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Responses of potential ammonia oxidation and ammonia oxidizers community to arsenic stress in seven types of soil

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ABSTRACT

Soil arsenic contamination is of great concern because of its toxicity to human, crops, and soil microorganisms. However, the impacts of arsenic on soil ammonia oxidizers communities remain unclear. Seven types of soil spiked with 0 or 100 mg arsenic per kg soil were incubated for 180 days and sampled at days 1, 15, 30, 90 and 180. The changes in the community composition and abundance of ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) were analyzed by terminal restriction fragment length polymorphism (T-RFLP) analysis, clone library sequencing, and quantitative PCR (qPCR) targeting *amoA* gene. Results revealed considerable variations in the potential ammonia oxidation (PAO) rates in different soils, but soil PAO was not consistently significantly inhibited by arsenic, probably due to the low bioavailable arsenic contents or the existence of functional redundancy between AOB and AOA. The variations in AOB and AOA communities were closely associated with the changes in arsenic fractionations. The *amoA* gene abundances of AOA increased after arsenic addition, whereas AOB decreased, which corroborated the notion that AOA and AOB might occupy different niches in arsenic-contaminated soils. Phylogenetic analysis of *amoA* gene-encoded proteins revealed that all AOB clone sequences belonged to the genus *Nitrosospira*, among which those belonging to *Nitrosospira* cluster 3a were dominant. The main AOA sequence detected belonged to *Thaumarchaeal* Group 1.1b, which was considered to have a high ability to adapt to environmental changes. Our results provide new insights into the impacts of arsenic on the soil nitrogen cycling.

Introduction

Heavy metal contamination in agricultural soil is one of the most important public concerns because these metals are considered to have persistent unfavorable impacts on the diversities and activities of soil microorganisms and their related ecological processes (Li et al., 2009; Mertens et al., 2009; Luo et al., 2019). Ammonia oxidation process, the first and rate-limiting step in the nitrification process, is reported to be highly sensitive to the heavy metal contamination and the potential ammonia oxidation (PAO) is usually used as indicators to reveal the impacts of heavy metal stress on soil nitrogen transformation function (Mertens et al., 2009; He et al., 2018). In recent years, the metalloid arsenic has

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received much attention for its high toxicity and prevalence in agricultural soil (Zhao et al., 2014). A joint pollution survey report issued in 2014 (http://www.mee.gov.cn/gkml/sthjbgw/qt/201404/t20140417_270670.htm) revealed that arsenic has become the third most abundant inorganic pollutant in all sampled soils from China (2.7% of the collected samples exceeded the limits published by the Ministry of Ecology and Environment of the P.R. China), implying that many types of arable soils throughout China possess a high risk of being contaminated with arsenic. However, studies insight into the responses of PAO to arsenic stress in agricultural soils, especially in different types of soils, remain scarce.

The toxicity of arsenic in soil not only depends on the total concentration of arsenic, but also on its chemical and combined forms. The available arsenic content in soils has been regarded as an alternative parameter to total arsenic content for assessing the potential biological effects of arsenic on plants and microorganisms because this form of arsenic can be easily taken up by plants and has direct toxic effects on microorganisms (Wang et al., 2017). Besides, it is well accepted that arsenic redistribution in soil take place after the soil is contaminated by arsenic (Tang et al., 2007). Our previous studies demonstrated that the spiked arsenic in different types of soils distributed into five fractionations of arsenic and their proportions changed with time (Wang et al., 2015, 2017). The fractionations of arsenic could reflect the degree of arsenic binding to soil colloids and the bioavailability of arsenic in soils (Tang et al., 2007), so understanding the influences of arsenic fractionations on soil PAO and the change in related microbial communities is a key issue to accurately evaluate and monitor the soil disturbances caused by the contamination of arsenic.

Ammonia oxidation plays a vital role in the global nitrogen cycle, which is biologically driven by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA). Recently, a single microorganism which could catalyze the entire process of oxidizing ammonia to nitrate (including ammonia oxidation and nitrite oxidation) was reported and named as complete ammonia oxidizer (Comammox) within the *Nitrospira* genus (Daims et al., 2015; Van Kessel et al., 2015). Ammonia monooxygenase α-subunit (*amoA*) is frequently used as a molecular marker to determine the diversity and abundance of AOB and AOA communities in various environments. Previously, numerous studies that used *amoA* as a marker particularly emphasized the response of ammonia oxidizing microorganisms to heavy metals (Mertens et al., 2009; Liu et al., 2010; Luo et al., 2019; Liu et al., 2019). The influence of heavy metals on AOB and AOA communities and functions has been controversial. He et al. (2018) demonstrated that differences in *amoA* gene transcript abundance indicated that AOA may be more resistant to copper stress than AOB. On the contrary, zinc and mercury seem to have a profound impact on AOA community composition, but only a slight effect on AOB communities (Mertens et al., 2009; Liu et al., 2014). AOB and AOA communities also have diverse responses to soils contaminated with compound heavy metal pollutants. For example, Ollivier et al. (2012) pointed out that high concentrations of arsenic and lead result in a clear reduction of AOA and AOB populations and pointed that some ecotypes of AOA could tolerate higher concentrations of lead and arsenic in soils than AOB. In two acidic alfisols, AOA were found to be more abundant than AOB in all heavy metal-treated soils (arsenic, copper, and arsenic + copper), and no apparent shift in AOA communities was observed even at higher concentrations of arsenic and copper (Subrahmanyam et al., 2014a). Clearly, remarkable differences in the responses of AOB and AOA communities to different kinds of heavy metal stresses have been observed. Unlike many kinds of cationic heavy metals, most of the inorganic arsenic in soils exists as anionic compound, which result in the disparate and complicated effects of arsenic on soil microbial-mediated process. Although certain microorganisms can adapt to arsenic toxicity, thrive in arsenic-enriched environments, or even biologically metabolize arsenic species (Rahman and Hassler, 2014; Zhu et al., 2017; Chen et al., 2020; Li et al., 2021), adverse effects of arsenic on soil nitrification processes and AOB and AOA community compositions and abundances have been reported (Turpeinen et al., 2004; Subrahmanyam et al., 2014a, 2014b). However, our knowledge about the impact of arsenic on AOB and AOA community composition and abundance in different soil types is still in infancy.

This study was to investigate the responses of ammonia oxidizing microorganisms to fractionations of arsenic in different types of arable soils during a 180-day incubation. Seven types of soil with different soil properties were selected from different regions throughout China, and potential ammonia oxidation (PAO) rate was evaluated for soils incubated with or without arsenic at different sampling time. Terminal restriction fragment length polymorphism (T-RFLP) analysis, clone library sequencing and real-time quantitative PCR (qPCR) approach targeting the *amoA* gene were used to describe the community composition and abundance of AOB and AOA at different sampling times. Simultaneously, the interactive relationships among soil physiochemical properties, contents of available arsenic and arsenic fractionations, and AOB and AOA community compositions were also analyzed. We postulated that AOB community might be more vulnerable than AOA community to the addition of arsenic during the incubation because of their different sensitivities to arsenic. The results of this study may help to improve our understanding of the ecological effects of arsenic on the community composition and abundance of AOB and AOA, as well as the ecological niches of AOB and AOA in different types of soil under arsenic stress.

1. Materials and methods

1.1. *Microcosm experiments*

Seven types of soils were collected from the 0–20 cm layer in cultivated upland fields located in six provinces of China. The main crops in these fields were maize. These soils from different geological regions were classified as cinnamon soil (CS), black soil (BS), brown soil (BNS), red soil derived from quaternary red clay (RS1), red soil derived from purple sandy shale (RS2), irrigated desert soil (IDS) and purplish soil (PS). The sampling sites and soil classification were described in detail in our previous study (Wang et al., 2015, 2017). The soils were air-

dried, sieved through a 2 mm nylon sieve, and stored at room temperature.

