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Title: Effects of nitrification inhibitors on gross N nitrification rate, ammonia oxidizers, and N_2O production under different temperatures in two pasture soils

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Abstract

Australian pasture soil for cattle and sheep industries constitute the principal land use with considerable N fertilizer consumption, which is one of the causes of local environmental problems. Nitrification plays a key role in regulating soil inorganic N concentration and its environmental diffusion. The effects of different nitrification inhibitors (NIs) on gross N nitrification (n_{gross}) rate and N_2O production under different temperatures in pasture soils remain unclear. A laboratory incubation experiment was conducted to determine the effect of NIs (dicyandiamide [DCD], 3,4-dimethylpyrazole phosphate [DMPP], and 3-methylpyrazol and 1H-1,2,4-triazol [3MP+TZ]) on N_2O emissions, n_{gross} and net N nitrification (n_{net}) rates, and the abundance of ammonia oxidizers, namely, ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB), in two Australian pasture soils incubated at temperatures of 15 °C, 25 °C and 35 °C. All NIs reduced both n_{gross} and n_{net} rates and N_2O production rate from the two pasture soils but to different extents. The inhibitory rates of NIs on n_{gross} and n_{net} reached 6.80%–63.8% and 5.91%–62.3%, respectively, whereas that on N_2O production rate totaled 4.5%–41.4% in the tested soils. NIs reduced nitrification and N_2O production by inhibiting the growth of AOB rather than AOA. The inhibitory effects of NIs were temperature-dependent, that is, decreasing with increasing temperature from 15 °C to 35 °C. In general, DMPP performed better than DCD and 3MP+TZ at 15 °C and 35 °C, whereas DCD performed more effectively than the other two NIs at 25 °C. Our results suggest that the utilization of NIs will depend on the conditions present, especially soil temperature. Additionally, AOB is the target of inhibition when mitigating nitrification and N_2O emission by applying NIs in pasture soils.

Keywords: Acid soils · AOA · AOB · Gross N nitrification rate · ^{15}N dilution

Introduction

Nitrification is a microbial process that plays important roles in regulating soil inorganic N concentration, NO_3^- leaching, and N_2O production (Bremner 1997). N_2O is a potent greenhouse gas that also contributes to stratospheric ozone destruction (IPCC 2007). Nitrification can be either autotrophic or heterotrophic. Ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) are involved in the first step of autotrophic nitrification, that is, the oxidation of NH_3 to NO_2^- in soil (Francis et al. 2005). AOA and AOB can be identified by the *amo*A gene, which is a functional marker encoding the ammonia mono-oxygenase (AMO) enzyme (McCarty 1999). Conversely, few heterotrophs carry out heterotrophic nitrification (oxidize either NH_3 or organic N to form NO_3^-) in soil, usually at much lower rates than those carried out by autotrophs (Sahrawat et al. 2008). However, complete ammonia oxidizers within the *Nitrospira* genus, which can convert ammonia to nitrate in a single organism (comammox), were discovered (Hu and He 2017).

Nitrification inhibitors (NIs) can decelerate the rate of soil nitrification by deactivating AMO and blocking the first and rate-limiting steps of autotrophic nitrification, specifically, NH_3 oxidization (Di et al. 2009; Di and Cameron, 2012). By suppressing autotrophic nitrification, NIs potentially reduce subsequent denitrification and N leaching. As a result, reduction in the N_2O produced through this pathway is expected to occur (Bauhus et al. 1996; Lan et al. 2013; McTaggart et al. 1997). A meta-analysis by Akiyama et al. (2010) showed that compared with conventional fertilizers application of NIs reduced N_2O emissions by 44% to 31%, with an average of 38%. Dicyandiamide (DCD, $C_2H_4N_4$) and 3,4-dimethylpyrazole phosphate (DMPP) are the two most commonly used NIs. DCD has been studied for over 80 years, and its effect in reducing both NO_3^- leaching and N_2O emission has been reported for

different agroecosystems, such as maize–wheat crop systems (Sharma and Prasad 1996), grazed pastures (Di and Cameron 2004; 2012), and paddy soils (Kumar et al. 2000; Lan et al. 2013). DMPP was demonstrated as one of the most efficient NIs to date and has undergone ecotoxicological and standard toxicological tests (Menendez et al. 2012; Benckiser et al. 2013). Zerulla et al. (2001) specified several advantageous properties of DMPP compared with DCD. For example, DMPP can be used with solid fertilizer granules at a lower rate than that of DCD. 3-Methylpyrazol + 1H-1,2,4-triazol (3MP+TZ) is a synthetic compound proposed as a NI (Hu et al. 2013). However, this compound is less commercially used than DCD and DMPP, and its inhibitory effects on nitrification or N_2O emission are unclear.

Evidence showed that the effect of NIs in reducing nitrification rate produce inconsistent results and is affected by environmental factors, such as soil temperature. For example, Merino et al. (2005) observed that inhibition by DMPP can last longer in autumn than in spring. Menéndez et al. (2012) discovered a declining inhibitory efficacy of DMPP with increasing soil temperature from 10 °C to 20 °C. According to Di and Cameron (2014), the half-life of DCD increased from 111 days to 116 days under a soil temperature of 8 °C but shortened to 18–25 days when the soil temperature reached 20 °C. These observations suggest that high soil temperatures can lower the NIs efficiency owing to the faster molecular degradation under these conditions. Therefore, soil temperature is an important factor affecting NI efficacy in systems with repeated NI applications.

