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Response of ammonia oxidizers and denitrifiers to repeated applications of a nitrification inhibitor and a urease inhibitor in two pasture soils

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Abstract

Purpose The nitrification inhibitor, 3,4-dimethylpyrazol-phosphate (DMPP) and the urease inhibitor *N*-(*n*-butyl) thiophosphoric triamide (nBTPT) can mitigate N losses through reducing nitrification and ammonia volatilization, respectively. However, the impact of repeated applications of these inhibitors on nitrogen cycling microorganisms is not well documented. This study aimed to investigate the changes in the abundance and community structure of the functional microorganisms involved in nitrification and denitrification in Australian pasture soils after repeated applications of DMPP and nBTPT.

Materials and methods Soil was collected in autumn and spring, 2014 from two pasture sites where control, urea, urea ammonium nitrate, and urea coated inhibitors had been repeatedly applied over two years. Soil samples were analyzed to determine the potential nitrification rates (PNRs), the abundances of *amoA*, *narG, nirK* and bacterial 16S rRNA genes, and the community structure of ammonia oxidizers.

Results and discussion Two years of urea application resulted in a significantly lower soil pH at Terang and a significant decrease in total bacterial 16S rRNA gene abundance at Glenormiston, and led to significantly higher PNRs and abundances of ammonia oxidizers compared to the control. Amendment with either DMPP or nBTPT significantly decreased PNRs and the abundance of *amoA* and *narG* genes. However, there was no fertilizer- or inhibitor-induced change in the community structure of ammonia oxidizers.

Conclusions These results suggest that there were inhibitory effects of DMPP and nBTPT on the functional groups mediating nitrification and denitrification, while no significant impact on the community structure of ammonia oxidizers was observed. The application of nitrification or urease inhibitor appears to be an effective approach targeting specific microbial groups with minimal effects on soil pH and the total bacterial abundance.

Keywords 3,4-dimethylpyrazole phosphate (DMPP) • Ammonia-oxidizing archaea • Ammonia-oxidizing bacteria • *N*-(*n*-butyl) thiophosphoric triamide (nBTPT)

1 Introduction

Australian dairy is characterized by pasture grazing throughout the year and has been recognized as an agricultural system with the lowest nitrogen (N) use efficiency (Rowlings et al. 2016). Given that 80 % of urine-N is urea-N, urine patches on grazed pastures are susceptible to substantial N loss, causing a range of environmental issues (Selbie et al. 2015). Nitrogen losses in pasture soils could occur through ammonia volatilization and nitrous oxide (N_2O) emission from the nitrification and denitrification processes (Chen et al. 2008; Selbie et al. 2015). Ammonia oxidation, as the rate-limiting step of nitrification, is catalyzed by ammonia monooxygenase (AMO) encoded by the *amoA* gene within ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) (Purkhold et al. 2000; Brochier-Armanet et al. 2008). The end product of nitrification, nitrate, is subjected to sequential denitrification processes carried out by nitrate reductase (encoded by the *narG* gene), nitrite reductase (encoded by the *nirK*/*nirS* genes) and nitrous oxide reductase (encoded by the *nosZ* gene) under anaerobic conditions (Philippot et al. 2007). Major efforts have been devoted to the development of enhanced fertilizer strategies to mitigate N losses and improve nitrogen use efficiency (Chen et al. 2008; Zaman et al. 2009; Di and Cameron 2016).

Nitrification inhibitors are defined as chemicals capable of retarding ammonia oxidation through deactivating AMO (Hatch et al. 2005; Chaves et al. 2006). 3,4-dimethylpyrazole phosphate (DMPP) has been recognized as one of the most widely used nitrification inhibitors. DMPP has also been reported to indirectly inhibit denitrification by affecting the formation of nitrate, which serves as the obligatory electron acceptor for denitrification (Ruser and Schulz 2015). Numerous studies have shown that application of DMPP in pasture could delay the conversion of ammonium to nitrate (Macadam et al. 2003; Merino et al. 2005; Di and Cameron 2012), maintain soil cations such as K^+, Ca^{2+}, Mg^{2+} and Na^+ (Wu et al. 2007; Yu et al. 2008), and decrease N_2O and nitric oxide emissions (Menéndez et al. 2006; Menéndez et al. 2012; Shi et al. 2016a). However, limited information is currently available regarding the effects and mechanisms of DMPP inhibition on soil microbial communities in pasture soils. A microcosm incubation with six pasture soils revealed that DMPP reduced the abundance of AOB (Di and Cameron 2011), while a more recent incubation experiment reported that DMPP substantially decreased the cell-specific activity of both AOA and AOB, but had no significant effect on their abundance (Kong et al. 2016). To our knowledge, no studies have been carried out to explore the effects of repeated uses of DMPP on soil microbial dynamics in pasture soils.

N-(*n*-butyl) thiophosphoric triamide (nBTPT) is recognized as a successful inhibitor to slow down soil urease activity, further reducing ammonia volatilization in pasture soils (Zaman et al. 2008; Singh et al. 2013), but the effect of nBTPT on soil microorganisms associated with nitrification and denitrification is unknown. In recent years, the presence of the *ureC* gene encoding the alpha subunit of urease in active ammonia oxidizers was reported (Lu and Jia 2013; Tolar et al. 2016), indicating the potential of nBTPT inhibiting the intracellular urea hydrolysis in ammonia oxidizers (Hu et al. 2014). Furthermore, ammonia is the natural substrate for AMO and the ammonia concentration has been recognized as a critical factor influencing the ecological differentiation of ammonia oxidizers (Hyman et al, 1983; Di et al. 2010; Verhamme et al. 2011). Therefore, it is hypothesized that nBTPT might inhibit ammonia oxidizers and following denitrification. An earlier study revealed that nBTPT inhibited nitrification when it was applied at a rate of 50 or 100 μg g^{-1} soil, while it showed no effect on denitrification (Bremner et al. 1986). By contrast, Giovannini et al. (2009) reported that nitrification activity was not affected by nBTPT addition, but nBTPT reduced the accumulation of NH_4^+-N. The effect of nBTPT on nitrification and associated ammonia oxidizers deserves further investigation. Meanwhile, another study revealed that nBTPT exhibited inhibitory effect on methane oxidation (Bronson 1994), but the underlying mechanism is unknown. Because of the similar structure of AMO as methane monooxygenase catalyzing methane oxidation (Hatch et al. 2005), there is a possibility that nBTPT could inhibit AMO as well.

