Humic Substances Facilitate Arsenic Reduction and Release in Flooded Paddy Soil

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ABSTRACT: Organic matter is important for controlling arsenic reduction and release under anoxic conditions. Humic substances (HS) represent an important fraction of natural organic matter, yet the manner in which HS affect arsenic transformation in flooded paddy soil has not been thoroughly elucidated. In this study, anaerobic microcosms were established with arsenic-contaminated paddy soil and amended with three extracted humic fractions (fulvic acid, FA; humic acid, HA; and humin, HM). The HS substantially enhanced the extent of arsenic reduction and release in the order FA > HA > HM. It was confirmed that microbially reduced HS acted as an electron shuttle to promote arsenate reduction. HS, particularly FA, provided labile carbon to stimulate microbial activity and increase the relative abundances of Azoarcus, Anaeromyxobacter, and Pseudomonas, all of which may be involved in the reduction of HS, Fe(III), and arsenate. HS also increased the abundance of transcripts for an arsenate-respiring gene (arra) and overall transcription in arsenate-respiring Geobacter spp. The increase in both abundances lagged behind the increases in dissolved arsenate levels. These results help to elucidate the pathways of arsenic reduction and release in the presence of HS in flooded paddy soil.

INTRODUCTION

Arsenic (As) contamination in paddy fields has gained considerable attention because rice is an important source of arsenic pollution in human diets.1–3 The biogeochemical processes and toxicity of arsenic vary considerably with seasons in paddy fields. During the flooding season, arsenic can be released into soil solution by reductive dissolution of arsenic-bearing Fe(III) minerals and reduction of arsenate [As(V)] to arsenite [As(III)], which increases the arsenic mobility and facilitates arsenic uptake and accumulation in rice plants.4,5 Microbes play a key role in arsenic mobilization in arsenic-contaminated aquifers, sediments, and soils,6–11 and organic matter is important in controlling the rate and magnitude of microbially mediated arsenic release from these arsenic-contaminated environments.10,12 Several microcosm-based and in situ studies used labile organic carbon (mainly acetate) to mimic the influx of easily degradable organic matter and to promote the activity of indigenous microbial communities potentially involved in Fe(III) reduction and arsenic release.10,11,13–15 Our recent study demonstrated that the introduction of lactate into arsenic-contaminated paddy soil stimulated microbial reduction of As(V), mainly by upregul-
ing transcription of the As(V) respiratory reductase gene *arrA* and overall transcription in As(V)-respiring *Geobacter* spp.

Humic substances (HS), which are operationally defined by their extraction from soils using a traditional alkaline–acid extraction technique, constitute one of the important fractions of soil organic matter (SOM). HS can influence the transformation of arsenic in soils by both abiotic and biotic processes. First, arsenic can interact with HS by adsorption and complexation due to the functional groups of HS, such as carboxylic, amino, hydroxyl, phenolic, and sulfhydryl groups. Second, owing to their redox properties, HS can act as an electron shuttle to accelerate the electron transfer from humic-reducing bacteria to solid electron acceptors, such as Fe(III) minerals. Fe(II) is substantially generated in an aquifer with more reduced forms of quinone-like moieties in HS, which is accompanied by an increase in arsenic release. The addition of anthraquinone-2,6-disulfonic acid, a proxy for the quinone moieties in HS, increases both Fe(III) and As(V) reduction in arsenic-rich sediments. It is reasonable to consider, therefore, that HS could also function as an electron shuttle between bacteria and As(V) to facilitate As(V) reduction. Third, HS may increase the abundance of microbial species [e.g., humic-, Fe(III)-, and As(V)-reducing bacteria], as HS can either be utilized as a carbon source or enhance interactions between bacteria and organic matter by forming ternary bacteria—HS—organic matter complexes. However, no study has extracted HS from soil and investigated their effect on arsenic transformation in paddy soil.

HS are highly complex, and differences in chemical properties and environmental effects between bulk HS and their fractions can be notable. The objectives of our study were (i) to isolate and characterize different fractions of HS (fulvic acid, FA; humic acid, HA; and humin, HM) to provide a better understanding of the pathways of arsenic reduction and release and their associated contributors in the presence of HS in flooded paddy soil.