The agricultural sector of Australia contributes 16% of the total greenhouse gas emissions, contributing approximately 85% of all N_2O emissions (20.7 Mt CO_2-eq.) (AGO 2010). Pasture soils for sheep and cattle industries constitute the principal land use and covers approximately 450 million ha of the lands in Australia (AGO 2010);

the industry is considered the largest N fertilizer user in the country. Previous studies have demonstrated that applying NIs can effectively reduce autotrophic nitrification and N_2O emissions from pasture soils (Friedl et al. 2017). For example, Di et al. (2016) reviewed the effects of DCD and DMPP in inhibiting NO_3^- leaching and N_2O emission in pasture soils, and the results showed that N_2O emission from animal urine–N can be reduced by 57%. Evidence showed that N_2O emission is significantly related to the amount of NO_3^-–N in pasture soils and to *amo*A gene copy numbers of AOB but not related to the population of AOA (Di et al. 2009). In addition, both AOB population growth and the amounts of soil NO_3^-–N concentration were significantly related to the amount of DCD application. However, AOA population abundance showed no relationship to the application rate of DCD (Guo et al. 2014). Although various studies have investigated the effect of different NIs on nitrification rate, N_2O emission, and ammonia oxidizers in pasture soils, the influence of temperature on the inhibitory efficacy of NIs in pasture soils with low pH and high organic C content remains unclear. Evidence showed that heterotrophic nitrification, a process that cannot be inhibited by NIs, may act as the predominant pathway in producing NO_3^- in soils at low pH and high recalcitrant organic C primarily in forest ecosystems and grassland (Müller et al. 2004; Zhang et al. 2013). In addition, most previous studies have been carried out by measuring the net changes in NH_4^+–N and NO_3^-–N concentrations in pasture soils. Net rate results from several opposing soil processes. We possess very limited knowledge about the effectiveness of different NIs in n_{gross} rate of pasture soils. The gross rates of NH_4^+ and NO_3^- production measured using the ^{15}N isotopic pool dilution technique provide considerable information about N transformation processes than net rates (Müller et al. 2004). However, the effects of temperature on the efficacy of NIs in inhibiting ammonia oxidizers (AOA and AOB)

in pasture soils remain unclear.

This study was designed to verify the following points: (i) the effects of NIs (DCD, DMPP, and 3MP+TZ) on n_{net} and n_{gross} rates, ammonia oxidizers (AOA and AOB), and N_2O production from pasture soils with low pH and high organic C content; (ii) the effects of temperature on the efficacy of NIs. We hypothesized that (i) NIs can only partly inhibit nitrification and N_2O production in pasture soils with low pH and high organic C content; (ii) the efficacy of NIs on n_{gross} rate in pasture may vary under different temperatures. The findings from the study can improve our understanding of the interactions of NIs with nitrification rate, N_2O production, and soil microbial communities in pasture soils under a range of temperatures.

Materials and methods

Soil collection

Two pasture soils were used in the incubation experiments. Soils were collected from two typical pasture lands, namely, Glenormiston (GN; 38.18° S, 142.97° E) and Terang in Victoria (TR; 33.73° S, 84.43° E), Australia. Soil samples were randomly collected from 10 locations to a depth of 15 cm. The collected samples were mixed into one composite sample. Soil samples were thoroughly homogenized, stones and plant roots were removed by hand, and the soil was sieved at <2 mm mesh before being air dried. Table 1 shows the physical and chemical properties of the two tested pasture soils.

Soil incubation experiment

A total of 60 g (dry weight) soil samples were introduced into a 500 ml plastic vial. To reactivate soil microorganisms, we rehydrated the soil and preincubated it at 15 °C,

25 °C, or 35 °C for 21 days. The following five treatments (each with four replicates) were included in this study:

1) Control (equivalent water, no fertilizer) (abbr.: control)
2) $NH_4Cl+K^{15}NO_3$ (containing 26% N) (abbr.: ACl)
3) $NH_4Cl+K^{15}NO_3$+DMPP (DMPP at 0.7% of applied NH_4^+–N) (abbr.: DMPP)
4) $NH_4Cl+K^{15}NO_3$+3MP+TZ (3MP [at 0.33%]+TZ [at 0.67%] of applied NH_4^+–N), (abbr.: 3MP+TZ)
5) $NH_4Cl+K^{15}NO_3$+DCD (DCD at 4.5% of applied NH_4^+–N) (abbr.: DCD)

A total of 600 vials (5 treatments × 4 replicates × 3 temperature × 5 destructively sampling time×2 soils=600 vials) were observed. The application rates of NIs were typical of the amount applied to pasture soils (Di et al. 2004; Di and Cameron 2012; Friedl et al. 2017). Fertilizer treatments were surface-applied to the soil on 0-day. The application rate was 100 mg kg^{-1} NH_4^+–N plus 50 mg NO_3^-–N kg^{-1} soil, and the ^{15}N atom% of NO_3^- was 10%. An equivalent volume of deionized water (instead of NIs and fertilizer) was applied on the control treatment, and soil water content of all treatments was adjusted to 60% water-filled pore space. Immediately after application, all vials were covered with parafilm, and four pin holes were created to facilitate air exchange. Vials were incubated at 15 °C, 25 °C and 35 °C for 28-day. Vials were weighed every two days to calculate water loss, and deionized water was replenished as needed.

Gas collection and analysis

Headspace gas was obtained from the 500 ml vials using gas-tight syringes 0, 1, 2, 3, 4, 7, 10, 14, 21, and 28 days after applying N fertilizer and NIs. Prior to each gas

collection, the air in vials was replaced with zero air (pure N_2) to balance the air pressure when performing gas sampling. On each sampling day, 20 ml gas samples were collected 0 and 12 h after closing the vials with a plastic syringe and storing them into a pre-evacuated exetainer (Exetainer®, Labco Ltd., Lampeter, Ceredigion, UK). Samples were analyzed for N_2O concentration by Agilent 7890 Gas Chromatograph equipped with ECD detector.

