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Response of ammonia oxidizing microbes to the stresses of arsenic and copper in two acidic alfisols

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ABSTRACT

Soil pollution by elevated heavy metals exhibits adverse effects on soil microorganisms. Ammonia oxidizing bacteria and ammonia oxidizing archaea perform ammonia oxidative processes in acidic soils. However, influence of heavy metal stress on soil ammonia oxidizers distribution and diversity is inadequately addressed. This study investigated the responses of ammonia oxidizing bacteria and archaea to heavy metals, Cu and As during short-term laboratory experiment. Two different acidic alfisols named as Rayka and Hangzhou spiked with different concentrations of As, Cu and As + Cu were incubated for 10 weeks. Significant reduction in copy numbers of archaeal-16S rRNA, bacterial-16S rRNA and functional *amoA* genes was observed along elevated heavy metal concentrations. Ammonia oxidizing archaea was found to be more abundant than ammonia oxidizing bacteria in all the heavy metal treatments. The potential nitrification rate significantly decreased with increasing As and Cu concentrations in the two soils examined. Denaturing gradient gel electrophoresis analysis revealed no apparent community shift for ammonia oxidizing archaea even at higher concentrations of As and Cu. Phylogenetic analysis of archaeal *amoA* gene from 4 clone libraries indicated that all the archaeal *amoA* sequences were placed within 3 distinct clusters from soil and sediment group 1.1b of Thaumarchaeota. Our results could be useful for the better understanding of the ecological effects of heavy metals on the abundance and diversity of soil ammonia oxidizers.

1. Introduction

Ammonia oxidation (AO) is the first and rate limiting step in nitrification (Di et al., 2009), and thus plays a decisive role in the global nitrogen cycle. For more than a century, it has been believed that AO is exclusively mediated by chemolitho-autotrophic ammonia oxidizing bacteria (AOB) (De Boer and Kowalchuk, 2001). The recent discovery of homologs of ammonia monooxygenase gene (*amoA*) in archaea and subsequent cultivation of autotrophic ammonia oxidizing archaea (AOA) affiliates to phylum Thaumarchaeota has shown the importance of AOA in the global nitrogen cycle (Könneke et al., 2005). Nevertheless, the key factors driving the abundance, diversity and activity of ammonia oxidizers are still unclear. Studies have demonstrated the changes in abundance and diversity of AOB and AOA in response to different abiotic stresses like temperature, pH, soil type, organic and inorganic fertilization (Chan et al., 2013; Chen et al., 2013; He et al., 2007; Nicol et al., 2008; Nyberg et al., 2012). However, the response of AOA to heavy metals in acidic soils remains poorly characterized. It has been estimated that over 50% of the world's potential arable lands are acidic (von Uexkull and Mutert, 1995). Currently about 12% of world land area in crop production is acidic in nature. Asian continent accounts for approximately 1044 million ha (26.4%) of the world's acidic soils. It is of paramount importance to know the effect of anthropogenic perturbations on nutrient cycling processes of acidic soils. For example, soil contamination by heavy metals is known to be one of the most important anthropogenic perturbations and is of great worldwide concern. Heavy metals adversely affect the activity and diversity of microbial community associating with soil elemental cycling (Dai et al., 2004; Khan et al., 2010). Among all metals, copper (Cu) and arsenic (As) have received much attention because of their high toxicity and solubility in agricultural soils (Sheldon and Menzies, 2005; Smedley et al., 1996). In addition to this, As has received a lot of attention in East and Southeast Asia because of the well-publicized catastrophes in India (West Bengal), Bangladesh, and Vietnam (Meharg and Hartley-Whitaker, 2002; Singh and Ma, 2007). Cu enters the soils due to several sources, e.g. mining, smelting, electroplating, biosolids, agricultural

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application of sludge, copper-containing pesticides and fertilizers (Lone et al., 2008). Elevated levels of As in soils on the other hand originate from the widespread use of arsenicals as pesticides, primary and secondary industrial inputs (Adriano, 2001). Numerous field and microcosm studies have demonstrated the adverse effects of As and Cu on soil microbial communities (Brandt et al., 2006; Chodak et al., 2013; Wightwick et al., 2013). However, little information is available on the effects of Cu and As on soil ammonia oxidizers (especially in acidic soils) which are an important functional groups within the N cycle.

Nitrification is an important soil function that could be reduced by heavy metals and therefore considered as sensitive microbial process with regards to heavy metal stress (Smolders et al., 2001). Studies have particularly emphasized the response of ammonia oxidizing bacteria to heavy metals such as Zn, Cu and Hg (Lee et al., 2011; Liu et al., 2010; Mertens et al., 2009; Vasileiadis et al., 2012). Some of these studies reported a sensitive response of the AOB community to heavy metals (Frey et al., 2008; Lee et al., 2011; Vasileiadis et al., 2012); AOA, on the other hand, appear to be tolerant to the stress of heavy metal such as Hg and Zn (Liu et al., 2010; Vasileiadis et al., 2012). Previously, AOA-*amoA* gene diversity profile in heavy metal polluted soils has been established by DGGE and T-RFLP based community shifts (Mertens et al., 2009; Ollivier et al., 2012; Vasileiadis et al., 2012). However, these studies could not offer detailed AOA phylogenetic information such as what are the AOA in heavy metal polluted soils. Moreover, little information is available on the responses of ammonia oxidizers to As and Cu in acidic soils, where AOA are considered to be a main contributor to ammonia oxidization (Lee et al., 2011; Zhang et al., 2012).