The objective of this study was to investigate the effects of repeated uses of the nitrification inhibitor, DMPP and the urease inhibitor, nBTPT on the abundances of nitrifiers and denitrifiers in two pasture soils under field conditions. We hypothesized that repeated application of DMPP or nBTPT would decrease the abundance and activity of ammonia oxidizers and denitrifiers.

2 Materials and methods

2.1 Site description and soil sampling

Soil samples were collected in autumn (15th April) and spring (16th October), 2014 from two existing field plot experiments that had repeatedly applied treatments since July 2012. The field experiments were established on grazed dairy pastures at Terang (TR, 38°14’ S; 142° 55’E) and Glenormiston (GM, 38°10’S; 142°58’E) in south-western Victoria, Australia. The TR soil is derived from quaternary basalt and is classified as a Brown Chromosol according to the Australian Soil Classification (Isbell 2002). The GM soil is derived from quaternary volcanic tuff deposits and classified as a Black Dermosol (Isbell 2002). Both sites were dominated by perennial ryegrass (*Lolium perenne* L.), and livestock were excluded during the period of the study. Soil samples were collected from the following treatments: control (C), urea (U), urea ammonium nitrate (UAN, including 35 % urea, 45 % ammonium nitrate and 20 % water), urea coated DMPP (UD), and urea coated nBTPT (UN). Nitrogen at a rate of 50 kg N ha^{-1} had been applied to the field plots at regular intervals since 2012 (For detailed application timings, see Table 1). The experiment consisted of five replicate plots (2.5 × 3 m^2 for each plot) for each treatment. At each sampling plot, five surface soil cores (2.5 cm diameter × 15 cm depth) were collected and mixed together to form a replicate sample. Soil samples were transported on ice to the laboratory and passed through a 2 mm sieve. Soil samples were stored at 4 °C for analysis of soil properties and at -20 °C for DNA extraction. All samples were processed within one week after soil collection in the field.

2.2 Soil physical and chemical properties

Details of the soil physical and chemical properties are shown in Table 2. Soil gravimetric water content was determined by oven-drying sub-samples at 105 °C for 24 h. Soil pH was determined using a ratio of 1:5 (fresh soil : water) with an Orion Star A211 pH-meter (ThermoFisher Scientific Inc., Melbourne, Australia). Soil organic matter was determined using the $K_2Cr_2O_7$ wet oxidation and colorimetric method as previously described (Walkley and Black 1934). Soil NH_4^+-N and NO_3^--N were extracted using a ratio of 1:5 (fresh soil : 1 M KCl) after shaking at 175 rpm for 1 h, and the solutions were filtered through Whatman No. 42 filter paper prior to analysis by a Segmented Flow Analyzer (SAN++, Skalar, Breda, Holland). Total N was determined using the classic Dumas method of combustion on the isotope ratio mass spectrometry (Sercon Hydra, Crewe, UK). Particle size analysis was measured using the sieve and hydrometer procedures. The temperature and rainfall data is from the closest Bureau of Meteorology station (see http://www.bom.gov.au/climate/cdo/about/rain-districts.shtml). The two sites are 15 km apart and thus belong to the same weather station (090077 Terang VIC).

2.3 Potential nitrification rates assay

Soil potential nitrification rates were determined according to the chlorate inhibition method (Hu et al. 2015). Briefly, fresh soil samples (5 g) were placed in 50 mL falcon tubes with 20 ml ammonium sulphate (1 mM). Potassium chlorate with a final concentration of 10 mM was added to the tubes to inhibit nitrite oxidation. The falcon tubes were covered with parafilm with small holes for aeration and were incubated in the dark at 25 °C for 24 h, and then nitrite was extracted with 10 ml of 2 M KCl. The supernatant was measured by spectrophotometry at a wavelength of 540 nm with N-(1-naphthyl) ethylenediamine and sulfonic acid.

2.4 DNA extraction and quantitative PCR (qPCR) analysis

Total genomic DNA was isolated from 0.25 g of soil using MoBio PowerSoil™ DNA Isolation Kits (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and quality of the extracted DNA were assessed using the NanoDrop ND2000c Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All extracted DNA was diluted with sterilized MilliQ water (1:10) to reduce potential PCR inhibition. Abundances of the key nitrifying and denitrifying genes were quantified on a Bio-Rad CFX96 Optical Real-Time PCR Detection System (Bio-Rad, Laboratories Inc, Herculers, CA, USA) using the primer sets shown in Table 3 (The abundance of the *nosZ* gene was below the detection limit in this study, therefore it was not shown here). The 10 μl reaction mixture contained 5 μl of SYBR Premix Ex Taq™ (TaKaRa Biotechnology, Otsu, Shiga, Japan), 0.4 μl of each primer (10 μM), and 2 μl of template DNA (2.5-5 ng μl^{-1}).

