

Effects of mercury on the activity and community composition of soil ammonia oxidizers

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Abstract

Purpose Experiments were conducted to examine the effects of mercury (Hg) on soil nitrification activities and the microbial communities of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA).

Methods The soil samples spiked with different Hg concentrations were incubated for a period of 1, 2, 4, and 8 weeks in triplicate and the potential nitrification rate (PNR) of the samples was determined. The abundance of AOB and AOA was measured after an 8-week incubation by real-time polymerase chain reaction (PCR) assay of the *amoA* genes, while the community compositions by cloning and sequencing approaches.

Results The soil PNR differed with different incubation periods. It tended to decrease with increasing soil Hg concentrations at week 1, basing on which the half-maximal effective concentration (EC₅₀) was 1.59 mg kg⁻¹. There was no significant difference in the abundance of AOB or AOA among the treatments. The AOB community was dominated by *Nitrosospira*-like sequences and more than 70% of the obtained clones were affiliated with the cluster 3a.2. The percentage of cluster 3a.1 in AOB community appeared to a consistent trend of decreasing with ascending soil Hg concentrations. While all the AOA sequences in the

clone libraries were grouped into cluster S (soil and sediment origin).

Conclusions This study revealed that Hg could inhibit soil potential nitrification and the extent varied with incubation periods. Soil Hg pollution changed the composition of soil AOB to some extent. These findings will be helpful to recognize the effects of Hg on the activity and community composition of soil ammonia oxidizers.

Keywords Ammonia-oxidizing bacteria · Ammonia-oxidizing archaea · Mercury · Potential nitrification rate · Soil function · Soil pollution assessment

1 Background, aim, and scope

Heavy metals are known to have a considerable effect on the activities of microorganisms and their mediated biogeochemical processes (Ehrlich 1997; Li et al. 2009). Mercury (Hg), as one of the most toxic heavy metals, has been widely studied, especially for its effects on soil microbes (Ranjard et al. 1997; Müller et al. 2002; Hayyis-Hellal et al. 2009). For example, the effect of Hg on the bacterial diversity has been demonstrated based on total community DNA, which indicated that microbial community in mercury-polluted soil diverged from the diversity in the control soil (Rasmussen and Sorensen 2001). However, it was also reported that an increase of mercury-resistant colonies was found in the Hg-treated soil (Ranjard et al. 1997). Some studies have also indicated that Hg could influence the soil particular functions which the microbes mediate, such as soil respiration, enzymatic activities, and nitrification through a combination of physiological and soil microcosm experiments (Rasmussen and Sorensen

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2001; Müller et al. 2002). Nitrification has been considered to be an important process in the nitrogen cycle and one of the most sensitive soil microbial processes with regard to heavy metal stress (Broos et al. 2005; Xu and Chen 2006; Xu et al. 2009). Therefore, the potential nitrification rate (PNR) has been used as an endpoint to evaluate the risk of contaminated soil (Smolders et al. 2001). However, little information is available for the effects of Hg on the PNR of soil.

Ammonia-oxidizing bacteria (AOB) are a group of autotrophic microbes which have a key position in the rate-limiting step of nitrification (Purkhold et al. 2000; Di et al. 2009). A number of studies have shown that the community structure of AOB would shift in response to different environmental conditions, such as temperature, fertilization, and pH (Phillips et al. 2000; He et al. 2007, 2009; Nicol et al. 2008). Some groups belonging to the AOB appeared to be particularly sensitive to heavy metals though it is still unclear how the contaminants affect these *Proteobacteria* (β -subgroup ammonia oxidizers; Frey et al. 2008). It was reported that the AOB were completely inhibited in the heavy metal-contaminated soil according to the measurement of the potential ammonia oxidation (Gremion et al. 2004). However, AOB populations from long-term contaminated soil samples were able to tolerate higher Zn concentrations than those from uncontaminated soil samples (Mertens et al. 2006). Recent study also found that the ammonia-oxidizing population had a trend of resilience after the relief of heavy metal stress although the soil remained depressed microbial activities (Frey et al. 2008). Both metal-sensitive and metal-tolerant AOB populations could be observed in agricultural soil amended with heavy metals (Stephen et al. 1999). There were markedly different responses of AOB community to the different pollution stress. However, little information is available regarding the effects of Hg pollution on the abundance and composition of AOB community in soil.