Before the microcosm incubation experiment, all soils were homogenized and adjusted to 60% maximum water holding capacity, then incubated at 25°C for a week to reduce the variability among soils. The effects of arsenic on soil ammonia oxidizers in different soils were explored at two arsenic levels. Each 30 g dry soil sample was placed in a 100 mL flask and then added with double-distilled water or 1000 mg As/L of Na_3AsO_4 solution to generate the arsenic-treated soils of 0 (control treatment, CK) or 100 mg As/kg. The flasks were mixed thoroughly then incubated in an artificial climate incubator at 25°C and maintained at 60% of soil maximum water holding capacity in the dark. The tops of the flasks were covered with filter membranes to allow air exchange and avoid evaporation. The flasks were weighed weekly and sterile distilled water was added to supplement the water lost. Finally, soil samples were destructively harvested from the flasks after 1, 15, 30, 90 and 180 days of incubation for subsequent analysis. Experiments were performed in triplicate for each arsenic level and each sampling time.

1.2. Soil physicochemical parameters and PAO rate analysis

Soil physicochemical characteristics were analyzed before the microcosm experiment in accordance with the standard methods recommended by Lu (2000). The available arsenic was determined using a 0.5 mol/L $NaHCO_3$ extraction method, and five fractionations of arsenic in soils (non-specifically adsorbed arsenic, F1; specifically adsorbed arsenic, F2; arsenic associated with amorphous and poor-crystalline Fe/Al oxides, F3; arsenic associated with well-crystalline Fe/Al oxides, F4; and residual arsenic fraction, F5) were determined using a sequential extraction method described by Wenzel et al. (2001) with some modifications. Detailed analytical procedures about the selected physicochemical parameters are presented in supplementary section. Briefly, the physicochemical parameters of seven selected soils were significant different and summarized in **Appendix A Table S1**. The available and fractionations of arsenic in sampled soils were also determined as shown in **Appendix A Figs. S1 and S2**. Although the available and fractionations of arsenic contents in seven types of soil were conspicuously different, obvious decreases in the contents of available arsenic and F1 with increasing incubation time were found in all the soils. Arsenic distribution in soils changed from more available forms to less available forms with time, which subsequently resulted in the contents of F3, F4 and F5 fractions in soils gradually increased.

The chlorate inhibition method (Kurola et al., 2005) was used to measure the soil PAO rate. In brief, 5.0 g of fresh soil was added to a 50 mL centrifuge tube containing 20 mL of phosphate buffer solution (g/L: KCl, 0.2; NaCl, 8.0; Na_2HPO_4, 0.2; NaH_2PO_4, 0.2) with 1 mmol/L $(NH_4)_2SO_4$ at pH 7.4, then potassium chlorate was added to a final concentration of 10 mg/L to inhibit nitrite oxidation. The suspension was incubated at 25°C for 24 hr and the nitrite concentrations in the suspensions were measured using the FIAstar™ 5000 flow analyzer (Foss, Denmark). The calculated rate of nitrite accumulation over time was used to represent the PAO rate. All analysis was performed in triplicate and values are presented as mean value ($n = 3$) with standard error.

1.3. Soil DNA extraction and T-RFLP analysis of amoA gene

DNA was extracted from 0.5 g of fresh soil samples using the FastDNA® SPIN Kit for soil (MP Biomedicals, Carlsbad, CA) according to the manufacturer's protocol. The concentration and quality of the DNA were estimated by running extracts on a 1.0% agarose gel and by NanoDrop™ One UV-Vis spectrophotometry (Nanodrop Technologies, Wilmington, USA).

The primers used for PCR amplification of the bacterial and archaeal *amoA* genes were amoA-1F/amoA-2R (Rotthauwe et al., 1997) and Arch-amoAF/Arch-amoAR (Francis et al., 2005), respectively. The 5' ends of the forward primers were labeled with 6-FAM before amplification. A detailed description of the PCR assay is provided in the supplementary materials and methods. T-RFLP analysis of bacterial and archaeal *amoA* genes was performed in triplicate as described previously (Wang et al., 2009). In brief, after amplification and purification, amplicons were digested with *BsaJI* (bacterial *amoA*) or *Hpy8I* (archaeal *amoA*) (Fermentas, St Leon Rot, Germany), and the products were size separated using an ABI 3730 Genetic Sequencer (Applied Biosystems, USA). The relative abundance of the terminal restriction fragments (TRFs) was calculated as the percentage of total peak area in the T-RFLP profiles. The TRFs with a relative abundance >1% were selected for analysis.

1.4. Cloning, sequencing, and phylogenetic analysis

Clone libraries were constructed using spiked RS1, IDS and PS soils collected at 1 day. DNA extracted form triplicate samples were mixed. These selected soils were significantly different in soil physiochemical properties and contained diverse types of T-RFs. Two clone libraries were constructed for bacterial *amoA* gene fragments (RS1-1d and IDS-1d) and three clone libraries were constructed for archaeal *amoA* gene fragments (RS1-1d, IDS-1d and PS-1d). The primers used were the same as those used for T-RFLP analysis but without 6-FAM. The purified PCR products were ligated into the pMD19-T vector (TaKaRa, Japan), and the ligation products were transformed into *Escherichia coli* JM109 competent cells according to manufacturer's instructions (TaKaRa, Japan). Finally, 52 positive clones for AOB and 111 positive clones for AOA were confirmed before being sequenced by an ABI 3730 sequencer using BigDye-terminator cycle sequencing chemistry (Applied Biosystems, USA).

Bellerophon was used to identify and delete sequences of chimeric origin in multiple sequence alignments (Huber et al., 2004). The aligned DNA sequences were translated into amino acid sequences using MEGA 7, and neighbor-joining trees were constructed as described by Wang et al. (2009) using p-distances and pairwise deletion of gaps and missing data with 1000 bootstrap replicates. The *amoA* gene sequences obtained in this study have been deposited in GenBank under the accession numbers MF508743-MF508794 for bacterial *amoA* genes and MF508795-MF508916 for archaeal *amoA* genes.

1.5. *Real-time quantitative PCR (qPCR) analysis*

The qPCR analysis of the bacterial and archaeal *amoA* genes was carried out with the iCycler iQ5 thermocycler (Bio-Rad, Hercules, CA, USA). Detailed thermal profiles of the amplification as well as standard curves for qPCR were performed as described by He et al. (2007). PCR efficiency (*E*) and the linear correlation (R^2) of the standard curves ranged from 84.7% to 94.1% and from 0.990 to 0.999, respectively, for AOB, and from 87.7% to 101.8% and from 0.990 to 0.995, respectively, for AOA.

1.6. *Statistical analysis*

Factorial analysis of variance (ANOVA) was carried out to detect significant effects of different soil types, arsenic levels, and incubation periods and their interactions on PAO rate. The differences in Shannon diversity indices, Simpson diversity indices and *amoA* gene abundances among samples were evaluated using one-way ANOVA. Post-hoc analysis (Duncan's multiple-range test) was performed to test for significant differences between treatments. $P < 0.05$ was considered significant. Pearson's correlation analysis was employed to quantify the relationships among the PAO rate, the contents of available arsenic and fractionations of arsenic, and abundances of AOB and AOA *amoA* genes. These statistical analyses were conducted with SAS v. 9.2.

To determine the influence of soil type, arsenic level, and incubation time on the T-RFLP profiles of both bacterial and archaeal *amoA* genes, permutational multivariate analysis of variance (PERMANOVA) was performed. Variation partitioning analysis (VPA) was carried out to quantify the relative contribution of soil physicochemical properties and arsenic fractionations to the responding of AOB and AOA community compositions. Canonical correlation analysis (CCA) was conducted using *R* (version 3.4.2) to reveal the relationships between functional *amoA* gene T-RFLP profiles and arsenic fractionations for AOB and AOA separately. The correlation heatmaps between TRFs and environmental factors were plotted using the R package "pheatmap", with the clustering distance "Euclidean" and clustering method "correlation". Structural equation modeling (SEM) was performed with AMOS (Amos 21.0, Development Corporation, Meadville, PA, USA) to test the hypothetical causal relationships among soil properties, soil arsenic fractions, AOB and AOA community composition and abundance, and soil PAO rate. The data matrix was fitted to the model using the maximum-likelihood estimation method. The overall goodness-of-fit of the model was displayed by a low chi-square/degree of freedom test (CHI/DF < 3), high adjusted goodness-of-fit index (AGFI > 0.80), and low root square mean errors of approximation (RMSEA < 0.09) and Akaike information criteria (AIC).