To prepare the standards used in the qPCR analysis, the PCR amplicons of each gene were purified using PCR Clean-Up System and subsequently cloned into the pGEM®-T EasyVector (Promega, San Luis Obispo, CA, USA). The constructs were then transferred into JM109 Competent Cells (Promega) and the plasmids were extracted from cultures of the positive clones using PureYield™ Plasmid Miniprep System (Promega) and used as the template for the standards. Standard curves were generated using ten-fold serial dilutions of plasmids containing correct inserts of the target genes. Each qPCR run contained corresponding standard curves, soil DNA samples and no-template controls. Melting curve analysis was performed between 75-94.5 °C at the end of each amplification assay to evaluate the specificity of qPCR products, and the amplification efficiencies for all qPCR runs ranged between 80-105 %.

2.5 Terminal restriction fragment length polymorphism (T-RFLP) analysis

The community structures of AOA and AOB were determined by T-RFLP analysis of the *amoA* genes, using the fluorescently labelled primers FAM-CrenamoA23f/CrenamoA616r and FAM-amoA1F/amoA2R, respectively (Hu et al. 2015). The 25 μl PCR reaction mixture contained 2 μl of diluted template DNA (5-10 ng), 0.3 μl of each primer (20 μM), 2.5 μl of 10 × NH_4 reaction buffer, 1 μl of $MgCl_2$ solution (50 mM), 1 μl of BSA (20 mM), 0.5 μl of dNTP mix (20 mM), and 1.5 units of BioTaq DNA polymerase (Bioline, Sydney, Australia). The thermal cycling condition for both AOA and AOB was as follows: 95 °C for 5 mins; 35 cycles of 30 s at 95 °C, 45 s at 55 °C, 45 s at 72 °C, followed by 10 min at 72 °C. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA, USA) and quantified using the NanoDrop ND2000c Spectrophotometer.

The restriction digestion was carried out in a 10 μl mixture containing 300 ng of purified PCR product, 0.1 μl of BSA, 1 μl of 10 × NEBuffer, and 5 units of the restriction enzymes RsaI (BioLabs, Sydney, Australia) for AOA and MspI for AOB. Digests were incubated at 37 °C for 3 h, and then denatured for 10 min at 95 °C. Terminal restriction fragments (TRFs) were size-separated with an ABI PRISM 3500 Genetic analyzer (Applied Biosystems, CA, USA), and analyzed using a local southern size calling method (peaks > 50 bp) and a peak amplitude threshold setting of 50, using Genemapper version 4.0 (Applied Biosystems). TRFs with peak height comprising less than 1 % of the total peak height were removed from the downstream analysis, and peaks that differed by less than 1 bp were combined into the same TRF (Singh and Thomas 2006). The relative fluorescence abundance of all TRFs were exported for the analysis of community structure.

2.6 Data analysis

The copy numbers of genes were log-transformed prior to statistical analysis to meet normality assumptions. One-way analysis of variance (ANOVA) based on the Duncan-test was used to test the treatment effects on potential nitrification rates or the abundances of microbial genes in SPSS 19.0 (IBM, USA). Spearman's correlation was performed to assess the correlation between the *amoA* gene copy numbers and NO_3^--N concentrations. Difference at $P < 0.05$ was considered to be statistically significant. Non-metric multidimensional scaling (NMDS) plots were used to visualize the Bray-Curtis dissimilarity matrices based on the T-RFLP data of AOA and AOB.

3 Results

3.1 Soil pH and potential nitrification rates (PNRs)

Soil pH at TR changed from 5.37 to 5.76, with no significant difference between treatments or sample times except for the significantly lower value observed in U in spring compared to C ($P < 0.05$) (Fig. 1A). Soil pH at GM ranged from 5.92 to 6.15 and no significant difference existed between treatments or sample times (Fig. 1B).

PNRs ranged from 0.15 to 0.66 mg NO_2^--N kg $^{-1}$ soil d^{-1} at TR and from 0.09 to 0.47 mg

NO_2^--N kg $^{-1}$ soil d^{-1} at GM (Figs. 1C and 1D). Compared with C, PNRs were significantly higher in U at TR for samples collected in autumn and at both sites for samples collected in spring ($P < 0.05$). PNRs were invariably the same between the fertilizer treatments at TR, with the exception of DMPP which significantly lowered the PNR compared to U in autumn.

3.2 Copy numbers of the *amoA* gene and bacterial 16S rRNA gene

The AOA *amoA* gene copy numbers changed from 4.89×10^5 (C) to 1.08×10^6 (U) copies g^{-1} soil at TR and from 1.64×10^7 (U) to 9.94×10^7 (U) copies g^{-1} soil at GM (Figs. 2A and 2B). Urea application significantly increased the abundance of AOA in comparison with C at TR in spring. Amendment with DMPP (UD) and nBTPT (UN) significantly decreased the AOA abundance in spring, by 33 % and 44 % ($P < 0.05$) compared with U. At GM, there was no significant difference in the AOA abundance between treatments, but significantly higher AOA abundances were observed in spring compared to autumn for all treatments, except for UD. A significant and positive correlation between the AOA abundance and PNRs was only observed at GM ($R^2 = 0.83$, $P < 0.01$).

The AOB *amoA* gene copy numbers changed from 1.12×10^6 (C) to 6.18×10^6 (U) copies g^{-1} soil at TR and from 7.83×10^6 (UD) to 5.82×10^7 (U) copies g^{-1} soil at GM (Figs. 2C and 2D). At both sites, the AOB abundances in U in spring were significantly ($P < 0.05$) higher than those of C, and treatment amended with DMPP (UD) resulted in significantly lower AOB abundance compared with U by 56 % and 72 %, respectively, at TR and GM in spring. However, a significant decrease by 41 % ($P < 0.05$) in the AOB abundance as a consequence of nBTPT addition was only observed at GM in spring, compared to U. In UAN, the AOB abundance was significantly higher relative to C at GM in spring. The AOB abundance showed significant and positive correlations with PNR at TR ($R^2 = 0.81$, $P < 0.01$) and GM ($R^2 = 0.93$, $P < 0.01$).

