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Responses of soil ammonia oxidizers to a short-term severe mercury stress

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ABSTRACT

The responses of soil ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) to mercury (Hg) stress were investigated through a short-term incubation experiment. Treated with four different concentrations of Hg (CK, Hg25, Hg50, and Hg100, denoting 0, 25, 50, and 100 mg Hg/kg dry soil, respectively), samples were harvested after 3, 7, and 28 day incubation. Results showed that the soil potential nitrification rate (PNR) was significantly inhibited by Hg stress during the incubation. However, lower abundances of AOA (the highest in CK: 9.20×10^7 copies/g dry soil; the lowest in Hg50: 2.68×10^7 copies/g dry soil) and AOB (the highest in CK: 2.68×10^7 copies/g dry soil; the lowest in Hg50: 7.49×10^6 copies/g dry soil) were observed only at day 28 of incubation ($P < 0.05$). Moreover, only the community structure of soil AOB obviously shifted under Hg stress as seen through DGGE profiles, which revealed that 2–3 distinct AOB bands emerged in the Hg treatments at day 28. In summary, soil PNR might be a very useful parameter to assess acute Hg stress on soil ecosystems, and the community structure of soil AOB might be a realistic biological indicator for the assessment of heavy metal stress on soil ecosystems in the future.

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Introduction

The ammonia oxidation process, as the first and key step in nitrification, plays a very important role in the global nitrogen (N) cycle (De-Boer et al., 1991). This process in soil is driven by both ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) (Leininger, 2006; Adair and Schwartz, 2008; Shen et al., 2008). The abundance and community structure of soil ammonia oxidizers could be affected by many soil properties and environmental stresses (Nicol et al., 2008; He et al., 2007; Li et al., 2009; Mertens et al., 2009). As the crucial functional microbes involved in the soil N-cycle, AOA and AOB also are generating increasing interest concerning their suitability as indicators to assess the

environmental stresses on soil ecosystems (Ritz et al., 2009; Wessen and Hallin, 2011), especially with the assistance of molecular techniques (Liu et al., 2010; Ruyters et al., 2010; Subrahmanyam et al., 2014).

Heavy metal contamination in soil has been a public concern for a long time due to their toxicity and persistency. Since the traditional risk assessment for soil heavy metals based on the total quantities was insufficient because it lacked measurement of bioavailability, soil microorganisms have been considered as important indicators for bioavailability measurement (Harms et al., 2006; Hinojosa et al., 2005; Stefanowicz et al., 2008). Previously, the study of the influence of heavy metals on soil microorganisms had been mainly focused on the community structure of bacteria and fungi

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(Gremion et al., 2004; Frey et al., 2006; Gomes et al., 2010). Recently, the responses of soil microbes involved in the N-cycle to heavy metal stress have been investigated (Li et al., 2009; Mertens et al., 2009; Subrahmanyam et al., 2014; Zhou et al., 2012). For example, using four different Chinese soils, Li et al. (2009) observed that copper (Cu) intensively inhibited the soil potential nitrification rate (PNR) and decreased the abundances of soil AOB and AOA within 20 days. Meanwhile, AOB were found to be more sensitive to heavy metals than AOA. Mertens et al. (2009) reported that the abundances of AOB and AOA decreased in Zn-spiked soil after 1 week, while only the community structure of AOB changed significantly after 2 years. It appeared that the abundances of soil AOB and AOA were sensitive to heavy metal stresses.

Mercury (Hg) as a typical heavy metal has pronounced negative effects on all living organisms. During the past two decades, almost 50% of Hg production worldwide was consumed in China (Jiang et al., 2006). It was also reported that the total Hg ranged from 5.1 to 790 mg/kg in agricultural fields near a mining area in Guizhou Province, Southwest of China (Qiu et al., 2004). It has been recognized that mercury can change the community structure of soil microorganisms and inhibit soil enzymatic activities (Ranjard et al., 1997; Muller et al., 2001; Rasmussen and Sorensen, 2001; Harris-Hellal et al., 2009). Liu et al. (2010) tested the responses of soil AOB and AOA to Hg at up to 35 mg/kg and no variation in the abundance of either AOA or AOB was exhibited. Considering the heavy contamination of Hg in farm fields (up to 790 mg/kg), it is essential to investigate the effect of more severe Hg stress on soil AOB and AOA, which also could provide some useful information for the application of ammonia oxidizers in the risk assessment of soil Hg pollution in the future.

In this study, the effect of Hg on the activity of soil ammonia oxidizers was evaluated for a series of Hg concentrations (0–100 mg/kg), as well as the abundance and community structures of soil AOA and AOB under Hg stress. The objective was to explore the response of the ammonia oxidizing activity and different ammonia oxidizers to severe Hg stress.

