

Bacteria Responsible for Nitrate-dependent Antimonite Oxidation in Antimony-contaminated Paddy Soil Revealed by the Combination of DNA-SIP and Metagenomics

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Research

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Abstract

Background: Antimonite (Sb(III)) oxidation (SbO) can decrease the toxicity of antimony (Sb) and its uptake into plants (*e.g.*, rice), thus serving an ecological role in bioremediation of Sb contamination. In some anoxic environments, Sb(III) can be oxidized coupled with nitrate as the electron acceptor. Here we investigate the potential for nitrate-dependent SbO in Sb contaminated rice paddies and identify nitrate-dependent Sb(III)-oxidizing bacteria (SbOB) using stable isotope probing (SIP) coupled with amplicon and shotgun metagenomic sequencing.

Results: Anaerobic SbO was exclusively observed in the paddy soil amended with both Sb(III) and NO_3^- , whereas no apparent SbO was detected in the soil amended with Sb(III) only. The increasing abundance of the arsenite oxidase (*aioA*) gene suggests that nitrate-dependent SbO was catalysed by microorganisms harbouring the *aioA* gene. After 60-day DNA-SIP incubation, obvious shift in the *aioA* gene to heavy DNA fractions only in the treatment amended with $^{13}\text{C-NaHCO}_3$, Sb(III) and NO_3^- suggested the incorporation of ^{13}C by nitrate-dependent SbOB. Accordingly, DNA-SIP identified a number of putative nitrate-dependent SbOB in the paddy soil, including *Azoarcus*, *Azospira* and *Chelativorans*. Metagenomic analysis further revealed that they contained *aioA* gene and genes involved in denitrification and carbon fixation, supporting their capability for nitrate-dependent SbO.

Conclusions: These observations in this study suggested the occurrence of nitrate-dependent SbO in paddy soils. A number of putative nitrate-dependent SbOB (*i.e.*, *Azoarcus*, *Azospira* and *Chelativorans*) were reported here, which expands our current knowledge regarding the diversity of nitrate-dependent SbOB. In addition, this study provides a proof of concept using DNA-SIP to identify nitrate-dependent SbOB.

Background

Antimony (Sb) is a toxic metalloid belonging to Group 15 in the periodic table and share similar chemical properties with arsenic (As) [1]. Sb has been classified as a suspected carcinogen [2] and chronic exposure can cause damage to various organ systems including the heart, lung, liver and kidney [3]. Although Sb is ubiquitously present at low concentrations ($0.2\text{--}1\ \mu\text{g g}^{-1}$) in nature [4], anthropogenic activities such as mining and smelting have led to Sb contamination in the environment [5–7]. Typically, two oxidation states of Sb, namely antimonite (Sb(III)) and antimonate (Sb(V)), are the main forms of Sb in nature [9, 10]. The mobility, bioavailability and toxicity of Sb strongly depends on its oxidation state, for instance, Sb(V) is less toxic than Sb(III) [11–13].

Microorganisms are major drivers changing Sb speciation in the environment [15]. Compared to our extensive knowledge regarding As microbiology [16, 17], microbially-mediated Sb cycling has only attracted attention recently [18–20]. Owing to the higher toxicity of Sb(III) than Sb(V) [12, 21], oxidation of Sb(III) to Sb(V) can decrease the toxicity of Sb and is thus of great interest. Previous studies reported that heterotrophic Sb-resistant bacteria can oxidize Sb(III) as cellular detoxification mechanism [22–24].

However, *Stibiobacter senarmontii* was reported to be capable of growing chemoautotrophically using the energy obtained from SbO [25]. Currently, over 60 bacterial strains have been reported to oxidize Sb(III) via arsenite oxidase AioAB or antimonite oxidase AnoA [26]. Southwest China is the major rice producing area and also a key Sb mining area. The Sb mining activities have caused frequent contamination of rice fields. For instance, it has been reported that rice is a major route for Sb exposure near Xikuangshan Sb mining area, contributing to over 30% of the daily intake of the Sb to exposed population [25]. Therefore, Sb contamination in rice paddies near Sb mining area is an important environmental issue. In addition, rice is found to be more efficient in uptake of Sb(III) than of Sb(V) [25, 26]. Hence, SbO may be an important biogeochemical process that attenuates Sb toxicity in rice paddies and decreases the translocation and accumulation of Sb in the rice. However, the anoxic conditions in some habitats such as river sediment and flooded rice paddies may hinder SbO because of the lack of O₂. Notably, nitrate is an alternative to O₂ as an oxidant that can support SbO under anoxic conditions. Indeed, three anaerobic Sb(III)-oxidizing bacteria (SbOB) (*i.e.*, *Hydrogenophaga taeniospiralis* strain IDSBO-1, *Ensifer* sp. NLS4 and *Sinorhizobium* sp. GW3) have been isolated using nitrate as the electron acceptor [13, 18, 27]. The anoxic conditions and high concentrations of nitrate caused by N fertilization would allow rice paddies to facilitate nitrate-dependent SbO. However, nitrate-dependent SbO has never been reported in rice paddies so far.

Xikuangshan Sb mine, designated as “the World Antimony Capital”, located in Lengshuijiang City in Hunan Province of China, is the world largest Sb deposit with over 120 years of mining history and environmental contamination. In order to investigate nitrate-dependent SbO in rice paddy soils and identify the bacteria responsible for this process, Sb-contaminated paddy samples were collected near the Xikuangshan mining area [28, 29]. It is proposed that the long-term Sb contaminated may enrich Sb-metabolising bacteria including nitrate-dependent SbOB and makes these Sb-contaminated rice paddy soils ideal for this study. Although three nitrate-dependent SbOB have been isolated and identified so far [13, 18, 27], their overall diversity and distribution in soil, especially in rice paddy soil, is not known. Culture-independent DNA-stable isotope probing (SIP) incubations can link microbial identity with function in environment and enable a greater understanding of active microbial communities involved in the process under study [30]. Indeed, SIP has been used to identify chemolithotrophs in various habitats such as rice fields [31], Karst caves [29], and marine sediments [32]. More specially, DNA-SIP has been used to identify microorganisms responsible for nitrate-dependent Fe(II) and As(III) oxidation [33, 34]. Therefore, DNA-SIP may be capable of identifying nitrate-dependent SbOB as well. Combining DNA-SIP with amplicon and shotgun metagenomics herein, we aimed to (i) investigate the potential of nitrate-dependent SbO in Sb-contaminated rice paddy soil; (ii) identify bacteria responsible for nitrate-dependent SbO; and (iii) explore the metabolic potentials of the putative nitrate-dependent SbOB.

Methods

The analyses carried out in this study included chemical characterization of the soil, microcosms incubation, DNA-SIP, and amplicon and shotgun metagenomic sequencings, which are described in detail

below.

Soil collection and geochemical analyses

Rice soils were sampled at 30–40 cm depth from the surface in various flooded rice paddy fields (27°44'38"N, 111°27'46"E) near Xikuangshan mining area, which were immediately sealed and transported at 4 °C to the laboratory. In addition, soils from Sb-contaminated arid fields were also collected near the mining area. A preliminary set of experiments were performed to monitor nitrate-dependent SbO in all of the soils collected from various rice fields and arid fields. The rice paddy soil showing the most rapid rate of nitrate-dependent SbO was selected for further experiments while no soils from arid fields showing nitrate-dependent SbO. The selected rice paddy soil was subsequently subsampled for geochemical analyses and for microcosm setup. The soil contained over 750 mg kg⁻¹ Sb, 200 mg kg⁻¹ As, and a pH of 6.89 (Table S1).

