Does long-term fertilization treatment affect the response of soil ammonia-oxidizing bacterial communities to Zn contamination?

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Abstract Experiments were conducted to examine the effects of long-term fertilization and acute Zn toxicity on the size, nitrification activity and community structure of autotrophic ammonia-oxidizing populations of the β -subgroup of the class *Proteobacteria* in arable soils. Plots under different long-term fertilization regimes were sampled, and then different concentrations of ZnCl_2 were spiked into soil samples for 8 weeks. It was found that long-term fertilization significantly increased nitrification rates and population size, and there was a positive correlation between them. A shift in the composition of AOB was also detected in samples fertilized with mineral N fertilizer (NPK) and organic matter (OM) as compared to unfertilized sample. EC50 values suggested that there was no significant difference in Zn toxicity to nitrification rates among the three fertilization treatments. Long-term fertilization did not improve the resilience of AOB activity to Zn toxicity.

Keywords AOB · Long-term fertilization · Zn toxicity · Bacterial populations · Nitrification

Introduction

Chemolitho-autotrophic ammonia-oxidizing bacteria (AOB) play an important role in the conversion of ammonia to nitrite and are responsible for the rate-limiting step of nitrification, making them a key to the global nitrogen cycle (Kowalchuk and Stephen 2001). Because of the importance of this functional group, understanding their ecology and physiology has become a subject of intensive research in recent years (Francis et al. 2003; Prosser and Embley 2002).

It is generally accepted that fertilization could change the resilience of soil ecosystems through improving soil fertility and physical structure, as well as stimulating the soil microflora and suppressing pathogens through antagonistic effects (Ouedraogo et al. 2001; Ros et al. 2003; Saison et al. 2006). Impacts of nitrogenous fertilizers on AOB ecology in arable soils have been reported in previous studies. Ammonium-based fertilizers increased AOB population size and nitrification activity (Okano et al. 2004), and affect the community (Avrahami et al. 2003; Phillips et al. 2000a). However, information on the fertilization-induced changes in the size and structure of AOB communities occurring concurrently with the activity changes is scarce.

Heavy metal contamination of soils originating from agricultural or industrial activities is one of the major environmental problems in many parts of the world (Salt et al. 1995). Elevated metal concentrations

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in soils can disturb soil microbial processes, including nitrification, which is one of the most sensitive soil microbial processes with regard to heavy metal stress (Broos et al. 2005; Mertens et al. 2006; Smolders et al. 2001). The background Zn concentration in soil with no observed adverse effect on several soil microbial processes (nitrification, glucose decomposition, phosphatase, respiration et al.) ranges from 2.6 to 226 mg/kg (McLaughlin and Smolders 2001). The range of total Zn concentration in contaminated sites, for example zinc contents in soils around the Fuquan in Sichuan Province of China is from 513 to 9145 mg/kg (Mao et al. 2007). The effect of toxicity of Zn in contaminated sites and in short-term spiked soil on nitrification has been well studied (Smolders et al. 2001, 2003, 2004). For example, the total Zn concentration at which the potential nitrification rate was reduced by 50% (=EC50 value) in freshly ZnCl₂ spiked soil was more than 10-fold below the corresponding concentration required to have a similar impact on C-mineralization endpoints (Smolders et al. 2004). Assessing the effects of fertilization-induced resilience of soil and microbial organisms on their response to acute heavy metal toxicity is necessary to properly characterize how fertilization affects soil quality.

In this study, we used *amoA*, the gene encoding the α -subunit of ammonia monooxygenase (Rotthauwe et al. 1997) as a functional marker to assess whether any changes in the population size and community structure of AOB could be induced by long-term fertilization and an 8-week exposure to Zn toxicity. The numbers of AOB were determined using quantitative polymerase chain reaction (qPCR) and related to potential nitrification rate (PNR), which was measured using the chlorate inhibition technique (Kurola et al. 2005). Specifically, the aims of this study were: (1) to determine whether all indigenous species of β -subgroup ammonia oxidizers were equally susceptible to acute toxic metals through observation of changes in the structure of *amoA* clone libraries, and to examine a dose response reaction in

both nitrification rates and reduction in AOB population; and (2) to assess whether the long-term application of fertilizers could protect the AOB from the acute toxicity of Zn contamination, and whether any changes in the β -subgroup ammonia oxidizers community were observed.

