The response of nitrifier, N-fixer and denitrifier gene copy numbers to the nitrification inhibitor 3,4-dimethylpyrazole phosphate

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ABSTRACT

A laboratory incubation experiment was conducted to examine the effects of 3,4-dimethylpyrazole phosphate (DMPP) on the transformation of urea-N and associated microbial communities in a low-fertility brown soil. The soil was supplied with urea at 180 kg N/ha and DMPP at 0, 1.8 or 18 kg/ha, while soil that was not supplied with urea and DMPP was treated as the control. Soil mineral nitrogen (NH_4^+-N and NO_3^--N) was measured at regular intervals, and temporal variations in the population sizes of nitrogen-cycling microbes were determined using real-time PCR. Compared to the urea-alone treatment, the additional application of DMPP maintained significantly higher ($P < 0.05$) NH_4^+-N concentrations and lower NO_3^--N ($P < 0.05$) concentrations in the soil and decreased the population sizes of ammonia oxidizing and denitrifying microbes but promoted the growth of nitrogen-fixing bacteria. The results suggest that the application of DMPP is beneficial in improving the availability of fertilizer-N for plant uptake. This study is the first comprehensive investigation to examine the effects of DMPP on the functional microorganisms that are important in nitrogen-cycling processes.

Keywords: ammonia oxidizing bacteria; ammonia oxidizing archaea; nitrogen cycling; RT-PCR

Through hydrolysis, urea applied to soil becomes ammonium (NH_4^+), which subsequently enters the process of soil nitrogen cycling. However, the relationship between the nitrogen-cycling soil microbial community and ecosystem processes is relatively poorly understood (Schimel et al. 2005). For example, nitrogen cycling processes require a diverse bacterial community possessing several functional genes responsible for nitrogen transformations, including fixation (*nifH*), ammonia oxidation (*amoA*), nitrite reduction (*nirS* and *nirK*) and others (Poly et al. 2001, Francis et al. 2005, Wallenstein et al. 2006). Therefore, a community approach is warranted because the study of a single species is insufficient for understanding soil nitrogen cycling processes. One approach to investigating the composition of functional guilds is to analyze the abundance of functional genes from whole communities in genomic DNA extracted from soils and environmental samples (Kolb et al. 2003). Recent developments in molecular-based approaches allowed researchers to survey the abundance and diversity of the nitrogen metabolism genes from environmental samples (Rosch et al. 2002, He et al. 2007, Shen et al. 2008). While most studies to date have focused on a single functional gene, analysis of a more complete suite of genes would enable us to explain better the role of community structure in controlling nitrogen cycling.

3,4-dimethylpyrazole phosphate (DMPP) is a new nitrification inhibitor that specifically influences the process of ammonia oxidation by depressing the

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activity of *Nitrosomas* bacteria in soils (Zerulla et al. 2001). The advantages of DMPP include lower application rates, greater efficiency and lower water solubility compared with dicyandiamide (DCD), and the application of DMPP-containing fertilizers was shown to improve yields (Weiske et al. 2001, Zerulla et al. 2001). The objectives of this laboratory study, as obtained using a 20-year non-fertilized aquic brown soil, were to examine the effects of applying DMPP on (1) the abundance of ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA), nitrogen-fixing bacteria and denitrifying bacteria, and (2) the dynamic changes in soil NH_4^+ and NO_3^- concentrations after urea application, as well as the relationships between bacterial or archaeral *amoA* abundance and soil NO_3^- concentration.

MATERIAL AND METHODS

Soil preparation. An aquic brown soil (0–20 cm) that had not been fertilized since 1998 was collected from a soybean-corn-corn rotated field at the Shenyang Ecological Experimental Station (41°32'N, 123°23'E) at the Chinese Academy of Sciences (SEES-CAS). The soil was sieved through a 2-mm sieve and maintained at 4°C and field moisture until use. The basic properties of the soil were total nitrogen, 0.93 g/kg; total carbon, 10.7 g/kg; available P 2.21 mg/kg; soil pH (water) 6.31 (Yu et al. 2010).

Experimental design. The soil received four treatments during the laboratory incubation experiment (Table 1), and three replicates were prepared for each treatment. Wet soil (wet weight 238 g, equivalent to air-dried weight of approximately 200 g) was blended with appropriate quantities of urea and/or DMPP (purity ≥ 97%), thoroughly mixed and placed into a column (12 cm in diameter and 10 cm in height). Next, the column was incubated at 25 ± 1°C, in darkness for 90 days. Deionized water was regularly added to maintain the water holding capacity (WHC) of the soil at 60%.