Soil microcosms incubations

Three sets of microcosms were performed: (i) *nitrate-dependent SbO activity incubation* - to examine the potential of nitrate-dependent SbO in the rice paddy; (ii) *nitrate-dependent SbO enrichment incubation* - to monitor the shift in the bacterial community after amending Sb(III) and NO₃⁻; (iii) *SIP incubation* - to identify nitrate-dependent SbOB by DNA-SIP.

Nitrate-dependent SbO activity incubation: Soil cultures were prepared by mixing 2 g of paddy soil and 40 ml of mineral salts medium [53] in serum bottles. The bottles were immediately sealed with aluminium caps and rubber septa after undergoing gas exchange with pure N₂ for 20 min. The headspace was then purged with N₂/CO₂ (80% : 20%, v/v) for 5 min. The redox potential of the cultures was further lowered by adding 60 µL sodium sulphide (Na₂S·9H₂O, 10 mM) solution. NaHCO₃ (8 mM) was provided as carbon source in all cultures. Three treatment sets were established with the following amendments: (i) 1 mM Sb(III) and 3 mM NO₃⁻ (NaNO₃); (ii) 1 mM Sb(III) only; or (iii) 3 mM NO₃⁻ only. Controls inoculated with autoclaved soils were also prepared by providing 1 mM Sb(III) and 3 mM NO₃⁻. All cultures were incubated at 30 °C in the dark. Cultures of each treatment were destructively sampled in triplicate after incubation for 0, 3, 6, and 12 days. Supernatant collected from each culture was subsampled for analyses of NO₃⁻, NO₂⁻ and Sb species after immediate centrifugation at 6,000 g for 15 min. Specifically, NO₃⁻ and NO₂⁻ concentrations were quantified by a Continuous Flow Analyser (SAN++, Skalar, Holland). Sb species (*i.e.*, Sb(III) and Sb(V)) were adapted from the methods for measurement of As species using LC-AFS (AFS-920, Haiguang, Beijing) equipped with a hollow cathode lamp for Sb (Shuguangming, Beijing) [54]. The remaining soil sample pellets were subsampled for analyses of Sb species adsorbed in the soil (stored at 4 °C) and for molecular microbial community analysis (stored at -80 °C). To extract Sb species adsorbed in the soil phase, 0.5 g of the remaining soil sample pellets were mixed with 50 mL 1 M H₃PO₄ and resuspended by 10 s ultrasonication, shaken for 2 h at 200 rpm, and followed by 10 s ultrasonication (modified from a method for As species extraction from soil [55]). The resuspended

mixture was immediately centrifuged and the resulting supernatant was subject to LC-AFS for Sb species measurement as mentioned above.

Nitrate-dependent SbOB enrichment incubation: To investigate the shift of microbial communities after amending Sb(III) and NO_3^- , cultures inoculated with approximately 2 g paddy soil were prepared, purged and sealed as described above. Two treatments were set up with the following amendments: (i) 1 mM Sb(III) and 3 mM NO_3^- ; or (ii) 3 mM NO_3^- only. These cultures were respiked with 8 mM NaHCO_3 , 3 mM NO_3^- and 1 mM Sb(III) every 12 days when Sb(III) was completely oxidized and NO_3^- was depleted. All the cultures were incubated for a total of 60 days as mentioned above. Triplicate cultures of these two treatments were destructively sampled after incubation for 0, 12, 30 and 60 days. Each culture sample was immediately centrifuged and the soil pellet was stored at -80°C for molecular analysis.

SIP incubation: The SIP experiment was established to target putative nitrate-dependent SbOB. Cultures were prepared by amending 2 g soil inoculum with 40 mL mineral salts medium [53], and then purged and sealed as described above. Either 8 mM ^{13}C - or ^{12}C - NaHCO_3 was supplied as carbon source. Four sets of treatments were performed by amended: (i) 1 mM Sb(III), 3 mM NO_3^- with either $\text{H}^{13}\text{CO}_3^-$ (designated as $^{13}\text{CSbN}$) or $\text{H}^{12}\text{CO}_3^-$ (designated as $^{12}\text{CSbN}$); (ii) 3 mM NO_3^- with either $\text{H}^{13}\text{CO}_3^-$ (designated as ^{13}CN) or $\text{H}^{12}\text{CO}_3^-$ (designated as ^{12}CN). These cultures were re-spiked every 12 days with 1 mM Sb(III), 3 mM NO_3^- and either 8 mM ^{13}C - or ^{12}C - NaHCO_3 . All cultures were incubated as mentioned above, and were destructively sampled in triplicate at day 0, 30 and 60.

DNA extraction and qPCR of *aioA* and *anoA* genes

Genomic DNAs were extracted from soils of three incubations at various time points: nitrate-dependent SbO activity incubation (day 0, 3, 6 and 12, for qPCR), the nitrate-dependent SbOB enrichment incubation (day 0, 12, 30 and 60, for amplicon sequencing), and the SIP incubation (day 0, 30 and 60, for SIP gradient fractionation and amplicon and shotgun metagenomic sequencing). DNA extractions were performed using the DNeasy PowerSoil DNA Kit (QIAGEN, Germany) by following the manufacturer's protocol. The copy numbers of the *aioA* gene were quantified by qPCR with the primer set *aoxBM1-2F* (5'-CCACTTCTGCATCGTGGGNTGYGGNTA-3')/*aoxBM2-1R* (5'-GGAGTTGTAGGCGGGCCKRTRTGDAT-3') by following the protocol published by Quemeneur, et al. [56]. The gene encoding antimonite oxidase (*anoA*) was enumerated with the primers *anoA-F* (5'-TCATGGTCGAAAGCATCGG-3')/*anoA-R* (5'-GCGTTCTGGACGATGTCATA-3') by following the protocol published by Li, et al. [20].

SIP gradient fractionation

Genomic DNAs from the ^{13}C - and ^{12}C - NaHCO_3 SIP incubations (day 0, 30 and 60) were separated into "heavy" (*i.e.*, ^{13}C -DNA) and "light" (*i.e.*, ^{12}C -DNA) fractions by isopycnic density gradient centrifugation as previous [34]. Briefly, approximately 2 μg of genomic DNA was added into CsCl solution (buoyant density (BD) = 1.714 g mL^{-1}) in an OptiSeal polyallomer tube (Beckman Coulter, Palo Alto, USA). The mixture was

then ultracentrifuged at 409,000 g for 24 h using a VTi 90 vertical rotor in an Optima XPN-100 Ultracentrifuge (Beckman Coulter). The resulting CsCl gradients were then fractionated into 24 equal volumes (~ 200 μ L) with a fraction recovery system (Beckman Coulter, USA) [57, 58]. The BD value of each fraction was immediately determined by measuring the refractive index using a digital refractometer (Palette, ATAGO, Japan). DNA in each fraction were precipitated with 6 μ L glycogen (ZOMANBIO, China) dissolved in 30% cold ethanol and then finally eluted in 30 μ L of TE buffer (pH 8.0) [57, 58]. From the eluted DNA, qPCR was used as described above to determine the copy numbers of *aioA* gene in each of the 24 fractions collected.