Soil collection and analysis

Soils were destructively sampled for the analysis of mineral N (NH_4^+ and NO_3^-) concentration and their ^{15}N isotopic abundance immediately after gas sampling on days 0, 7, 14, 21, and 28. A total of 2 g soil subsamples were obtained on days 0, 14, and 28 from each vial for molecular analysis and stored at −80 °C prior to DNA extraction. The remaining soil was extracted with 2 M KCl (soil:solution=1:5) by shaking for 1 h, and KCl extracts were filtered through a quantitative filter paper. Extract samples were maintained at −20 °C until analysis for mineral N with a continuous-flow autoanalyzer (Skalar, SAN++) and ^{15}N abundance in mineral N (100 ml) after microdiffusion. The detailed procedure of the microdiffusion methods has been reported by Saghir et al. (1993) and Liu et al (2015a, b). The isotope composition of mineral N was performed by isotope-ratio mass spectrometry (Crewe, UK). The abundances of AOA and AOB *amo*A genes were quantified by real-time quantitative polymerase chain reaction (qPCR) with primers targeting the archaeal and bacterial *amo*A genes (Francis et al. 2005). Details of the methods of soil DNA extraction and qPCR analysis have been reported (Shi et al. 2016).

Calculations

The n_{net} rates during the 28-day incubation were calculated from the changes in NO_3^- concentrations by using the following equation:

$n_{net}=([NO_3^-–N]t_1–[NO_3^-–N]t_0)/(t_1–t_0)$

where t represents the incubation time (day).

According to the principle of ^{15}N dilution, n_{gross} rates were calculated using the data obtained in 0–7 day of incubation. This process was performed as soil N transformation will complicate if the incubation time exceeds 7-day, processes such as remineralization will occur, thereby leading to inaccuracy in n_{gross} rates. The classical ^{15}N isotopic dilution equation was used to calculate the n_{gross} rates (Kirkham and Bartholomew 1954).

$$n=\frac{\left[(NT_1\text{-}NT_2)/\Delta t\right]\log(NL_1NT_2/NL_2NT_1)}{\log(NT_1/NT_2)}$$

where NL represents labeled NO_3^-; NT denotes the total NO_3^- (labeled+unlabeled); Δ t is the time interval. Subscripts 1 and 2 denote the 0- and 7-day values, respectively.

N_2O fluxes were calculated according to the following equation:

$$F=\rho\times\frac{V}{A}\times\frac{\Delta c}{\Delta t}\times\frac{273}{273+T}$$

where F is the gas flux in ng N_2O-N m^2 h^{-1}, ρ stands for the density of N_2O in the standard state (g ml^{-1}), V species the volume of the head space (ml), A is the area of the vial (cm^2), $\Delta c/\Delta t$ indicates the change in N_2O concentration per unit of time (ppm d^{-1}), and T is the air temperature within the vials (^oC).

Statistical analyses

Statistical analyses were performed using SPSS 18.0 software. One-way ANOVA was used to compare differences in the average rates of N_2O flux, n_{net} and n_{gross} among the

different treatments (P<0.05). The relationships of n_{net} rate and NO_3^-–N concentration to N_2O production rate were investigated by redundancy and correlation analyses.

Results

Mineral N

In the control treatments, the exchangeable NH_4^+–N concentrations increased with the increase in incubation time for both soils at three temperatures (Figs. 1 and 2). However, in ACl treatments, the concentration of exchangeable NH_4^+ decreased with prolonged incubation, showing a decline toward the control levels within four weeks in both soils (Figs. 1 and 2). The amendment of NIs retarded the decrease in NH_4^+ concentrations, and such effects were more evident at 15 °C than at 25 °C and 35 °C. Compared with the control treatment, adding NH_4Cl significantly increased the NO_3^-–N concentrations over time (P<0.05), indicating nitrification occurrence. NO_3^- concentration increased more slowly at low temperature (Figs. 1 and 2), that is, 15 °C, than at high temperature, that is, 35 °C (Figs. 1 and 2). The amendment of NIs significantly retarded the accumulation of NO_3^-–N in both soils, and such effects were more significant in DMPP treatment than in DCD and 3MP+TZ, regardless of the temperature treatment and soil used (Figs. 1 and 2).

Nitrification rate

Fig. 3 shows the n_{gross} rates at days 0–7 and the time-weighted averages of n_{net} rates during the 28-day incubation. ACl treatments yielded the highest n_{net} values in both soils (2.94–5.95 and 1.31–2.32 mg kg^{-1} day^{-1} in GN and TR soil, respectively). All NIs also significantly inhibited the n_{net} rates, with inhibition rates varying from 5.91% to 62.3% (Fig. 3). In general, the inhibitory effect of NIs on n_{net} rates decreased with

increasing soil temperature regardless of the NI type. In GN soil, DMPP performed better than DCD and 3MP+TZ under the three temperatures (Fig. 3). However, no statistical difference in n_{net} was detected between DMPP, DCD, and 3MP+TZ at the three temperatures in TR soil (P>0.05, Fig. 3).

The n_{gross} rates of ACl treatments (7.12–15.2 and 9.82–12.4 mg kg^{-1} d^{-1} in GN and TR soil, respectively) significantly increased when the temperature was increased from 15 °C to 25 °C in both GN and TR soils (P<0.05, Fig. 3). However, no difference in n_{gross} rate was observed between 25 °C and 35 °C (P>0.05, Fig. 3). The n_{gross} rates were in agreement with the rapid consumption of NH_4^+ in soil at 25 °C and 35 °C, and they were generally higher in GN soil than in TR soil during incubation at the same temperature (Fig. 3). The n_{gross} rates were inhibited by NIs from 6.80% to 63.8% (Fig. 3), although the inhibitory effects of DMPP, DCD, and 3MP+TZ on n_{gross} rate declined with increasing soil temperature from 15 °C to 35 °C. DMPP worked better than DCD and 3MP+TZ in both GN and TR soils at 15 °C and 35 °C, but DCD presented the best performance among the studied NIs at 25 °C (Fig. 3).