2. Results

2.1. *PAO and its relationship with available arsenic and fractionations of arsenic*

Factorial ANOVA revealed that the soil PAO rate was significantly affected by soil type ($P < 0.001$), by arsenic level ($P < 0.001$) and by incubation time ($P < 0.001$). The interaction effects of soil type, incubation time and arsenic level was also significant ($P < 0.001$, **Appendix A Table S2**). As shown in Fig. 1, the PAO rates in different types of soils were dramatically different due to the conspicuous differences in soil properties. In all soils except for PS, the change trend in PAO rate was basically consistent. Generally, the PAO rate in soils increased sharply during the first 30 days of incubation and then decreased, regardless of whether the soils were spiked with arsenic or not. However, the effects of arsenic stress on PAO rate in different soils were diverse. Arsenic addition had a positive effect on the PAO rate in PS soil during the entire incubation period. Although the PAO rate in RS1 soil was

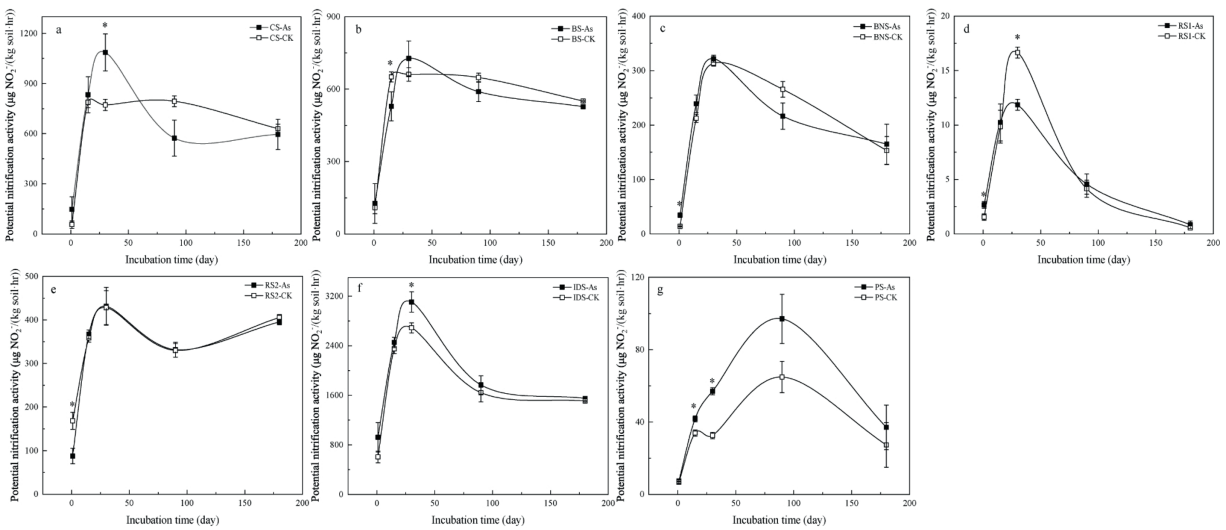

Fig. 1 – Response of PAO rate to arsenic (As) stress in different soils during the 180-day incubation. Results list for CS (a), BS (b), BNS (c), RS1(d), RS2 (e), IDS (f) and PS (g) soils, respectively. Error bars indicate standard deviation ($n = 3$). Asterisk (*) in the in figures indicate that there are significant differences in the PAO rate between CK and As-treated soils at that time.

consistently low, an adverse effect of arsenic addition could be observed especially at 60 days.

Information about the variation in available arsenic and fractionations of arsenic in these soils during the incubation period was described in detail in our previous study (Wang et al., 2015, 2017), and a summary of the results relevant to the present study are provided in the supplementary material (**Appendix A Figs. S1 and S2**). Correlation analysis between PAO rate and the contents of fractionations of arsenic showed that PAO rate was not significantly correlated with the available arsenic content in these soils. However, a significant positive correlation was found in the relationship between PAO rate with F1 ($r = 0.39$, $n = 35$, $P < 0.05$), but not with the other arsenic fractionations.

2.2. *Changes in AOB and AOA* amoA *gene compositions in response to arsenic stress*

The AOB and AOA *amoA* gene compositions in all tested soils at 1, 15, 30, 90 and 180 days were analyzed based on the T-RFLP profiles as shown in **Appendix A Figs. S3** and **S4**.

2.2.1. *AOB* amoA *gene composition*

Seven TRFs of the AOB *amoA* gene were detected in all soil samples using the *BsaJI* restriction enzyme. There were conspicuous differences in the relative proportions of individual TRFs in different treatment soils (**Appendix A Fig. S3**). PERMANOVA showed that the effect of soil type on AOB *amoA* gene T-RFLP profiles was significant (Table 1). Based on analysis of α-diversity indices, the highest average Shannon-Wiener diversity index (*H*, 1.71) and lowest mean average Simpson's Dominance diversity index (*S*, 0.2) values were observed in IDS soil. Conversely, the lowest average *H* (0.94) and highest average *S* (0.51) values were observed in BNS soil (**Appendix A Table S3**). A considerable change in AOB community composition could be observed in RS1 and PS arsenic-treated soils during the incubation (**Appendix A Figs. S3** and **S5**). Correlation analysis was conducted to explore the correlation between environmental factors (including soil physicochemical properties and arsenic fractions) and the relative abundances of individual TRFs (Fig. 2a). The contents of TP, available arsenic, F1 and pH were significantly negatively correlated with the relative abundance of the 60-bp TRF, but positively correlated with the relative abundances of the 67-,118- and 428-bp TRFs. The contents of Fe_o and F3 in soils were also important, which were significantly positively correlated with the relative abundance of the 60-bp TRF and negatively correlated with the relative abundances of the 118- and 428-bp TRFs. The relative abundance of the 155-bp TRF was affected by the contents of Al oxides (Al_o and Al_d), AN, F2, Fe_o, F1 and pH in the soil.

VPA was conducted to ascertain the relative contributions of soil properties and arsenic fractions to the changes of AOB community composition. Soil properties and arsenic fractions explained 20.3% and 18.9% of the variations in the AOB community composition, respectively. The interaction between soil properties and arsenic fractions explained 32.0% of the variation, and 28.8% of the variations remained unexplained. (Fig. 3a). Next, CCA illustrated that the available arsenic and five fractionations of arsenic in soils could explain 46.8% of the total variations in AOB community composition (Fig. 4a). The AOB community composition in IDS soil was distinct from that in the other soils, which was not affected by arsenic fractionations. The compositions of AOB in CS and RS2 soils were similar, which were mainly affected by the contents of available arsenic and F1. The contents of F2, F3 and F5 were crucial factors altering the AOB community compositions in the other four soils. Additionally, an obvious temporal variation in AOB community composition was observed in PS soils.

2.2.2. *AOA* amoA *gene composition*

For the AOA *amoA* gene, there were eight TRFs after digestion with the *Hpy8I* restriction enzyme in all soil samples (**Appendix A Fig. S4**). PERMANOVA showed that the effects of soil type as well as incubation time on AOA *amoA* gene T-RFLP profiles were significant (Table 1). Based on α-diversity parameters of AOA, the arsenic-treated IDS soil at 180 d had the highest *H* value (1.95, **Appendix A Table S3**). Arsenic addition apparently increased the *H* value in BS and RS1 soils, which meant that elevated levels of arsenic might stimulate the growth of specific AOA species in these soils. The increase in AOA community diversity indicated that the negative effect of arsenic addition on the AOA community was slight, suggesting that the species in AOA community were more tolerant to arsenic stress than those in AOB community.