Illumina Miseq sequencing and analysis

Genomic DNAs from the nitrate-dependent SbOB enrichment incubations (day 0, 12, 30 and 60) were subject to amplicon sequencing of the partial 16S rRNA gene with the primers 520f(5'-AYTGGGYDTAAAGNG-3')/802r(5'-TACNVGGGTATCTAATCC-3') [59]. In addition, three representative heavy or light fractions containing the highest *aioA* gene abundance from each culture of the $^{13}\text{CSbN}$ (H1-H3), $^{12}\text{CSbN}$ (L1-L3) and ^{13}CN (H1-H3) treatments (day 60) were pooled as composite DNA samples respectively, and then the nine composite DNA samples (one composite DNA sample per culture, triplicate cultures per treatment) were sequenced for the partial 16S rRNA gene with the primer set 520f/802r. The amplicon sequencing was performed on an Illumina MiSeq platform (Personalbio, Shanghai, China) [60]. Approximately 22.9 Mb raw paired-end reads per culture were generated and then analysed with the QIIME2-201904 toolkit [61]. Briefly, all the raw reads were qualified, merged and Chimera-removed (-p-trim-left-f 0, -p-trim-left-r 228, -p-trunc-len-f 0, -p-trunc-len-r 219). The filtered reads (approximately 21.9 Mb per culture) were then clustered into amplicon sequence variants (ASV) with DADA2 [62]. The obtained ASV features (> 0.01%) were assigned into taxonomy against the SILVA database [63].

Shotgun metagenomic sequencing and analysis

The heavy DNA fractions with the highest *aioA* gene abundance from triplicate cultures of $^{13}\text{CSbN}$ (i.e., H2 from triplicate microcosms of $^{13}\text{CSbN}$ at day 60) were pooled as one composite DNA sample, which was sequenced for the metagenome on an Illumina HiSeq 4000 platform (Personalbio, Shanghai, China). All the raw reads (17.9 Gb) were trimmed and qualified using Trimmomatic 0.36 (SLIDINGWINDOW:4:20 MINLEN:70) [64]. The qualified reads (17.1 Gb) were assembled into contigs using Megahit ($k = 21 - 121$, step = 10) [65] and then taxonomically assigned by comparing against the NCBI database using Kraken2 with default settings [66]. Subsequently, binning of the obtained contigs was performed and further refined using MaxBIN2 (default settings) [67] and metaBAT2 (default settings) [68] integrated in MetaWRAP [67]. Contamination and completion of all bins were determined by CheckM [69]. Only high-quality bins with completion > 70% and contamination < 5% were selected for further analyses. Taxonomy of the bins was classified using *Taxator-tk* (default settings) [70]. Annotation of the bins was performed using KofamKOALA online (score > 60, evaluate = 1e-5) (<https://www.genome.jp/tools/kofamkoala/>) [71]. In addition, the bins phylogenetic tree was generated based on conserved protein sequences with PhyloPhlAn [72].

Results

Activity of nitrate-dependent SbO

Oxidation of Sb(III) to Sb(V) was only observed in the treatment amended with both Sb(III) and NO_3^- but not in the treatment amended with Sb(III) or NO_3^- only (Fig. 1a and b). Approximately 0.90 ± 0.08 mM Sb(III) was fully oxidized to Sb(V) (0.87 ± 0.04 mM) after an incubation period of 12 days, with 2.26 ± 0.06 mM NO_3^- reduced. In contrast, 0.11 ± 0.00 mM Sb(V) (probable carryover from the paddy soil) was detected at day 0 in the treatments amended with Sb(III) only, but no discernable SbO was detected over the course of incubation (Fig. 1a and b). Nitrate reduction (1.41 ± 0.03 mM) was also observed in the treatment amended with NO_3^- only, but was significantly less than that in the treatment amended with both Sb(III) and NO_3^- ($P < 0.05$) (Fig. 1c). These observations suggest that anaerobic SbO in these cultures was nitrate-dependent. In addition, neither SbO nor NO_3^- reduction was detected in the sterile controls inoculated with autoclaved soil (Fig. 1), indicating that anaerobic SbO was mainly driven by microorganisms in the paddy soil.

Increase in the abundance of the *aioA* gene over the course of nitrate-dependent SbO

Given that arsenite oxidase AioA has been reported to catalyze SbO in previous studies [20, 35], the *aioA* gene was quantified in all treatments from nitrate-dependent SbO activity incubations at day 0, 3, 6 and 12. The copies of the *aioA* gene significantly increased by 1.4-fold throughout the 12-day incubation in the treatments amended with Sb(III) and NO_3^- ($P < 0.05$), while no such change in *aioA* genes was observed in the treatments amended with Sb only (Fig. 2a). A significant decrease in the abundance of the *aioA* gene was observed in the treatments amended with NO_3^- only ($P < 0.05$) (Fig. 2a), suggesting that *aioA* gene-containing microorganisms might be outcompeted by others when no stress of Sb contamination occurred. In addition, a significantly positive correlation between the copies of the *aioA* gene and concentrations of Sb(V) was found in the treatment amended with Sb(III) and NO_3^- ($R = 0.88$, $P < 0.05$) (Fig. 2b), while no such correlations were found in treatments amended with Sb or NO_3^- only (data not shown). No PCR products were obtained by amplifying genes encoding antimonite oxidase (*anoA*) in any of these three treatments (data not shown).

Shift of bacterial communities over the course of nitrate-dependent SbO

The bacterial communities of two treatments (treatment amended with Sb(III) and NO_3^- and treatment amended with NO_3^- only) were further characterized. Accordingly, an obvious shift in the bacterial

communities was detected in the treatment amended with Sb(III) and NO_3^- over the course of the incubation (day 0, 12, 30 and 60) (Fig. 3). Specifically, the relative abundance of *Azoarcus* significantly increased from undetectable at day 0 to $78 \pm 2\%$ at day 60 in treatments amended with Sb(III) and NO_3^- ($P < 0.05$) (Fig. 3). The proportion of bacteria associated with *Azospira* reached their peak ($42 \pm 6\%$) at day 30 then decreased to $5 \pm 2\%$ at day 60 in treatments amended with Sb(III) and NO_3^- ($P < 0.05$) (Fig. 3). In contrast, bacterial communities were relatively stable in treatments amended with NO_3^- only, with *Herbaspirillum* and *Gemmatimonas* gradually increasing from undetectable at day 0 to $10 \pm 2\%$ at day 60 and from $4 \pm 0\%$ at day 0 to $9 \pm 1\%$ at day 60, respectively (Fig. 3). Further, PERMANOVA demonstrated that the bacterial communities were significantly different between the treatment amended with Sb(III) and NO_3^- and the treatment amended with NO_3^- only ($P = 0.001$), suggesting that Sb(III) plus NO_3^- shapes the bacterial communities and possibly enriched those involved in nitrate-dependent SbO. Therefore, DNA-SIP combined with amplicon sequencing was subsequently performed to identify the microorganisms responsible for nitrate-dependent SbO.

SbOB identified by DNA-SIP and amplicon sequencing of 16S rRNA gene

The maximum abundance of *aiOA* gene was detected in the light fraction ($\text{BD} = 1.708 \text{ g ml}^{-1}$) of $^{12}\text{CSbN}$ during the SIP incubation (Fig. 4). Compared to $^{12}\text{CSbN}$, the highest abundance of the *aiOA* gene gradually shifted to the heavier fractions ($\text{BD} = 1.712$ at day 30, $\text{BD} = 1.727 \text{ g ml}^{-1}$ at day 60) in the $^{13}\text{CSbN}$ treatment only (Fig. 4), implying that nitrate-dependent SbOB incorporated ^{13}C over the course of the incubation. In contrast, no obvious shifts in abundance of the *aiOA* gene to the heavy fractions were detected in $^{12}\text{CSbN}$, ^{12}CN or ^{13}CN treatments (Fig. 4).

