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Diversity and metabolic potentials of As(III)-oxidizing bacteria in activated sludge

Rui Xu[a,b,c,d,1], Duanyi Huang[c,d,1], Xiaoxu Sun[a,b], Miaomiao Zhang[a,b], Dongbo Wang[c,d], Zhaohui Yang[c,d], Feng Jiang[e], Pin Gao[a,b], Baoqin Li[a,b], Weimin Sun[a,b,f,g,#]

[a] National-Regional Joint Engineering Research Center for Soil Pollution Control and Remediation in South China, Guangdong Key Laboratory of Integrated Agro-environmental Pollution Control and Management, Institute of Eco-environmental and Soil Sciences, Guangdong Academy of Sciences, Guangzhou 510650, China

[b] Guangdong-Hong Kong-Macao Joint Laboratory for Environmental Pollution and Control, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China

[c] College of Environmental Science and Engineering, Hunan University, Changsha 410082, P.R. China

[d] Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, P.R. China

[e] Guangdong Provincial Key Lab of Environmental Pollution Control and Remediation Technology, School of Environmental Science and Engineering, Sun Yat-sen University, Guangzhou, China

[f] School of Environment, Henan Normal University

[g] Key Laboratory of Yellow River and Huai River Water Environment and Pollution Control, Ministry of Education

Running Head: *Structure and function of As(III)-oxidizing bacteria*

[1] These authors contributed equally.

[#] Correspondence to Weimin Sun, wmsun@soil.gd.cn

808 Tianyuan Road, Guangzhou, Guangdong, China.

Abstract

Biological arsenite (As(III)) oxidation is an important process in the removal of toxic arsenic (As) from contaminated water. However, the diversity and metabolic potentials of As(III)-oxidizing bacteria (AOBs) responsible for As(III) oxidation in wastewater treatment facilities are not well documented. In this study, two groups of bioreactors inoculated with activated sludge were operated under anoxic or oxic conditions to treat As-containing synthetic wastewater. Batch tests of inoculated sludges from the bioreactors further indicated that microorganisms could use nitrate or oxygen as electron acceptors to stimulate biological As(III) oxidation, suggesting the potentials of this process in wastewater treatment facilities. In addition, DNA-based stable isotope probing (DNA-SIP) was performed to identify the putative AOBs in the activated sludge. Bacteria associated with *Thiobacillus* were identified as nitrate-dependent AOBs, while bacteria associated with *Hydrogenophaga* were identified as aerobic AOBs in activated sludge. Metagenomic binning reconstructed a number of high-quality metagenome-assembled genomes (MAGs) associated with the putative AOBs. Functional genes encoding for As resistance, As(III) oxidation, denitrification, and carbon fixation were identified in these MAGs, suggesting their potentials for chemoautotrophic As(III) oxidation. In addition, the presence of genes encoding secondary metabolite biosynthesis and extracellular polymeric substance metabolism in these MAGs may facilitate the proliferation of these AOBs in activated sludge and enhance their capacity for As(III) oxidation.

Importance

AOBs play an important role in the removal of toxic arsenic from wastewater. Most of the AOBs have been isolated from natural environments. However, knowledge regarding the structure and functional roles of As(III)-oxidizing communities in wastewater treatment facilities are not well documented. The combination of DNA-SIP and metagenomic binning provides an opportunity to elucidate the diversity of *in situ* AOBs community inhabited the activated sludges. In this study, the putative AOBs responsible for As(III) oxidation

in wastewater treatment facilities were identified, and their metabolic potentials including As(III) oxidation, denitrification, carbon fixation, secondary metabolites biosynthesis, and extracellular polymeric substances metabolisms were investigated. This observation provides an understanding of anoxic and/or oxic AOBs during the A(III) oxidation process in wastewater treatment facilities, which may contribute to the removal of As from contaminated water.

Keywords: DNA-stable isotope probing; Metagenomic binning; As(III)-oxidizing bacteria; Arsenic-contaminated water; Biological As(III) oxidation

Introduction

Arsenic (As) is a toxic and carcinogenic metalloid belonging to group 15 of the periodic table. It is generally accepted that arsenite (As(III)) and arsenate (As(V)) are the predominant forms of inorganic As in the environment (1). Of these two As species, As(V) is less toxic and mobile than As(III) (1). Chronic exposure to As can cause various adverse health effects to humans (2). It has been estimated that hundreds of millions of people in more than 70 countries are threatened by As pollution (3). High amounts of As in aquifers have been found in various natural environments, such as groundwater and surface water (4-6). Wastewater may also contain a significant amount of As due to the widespread usage of As in herbicides, pesticides, and wood preservatives (4, 5, 7-9). This adds to the contamination of water supplies and poses a global health problem (6, 10). Therefore, the removal of As from contaminated water is a critical issue worldwide (3). As(III) oxidation is considered as a key step in the removal of As from the aqueous phases because As(V) is more readily removed by the following units, such as coagulation and precipitation (7-9). It is generally recognized that microbiologically mediated As(III) oxidation is more environmentally friendly than chemical As(III) oxidation and does not generate oxidant residuals and harmful byproducts(11). In fact, many As-contaminated wastewater have been treated by biological As(III) oxidation (9, 11, 12).

As(III)-oxidizing bacteria (AOBs) are responsible for biological As(III) oxidation. These bacteria are widespread in the environment (4, 13, 14) and are broadly classified into two categories: heterotrophic AOBs and chemoautotrophic AOBs (15). Heterotrophic As(III) oxidation is a bacterial detoxification process. Heterotrophic As oxidizers can convert As(III) to As(V) but they can not derive major energy for growth from this process (16). In contrast, chemoautotrophic AOBs use As(III) as the electron acceptor and gain energy from As(III) oxidation for CO_2 fixation and cell growth under aerobic (17), nitrate-reducing (14), or chlorate-reducing (18) conditions. Generally, chemoautotrophic AOBs are more beneficial than heterotrophic bacteria for As removal because of their lower nutritional requirements (5, 11, 14, 19). To date, most of the known chemoautotrophic AOBs have been isolated from natural environments, such as pyrite mines (20), As-contaminated soils (13), or lakes (14). However, knowledge regarding As(III)-oxidizing communities in wastewater treatment facilities remains limited. Therefore, it is necessary to elucidate the diversity and metabolic potentials of the *in situ* microbial community responsible for As(III) oxidation in wastewater treatment facilities. The emergence of cultivation-independent techniques, such as DNA-based stable isotope probing (SIP) and omics-related approaches, have been shown to reveal the activities of environmental microorganisms and their metabolic potentials without isolation (21, 22). The analysis of stable isotope-labeled DNA followed by molecular techniques links microbial identities to functions in complex environmental samples (19, 23).

In this study, two groups of bioreactors were operated to treat As-containing synthetic wastewater under oxic and anoxic conditions. The phylogeny of putative AOBs that actively participated in As(III) oxidation were identified in the ^{13}C-labelled batch incubations. Metagenomic binning was used to reconstruct the metagenome assembled genomes (MAGs) of AOBs from these complex microbial communities. Genomic potentials with regard to As(III) oxidation, carbon fixation, and microbial interactions were investigated in the AOB-associated MAGs. The current study aims to investigate (1) Which microorganism are responsible for

As(III) oxidation in activated sludge? (2) What are the metabolic potentials of these AOBs? The results provide an understanding of the diversity and metabolic potentials of AOBs during the As(III) oxidation process in wastewater treatment facilities, which may contribute to the removal of As from contaminated water.

Results

Performances of the As(III)-oxidizing bioreactors under oxic and anoxic conditions

Two types of lab-scale bioreactors were set up under oxic conditions (RO) and anoxic conditions (RN) to treat As-containing synthetic wastewater (**Figure 1**). As(III) was supplemented as the sole electron donor to these reactors for five operation cycles to select the chemoautotrophs. The concentrations of aqueous As(III) and As(V) were monitored over the course of operation. It was observed that concentrations of As(III) constantly decreased with the concomitant generation of As(V), suggesting that As(III) could be oxidized to As(V) under both oxic and anoxic conditions. Moreover, the As(III) oxidation rates in the two bioreactors had a good linear fit with the pseudo-first-order and pseudo-second-order kinetic models as $R^2 > 0.8$ (in all cases, **Figure S2**). Within the five cycles of As(III) oxidation, the k_1 values of the RO (0.06, 0.62, 0.60, 0.35, and 0.86, averaged as 0.50 d^{-1}) were higher than those of the RN (0.04, 0.03, 0.09, 0.09, and 0.09, averaged as 0.07 d^{-1}). In addition, the k_2 values of the RO (0.09, 1.42, 1.17, 0.78, and 2.29, averaged as 1.15 mM^{-1} d^{-1}) were also higher than those of the RN (0.05, 0.04, 0.11, 0.13, and 0.12, averaged as 0.09 mM^{-1} d^{-1}). The kinetic model suggested that the As(III) oxidation rates in the RO were higher than those in the RN as reflected by the higher slopes. In addition, the As(III) oxidation gene (*aioA*) was quantified from DNA extracted from the two bioreactors as a proxy of the potential activity of As(III) oxidation. It is observed that the ratio of the *aioA* gene to 16S rRNA gene in the RO was higher than those in the RN (**Figure S3**), suggesting higher potential of As(III) oxidation in RO than that in RN.