**MATERIALS AND METHODS**

**Soil Sampling and Characterization.** Soil was sampled from a drained paddy field contaminated by arsenic-containing acid mine drainage in Shantou City, China. The soil contains 0.3 g kg⁻¹ total arsenic, 30.5 g kg⁻¹ total iron, and 17.3 g kg⁻¹ dithionite–citrate–bicarbonate (DCB)-extractable Fe, which represents crystalline and amorphous Fe oxides.

**Extraction and Characterization of HS.** The fractions of HS used in this study were extracted from a subtropical peat soil (Zhongxiang International Ltd., Beijing) that had a lower content of iron than the tested paddy soil, minimizing the interference of iron in the electron-shuttling of HS. HA and FA were extracted following the International Humic Substances Society (IHSS) method, and HM was prepared on the basis of previously described procedures. For purification, each fraction of HS was concentrated in a rotating evaporator and purified using a dialysis membrane (molecular weight cutoff 10 000 Da, Sigma) against deionized water according to a procedure described previously. The water collected outside the dialysis membrane was subjected to a Cl⁻ test with AgNO₃ (0.10 M) with a detection limit of 0.81 μM. The fractions of HS were freeze-dried and then stored in a dessicator at 25 °C in the dark until use.

**Microcosm Experiments.** Anaerobic microcosms were set up as previously described. 7 g of anoxic paddy soils (wet weight) were placed into sterile serum vials containing 70 mL of 30 mM bicarbonate buffer (pH 7.3), 1 mL L⁻¹ trace element solution, and 1 mL L⁻¹ vitamin solution, followed by the addition of 0.07 g of individual HS. After flushing with a mixture of N₂:CO₂ (80:20) for 40 min, the microcosms were sealed with butyl rubber stoppers. Incubation and sampling were carried out at 30 °C in the dark without shaking in an anaerobic chamber (Coy Laboratories, Grass Lake, MI). All microcosms, including abiotic controls, were processed in triplicate (Table S1, SI). For the abiotic control, the fractions of sterile soil and HS were prepared by irradiation with 50 kGy γ-rays from a 60Co radioactive source at a γ-irradiation facility (Guangzhou Huada, China). The uncertainties in γ-ray dosimetry agreed to within 2% by both alanine and dichromate dosimeters, which gives confidence in the γ-dose applied. To evaluate the potential effect of microbially reduced HS on arsenic reduction in paddy soil, additional experiments were conducted with *Shewanella oneidensis* MR-1 and with incubation with sterile soil and HS in the presence and absence of acetate. Details of the experiments are in the SI.

**Arsenic and Iron Speciation.** When sampling at intervals, three vials of each treatment were centrifuged at 8000g for 10 min at room temperature. A total of 5 mL of the supernatant was then filtered through a sterile 0.22-μm filter for determination of dissolved As(III) and As(V) concentrations using atomic fluorescence spectroscopy (SA-20, Jitian, Beijing, China). Soil pellets were divided into two subsamples. One subsample was immediately frozen in liquid nitrogen and stored at −80 °C until total RNA extraction could occur; the other was used to sequentially fractionate arsenic in soil using 1 M KH₂PO₄ + 0.1 M ascorbic acid [mainly targeting As adsorbed on iron (oxyhydr)oxides], 33 0.2 M NH₄⁺–oxalate [predominantly targeting As incorporated into iron (oxyhydr)oxides], and HNO₃ + H₂O₂ (dissolving the residual As remaining in the soil). Detailed descriptions of the PO₄-As, oxalate-As, and residual As extraction are available in the SI. Dissolved Fe(II) and 0.5 M HCl-extractable Fe(II), namely,
HCl-Fe(II), were quantified by the methods previously described.\textsuperscript{35} 16S rRNA High-Throughput Sequencing. Total community RNA was extracted using the PowerSoil Total RNA Isolation Kit (Mio Bio Laboratories) according to the manufacturer’s instructions. Elimination of genomic DNA and reverse transcription were performed with the PrimeScript RT reagent kit with gDNA Eraser (Takara). The cDNA from three replicates of experiments at each sampling time was amplified with primers 515F and 806R and then subjected to Illumina sequencing.\textsuperscript{36} Details of the PCR amplification and sequence analysis are in the SI. The sequence data have been deposited by qPCR using primers Geo494F and Geo825R.\textsuperscript{39} Details of PCR conditions and the nested PCR).\textsuperscript{38} Details of PCR conditions and the construction and further analysis of the clone library are in the SI. The sequence data have been deposited by the methods previously described.\textsuperscript{25,41} The maximum possible contribution of the Fe to the measured EAC is 0.7\%, 0.9\%, and 13.2\% for FA, HA, and HM, respectively, if all Fe in the HS is present as Fe(III) and each Fe(III) accepts one electron. Humic-bound iron can act as an electron shuttle for microbial Fe(III) reduction,\textsuperscript{42} although the complexed iron alone might not be sufficient to be responsible for the amount of electrons transferred by the HS samples.\textsuperscript{43,44} Nevertheless, our results implied that HA and FA should accept and donate electrons more efficiently than HM when acting as an electron shuttle on a per gram basis.