Non-thermophilic crenarchaea have a potential role in soil ammonia oxidization (Nicol et al. 2008). Some studies had showed that there was a higher transcriptional activity of archaeal over bacterial ammonia oxidizers in soils (Chen et al. 2008; Nicol et al. 2008), which provides increasingly convincing evidences for the importance of ammonia-oxidizing archaea (AOA) in the global nitrogen cycle. Recently, the impacts on AOA community by soil factors, such as fertilization, pH, rhizosphere, and addition of antibiotics have been reported (He et al. 2007; Shen et al. 2008; Chen et al. 2008; Schauss et al. 2009), which presented considerable information recognizing the community characteristics of AOA in soils.

The aim of this study was to investigate the responses of ammonia-oxidizing prokaryotes to the toxicity of Hg in soils during an 8-week incubation period. To achieve this goal, the soil PNRs were determined in the soils with series of Hg

concentrations at different incubation periods. Then, the real-time polymerase chain reaction (PCR) and clone-library sequencing were used to characterize the abundance and community composition of AOB and AOA in the treatments.

2 Materials and methods

2.1 Soil samples and experimental design

The soil sample was collected from a vegetable field in a suburb of Beijing, China, and its characteristics are listed in Table 1. All the samples were air-dried and ground to a size finer than 2.0 mm. The samples were spiked with HgCl_2 solutions to give the final Hg concentrations of 0, 4, 8, 12, 15, and 30 mg kg^{-1} dry soil. A total of 60 g of each sample was placed in a 300-ml-plastic jar microcosm in triplicate for each Hg concentration and each harvest time. The microcosms were incubated at $22 \pm 1^\circ\text{C}$ for 8 weeks with 55% of soil water holding capacity and destructively harvested at week 1, 2, 4, and 8 in triplicate for soil analyses. Soil samples harvested at week 8 were stored at -80°C for subsequent DNA extraction and molecular analysis.

2.2 Soil chemical analysis and potential nitrification rates

Soil pH was determined with a soil to water ratio of 1:2.5, and soil organic matter was determined by $\text{K}_2\text{Cr}_2\text{O}_7$ oxidation method (Walkley and Black 1934). Available P was analyzed according to Olsen P method. Soil samples were digested with $\text{HNO}_3 + \text{HCl}$ (10 ml, 1:1 v/v) for analysis of Hg, while soil samples were digested with HNO_3 and H_2O_2 for analysis of other elements. The concentrations of Mn, Fe, Cu, Zn, and Cr were analyzed by an inductively coupled plasma optical emission spectrometer (ICP-OES, PerkinElmer Co., America), and Hg was analyzed by an atomic fluorescence spectrometer (AFS-2202E Haiguang Analytical Instrument Co., Beijing, China). Two standard reference materials, GBW-07401 (GSS-1) and GBW-07405 (GSS-5), obtained from the Center for National Standard Reference Materials of China, were inserted to the analytical process for quality assurance/quality control. PNR was measured using the chlorate inhibition method (Kurola et al. 2005). Briefly, 5.0 g of fresh soil was added to 50 ml centrifuge tube containing 20 ml of phosphate buffer solution (g l^{-1} : NaCl, 8.0; KCl, 0.2; Na_2HPO_4 , 0.2; NaH_2PO_4 , 0.2; pH 7.4) with 1 mM $(\text{NH}_4)_2\text{SO}_4$. Potassium chlorate with a final concentration of 10 mM was added to inhibit nitrite oxidation. The suspension was incubated in a dark incubator at 25°C for 24 h. And then nitrite was extracted with 5 ml of 2 M KCl and determined spectrophotometrically at 540 nm with *N*-(1-naphthyl) ethylenediamine dihydrochloride.

Table 1 Selected basic characteristics of the tested soil

pH (H ₂ O)	Organic matter (%)	Total N (g kg ⁻¹)	Available P (mg kg ⁻¹)	Total metal concentration in the soil (mg kg ⁻¹)					
				Mn	Fe	Cu	Zn	Cr	Hg
7.14	2.05	0.83	9.51	458	24,525	2.36	89.23	4.53	0.05

2.3 DNA extraction

DNA was extracted from 0.5 g fresh soil samples using Ultra-clean™ soil DNA Isolation Kits (MoBio Laboratory, USA) according to the manufacturer’s protocol. The extracted DNA was evaluated on a 1% agarose gel and the concentration and purity of the extracts were estimated by spectrophotometry (NanoDrop, USA).