The bacterial 16S rRNA gene copy numbers changed from 1.10×10^9 (U) to 4.85×10^9 (UN) copies g^{-1} soil at TR and from 3.15×10^9 (U) to 1.06×10^{10} (UD) copies g^{-1} soil at GM (Figs. 2E and 2F). There was no significant difference between fertilizer treatments with an exception that a significantly lower abundance of the bacterial 16S rRNA was observed in U compared to C at GM in autumn ($P < 0.05$).

3.3 Copy numbers of the *narG* and *nirK* genes

In terms of the denitrifiers, the *narG* gene copy numbers varied from 8.25×10^4 (UN) to 4.51×10^5 (C) copies g^{-1} soil at TR and from 1.12×10^5 (U) to 1.19×10^6 (UAN) copies g^{-1} soil at

GM (Figs. 3A and 3B). There was no difference in the *narG* gene abundance at TR between treatments or sample times. At GM, urea amended with DMPP and nBTPT significantly lowered the *narG* gene abundance by 43 % and 33 %, respectively, compared with UAN in spring ($P < 0.05$). The *nirK* gene copy numbers varied from 5.67×10^4 to 1.99×10^5 copies g^{-1} soil at TR and from 2.39×10^5 to 1.30×10^6 copies g^{-1} soil at GM (Figs. 3C and 3D). There was no significant difference observed in the *nirK* gene abundances across treatments at either sampling time. Significant seasonal effect was observed in the abundances of both *narG* and *nirK* genes in C, U and UAN at GM (Figs. 3B and 3D).

3.4 Community structure of ammonia oxidizers

The T-RFLP analysis of AOA *amoA* gene by the RsaI enzyme yielded six and eight distinct terminal restriction fragments (TRFs) across all the treatments, at TR and GM, respectively, of which TRF-56 and TRF-296 were the most dominant genotypes at both sites (Fig. 4). NMDS ordinations according to the Bray-Curtis dissimilarity matrices showed that the AOA assemblages clustered together based on sampling time rather than treatment (Figs. 5A and 5B). As for AOB, eight distinct TRFs were obtained across all the treatments at both sites, with TRF-154 and TRF-489 as the dominant genotypes at TR, and TRF-233 and TRF-483 as the most dominant TRFs at GM (Figs. 4C and 4D). NMDS ordinations based on the Bray-Curtis dissimilarity matrices revealed a clear divergence of AOB assemblage based on sampling time (Figs. 5C and 5D).

4 Discussion

4.1 Effect of treatments on soil pH and total bacteria abundance

Urea is the most widely used form of synthetic N fertilizer for dairy pasture systems in Australia, mostly due to the high N content (46 %) and low transportation cost (Chen et al. 2008; Di and Cameron 2016; Suter et al. 2016). In this study, the continuous application of urea over the two years resulted in lower values of soil pH at TR (Fig. 1A) and bacterial abundance at GM (Fig. 3F). The mechanism of generating protons and soil acidification due to N fertilizer application in pasture soils was reviewed in detail by Bolan et al. (1991). Bolan et al. (1991) found that two net moles of protons (H^+) are generated with each mole of NH_4^+ nitrified (nitrification), while one mole of H^+ is consumed per mole of NO_3^- reduced to N_2 (denitrification), and as a consequence, the extent of soil acidification is generally higher with ammoniacal fertilizers than with nitrate fertilizers. The addition of DMPP and nBTPT relieved the decrease in soil pH and showed no significant difference with C (control), indicative of their inhibition on nitrification (Fig. 1). Previous studies suggested that the distribution and activity of soil bacteria could be negatively affected by soil pH (Hu et al. 2013; Ernfors et al. 2014). Intriguingly, the observed significantly lower abundance of soil total bacteria in U was found at GM, instead of TR, where the significant decrease in soil pH was observed relative to the control. This result indicates there are other factors influencing soil bacterial abundance.

4.2 Effects of environmental factors on nitrifiers and denitrifiers

Climatic and seasonal factors have been proposed as variables that strongly influence the distribution and abundances of AOA and AOB based on the multivariate analysis of 45 sites within the cropping regions in southern Australia (O'Sullivan et al. 2013). In our study, the mean temperature of April (Autumn) and October (Spring) is similar, at 18.7 and 17.7 °C, respectively, however, the mean rainfall in April (Autumn), 41.1 mm is much lower than that of October (Spring), 62.2 mm (Table 2). The same climatic pattern was reported by Suter et al. (2016) that winter was subjected to higher rainfall, as a result, the soil moisture in spring was at higher level than in the dry autumn in a temperate Australian pasture located at the site close to this study. Soil moisture was suggested as a more important factor influencing the dynamics of ammonia oxidizers than temperature (Osborne et al. 2016). The higher soil moisture content could significantly enhance the growth and activity of nitrifiers and denitrifiers in grassland soils (Di et al. 2014; Suter et al. 2016). A field investigation conducted by Hu et al. (2015) suggested that water addition significantly increased soil PNRs and the functional genes of ammonia oxidizers. In accordance with prior studies, substantially higher PNRs (Fig. 1) and abundances of functional genes of nitrifiers and denitrifiers (Figs. 2 and 3) were observed in the field samples collected in spring than autumn in this study. Intriguingly, the seasonal effect could be offset by the application of inhibitors. For instance, the significant seasonal effect was not observed in the abundances of the *narG* or *nirK* genes in UD and UN at GM (Fig. 3B). In this study, the significant inhibition of DMPP on the abundances of AOA and AOB mainly occurred in samples collected in spring (Figs. 2A, 2C and 2D). This is not surprising as the samples collected in spring is more close to the date of treatment application (Table 1) than autumn. It should be noted that no significant change in the community composition of ammonia oxidizers was observed due to DMPP application at either sampling time, which is in accordance with the short-term microcosm incubation experiment that DMPP showed no impact on the community composition of ammonia oxidizers in pasture soils (Shi et al. 2016b).