The objective of the present study was to assess the responses of ammonia oxidizing microbes (AOA and AOB) to the Cu and As stresses particularly in acidic soils after 10 weeks of incubation. The effects of heavy metal on abundance and community diversity were studied based on the q-PCR, DGGE and clone library sequencing analysis. The results may help to improve our understanding of the ecological effects of heavy metals on the abundance and diversity of ammonia oxidizers in acidic alfisols.

2. Experimental procedures

2.1. Soil samples and experimental setup

Two different types of pristine alfisols (from both India and China) were collected for short-term incubation experiments. Triplicate composite sampling was performed for soil collection. In brief, each top soil sample (10 cm in depth) was composed by pooling three subsamples obtained in a 1 × 1-m area. Alfisol-RA was collected from Rayka, a site located in semiarid region near the Mahi River basin, Gujarat, western India. 'RA' is an oligotrophic silty-loam soil, with low organic matter (0.86%), low moisture content (8%, equivalent to 40% of maximum WHC) and pH of 5.1. Alfisol-HZ was collected from Hangzhou, Zhejiang province, eastern China. 'HZ' alfisol is a clay loam, consisting of relatively more organic matter (2.88%) with a moisture content of 18.9% (equivalent to 55% of maximum WHC) and a pH of 5.6. The observed field values of NH_4^+–N for RA and HZ soils at the time of sampling were 9.05 (±2.38) and 9.37 (±1.72) mg kg^{-1} dry soil, respectively. The Cu concentrations in these soils (4.2 mg kg^{-1} dry soil in HZ and 8.6 mg kg^{-1} dry soil in RA) were below the permissible limits established by the Indian Standard guidelines, while As was not detected in any of the soils.

We constructed series of short-term laboratory experiments with well mixed samples of RA and HZ (Liu et al., 2010). Soil samples were ground to a size finer than 2.5 mm and stored at 4 °C. Before conducting the incubation experiments, soils moisture content was adjusted to the original soil moisture content at the time of sampling (40% WHC for RA soils and 55% WHC for HZ soils). Two different series of short-term experimental setups were constructed with RA and HZ soils. Three different types of metal treatments viz. As-spiked, Cu-spiked and combination of As and Cu-spiked (As + Cu) were chosen for each short-term experimental series (RA and HZ series). Soil without addition of heavy metal contamination was considered as background control treatment (CK). Aqueous solutions of $CuCl_2$ (Sigma-Aldrich, ≥99.9% purity) and $Na_2HAsSO_4{\cdot}7H_2O$ (Sigma-Aldrich ≥98% purity) were used as source of Cu and As, respectively, and pH of the soils maintained to their natural values. The type of experimental design was completely randomized block. In Cu amended experiments the final concentrations of Cu were adjusted to 0, 125, 250 and 500 mg kg^{-1} dry soil, while in As amended soils the concentrations were adjusted to 0, 17.5, 35, and 70 mg kg^{-1} dry soil. Concentrations for combined As and Cu (As + Cu) treatment were as follows: As-35 + Cu-250, As-35 + Cu-500, As-70 + Cu-250 and As-70 + Cu-500. Short-term experiments were named according to their nominal As and Cu concentrations. A total of 20 g (dry weight) of each sample was placed in a 200 ml plastic jar. For each concentration of heavy metal three independent replicates were performed. The experimental setups were incubated at 25 °C for 10 weeks in the dark. The samples were destructively harvested at the end of 10th week.

2.2. Soil chemical analysis and potential nitrification rates (PNRs)

Soil chemical characteristics were analyzed before and after incubation periods. Soil gravimetric water content was determined after drying 10 g subsamples at 105 °C for 24 h. Soil pH was measured at the soil to water ratio of 1:2.5 and soil organic matter content was determined with $K_2Cr_2O_7$ oxidation method. Soil samples were digested with HNO_3 + HCl (10 ml, 1:1 v/v) for analysis of heavy metals by ICP-OES (PerkinElmer, California, USA). Soil ammonium and nitrate contents were extracted from fresh soil samples with 2 M KCl and determined by a continuous flow analyser (SAN++, Skalar, Holland). Particle size distribution was measured using the rapid sieving procedure. PNRs were measured using the chlorate inhibition method (Kurola et al., 2005). All the analysis was performed in triplicate samples and values are represented as mean value (n = 3) with standard error.

2.3. Soil DNA extraction and quantification of genes by real-time quantitative PCR (q-PCR) assays

DNA was extracted from 0.5 g of soil samples using Ultraclean™ soil DNA Isolation Kits (MoBio Laboratory, Carlsbad, CA, USA). Abundance of bacterial-16S rRNA, archaeal-16S rRNA, bacterial-*amoA* and archaeal-*amoA* genes was determined by q-PCR with the iCycler iQ5 thermocycler (Bio-Rad, Hercules, CA, USA) by the method described by Zhang et al. (2009). Quantification of bacterial-16S rRNA gene was performed using primers BACT1369F/PROK1541R and probe TM1389F (Suzuki et al., 2000). Primers A364Af and A934bR were used for quantification of archaeal-16S rRNA genes (Kemnitz et al., 2005). Primers Arch-*amoA*F/Arch-*amoA*R (Francis et al., 2005) and *amoA*1F/*amoA*2R (Rotthauwe et al., 1997) were used for quantification of archaeal-*amoA* genes and bacterial-*amoA* genes, respectively, with SYBR® Premix Ex Taq™ (TaKaRa). Detailed thermal profiles of all the primers as well as standard curves for q-PCR were reported in earlier studies (He et al., 2007; Shen et al., 2008).

2.4. Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE of archaeal-*amoA* gene community was performed with a DCode Universal Mutation Detection System (Bio-Rad, Germany). Archaeal-*amoA* genes were amplified using primers Cren-*amoA*23f