N_2O production rate

Soil temperature and NI application significantly affected the average daily N_2O production rates in both GN and TR soils (Fig. 4). The N_2O production rates over the 28-day incubation time increased with increasing soil temperature, and the highest N_2O production rates were observed in ACl treatment regardless of the temperature and soil sample used (Fig. 4). N_2O production was reduced by 23.9%–42.6%, 16.3%–41.4%, and 13%–27.8% in GN soil and by 4.5%–11.6%, 20.3%–25.3%, and 20.7%–25.8% in TR soil by applying DCD, DMPP, and 3MP+TZ, respectively. However, the

inhibitory effects of NIs were affected by incubation temperature. The highest inhibition effect by DCD was achieved at 25 °C and 35 °C in GN and TR soil, respectively. On the other hand, the highest inhibition effects of DMPP and 3MP+TZ were achieved at 35 °C and 25 °C for both soils, respectively.

AOA and AOB populations

In GN soil, both AOA and AOB *amo*A gene copy numbers increased with incubation time but decreased as incubation temperature increased from 15 °C to 35 °C (Fig. 5). The application of NIs affected AOB abundance with respect to the ACl treatment (10.1%–44.8% inhibition rate on 14-day; 10.9%–39.4% inhibition rate on 28-day) but not AOA abundance. Throughout the entire incubation period, the largest average reduction effect of NIs on bacterial *amo*A gene abundance was observed in DMPP treatment at 15 °C (29.1%), followed by DCD and DMPP treatments at 25 °C (25.8%) and 35 °C (11.9%), respectively.

In TR soil, both AOA and AOB *amo*A gene copy numbers under ACl treatment increased from 2 h on 14-day and then decreased for the rest of the incubation time. This finding was true except for AOA at 25 °C and AOB at 15 °C, which both decreased from 0-day to 14-day and increased from 14-day to 28-day (Fig. 6). The variation ranges of AOA and AOB *amo*A gene copy numbers during three sampling dates were significantly larger at the incubation temperature of 15 °C than at 25 °C and 35 °C (Fig. 6). Similar to that in GN soil, adding NIs caused no effect on AOA growth in TR soil. This observation was obtained except for DCD and 3MP+TZ treatment at 15 °C on 14-day (Fig. 6). By contrast, applying the three NIs to TR soil significantly inhibited AOB population under 25 °C and 35 °C on 14-day and under 15 °C, 25 °C, and 35 °C on 28-day. The highest inhibition effect on AOB population

was achieved at 15 °C in DMPP treatment (42.8%), followed by DCD treatment at 25 °C (24.7%) and DMPP treatment at 35 °C (20.2%).

Discussion

Effects of temperature on NIs efficacy

DCD, DMPP, and 3MP+TZ can inhibit the n_{net} and n_{gross} in the two studied pasture soils (Fig. 3). However, soil temperature significantly affected their efficacy. The increased incubation temperature lowered the efficacy of these NIs (Fig. 3). This phenomenon may be due to at least three reasons. The first reason is the positive effect of temperature on soil nitrification activity. Sahrawat (2008) reported that nitrification generally follows a bell-shaped temperature response curve with an optimum temperature ranging from 25 °C to 35 °C. According to Avrahami and Bohannan (2007), nitrification activity intensifies with rising soil temperature, and evidence revealed that temperature negatively affects the abundance of ammonia oxidizers but positively affects their activity (Gubry-Rangin et al. 2017). The second reason is the rapid decomposition of NIs by soil microorganisms when the temperature was increased. Previous studies reported the hastened NI degradation in soil at increased temperatures (Di and Cameron 2014; Menéndez et al. 2012; Puttanna et al. 1999) and accelerated microbiological degradation of NIs with increasing temperature, with the maximum values reached between 25 °C and 33 °C. Additionally, half-life dissipation decrease was higher under laboratory incubation conditions than under field practice conditions because of the presence of other possible dissipation pathways, such as NI leaching, in the field practice (Kelliher et al. 2014). The third reason is that the substrate affinity or capacity to access substrates elicited by varying microbial activities may change along with the changes in

temperature (Wallenstein and Hall 2012). These findings demonstrated that NH_4^+-based fertilizers with added NI can preserve NH_4^+–N in soils, but the efficiency of NIs drastically reduces with rising temperature. Consequently, the possible effect on the environment and crops treated with fertilizers coupled with NI application can be dramatically diminished if the temperature is close to or beyond 30 °C. Therefore, soil temperature should be considered to efficiently use NIs.

Effects of NIs on nitrification rate

The n_{gross} rates in two pasture soils with ACl treatment ranged from 7.12 mg N kg^{-1} day^{-1} to 15.2 mg N kg^{-1} day^{-1}. Booth et al. (2005) assembled n_{gross} data from 100 studies conducted in grassland, forest, shrubland, and agricultural systems. Most of the rates were lower than 10 mg N kg^{-1} day^{-1}. The high nitrification rates in the two ACl-treated pasture soils will increase the risk of NO_3^- loss via leaching or denitrification or N_2O emission in field practice. Applying NIs effectively decreased the n_{gross} and n_{net} rates (Fig. 3). The decrease in NO_3^- accumulation approximated 10.8% to 56.2% in the two soils (Figs. 1 and 2). Our results demonstrated that NIs exhibited efficacy in inhibiting both n_{gross} and n_{net} rates in the soils used under laboratory conditions, and this finding may be useful in reducing NO_3^-–N loss via denitrification and leaching processes. Consistently, Di and Cameron (2016) reviewed NIs, including DCD and DMPP, in decreasing N_2O emission and NO_3^- leaching in pasture soils. Their study revealed that NO_3^- leaching from grazed pastures can be decreased by 30% to 50%.

NH_4^+–N concentrations decreased, whereas NO_3^-—N concentrations increased as incubation progressed. NI application slowed down the variations of both NH_4^+–N and NO_3^-–N (Figs. 1 and 2). However, the observed increases in NO_3^-–N

concentrations were much faster than the declines in NH_4^+–N concentrations. These results occurred possibly because NO_3^-–N in the two pasture soils was probably not only produced through autotrophic nitrification (NH_4^+–N to NO_3^-–N) but also by other N transformation processes, such as heterotrophic nitrification. Previous studies showed that heterotrophic nitrification is the dominating NO_3^- production process in acid soils with high organic C (Zhang et al. 2015a). Therefore, partial inhibition of NIs on NO_3^- production in the two pasture soils may be ascribed to heterotrophic nitrification.