The EAC of HA was higher than that of FA, which is similar to earlier studies.\textsuperscript{25,45} Generally, the EAC of HA and FA correlated positively with their C/H elemental ratios and aromaticity data from \textsuperscript{13}C NMR.\textsuperscript{25,45,46} In this study, HA also showed higher aromaticity (50.0\%) than FA (43.5\%), but FA had a higher C/H ratio and contained higher percentages of carbon in carbonyl groups relative to HA (Table 1), which could lead to high electron transfer capacity. HM usually has a high aromaticity, but the aromaticities calculated from \textsuperscript{13}C NMR spectral data cover a wide range, from 35\% to 92\%.\textsuperscript{47} Jin et al. found that while the HM of a grassland soil had a higher C/H than the corresponding HA, the HM of an agriculture soil showed a lower C/H ratio than the corresponding HA.\textsuperscript{48} Hence, the C/H ratios and aromaticities of HM could vary depending on the HM’s origins. In this study, HM had the lowest C/H ratio and aromaticity, which thus resulted in its low EAC and EDC (Table 1). The ratios of total carbon to labile carbon were 11:1, 5:1, and 63:1 for HA, FA, and HM, respectively (Table 1), suggesting that FA can provide a greater amount of bioavailable carbon as an electron donor for microbial respiration than FA and HM.

Arsenic and Iron Transformation. Figure 1 illustrates the levels of soluble and phosphate- and oxalate-extractable arsenic species over time as affected by the different treatments. Dissolved As(III) increased over time and reached 4.7, 4.2, and 3.9 mg L\textsuperscript{−1} in the FA-, HA-, and HM-amended microcosms on day 30, respectively, which was at least twice the concentration in the soil control (1.7 mg L\textsuperscript{−1}) (Figure 1a). Dissolved As(V) levels rapidly increased to 0.4–1.1 mg L\textsuperscript{−1} (FA > HA ≈ HM ≈ control) at day 6 and then gradually declined to approximately 0.2 mg L\textsuperscript{−1} at day 30 (Figure 1b). Compared with the control treatment, the addition of HS resulted in higher PO\textsubscript{4}\textsuperscript{−} and oxalate-As(III) and lower PO\textsubscript{4}\textsuperscript{−} and oxalate-As(V) at day 30 (Figure 1c–f), indicating that HS enhanced the reduction of As(V) adsorbed on and incorporated into iron (oxyhydr)-oxides. As shown in Figure S3 (SI), the molar mass of different arsenic fractions suggested that the dominant arsenic species was PO\textsubscript{4}-As(V) at the beginning and dissolved As(III) at the