Microbial community in the As(III)-oxidizing bioreactors

Microbial communities in the RO and RN were characterized at the end of cycle 1, cycle 3, and cycle 5, as well as in the raw inoculum sludge. The Bray-Curtis distances based PCoA plots indicated that the microbial communities from the RO samples were distinct from the RN samples (**Figure S4**). This result indicated that redox conditions substantially impacted the innate microbial community. Indeed, different microbial taxa were enriched in the RO and RN bioreactors (**Figure 2**). At the phylum level, Proteobacteria dominated in all of the reactors (over 80%) from cycle 1 to 5, while Chloroflexi (2%–6%) and Bacteroidetes (3%–6%) were the second most dominant phylum in the RN and RO bioreactors, respectively. At the genus level, *Pseudoxanthomonas* dominated in the RN bioreactor, with relative abundances ranging from 5% to 40%, followed by *Pseudomonas* (1%–13%) and *Thermomonas* (4%–8%). Other genera, such as *Thiobacillus*, *Acinetobacter*, and *Nitrosomonas*, had a abundances of less than 1%. In the RO bioreactor, the dominant genera were *Comamonas* (21%–32%), followed by *Stenotrophomonas* (11%–18%), *Hydrogenophaga* (3%–9%), *Delftia* (5%–6%), and *Pseudoxanthomonas* (1%–4%).

Batch tests for the As(III) oxidation

The batch incubations were further established using acclimated activated sludges obtained from the two types of bioreactors and were incubated under oxic and anoxic conditions (**Figure S5**). Under anoxic conditions, As(III) oxidation only occurred in the treatments amended by nitrate and As(III), while no As(III) oxidation was observed in the treatments amended by As(III) only or in the sterile controls. The concentrations of nitrate (NO_3^-) constantly decreased with the concomitant generation of nitrite (NO_2^-). The results suggested that As(III) could be oxidized to As(V) by coupling with the reduction of nitrate. Under oxic conditions, As(III) oxidation was only observed in the treatments amended by oxygen and As(III), while As(III) oxidation did not occur in the absence of oxygen or sterilized treatments.

Furthermore, DNA-SIP batch incubations were performed to identify the AOBs by feeding the ^{13}C-labeled $NaH^{13}CO_3$ as the sole carbon source. Genomic DNA from different gradient fractions, including the ^{13}CAsN/^{13}CAsO, ^{12}CAsN/^{12}CAsO, and ^{13}CAs treatments, were separated into heavy and light fractions using the CsCl gradient ultracentrifugation. The copy numbers of the 16S rRNA gene were measured across each fraction of these treatments from three time points (i.e., days 7, 15, and 30) (**Figure 3**). The copies of 16S rRNA were initially observed at the maximum relative abundance in the "light fractions (BD values of 1.707 g mL^{-1})" in all treatments at day 7. The "peak" of 16S rRNA gradually shifted to the "heavy fractions" (BD values of 1.73–1.75 g mL^{-1}) in either the ^{13}CAsN or the ^{13}CAsO treatments at latter time points on days 15 and 30. This suggested that the AOBs gradually incorporated $NaH^{13}CO_3$ under both anoxic and oxic conditions. In a comparison, no significant enrichment of the 16S rRNA genes in the "heavy fractions" was observed in either the ^{12}CAsN/^{12}CAsO or the ^{13}CAs treatments.

Phylogeny of the AOBs

The genomic DNA of the representative fractions (i.e., "heavy" or "light") from the three treatments (i.e., ^{13}CAsN/^{13}CAsO, ^{12}CAsN/^{12}CAsO, and ^{13}CAs) at the three time points (i.e., day 7, 15, and 30) were collected for 16S rRNA amplicon sequencing. Specifically, the bacterial taxa enriched in the heavy fractions of the either^{13}CAsN or ^{13}CAsO treatments were considered as the putative AOBs because they may have incorporated ^{13}C from the $NaH^{13}CO_3$ for autotrophic As(III) oxidation. The details on the sample collections are shown in **Table S1**.

The microbial communities of each fraction are shown in **Figure 4**. Under anoxic conditions, bacteria affiliated with *Thiobacillus* were identified as one of the most abundant bacteria in the ^{13}CAsN_heavy fractions, suggesting that they may have been responsible for nitrate-dependent As(III) oxidation. The relative abundance of *Thiobacillus* was found to be the highest in the ^{13}CAsN_heavy fractions on day 15 (accounting

for 56%) and day 30 (69%). In contrast, the relative abundances of *Thiobacillus* were only 0.4% (day 15) and 1% (day 30) in the corresponding ^{13}CAsN_light fractions. Additionally, *Serratia* and *Acidovorax* were also detected in the ^{13}CAsN_heavy fractions but with lower relative abundances (less than 0.6%).

Under the oxic conditions, bacteria affiliated with *Hydrogenophaga* dominated in the ^{13}CAsO_heavy fractions, followed by *Serratia*, *Brucella*, and *Stenotrophomonas*. The relative abundances of *Hydrogenophaga* accounted for 9% and 23% in ^{13}CAsO_heavy fractions on day 15 and day 30, respectively. The relative abundances of the other genera only accounted for a small proportion, such as *Serratia* (1%–3%), *Brucella* (0.2%–2%), and *Stenotrophomonas* (2%–3%). Notably, *Serratia* were detected in the heavy fractions of both ^{13}CAsN and ^{13}CAsO, suggesting that they may be capable of oxidizing As(III) under two redox conditions. In addition, the putative AOBs identified by the DNA-SIP, *Thiobacillus* and *Hydrogenophaga*, were also identified as the keystone taxa of the bacterial communities in the RN and RO bioreactors, respectively (**Figure 5**). Although their percentages in the bioreactors were relatively low (*Thiobacillus* in RN: <1%, *Hydrogenophaga* in RO: 3%–9%, **Figure 2**), high connection degrees between these two AOBs and other bacteria were observed in the co-occurrence networks, suggesting that they may have played critical ecological roles in both the anoxic and oxic bioreactors.

Metagenomic binning of the AOBs

Four metagenomes from representative heavy and light fractions of either ^{13}CAsN or ^{13}CAsO were assembled by metagenomic binning. Metagenomic binning reconstructed 71 high-quality metagenome-assembled genomes (MAGs) with > 60% completeness and < 10% contamination from the four metagenomes. The coverage, completeness, contamination, abundance, and taxonomy of the qualified MAGs were evaluated (**Table S2**, **Figures S6** and **S7**). Among the recovered MAGs, three MAGs associated with putative AOBs, including *Thiobacillus* sp. (bin.9), *Hydrogenophaga* sp. (bin.59), and *Serratia* sp. (bin.30), were obtained and

selected for further investigation.

Metabolic potentials of the AOBs

The functional genes of the MAGs associated with the three AOBs were annotated (**Figure 6**). The key genes involved in the As(III) oxidation, (i.e., *aioA* and *aioB*), were detected, suggesting their capability to oxidize As(III). In addition, genes encoding for As resistance (*ACR3* and *arsBC*) and methylation (*arsM*) were also observed in these MAGs. In contrast, the As(V) respiratory reductase gene (*arrA*) was not detected.

Because anoxic As(III) oxidation was coupled with nitrate reduction in this study, the genes responsible for denitrification, including nitrate (NO_3^-) reduction (*nasAB*, *narGHIJ*, and *napAB*), nitrite (NO_2^-) reduction (*nirAB*, *nirK*, and *nirS*), nitric-oxide (NO) reduction (*norB* and *norC*), and nitrous-oxide (N_2O) reduction (*nosZ*), were searched for in the MAGs. The MAGs associated with the two anaerobic AOBs (i.e., *Thiobacillus*-associated bin.9 and *Serratia*-associated bin.30) had all of the necessary genes for denitrification, such as *napAB*, *narG*, *nirK*, *norB*, and *nosZ*. In addition, all three AOB-associated MAGs contained genes encoding for six major carbon fixation pathways, suggesting their capacity for chemoautotrophic growth.

In addition, a set of genes encoding for secondary metabolites biosynthesis and extracellular polymeric substance (EPS) biosynthesis, regulation, and secretion were investigated in the three AOB-associated MAGs (**Figure 6**). Details on the detected genes can be found in **Tables S2 and S3**. For the secondary metabolite biosynthesis, genes encoding dihydroanticapsin dehydrogenase (*bacC*), 1-deoxy-11beta-hydroxypentalenate dehydrogenase (*ptlF*), and secoisolariciresinol dehydrogenase (*SDH*) were ubiquitously identified in the three AOB-MAGs. Additionally, a number of genes encoding for antibiotic biosynthesis, such as the puromycin biosynthesis protein (*Pur7* and *Pur3*) and bacilysin biosynthesis (*bacF* and *bacG*), were also detected. Additionally, a high abundance of genes involved in EPS metabolism (e.g., glycosyltransferase (*EpsD*),

sensor histidine kinase (*KdpD*), glycosyltransferase 2 family protein, and UDP-glucose 4-epimerase) and polysaccharide biosynthesis (polysaccharide biosynthesis proteins (*PslH*, *PslF*, and *PelF*)) were detected in three AOB-associated MAGs.