As shown in Fig. 2b, the relative abundances of AOA TRFs were also significantly affected by both soil properties and arsenic fractions. The contents of F1 and pH were significantly positively correlated with the relative abundance of the 196- and 614-bp TRF and negatively correlated with that of the 572-bp TRF. The CEC, contents of AN, SOM, TN, AK were significantly positively correlated with the relative abundance of the 116-bp TRF and negatively correlated with that of the 614-bp TRF. An increase in available arsenic content was associated with the decreases in the relative abundances of the 149- and 572-bp TRFs.

VPA result revealed that soil properties and arsenic fractions could explain 22.5% and 15.0% of the variations in the AOA community composition, respectively. The interaction between soil properties and arsenic fractions explained 28.4% of the AOA community composition variations, and 34.1% of the variations remained unexplained (Fig. 3b). CCA demon-

Table 1 – Permutation multivariate (PERMANOVA) analyses of the effect of soil type (Soil), arsenic level (As) and incubation time (Time) on bacterial and archaeal *amoA* genes T-RFLP profiles.

	AOB		AOA	
	R^{2a}	*Pr* (>*F*)[b]	R^2	*Pr* (>*F*)
Soil	0.796	**0.001**	0.554	**0.001**
As	0.006	0.154	0.020	0.238
Time	0.024	0.045	0.151	**0.003**
Soil × As	0.021	0.354	0.020	0.962
Soil × Time	0.080	0.299	0.114	0.644

[a] R^2-value (effect size) shows the percentage of variation explained by the factors.
[b] Significant differences ($P < 0.05$) are indicated in bold.

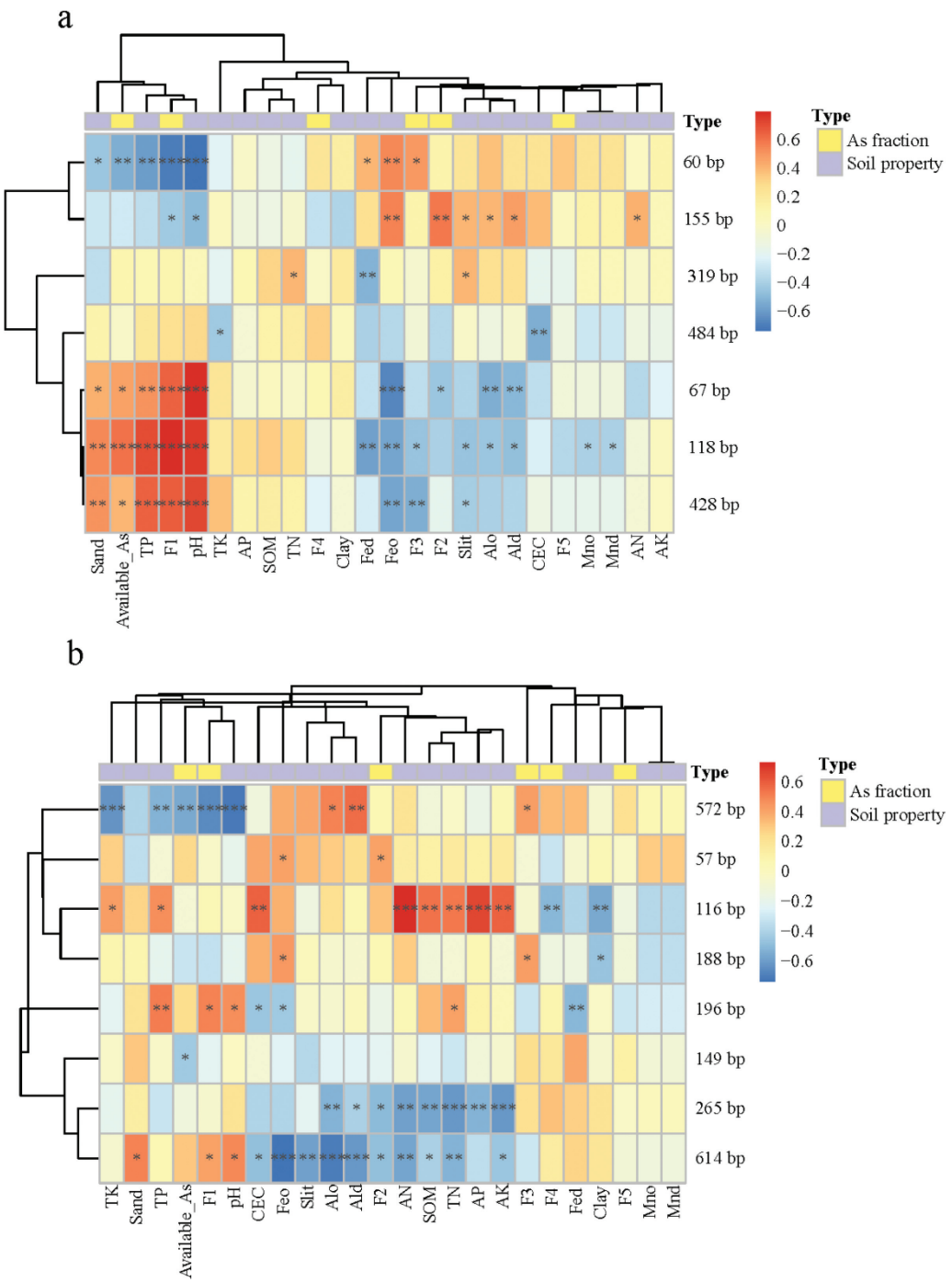

Fig. 2 – Correlation heatmap analysis of the relative abundance of TRFs and different environmental factors for AOB (a) and AOA (b). *P < 0.05, **P < 0.01, ***P< 0.001; SOM: soil organic matter; CEC: cation exchange capacity; TN/TP/TK: soil total N, P and K; AN/AP/AK: soil available N, P and K; $Fe_d/Al_d/Mn_d$: soil free Fe, Al and Mn oxides; $Fe_o/Al_o/Mn_o$: soil amorphous Fe, Al and Mn oxides; Clay/Slit/Sand: the percentage contents of soil clay, slit, and sand; F1: non-specifically absorbed As; F2: specifically absorbed As; F3: As associated with amorphous and poor-crystalline hydrous oxides of Fe and Al; F4: As associated with well-crystalline hydrous oxides of Fe and Al; F5: residual As.

strated that F1 and F2 were the two most key factors driving the AOA community (Fig. 4b). Apparently, the samples collected from the same soil type were not closely grouped together, suggesting a considerable temporal variation in the AOA community composition at sampling time (**Appendix A Fig. S5**).

2.3. *Changes in AOB and AOA* amoA *gene abundances in response to arsenic stress*

There was a considerable variation in the abundances of the AOB and AOA *amoA* genes in the seven types of soils at each sampling time (Fig. 5). For AOB, *amoA* gene abundance among

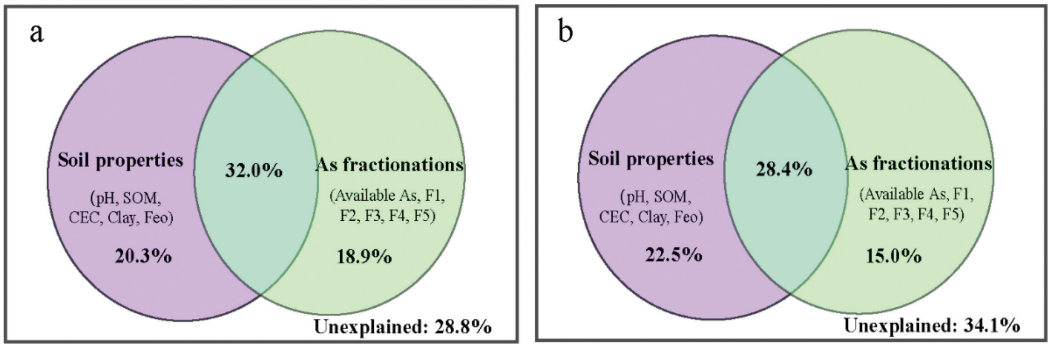

Fig. 3 – Variance partitioning analysis (VPA) of the relationships of soil properties, arsenic fractions and AOB (a) and AOA (b) community composition. SOM: soil organic matter; CEC: cation exchange capacity; Clay: the percentage contents of soil clay; Fe_o: soil amorphous Fe oxides; F1: non-specifically adsorbed arsenic; F2: specifically adsorbed arsenic; F3: arsenic associated with amorphous and poor-crystalline Fe and Al oxides; F4: arsenic associated with well-crystalline Fe and Al oxides; F5: residual arsenic.

