J Soils Sediments (2015) 15:1113–1118
DOI 10.1007/s11368-015-1086-6

SOILS, SEC2 • GLOBAL CHANGE, ENVIRON RISK ASSESS, SUSTAINABLE LAND USE • SHORT ORIGINAL COMMUNICATION

The effect of nitrification inhibitors in reducing nitrification and the ammonia oxidizer population in three contrasting soils

Rui Liu · Helen Hayden · Helen Suter · Jizheng He · Deli Chen

Received: 10 December 2014 / Accepted: 3 February 2015 / Published online: 13 February 2015

Abstract
Purpose 3,4-Dimethylpyrazole phosphate (DMPP) and acetylene (C_2H_2) are widely used nitrification inhibitors. These nitrification inhibitors have shown inconsistent efficacy in different soils, demonstrating the importance of determining which soil and microbial factors cause this variability. The aim of the present study was to investigate the efficacy of DMPP and C_2H_2 to inhibit nitrification and the ammonia oxidizer population in three contrasting soil types from Australia.
Materials and methods Three contrasting soils of different pH_{Water} (4.6, 7.0, and 8.0) collected from different agriculture systems in Australia were used in a laboratory incubation experiment for 28 days to compare the efficacy of DMPP and C_2H_2 to inhibit nitrification. We measured mineral nitrogen (N) concentrations during the incubation. In addition, quantitative PCR was applied to quantify the ammonia oxidizer population and to investigate the population change in response to DMPP and C_2H_2 addition.
Results and discussion Acetylene completely blocked nitrification in the three soils while DMPP was more effective in inhibiting nitrification in the neutral soil (93.5 %) than in the alkaline soil (85.1 %) and acid soil (70.5 %). Ammonia-oxidizing archaea (AOA) were more abundant than ammonia-oxidizing bacteria (AOB) in all three control soils, with the highest AOA abundance found in the acid soil. The addition of DMPP and C_2H_2 significantly decreased AOA abundance in all soils ($P<0.05$) and significantly suppressed AOB abundance in the neutral soil and slightly blocked AOB growth in the alkaline soil, though it had no effect on AOB abundance in the acid soil.
Conclusions Our results show C_2H_2 completely inhibited nitrification and performed better than DMPP in our study. DMPP was more effective in the neutral soil than other two soils. Neither DMPP nor C_2H_2 was a selective nitrification inhibitor in neutral and alkaline soils in which both AOA and AOB were inhibited. Neither DMPP nor C_2H_2 had any effect on AOB abundance in the acid soil. Soil pH plays an important role in the effectiveness of DMPP and C_2H_2 in inhibiting nitrification and ammonia oxidizer population.

Keywords 3,4-Dimethylpyrazole phosphate (DMPP) · Acetylene · AmoA · Archaea · Bacteria · Soil pH

Responsible Editor: Hailong Wang

R. Liu (✉) · H. Suter · J. He · D. Chen
Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Melbourne, Victoria 3010, Australia
e-mail: ruimelb@gmail.com

R. Liu
e-mail: ruiliu@student.unimelb.edu.au

H. Hayden
Department of Environment and Primary Industries, AgriBio, 5 Ring Rd, Bundoora, Melbourne, Victoria 3083, Australia

1 Introduction

Nitrification is an important nitrogen transformation process in soils, which converts a relatively immobile form of nitrogen, ammonium (NH_4^+), into a mobile form (nitrate, NO_3^-) (Joye and Hollibaugh 1995). Nitrate is subjected to losses by leaching and denitrification when oxygen is limited. Nitrification and denitrification can also lead to the emission of potent greenhouse gas, nitrous oxide (N_2O). The application of nitrification inhibitors is one possible way to reduce nitrogen losses and thereby increase nitrogen fertilizer efficiency for ammonium-based fertilizers. Ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) are responsible for the first and rate-limiting step of autotrophic

nitrification. Many studies have found that nitrification inhibitors blocked nitrification by stopping AOA or AOB growing (Di et al. 2009, 2010; Gubry-Rangin et al. 2010).