Materials and methods

Soil characteristics and sample preparation

The long-term field fertilization experiment was carried out in Fengqiu National Agro-Ecological Experimental Station (Fengqiu County, Henan Province, North China Plain, 35° N, 114° 24' E). The soil is calcareous fluvo-aquic with a sandy loam texture. Seven treatments (four replicates of each) were established in completely randomized blocks under a rotation of winter wheat and summer maize from 1989. The three selected treatments were (1) control (without fertilization); (2) mineral N fertilizer (NPK); (3) half mineral N fertilizer plus half organic matter N (1/2NPK+1/2 OM). The amount of NPK applied each year was 200 kg N/hm² with urea, 135 kg P₂O₅/hm² with super phosphate and 349 kg K/hm² with potassium sulphate. The amount of organic fertilization was 18,000 kg/hm², and contained N, P and K contents equal to NPK treatments. The raw materials for organic compounds were mixture of ground wheat straw, oil cake and cotton cake. In this study, three of these treatments, control, NPK and 1/2(NPK+OM), were chosen for further analysis. The soil consisted of 17% sand, 72% silt and 11% clay. Other properties (Ding et al. 2007) of the soils are summarized in Table 1. Sieved soil (<2 mm) was used for microcosm preparation.

Microcosm preparation

Soil collected from each of the three fertilizer treatments (control, NPK, 1/2(NPK+OM)) was spiked

Table 1 Selected characteristics of the treatments

Treatment	pH (in water)	Organic C (g/kg)	Total N (mg/kg)	NO ₃ ⁻ -N (mg/kg)
Control	8.55 ^b	6.38 ^a	0.397 ^a	5.04 ^a
NPK	8.38 ^{ab}	7.28 ^b	0.573 ^b	10.27 ^b
1/2(NPK+OM)	8.03 ^a	7.96 ^c	0.660 ^c	14.66 ^c

Values within the same column not followed by the same letter differ significantly ($p < 0.05$).

with ZnCl_2 solutions to give final Zn concentrations of 0, 200, 500, 1,000, 1,500 and 2,000 mg/kg dry soil. Thorough mixing work was done to ensure the ZnCl_2 was evenly distributed within the soil samples. A total of 50 g of dry soil was placed in 300 ml plastic jar microcosms in triplicate for each Zn concentration and each harvest time. Microcosms were incubated at $20 \pm 2^\circ\text{C}$ and destructively harvested at 2, 4, 6 and 8 weeks in triplicate for soil analyses (see below). The microcosms were kept in the dark over 8 weeks' incubation, and soil moisture content was kept at 15% of dry weight soil during the period. After sampling, soil pH was measured in CO_2 -free diluted water in a 1:2.5 solid/liquid ratio.

Potential nitrification rate

Potential nitrification rates were measured using the chlorate inhibition method of Kurola et al. (2005). Sodium chlorate was used to inhibit NO_2^- from being oxidized to NO_3^- . In brief, 5 g (fresh weight) of soil was incubated in 20 ml of 1 mM phosphate-buffered saline (PBS) (g/l: NaCl, 8.0; KCl, 0.2; Na_2HPO_4 , 1.44; KH_2PO_4 , 0.24; pH 7.4) containing 1 mM of $(\text{NH}_4)_2\text{SO}_4$ and NaClO_3 (final concentration 50 mg/l) at room temperature in the dark. After moderately shaking for 24 h, 5 ml of 2 M KCl was added to the tubes for extracting NO_2 -N. After centrifugation, the optical density of the supernatant was analyzed for the presence of NO_2 -N at 540 nm using sulphonamide and naphthylethylene diamide as reagents (Kandeler 2002).

DNA extraction from soil

DNA was extracted from 0.5 g (fresh weight) of soil using a Bio 101 FastDNA SPIN Kit (for soil) as described by the manufacturer (Bio 101, Inc., USA). Extracted DNA was dissolved in 50 μl double distilled H_2O . The quantity (relative to DNA size marker) and quality of the extracted DNA were analyzed by electrophoresis on a 1.0% agarose gel and by spectroscopic analysis (Nanodrop).