1. Materials and methods

1.1. Microcosms and experimental design

The soil sample for the experiment was collected from the topsoil (0–20 cm) of a wheat–maize rotation field in Shunyi District of Beijing (China) in July 2010. The soil, classified as Cinnamon soil (Semi-Luvisols), was air-dried and ground to pass through a 2 mm sieve for the incubation experiment. Some basic properties were pH, 7.8; organic matter, 22.8 g/kg; NO_3^- , 21.3 mg/kg; NH_4^+ , 11.3 mg/kg; and Hg, 0.02 mg/kg.

The effect of Hg on soil ammonia oxidizers was studied using four different Hg contents (0, 25, 50, and 100 mg Hg/kg dry soil, represented as CK, Hg25, Hg50, and Hg100, respectively) with three replicates. The soil samples were spiked with Hg by application of HgCl_2 solutions to dry soil. Each soil sample (60 g) was placed in a tube (8 cm diameter, 15 cm height) in triplicate for each Hg concentration and each

harvest time. In each tube, the water content in soil was adjusted to 35% of the maximum water holding capacity and maintained throughout the experiment. Each tube was weighed weekly and the loss of weight was supplemented with sterile distilled water if needed. Finally, each tube was loosely covered with a plastic cap allowing air-exchange and incubated in a dark incubator at 25°C for 28 days. Soil samples were collected from the tubes at 3, 7 and 28 days for PNR, real-time PCR and DGGE analysis.

1.2. Measurement of soil potential nitrification rate (PNR)

The chlorate inhibition method (Kurola et al., 2005) was used to measure soil PNR. Briefly, 5.0 g of fresh soil, 20 mL of phosphate buffer solution (PBS) (g/L: NaCl, 8.0; KCl, 0.2; Na_2HPO_4 , 0.2; NaH_2PO_4 , 0.2) with pH 7.4, and 1 mmol/L $(\text{NH}_4)_2\text{SO}_4$ were added into a 50 mL centrifuge tube successively, and then Potassium chlorate (10 mol/L) was added to inhibit nitrite oxidation. The suspension was incubated in a dark incubator at 25°C for 24 hr, after which the nitrite was extracted with 5 mL of 2 mol/L KCl and determined spectrophotometrically at 540 nm with N-(1-naphthyl) ethylenediamine dihydrochloride.

1.3. Soil DNA extraction and real-time PCR assay

Soil DNA was extracted from 0.5 g fresh soil using MoBio UltraClean™ soil DNA isolation kits (San Diego, CA, USA) according to the manufacturer's protocol with previously described modifications (He et al., 2007).

Real-time PCR was performed on an iCycler iQ5 thermocycler (Bio-Rad, USA). Amplification was performed in 25 μL reaction mixtures by using SYBR® Premix Ex Taq™ as described by the suppliers (Takara Bio, Otsu, Shiga, Japan). The DNA extracts were diluted 5-fold and used as template, with a final content of 1–10 ng in each reaction mixture. The primer sets (AOA: Arch-amoAF/Arch-amoAR; AOB: amoA-1F/amoA-2R) and thermal profiles used to amplify each target gene with real-time PCR are listed in Table 1. Following the thermal profile, determined by measuring the fluorescence continuously as the temperature increased from 55 to 95°C, a melting curve analysis was performed to confirm the specificity of the PCR product for each real-time PCR amplification. Data analysis was carried out with iCycler software (version 1.0.1384.0 CR). The parameter C_t (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected.

Standard curves for real-time PCR assays were developed as described previously (He et al., 2007). Briefly, the AOA and AOB amoA genes were PCR-amplified from extracted DNA with the primer sets (AOA: Arch-amoAF/Arch-amoAR; AOB: amoA-1F/amoA-2R) listed in Table 1, and then the PCR products were cloned into the pGEM-T Easy Vector (Promega Madison, USA). Plasmids used as standards for quantitative analyses were extracted from the correct insert clones of each target gene. The concentration of plasmid DNA was determined on a Nanodrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, USA), then the copy numbers of the target genes were calculated. To generate an external standard curve, ten-fold serial dilutions of a known copy number of the plasmid DNA were subjected to real-time PCR assay in triplicate.

Fig. 1 – Soil potential nitrification rate (PNR) in the treatments with different concentrations of Hg (CK, Hg25, Hg50, and Hg100, representing 0, 25, 50, and 100 mg Hg/kg dry soil, respectively) during the incubation. Error bars indicate standard deviations ($n = 3$). The different letters above bars indicate significant differences ($P < 0.05$) among the treatments for the same sampling day.