3,4-Dimethylpyrazole phosphate (DMPP) is a widely used nitrification inhibitor which in some cases has been very effective in reducing nitrification at low application rates of 0.5–1.0 kg active compound ha^{-1} (Zerulla et al. 2001; Barth et al. 2008). However, its efficacy at inhibiting nitrification varies markedly with soils (Barth et al. 2001; Shi et al. 2011), temperature, and moisture (Chen et al. 2010). Acetylene (C_2H_2) is another effective inhibitor of nitrification (Bremner and Blackmer 1978). Moreover, C_2H_2 is bacteriostatic (Juliette et al. 1993) and commonly works on autotrophic nitrification at a low concentration (e.g., 10 Pa) (De Boer and Kowalchuk 2001). Previous studies have shown that C_2H_2 is very effective in inhibiting nitrification in acid soils (Hynes and Knowles 1982; Gubry-Rangin et al. 2010), but the interactions between C_2H_2 and soil properties are rarely reported. Therefore, it is essential to determine what causes the inconsistent efficacy of nitrification inhibitors. Soil pH is believed to be a key factor that affects biological processes in soils (Šimek and Cooper 2002) by affecting the chemical form, concentration, and availability of substrates (Kemmitt et al. 2006) and influencing bacterial diversity and community structure on a global scale (Fierer and Jackson 2006). It has been reported that soil pH was the main factor driving the community changes of AOA and AOB in a series of soil pH gradient plots (He et al. 2007; Nicol et al. 2008; Shen et al. 2008). However, the relationship between soil pH and the effectiveness of nitrification inhibitors has rarely been studied. There are several studies on the responses of AOA and AOB to the addition of nitrification inhibitors (Shen et al. 2008; Di et al. 2009, 2010; Gubry-Rangin et al. 2010; Zhang et al. 2011), though their findings are often contradictory. A comprehensive study is needed to determine the effects of nitrification inhibitors on nitrification and ammonia oxidizer population in different soils.

The objectives of this study were (i) to investigate the efficacy of two types of nitrification inhibitors (DMPP and C_2H_2) to inhibit nitrification in three contrasting soil types of different pH from Australia and (ii) to determine the effects of DMPP and C_2H_2 on ammonia oxidizers in these soils.

2 Materials and methods

Surface soil samples (0–10 cm) of three soil types from different regions and industries and with different pH values were collected from Clare (sugarcane, pH 7.0), Queensland (19.78°S, 147.23°E), Tamworth (pasture, pH 8.0), New South Wales (31.09°S, 150.93°E), and Hamilton (cropping, pH 4.6), Victoria (38.32°S, 142.07°E), air-dried, and ground to pass through a 2-mm sieve prior to analysis. Details of selected soil properties are shown in Table 1. The soils were stored at 4 °C prior to incubation experiments.

Laboratory incubation experiments were conducted in the dark. Sixty grams of air-dried soil from each of the three soil types was placed in capped 500-ml vials at 25 °C and 60 % water filled pore space (WFPS). Samples were pre-wetted and incubated for 3 weeks under 25 °C and just below 60 % WFPS to equilibrate the soil before the application of treatments. Ammonium chloride (NH_4Cl) was applied to all soils at the rate of 100 μg N g^{-1} soil. The treatments applied were as follows: control (NH_4Cl), DMPP (3.37 ml of 29 % DMPP per g NH_4Cl), and C_2H_2 (1 % of the headspace in the vials). The vials were aerated every 3 days when water content and C_2H_2 were replenished.

Triplicate samples were extracted for NH_4^+ and NO_3^- analyses on days 0, 7, 14, 21, and 28 with 2 M KCl (soil-to-solution ratio 1:5) by shaking for 1 h. The soil extracts were filtered through Whatman number 42 filter papers and analyzed for NH_4^+ and NO_3^- using a segmented flow analyzer (Skalar SAN++).

The *amo*A gene copy numbers were quantified from triplicate samples on day 28 using real-time polymerase chain reaction (PCR) with two different primer sets to target the AOA (Francis et al. 2005) and AOB (Rotthauwe et al. 1997). Each archaeal *amo*A real-time PCR reaction was performed in a 20-μl volume containing 10 μl SensiFAST (Bio-Rad Laboratories, USA), 0.5 μM of each primer, and 2 μl of 10-fold dilution DNA template (1–10 ng). Amplification conditions were as follows: 95 °C for 3 min, 40 cycles of 5 s at 95 °C, 30 s at 60 °C, and 45 s at 72 °C. Each bacterial *amo*A real-time PCR reaction was performed in a 10-μl volume containing 5 μl iTaq Universal SYBR GREEN Supermix (Bio-Rad Laboratories, USA), 0.6 μM of each primer, and 2 μl of 10-fold dilution DNA template (1–10 ng). Amplification conditions were the same as the AOA QPCR assay. A known copy number of plasmid DNA for AOA or AOB was used to create a standard curve. For all assays, PCR efficiency was 90–100 % and r^2 was 0.96–0.99.