2.4 Quantification of *amoA* genes by real-time PCR assays

Abundances of AOB and AOA were determined by quantitative real-time PCR method targeting on *amoA* gene with the iCycler iQ5 thermocycler (Bio-Rad, USA; He et al. 2007). The 25 µl reaction mixture included 12.5 µl Premix Ex Taq™ (Takara Biotechnology, Japan), 1 µl of BSA, 0.5 µl of each primer (10 µM), and 2 µl of tenfold diluted extracted DNA as template. Bacterial *amoA* gene was quantified using the primers amoA1F (5'-GGGGTTTCTACTGGTGGT-3') and amoA2R (5'-CCCCTCKGSAAAGCCTTCTTC-3'; Rotthauwe et al. 1997), and the primers for archaeal *amoA* gene were arch-amoAF (5'-STAATGGCTGGCTTAGACG-3') and Arch-amoAR (5'-GCGGCCATCCATCTGTATGT-3'; Francis et al. 2005).

2.5 Construction of the *amoA* gene clone library and restriction fragment length polymorphism analysis

In order to investigate the effects of Hg on the community composition of soil AOB and AOA, three soil treatments spiked with 0, 8, and 30 mg kg⁻¹ Hg were selected and the DNA extracts from three replicated samples were mixed. The products of bacterial and archaeal *amoA* genes amplified with the primers for the real-time PCR assays were purified and cloned, which produces a 490 bp fragment for bacterial *amoA* gene and 635 bp for archaeal *amoA* gene. Clones were randomly chosen for PCR reamplification using the primers T7 and SP6. For restriction fragment length polymorphism (RFLP) and sequencing, the inserted fragments were digested with restriction endonuclease *HhaI* (Takara Biotechnology, Japan) and incubated at 37°C for 2 h. The restricted fragments were separated by gel electrophoresis on 3% agarose gel with ethidium bromide and observed under UV illumination. The fingerprint images of the RFLP band types in each replicate of three HgCl₂ concentrations treatments were analyzed by the software Quantity One (Bio-

Rad, USA) and grouped based on different band patterns. Evaluation of the clone libraries was performed by a rarefaction analysis using software aRarefactWin Version 1.3 (Steven Holland, Stratigraphy Lab, University of Georgia [<http://www.uga.edu/strata/software/Software.html>]).

2.6 Sequencing and phylogenetic analysis

The clones of unique RFLP band types in each library were sequenced. All *amoA* gene sequences were deposited in the GenBank nucleotide sequence database under accession no. FJ940180 to FJ940192, FJ999545 to FJ999552 for AOB and FJ940130 to FJ940179 for AOA. The *amoA* nucleotide sequences were trimmed and assembled using SeqMan (DNAStar, Madison, WI, USA). Homology analysis of the *amoA* sequences was performed using the software DNAMAN version 4.0 (Lynnsoft Biosoft, USA). Sequences displaying more than 98% identity with each other were grouped into the same operational taxonomic units (OTUs). Phylogenetic analysis was performed using MEGA version 4.0 and the neighbor-joining trees were constructed using the Poisson correction model with bootstrap value of 1,000.

2.7 Statistical analysis

Copy numbers for the *amoA* gene analysis were log-transformed as needed to normalize the distributions prior to statistical analysis. All statistical analyses were per-

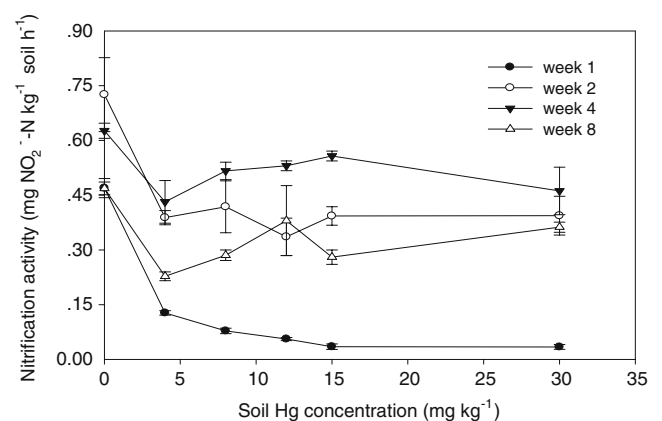


Fig. 1 The potential nitrification rates (±standard error) of soil samples related to the total amount of added Hg at different incubation periods (n = 3)