Effects of NIs on N_2O emission rate

N_2O flux was correlated with the n_{net} rate in soil (Fig. 7), thereby suggesting that NIs can partly inhibit nitrification-related N_2O emission. Moreover, the different NIs reduced N_2O emissions to different extents. DMPP generally reduced N_2O emission more than DCD and 3MP+TZ at 15 °C and 35 °C, whereas DCD performed better than DMPP and 3MP+TZ at 25 °C. Weiske et al. (2001) observed a more significant inhibition in N_2O emissions under field conditions after DMPP application than after DCD application. By contrast, Di and Cameron (2012) cannot find any difference between DCD and DMPP in terms of inhibition of N_2O emission under field conditions. In the present study, the effects of NIs on inhibiting cumulative N_2O emission were lower than that on nitrification rate. This result may be attributed to the high ratio of C and NO_3^- in dairy pasture soil (Table 1); this condition reduced the efficiency of inhibitory effect on N_2O production (Davidson et al. 1986). Another possible explanation is that N_2O release was due to processes, aside from autotrophic nitrification, such as denitrification or heterotrophic nitrification, where NIs caused less effect on N_2O emission. N_2O flux was positively correlated with NO_3^-–N

concentrations (Fig. 7). This result suggested that NO_3^-, which is a product of nitrification and a substrate for denitrification, was an important indicator for N_2O emission from the studied pasture soils. Furthermore, previous studies have shown that the contribution of heterotrophic nitrification to N_2O emission is much higher than that of autotrophic nitrification in several acidic soils with high soil organic C contents (Liu et al. 2015a; Zhang et al. 2011; Zhang et al. 2015b). Therefore, the importance of heterotrophic nitrification to N_2O emission in tested pasture soils requires further exploration, and this knowledge is valuable for the effective use of NIs in pasture soils with high soil C content.

In contrast to the lack of significant difference in n_{gross} rates between 25 °C and 35 °C, the average N_2O production rate was much larger at 35 °C than at 25 °C and 15 °C regardless of treatment. High N_2O production rate at 35 °C may be ascribed to the increased contribution of denitrification to N_2O emission. Evidence has shown that variation in incubation temperatures may result in changes in soil microbial populations for denitrification (Braker et al. 2010) or differential expression of the enzymes responsible for different N_2O/N_2 emissions during denitrification (Saleh-Lakha et al. 2009). According to Phillips et al. (2015), the denitrified N shifts toward N_2O when temperature increases. Therefore, the temperature dependence of N_2O emission is an important environmental factor that will benefit the successful use of NIs in systems with repeated NI applications.

Effects of NIs on AOA and AOB abundance

Our study revealed that AOB, but not AOA, significantly decreased after NI application (Figs. 5 and 6). This result is in agreement with the findings of previous studies (Liu et al. 2015b; Shi et al. 2016). The different responses of AOB and AOA

to NI addition may be due to the following reasons. First, nitrification in soils containing high levels of NH_4^+–N is mainly followed by the dynamics of AOB abundance rather than AOA abundance (Di et al. 2009). Second, the different compositions of the cell membranes of AOB and AOA (fatty acids as side chains in AOB cell walls versus isoprenoid side chains in the membranes of AOA) influence the cell membrane permeability of NIs (Benckiser et al. 2013; Ruser and Schulz 2015). However, all the three NIs inhibited AOB growth but to different extents. This result indicated that the responses of AOB to different NIs may vary.

In the current study, AOA were generally the predominant ammonia oxidizer (Figs. 5 and 6) in the two examined pasture soils with soil pH <6.0. Previous findings suggested that soil pH is a key factor driving the niche partitioning of AOA and AOB (Liu et al. 2015b; Hu et al. 2014; Shi et al. 2016). AOA and AOB populations in the two pasture soils decreased in size with increasing soil temperature from 15 °C to 35 °C (Figs. 5 and 6, respectively). Soil bacterial ammonia oxidizer communities were affected by temperature, similar to the results of previous studies (Avrahami et al. 2003; Avrahami et al. 2011; Gubry-Rangin et al. 2017). According to Gubry-Rangin et al. (2017), temperature influences AOA and AOB community compositions in most soils. This effect may be due to the rapid growth of thaumarchaeotal populations at different temperature optima, whereas increasing temperature may increase the death of susceptible community members. The essential differences in temperature-influenced properties of nitrification driven by AOA and AOB support the hypothesis that the biochemical processes associated with NH_3 oxidation in AOA and AOB differ thermodynamically from each other.

Conclusion

DCD, DMPP, and 3MP+TZ can decrease the n_{net} and n_{gross} rates and N_2O productions from pasture soils but to different extents. NIs reduced nitrification rates by inhibiting AOB growth but not AOA growth. However, the inhibitory effects of NIs on nitrification rates were temperature-dependent, that is, decreasing with increasing temperature. N_2O production in the two pasture soils was only partly inhibited by NIs. Hence, high organic C in two pasture soils may lead to denitrification and heterotrophic nitrification, which can contribute to N_2O production. This aspect requires further study. We also measured AOA and AOB *amo*A gene abundances on the basis of soil DNA. Measuring active communities using soil RNA is highly desirable in future studies.