Soil sampling and analysis. During the incubation period, soil sub-samples were taken from each treatment at specific time intervals (1, 3, 7, 14, 28, 49 and 90 days). All of the samples were divided into two parts; one part was stored at 4°C for the determination of moisture content and mineral N (NH_4^+-N and NO_3^--N), while the other was stored at –70°C for DNA extraction.

Soil NH_4^+ and NO_3^- concentrations were measured according to the Keeney's (1982) method. Total genomic DNA was extracted using the Fast DNA SPIN Kit for Soil (MP Biomedicals, Solon, USA) according to the manufacturer's instructions. Numbers of copies of AOB *amoA*, AOA *amoA*, *nifH* and *nirS* genes were determined by real-time PCR using a SYBR® Premix Ex Taq™ II (Takara, Japan) on an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, USA). The 20 µL of reaction solution contained 10 µL of SYBR® Premix EX Taq II, 0.4 µmol of each primer (Table 2), 0.4 µL of ROX Reference Dye II and 20 ng of template DNA. Thermal cycling for the real-time PCR consisted of 95°C for 1 min followed by 30~40 cycles at 95°C for 10 s, annealing temperature for 30 s (Table 2) and 72°C for 1 min. To prepare the standard curve for the real-time PCR assay, *amoA* (AOB and AOA), *nifH* and *nirS* genes were amplified using the primers listed in Table 2. The PCR products were purified with a Gel Extraction B system (BioDev-Tech, Beijing, China), ligated into pMD19-T vectors (Takara, Japan) and subsequently transformed into competent *Escherichia coli* DH5α cells following the manufacturer's instructions. Positive clones were selected for plasmid DNA extraction with an AxyPrep™ Plasmid Miniprep Kit (Axygen Bioscience, USA). The plasmid DNA concentration was determined on a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). For each gene, a high amplification efficiency of 95–100% was obtained, the R^2 values were > 0.992 and no signal was observed in the negative controls.

Statistical analysis. All data were calculated based on oven-dried (105°C) soil weight. The experimental results were statistically analyzed using a one-way analysis of variance (ANOVA) followed by an SNK test using SPSS 16.0.

Table 1. Experimental design

Treatment	Urea (kg N/ha)	DMPP (kg/ha)
Control	0	0
Treatment 1 – U	180	0
Treatment 2 – UD	180	1.8
Treatment 3 – 10UD	180	18

U – soils supplied with urea; UD – soils supplied with urea + 1% 3,4-dimethylpyrazole phosphate (DMPP); 10UD – soils supplied with urea + 10% DMPP

Table 2. RT-PCR primers used for amplification of functional target genes

Primer	Target gene	Sequence (5'–3')	Annealing temperature (°C)	Reference
Arch-*amoAF*	AOA *amoA*	STAATGGTCTGGCTTAGACG	55	Francis et al. (2005)
Arch-*amoR*	AOA *amoA*	GCGGCCATCCATCTGTATGT	55	
amoA-1F	AOB *amoA*	GGGGTTTCTACTGGTGGT	58	Rotthauwe et al. (1997)
amoA-2R	AOB *amoA*	CCCCTCKGSAAAGCCTTCTTC	58	
nifH-F	*nifH*	AAAGGYGGWATCGGYAARTCCACCAC	53	Rosch et al. (2002)
nifH-R	*nifH*	TTGTTSGCSGCRTACATSGCCATCAT	53	
nirS-cd3aF	*nirS*	AACGYSAAGGARACSGG	58	Throbäck et al. (2004)
nirS-R3cd	*nirS*	GASTTCGGRTGSGTCTTSAYGAA	58	

AOB – ammonia oxidizing bacteria; AOA – amomonia oxidizing archaea

RESULTS AND CONCLUSIONS

A low-fertility soil should be the best soil for quantifying the soil transformation of urea-N. In this experiment, the application of DMPP to soil, however, led to the effective inhibition of nitrification, resulting in the long-term maintenance of soil NH_4^+-N and a low production of soil NO_3^--N (Zerulla et al. 2001). NH_4^+ concentrations were maintained at higher levels in the urea + DMPP treatments than in the urea-alone treatments during the first 49 days ($P < 0.05$). The results were in substantial agreement with those of Zerulla et al. (2001), who described an effect of DMPP on soil NH_4^+-N content lasting for approximately 40 days. Moreover, we found a strong decrease in NH_4^+ concentrations in all of the treatments from day 1 to day 3, but soil NO_3^- concentrations increased only slightly during this period. One reason for these results might be not only the process of nitrification but also the assimilation of soil NH_4^+. Another reason might be that the rapid hydrolysis of urea caused an instantaneously higher pH, which led to gaseous NH_3 losses. Barth et al. (2001) reported that DMPP was able to retard nitrification over a period of 28 to 70 days, depending on the climatic conditions and site characteristics. In cases without nitrate leaching and crop uptake, soil NO_3^- was maintained at lower levels in the urea + DMPP treatments than in the urea-alone treatments for nearly 90 days.