Discussion

Biological As(III) oxidation under different redox conditions

Biological As(III) oxidation driven by AOBs has important environmental implications for treating As-contaminated water. Microbially mediated As(III) oxidation has been extensively studied in natural habitats such as paddy soils (13), sediments (24), and lakes (14). This study further investigated the diversity and metabolic potentials of AOBs inhabiting engineering environments (e.g., activated sludge). Although the activated sludge was inoculated from municipal wastewater treatment plants with low As concentrations, the performances of the two bioreactors indicated that As(III) could be readily oxidized under both anoxic and oxic conditions with the enrichment of AOBs (**Figure 1**). In addition, by fitting the kinetic models, the oxic As(III) oxidation had a relatively higher rate than the anoxic As(III) oxidation (**Figure S2**).

As(III) could be oxidized in the wastewater treatment facilities using different electron acceptors. The succession of the electron acceptors for As(III) oxidation generally follows the order of oxygen > nitrate > manganese > iron > sulfate > methanogenesis (25). Dissolved oxygen (DO) may be easily exhausted by various reduced compounds in the bioreactors. Thus, anoxic As(III) may be another important pathway for As(III) oxidation. Given the abundant nitrate in wastewater (26) and the high position of nitrate in the redox tower, microorganisms are likely to use nitrate as electron acceptor to oxidize As(III) in the absence of DO. In this study, the batch incubation further that confirmed the concomitant consumption of As(III) and the generation of As(V) were driven by microorganisms using nitrate or oxygen as the terminal electron acceptors (**Figure S5**). Taken together, these findings suggested that biological As(III) oxidation coupled with the

reduction of nitrate or oxygen may have occurred in the As-containing wastewater treatment process.

Putative AOBs and their metabolic potentials

The enrichment of AOBs has successfully promoted the removal of As from many full-scale wastewater treatment plants (7, 9, 11, 27). In this study, the microbial communities inhabiting the RO and the RN were characterized using 16S rRNA gene sequencing (**Figure 2**). A distinct microbial cluster formed during the course of incubation, suggesting that the redox conditions shaped the microbial communities of the activated sludge (**Figure S4**). For instance, *Pseudoxanthomonas* was enriched as the most abundant genus in the RN while *Comamonas* was enriched as the most abundant genus in the RO (**Figure 2B**). However, the dominance of specific taxa in the bioreactors may not indicate that they are responsible for As(III) oxidation (28). For example, He et al. (9) found that *Bacillus* and *Pseudomonas* were the As(III) oxidation related genera, but the percentages of these bacteria in the microbial community were less than 0.1%. Li et al. (11) isolated a number of AOBs from bioreactors and found that their abundances in the bioreactor were low. Therefore, DNA-SIP was used here to identify the phylogeny of the putative AOBs (**Figures 3** and **4**). In our previous work, this method was successfully applied to identify the nitrate-dependent AOBs in paddy soils, such as *Azoarcus*, *Rhodanobacter*, *Pseudomonas*, and Burkholderiales-related bacteria (23). However, none of these AOBs were identified in this study. Instead, bacteria associated with *Thiobacillus* were identified as nitrate-dependent AOBs in sludge, while bacteria associated with *Hydrogenophaga* were identified as aerobic AOBs. *Serratia* spp. were identified as AOBs under both redox conditions, suggesting that the inoculum may also influence the diversity of AOBs.

Thiobacillus spp. have been previously described as anaerobic denitrifying bacteria with the capability to oxidize Fe(II) (29), FeS (30), and U(IV) oxide minerals (31). Garcia-Dominguez et al. (24) reported that *Thiobacillus* spp. were able to oxidize As(III) aerobically. The current study suggested that *Thiobacillus* is

able to anaerobically oxidize As(III) using nitrate as the electron acceptor as well. Metagenomic binning further indicated that a set of key genes involved in As(III) oxidation and denitrification were identified in the *Thiobacillus*-associated bin.9, suggesting their ability to couple As(III) oxidation with nitrate reduction (**Figure 6A**). In addition to As(III) oxidation, genes associated with As resistance were detected in *Thiobacillus*-associated bin.9, which could enable these bacteria to survive under a high concentration of As (32). Moreover, genes encoding six carbon fixation pathways also were identified, suggesting their capability for chemoautotrophic growth.

Under oxic conditions, bacteria associated with *Hydrogenophaga* were identified as putative AOBs. *Hydrogenophaga* spp. have been reported to have versatile abilities to oxidize As(III). For example, *Hydrogenophaga* sp. NT-14 was found to heterotrophically oxidize As(III) (33). *Hydrogenophaga* sp. CL-3 was able to oxidize As(III) under oxic conditions autotrophically (24). *Hydrogenophaga* sp. H7 can simultaneously oxidize As(III) and degrade aromatic compounds, which is helpful to co-remediate metals and aromatic compounds (34). In addition, members of *Hydrogenophaga* are well known for their capacity for autotrophic growth using CO or hydrogen as electron donors (35). Indeed, the presence of genes encoding As(III) oxidation and carbon fixation further confirmed their roles in autotrophic As(III) oxidation (**Figure 6B**). In addition, *Serratia* spp. were identified as AOBs under both anoxic and oxic conditions. *Serratia* spp. have been previously identified as putative As(III)-resistant bacteria (36, 37), but have not been reported as AOBs. In this study, a number of genes encoding As resistance, As(III) oxidation, denitrification, and carbon fixation were identified in the *Serratia*-associated bin.30, suggesting their metabolic versatility to autotrophically oxidize As(III) under different redox potentials (**Figure 6C**).

Ecological roles of AOBs in the bacterial community

Given the complexity of the microbial communities residing in activated sludge, it is of great interest to

268 investigate the interactions of these AOBs with other microorganisms inhabiting the sludge. Notably,
269 *Hydrogenophaga* and *Thiobacillus* were identified as "keystone taxa" within the microbial community due to
270 their high connections with other taxa inhabiting the bioreactors (**Figure 5**) (38). These keystone taxa are
271 critical in structuring the microbial interactions and functions by mediating the microbial community
272 composition in response to both environmental and biological factors (38, 39). It has been reported that
273 keystone taxa may use secondary metabolites to regulate other microorganisms in the community (40).
274 Secondary metabolites are small organic molecules produced by an organism that are not necessary for their
275 growth and reproduction, but are responsible for multiple functions in interactions with other organisms in the
276 microbial communities (41). Thus, secondary metabolite production has been suggested to regulate
277 community dynamics (40, 42). Indeed, a number of genes associated with secondary metabolite biosynthesis
278 were observed in these keystone taxa (**Figure 6**). In particular, the detection of genes encoding the
279 biosynthesis of antibiotics here might be important for the successful competition and niche establishment of
280 these AOBs by outcompeting other microorganisms in the bioreactors (**Table S3**). Members of *Thiobacillus*,
281 *Hydrogenophaga*, and *Serratia* have consistently been reported to encode or produce diverse secondary
282 metabolites (43-46). Therefore, further investigation of the metabolic traits of the secondary metabolites
283 synthesized by these "keystone taxa" AOBs could benefit the biological process of As(III) oxidation in
284 wastewater treatment facilities.

285 In addition, a variety of genes encoding key proteins for EPS biosynthesis, regulation, and secretion were
286 detected in these three putative AOBs (**Figure 6** and **Table S4**). EPS is a complex high-molecular-weight
287 mixture of polymers in activated sludge that has a significant influence on the formation of microbial
288 aggregates by providing protective shields (47). The formation of microbial aggregates is an adaptation that
289 enhances the metabolism, survival, and propagation of the microorganisms (48). Likewise, Li et al. (11)
290 enriched the microbial aggregates in biofilm reactors and achieved a higher As(III) oxidation capability and

operation stability as compared to those that only used single AOB strains. It is thought that many groups of bacteria coordinate their activities in spatial proximity, which promotes resilience as well as the efficiency of the community. Therefore, the EPS synthesized by AOBs has been suggested to enhance the stability of As(III)-oxidizing communities in activated sludge, which promotes biological As(III) oxidation.

In summary, As(III) could be consistently oxidized to As(V) by microorganisms in activated sludge under oxic or anoxic conditions. The biological As(III) oxidation was mediated by AOBs using oxygen or nitrate as the electron acceptors, suggesting the potentials of this process in wastewater treatment facilities. Metagenomic binning reconstructed several high-quality MAGs associated with the putative AOBs, such as *Thiobacillus*, *Hydrogenophaga*, and *Serratia*. The presence of key genes associated with As(III) oxidation, As resistance, denitrification, and carbon fixation in these MAGs suggested their capability for chemoautotrophic As(III) oxidation. In addition, many genes involved in secondary metabolite biosynthesis and EPS metabolism were identified in these MAGs, which may facilitate the proliferation of the AOBs and enhance the As(III) oxidation process. This information provides an understanding of chemoautotrophic AOBs, which may contribute to the removal of As from contaminated water.