4.3 Potential reasons for the inhibition of nBPTP on nitrifiers

nBTPT is assumed to be rapidly converted to an oxygen analogue *N*-(*n*-butyl) phosphoric triamide (nBPT) when applied to the soil, and nBPT could form a tridentate ligand with urease enzyme and thus inhibit the urease activity (Manunza et al. 1999; Chen et al. 2008). However, limited studies have been conducted to explore the possible effect of nBTPT on nitrification. In the present study, addition of nBTPT significantly decreased the copy numbers of the AOA and AOB *amoA* genes at TR and GM respectively, in spring (Figs. 2A and 2D). A possible explanation is that nBTPT has the capacity to inhibit urease within the cells of ammonia oxidizers, and thereby limits NH_3 availability for the intracellular nitrification. Previous studies showed that AOB strains belonging to the *Nitrosomonas*, *Nitrosospira* and *Nitrosococcus* lineages are capable of hydrolyzing urea and growing with urea as the sole substrate source (De Boer and Laanbroek 1989; Allison and Prosser 1991; Koper et al. 2004). Results from culture-dependent studies showed that urea could enter the cells of AOB by diffusion, and intracellular urea hydrolysis and ammonia oxidation can occur to support nitrification under soil pH conditions between 4-7.5 (Burton and Prosser 2001). In addition, the urease genes have been recently found in isolated AOA strains of *Nitrososphera viennensis* (Tourna et al. 2011) and *Nitrososphaera gargensis* (Spang et al. 2012). Microcosm studies using DNA-stable isotope labelling have further demonstrated the growth of autotrophic urease-containing AOA that were dependent on intracellular urea hydrolysis as a substrate for ammonia oxidiation (Lu and Jia 2013). Collectively, the results from culture, genome and microcosm studies indicate that AOA and AOB have the capacity to use urea as an energy source. Prosser and Nicol (2012) indicated that ammonia oxidizers, with mixotrophic or heterotrophic growth, are able to transport low molecular-weight organic compounds. Therefore, it could be postulated that nBTPT could inhibit the intracellular nitrification of ammonia oxidizers.

4.4 Effects of DMPP and nBTPT on denitrifiers

Nitrification and urease inhibitors are generally expected to be ineffective at inhibiting denitrification, and it is therefore not likely that the significant decrease in the *narG* gene abundance observed at GM in spring (Fig. 3B) was caused by the direct inhibition by DMPP and nBTPT, but it might be ascribed to the inhibitory effect of these inhibitors on the formation of nitrate, as suggested by the inhibition of DMPP and nBTPT on the abundances of ammonia oxidizers in this study. Therefore, the inhibition of denitrification was most likely due to the limited substrate available. The inhibition by nitrification inhibitors of nitrification- and denitrification-derived N_2O was reported by Ruser and Schulz (2015). Several studies have reported that the application of nitrification inhibitors reduced denitrification-produced N_2O through decreasing the functional gene abundances of denitrifiers (Dong et al. 2013a; Kou et al. 2015; Wang et al. 2015). Further research is highly desirable to relate the changes in microbial functional genes abundance to process rates.

5 Conclusions

In conclusion, through the quantification of functional genes involved in nitrification and denitrification, and the exploration of the community structure of ammonia oxidizers in two grazed pastures following two years of application of urea, UAN and urea coated inhibitors, we provide evidence that the use of these inhibitors could specifically target the *amoA* and *narG* genes and have great genetic capacity to reduce N losses during nitrification and denitrification. Furthermore, the amendment with DMPP and nBTPT could slow down the trend in soil acidification and consequently maintain the level of soil indigenous bacteria, while shows no effect on the community structure of ammonia oxidizers.

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Figure legends

Fig. 1 Soil pH and PNRs across the five treatments (C, control; U, urea; UAN, urea ammonium nitrate; UD, urea coated DMPP; UN, urea coated nBTPT) at Terang (TR) and Glenormiston (GM) in autumn and spring, 2014. Error bars represent standard errors of five replicates. Different letters above the bars indicate a significant difference at $P < 0.05$

Fig. 2 Abundances of the AOA and AOB *amoA*, and bacterial 16S rRNA genes across the five treatments (C, control; U, urea; UAN, urea ammonium nitrate; UD, urea coated DMPP; UN, urea coated nBTPT) at Terang (TR) and Glenormiston (GM) in autumn and spring, 2014. Error bars represent standard errors of five replicates. Different letters above the bars indicate significant difference at $P < 0.05$. Note: Y axes scales differ between charts

Fig. 3 Abundances of the *narG* and *nirK* genes across the five treatments (C, control; U, urea; UAN, urea ammonium nitrate; UD, urea coated DMPP; UN, urea coated nBTPT) at Terang (TR) and Glenormiston (GM) in autumn and spring, 2014. Error bars represent standard errors of five replicates. Different letters above the bars indicate a significant difference at $P < 0.05$. Note: Y axes scales differ between charts

Fig. 4 Terminal restriction fragment length polymorphism fingerprints of the AOA *amoA* gene digested by the RsaI enzyme and the AOB *amoA* gene digested using the MspI enzyme at Terang (TR) and Glenormiston (GM) across the five treatments (C, control; U, urea; UAN, urea ammonium nitrate; UD, urea coated DMPP; UN, urea coated nBTPT) in autumn (A) and spring (S), 2014. Error bars represent standard errors of five replicates