Real-time PCR

Quantitative PCR of *amoA* was used to estimate the abundance of AOB. The procedures for the real-time PCR followed those described by Horz et al. (2004)

with minor modifications. The quantification was based on the fluorescent dye SYBR-Green I, which binds to double-stranded DNA during PCR amplification. The primers *amoA*-1F* and *amoA*-2R (Rotthauwe et al. 1997) used for amplification of an *amoA* gene fragment were described previously by Stephen et al. (1999). Real-time PCR was performed in 25 μl reaction mixture that consisted of 2 μl (approximately 5 ng/ μl) of template DNA which was diluted 10-fold, 12.5 μl of SYBR *Premix Ex Taq* (TaKaRa), 500 nM of each primer. The template DNA was amplified and monitored using an iQ5 instrument (Bio-rad, USA). MicroAmp optical caps and tubes were used. The PCR protocol was as the following program: 3 min at 94°C , and then 40 cycles consisting of 30 s at 94°C , 30 s at 55°C , and 45 s at 72°C . The temperature at which the fluorescence was read during each cycle was 83°C , a temperature above the melting point of primer dimers (Horz et al. 2004). The protocol for melting curve analysis was from 65 – 98°C (0.2°C per read, 6 s hold). All samples were analyzed in triplicate. The amount of initial template DNA was estimated by determining the threshold cycle (C_T).

An external standard curve to show the relationship between *amoA* copy numbers and C_T values was generated as described by Okano et al. (2004) with minor modification. PCR primers *amoA*-1F* and *amoA*-2R (Stephen et al. 1999; Rotthauwe et al. 1997) were used to amplify the *amoA* gene from the total DNA extracted from soil samples. PCR product was ligated into p-GEM T easy vector (Promega, Corp., Madison, Wis.) and then cloned into *E. coli* JM109 (TaKaRa) in accordance with the manufacturer's instructions. Clones were picked and the *amoA* gene inserts were sequenced. One clone that had the right *amoA* gene insert was chosen as the standard for real time PCR. Plasmid DNA was extracted with Plasmid Kit, and the plasmid concentration was measured with a spectrophotometer (Nanodrop). Since the sequences of the vector and PCR inserts were known, the copy numbers of *amoA* were calculated directly from the concentration of extracted plasmid DNA. There was a strong linear ($R^2=0.996$) inverse relationship between C_T and Log_{10} number of *amoA* copies over seven orders of magnitude. The equation of the curve was $y=-3.41x+37.25$.

PCR efficiency was tested according to the method of Horz et al. (2004). In short, a defined amount of pUC19 DNA (TaKaRa) was mixed with the same

volume of soil DNA that was used for the regular real-time PCR. A fragment was then amplified by using primers M12F and M12R under the PCR conditions described above. Then PCR efficiencies could be determined by subtracting the C_T of the spiked control gene from the C_T of the target gene for each soil sample.

Construction and analysis of *amoA* gene fragment libraries

Clone libraries of control, NPK, 1/2(NPK+OM) and control-1,500 (treated with 1,500 mg Zn/kg), NPK-1,500, 1/2(NPK+OM)-1,500 sample were constructed to compare the community structure. Briefly, after PCR amplification of the *amoA* gene, gel slices of an agarose gel containing the PCR products were excised, and DNA was purified by using Agarose Gel DNA Purification Kit (Ver. 2.0, TaKaRa). Purified *amoA* PCR products were cloned as described above. About 50 representative clones from each library bearing inserts of the correct size (491 bp) were randomly selected for sequencing.

Sequencing and phylogenetic analysis

Representative clones were sent to Sangon Company (Shanghai, China, <http://www.sangon.com/>) for sequencing (ABI PRISM 3730 sequencer). We confirmed that the sequences were from AOBs by searching international sequence database via the blast program in Mega software (Version 3.1, Kumar et al. 2004). The environmental *amoA* sequences generated in this study have been submitted to the GenBank databases under accession numbers EF122009 to EF122073. Clones from control, NPK, 1/2(NPK+OM) and control-1,500, NPK-1,500, 1/2(NPK+OM)-1,500 were labelled with prefix A, B, C, D, E, F in this paper.