Materials and methods

Sample collection

Fresh sludge was collected from a local wastewater treatment plant (WWTP), which was operated under a conventional activated sludge process. The WWTP received domestic sewage was not contaminated by As. The concentrations of As in the influent and effluent were less than 0.2 mg L^{-1}). The sludge samples were transported to the lab immediately and stored at 4°C for further incubation or analysis.

Operation of the As(III)-oxidizing bioreactors

Two groups of bioreactors were operated in triplicate to treat the As-containing synthetic wastewater (represented by As(III) solution) under anoxic or oxic conditions (1.5 L working volume; **Figure S1, Design 1**). One group of reactors was purged with N_2 and sealed to create anoxic condition (DO < 0.5 mg L^{-1}, denoted as "RN"). Another group of reactors was purged with compressed air to create oxic condition (DO > 2 mg L^{-1}, "RO"). All of the reactors were seeded with a 100 mL volume of sludge as the inoculum. The RN was fed with synthetic wastewater primarily consisting of 1 mM As(III) and 3 mM nitrate. In comparison, the RO was only fed with 1 mM As(III)-containing synthetic wastewater. All of the reactors were mixed using a magnetic stirrer and equipped with probes to monitor the DO, pH (controlled at 7.0 ± 0.2), and temperature (controlled at 25 ± 1°C). The effluent of each reactor was collected twice a week to measure the concentrations of As(III) and As(V). Once the As(III) was completely oxidized, the reactors were settled and the supernatant was replaced by fresh As(III)-containing synthetic wastewater (denoted as "one cycle"). A total of five cycles (over 40 days) were conducted. In addition, a kinetic model was calculated to evaluate the oxidation rates based on the pseudo-first-order kinetic model and pseudo-second-order kinetic model. Details of the formula can be found in the supporting information.

DNA extraction from the bioreactors and quantitative PCR

The sludge samples (ca. 2 mL) were collected in triplicate from the RO and RN at the end of running cycle 1 (day 15), cycle 3 (day 28), and cycle 5 (day 42). Genomic DNA was immediately extracted from the sludge samples using the FastDNA spin kit (MP Biomedicals, USA) (49). The qualified DNA was sent for 16S rRNA genes sequencing (Illumina MiSeq PE250) to characterize the microbial community composition in the bioreactors (50). The copy number of As(III) oxidation gene (*aioA*) in the bioreactors were measured using qPCR with the primers aoxBM1-2F/aoxBM2-1R (51). Details of the qPCR mix cocktail can be found in the supporting information.

Batch tests of the As(III) oxidation

The AOB communities in the sludge were expected to be enriched after the long-term operation of the bioreactors, which could be used as acclimated inoculum in batch tests. Two sets of batch tests were established to characterize the biological As(III) oxidation under the control conditions: one was for the As(III)-oxidizing incubation and the other was for the DNA-SIP incubation.

As(III)-oxidizing incubation (**Figure S1, Design 2**): Two types of As(III)-oxidizing incubations were set up to investigate the As(III) oxidation under oxic or anoxic (nitrate-reducing) conditions. The anoxic batch incubations were seeded with acclimatized activated sludge (collected from the RN), $NaHCO_3$ (as a carbon source), and autoclaved mineral salt medium (MSM) in 100-mL serum bottles (52). The batch bottles were purged with N_2 to exclude oxygen. A total of three anoxic treatments were set up by adding (A) 1 mM As(III) and 3 mM nitrate, and (B) 1 mM As(III) only. Autoclaved controls (C) were also established using sterilized sludge and 1 mM As(III) and 3 mM nitrate additions.

For the oxic batch incubation, acclimatized activated sludge (collected from the RO) was mixed with $NaHCO_3$ and MSM in serum bottles as mentioned above. Three oxic treatments were established by adding (A) 1 mM As(III) and constantly purging with compressed air, or (B) 1 mM As(III) only without oxygen. Autoclaved controls (C) were also prepared. For each treatment, triplicate bottles were destructively sampled after 0−7 days of incubation. The supernatant was collected for analyses of As(III), As(V), nitrate, and nitrite. Details of the batch incubation and analytical methods can be found in the supporting information.

DNA-SIP incubation (**Figure S1, Design 3**)*:* Batch incubations for DNA-SIP (DNA-SIP incubation) were set up under oxic and anoxic conditions to identify putative AOBs. The DNA-SIP incubations were prepared by mixing 4 mL of the acclimatized activated sludge, 1 mM As(III), and 46 mL of autoclaved MSM under anoxic

or oxic conditions. Because aerobic- or nitrate-dependent AOBs utilize HCO_3^- as their carbon source, the assimilation of ^{13}C-labeled $NaH^{13}CO_3$ was used to discern the putative AOBs. Two treatments with different terminal electron acceptors were amended by either 8 mM ^{13}C-labeled $NaH^{13}CO_3$ (denoted as "^{13}CAsN" for nitrate-dependent or "^{13}CAsO" for oxic), or 8 mM unlabeled $NaH^{12}CO_3$ (denoted as "^{12}CAsN" for nitrate-dependent or "^{12}CAsO" for oxic). As a comparison, treatments without terminal electron acceptors were amended by 8 mM ^{13}C-labeled $NaH^{13}CO_3$ (denoted as "^{13}CAs"). All of the batch bottles were incubated in the dark and were destructively sampled at days 7, 15, and 30.

DNA extraction from the DNA-SIP gradient fractionation

Genomic DNA was extracted from the DNA-SIP batch incubations from the different treatments (i.e., ^{13}CAsN/^{13}CAsO, ^{12}CAsN/^{12}CAsO, and ^{13}CAs) at three time points (days 7, 15, and 30). The labelled ^{13}C-DNA and unlabeled ^{12}C-DNA were separated into "heavy" fractions and "light" fractions using CsCl gradient ultracentrifugation. Details of the ultracentrifugation can be found in the supporting information.

Quantitative PCR and high-throughput sequencing of the DNA-SIP gradient fractionation

The relative abundances of 16S rRNA gene copies in each fraction were measured using qPCR. The gene copies were quantified using the primers 338F/518R (53). Details of the qPCR mix cocktail can be found in the supporting information. DNA samples from the representative gradient fractions (e.g., heavy or light fractions) in each treatment were selected for 16S rRNA amplicon sequencing (V4 region with the primer set 515F/806R) to characterize the microbial communities. In addition, DNA samples from the representative gradient fractions (e.g., heavy or light fractions) in the either ^{13}CAsN or ^{13}CAsO cultures were sent for shotgun metagenomic sequencing (Illumina Hiseq 4000) (54). Detailed information about the selected fractions for amplicon and shotgun metagenomic sequencing can be found in **Table S1**.

Bioinformatic analysis

Raw amplicon sequencing data (19 samples from bioreactors and 75 samples from the DNA-SIP incubations) were merged, trimmed, and filtered using USEARCH. The non-redundant sequence was denoised to obtain the unique amplicon sequence variants (ASV) for the classification of taxonomy. Details of the analysis can be found in the supporting information.

For the shotgun metagenomic sequencing data, a total of four metagenomes with 140 Gb raw reads were initially checked using Trimmomatic for quality control with options "SLIDINGWINDOW:4:20 MINLEN:70" (49, 55, 56). The trimmed reads were then evaluated using the FastQC toolkit. The clean data were *de novo* assembled using MEGAHIT (k-mer: 21−121, at the step of 10) (57). Metagenomic binning of the assembled contigs was conducted by MetaWRAP using MetaBAT2, MaxBin2, and CONCOT software (58). The recovered MAGs were refined and reassembled to improve their quality. CheckM was used to retain the qualified MAGs with a completion of > 60% and contamination of < 10% (59, 60). The taxonomy of the MAGs was annotated using the Genome Taxonomy Database (GTDB) (61). The functional genes of the MAGs were annotated using the KofamKOALA with default parameters (62).

Statistical methods

Co-occurrence networks were constructed to explore the microbial correlations within the microbial communities (63). The co-occurrence networks were generated using the Spearman's correlations with $|R| > 0.6$ and $P < 0.05$ in R ("ggcorrplot") (53). The calculated node-edge pairs were then used to construct the different networks in Gephi v. 0.92 software (64). The node size was proportional to the number of connections (65). Data visualization was performed using GraphPad Prism v. 8.

Figure legends

Figure 1. Bioreactor performances under anoxic (RN) or oxic (RO) conditions: (A) concentrations of As(III) and (B) concentrations of As(V). Triplicated bioreactors of each condition were operated for five cycles by feeding with the As(III) synthetic wastewater. The triangles represent the sampling times for the microbial community analysis.