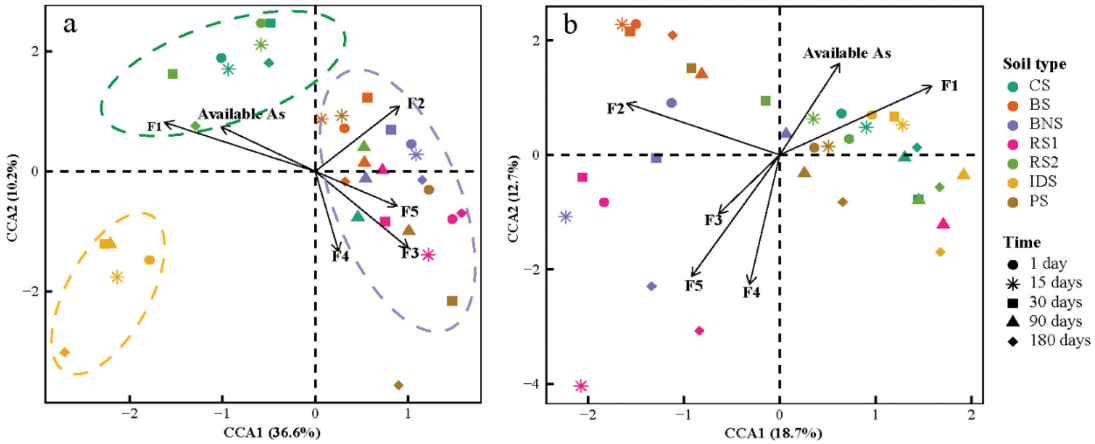

Fig. 4 – CCA ordination plots for available and fractionations of arsenic and the community compositions of AOB (a) and AOA (b). F1: non-specifically adsorbed arsenic; F2: specifically adsorbed arsenic; F3: arsenic associated with amorphous and poor-crystalline Fe and Al oxides; F4: arsenic associated with well-crystalline Fe and Al oxides; F5: residual arsenic.

different soils differed by approximately two orders of magnitude. AOB *amoA* gene abundance in IDS soil was high, ranging from 6.85×10^8 to 1.02×10^9 copies per gram of dry soil. By contrast, the *amoA* gene abundance in RS1 and PS soil was low, with copy numbers ranging from 1.64×10^6 to 1.81×10^7 and 6.47×10^6 to 2.32×10^7 copies per gram of dry soil, respectively. For AOA, the *amoA* gene abundances in CS and IDS were notably higher than those in the other soils and ranged from 2.95×10^8 to 7.89×10^8 and 3.07×10^8 to 5.04×10^8 copies per gram of dry soil, respectively. On the contrary, the AOA *amoA* gene abundance in BNS soil was much lower. In this study, the AOA *amoA* gene abundances in the soils were not consistently greater than those of AOB in all soils examined, with AOA/AOB ratios ranging from 0.20 to 19.29. This may be due to the large variation in pH value and soil nitrogen availability and contents (**Appendix A Table S1**).

As shown in Fig. 5, the changes in AOB and AOA *amoA* gene abundance in response to arsenic stress were different. In most cases, arsenic addition decreased the abundance of AOB *amoA* compared with CK soil, but increased the abundance of AOA *amoA*, indicating that AOA were more tolerant to arsenic contamination than AOB. Incubation time obviously influenced the effect of arsenic on both AOB and AOA *amoA* gene abundance. Generally, the AOA *amoA* gene abundances increased with prolonged incubation time, whereas the AOB *amoA* gene abundances presented the opposite responses. Moreover, with increasing incubation time, the AOA/AOB ratios in arsenic-treated soil increased (Table 2).

2.4. *Linkages among soil PAO rates and soil properties, arsenic fractions, AOB and AOA community compositions and abundances*

Correlation analysis indicated that the PAO rate was significantly positively correlated with both AOB ($r = 0.84$, $P < 0.01$, $n = 70$) and AOA ($r = 0.61$, $P < 0.01$, $n = 70$) *amoA* gene abundance, indicating that both AOB and AOA could perform ammonia oxidation, whereas their relative contributions were different. SEM was conducted to explore the direct and indirect effects of soil properties (pH, CEC, Fe_o), arsenic fractions (available As, F1, F2, F3, F4 and F5), AOB and AOA community compositions and abundances on the PAO rate (Fig. 6). 74% of

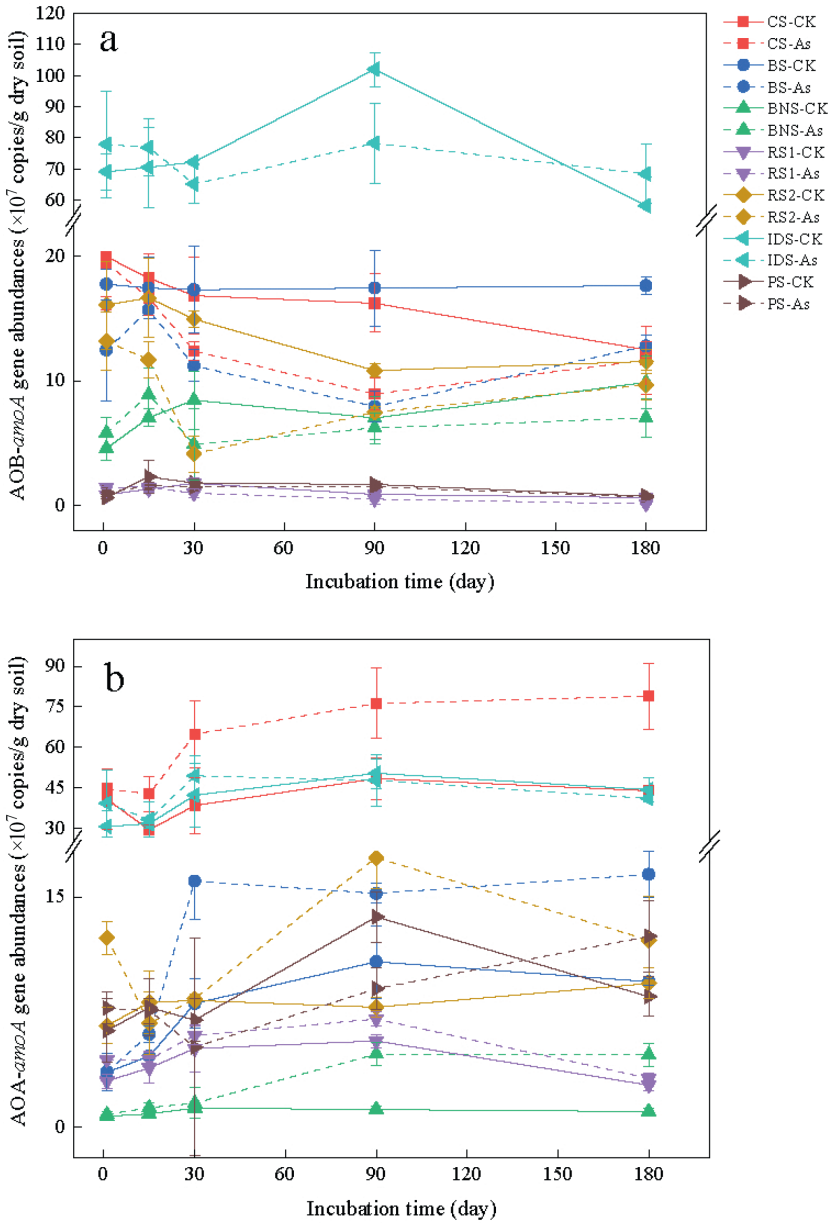

Fig. 5 – Abundances of AOB (a) and AOA (b) *amoA* genes in seven types of soil at five sampling times. Vertical bars indicate the standard deviation. Different colors represent different soil type. The solid lines show the soils without arsenic addition, and the dash lines show the soils spiked with 100 mg As/kg.

the variability in the PAO rate could be explained by these parameters.