Similarity analysis was performed by using Dnadist and Prodist in Phylip (version 3.66) (Felsenstein 1993). For sequence types that exhibited at least 99% amino acid identity to each other, only one representative was considered for construction of trees. Phylogenetic analyses were performed based on 150 deduced amino acid positions with Mega software. Trees were reconstructed using neighbour-joining method. Robustness of derived groupings was tested by bootstrap using 1,000 replications.

For classification analysis, operational taxonomic units (OTUs) were defined as *amoA* sequence groups in which sequences differed by $\leq 5\%$ (Francis et al. 2003).

Data analysis

Effects of different fertilization and metal concentrations on nitrification and *amoA* gene number were analyzed with a multivariate general linear model using SPSS 11.5 (SPSS, Inc. Chicago, IL). If significant differences were detected ($p < 0.05$), pairwise comparisons of means were made using LSD (Least Significant Difference) test. Triplicate results of real-time PCR measurements were averaged, and standard errors were calculated. When the standard error (in the units of C_T value) for a soil sample was greater than 0.5, the real-time PCR assay of that sample was repeated. Dose–response curves were well fitted by a three-parameter log-logistic model (Streibig 1988) using Sigma Plot 9.0 (SPSS, Inc. Chicago, IL). Zn concentrations causing 50% inhibition of the nitrification (EC50) were derived from the appropriate parameters.

Results

Standard curve and effect of PCR inhibitors in soil

A standard curve (Fig. 1) relating the C_T value and the number of *amoA* gene copies was generated with a fivefold dilution series of a plasmid containing the *amoA* gene.

Fig. 1 Standard curve for real-time PCR of *amoA*

The test for the potential presence of PCR inhibitors in soil DNA samples was conducted by real-time PCR using the defined pUC 19 DNA, with and without the addition of soil DNA, by real-time PCR. There was no significant difference in the quantified pUC 19 DNA between the samples with and without soil DNA addition (data not shown). Therefore, it was assumed that soil PCR inhibitors did not interfere with the real-time PCR assay.

amoA gene number estimated by real-time PCR assay

Samples that were taken at 8 weeks were used for measuring *amoA* gene numbers. In the samples with fertilization treatments but no Zn addition, the estimates of total *amoA* gene numbers are 4.1×10^7 and 3.7×10^7 copies/g d.w. respectively for NPK and 1/2(NPK+OM), which are almost four times higher than the AOB numbers in control samples (Fig. 2). The *amoA* gene numbers in all treatments decreased with increasing Zn addition in the range of 0–1,500 mg/kg, except the point of 1/2(NPK+OM)-1,000 (added with 1,000 mg/kg concentration of Zn). In control samples, the *amoA* copy number in the sample amended with 1,500 mg/kg Zn was approximately 25% of the number in the treatment with no Zn addition. A similar effect was seen in the 1/2(NPK+OM) treatment. The effect of Zn addition on *amoA* gene number was greatest in the NPK fertilized treatment, which

Fig. 2 *amoA* gene number ($\pm$ standard error) of soil with long-term fertilization and different Zn concentrations ($n=3$) Control: unfertilized control soil, NPK: treated with N, P, K fertilizers, 1/2(NPK+OM): fertilized with half amount of NPK and organic matter

showed 4.1×10^7 copies/g dry soil when no Zn was applied and 4.8×10^6 when 1,500 mg/kg Zn was applied. The difference of AOB population size among the treatments were all significant ($p < 0.05$).

Potential nitrification rate (PNR) and pH in Zn spiked soil

Potential nitrification rates were measured at 2, 4, 6 and 8 weeks after the addition of ZnCl_2 . Potential nitrification rates varied significantly between different fertilization treatments ($p < 0.05$), with the decreasing order: NPK > 1/2(NPK+OM) > control (Fig. 3). The higher potential nitrification rates appeared in the NPK and 1/2(NPK+OM) treatments, however, when the Zn addition reached 1,500 and 2,000 mg/kg, the potential nitrification rates all dropped to a similar level.