Figure 2. Microbial communities in the anoxic bioreactors (RN) and oxic bioreactors (RO) for As(III) oxidation: (A) the major phyla (top 5) and (B) the major genera (top 20). Sludge samples were collected in triplicate from the raw inoculum sludge (0 day) and at the end of operation cycle 1 (15 days), cycle 3 (28 days), and cycle 5 (42 days).

Figure 3. 16S rRNA quantification by the quantitative PCR (qPCR) indicated that the As(III)-oxidizing bacteria (AOBs) were labeled in the (A) anoxic and (B) oxic DNA-SIP incubations. The distribution of the 16S rRNA genes were normalized to the highest 16S rRNA gene copies. The legends "^{13}CAsN" or "^{13}CAsO" represent the treatments amended with the $NaH^{13}CO_3$ and terminal electron acceptors (nitrate for ^{13}CAsN or oxygen for^{13}CAsO). As a comparison, "^{12}CAsN" or "^{12}CAsO" represent the treatments amended with the $NaH^{12}CO_3$ and terminal electron acceptors (nitrate for ^{12}CAsN or oxygen for ^{12}CAsO). "^{13}CAs" represent the treatment amended with $NaH^{13}CO_3$ without any electron acceptors.

Figure 4. Relative abundances of the bacterial communities (genus level) in the heavy and light fractions from either (A) anoxic or (B) oxic DNA-SIP incubations, including three treatments (i.e., ^{13}CAsN/^{13}CAsO, ^{12}CAsN/^{12}CAsO, and ^{13}CAs) at three time points (i.e., days 7, 15, and 30). The ^{13}C-labeled bacteria represent the putative As(III)-oxidizing bacteria in the ^{13}CAsN_heavy and ^{13}CAsO_heavy fractions, which are highlighted with red bubbles.

Figure 5. The co-occurrence networks of the bacteria communities in the (A) anoxic bioreactors (RN) and (B) oxic bioreactors (RO). The ^{13}C-labeled As(III)-oxidizing bacteria by DNA-SIP are highlighted with red and navy nodes in the two networks, respectively. The size of the node denotes the connection degree.

Figure 6. Genes encoding As cycling (blue), denitrification (green), carbon fixation (purple), extracellular polymeric substance (EPS) metabolisms (red), and secondary metabolites (SMs) biosynthesis (yellow) in the three metagenome-assembled genomes (MAGs) associated with the putative As(III)-oxidizing bacteria: (A) *Thiobacillus* sp. (bin.9), (B) *Hydrogenophaga* sp. (bin.59), and (C) *Serratia* sp. (bin.30). The height of the bar indicates the gene abundance.

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Conflict of interest

The authors declare they have no conflict of interest to report.

Availability of data

The datasets of the 16S rRNA amplicon sequencing and shotgun metagenomic sequencing generated and

analyzed during the current study have been submitted to the NCBI GenBank database with the project number PRJNA713939.

References

1. Zhu Y, Xue X, Kappler A, Rosen BP, Meharg AA. 2017. Linking genes to microbial biogeochemical cycling: lessons from arsenic. ***Environmental Science & Technology*** 51:7326-7339.

2. Rahman MM, Ng JC, Naidu R. 2009. Chronic exposure of arsenic via drinking water and its adverse health impacts on humans. ***Environmental Geochemistry and Health*** 31:189-200.

3. Singh R, Singh S, Parihar P, Singh VP, Prasad SM. 2015. Arsenic contamination, consequences and remediation techniques: a review. ***Ecotoxicology and Environmental Safety*** 112:247-270.

4. Sun W, Sierra R, Field JA. 2008. Anoxic oxidation of arsenite linked to denitrification in sludges and sediments. ***Water Research*** 42:4569-4577.

5. Lièvremont D, Bertin PN, Lett M-C. 2009. Arsenic in contaminated waters: Biogeochemical cycle, microbial metabolism and biotreatment processes. ***Biochimie*** 91:1229-1237.

6. Bhattacharya P, Welch AH, Stollenwerk KG, McLaughlin MJ, Bundschuh J, Panaullah G. 2007. Arsenic in the environment: Biology and Chemistry. ***Science of The Total Environment*** 379:109-120.

7. Andrianisa HA, Ito A, Sasaki A, Aizawa J, Umita T. 2008. Biotransformation of arsenic species by activated sludge and removal of bio-oxidised arsenate from wastewater by coagulation with ferric chloride. ***Water Research*** 42:4809-4817.

8. Sullivan C, Tyrer M, Cheeseman CR, Graham NJD. 2010. Disposal of water treatment wastes containing arsenic — A review. ***Science of The Total Environment*** 408:1770-1778.

9. He Z, Zhang Q, Wei Z, Wang S, Pan X. 2019. Multiple-pathway arsenic oxidation and removal from wastewater by a novel manganese-oxidizing aerobic granular sludge. ***Water Research*** 157:83-93.

10. Song P, Yang Z, Zeng G, Yang X, Xu H, Wang L, Xu R, Xiong W, Ahmad K. 2017. Electrocoagulation treatment of arsenic in wastewaters: A comprehensive review. ***Chemical Engineering Journal*** 317:707-725.

11. Li H, Zeng X-C, He Z, Chen X, E G, Han Y, Wang Y. 2016. Long-term performance of rapid oxidation of arsenite in simulated groundwater using a population of arsenite-oxidizing microorganisms in a bioreactor. ***Water Research*** 101:393-401.

12. Sun W, Sierra-Alvarez R, Hsu I, Rowlette P, Field JA. 2010. Anoxic oxidation of arsenite linked to chemolithotrophic denitrification in continuous bioreactors. ***Biotechnology and Bioengineering*** 105:909-917.

13. Zhang J, Zhao S, Xu Y, Zhou W, Huang K, Tang Z, Zhao F-J. 2017. Nitrate stimulates anaerobic microbial arsenite oxidation in paddy soils. ***Environmental Science & Technology*** 51:4377-4386.

14. Oremland RS, Hoeft SE, Santini JM, Bano N, Hollibaugh RA, Hollibaugh JT. 2002. Anaerobic oxidation of arsenite in Mono Lake water and by a facultative, arsenite-oxidizing chemoautotroph, strain MLHE-1. ***Applied and Environmental Microbiology*** 68:4795-4802.

15. Oremland RS, Stolz JF. 2005. Arsenic, microbes and contaminated aquifers. ***Trends in Microbiology***

13:45-9.

16. Silver S, Phung LT. 2005. Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. ***Applied and Environmental Microbiology*** 71:599-608.

17. Inskeep WP, Macur RE, Hamamura N, Warelow TP, Ward SA, Santini JM. 2007. Detection, diversity and expression of aerobic bacterial arsenite oxidase genes. ***Environmental Microbiology*** 9:934-43.

18. Sun W, Sierra-Alvarez R, Milner L, Field JA. 2010. Anaerobic oxidation of arsenite linked to chlorate reduction. ***Applied and Environmental Microbiology*** 76:6804.

19. Xing W, Li J, Li D, Hu J, Deng S, Cui Y, Yao H. 2018. Stable-isotope probing reveals the activity and function of autotrophic and heterotrophic denitrifiers in nitrate removal from organic-limited wastewater. ***Environmental Science & Technology*** 52:7867-7875.

20. Santini JM, Sly LI, Schnagl RD, Macy JM. 2000. A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: phylogenetic, physiological, and preliminary biochemical studies. ***Applied and Environmental Microbiology*** 66:92-97.

21. Alneberg J, Bjarnason BS, de Bruijn I, Schirmer M, Quick J, Ijaz UZ, Lahti L, Loman NJ, Andersson AF, Quince C. 2014. Binning metagenomic contigs by coverage and composition. ***Nature Methods*** 11:1144-1146.

22. Xu R, Sun X, Häggblom MM, Dong Y, Zhang M, Yang Z, Xiao E, Xiao T, Gao P, Li B, Sun W. Metabolic potentials of members of the class Acidobacteriia in metal-contaminated soils revealed by metagenomic analysis. ***Environmental Microbiology*** n/a.

23. Zhang M, Li Z, Häggblom MM, Young L, He Z, Li F, Xu R, Sun X, Sun W. 2020. Characterization of nitrate-dependent As(III)-oxidizing communities in arsenic-contaminated soil and investigation of their metabolic potentials by the combination of DNA-stable isotope probing and metagenomics. ***Environmental Science & Technology*** 54:7366-7377.

24. Garcia-Dominguez E, Mumford A, Rhine ED, Paschal A, Young LY. 2008. Novel autotrophic arsenite-oxidizing bacteria isolated from soil and sediments. ***FEMS Microbiology Ecology*** 66:401-410.

25. Tratnyek PG, Grundl TJ, Haderlein SB. 2011. Aquatic redox chemistry. ACS Publications.

26. Hu Y, Wu G, Li R, Xiao L, Zhan X. 2020. Iron sulphides mediated autotrophic denitrification: An emerging bioprocess for nitrate pollution mitigation and sustainable wastewater treatment. ***Water Research*** 179:115914.