The soil properties affected arsenic fractions through a positive path as well as affected AOB and AOA community compositions and abundances through a negative path, but the no significant direct effects of soil properties on the PAO rate (Fig. 6). Though no significant effects of AOB and AOA community compositions on the PAO rate, AOB and AOA abundances induced strong changes in the PAO rate, and the positive effects of AOB abundances ($r = 0.75$, $P < 0.001$) on the PAO rate were more significant than those of AOA abundances ($r = 0.28$, $P < 0.05$), suggesting that the greater contribution of AOB to nitrification than AOA in arsenic-treated soils.

2.5. *Phylogenetic analysis of AOB and AOA communities*

Soils spiked with arsenic were considered as candidates to construct the clone libraries. Twenty-five AOB *amoA* clones from IDS-1d and twenty-seven clones from RS1-1d were sequenced for phylogenetic analysis. Thirty-two AOA *amoA* clones from IDS-1d, forty clones from RS1-1d and forty clones from PS-1d were sequenced for phylogenetic analysis. Finally, twelve representative AOB *amoA* gene sequences and eleven

Table 2 – Ratios of AOA/AOB in all soils incubated with or without arsenic sampled at different times during incubation.

Soil Type	Treatment	Ratio of AOA-*amoA* abundance/AOB-*amoA* abundance				
		1 day	15 days	30 days	90 days	180 days
CS	CK	2.04	1.62	2.29	2.99	3.52
	As treated	2.30	2.56	5.23	8.51	6.77
BS	CK	0.20	0.26	0.47	0.62	0.54
	As treated	0.29	0.38	1.43	1.92	1.29
BNS	CK	0.15	0.12	0.14	0.16	0.10
	As treated	0.14	0.14	0.31	0.76	0.67
RS1	CK	3.32	2.90	2.83	6.10	4.11
	As treated	2.97	2.96	5.99	12.96	19.29
RS2	CK	0.41	0.49	0.55	0.72	0.81
	As treated	0.94	0.58	2.01	2.35	1.26
IDS	CK	0.44	0.45	0.58	0.49	0.76
	As treated	0.50	0.43	0.76	0.61	0.60
PS	CK	9.69	3.36	3.83	8.07	11.15
	As treated	7.45	4.71	3.49	5.95	17.98

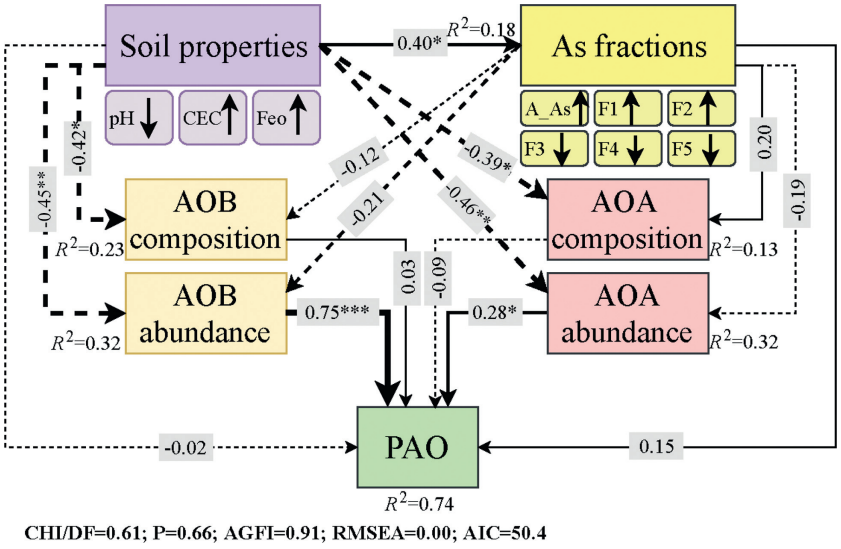

Fig. 6 – The SEM showing the hypothesized causal relationships among soil properties, soil arsenic fractions, AOB and AOA community composition and abundance, and soil PAO rate. CEC: cation exchange capacity; Fe_o: soil amorphous Fe oxides; A_As: available As content; F1: non-specifically adsorbed arsenic; F2: specifically adsorbed arsenic; F3: arsenic associated with amorphous and poor-crystalline Fe and Al oxides; F4: arsenic associated with well-crystalline Fe and Al oxides; F5: residual arsenic. The strength of the path standardized coefficients is expressed as the width of the arrows. Dashed and solid lines represent negative and positive paths, respectively. The R^2 represent the proportion of variances explained by the listed variables in the structural equation model. Significant levels are as follows: *P < 0.05, **P < 0.01 and ***P < 0.001. Goodness-of-fit statistics are shown under the modeling frame.

representative AOA *amoA* gene sequences (with at least 97% nucleotide similarity) and related sequences retrieved from NCBI were used for constructing phylogenetic trees of the deduced amino acid sequences.

For AOB *amoA*, only *Nitrosospira*-like sequences were present, and no *Nitrosomonas*-like sequence was found. Phylogenetic analyses showed that 52 deduced amino acid sequences of AOB *amoA* genes were distributed into three clusters (**Appendix A Fig. S6**). *Nitrosospira* cluster 3a (37 out of 52 clones) were the major AOB groups and made up a large proportion of the AOB community in both IDS (68% of the clones) and RS1 (74.1% of the clones) soil. This cluster was grouped with the *Nitrosolobus multiformis* and *Nitrosospira* sp. NI5 strains. *Nitrosospira* cluster 3b was comprised of eight *amoA* sequences closely grouped with the cultured *Nitrosospira briensis* strain. The other seven AOB *amoA* sequences belonged to *Nitrosospira* cluster 10. Notably, sequences affiliated with cluster 10 were only found in RS1 soil, indicating there

were substantial differences in AOB community composition among different types of soil. Unfortunately, some TRFs with the same size (e.g., the 60-bp TRF) belonged to different clusters, so it was impossible to classify AOB clusters based on the TRFs.

Phylogenetic analysis demonstrated that all the detected AOA *amoA* sequences fell within the previously characterized group 1.1b *Nitrosopheara* cluster and were designated as belonging to soil and sediment clusters 1, 2, 3 and 4 according to the classification described by Zhang et al. (2009). No sequence was classified into the group 1.1a *Nitrosopumilus* cluster or group 1.1a-associated cluster (**Appendix A Fig. S7**). Soil and sediment cluster 2 was the dominant AOA group (42.9% of the sequencing clones) in the tested soils, and the sequences in this cluster were grouped with the *Candidatus Nitrososphaera gargensis* strain. 39 clones (34.8%) were less abundant and belonged to soil and sediment cluster 1, and these sequences were affiliated with uncultured crenarchaeote clone P2-25 (HM803773) and clone AOAC-s sH09 (EU339459). Soil and sediment clusters 3 and 4 contained 15 (13.3%) and 3 (2.7%) *amoA* sequences, respectively.