### Table 1. Characteristics of the FA, HA, and HM Fractions

<table>
<thead>
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<th></th>
<th>bulk elemental composition</th>
<th>\textsuperscript{13}C NMR</th>
<th>ETC (mmol\textper百万\textsuperscript{g}\textsuperscript{−1})</th>
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<tr>
<td></td>
<td>C (%)</td>
<td>O (%)</td>
<td>N (%)</td>
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<tr>
<td>FA</td>
<td>41.0</td>
<td>31.2</td>
<td>1.1</td>
</tr>
<tr>
<td>HA</td>
<td>40.2</td>
<td>30.0</td>
<td>0.9</td>
</tr>
<tr>
<td>HM</td>
<td>47.8</td>
<td>28.0</td>
<td>0.4</td>
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\textsuperscript{a}Aromaticity = 100 × aromatic C (93–165 ppm)/[aromatic C (93–165 ppm) + aliphatic C (0–93 ppm)].\textsuperscript{b}The electron-accepting and -donating capacities (EAC and EDC) values were the average of replicates (n ≥ 3) of the data in Figure S1 (SI). The labile carbon was extracted by 2.5 M H\textsubscript{2}SO\textsubscript{4} at 105 °C for 30 min according to the method of Rovira and Vallejo.\textsuperscript{22}
As(III) concentrations were 140 ± 7.6 mg L−1 in the FA, HA, and HM amendments, which results in its stronger electrostatic interaction with the iron (hydr)oxide surfaces, adsorption with arsenic than HA because FA has a smaller size and closer proximity to the iron (hydr)oxide surfaces, among these abiotic effects, competitive adsorption due to electrostatic interaction is considered to be the main mechanism, and FA likely competes more strongly for arsenic release than HA because FA has a smaller size and closer proximity to the iron (hydr)oxide surfaces, which results in its stronger electrostatic interaction with arsenic. FA did not show a stronger effect on arsenic release than HA and HM in the abiotic controls (Figure S4, SI), probably because γ-ray irradiation can increase its average molecular weight due to condensation of carboxylic groups and CO2 liberation. Nevertheless, FA without any irradiation treatment likely contributes in part to the facilitation of arsenic release in the biotic microcosms.

The formation of dissolved Fe(II) and HCl-Fe(II) was higher in all biotic microcosms than in the abiotic controls (Figure S5a, SI). HS facilitated As(V) reduction in the biotic microcosms, the initial increases in dissolved As(V) (Figure 1b) are predominantly due to microbial Fe(III) reduction, and As(V) and Fe(III) reduction proceeded simultaneously (Figures 1 and S5 (SI)). Regarding the substantial increase in dissolved As(III) concentration, it is necessary to clarify how HS facilitated As(V) reduction in flooded paddy soil.

**Arsenic Reduction by Microbiologically Reduced HS.** To generate microbially reduced HS, another experiment with sterile HS and sterile soil was incubated with *S. oneidensis* MR-1 and supplied with acetate. MR-1 is a humic- and Fe(III)-reducing bacterium that cannot reduce As(V). In the treatment of acetate + MR-1 + sterile soil, both dissolved Fe(II) and dissolved As(V) levels increased after day 6 and remained at 28–36 and 3.1–4.6 mg L−1 during days 6–30, respectively (Figure 2d). Thus, As(V) release from the soil into solution is mainly due to the microbial Fe(III) reduction by MR-1. Meanwhile, dissolved As(III) was barely detected in the soil solution (Figure 2d), confirming that MR-1 cannot reduce As(V).

With the addition of sterile HS, dissolved As(III) increased over time, and dissolved As(V) decreased after day 6. The dissolved As(III) and dissolved Fe(II) concentrations were 3.0–3.4 and 21–30 mg L−1 on day 30, respectively (Figure 2a–c). It seems that HS facilitate As(V) reduction by functioning as an electron shuttle, in which HS are reduced by MR-1 and the reduced HS transfer electrons to As(V) for As(V) reduction. The decrease in dissolved As(V) being larger than the increase in dissolved As(III) in Figure 2a–c and the slight decrease in dissolved As(V) in Figure 2d are likely due to readsoption on and reincorporation into secondary Fe(II)–Fe(III) minerals.

To confirm the electron shuttle effect of HS, two supplementary experiments were performed. (i) MR-1 was...
incubated with sterile soil and sterile HS but without acetate to verify whether the HS can serve as an electron donor for MR-1. The results shown in Figure S6 (SI) demonstrate that the electron donor effect of HS on facilitating anaerobic respiration of MR-1 for Fe(III) and HS reduction was small because HS only slightly increased the dissolved Fe(II), As(III), and As(V) levels; these concentrations were negligible relative to their higher production levels with acetate first (Figure 2). (ii) FA and HA were incubated with MR-1, and the reduced FA and HA (without any microorganisms) were then collected and mixed with dissolved As(V) for reaction. It is confirmed that microbially reduced FA and HA can abiotically reduce As(V) to As(III) (Figure S7, SI).