There were significant increases in the population sizes of AOB and AOA in the soil supplied only with urea, and as expected, these populations did not increase in the soil supplied with urea and DMPP. As can be observed in Figure 2a, a significant and continuous increase in AOA abundance was observed in the U treatment over the

Table 3. The copy numbers of *nifH* and *nirS* genes during incubation of microcosms

Treatment	*nifH* gene			*nirS* gene		
	copy numbers/g dry mass ($\times 10^7$)					
	7 days	14 days	49 days	7 days	14 days	49 days
CK	1.44 (0.13)^b	5.86 (0.82)^a	3.17 (0.34)^b	5.54 (0.53)^a	11.16 (0.31)^a	10.32 (0.56)^a
U	1.29 (0.17)^b	2.88 (0.65)^b	4.37 (0.08)^a	4.81 (0.56)^{ab}	9.69 (0.84)^a	10.89 (1.05)^a
UD	2.23 (0.12)^a	5.63 (0.41)^a	1.01 (0.31)^c	3.64 (0.35)^{cb}	9.52 (0.19)^a	4.96 (0.01)^b
10UD	1.41 (0.26)^b	4.89 (0.16)^a	3.34 (0.07)^b	3.13 (0.31)^c	7.55 (2.40)^a	3.98 (0.22)^b

CK – control; U – soils supplied with urea; UD – soils supplied with urea + 1% 3,4-dimethylpyrazole phosphate (DMPP); 10UD – soils supplied with urea + 10% DMPP. Values are means + standard error ($n = 3$). Values within the same column followed by the different letters are significantly different ($P < 0.05$)

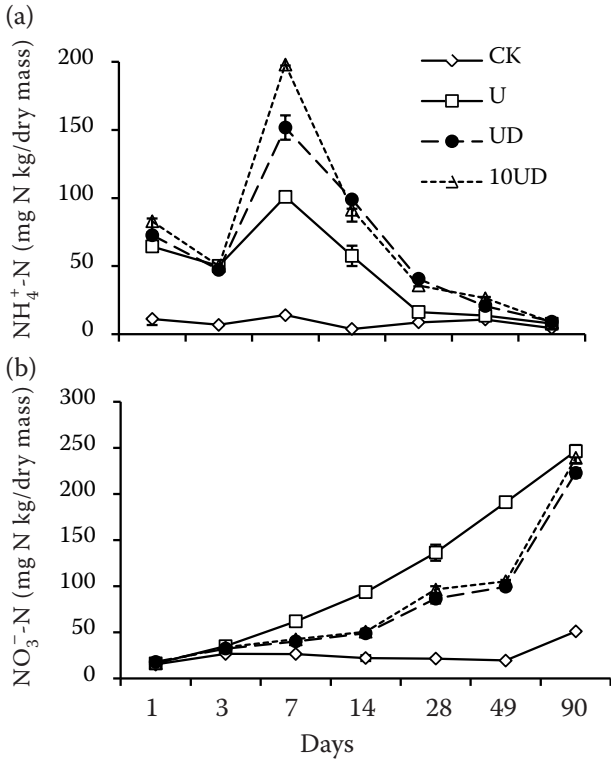

Figure 1. Soil ammonia (a) and nitrate (b) concentrations during incubation of microcosms. CK – control; U – soils supplied with urea; UD – soils supplied with urea + 1% 3,4-dimethylpyrazole phosphate (DMPP); 10UD – soils supplied with urea + 10% DMPP. Error bars indicate standard deviations