Fig. 5 Nonmetric multi-dimensional scaling ordinations based on the Bray-Curtis dissimilarity matrices of the T-RFLP data of AOA and AOB TRFs across the five treatments (C, control; U, urea; UAN, urea ammonium nitrate; UD, urea coated DMPP; UN, urea coated nBTPT) at Terang (TR) and Glenormiston (GM) in autumn (A) and spring (S), 2014

Table 1 Treatment application times at Terang (TR) and Glenormiston (GM)

Year	Terang (TR)	Glenormiston (GM)
2012	18th July 2012	18th July 2012
	20th September 2012	20th September 2012
2013	18th July 2013	19th September 2013
	19th September 2013	7th November 2013
	7th November 2013	
2014	22th May 2014	19th June 2014
	17th July 2014	21th August 2014
	18th September 2014	

Table 2 Relevant soil physical and chemical properties (0-15 cm) at Terang (TR) and Glenormiston (GM) before the experiment

Soil property	Terang (TR)	Glenormiston (GM)
Soil Order	Chromosol	Dermosol
Soil pH	5.7	6.1
Organic carbon (%)	4.3	5.8
NH_4^+-N (mg kg^{-1} soil)	33.2	11.0
NO_3^--N (mg kg^{-1} soil)	35.8	33.3
Exchangeable Al (cmol kg^{-1})	0.35	0.05
Exchangeable Ca (cmol kg^{-1})	6.92	25.59
Exchangeable Mg (cmol kg^{-1})	2.87	7.02
Exchangeable K (cmol kg^{-1})	0.71	1.80
Particle size (%)		
Clay (< 0.002 mm)	16	19
Silt (0.002-0.02 mm)	24	14
Sand (0.02-2 mm)	60	67
Monthly mean temperature (°C):		
April (autumn)	18.7	
October(spring)	17.7	
Monthly mean rainfall (mm):		
April(autumn)	41.1	
October(spring)	62.2	

Table 3 The primer sets used for the quantification of the *amoA*, *narG*, *nirK* and bacterial 16S rRNA genes

Primers	Sequence (5'-3')	Length (bp)	Reference
AOA *amoA*:			
CrenamoA23f	ATGGTCTGGCTWAGACG	629	Tourna et al. 2008
CrenamoA616r	GCCATC CATCTGTATGTCCA		
AOB *amoA*:			
amoA1F	GGGGTTTCTACTGGTGGT	491	Rotthauwe et al. 1997
amoA2R	CCCCTCKGSAAAGCCTTCTTC		
narG:			
narGG-F	TCGCCSATYCCG GCSATGTC	173	Bru et al. 2007
narGG-R	GAGTTGTACCAGTCRGCSGAYTCSG		
nirK:			
FlaCu	ATCATGGTSCTGCCGCG	470	Hallin and Lindgren 1999
R3Cu	GCCTCGATCAGRTTGTGGTT		
Bacterial 16S rRNA:			
1369F	CGGTGAATACGTTCYCGG	100	Suzuki et al. 2000
1492R	GGWTACCTTGTTACGACTT		

Fig. 1

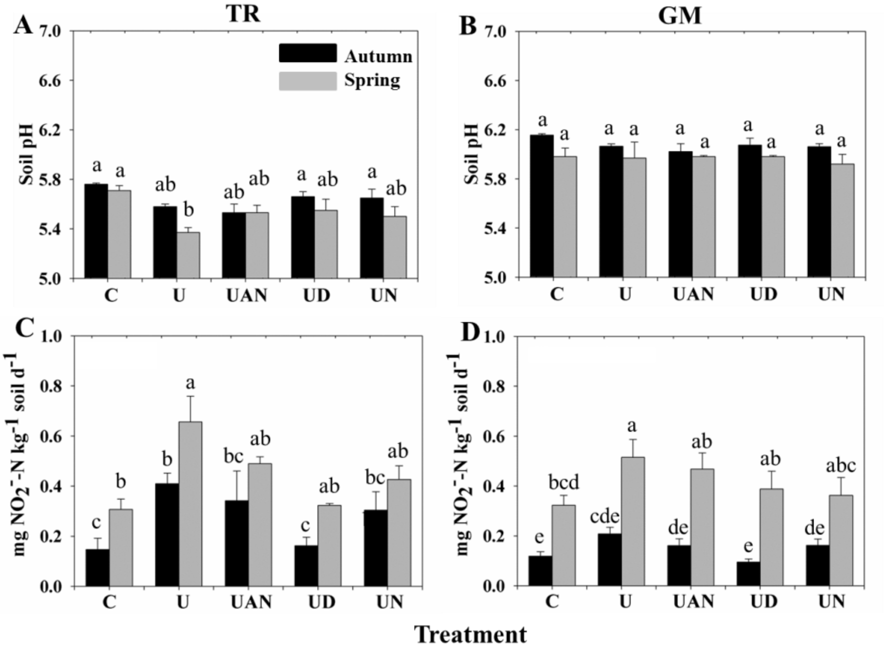

A
TR
Autumn
Spring
Soil pH
7.0
6.6
6.2
5.8
5.4
5.0
a a
ab b
ab ab
a ab
a ab
C
U
UAN
UD
UN
B
GM
Soil pH
a a
a a
a a
a a
a a
C
U
UAN
UD
UN
C
mg NO_2^--N kg^{-1} soil d^{-1}
1.0
0.8
0.6
0.4
0.2
0.0
c b
b a
bc ab
c ab
bc ab
C
U
UAN
UD
UN
D
mg NO_2^--N kg^{-1} soil d^{-1}
e bcd
cde a
de ab
e ab
de abc
C
U
UAN
UD
UN
Treatment