Data were analyzed using SPSS 19, and means were compared using one-way ANOVA between treatments to test the variance with a level of significance of $P<0.05$.

Table 1 Properties of the surface soil (0–10 cm) collected at field sites

Location	Clare, SA	Tamworth, NSW	Hamilton, VIC
Soil type	Clay	Clay loam	Loam
Clay (%)	53	39	19
Silt (%)	21	24	44
Sand (%)	26	37	38
pH_{Water}	7.0	8.0	4.6
Organic C (%)	4.7	1.5	6.2
Total N (%)	0.9	0.19	0.52

3 Results

The inhibition of nitrate production by C_2H_2 and DMPP varied with soil type. Nitrate concentrations in the three control soils increased gradually during the incubation, with more NO_3^- being produced in the alkaline soil than the other two soils (Fig. 1b, d, f). The addition of C_2H_2 completely blocked the production of NO_3^- in all soils (Fig. 1b, d, f). DMPP completely inhibited the production of NO_3^- in the neutral soil (Fig. 1b) and markedly slowed its formation in the other two soils (Fig. 1d, f and Table 2). In the first 7 days, the NH_4^+ concentrations in the control soils decreased from 105, 106, and 113 mg N kg^{-1} soil to 60, 28, and 90 mg N kg^{-1} soil for the neutral, alkaline, and acid soils, respectively. After that, the rate at which the NH_4^+ concentrations decreased was slower, and overall, more NH_4^+ was lost from the alkaline soil and less from the acid soil (Fig. 1a, c, e). In the inhibitor treatments, a slight decrease in NH_4^+ concentration was observed during the first 7 days, but after that, the concentrations were generally increased. The largest increase occurred in the acid soil treated with C_2H_2 (Fig. 1a, c, and e).

Table 2 Mean nitrification rate and inhibition by DMPP and C_2H_2 during incubation at 25 °C and 60 % WFPS for 28 days

Soil	Nitrification rate (mg N kg^{-1} day^{-1})			Inhibition[a] (%)	
	Control	DMPP	C_2H_2	DMPP	C_2H_2
Neutral clay	1.7	0.11	0.05	93.5	97.1
Alkaline clay loam	3.82	0.57	0	85.1	100
Acid loam	1.93	0.57	0	70.5	100

[a] Inhibition of nitrification = ((NO_3-N produced in control soil) − (NO_3-N produced in inhibitor-treated soil)) / (NO_3-N produced in control soil) × 100

AOA and AOB populations changed with incubation time. After incubation of the soils with NH_4Cl (100 μg N g^{-1} soil)