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Table 1 Soil properties

Soil	Organic matter (%)	Organic C (%)	pH (1:5 water)	CEC (c mol kg^{-1})	Clay (<2 μm, %)	Silt (2–60 μm, %)	Sand (60–2000 μm, %)	Soil texture	NH_4^+–N (mg kg^{-1})	NO_3^-–N (mg kg^{-1})
Terang (TR)	7.9	4.60	5.50	7.67	8	64	29	Sandy loam	7.4	16.0
Glenormiston (GN)	10.0	5.90	6.00	24.0	11	52	36	Sandy loam	7.7	44.0

Fig. 1 Dynamics of NH_4^+–N and NO_3^-–N under different temperatures in GN soil during the 28-day incubation. Error bars indicate the standard errors of four replicates. GN: soil from Glenormiston, TR: soil from Terang, ACl: $NH_4Cl+K^{15}NO_3$, DMPP: $NH_4Cl+K^{15}NO_3$+DMPP (DMPP at 0.7% applied NH_4^+–N), 3MP-TZ: $NH_4Cl+K^{15}NO_3$+3MP+TZ (3MP at 0.33% applied NH_4^+–N+TZ at 0.67% applied NH_4^+–N), DCD: $NH_4Cl+K^{15}NO_3$+DCD (DCD at 4.5% applied NH_4^+–N). Numbers 15, 25, and 35 represent the incubation temperatures of 15 °C, 25 °C, and 35 °C, respectively

Fig. 2 Dynamics of NH_4^+–N and NO_3^-–N under different temperatures in TR soil during 28-day incubation. Error bars indicate the standard errors of four replicates. The legend from Fig. 1 also applies to this figure.

Fig. 3 Average net (0−28 day) and n_{gross} rates (0−7 day) from GN and TR soils treated with different NIs. Error bars indicate the standard errors of four replicates. Different letters indicate significant difference among treatments at the same temperature (P<0.05).

Fig. 4 Average daily N_2O production rate from GN and TR soils treated with different NIs. Error bars indicate the standard errors of four replicates. Different letters indicate significant difference among treatments at the same temperature (P<0.05).

Fig. 5 Changes in AOA and AOB *amo*A gene copy numbers in GN soil during the 28-day incubation after treatment with different NIs and temperatures. Error bars indicate the standard errors of four replicates.

Fig. 6 Changes in AOA and AOB *amo*A gene copy numbers in TR soil during 28-day incubation after treatment with different NIs and temperatures. Error bars indicate the standard errors of four replicates.

Fig. 7 Relationships between the average daily N_2O production rate and (a) NO_3^-N and (b) n_{net} rates. Dashed curves correspond to 95% confidence interval for linear regression.

Fig. 1

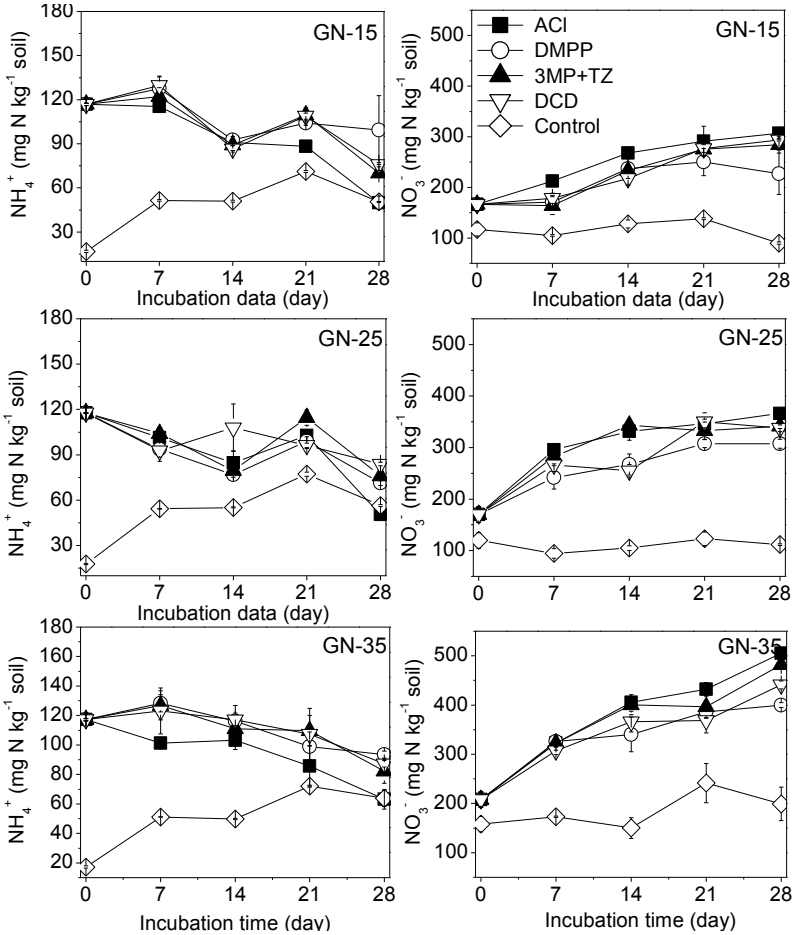

GN-15
GN-25
GN-35
ACl
DMPP
3MP+TZ
DCD
Control
NH4+ (mg N kg-1 soil)
NO3- (mg N kg-1 soil)
Incubation data (day)
Incubation time (day)