27. Hayat K, Menhas S, Bundschuh J, Chaudhary HJ. 2017. Microbial biotechnology as an emerging industrial wastewater treatment process for arsenic mitigation: A critical review. ***Journal of Cleaner Production*** 151:427-438.

28. Jousset A, Bienhold C, Chatzinotas A, Gallien L, Gobet A, Kurm V, Küsel K, Rillig MC, Rivett DW, Salles JF, van der Heijden MGA, Youssef NH, Zhang X, Wei Z, Hol WHG. 2017. Where less may be more: how the rare biosphere pulls ecosystems strings. ***The ISME journal*** 11:853.

29. Straub KL, Benz M, Schink B, Widdel F. 1996. Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. ***Applied and Environmental Microbiology*** 62:1458.

30. Schippers A, Jørgensen BB. 2002. Biogeochemistry of pyrite and iron sulfide oxidation in marine sediments. ***Geochimica et Cosmochimica Acta*** 66:85-92.

31. Beller HR. 2005. Anaerobic, nitrate-dependent oxidation of U(IV) oxide minerals by the chemolithoautotrophic bacterium Thiobacillus denitrificans. ***Applied and Environmental Microbiology***

71:2170-4.

32. Qin J, Rosen BP, Zhang Y, Wang G, Franke S, Rensing C. 2006. Arsenic detoxification and evolution of trimethylarsine gas by a microbial arsenite S-adenosylmethionine methyltransferase. ***PNAS*** 103:2075.

33. vanden Hoven RN, Santini JM. 2004. Arsenite oxidation by the heterotroph Hydrogenophaga sp. str. NT-14: the arsenite oxidase and its physiological electron acceptor. ***Biochimica et Biophysica Acta*** 1656:148-155.

34. Fan X, Nie L, Shi K, Wang Q, Xia X, Wang G. 2019. Simultaneous 3-/4-Hydroxybenzoates Biodegradation and Arsenite Oxidation by Hydrogenophaga sp. H7. ***Frontiers in Microbiology*** 10.

35. Willems A, Gillis M. 2015. Hydrogenophaga. ***Bergey's Manual of Systematics of Archaea and Bacteria***:1-15.

36. Yoon I-H, Chang J-S, Lee J-H, Kim K-W. 2009. Arsenite oxidation by Alcaligenes sp. strain RS-19 isolated from arsenic-contaminated mines in the Republic of Korea. ***Environmental Geochemistry and Health*** 31:109.

37. Escalante G, Campos VL, Valenzuela C, Yañez J, Zaror C, Mondaca MA. 2009. Arsenic resistant bacteria isolated from arsenic contaminated river in the Atacama Desert (Chile). ***Bulletin of Environmental Contamination and Toxicology*** 83:657-661.

38. Banerjee S, Schlaeppi K, Mga VDH. 2018. Keystone taxa as drivers of microbiome structure and functioning. ***Nature Reviews Microbiology*** 16:1.

39. Xu R, Li B, Xiao E, Young LY, Sun X, Kong T, Dong Y, Wang Q, Yang Z, Chen L, Sun W. 2020. Uncovering microbial responses to sharp geochemical gradients in a terrace contaminated by acid mine drainage. ***Environmental Pollution*** 261:114226.

40. Sun X, Xu R, Dong Y, Li F, Tao W, Kong T, Zhang M, Qiu L, Wang X, Sun W. 2020. Investigation of the ecological roles of putative keystone taxa during tailing revegetation. ***Environmental Science & Technology*** 54:11258-11270.

41. Tyc, Olaf, Song C, Dickschat JS, Vos M, Garbeva P. 2017. The ecological role of volatile and soluble secondary metabolites produced by soil bacteria. ***Trends in Microbiology*** 25:280-292.

42. Riley MA, Wertz JE. 2002. Bacteriocins: evolution, ecology, and application. ***Annual Review of Microbiology*** 56:117-137.

43. Hutt LP, Huntemann M, Clum A, Pillay M, Palaniappan K, Varghese N, Mikhailova N, Stamatis D, Reddy T, Daum C. 2017. Permanent draft genome of Thiobacillus thioparus DSM 505 T, an obligately chemolithoautotrophic member of the Betaproteobacteria. ***Standards in Genomic Sciences*** 12:10.

44. Martin-Creuzburg D, Beck B, Freese HM. 2011. Food quality of heterotrophic bacteria for Daphnia magna: evidence for a limitation by sterols. ***FEMS Microbiology Ecology*** 76:592-601.

45. Mishra J, Arora NK. 2018. Secondary metabolites of fluorescent pseudomonads in biocontrol of phytopathogens for sustainable agriculture. ***Applied Soil Ecology*** 125:35-45.

46. Clements T, Ndlovu T, Khan W. 2019. Broad-spectrum antimicrobial activity of secondary metabolites produced by Serratia marcescens strains. ***Microbiological Research*** 229:126329.

47. Sheng G, Yu H, Li X. 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. ***Biotechnology Advances*** 28:882-894.

48. Hansen SK, Rainey PB, Haagensen JA, Molin S. 2007. Evolution of species interactions in a biofilm

community. ***Nature*** 445:533-6.

49. Xu R, Zhang Y, Xiong W, Sun W, Fan Q, Zhaohui Y. 2020. Metagenomic approach reveals the fate of antibiotic resistance genes in a temperature-raising anaerobic digester treating municipal sewage sludge. ***Journal of Cleaner Production*** 277:123504.

50. Xu R, Yang Z, Zheng Y, Zhang H, Liu J, Xiong W, Zhang Y, Ahmad K. 2017. Depth-resolved microbial community analyses in the anaerobic co-digester of dewatered sewage sludge with food waste. ***Bioresource Technology*** 244:824-835.

51. Quéméneur M, Cébron A, Billard P, Battaglia-Brunet F, Garrido F, Leyval C, Joulian C. 2010. Population structure and abundance of arsenite-oxidizing bacteria along an arsenic pollution gradient in waters of the Upper Isle River Basin, France. ***Applied and Environmental Microbiology*** 76:4566-4570.

52. Zhang J, Zhou W, Liu B, He J, Shen Q, Zhao F-J. 2015. Anaerobic arsenite oxidation by an autotrophic arsenite-oxidizing bacterium from an arsenic-contaminated paddy soil. ***Environmental Science & Technology*** 49:5956-5964.

53. Xu R, Yang Z, Wang Q, Bai Y, Liu J, Zheng Y, Zhang Y, Xiong W, Ahmad K, Fan C. 2018. Rapid startup of thermophilic anaerobic digester to remove tetracycline and sulfonamides resistance genes from sewage sludge. ***Science of The Total Environment*** 612:788-798.

54. Sun W, Sun X, Li B, Xu R, Young LY, Dong Y, Zhang M, Kong T, Xiao E, Wang Q. 2020. Bacterial response to sharp geochemical gradients caused by acid mine drainage intrusion in a terrace: Relevance of C, N, and S cycling and metal resistance. ***Environment International*** 138:105601.

55. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. ***Bioinformatics*** 30:2114-2120.

56. Xu R, Zhang M, Lin H, Gao P, Yang Z, Wang D, Sun X, Li B, Wang Q, Sun W. 2022. Response of soil protozoa to acid mine drainage in a contaminated terrace. ***Journal of Hazardous Materials*** 421:126790.

57. Li D, Liu C-M, Luo R, Sadakane K, Lam T-W. 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. ***Bioinformatics*** 31:1674-1676.

58. Gherman U, Jocelyne D, James T. 2018. MetaWRAP-a flexible pipeline for genome-resolved metagenomic data analysis. ***Microbiome***.

59. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. ***Genome Research*** 25:1043-1055.

60. Sun X, Qiu L, Kolton M, Ha¨ggblom M, Xu R, Kong T, Gao P, Li B, Jiang C, Sun W. 2020. VV reduction by Polaromonas spp. in vanadium mine tailings. ***Environmental Science & Technology***.

61. Chaumeil PA, Mussig AJ, Hugenholtz P, Parks DH. 2019. GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database. ***Bioinformatics*** 36:1925-7.