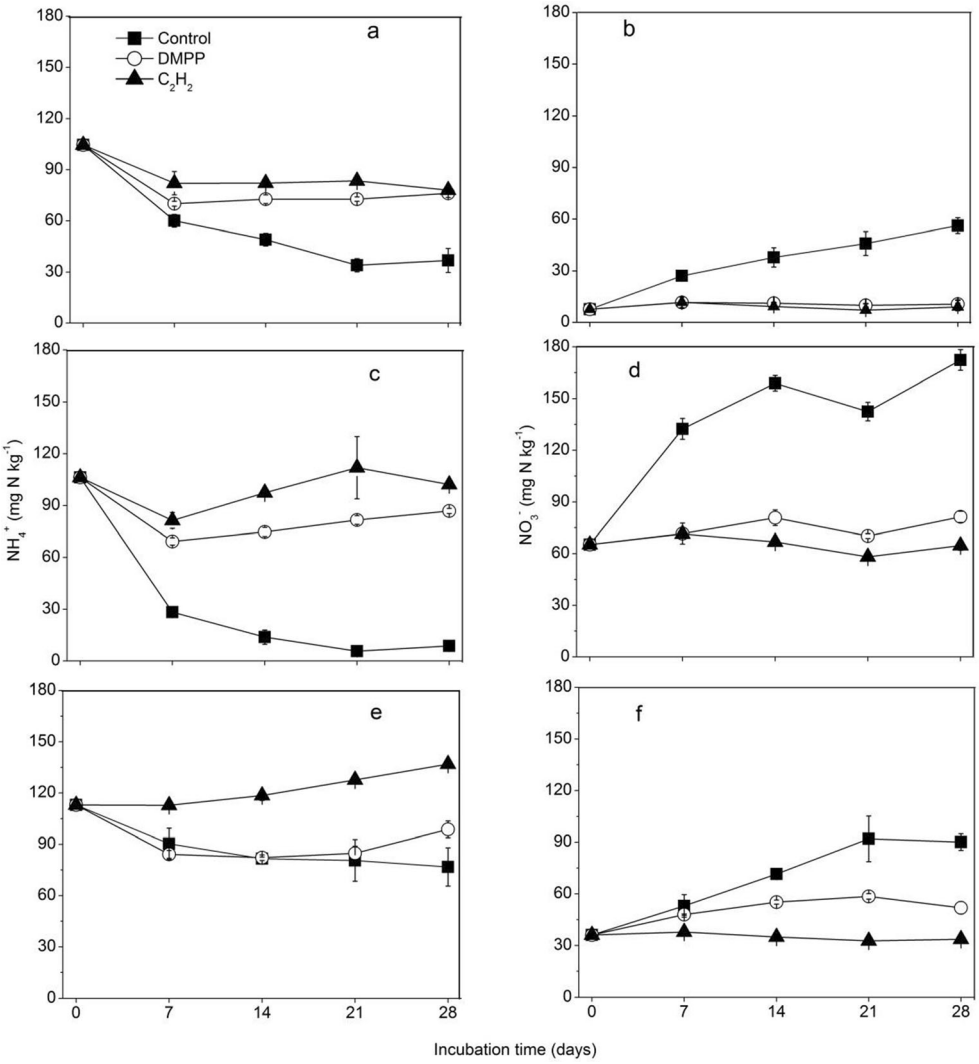

Fig. 1 Ammonium and nitrate concentrations in a neutral soil (**a**, **b**), alkaline soil (**c**, **d**), and acid soil (**e**, **f**) incubated at 25 °C and 60 % WFPS. *Error bars* indicate standard errors of three replicates

for 28 days, the AOA *amo*A gene copy numbers in the control soils ranged from 5.2 to 39×10^8 g^{-1} dry soil. The AOA population in the acid soil was greater (39×10^8 g^{-1} dry soil) than that that in the alkaline soil (18×10^8 $g^{-1}$of dry soil) and neutral soil (5.2×10^8 $g^{-1}$of dry soil) ($P<0.05$) (Fig. 2). The AOB population was smaller than that of the AOA in all three soils (Fig. 2). There were more AOB *amoA* gene copy numbers in the alkaline soil (3.7×10^8 g^{-1} dry soil) than in the acid soil (2×10^8 g^{-1} dry soil) and neutral soil (0.4×10^8 g^{-1}dry soil). In the acid control soil, the ratio of AOA to AOB was highest at 19.5 followed by neutral control soil at 13 and alkaline control soil at 4.9.