Fig. 2

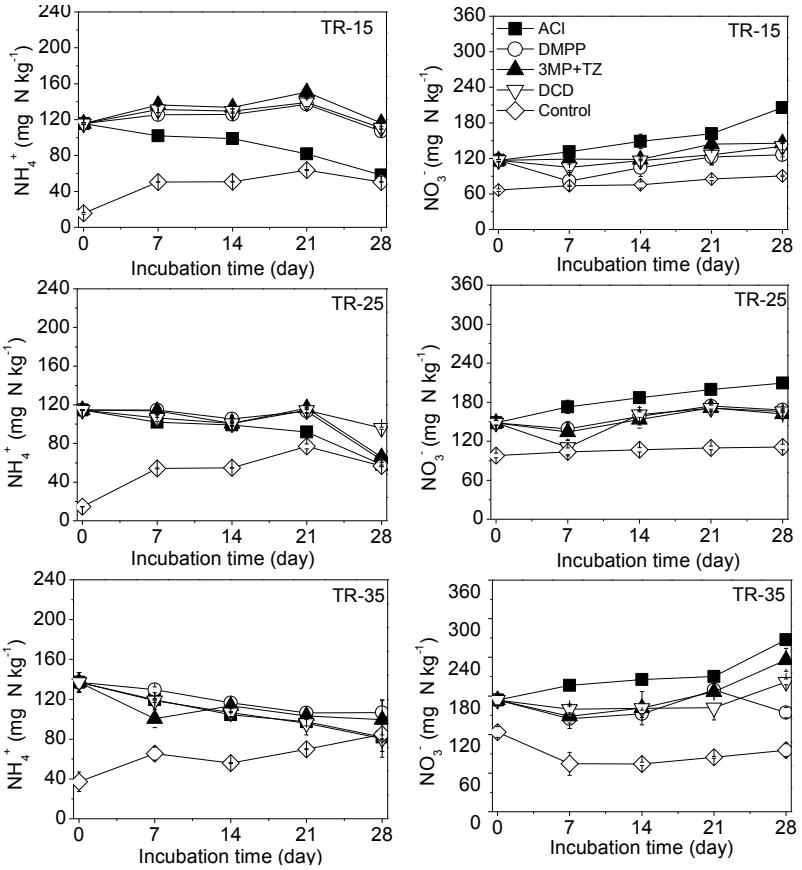

TR-15
TR-25
TR-35
ACl
DMPP
3MP+TZ
DCD
Control
NH_4^+ (mg N kg^{-1})
NO_3^- (mg N kg^{-1})
Incubation time (day)

Fig. 3

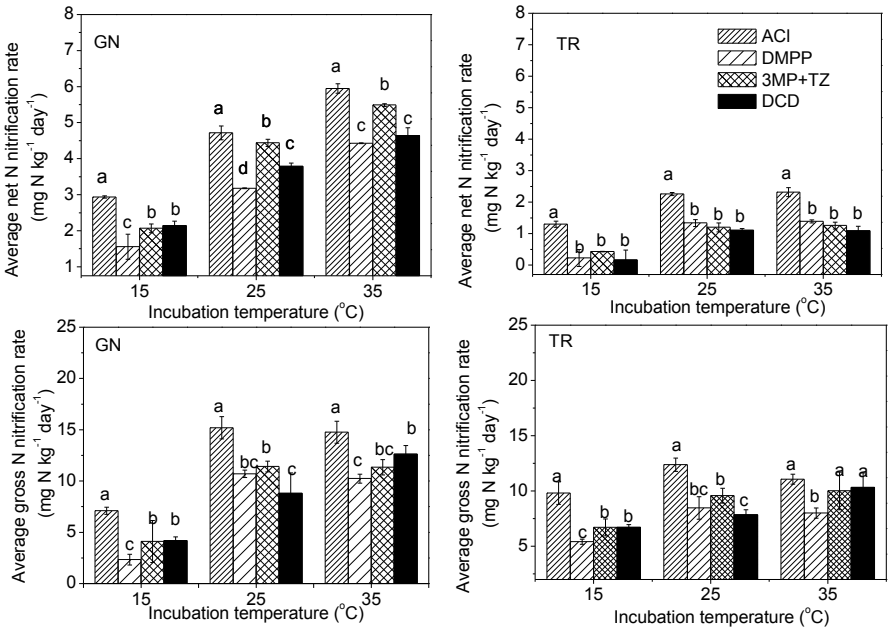

GN
TR
ACl
DMPP
3MP+TZ
DCD
Average net N nitrification rate (mg N kg-1 day-1)
Average gross N nitrification rate (mg N kg-1 day-1)
Incubation temperature (°C)
15
25
35

Fig. 4

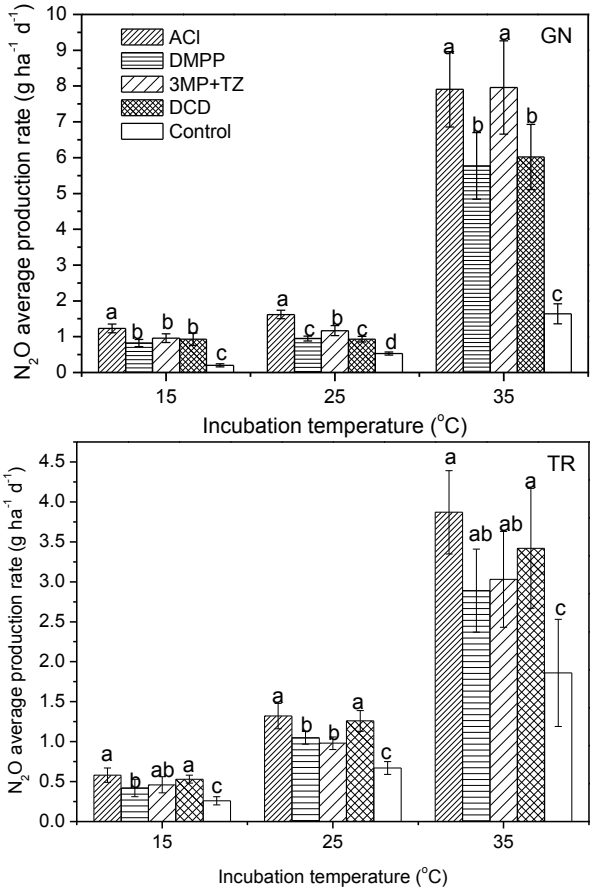

ACl
DMPP
3MP+TZ
DCD
Control
GN
TR
N2O average production rate (g ha-1 d-1)
Incubation temperature (°C)
15
25
35