In Figure 2, the sterile HS seem to facilitate only As(V) reduction, not Fe(III) reduction. In this experiment, acetate and MR-1 were supplied at concentrations as high as 20 mM and 10^10−10^11 cells mL^−1, respectively, both of which can provide a sufficient number of electron donors and cells to maximize the microbial activity for Fe(III) and HS reduction. Since MR-1 cannot reduce As(V), the reduced HS mainly facilitated As(V) reduction when acting as an electron shuttle, given that microbially available Fe(III) and HS could be fully reduced by MR-1. The extracellular electron transfer performance of HS is positively correlated with their EAC and aromaticity. Different HS with MR-1 led to similar production of dissolved As(III) (Figure 2) because reduced HS could be provided repeatedly by MR-1, maximizing the electron shuttle effect on As(V) reduction. In the biotic microcosms with soil (Figure 1), however, HS resulted in different levels of As(III) production, which should be associated not only with their EAC and aromaticity (Table 1) but also with the microorganisms stimulated by HS.

**Bacterial Communities Stimulated by HS.** An overall summary of high-throughput sequencing libraries is provided in the SI. The profiling of bacterial 16S rRNA genes identified 13 distinct phyla/classes (Figure S8, SI) and 12 genera (Figure 3) at a relative abundance >1%. At the genus level, *Variovorax* (1−22%), *Flavisolibacter* (0.1−6%), and *Azoarcus* (0.4−6%) represented the dominant genera in the soil control, and their abundances greatly increased during days 0−6 (Figure 3d). With HA and HM, the abundance of *Variovorax* was constant at 16−20% during days 2−30, which was similar to its level in the soil control (Figure 3b−d). *Azoarcus* was also one of the dominant genera in samples with HA and HM, and its abundance (1−10%) was slightly higher in these samples than in the soil control (Figure 3b−d). In addition, a gradual increase in *Aeranomyxobacter* (from 0.7% to 4%) was observed over time in samples with HA (Figure 3b). Compared with amendments with the other HS, FA amendment resulted in a lower abundance of *Variovorax* (1.2−12%) during incubation and a higher abundance of *Azoarcus*, particularly during days 2−6 (14−17%) (Figure 3a). Meanwhile, FA amendment increased the abundance of *Pseudomonas* (0.5−8%), *Syrnecococcus* (0−7%), and *Aeranomyxobacter* (1−4%).

The genus *Variovorax* contains a large number of heavy-metal-tolerant strains. The sequenced genome of *Variovorax paradoxus* S110 provides some clues to the organism’s arsenic tolerance by identifying the operon coding for an arsenic reductase complex. Similarly, whole-genome analyses find a putative arsenic reduction operon in the genomes of *Azoarcus* members. Both *Pseudomonas* and *Aeranomyxobacter* have been frequently detected in arsenic-contaminated sites, and some of them can respire humics, Fe(III), or As(V) under anoxic conditions. *Variovorax* and *Aeranomyxobacter* might participate in As(V) reduction without HS, and HS, particularly FA, might stimulate *Aeranomyxobacter, Aeranomyxobacter, Anaeromyxobacter*, and *Pseudomonas* to reduce HS, Fe(III), and/or As(V).

**Identification and Quantification of As(V)-Respiring Gene and Bacteria.** This study focused on transcriptional copy numbers of the As(V)-respiration-related *arrA* gene and associated bacteria, since As(V)-respiring bacteria play a more important role in arsenic reduction than arsenic-resistant microorganisms in anoxic arsenic-contaminated environments. Following the addition of HS, the transcription of bacterial 16S rRNA and *arrA* genes was stimulated overall (Figure S9a,b, SI).

The *arrA* gene transcripts clone library showed that more than 50% of the *ArrA* sequences were related to putative *ArrA* sequences of *Geobacter* spp. and 46% of them were related to *ArrA* sequences of uncultured bacteria (Figure S10 and Table S3, SI). Transcriptional patterns of *Geobacter* spp. revealed that FA stimulated the transcription of *Geobacter* spp. throughout the incubation the most (Figure S9c, SI). *Geobacter* was also detected in the bacterial communities of all microcosms and at relatively high levels (0.5−1.3%) during days 6−30 (Figure 3). These results emphasized the importance of *Geobacter* spp. in respiratory arsenic reduction in the overall As(V) reduction in arsenic-contaminated paddy soil, which can be stimulated by FA.