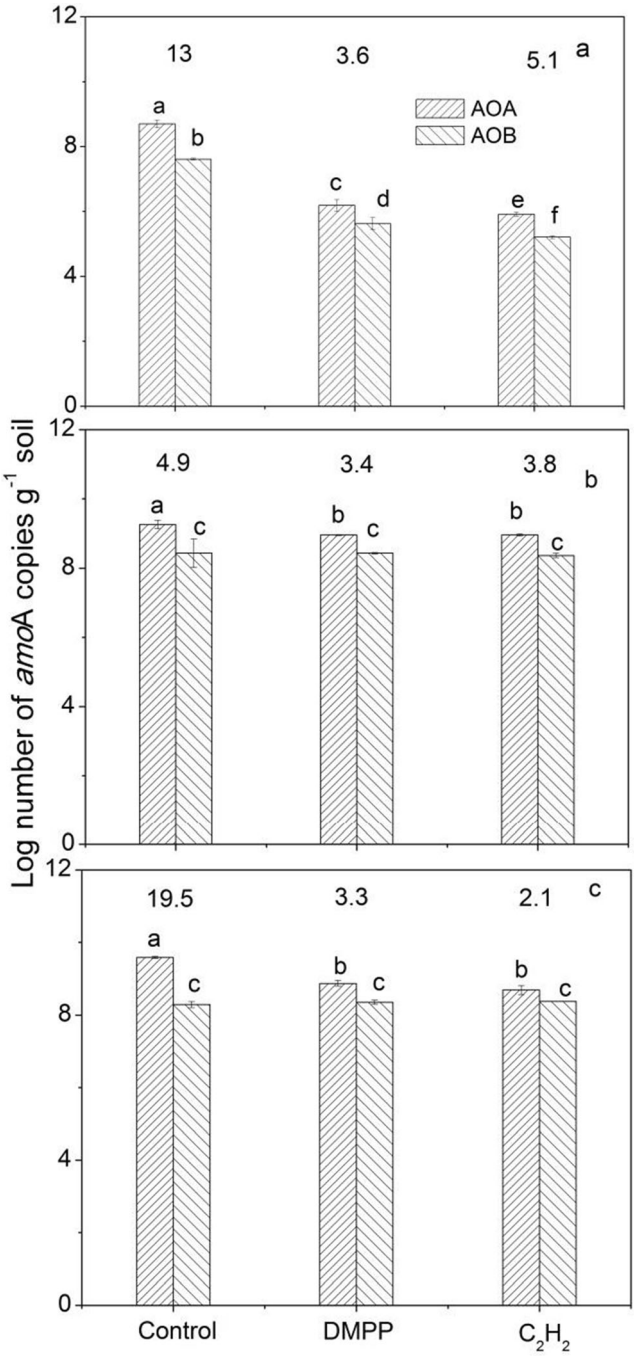

Fig. 2 AOA and AOB amoA gene copy numbers at day 28 in neutral soil (**a**), alkaline soil (**b**), and acid soil (**c**). The *number above the bar* indicates the ratio of AOA to AOB. *Error bars* indicate standard errors of three replicates

The inhibition of AOA and AOB populations by C_2H_2 and DMPP varied with soil type. The addition of DMPP and C_2H_2 significantly reduced the AOA population in all soils, but the effect on the neutral soil was much greater than that on the acid and alkaline soils. Acetylene significantly inhibited AOB population in the neutral soil ($P<0.05$) and slightly blocked AOB growth in alkaline soil (Fig. 2). However, the AOB population in the acid soil was not affected by the application of the nitrification inhibitors (Fig. 2). The reduction in the ratio of AOA to AOB was greatest (from 19.5 to 2.1) with C_2H_2 in the acid soil and least (from 4.9 to 3.4) with DMPP in the alkaline soil.

4 Discussion

Our study indicated that application of DMPP and C_2H_2 reduced nitrification for all three soil types, but to different extents. Acetylene was much more effective than DMPP in inhibiting nitrification in all three soil types. DMPP performed better in the neutral soil than the other two soils. There was no effect on the rate of NH_4^+ immobilization after inhibitor addition (Fig. 1), although the inhibitors blocked transformation of NH_4^+ to NO_3^- supporting previous reports by Chalk (1990) and Crawford and Chalk (1993). In our study, soil pH might be a key factor influencing the effectiveness of DMPP and C_2H_2 on nitrification; however, their effectiveness might also have been affected by other soil properties such as soil texture (Barth et al. 2001). A multiple regression including more soil physico-chemical properties is therefore necessary. Although C_2H_2 completely inhibited nitrification, we still measured N_2O emission (data not reported) which we hypothesize must have originated from denitrification of the original NO_3^- or heterotrophic nitrification.