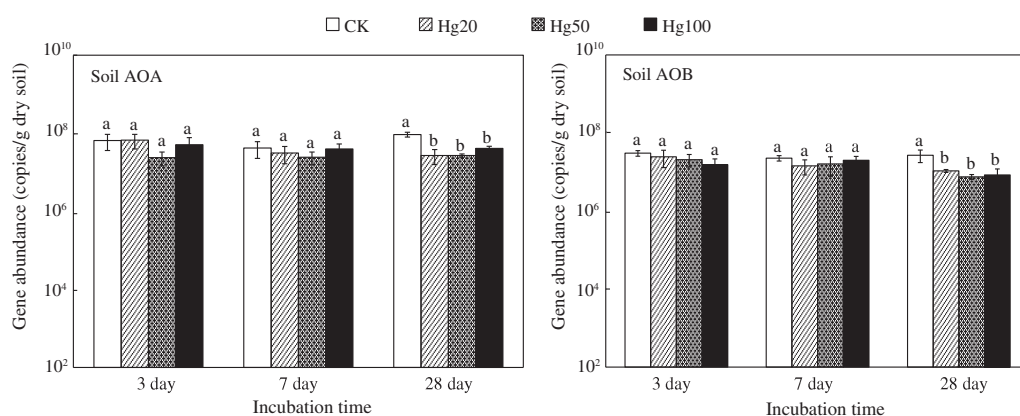


Fig. 2 – Quantification of soil ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) *amoA* gene copy numbers in the treatments with different concentrations of Hg during the incubation. Error bars indicate standard deviations ($n = 3$). The different letters above bars indicate significant differences ($P < 0.05$) among the treatments for the same sampling day.

the treatments (Fig. 3 for soil AOA). However, distinct differences were exhibited in the community structure of soil AOB (Fig. 3B for soil AOB) between CK and all the Hg treatments. Several bands disappeared in the Hg-amended soil at days 7 and 28 (top rectangle in Fig. 3B), and some special bands were detected in some Hg treatments (Hg25, Hg50) at day 28 (bottom rectangle in Fig. 3 for soil AOB).

3. Discussion

The inhibiting effect of heavy metals on soil PNR had been reported previously (Rother et al., 1982; Smolders et al., 2001; Broos et al., 2005; Yang et al., 2005). For example, Broos et al. (2005) demonstrated that soil PNR was a more sensitive endpoint than SIR (substrate-induced respiration) or basal respiration (BR) under cadmium (Cd) and Zn stress. In this study, the intensive inhibition of soil PNR by Hg after 3-day

exposure indicated the influence of Hg on the activity of soil ammonia oxidizers. The soil PNR showed a clear dose–response relationship with EC50 (22.59 mg/kg) at day 28, which further confirmed the negative effects of Hg on those soil key functional microbes. It seemed that soil PNR had different responses to severe and mild Hg stress. The PNR was partially restored at day 28 at low Hg concentration, but not for the treatment of Hg100 (Fig. 1). Liu et al. (2010) reported that soil PNR was inhibited by Hg (30 mg/kg) after 1 week, and subsequently recovered after 2 weeks. Additionally, only the PNRs at 1 week could be well fitted with the logistic dose–response model (EC50, 1.59 mg/kg). The mild Hg stress inhibited soil PNR with an obvious dose–response relationship at 1 week, but severe Hg stress could be evaluated after 4 weeks as the soil PNR began to recover. Other researchers also reported that Zn inhibited the soil PNR after 1 week, but that the soil PNR recovered gradually after 1 year (Ruyters et al., 2010). Li et al. (2009) found that soil PNR was inhibited by

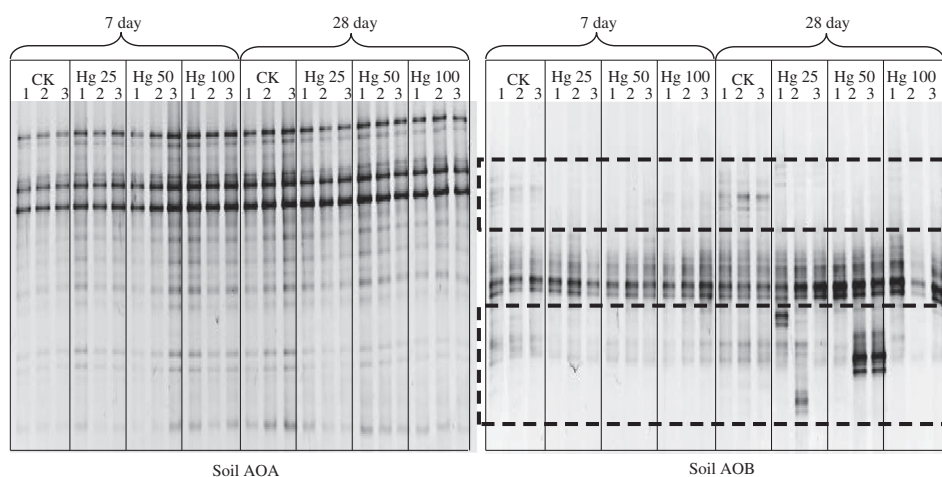


Fig. 3 – DGGE profiles of soil ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) *amoA* gene amplified from the treatments with different concentrations of Hg at days 7 and 28. The districts in the rectangles marked with dotted lines show the obviously different bands in the CK and Hg treatments.