Fractions containing the highest abundances of *aiOA* genes from each culture of the treatments $^{13}\text{CSbN}$ (H1-H3, Fig. 4), $^{12}\text{CSbN}$ (L1-L3, Fig. 4), and ^{13}CN (H1-H3, Fig. 4) at day 60 were pooled as composite DNA samples and were then subject to 16S rRNA amplicon sequencing. As shown in Fig. 5a, bacteria affiliated with *Azoarcus* ($45 \pm 6\%$) dominated the heavy DNA fractions in the $^{13}\text{CSbN}$ treatment, followed by *Gemmatimonas* ($18 \pm 4\%$), *Ramlibacter* ($5 \pm 0\%$), *Halomonas* ($4 \pm 1\%$) and *Chelativorans* ($2 \pm 0\%$). Notably, *Gemmatimonas* ($9 \pm 2\%$) was also the most abundant genus in the heavy fractions of the ^{13}CN treatment (Fig. 5a), implying that they were more likely autotrophic denitrifiers without the capability to oxidize Sb(III). In addition, linear discriminant analysis effect size (LEfSe) analysis identified 19 genera that showed significantly different abundances in the heavy DNA fractions between the $^{13}\text{CSbN}$ and ^{13}CN treatments ($P < 0.05$ and LDA score > 2.0) (Fig. 5b). Specifically, *Azoarcus*, *Halomonas*, *Geobacter*, *Azospira*, *Pelagibacterium* and *Chelativorans* were significantly enriched in the heavy fractions of the $^{13}\text{CSbN}$ treatment, implying that bacteria associated with these six genera might play a role in nitrate-dependent SbO. Their exact role was further examined by metagenomic-binning to detect the presence of some key genes for nitrate-dependent SbO in bins associated with these bacteria.

Shotgun metagenomic analysis of the heavy fractions from $^{13}\text{CSbN}$ treatment

Taxonomic assignment of the metagenome from the heavy DNA fractions in the $^{13}\text{CSbN}$ treatment revealed that *Azoarcus* (42%) was the most dominant genus, followed by *Thauera* (6%), *Streptomyces* (2%), *Pseudomonas* (2%), *Burkholderia* (2%) and *Ramlibacter* (2%) (Fig. S1). Further, metagenomic-binning was performed. Assembled bins were affiliated with *Proteobacteria*, *Gemmatimonadetes* and *Verrucomicrobia* (Fig. S2). Bins associated with putative nitrate-dependent SbOB identified by the above-mentioned SIP experiments, such as *Azoarcus* sp. (bin9), *Azospira* sp. (bin10) and *Chelativorans* sp. (bin14), were obtained (Fig. S2), whereas bins associated with *Halomonas*, *Geobacter*, and *Pelagibacterium* were not obtained. Also, bins associated with the genera showing relatively higher abundance in the metagenome were obtained, including *Thauera* (bin4) and *Ramlibacter* (bin3) (Fig. S2). Genetic annotations of these bins showed that most of the bins (except for *Devosia*-associated bin6, and *Gemmatimonas*-associated bin8 and bin15) harboured *aioA* genes, while *aioB* genes were observed in only three bins (*Thauera*-associated bin4, *Vulgatibacteraceae*-associated bin12, and *Chelativorans*-associated bin14) (Fig. 6). Genes involved in denitrification, including those responsible for NO_3^- , NO_2^- , NO - and N_2O -reduction, were investigated. Generally, nitrate-reducing genes were the most abundant genes detected in all bins, followed by NO_2^- -reducing and NO -reducing genes (Fig. 6). Among them, *Ramlibacter*-associated bin3, *Azoarcus*-associated bin9, *Azospira*-associated bin10 and *Anaeromyxobacter*-associated bin13 were observed to encompass the genes driven the whole denitrification process from NO_3^- to N_2 . In addition, all of the bins contained a number of genes involved in carbon fixation, suggesting their capability of autotrophic growth (Fig. 6).

Discussion

Rice has been suggested as a major route for Sb exposure, especially in mining areas [14, 36], and is reported to be more efficient in taking up Sb(III) than Sb(V) [14]. SbO will generate the less mobile Sb(V) and thus reduce the uptake of Sb by the rice. Therefore, SbO may be beneficial to attenuate the consequences Sb contamination in rice paddies. The anoxic conditions and the high levels of nitrate in rice paddies may facilitate nitrate-dependent SbO, which, however, has never been reported in rice paddies. Therefore, the current study tackles this important but less understood environmental issue to investigate the potential of nitrate-dependent SbO in the Sb-contaminated rice paddies.

Our current understanding of anaerobic Sb(III) oxidizers is mainly based on three isolates [13, 18, 27]. Culture-independent tools such as DNA-SIP may be available to expand the list of nitrate-dependent SbOB. However, clear Sb-dependent growth is difficult to observe because SbOB require high concentrations (millimolar range) of Sb(III) to prompt significant increases in biomass [26]. The slow growing nature of SbOB may increase the required duration of ^{13}C incorporation and thus incur cross-feeding, which complicates the interpretation of SIP data. The current study aims to examine the proof of

concept of using DNA-SIP combined with 16S rRNA gene amplicon sequencing to reveal nitrate-dependent SbOB. Given that the long incubation time for DNA-SIP may cause cross-feeding among microorganisms, shotgun metagenomic sequencing was further performed on the heavy DNA fractions of $^{13}\text{CSbN}$ to examine whether some key genes responsible for nitrate-dependent SbO (*i.e.*, Sb(III) oxidation, nitrate reduction, and carbon fixation) were present in the putative nitrate-dependent SbOB to confirm their role in nitrate-dependent SbO.

Nitrate-dependent SbO potential of the Sb-contaminated rice paddy soil

Anaerobic Sb(III) oxidation to Sb(V) was clearly demonstrated in the anoxic rice-paddy cultures amended with Sb(III) and NO_3^- , but not in the cultures amended with NO_3^- or Sb(III) only (Fig. 1), suggesting that the addition of nitrate may facilitate SbO. Nitrate reduction with concomitant SbO is further supported by the conversion of nitrate to nitrite (Fig. 1c and d). Sterile controls showed no formation of Sb(V) supporting that nitrate-dependent SbO is a biotic process. Overall, this observation confirms that bacteria can mediate nitrate-dependent SbO in rice paddy soils.