In the neutral soil, the two inhibitors significantly suppressed both AOA and AOB and decreased NO_3^- content (by 57–85 %). In contrast, the effect of these inhibitors on nitrification differed in the other two soil types. This difference may be attributed to soil pH or other properties such as organic matter content (Table 1). It has also been shown that the relative abundance of these organisms is affected by NH_4^+ concentration (Di et al. 2009; Martens-Habbena et al. 2009; Verhamme et al. 2011), pH (Nicol et al. 2008; Hu et al. 2013), soil type (Girvan et al. 2003; Suzuki et al. 2009), and nutrient content (Di et al. 2009; Erguder et al. 2009). In the alkaline soil, both DMPP and C_2H_2 halved the AOA abundance and decreased AOB gene copy numbers by 27 and 35 %, respectively. Our results differ from the study conducted by

Kleineidam et al. (2011) who observed that DMPP only reduced AOB but not AOA abundance in an acid soil 8 weeks after fertilizer application. He et al. (2007) found that AOB and AOA population sizes were the lowest in the N treatment with the lowest soil pH and that soil pH was significantly correlated with the abundance of AOB and AOA. However, this study found no clear relationship between soil pH and AOB abundance. Over the incubation time, we measured soil pH at each sample time and found there was no obvious difference between day 0 (Table 1) and day 28.

In the acid soil, both inhibitors inhibited only AOA but not AOB indicating that nitrification in acid soil was mainly associated with the dynamics of the AOA populations rather than with that of AOB. DMPP was less effective in lowering AOA, indicating AOA may be more sensitive to C_2H_2 than DMPP in acid soil. Offre et al. (2009) demonstrated a similar result in which AOA was inhibited by C_2H_2 in acid soil. It has been reported that soil environmental factors can determine the ecological niche of AOA and AOB (Girvan et al. 2003; Suzuki et al. 2009). Compared to AOB, AOA is better adapted to low NH_4 availability (Martens-Habbena et al. 2009) and low pH (Nicol et al. 2008). Studies have shown that AOA were more abundant in unfertilized agricultural soils with low NH_4^+ content (Offre et al. 2009), while more AOB were found in fertilized soils or grazed pastures receiving additional N from animal excrement (Di et al. 2009; Jia and Conrad 2009). Soil heterogeneity and physiological differences between AOA and AOB may explain why they can coexist in the same soil despite competing for NH_4^+. AOA and AOB have different niches and may therefore respond differently to inhibitors having specific targets.

5 Conclusions

Acetylene and DMPP effectively inhibited nitrification in all three soil types. C_2H_2 provided better inhibition than DMPP in our study, and DMPP was most effective when applied to neutral soil than alkaline and acid soils. AOA were significantly inhibited by C_2H_2 and DMPP in all soils; however, AOB were significantly inhibited by both inhibitors in neutral soil, slightly inhibited in alkaline soil, and was not affected in acid soil. Therefore, we propose that AOA might play a more important role than AOB in autotrophic nitrification in alkaline and acid soils in Australia. DMPP and C_2H_2 were effective in inhibiting both AOA and AOB in neutral soil.

Acknowledgments The authors would like to acknowledge the financial support by Incitec Pivot Limited and the Australian Government Department of Agriculture through the Grains Research and Development Corporation. Dr. John Freney, Dr. Shu Kee (Raymond) Lam, and Dr. Hangwei Hu provided their assistance during manscript preparation.

References

Barth G, Von Tucher S, Schmidhalter U (2001) Influence of soil parameters on the effect of 3, 4-dimethylpyrazole-phosphate as a nitrification inhibitor. Biol Fertil Soils 34:98–102

Barth G, Von Tucher S, Schmidhalter U (2008) Effectiveness of 3, 4-dimethylpyrazole phosphate as nitriflcation inhibitor in soil as influenced by inhibitor concentration, application form, and soil matric potential. Pedosphere 18:378–385

Bremner J, Blackmer AM (1978) Nitrous oxide: emission from soils during nitrification of fertilizer nitrogen. Science 199:295–296

Chalk PM (1990) Effect of a nitrification inhibitor on immobilization and mineralization of soil and fertilizer nitrogen. Vol-22

Chen D, Suter HC, Islam A, Edis R (2010) Influence of nitrification inhibitors on nitrification and nitrous oxide (N_2O) emission from a clay loam soil fertilized with urea. Soil Biol Biochem 42:660–664

Crawford D, Chalk P (1993) Sources of N uptake by wheat (Triticum aestivum L.) and N transformations in soil treated with a nitrification inhibitor (nitrapyrin). PLSO 149:59–72