more than 50% at a Cu concentration of 600 mg/kg in four different Chinese soils during a incubation of 20 days, and different soil had different ED50 (50% effective doses) under Cu stress. Meanwhile, the heavy metal concentration and the soil type could affect the dose–response relationship between soil PNR and heavy metal concentration, especially the former, which showed that a clear dose–response relationship occurred in the inhibition or initial recovery period. Moreover, the PNR values of CK at days 3 and 7 were notably lower than that at day 28, indicating the recovery process of soil PNR during the incubation, which might be due to the influence of soil pretreatment (air-drying and sieving) on the activity of ammonia oxidizers and the low growth rate of ammonia oxidizers (obligate chemoautotrophic organisms).

Significant inhibition effects of Hg on the abundance of soil AOB and AOA were only observed at the end of the incubation (at day 28). This is the probably the reason for PNR decrease. Li et al. (2009) studied the responses of soil AOA and AOB gene copies to Cu stress and observed much lower numbers of AOB *amoA* gene copies in the treatment with Cu concentration of 1000 mg/kg at day 21, indicating the inhibiting effect of Cu on the abundance of AOB. However, other researchers reported different results. The abundance of soil AOB and AOA had no changes under Hg (0–30 mg/kg soil) stress during an incubation experiment of 8 weeks (Liu et al., 2010). It is generally considered that there is a positive relationship between soil PNR and AOB *amoA* gene abundance (He et al., 2007; Di et al., 2009; Tian et al., 2014). By contrast, according to our results, soil PNR exhibited no obvious correlation with *amoA* gene abundance under short-term Hg stress at days 3 and 7, which might be explained by the following reason. Short-term Hg stress on the abundance of soil ammonia oxidizers is likely to be difficult to detect by real-time PCR based on soil DNA, since the DNA from the cells that were killed or inhibited under Hg stress might not be completely digested in a short period of time, and thus also might be extracted from the soil and amplified by real-time PCR. With the extension of incubation time, compared with CK, relatively lower PNRs and *amoA* gene abundances were simultaneously detected in the Hg treatments at day 28.

The result of DGGE indicated that Hg had no effect on the community structure of soil AOA, but significantly changed the community structure of soil AOB, especially at day 28. This result suggested that soil AOA and AOB had different sensitivity to Hg stress, and AOB were more sensitive than AOA. The results were supported by the previous investigation (Liu et al., 2010), in which the community structure of soil AOB but not AOA changed dramatically under Hg stress. The percentage of AOB community in cluster 3a.1 decreased with ascending soil Hg concentration. Mertens et al. (2009) investigated the community structures of soil AOA and AOB under Zn stress, and suggested that only the community structure of soil AOB was intensively affected by 2-year exposure to Zn. These results confirmed that the community structure of soil AOB was more sensitive to heavy metals than that of soil AOA, which might be due to the distinctions of cell structure and physiology between bacterial and archaea, although the exact mechanism of the different responses of AOB and AOA to environmental stresses was still unclear. It was reported that AOB also were more susceptible than AOA to some

organic inhibitors, such as allylthiourea and sulfadiazine, possibly because of the differences in cell walls and membranes (Hatzenpichler et al., 2008; Schauss et al., 2009). On the other hand, soil AOA might be the primary participant in the N-cycling under some harsh environmental conditions, such as low pH, extremely low or high temperature, high salinity, low oxygen, and low NH_4^+ concentration (Erguder et al., 2009; He et al., 2012). The soil used in this study had a rich NH_4^+ content with a neutral pH, which should be not suitable for the growth of AOA, and the soil AOA in a dormant state might be not sensitive to Hg stress.

The band numbers in the Hg treatments were less than that in CK at day 7, but some new bands emerged in the Hg treatments (Hg20 and Hg50) at day 28 (Fig. 3B). So the results indicated that severe Hg stress might have a strong selectivity on the soil AOB species that might be related with the restoration of soil PNR. Severe and mild Hg stress had different effects on the community structure of soil AOB. In addition, the community structure of soil AOB might be more suitable for the assessment on soil Hg stress both in short or long-term than that of soil AOA.

4. Conclusions

In conclusion, soil PNR was inhibited rapidly by Hg stress (day 3), and restored with the decrease of Hg levels. Significant inhibiting effects of Hg on the abundance of soil AOB and AOA were observed at the end of the incubation (day 28), which might be responsible for the reduced PNR. Only the community structure of soil AOB was changed, suggesting AOB are more sensitive than AOA to Hg stress.

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