3. Discussion

3.1. *Different responses of PAO rate to arsenic in seven types of soils*

The inhibitory effects of heavy metals on soil nitrification have been reported in many previous studies (Li et al., 2009; Liu et al., 2010; Mertens et al., 2010; Zhou et al., 2015). Ammonia oxidizer communities might exhibit distinct sensitivities to the stress of heavy metals or combinations of heavy metals, resulting in the different responses of PAO rate in soils (Mertens et al., 2010). In this study, although the arsenic spiked concentration in different soils was the same, there were contradictory findings regarding the effects of arsenic addition on PAO rates in different types of soils (Fig. 1). This meant that the different changes of PAO in soils were not only depended on the types of heavy metal, but were also related with the soil physicochemical properties, the bioavailability of arsenic, as well as the shifts in AOA and AOB communities and abundances.

It is well known that the arsenic present in soil is not completely bioavailable because it can be chelated by organic molecules and occurs as different chemical forms (Wang et al., 2017). Correlation analysis revealed that the PAO rate was significantly related to non-specifically adsorbed arsenic (F1), which is generally considered to consist of easily exchangeable, out-sphere complexes of arsenic (Wenzel et al., 2001). This arsenic banding state is the highly labile arsenic form in soil and can cause toxicity whenever it is present in excess. In the present study, due to the low spiked arsenic concentration in the soil as well as differences in soil adsorption characteristics, the non-specifically adsorbed arsenic in soils were less than 52.3 mg/kg and concurrently decreased with incubating time (**Appendix A Fig. S2**). This concentration did not reach the toxicity value to significantly inhibit the soil PAO as reported by Subrahmanyam et al. (2014a), who demonstrated the arsenic concentration inhibited the soil PAO by 50% (EC_{50}) in two acidic soils were proved to be 61.79 and 85.73 mg As/kg. Moreover, the toxic effects of arsenic on microbial processes are related to the capability of microbes to adapt through mechanisms such as resilience. Resilience is similar with the concept of community recovery, indicating that a microbial community restores to its primary composition after being disturbed (Shade et al., 2011; Tao et al., 2021). Microbial resilience ability might manifest as the selective recession of sensitive colonies and selective growth of tolerant microbial communities (Berg et al., 2012; Luo et al., 2019). Consequently, microbial community acclimation led to a reduction in the negative effects of arsenic toxicity on PAO. Arsenic-adapted ammonia oxidizers communities might gradually recover their diversities or activities over time, which resulted in little differences in the PAO rates between spiked and control soils prolonged with incubation time.

3.2. *Factors affecting AOA and AOB community structure and abundance*

As discussed in many previous studies, the changes in AOA and AOB community structure and abundance could be used as cost-effective and sensitive biological markers for soil monitoring (Vasileiadis et al., 2012; Subrahmanyam et al., 2014b). Our results also provide evidence for this hypothesis. It is well known that niche differentiation exists between AOA and AOB populations (Prosser et al., 2020). Some soil properties and environmental factors are thought to shape and dictate the differential growth and ecological niches of AOA and AOB, such as the ammonia substrate availability, pH value, temperature, soil moisture, salinity, oxygen levels, phosphate levels, and the availability of metals or metalloids (Leininger et al., 2006; Verhamme et al., 2011; He et al., 2012; Dodsworth et al., 2011; Bouskill et al., 2012; Zhalnina et al., 2012; Hatzenpichler, 2012; He et al., 2018). The present study showed that pH, CEC, clay content, available arsenic content, and the contents of Fe and Al oxides were all vital factors shaping the AOA and AOB community structure (Fig. 2). Among these, pH value and the available arsenic content contributed most to the changes in AOA and AOB communities. pH is important because it determines the availability of NH_3, which is the sole energy source for slow-growing ammonia oxidizing microorganisms (Hu and He, 2017). AOA and AOB possessed diverse ammonia affinities which resulted in their disparate contributions in different ecosystems (Zhang et al., 2012; Ouyang et al., 2018). It was reported that AOB were the main contributors to ammonia oxidation in neutral and alkaline agricultural soils, while AOA were more important in relatively harsh environments such as acidic soils (Xia et al., 2011; Zhang et al., 2012; Li et al., 2019). The pH values of the selected soils were in the range of 4.94–8.29, which resulted in the distinct niche differentiation of ammonia oxidizers in these soils (Fig. 5). The variations in the ratio of AOA to AOB also supposed this idea (Table 2). Moreover, pH could influence the availability of arsenic in the soil (Wang et al., 2017). pH affected the arsenic adsorption behavior by controlling the interaction between arsenic and its bearing phases, demonstrating a general increase in the arsenic availability with increasing soil pH (Smith et al., 1999; Yang et al., 2002). Hence, pH and available arsenic content are dependent variables affecting AOA or AOB community.

The importance of available arsenic and arsenic fractionations on AOA and AOB community structure were further verified by CCA (Fig. 4). Based on the results, the AOB community in the seven types of soil was classified into three groups depending on the contents of arsenic fractionations, while there was no obvious clustering of AOA community in different treatments, indicating that arsenic fractionations were more influential in shaping the AOB community than the AOA community. This was consistent with numerous studies indicated that heavy metals had a slight or even no obvious effect on AOA community structure in soils (Liu et al., 2010; Subrahmanyam et al., 2014a). The arsenic fractionations not only represent the distribution of arsenic in the soil, but they are also related to the toxicity and mobility of arsenic in soil (Gebel, 1997). These fractionations were extracted using a series of extraction chemicals and the first two combined forms were supposed to be more bioaccessible (Wenzel et al., 2001). Notably, due to the exogenous spiked arsenate solution in soils, the bioavailability of arsenic in soil was certainly high and the sum of F1 and F2 fractions accounted for 35.3%–90.4% of the total arsenic contents during the incubation period (**Appendix A Figs. S1** and **S2**). Consequently, these two fractions were proved to make more contributions to variations in AOB and AOA communities than the other fractions, which was in corroboration with the previous literature, demonstrating that these two arsenic fractions were considered as bioavailable and could be more accessible to microbes (Sun et al., 2019).

In this study, the abundances of AOA and AOB *amoA* genes were comparable to those observed in arable soils, grassland soils and arsenic-contaminated soils (He et al., 2007; Zhang et al., 2012; Di et al., 2010; Liu et al., 2014). A reduction in AOB *amoA* gene abundance was observed in arsenic amended soils, while AOA *amoA* gene abundance was found to increase in these soils, resulting in the AOA/AOB ratio increasing to 19.29 in RS1 (180 days, Table 2). Thus, these results further corroborate the idea that AOA and AOB may occupy different niches in arsenic-polluted soils due to their physiological distinctiveness. The negative effect of arsenic on AOB abundance was in concordance with some earlier studies, which reported that AOB were sensitive to arsenic and that the AOB abundance was negatively correlated with arsenic in sediments of estuarine tidal flats (Gong et al., 2002; Ollivier et al., 2012; Yang et al., 2015). A possible reason for this is that arsenic could inhibit the basic cellular functions of bacteria. For example, arsenic was supposed to lead to a lesser incorporation of organic carbon into microbial cells, which could result in the reducing in substrate utilization efficiency by bacteria followed by the anabolic processes to reduce microbial abundances (Ghosh et al., 2004).

The finding that arsenic increased the abundance of the AOA *amoA* gene provides further evidence that archaea adapt better to chronic environmental stress than bacteria (Ollivier et al., 2012). The tolerance of AOA under arsenic conditions might be attributed to the stability and exceptional chemical structure of AOA membranes under chronic stresses (Schleper et al., 2005). It was reported that the lower membrane permeability of AOA cells, which is a direct consequence of their preference for tetraether lipids, could result in a reduction in ion cycling and lower levels of maintenance energy compared with AOB (Valentine, 2007). In addition, the hypothesis that AOA possesses heavy metal resistance genes was verified by whole genome sequence analysis of the soil AOA species *Nitrososphaera gargensis* (Spang et al., 2012). Another possible reason for the stimulation effect on AOA might be caused by the hormesis effect, which suggested relatively lower doses of toxicity might virtually promote the AOA activities (Stebbing, 1982). Additionally, AOA community were supposed to survive and maintain diversity in terms of spores or in dormant states under heavy metal stress conditions (Sheik et al., 2012; Tang et al., 2019). However, because of the limited number of cultivated members of the AOA cluster, information concerning the specific mechanisms that confer arsenic resistance or arsenic stimulating effect in AOA remains ambiguous and requires further verification (He et al., 2012).