aioA may be the key gene for nitrate-dependent SbO

Given similar chemical properties shared by Sb and As elements, it has been proposed that microbes may drive Sb transformation by using similar metabolic pathways with As. Previous studies suggest that arsenite oxidase (encoded by the *aioA* gene) may be responsible for SbO. For example, the *aioA* gene has been detected in some known nitrate-dependent SbOB including *Hydrogenophaga taeniospiralis* strain IDSB0-1 and *Sinorhizobium* sp. GW3 [13, 27]. In addition, the transcription level of the *aioA* gene in Sb(III)-oxidizing *Sinorhizobium* sp. GW3 significantly increased upon Sb(III) addition under anaerobic conditions and a mutation in the *aioA* gene reduced the anaerobic SbO rate by over 70% [13]. Consistently, several observations in this study also support that the *aioA* gene is involved in anaerobic SbO: (i) the copy numbers of the *aioA* gene increased only in the cultures with nitrate-dependent SbO (Fig. 2a). The abundance of the *aioA* gene showed significant positive correlations with the concentration of Sb(V) produced over the course of nitrate-dependent SbO in the treatment amended with both Sb(III) and NO_3^- ($R = 0.88$, $P < 0.05$) (Fig. 2b), whereas such correlation was not detected in two other treatments where nitrate-dependent SbO was not observed (data not shown); (ii) following a 60-day incubation period, the highest relative abundance of the *aioA* gene was observed to gradually shift to the heavier DNA fractions only in the $^{13}\text{CSbN}$ treatment where nitrate-dependent SbO occurred, while no obvious shifts to the heavier fractions were found in other treatments (*i.e.*, $^{13}\text{CSbN}$, ^{13}CN and ^{12}CN). These observations collectively support that the *aioA* gene is responsible for nitrate-dependent SbO. A Sb(III) oxidase, encoded by *anoA* gene, belonging to the short-chain dehydrogenase/reductase family was recently identified and proposed to be responsible for aerobic SbO [20]. In this study, the *anoA* gene, however, was

neither successfully amplified from any of the treatments nor observed in the metagenome, implying that *anoA* may not be responsible for nitrate-dependent SbO in this rice paddy soil.

Putative nitrate-dependent SbOB identified by DNA-SIP

A number of genera, such as *Azoarcus*, *Azospira* and *Chelativorans*, were proposed as putative nitrate-dependent SbOB in the current study. The relative abundance of *Azoarcus* spp. increased from undetectable in the original rice paddy soil inoculum to 78% at day 60 in the cultures amended with Sb(III) and NO_3^- (Fig. 3). Since *Azoarcus* was not enriched in the cultures amended with NO_3^- only, it suggests that SbO likely supported its growth. Furthermore, as seen in the DNA-SIP result (Fig. 5), *Azoarcus* dominated (close to 50%) in the heavy DNA fractions of the $^{13}\text{CSbN}$ treatment, but was not found in the ^{13}CN treatments, thus demonstrating that *Azoarcus* incorporated $^{13}\text{C-NaHCO}_3$ only during nitrate-dependent SbO. *Azoarcus* spp. are well known for their capability to mediate nitrate-dependent As(III) oxidation via *AioA* in paddy soils and other environments [34, 38]. Consistently, *aioA* genes were observed in the bin associated with *Azoarcus* (bin9), supporting their role also in SbO. In addition, genes for denitrification and carbon fixation were observed in the *Azoarcus*-associated bin9, suggesting its capability for denitrification and autotrophy (Fig. 6). Collectively, these results support that *Azoarcus*-associated bacteria are responsible for the autotrophic oxidation of Sb(III) linked to nitrate reduction in the paddy soil. Bacteria associated with *Azospira* dominated ($42 \pm 6\%$) the bacterial communities in the treatment amended with Sb(III) and NO_3^- at day 30 (Fig. 3) and was observed to be significantly enriched in the heavy DNA fractions of $^{13}\text{CSbN}$ treatment compared to ^{13}CN (Fig. 5). In addition, a bin associated with *Azospira* containing the *aioA* gene was detected in the ^{13}C -heavy-fraction metagenome (Fig. 6). These observations suggest that *Azospira* may be a putative nitrate-dependent SbOB. The detection of genes for denitrification and carbon fixation in the *Azospira*-associated bin also supported its capability for nitrate-dependent SbO. Nitrogen cycling by the genus *Azospira* has been previously described, including nitrogen fixation and denitrification [39, 40]. Although *Azospira* spp. has not previously been shown to oxidize Sb(III) under either aerobic or denitrifying conditions, autotrophic *Azospira* sp. strain ECC1-pb2 isolated from sludge and sediment samples was capable of As(III) oxidation linked to chlorate reduction [41]. Our current study identified *Azospira* spp. as putative nitrate-dependent SbOB. *Chelativorans*-affiliated bacteria were identified as putative nitrate-dependent SbOB in this study because of two reasons: (i) they were significantly enriched in the heavy fractions of $^{13}\text{CSbN}$ than their counterparts in ^{13}CN (Fig. 5); (ii) *aioAB* genes and denitrifying genes were observed in the *Chelativorans*-associated bin (Fig. 6), supporting their potential ability for nitrate-dependent SbO. Members of *Chelativorans* have been extensively identified as heterotrophic denitrifiers and have been enriched in uranium-contaminated soil [42–44]. However, the detection of genes for carbon fixation suggested that they hold the potential to oxidize Sb(III) autotrophically.

Metagenomic-binning of the ^{13}C -heavy-fraction metagenome provides an additional method to examine the physiological traits of the nitrate-dependent SbOB community. Many bins, such as those associated

with *Thauera*, *Ramlibacter* and *Anaeromyxobacter*, contained an *aioA* gene. Although *Thauera*, *Ramlibacter* and *Anaeromyxobacter* have been detected in As-contaminated sites previously [45–47], their role in either As(III) or Sb(III) oxidation has not been reported. The presence of *aioA* and the genes responsible for denitrification and carbon fixation in the bins related with these genera (Fig. 6), suggests that they have the potential for nitrate-dependent SbO.

Relatively higher abundance of *Gemmatimonas* was observed in the heavy DNA fractions of both $^{13}\text{CSbN}$ and ^{13}CN than those in corresponding light fractions. Neither *aioA* nor *aioB*, however, was detected while genes involved in denitrification and carbon fixation were observed in the *Gemmatimonas*-associated bins (bin8). These observations suggested that *Gemmatimonas* may be more likely autotrophic denitrifier without the capability to oxidize Sb(III). In addition, bacteria associated with *Halomonas*, *Geobacter* and *Pelagibacterium* were significantly enriched in the heavy fractions in the $^{13}\text{CSbN}$ treatment than that of ^{13}CN (Fig. 5). Although *Halomonas* was identified as As(III) oxidizers [48], *Geobacter* spp. are notable for their capability for metal reduction [49] and *Pelagibacterium* has never been associated with As or Sb transformation. Unfortunately, bins associated with these three genera were not detected by the metagenomic-binning, thus we cannot determine whether they are potentially nitrate-dependent SbOB. Further investigation, such as isolation of members of these genera, are necessary to reveal their role in nitrate-dependent SbO.

The current study provided a proof of concept of using DNA-SIP to identify nitrate-dependent SbOB. The long incubation time (60 day) are necessary to observe obvious shift of ^{13}C -incorporating microbial communities. Because long incubation time may incur cross-feeding [50], shotgun metagenomics followed by DNA-SIP is suggested to provide the physiological traits of the putative nitrate-dependent SbOB and identify the scavenging denitrifiers or other microorganisms incorporating ^{13}C from cross-feeding.

Conclusions

Rice is a staple food in China and is a major route (over 30% of the daily intake) for Sb exposure in some Sb mining areas [8], especially when rice is grown close to Sb mines. Since Sb(III) is more efficiently taken up by rice than Sb(V) [8, 14], oxidation of Sb(III) in the rice paddies can have health and environmental benefits by reducing the uptake of Sb by rice. The current study showed that: (i) nitrate-dependent SbO can take place and carried out by the innate microbiota in rice paddy soils; (ii) the *aioA* gene may be the key gene responsible for nitrate-dependent SbO; (iii) a number of novel putative nitrate-dependent SbOB including bacteria associated with the genera *Azoarcus*, *Azospira*, and *Chelativorans* were identified by DNA-SIP.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The nucleotide sequences generated by amplicon and shotgun metagenome sequencing in this study have been deposited in GenBank database (Bioproject: PRJNA640466).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

M.Z. is the main author of this paper. M.Z. and L.Z. performed all the incubation experiments, DNA extraction, PCR experiments, and sequencings. Z.H. and Q.L. performed the chemical analysis. M.Z., Z.H., and G.L. performed SIP gradient fractionation. X.S. and R.X. helped interpret the data. M.Z. wrote the manuscript, and M.M.H., L.Y., F.L., and W.S. revised the manuscript. This project was conceived and designed by M.Z. and W.S. All authors have read, revised, and approved the manuscript.