Fig. 2

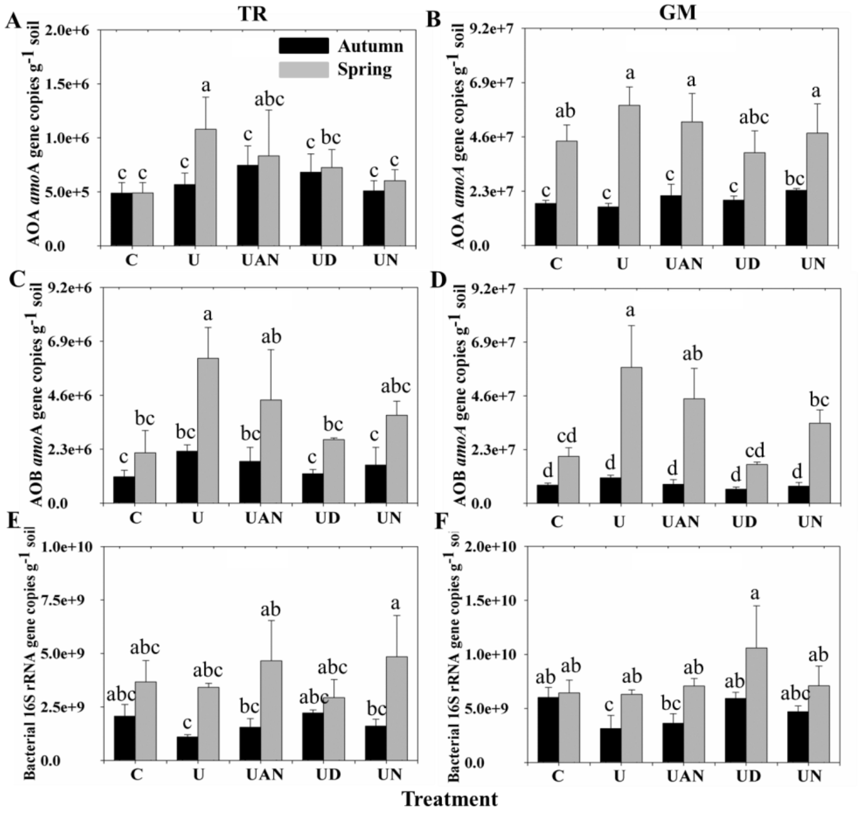

A
TR
AOA amoA gene copies g^{-1} soil
2.0e+6
1.5e+6
1.0e+6
5.0e+5
0.0
Autumn
Spring
c
c
c
a
c
abc
c
bc
c
c
C
U
UAN
UD
UN
B
GM
AOA amoA gene copies g^{-1} soil
9.2e+7
6.9e+7
4.6e+7
2.3e+7
0.0
c
ab
c
a
c
a
c
abc
bc
a
C
U
UAN
UD
UN
C
AOB amoA gene copies g^{-1} soil
9.2e+6
6.9e+6
4.6e+6
2.3e+6
0.0
c
bc
bc
a
bc
ab
c
bc
c
abc
C
U
UAN
UD
UN
D
AOB amoA gene copies g^{-1} soil
9.2e+7
6.9e+7
4.6e+7
2.3e+7
0.0
d
cd
d
a
d
ab
d
cd
d
bc
C
U
UAN
UD
UN
E
Bacterial 16S rRNA gene copies g^{-1} soil
1.0e+10
7.5e+9
5.0e+9
2.5e+9
0.0
abc
abc
c
abc
bc
ab
abc
abc
bc
a
C
U
UAN
UD
UN
F
Bacterial 16S rRNA gene copies g^{-1} soil
2.0e+10
1.5e+10
1.0e+10
5.0e+9
0.0
ab
ab
c
ab
bc
ab
ab
a
abc
ab
C
U
UAN
UD
UN
Treatment

Fig. 3

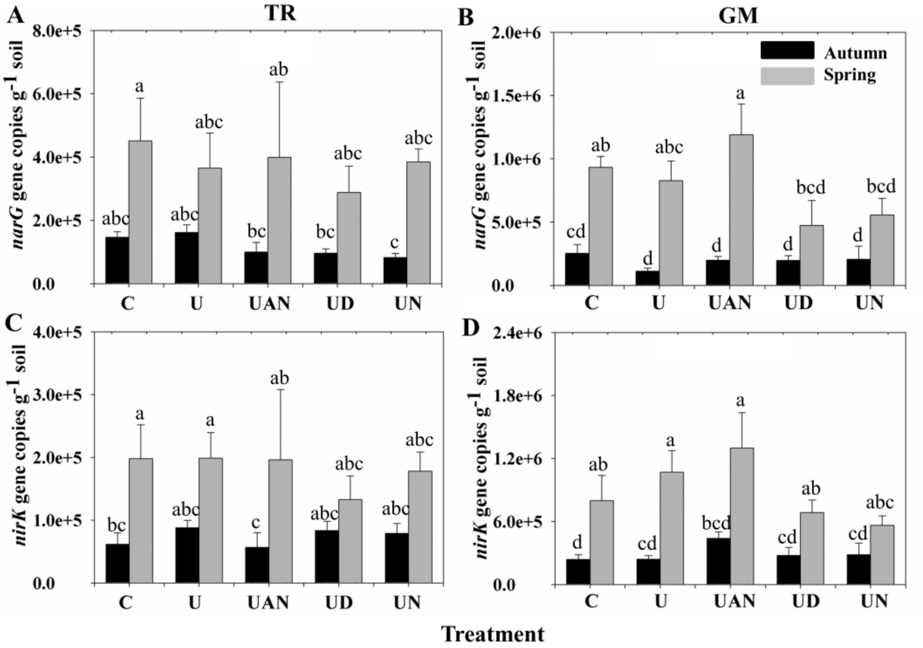

A
TR
B
GM
Autumn
Spring
narG gene copies g-1 soil
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0
abc
a
abc
abc
bc
ab
bc
abc
c
abc
2.0e+6
1.5e+6
1.0e+6
5.0e+5
0.0
cd
ab
d
abc
d
a
d
bcd
d
bcd
C
U
UAN
UD
UN
C
nirK gene copies g-1 soil
4.0e+5
3.0e+5
2.0e+5
1.0e+5
0.0
bc
a
abc
a
c
ab
abc
abc
abc
abc
D
2.4e+6
1.8e+6
1.2e+6
6.0e+5
0.0
d
ab
cd
a
bcd
a
cd
ab
cd
abc
Treatment