There was a marked fertilization effect on AOB activity, with the potential nitrification values in NPK more than five times those in the control. The effect of Zn toxicity on AOB activity was far more significant in all three treatments ($p < 0.05$). In order to detect if there was significant differences of Zn toxicity on PNR under different fertilizations, the Zn median effective concentration (EC_{50}) was calculated and statistical analysis of inhibitory effects was evaluated (Fig. 4). The EC_{50} values changed, and most of them decreased over time. It showed the Zn toxicity increased over a few weeks' time, which suggested that there was no adaptation of AOB activity to the Zn toxicity. After 8 weeks' incubation, the pH of samples spiked by different Zn concentrations varied between 7.3–6.79, 7.42–6.61 and 7.47–6.7 in the three treatments. The significance test showed that PNR was also significantly correlated with pH value.

Phylogenetic analysis of *amoA* sequences

PCR amplification of a 491-bp *amoA* fragment was obtained from DNA extracts of six microcosms (control, NPK, 1/2(NPK+OM), control-1,500, NPK-1,500 and 1/2(NPK+OM)-1,500). Between 45 and 50 clones were selected from each library and sequenced successfully, except sample B (NPK clone library) that contained 35 clones because of sequencing errors. BLAST searches of the GenBank database confirmed that all sequenced clones represented *amoA*-like sequences.

Fig. 3 PNR ($\pm$ standard error) of soil samples related to the total amount of added Zn after 8 weeks incubation ($n=3$). See the legend to Fig. 2 for explanations of abbreviations

A total of 271 AOB sequences were obtained, for sequence types that exhibited at least 99% amino acid similarity to each other, only one representative was selected for the construction of trees. Sixty-five novel types were chosen for subsequent phylogenetic analysis. The similarity values of *amoA* (AMO) varied

from 84 to 100% (90–100%). Based on their *amoA* gene sequences and 5% cutoff standard, 65 types were grouped into 14 OTUs, eight of which were singletons or doubletons. The most abundant OTU contained 31 novel types and was closely affiliated with *Nitrosospira* sp. Nsp 17 (AY 123825), which has been isolated from soil (Koops and Harms 1985).

Comparison with the available data from cultured species suggested that all the sequences recovered were derived from the members of the genus *Nitrosospira*. The diversity comparison between treatments was based on the measurement of OTU number. It indicated that the control sample showed the greatest *amoA* diversity, and that the lower diversity and richness were observed in the NPK and 1/2(NPK+OM) samples. After 8 weeks of Zn addition, the diversity dropped drastically in the control-1,500 sample.

For comparison, the grouping of the clones was basically according to the study of Avrahami et al. (2002), in addition, clusters 3b, 3d and 9 were created in this study because of phylogenetic differences and illustration need (Fig. 5). Most of the clones belong to 16S rRNA cluster 3 that is defined by Stephen et al.

Fig. 4 EC50 values ($\pm$ standard error) of different treatments changed over time. See the legend to Fig. 2 for explanations of abbreviations

Fig. 5 Distance neighbour-joining tree based on partial *AmoA* sequences (150 derived amino acid positions) of clone sequences retrieved from six samples. Abbreviation of the clones includes the following information: A, sample of control treatment; B, NPK; C, 1/2(NPK+OM); and D, control-1,500; E, NPK-1,500; F, 1/2(NPK+OM)-1,500. Scale bar indicates 1 change per 100 nucleotide positions

(1996). The majority of clones occurred within cluster 3a and they originated from each of the treatments, while the clones of cluster 3b were only from sample B, C, E, F to which fertilizers were applied. In contrast, the cluster 9 included only clones from A and D.

Discussion

AOB population size and nitrification rates

The population dynamics of AOB, and their relation to potential nitrification rates, in response to different fertilizer treatments and levels of Zn contamination were studied using quantitative real-time PCR analysis of *amoA* genes. Assuming that each AOB cell carries two or three nearly identical copies of the *amoA* gene (Norton et al. 2002), we then assumed that AOB cell numbers could be obtained by dividing the copy numbers of *amoA* gene by 2.5 (Okano et al. 2004). AOB in the fertilized samples had a much larger population size than that in the unfertilized sample (Fig. 2), which was consistent with earlier studies using cPCR (Phillips et al. 2000b) and real-time PCR (Okano et al. 2004). Okano et al. (2004) showed strong relationships among AOB densities and net nitrification, which suggests that the increased AOB population size was the result of more available nitrogenous fertilizers.