As shown in Fig. 1, the differences in the PAO rates between spiked soils and CK soils were not conspicuous in some soils especially in the late stage of incubation. Briefly, the changes of PAO rate after arsenic pollution were the results of the integrated performances of microorganism and largely depended on ammonia oxidizers species replacement. Sensitive species were replaced by the more tolerant ones, thus to a certain degree altering the AOA or AOB community associated with soil PAO rates (Tipayno et al., 2018). Because both AOA and AOB community abundances were positively correlated with the soil PAO rates (Fig. 6), the existence of functional redundancy of microbial guilds could delay the unfavorable impact of environmental disturbances on ecosystem function (Tao et al., 2021). The AOB community abundances were obviously decreased when soils were spiked with arsenic, whereas the AOA community abundances were substantially increased, which was accompanied by little difference in PAO rates between spiked and CK soils, suggesting that the increased contribution of AOA to PAO offset the reduced function of AOB to PAO as affected by arsenic. Some AOA species that are functionally redundant in unpolluted condition may become vital in soils disturbed by arsenic. It is supported the idea that the degree of functional redundancy varies with the role of species in different environment (Fetzer et al., 2015). Therefore, it was necessary to use selective inhibitor to distinguish the contributions of AOB and AOA to PAO rate to explore the functional contribution of each ammonia oxidizer under arsenic stress in the further work. Also, as discussed in several previous studies (Liu et al., 2010; Luo et al., 2019; Tang et al., 2019), the number of *amoA* gene copies may not accurately portray the *in-situ* PAO. Studies of the transcriptional activity of AOB and AOA in contaminated soils and whether specific AOA or AOB can adapt to arsenic contaminated soils may be especially meaningful in further investigations of the actual functions of ammonia oxidizing microorganisms.

3.3. *Phylogeny of AOB and AOA* amoA *proteins in arsenic-treated soil*

Phylogenetic analysis of the deduced amino acid sequences of the AOB *amoA* genes demonstrated that the AOB sequences in the IDS and RS1 clone libraries all belong to the genus *Nitrosospira* (**Appendix A Fig. S6**), with no *Nitrosomonas* species were found. This finding is consistent with a great many previous studies, which demonstrated that several different *Nitrosospira*-like sub-clusters are usually found in soils

(He et al., 2007;Fan et al., 2011; Xia et al., 2011). *Nitrosospira*-like AOB were found to be exceptionally tolerant to heavy metals and were hypothesized to be the dominant species in soils with elevated heavy metal levels because of their low substrate affinities (Frey et al., 2008; Mertoglu et al., 2008; Mertens et al., 2009), implying that *Nitrosospira* sp. may more actively contribute to PAO in contaminated soil. In this study, *Nitrosospira*-like AOB sequences, especially those in *Nitrosospira* cluster 3a and cluster 3b, were the dominant AOB sequences in arsenic-contaminated soils, suggesting the importance of *Nitrosospira*-like AOB in heavy metal-contaminated soil. The relative proportions of these sequences changed with soil type and arsenic treatment. However, the *Nitrosospira* cluster 3a group was also reported to be more sensitive to heavy metal pollution than other *Nitrosospira* clusters. For instance, Liu et al. (2010) found that the sequences affiliated with *Nitrosospira* cluster 3a were the dominant AOB sequences in uncontaminated soil, but that these sequences decreased with increased Hg concentrations. Luo et al. (2019) demonstrated that *Nitrosospira* cluster 3a sequences were found in agricultural soil but disappeared in soil polluted with multiple heavy metals. Taken together, based on the shifts in AOB community composition and the changes in AOB *amo*A gene abundance, it can be inferred that the decrease in AOB *amo*A gene abundance during incubation in arsenic-treated soils might be associated with the sensitivity of *Nitrosospira* cluster 3a species to arsenic. Additionally, some AOB species (or TRFs) were abundant in arsenic-treated soil as well as in CK soils, suggesting that there were probably some native arsenic-tolerant species living in uncontaminated soil. However, whether the resistance capability of AOB species in arsenic-contaminated soils depended on intrinsic tolerance to arsenic or was conferred by horizontal gene transfer is still unclear.

As for AOA species, clones belonging to the *Thaumarchaeal* Group 1.1b *Nitrosopheara* cluster were found to be dominant (**Appendix A Fig. S7**). It is generally accepted that *Thaumarchaeal* Group 1.1b is the major AOA lineage in a wide range of soils (Nicol et al., 2008; Yao et al., 2011; Zhang et al., 2012; Wu et al., 2019), which indicates the important role of this AOA lineage in ammonia oxidization in terrestrial ecosystems. Studies demonstrated that *Thaumarchaeal* Group 1.1b AOA species have a high ability to adapt to environmental changes and that this ability depends on the large number of genes for two-component systems, chemotaxis, and flagella-mediated motility in these species (Spang et al., 2012). Consistent with this, in some heavy metal-contaminated environments, Group 1.1b AOA species were shown to participate in ammonia oxidation (Subrahmanyam et al., 2014b; Zhang et al., 2016; Liu et al., 2019). The reason for these species being well adapted to living in soils containing heavy metals is that they encode multiple resistance proteins including at least 21 putative metal ion efflux proteins (Spang et al., 2012). Therefore, we infer that *Thaumarchaeal* Group 1.1b species potentially play a role in the arsenic-contaminated soils included in our study. Still, further studies are also needed to decipher the mechanisms underlying the arsenic resistance and adaptive mechanisms of *Thaumarchaeal* Group 1.1b AOA species.

It was worth noting that the responses of microbial community to arsenic stress in this study were mainly focused on the canonical ammonia oxidizers, comammox was not investigated. Comammox is a crucial member in soil ammonia oxidizing process, the taxonomy, ecophysiology, and metabolic versatilities of comammox is different from canonical ammonia oxidizers. So, further study is needed to quantify and partition the actual contribution of comammox to soil nitrification. In addition, more arsenic spiked levels should be considered to enlarge our comprehension of the niche differentiation and the recovery process of different ammonia oxidizers in soils polluted by arsenic.

4. Conclusions

In this study, the effects of exogenous arsenic on the soil PAO rate and AOB and AOA community composition and abundance were examined in seven types of soil. Incubation with arsenic distinctly changed the community compositions of AOB and AOA in all soils. The abundance of AOA was increased by the addition of exogenous arsenic, while AOB seemed to be more vulnerable to arsenic stress. The different responses of AOB and AOA community to arsenic disturbance further supports the previously stated hypothesis that AOB and AOA occupy different ecological niches in arsenic-contaminated soils. Several physicochemical factors, especially the contents of available arsenic and arsenic fractionations, were involved in shaping the dynamics of AOB and AOA communities in arsenic-contaminated soils (Figs. 2–4). All the AOB sequences identified in this study belonged to the genus *Nitrosopira*. Those in *Nitrosopira* cluster 3a seemed to be sensitive to arsenic stress. *Thaumarchaeal* group 1.1b AOA species were found to be dominant in arsenic-contaminated soils, highlighting the importance of this group for nitrification in contaminated soil. Taken together, these results highlight the fact that the community composition and abundance of AOB and AOA could be used as sensitive biological markers for assessing soils contaminated by arsenic. Assaying the transcriptional activity of AOB and AOA in contaminated soils in future studies will be necessary to reveal the potential impact of heavy metal pollution on soil nitrogen cycling.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jes.2022.02.038.

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