Fig. 4

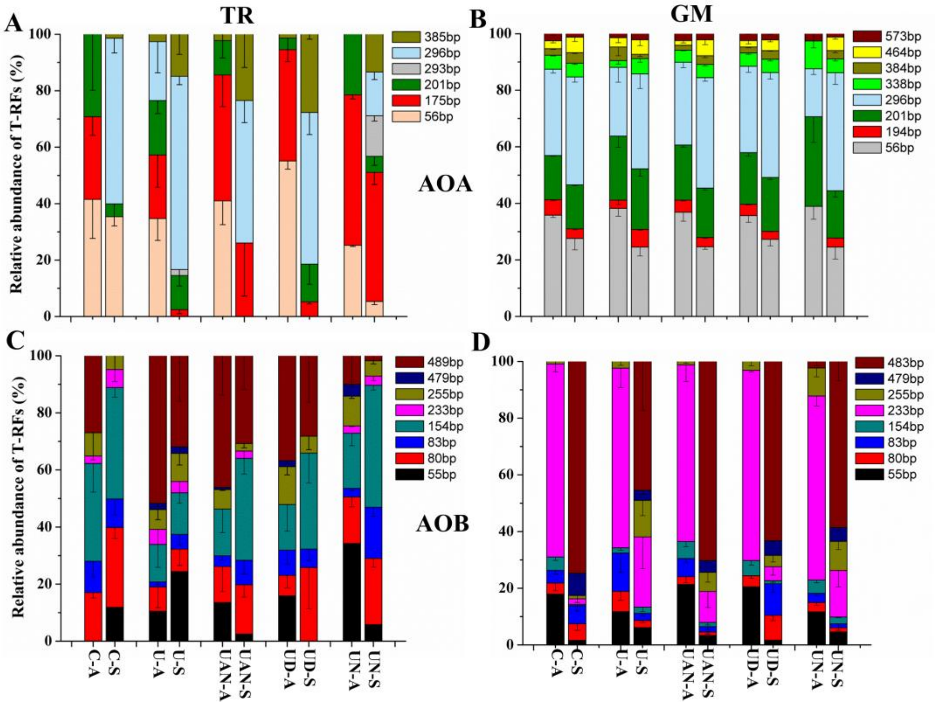
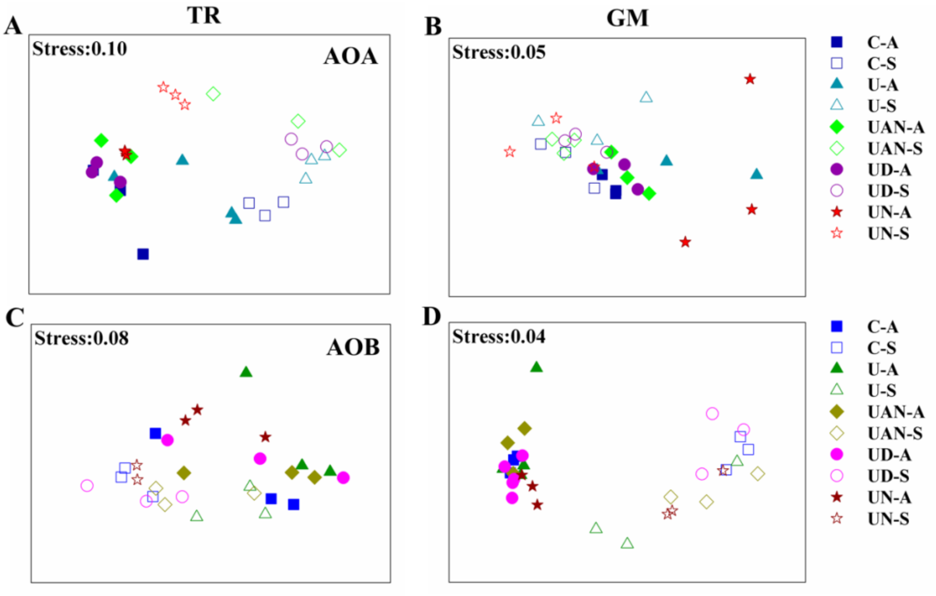

A
TR
B
GM
C
D
AOA
AOB
Relative abundance of T-RFs (%)
0
20
40
60
80
100
385bp
296bp
293bp
201bp
175bp
56bp
573bp
464bp
384bp
338bp
296bp
201bp
194bp
56bp
489bp
479bp
255bp
233bp
154bp
83bp
80bp
55bp
483bp
479bp
255bp
233bp
154bp
83bp
80bp
55bp
C-A
C-S
U-A
U-S
UAN-A
UAN-S
UD-A
UD-S
UN-A
UN-S

Fig. 5


A
TR
Stress:0.10
AOA
B
GM
Stress:0.05
C-A
C-S
U-A
U-S
UAN-A
UAN-S
UD-A
UD-S
UN-A
UN-S
C
Stress:0.08
AOB
D
Stress:0.04
C-A
C-S
U-A
U-S
UAN-A
UAN-S
UD-A
UD-S
UN-A
UN-S