slow rates of nitrification in the subsoils (Figs 2b, d, f and 5b, d, f). Therefore, it is the topsoil that should be the target of treatment when developing practical nitrification inhibitor technologies to reduce NO₃ leaching and N₂O emissions in grazed grassland (Di & Cameron, 2002b, 2005; Di et al., 2007).

**Acknowledgements**

We would like to thank the New Zealand Foundation for Research, Science and Technology (FRST) for funding, Drs Ross Monaghan, Stewart Ledgard and Mark Sheppard of AgResearch and Dr Bruce Thorrold and Deanne Waugh of Dairy NZ for assistance with soil sampling, Emily Gerard and Shona Brock of AgResearch and Jie Lei, Steve Moore, Carole Barlow, Trevor Hendry and Neil Smith of Lincoln University for technical support.

**References**


© 2010 Federation of European Microbiological Societies Published by Blackwell Publishing Ltd. All rights reserved