The increase in *arrA* gene and overall *Geobacter* spp. transcripts lagged behind the increase in dissolved As(V) levels in all biotic microcosms after being normalized against the copy numbers of 16S rRNA genes (Figure 4). This finding is different from the results of our previous study, which showed
species. However, HS are a mixture of complex organic compounds, and the majority of HS (i.e., HA and HM) are insoluble in water at circumneutral pH, which leads to the low bioavailability of HS for microbes. This effect is supported by the following results: (i) HS had a limited electron donor effect on anaerobic respiration of MR-1 in Fe(III) and HS reduction (Figure S6, SI), and (ii) the transcription levels of bacterial 16S rRNA gene and 

Environmental Implications. In summary, our results demonstrate that HS can facilitate arsenic reduction and release via three main pathways: (1) functioning as an electron shuttle, (2) providing labile carbon as an electron donor and increasing the abundance of indigenous soil bacteria (i.e., Arthrobacter, Anaeromyxobacter, and Pseudomonas), and (3) stimulating the transcriptions of the As(V)-respiration-related arrA gene and As(V)-respiring Geobacter species. Among the three fractions of HS, FA is the most effective because it has a greater amount of labile carbon and higher solubility and ETC. These characteristics not only favor the stimulation of microbial activity but also accelerate electron transfer from microbes to both dissolved and adsorbed As(V).

Since SOM is thoroughly mixed with and often adheres to soil minerals, it has traditionally been separated into several operationally defined fractions to better understand its properties. Similarly, the fractions of HS in this study were subject to traditional alkaline–acid extraction, but some conceptual problems have been raised when defining “humic substances” by this extraction procedure. For example, 50–70% of the soil organic carbon is unextracted due to incomplete extraction, and the alkaline solution at pH 13 can ionize compounds that would never dissociate within the pH range of soil (3.5–8.5). As such, the fractions of HS may not be the best representatives of SOM.

Recently, HA and FA have been found to share major chemical groups irrespective of their sources. However, their molecular architectures may differ substantially, which would influence their binding properties. In this study, the ETCs of HS differed from each other, which was associated with the functional groups involved in their ETC. Before an alternative approach of SOM isolation is developed, the operational separation of HS into three fractions, in combination with characterization of the individual fractions, could provide more information for a better understanding of their environmental effects on arsenic transformation.

It is important to note that the relative contributions of FA, HA, and HM to the electron-shuttling effect on arsenic reduction and release may differ among soils. The relative contributions of these fractions may also be different for the native FA, HA, and HM fractions in the studied paddy soil, as the observations in this study are based on added fractions of HS that were isolated from a different soil system. Nevertheless, paddy soil with a high content of HS, particularly with high FA levels, can be predicted to highly elevate As(III) concentrations into soil solution during flooding seasons. FA and HA are also commonly used as soil supplements in agriculture. Therefore, introducing HS into arsenic-contaminated paddy soil, particularly during planting season, should be considered very carefully. However, an arsenic remediation strategy using HS can still be developed and applied during the slack season to respond to the demand for safe food and sustainable agriculture because this environmentally friendly substance can effectively mobilize arsenic from arsenic-contaminated paddy soil.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b06333.

Additional experimental methods and discussion, current responses of HS in EAC and EDC measurement (Figure S1), 13C NMR spectra (Figure S2), molar mass of different As(III)/As(V) species and total As (Figure S3), different As(III)/As(V) species in abiotic controls (Figure S4), dissolved Fe(II) and HCl-Fe(II) (Figure S5), dissolved Fe(II), As(III), and As(V) in MR-1 +...
sterile soil + sterile HS (Figure S6), dissolved As(III) in microbially reduced FA/HA + dissolved As(V) (Figure S7), bacterial community at the phyllum and class levels (Figure S8), transcriptions of 16S rRNA, arrA gene, and Geobacter spp. (Figure S9), phylogenetic tree of putative ArrA sequences (Figures S10), experimental treatments conducted (Table S1), molar mass of different As(III)/As(V) species and total As (Table S2), summary of arrA gene transcribes clone library (Table S3), and additional references (PDF)

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J.Q. and X.L. contributed equally to this work.

**Notes**
The authors declare no competing financial interest.

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