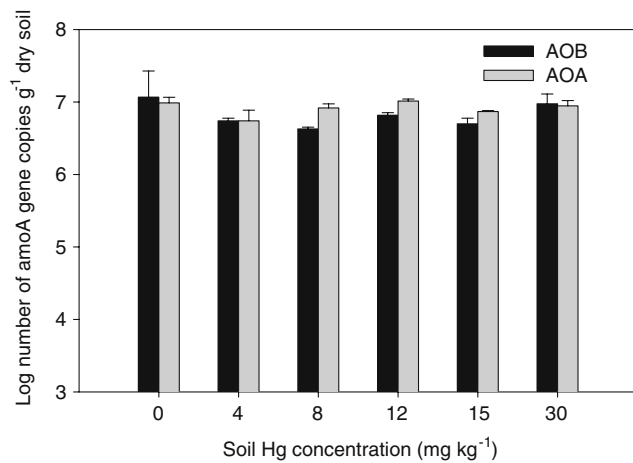


Fig. 2 Quantification of bacterial and archaeal *amoA* genes in the soils with different Hg concentrations. Error bars represent standard errors

formed using SPSS version 11.5 and one-way analysis of variance followed by S-N-K-test was used to check for quantitative differences between treatments. $P < 0.05$ was considered to be statistically significant. Effects on the PNR were plotted against added Hg concentration in the soil samples. A logistic response model was fitted to the data (Vanewijk and Hoekstra 1993):

$$y = \frac{k}{1 + (x/x_{50})^s}$$

Where y = response of measured endpoint (PNR), k = response of measured endpoint at $x = 0$, s = slope parameter, x = concentration (on a linear scale), and x_{50} = median effective concentration (EC50).

3 Results

3.1 Potential nitrification rates in the Hg-spiked soils

Soil PNRs were measured at week 1, 2, 4, and 8 after addition of HgCl₂ (Fig. 1). The PNRs in all Hg treatments were significantly lower than those in control in the respective

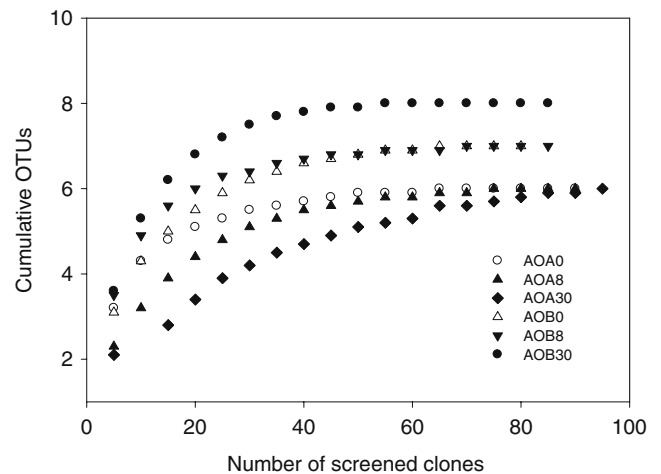


Fig. 3 Rarefaction analysis of the *amoA* clones obtained from the different treatments of Hg concentrations using software aRarefactWin Version 1.3. Treatment: bacterial *amoA* genes without Hg (AOB0), bacterial *amoA* genes with 8 (AOB8) and 30 (AOB30) mg kg⁻¹ soil Hg, archaeal *amoA* genes without Hg (AOA0), archaeal *amoA* genes with 8 (AOA8) and 30 (AOA30) mg kg⁻¹ soil Hg

incubation period, and the PNRs at week 1 were much lower than those at week 2, 4, and 8. No significant differences were found between the treatments with soil Hg concentrations higher than 8 mg kg⁻¹. Only the PNR curve of week 1 could be fitted well with logistic dose-response model ($P < 0.05$), from which the EC50 of PNRs was 1.59 mg kg⁻¹.