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Figures

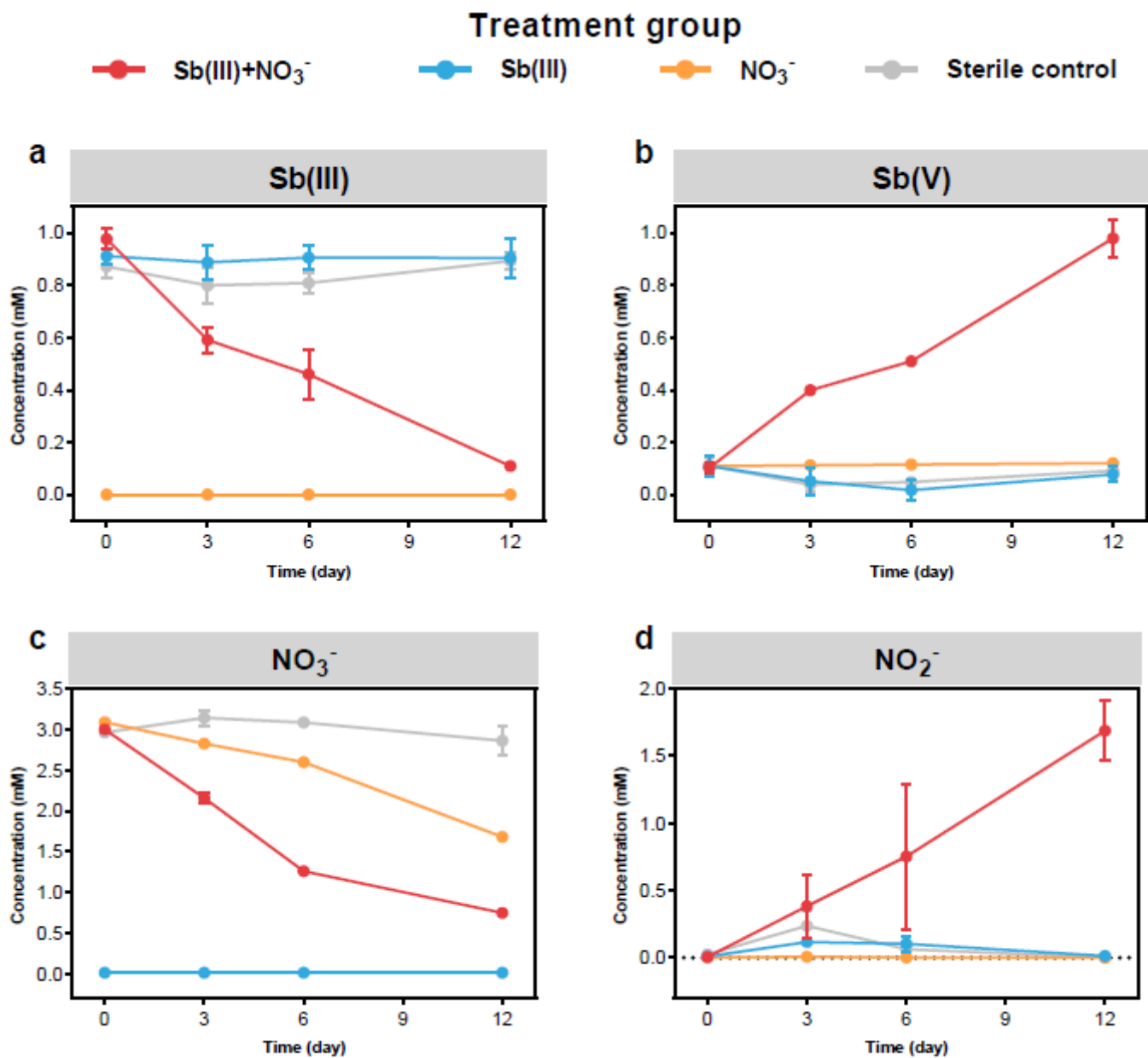


Figure 1

Transformation of Sb(III) (a) to Sb(V) (b) and NO₃⁻ (c) to NO₂⁻ (d) in paddy soils amended with Sb(III) and/or NO₃⁻. Sterile controls were performed with soils autoclaved before incubation. Data are shown in mean ± SE (n =3).

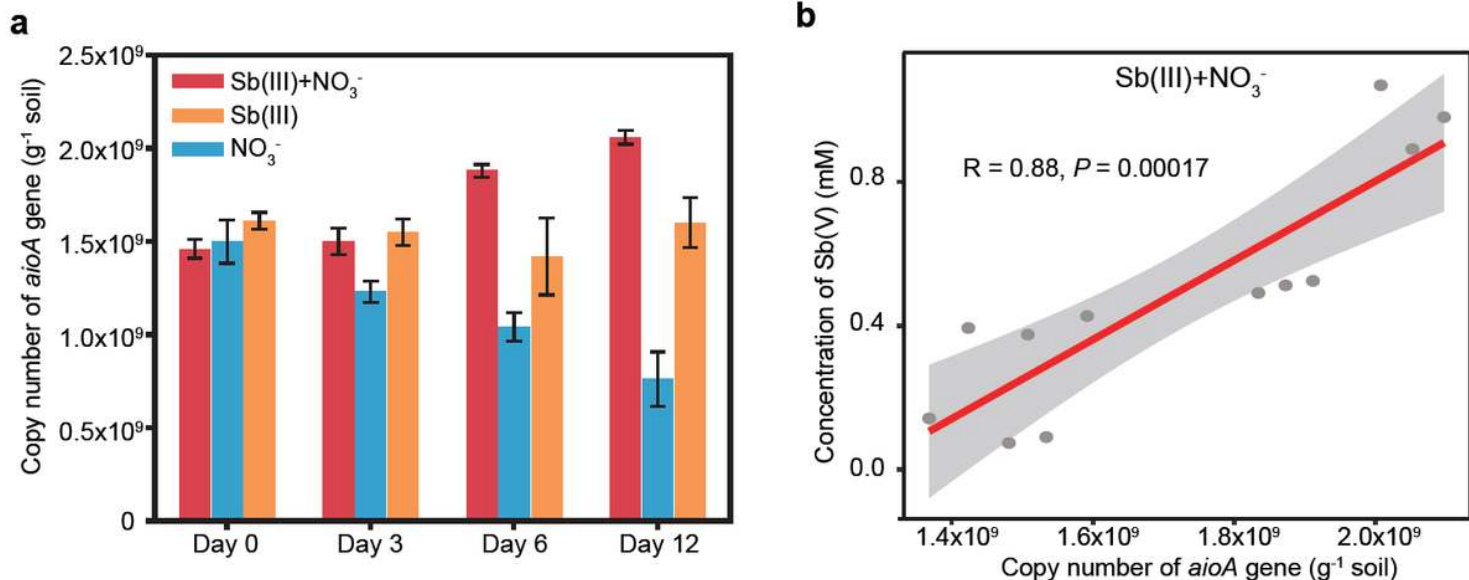


Figure 2

Abundance of the *aioA* gene (mean \pm SE (n =3)) in treatments amended with Sb(III) and/or NO₃⁻ (a) and the correlation between copy number of the *aioA* gene and the concentration of Sb(V) in treatment amended with both Sb(III) and NO₃⁻ (b) under anoxic conditions.