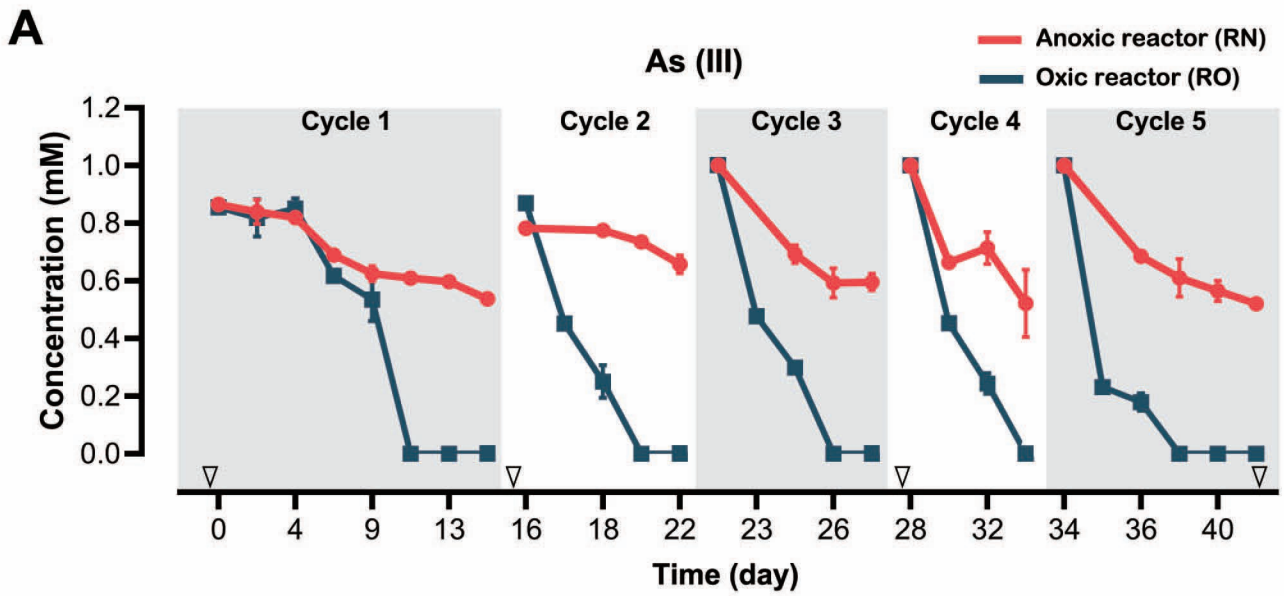
62. Aramaki T, Blanc-Mathieu R, Endo H, Ohkubo K, Kanehisa M, Goto S, Ogata H. 2020. KofamKOALA: KEGG Ortholog assignment based on profile HMM and adaptive score threshold. ***Bioinformatics*** 36:2251-2252.

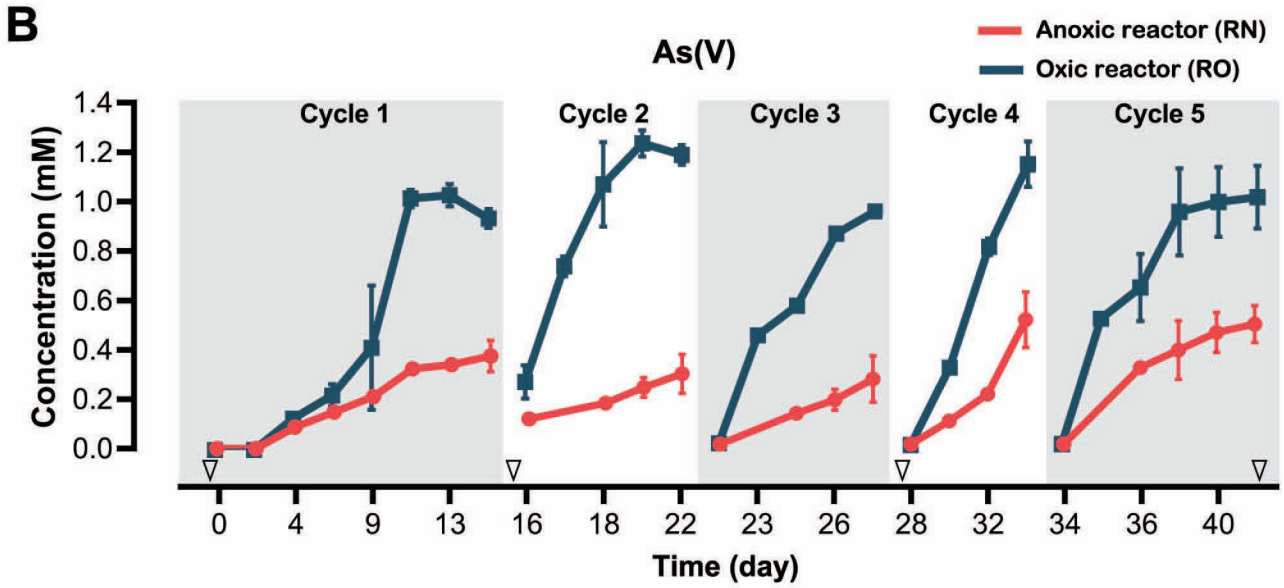
63. Sun X, Song B, Xu R, Zhang M, Gao P, Lin H, Sun W. 2021. Root-associated (rhizosphere and endosphere) microbiomes of the Miscanthus sinensis and their response to the heavy metal contamination. ***Journal of Environmental Sciences*** 104:387-398.

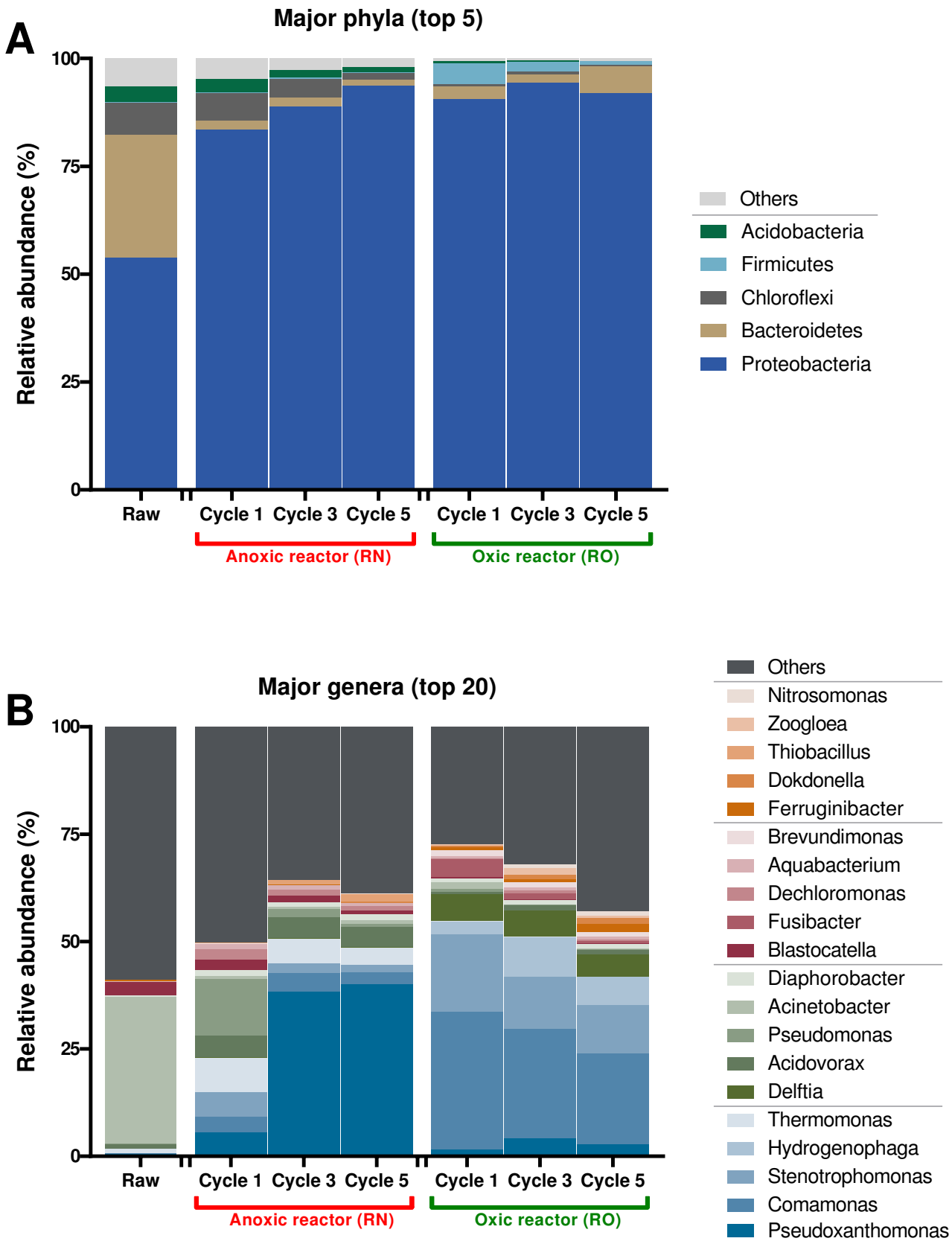
64. Xu R, Yang Z, Zheng Y, Liu J, Xiong W, Zhang Y, Lu Y, Xue W, Fan C. 2018. Organic loading rate and