3.2 Abundance of AOB and AOA in the soils

There was no significant difference for the bacterial or archaeal *amoA* gene copy numbers among all the treatments, respectively (Fig. 2). The average bacterial *amoA* gene copy numbers of 1.63×10^7 copies per gram of dry soil was found in all the tested soils, in which the average archaeal *amoA* gene copy numbers was 7.73×10^6 copies.

3.3 Analysis of *amoA* gene clone libraries

Each of the bacterial *amoA* gene clone libraries was clustered into seven to eight RFLP groups based on the

Table 2 Analysis of the bacterial and archaeal *amoA* gene clone libraries

Treatment ^a	AOB			AOA		
	Number of clones	RFLP patterns	OTU (2%)	Number of clones	RFLP patterns	OTU
Hg0	86	7	7	96	6	11
Hg8	86	8	13	95	6	6
Hg30	89	7	8	93	6	9

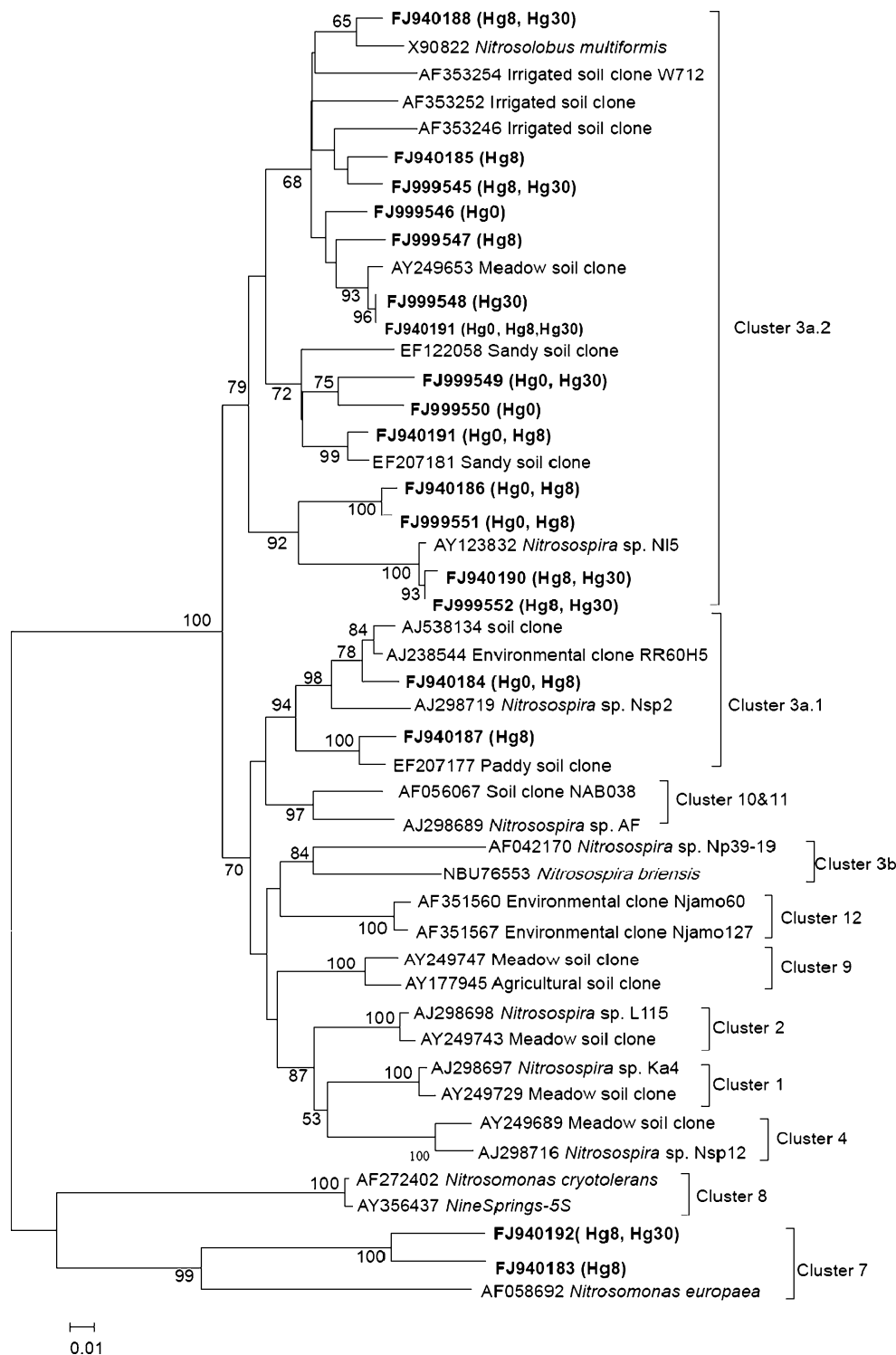
OTU operational taxonomic unit (DNA ≥ 98% identity)