The PNR test showed clearly that the activity of the AOB was much higher in NPK and 1/2(NPK+OM) samples than that in the control. However, this positive effect was not sufficient to compensate for the deleterious effects of Zn contamination, as shown in Fig. 3, because both the AOB population sizes and PNR decreased with increasing Zn concentrations in the range 0–2,000 mg/kg. The EC50 value also suggested that the PNR in three fertilization treatments could be reduced by 50% with the addition of Zn at the same level. Thus, the fertilization effect did not improve the community resilience to the Zn contamination.

With increasing Zn concentrations, the AOB population size declined and so did the ammonia oxidation activity. Previous studies have reported that AOB nitrification rates decreased because of Zn contamination. However, these studies did not give the measurement of AOB population size; thus it was

undefined if the decreased activity of AOB was related to AOB population size or its community structure. In this study, results showed a positive correlation ($R^2 = 0.783$, $p < 0.001$) between AOB population size and its nitrification activity (Fig. 6), suggesting that the driving force for the observed decrease in AOB activity is mainly the decline in population size. It also should be noticed that the existence of large amounts of archaeal ammonia oxidizers might have contributed to the detected nitrification activity based on a recent report (Leininger et al. 2006), although this remains to be shown.

Functional diversity and composition of AOB

No sequences related to the genus *Nitrosomonas* were recovered, which may reflect that the *Nitrospira* species are apparently predominant, as previously recorded in a variety of soils (Prosser and Embley 2002). A community shift between fertilized and unfertilized samples was detected. The most obvious difference is between clusters 3b and 9, although there were few clones presented in the phylogenetic tree (Fig. 5) in each cluster, they represented more clones of the same OTU type. This suggests that there was a change in the community structure of AOB, in which cluster 3b was less abundant at low fertilization input, and cluster 9 was more resistant to fertilizers, perhaps because different clusters have different sensitivity to fertilizations.

The diversity in each treatment was reduced but there were no apparent community shifts after Zn addition, as ammonia oxidizers are known to be slow-growing bacteria, longer incubation may be necessary for the detection of a community shift.

Fig. 6 The relationship between *amoA* gene number and potential nitrification rate ($\pm$ standard error)

Collectively, these results give no indication of the selection of specific *amoA* populations between the sample B and C. AOB community composition shift was detected between control and NPK/OM samples. The diversity in each treatment was reduced but there was no apparent community shifts after Zn addition.

Links between AOB population size, activity and diversity

Results from this study demonstrated that AOB nitrification activity was significantly correlated with population size, and Zn addition could significantly decrease AOB population. Differences in fertilization can affect soil AOB through changes in ammonium availability and amount of organic matter input to soils or indirectly through soil characteristics that influence the rates of N cycling, including soil pH and soil moisture (Kowalchuk and Stephen 2001; Phillips et al. 2000a). Nevertheless, in this study, treatments with fertilization showed high nitrification activity but low diversity, thus it is hard to attribute the increased nitrification rate to the diversity in these samples. O' Mullan and Ward (2004) and Avrahami et al. (2003) also reported that the rate of nitrification was decoupled to the community composition. In the three treatments, both diversity and nitrification rates decreased with Zn addition; however, there was no apparent transition trend of the AOB community composition before and after the addition of high concentration of Zn (only 1,500 mg/kg was analyzed). It seemed that the effect of Zn contamination on AOB activity is most probably via reducing population size.

Nitrification rate is sensitive to added Zn in laboratory-spiked soils that are not leached (Smolders et al. 2003). However, it is also believed that PNR is sensitive towards pH changes. Adding Zn in high doses to the soil could change the pH of the soil, and this would have an influence on the observed Zn toxicity. Using field contaminated soils could be an effective way to avoid the pH disturbance when evaluating the Zn toxicity on nitrification activity.

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