Fig. 5

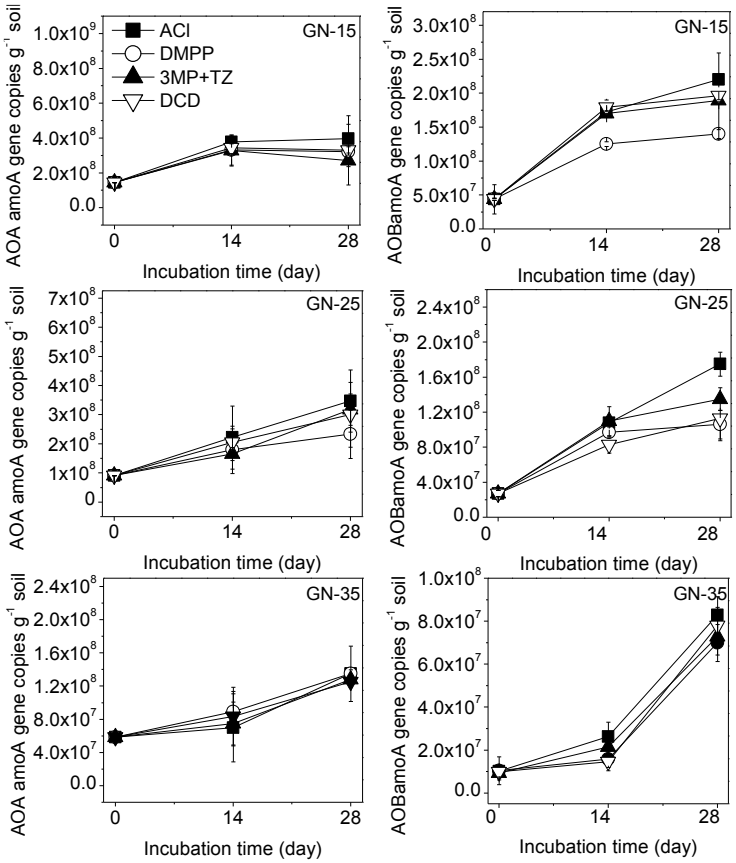

ACl
DMPP
3MP+TZ
DCD
GN-15
GN-25
GN-35
AOA amoA gene copies g-1 soil
AOBamoA gene copies g-1 soil
Incubation time (day)
0
14
28

Fig. 6

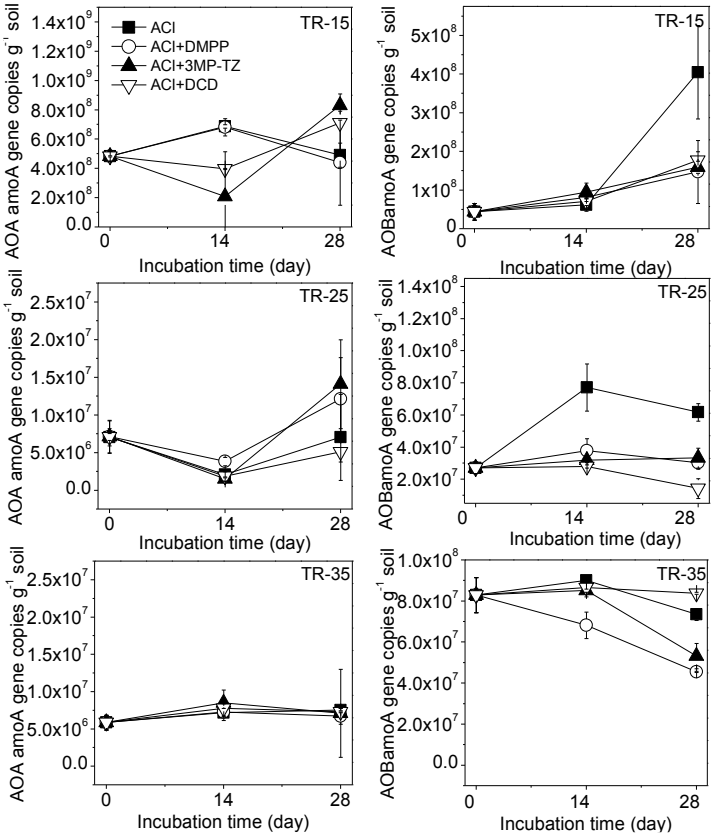
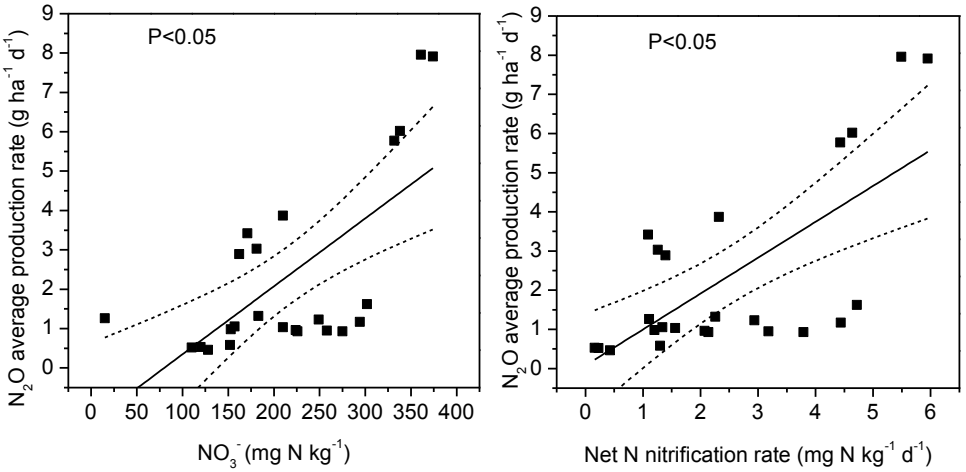

ACI
ACI+DMPP
ACI+3MP-TZ
ACI+DCD
TR-15
TR-25
TR-35
AOA amoA gene copies g-1 soil
AOBamoA gene copies g-1 soil
Incubation time (day)

Fig. 7


P<0.05
N2O average production rate (g ha-1 d-1)
NO3- (mg N kg-1)
P<0.05
N2O average production rate (g ha-1 d-1)
Net N nitrification rate (mg N kg-1 d-1)

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