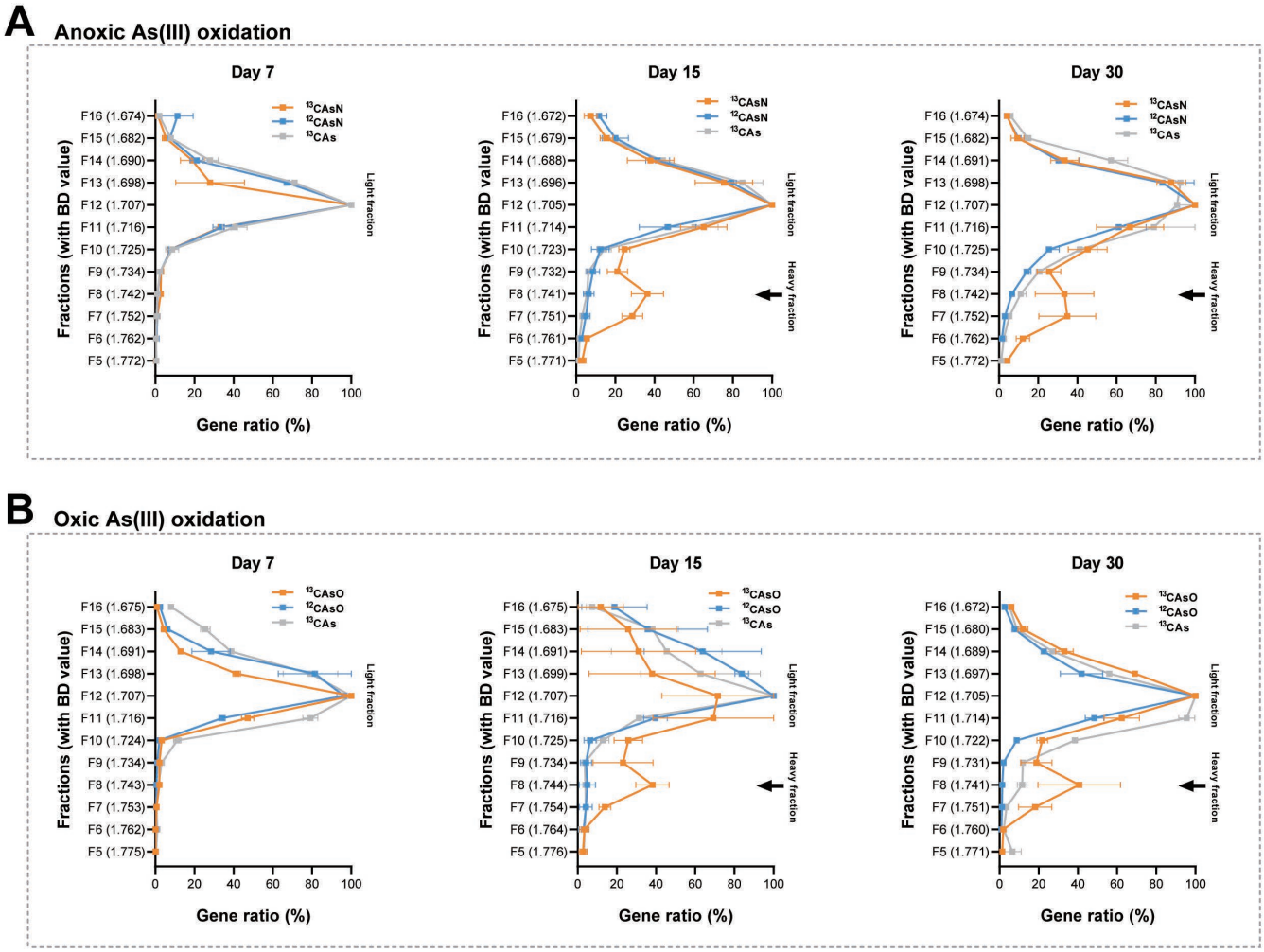
hydraulic retention time shape distinct ecological networks of anaerobic digestion related microbiome. ***Bioresource Technology*** 262:184-193.

65. Xu R, Sun X, Lin H, Han F, Xiao E, Li B, Qiu L, Song B, Yang Z, Sun W. 2020. Microbial adaptation in vertical soil profiles contaminated by an antimony smelting plant. ***FEMS Microbiology Ecology*** 96:1-12.









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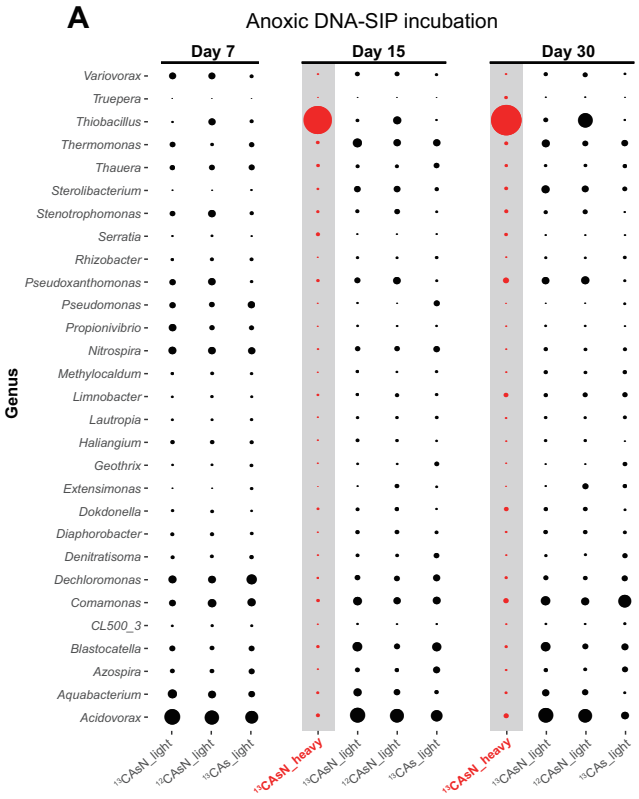

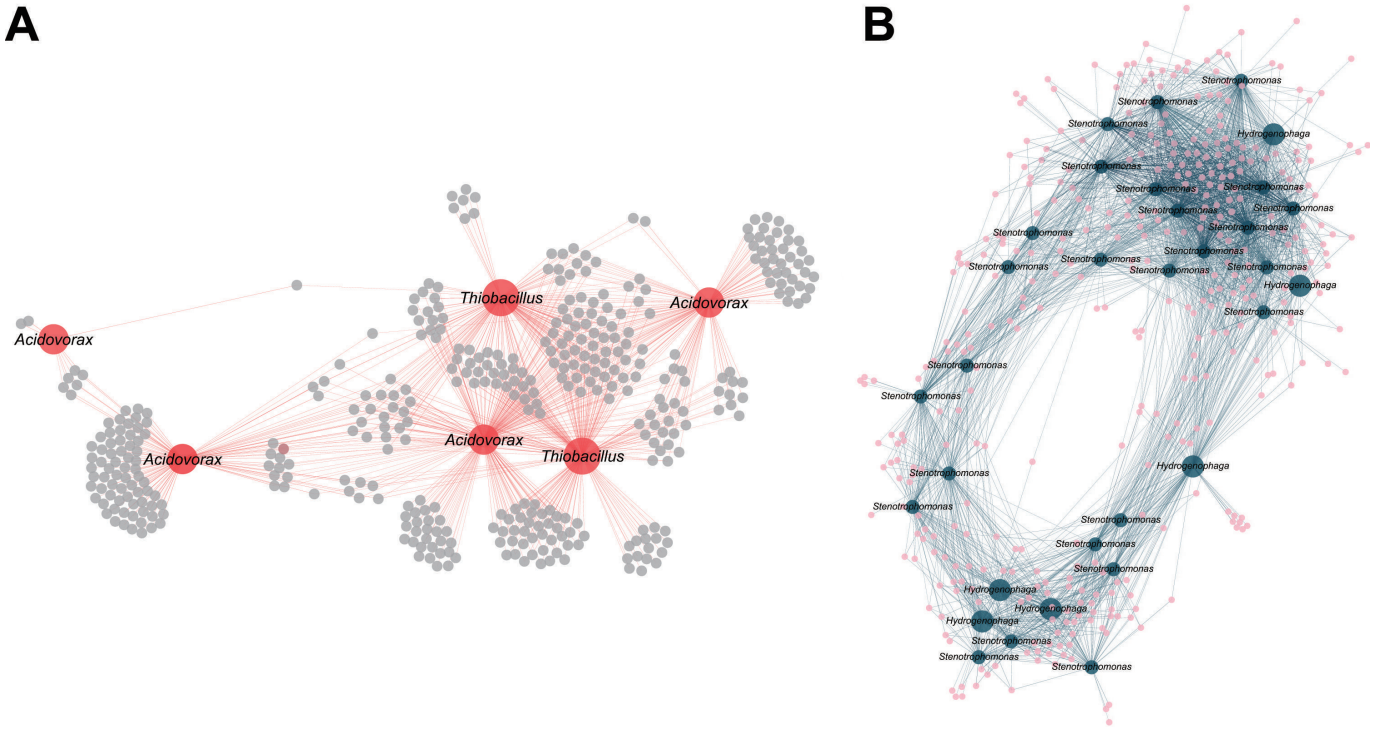

Anoxic network **Oxic network**

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