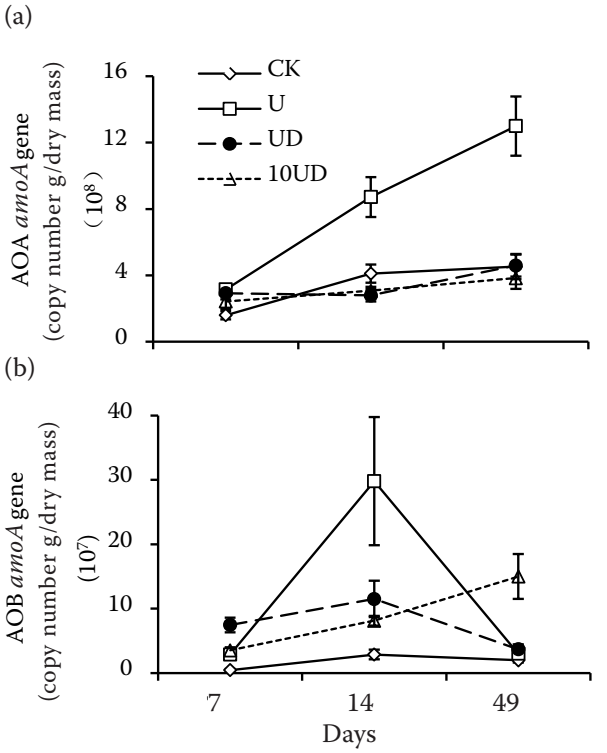

Figure 2. The copy numbers of archaeal (a) and bacterial (b) *amoA* genes during incubation of microcosms. CK – control; U – soils supplied with urea; UD – soils supplied with urea + 1% 3,4-dimethylpyrazole phosphate (DMPP); 10UD – soils supplied with urea + 10% DMPP. Error bars indicate standard deviations. AOB – ammonia oxidizing bacteria; AOA – amomonia oxidizing archaea

entire 49 day period. The abundance of AOA in the U treatment was approximately 2–3.4 times higher than that in the UD and 10UD treatments at days 14 and 49 ($P < 0.05$), respectively. Figure 2b demonstrates that a rapid increase in the abundance of AOB was found in the U-treated soil and that the maximum was 3.0×10^8 copies/g dry soil at day 14, 2.6 to 3.7 times higher than the AOB values in the UD, 10UD and control treatments. This result indicated that DMPP effectively inhibited the growth of AOA and AOB (Di et al. 2011), resulting in a lower accumulation of NO_3^--N in the soil (Figure 1). Meanwhile, a significant relationship existed between soil NO_3^- concentrations and the population of AOA (Figure 3). AOA was more effectively concerned with the inhibition activity of DMPP for a longer period and was the primary driver of nitrification, rather than AOB (Valentine et al. 2007, Erguder et al. 2009, Di et al. 2010). The contribution of AOA and AOB to soil nitrification displayed certain selectivity, depending on such factors as soil nutrients, pH and soil management (Erguder et al. 2009). Previous studies indicated that AOA grew preferentially in soils with lower nutrient availability and a low pH (Leininger et al. 2006, Erguder et al. 2009, Di et al. 2010). In the present research, we also confirmed that AOA but not AOB dominated in this low-fertility soil.

Nitrification inhibitors (NIs) should be bacteriostatic, rather than bactericidal, which restrains certain microorganisms in soils, such as *Nitrosomonas* sp. In fact, NIs had no negative influence on the activity of other microbes in soils (Pasda et al. 2001). In the present study, the *nifH* gene copy numbers in the urea treatments were always lower during the initial period of incubation than those in the CK and DMPP treatment soils, although they increased linearly from 1.3×10^7 to 4.4×10^7. Also, DMPP increased the population size for nitrogen-fixing bacteria by as much as 1.8–2.2-fold by day 14 compared with urea treatments ($P < 0.05$). Several previous studies indicated that nitrogen-fixing bacteria were notably sensitive to soil pH, that either lower or higher pH in soil