^a Treatment: Hg0 represents the soil without addition of Hg, Hg8 and Hg30 represent the soils with addition of 8 and 30 mg kg⁻¹ Hg

band patterns, while all the three archaeal *amoA* gene clone libraries were clustered into six RFLP groups (Table 2). Rarefaction curves were constructed for each *amoA* gene library. All of the *amoA* gene libraries were sampled adequately as the rarefaction curves approached a plateau (Fig. 3). To determine the effects of Hg addition on soil

AOB and AOA populations, the clones from *amoA* gene clone libraries that representing different RFLP patterns were partially sequenced. The obtained sequences from clone libraries were grouped into 18 bacterial *amoA* gene OTUs and 19 archaeal *amoA* gene OTUs by the Blast comparisons of nucleotide sequences in the NCBI database.

Fig. 4 Phylogenetic relationships among bacterial *amoA* sequences retrieved from the three Hg levels soil samples. Designation of the clones in *bold* includes the following information: accession number in the Genbank with Hg treatments in the *parentheses*. Bootstrap values (>50%) are indicated at *branch points*. The *scale bar* represents 1% estimated sequence divergence. Treatment: bacterial *amoA* genes without Hg (*AOB0*), with 8 mg kg⁻¹ Hg (*AOB8*), and with 30 mg kg⁻¹ Hg (*AOB30*)



3.4 Phylogenetic analysis of the bacterial and archaeal *amoA* sequences

Phylogenetic trees of the bacterial *amoA* gene sequences and related NCBI sequences are showed in Fig. 4. All AOB sequences were affiliated with *Nitrosospira amoA* cluster 3a.1, 3a.2 and *Nitrosomonsa amoA* cluster 7 (Avrahami and Conrad 2003). Sequences in cluster 3a fell within two sub-cluster 3a.1 and 3a.2 with bootstrap value at 90%. The percentage of the sequences with similarity less than 98% in all 50 tested clones was calculated (Table 3). The sequences affiliated with 3a.2 had high proportions (more than 70%) in the three clone libraries. The proportion of cluster 3a.1-sequence in the control was 28.0%, while it reduced to 9.5% in the treatment of 8 mg kg⁻¹. No sequence affiliated with cluster 3a.1 was detected in the highest Hg concentration treatment (30 mg kg⁻¹). In addition, no sequence affiliated with cluster 7 in the control but there was in the treatment of 8 or 30 mg kg⁻¹ soil Hg. These data indicated substantial differences in AOB community composition among the soil samples with different Hg concentrations.

All the detected AOA sequences fell within the previous characterized soil and sediment and were grouped into two distinct clades (data not shown). These were designated as soil and sediment cluster 1 and 2 according to the latest classification (Zhang et al. 2009). Soil and sediment cluster 1 and 2 represented all the AOA clones, presenting in all soils at slightly different proportions (Table 3). Eighteen AOA OTUs belonged to soil and sediment cluster 2 while only one OTU were grouped into soil and sediment cluster 1.

4 Discussion

The soil PNR curve of week1 could be fitted well with the logistic model, suggesting that the inhibition of soil Hg to the oxidation of ammonia could be predicted with dose-response relation in toxicology. The EC50 value calculated from the model was 1.59 mg kg⁻¹, which was lower than another EC50 (3.26 mg kg⁻¹) based on the reproduction of

soil animal *Folsomia candida* stressed by soil Hg (Lock and Janssen 2001). According to the previous report (Fountain and Hopkin 2005), the *F. candida* was very sensitive to the soil pollution and used as indicator to assess soil quality. Therefore, PNRs could be a sensitive parameter in the soil Hg pollution diagnostics as well, especially in the early diagnostics since there were no dose-response relations found in the treatments over 1 week incubation in this study. Mertens et al. (2006) mentioned the same phenomena in the study using Zn as a stressor, stating that the long-term Zn pollution could hardly change the PNRs in the soil.