De Boer W, Kowalchuk G (2001) Nitrification in acid soils: microorganisms and mechanisms. Soil Biol Biochem 33:853–866

Di HJ, Cameron KC, Shen JP (2009) Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. Nat Geosci 2:621–624

Di HJ, Cameron KC, Shen JP, Winefoeld CS, O'callaghan M, Bowatte S, He JZ (2010) Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. FEMS Microbiol Ecol 72:386–394

Erguder TH, Boon N, Wittebolle L, Marzorati M, Verstraete W (2009) Environmental factors shaping the ecological niches of ammonia oxidizing archaea. FEMS Microbiol Rev 33:855–869

Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. Proc Natl Acad Sci U S A 103:626–631

Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc Natl Acad Sci U S A 102:14683–14688

Girvan MS, Bullimore J, Pretty JN, Osborn AM, Ball AS (2003) Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Appl Environ Microbiol 69:1800–1809

Gubry-Rangin C, Nicol GW, Prosser JI (2010) Archaea rather than bacteria control nitrification in two agricultural acidic soils. FEMS Microbiol Ecol 74:566–574

He JZ, Shen JP, Zhang LM, Zhu YG, Zheng YM, Xu MG, Di HJ (2007) Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environ Microbiol 9:2364–2374

Hu HW, Zhang LM, Dai Y, Di HJ, He JZ (2013) pH-dependent distribution of soil ammonia oxidizers across a large geographical scale as revealed by high-throughput pyrosequencing. J Soils Sediments 13:1439–1449

Hynes RK, Knowles R (1982) Inhibition by acetylene of ammonia oxidation in Nitrosomonas europaea. FEMS Microbiol Lett 4:319–321

Jia Z, Conrad R (2009) Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environ Microbiol 11:1658–1671

Joye SB, Hollibaugh JT (1995) Influence of sulfide inhibition of nitrification on nitrogen regeneration in sediments. Science 270:623–625

Juliette LY, Hyman MR, Arp DJ (1993) Mechanism-based inactivation of ammonia monooxgenase in Nitrosomonas europaea by Allysulfide. Environ Microbiol 59:3728–3735

Kemmitt SJ, Wright DG, Keith WT, Jones DL (2006) pH regulation of carbon and nitrogen dynamics in two agricultural soils. Soil Biol Biochem 38:898–911

Kleineidam K, Kosmrlj K, Kublik S, Palmer I, Pfab H, Ruser P, Fiedler S, Schloter M (2011) Influence of the nitrification inhibitor 3, 4-dimethylpyrazole phosphate (DMPP) on ammonia-oxidizing bacteria and archaea in rhizosphere and bulk soil. Chemosphere 84:182–186

Martens-Habbena W, Berube PM, Urakawa H, Jose R, Stahl DA (2009) Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature 461:976–979

Nicol GW, Leininger S, Schleper C, Prosser JI (2008) The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environ Microbiol 10: 2966–2978

Offre P, Prosser JI, Nicol GW (2009) Growth of ammonia-oxidizing archaea in soil microcosms is inhibited by acetylene. FEMS Microbiol Ecol 70:99–108

Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Environ Microbiol 63:4704–4712

Shen JP, Zhang LM, Zhu YG, Zhang JB, He JZ (2008) Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. Environ Microbiol 10:1601–1611

Shi M, Zhang MT, Shen F, Liang DL, Dang HL (2011) Effects of nitrification inhibitors on nitrification inhibition and nitrite accumulation in calcareous soil. Sci Agric Sin 3:010

Šimek M, Cooper J (2002) The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. Eur J Soil Sci 53:345–354

Suzuki C, Nagaoka K, Shimada A, Takenaka M (2009) Bacterial communities are more dependent on soil type than fertilizer type, but the reverse is true for fungal communities. Soil Sci Plant Nutr 55:80–90

Verhamme DT, Prosser JI, Nicol GW (2011) Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. ISME J 5:1067–1071

Zerulla W, Barth T, Dressel J, Erhardt K, Klaus H, Pasda G, Radle M, Wissemeier A (2001) 3, 4-Dimethylpyrazole phosphate (DMPP)—a new nitrification inhibitor for agriculture and horticulture. Biol Fertil Soils 34:79–84

Zhang LM, Hu HW, Shen JP, He JZ (2011) Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. ISME J 6:1032–1045