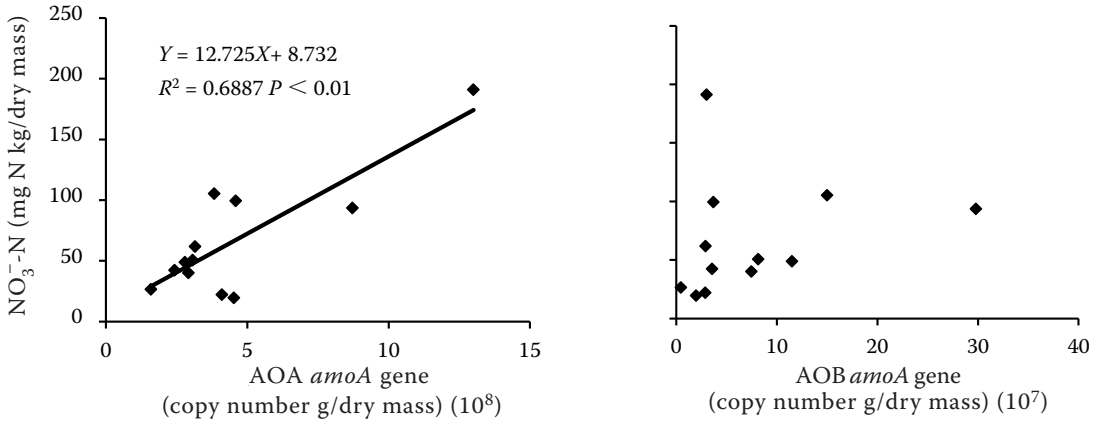

Figure 3. Relationships between nitrate concentration and archaeal or bacterial *amoA* gene abundance. AOB – ammonia oxidizing bacteria; AOA – amomonia oxidizing archaea

would result in the strong inhibition of the growth of nitrogen-fixing bacteria and that nitrogen-fixing bacteria grew preferentially in soils with higher pH environments (Nelson et al. 2006). In our study, the soil we used had a pH of 6.3, and the number of *nifH* genes was higher than in the UD and 10UD treatments. A possible reason for this finding might be that the higher and more stable pH conditions caused by urea + DMPP (O'Callaghan et al. 2010) resulted in the rapid proliferation of nitrogen-fixing bacteria. In addition, the lowest values were observed in the urea treatments, in which the soil pH was decreased because of the rapid conversion of urea-N. We could therefore infer that the pH was the key factor that influenced the growth of nitrogen-fixing bacteria.

In our study, DMPP resulted in reductions in *nirS* gene abundance compared with the urea-only treatment. At day 49, the number of copies of *nirS* genes in the UD and 10UD treatments was approximately 2.2 to 2.9 times lower than that observed in the control and U treatments ($P < 0.05$). From an enzymatic point of view, Li et al. (2008) demonstrated that DMPP reduced both nitrate reductase (*NaR*) activity and nitrite reductase (*NiR*) activity. However, in Müller et al. (2002), DMPP did not affect *NaR* or N_2O reductase capacity in a silty clay soil, even at concentrations 14 times higher than the recommended concentration. Several studies (Rich et al. 2004, Boyle et al. 2006) indicated that there were no obvious relationships between the activities of denitrifying enzymes and the abundance of denitrifiers in certain environments. Also, to date, there was no published evidence for the effects of NIs on denitrifiers. Soil environments such as nitrate content and water availability are two important factors affecting denitrifying activities in soil (Rich et al. 2004). Tindaon et al. (2011) indicated that denitrification potential was affected by the application of NIs at 10–50 times the recommended dose in various agricultural soils, owing to the inhibition of NO_3^- production. During the experimental incubation period, we regularly adjusted soil water content to maintain the same water conditions among the various treatments, and we adopted destructive sampling for obtaining homogeneous soil samples. Therefore, instead of soil water, the lower NO_3^- content owing to the application of DMPP became the important limiting factor affecting the size of populations of denitrifying bacteria among the different fertility treatments (Wallenstein et al. 2006) in the study.

REFERENCES

Barth G., von Tucher S., Schmidthalter U. (2001): Influence of soil parameters on the effect of 3,4-dimethylpyrazole-phosphate as nitrification inhibitor. Biology and Fertility of Soils, *34*: 98–102.

Boyle A.S., Rich J.J., Bottomley P.J., Cromack K. Jr., Myrold D.D. (2006): Reciprocal transfer effects on denitrifying community composition and activity at forest and meadow sites in the Cascade Mountains of Oregon. Soil Biology and Biochemistry, *38*: 870–878.

Braker G., Fesefeldt A., Witzel K.P. (1998): Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. Applied and Environmental Microbiology, *64*: 3769–3775.

Di H.J., Cameron K.C., Shen J.P., Winefield C.S., O'Callaghan M., Bowatte S., He J.Z. (2010): Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. FEMS Microbiology Ecology, *72*: 386–394.

Di H.J., Cameron K.C. (2011): Inhibition of ammonium oxidation by a liquid formulation of 3,4-dimethylpyrazole phosphate (DMPP) compared with a dicyandiamide (DCD) solution in six new Zealand grazed grassland soils. Journal of Soils and Sediments, *11*: 1032–1039.

Erguder T.H., Boon N., Wittebolle L., Marzorati M., Verstraete W. (2009): Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. FEMS Microbiology Reviews, *33*: 855–869.