No difference of AOB abundance was observed between treatments in this study, which was in agreement with the previous report suggesting that heavy metal contaminated soil did not decrease the abundance of AOB (Stephen et al. 1999; Frey et al. 2008). It was also reported that there was no significant difference in the number of 16S rRNA gene fragment of AOB using most probable number method (Mertens et al. 2006). Phylogenetic analysis of the bacterial *amoA* gene sequences indicated that *Nitrosospira* cluster 3 was the dominant AOB group and the community composition changed with different soil Hg concentrations. The sequence affiliated with cluster 3a.1 decreased when soil Hg was 8 mg kg⁻¹, and no cluster 3a.1 sequence was found when soil Hg was increased to 30 mg kg⁻¹. On the other hand, cluster 7, which was absent in the soil without addition of Hg, could be detected in the other two soils with Hg spiked. Intriguingly, based on the current phylogenetic analysis, it might be possible to reveal some dose-response relation in some populations from cluster 3a.1 or to do some screening in cluster 7 for possible discovery of resistant strains or genes of AOB. As for the sequences affiliated with cluster 3a.2, which had a high percentage in community composition and only slight difference between the treatments, they could hardly be affected by the addition of Hg. Although it was unclear whether these populations were intrinsically tolerant to Hg or tolerance was only induced by the extrinsic addition of Hg, this resistance could account for minimal difference of AOB abundance in the different treatments.

Meanwhile, the addition of Hg did not change the abundance of AOA. And there was no remarkable shift of AOA community between the treatments. The information about the effect of heavy metal on AOA was still limited up to now. Although it was reported that AOA might be in general less susceptible than AOB to inhibitor because of the remarkably different physiological characteristics of archaea compared with bacteria (Schauss et al. 2009), the mechanistic information about the response of AOA to environmental stress remains to be unclear due to limited cultivated AOA cluster so far (Konneke et al. 2005; de la Torre et al. 2008).

Table 3 Distribution of sequence types affiliated with *amoA* clusters in the clone libraries

Treatment ^a	AOB			AOA	
	Cluster 3a.1 (%)	Cluster 3a.2 (%)	Cluster 7 (%)	S1 (%)	S2 (%)
Hg0	28.0	72.0	0.0	28.0	72.0
Hg8	9.5	71.5	19.0	30.0	70.0
Hg30	0.0	91.7	8.3	38.5	61.5

^a Treatment: Hg0 represents the soil without addition of Hg, Hg8 and Hg30 represents the soils with addition of 8 and 30 mg kg⁻¹ Hg

According to the results in this study, it was not an appropriate option to use the *amoA* gene copy number of AOB or AOA for the purpose of soil pollution assessment. No significant correlation was found between PNRs and the abundance of AOB or AOA, which also proved the difficulties to build quantity relations between the abundance of AOB or AOA and the ammonia-oxidizing function of the soil. Then some new techniques or concepts should be introduced to assess the effects of pollutants on the soil nitrification function, for example exploration on the species level of microbes as mentioned above. How to link the soil function and the ecotoxicology still needs a lot of studies.

5 Conclusions

Our results revealed that the PNR in the soil incubated for 1 week was very sensitive to Hg stress and accorded with logistic dose-response model. The addition of Hg distinctly changed the composition of soil AOB, whereas the AOA presented minimal shift. Most of AOB sequences belonged to the cluster 3a.2, and the percentage of cluster 3a.1 in AOB community appeared to a consistent trend of decreasing with ascending soil Hg concentrations. Therefore, the addition of Hg impacted the soil potential nitrification activity and community composition of AOB in this study.

6 Recommendations and perspectives

These findings demonstrated the effects of Hg on the activity and community composition of soil ammonia oxidizers.

Further studies should be performed to determine how those sensitive markers respond to soil heavy metal stress at molecular level. Thus, studies of transcriptional activity of ammonia oxidizers in the contaminated soils may be especially valuable in further exploring this issue. Furthermore, more detailed studies on the effects of contaminants on soil ammonia oxidizers should be conducted and thus to collect more toxicological data for evaluating their ecological effects.

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