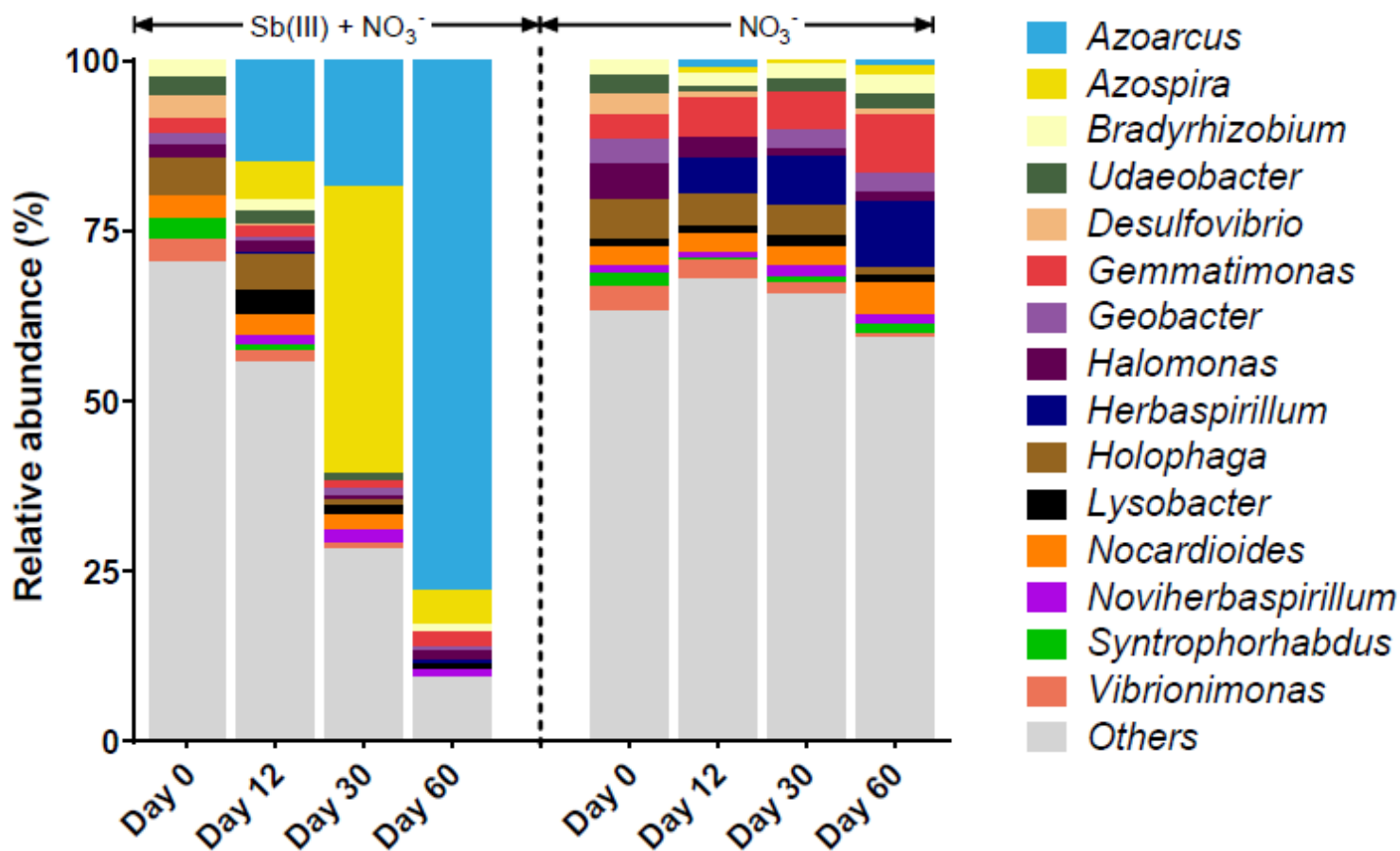


Figure 3

Effects of Sb(III) and NO₃⁻ amendment on bacterial community composition based on partial 16S rRNA gene sequencing represented by the most abundant genera (top 15). Data are shown as average of triplicates (the standard deviations are listed in the text).