Francis C.A., Roberts K.J., Beman J.M., Santoro A.E., Oakley B.B. (2005): Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proceedings of the National Academy of Sciences of the United States of America, *102*: 14683–14688.

Kolb S., Knief C., Stubner S., Conrad R. (2003): Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time pcr assays. Applied and Environmental Microbiology, *69*: 2423–2429.

Keeney D.R., Nelson D.W. (1982): Nitrogen-inorganic forms. In: Page A.L., Miller R.H., Keeney D.R. (eds.): Methods of Soil Analysis. Soil Science Society of America. Madison, 643–693.

Leininger S., Urich T., Schloter M., Schwark L., Qi J., Nicol G.W., Prosser J.I., Schuster S.C., Schleper C. (2006): Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature, *442*: 806–809.

Li H., Liang X.Q., Chen Y.X., Lian Y.F., Tian G.M., Ni W.Z. (2008): Effect of nitrification inhibitor DMPP on nitrogen leaching, nitrifying organisms, and enzyme activities in a rice-oilseed rape cropping system. Journal of Environmental Sciences, *20*: 149–155.

Müller C., Stevens R.J., Laughlin R.J., Azam F., Ottow J.C.G. (2002): The nitrification inhibitor DMPP had no effect on denitrifying enzyme activity. Soil Biology and Biochemistry, *34*: 1825–1827.

Nelson D.R., Mele P.M. (2006): The impact of crop residue amendments and lime on microbial community structure and nitrogen-fixing bacteria in the wheat rhizosphere. Soil Research, *44*: 319–329.

O'Callaghan M., Gerard E.M., Carter P.E., Lardner R., Sarathchandra U., Burch G., Ghani A., Bell N. (2010): Effect of the nitrification inhibitor dicyandiamide (DCD) on microbial communities in a pasture soil amended with bovine urine. Soil Biology and Biochemistry, *42*: 1425–1436.

Pasda G., Hähndel R., Zerulla W. (2001): Effect of fertilizers with the new nitrification inhibitor DMPP (3,4-dimethylpyrazole phosphate) on yield and quality of agricultural and horticultural crops. Biology and Fertility of Soils, *34*: 85–97.

Rich J.J., Myrold D.D. (2004): Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. Soil Biology and Biochemistry, *36*: 1431–1441.

Rösch C., Mergel A., Bothe H. (2002): Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Applied and Environmental Microbiology, *68*: 3818–3829.

Schimel J., Bennett J., Fierer N. (2005): Microbial community composition and soil N cycling: Is there really a connection. In: Bardgett R.D., Hopkins D.W., Usher M.B. (eds.): Soil Biodiversity and Ecosystem Function. Cambridge University Press, Cambridge, 171–188.

Shen J.P., Zhang L.M., Zhu Y.G., Zhang J.B., He J.Z. (2008): Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. Environmental Microbiology, *10*: 1601–1611.

Tindaon F., Benckiser G., Ottow J.C.G. (2011): Side effects of nitrification inhibitors on non target microbial processes in soils. Journal of Tropical Soils, *16*: 7–16.

Valentine D.L. (2007): Adaptations to energy stress dictate the ecology and evolution of the Archaea. Nature Reviews Microbiology, *5*: 316–323.

Watkins N.L. (2007): The Ability of Nitrification Inhibitors to Decrease Denitrification Rates in Dairy Farm Soils. The University of Waikato, Hamilton.

Wallenstein M.D., Myrold D.D., Firestone M., Voytek M. (2006): Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. Ecological Applications, *16*: 2143–2152.

Weiske A., Benckiser G., Herbert T., Ottow J.C.G. (2001): Influence of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) in comparison to dicyandiamide (DCD) on nitrous oxide emissions, carbon dioxide fluxes and methane oxidation during 3 years of repeated applications in field experiments. Biology and Fertility of Soil, *34*: 109–117.

Yu W.T., Xu Y.G., Bi M.L., Ma Q., Zhu H. (2010): Activity and composition of ammonia-oxidizing bacteria in an aquic brown soil as influenced by land use and fertilization. Pedosphere, *20*: 789–798.

Zerulla W., Barth T., Dressel J., Erhardt K., von Locquenghien K.H., Pasda G., Rädle M., Wissemeier A.H. (2001): 3,4-Dimethylpyrazole phosphate (DMPP) – a new nitrification inhibitor for agriculture and horticulture. Biology and Fertility of Soils, *34*: 79–84.

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