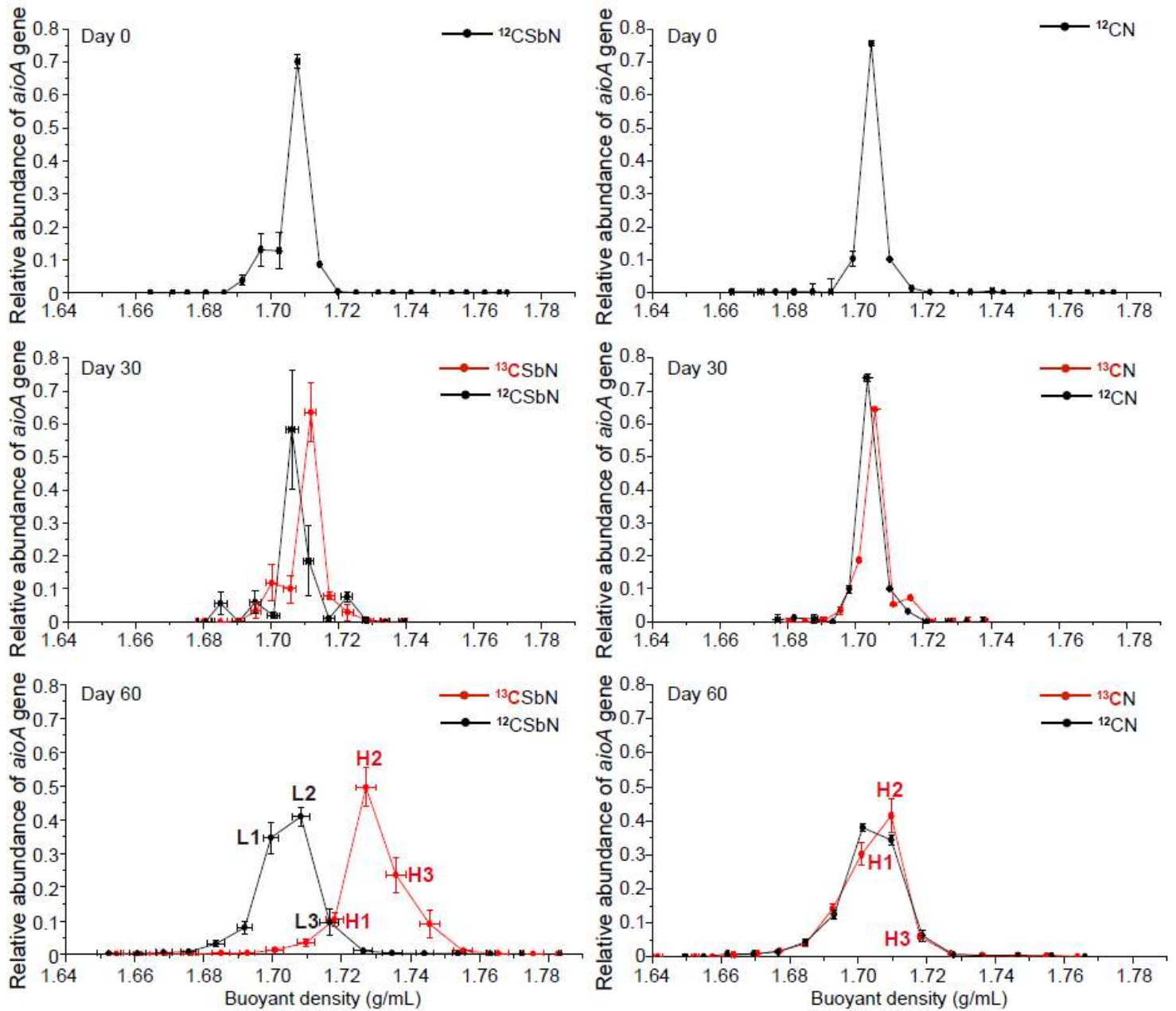
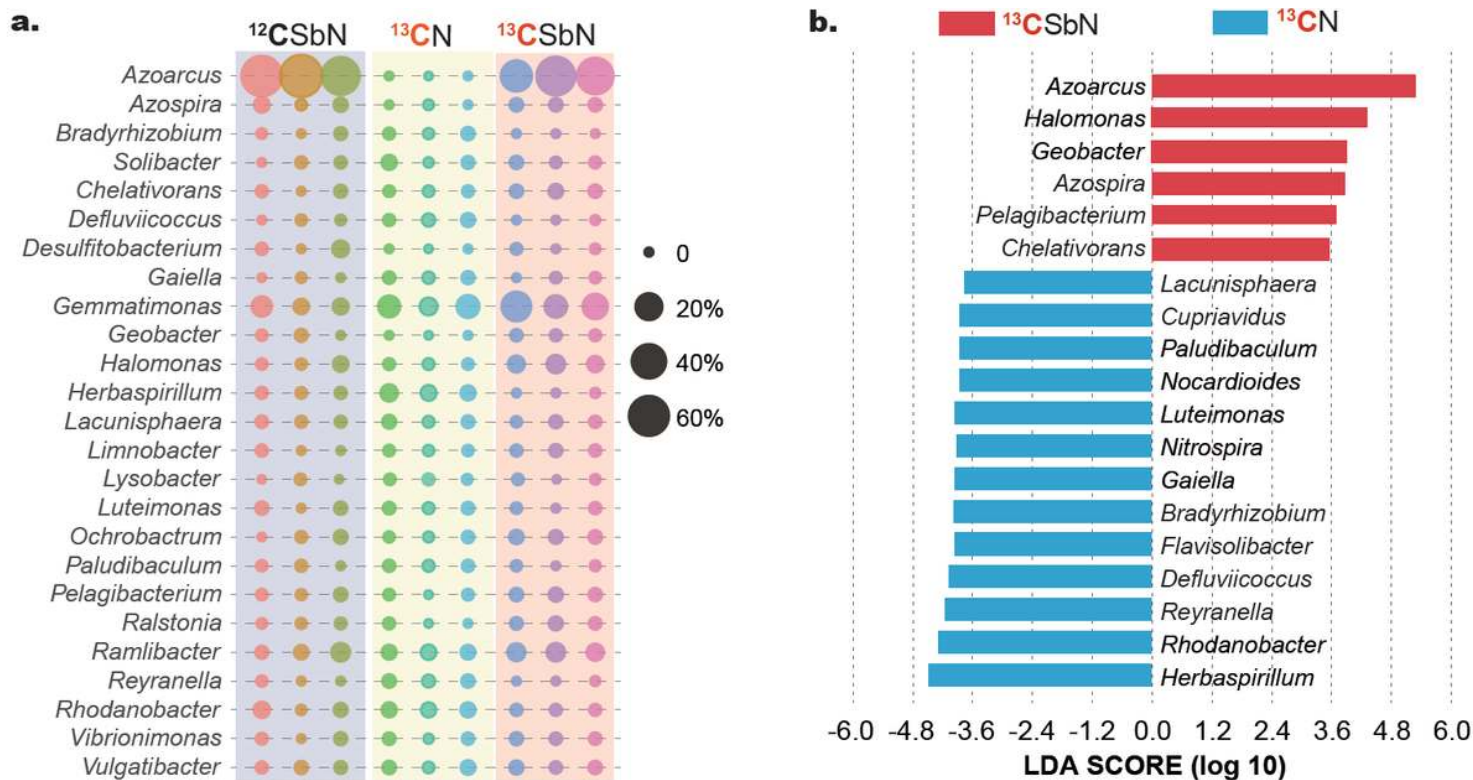


Figure 4

Relative abundance of the *aioA* gene in fractions collected from the 13/12CSbN and 13/12CN treatments at day 0, 30 and 60. L1-L3 represent the representative light fractions with the maximum *aioA* genes from the 12CSbN treatment, and H1-H3 represent the representative heavy fractions with the maximum *aioA* genes from the 13CSbN and 13CN treatments. Vertical and horizontal bars represent standard errors (n=3) of the relative abundance of the *aioA* gene and the buoyant density of the fractions, respectively.



	aioA	aioB	Denitrification				Carbon fixation					
			NO ₃ ⁻ -reducing genes	NO ₂ ⁻ -reducing genes	NO-reducing genes	N ₂ O-reducing genes	rTCA cycle	Calvin cycle	Wood-Ljungdahl pathway	3-HP bicycle	3-HP/4-HB cycle	DC/4-HB cycle
bin1 (<i>Propionivibrio</i> sp.)	9	0	17	7	3	0	22	13	3	10	3	12
bin2 (<i>Rhodanobacter denitrificans</i>)	1	0	4	3	4	1	9	6	2	7	4	12
bin3 (<i>Ramlibacter</i> sp.)	3	1	5	8	4	0	17	12	2	6	7	16
bin4 (<i>Thauera</i> sp.)	6	0	8	3	2	0	13	2	2	9	7	12
bin5 (Bacteria)	3	0	5	3	2	0	14	5	3	11	5	9
bin6 (<i>Devosia</i> sp.)	0	0	2	3	0	1	20	5	2	12	7	16
bin7 (<i>Rhizobiales</i> bacterium)	1	0	2	2	0	0	6	7	1	7	11	12
bin8 (<i>Gemmatimonas</i> sp.)	0	0	1	3	3	0	8	5	2	11	7	10
bin9 (<i>Azoarcus</i> sp.)	5	0	7	3	3	1	11	8	2	6	3	11
bin10 (<i>Azospira</i> sp.)	4	0	9	4	1	1	17	7	2	10	7	15
bin11 (<i>Lacunisphaera</i> sp.)	1	0	1	3	0	0	13	6	3	7	1	7
bin12 (<i>Vulgatibacteraceae</i> bacterium)	1	1	2	1	0	1	11	6	2	11	5	12
bin13 (<i>Anaeromyxobacter</i> sp.)	4	0	11	5	3	1	25	4	2	11	10	19
bin14 (<i>Chelativorans</i> sp.)	5	1	10	5	0	1	12	15	3	11	11	19
bin15 (<i>Gemmatimonas</i> sp.)	0	0	2	0	1	1	11	5	2	12	7	12

Figure 6

Counts of genes responsible for Sb(III) oxidation, denitrification and carbon fixation detected in the assembled bins according to shotgun metagenomic sequencing of heavy DNA fractions from the 13CSbN treatment.