Arsenite Oxidation by the Phyllosphere Bacterial Community Associated with *Wolffia australiana*

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Supporting Information

ABSTRACT: Speciation is a key determinant in the toxicity, behavior, and fate of arsenic (As) in the environment. However, little is known about the transformation of As species mediated by floating macrophytes and the phyllosphere bacteria in aquatic and wetland environment. In this study, *Wolffia australiana*, a rootless floating duckweed, was cultured with (*W+B*) or without (*W−B*) phyllosphere bacteria to investigate its ability in arsenite (As(III)) oxidation. Results showed that sterile *W. australiana* did not oxidize As(III) in the growth medium or in plant tissue, whereas *W. australiana* with phyllosphere bacteria displayed substantial As(III) oxidation in the medium. Quantitative PCR of As redox-related functional genes revealed the dominance of the arsenite oxidase (*aioA*) gene in the phyllosphere bacterial community. These results demonstrate that the phyllosphere bacteria were responsible for the As(III) oxidation in the *W+B* system. The rapid oxidation of As(III) by the phyllosphere bacterial community may suppress As accumulation in plant tissues under phosphate rich conditions. The *aioA* gene library showed that the majority of the phyllosphere arsenite-oxidizing bacteria related either closely to unidentified bacteria found in paddy environments or distantly to known arsenite-oxidizing bacteria. Our research suggests a previously overlooked diversity of arsenite-oxidizing bacteria in the phyllosphere of aquatic macrophytes which may have a substantial impact on As biogeochemistry in water environments, warranting further exploration.

INTRODUCTION

The ubiquitously distributed arsenic (As) is a nonthreshold carcinogen, ranking at the top of the Priority List of Hazardous Substances (ATSDR, 2011, http://www.atsdr.cdc.gov/SPL/index.html). Elevated levels of As in paddy environments through anthropogenic activities, such as mining, and irrigation with As contaminated water pose a serious threat to human health and sustainable agriculture, especially in South and Southeast Asia where rice is the staple food.1–4 Arsenic speciation plays an important role in determining its toxicity, behavior, and fate.5 Therefore, it is vital to understand the pathways and mechanisms of As species transformation in paddy ecosystems.

In fresh water environments, arsenate (As(V)) and arsenite (As(III)) are the major species.5 Arsenite occurs predominantly underoxic conditions, while in suboxic environments, As(III) prevails. Plants can take up As(III) and As(V) through aquaporins in the NIP (nodulin 26-like intrinsic protein) subfamily and phosphate transporters, respectively.6 As an essential pathway of detoxification in nonhyperaccumulators, most of the intracellular As(V) can be reduced by plant arsenate reductase (ACR2) efficiently to As(III).6,7 Some portion of the As(III) can be excreted back into the environments partly by members of NIP aquaporins.8 Therefore, plants can reduce As(V) in water or soil porewater.8,9 In contrast to speciation by plants, arsenic metabolism by microorganisms is much more diverse, including reduction, oxidation, methylation, and demethylation.10 The ubiquitous coexistence of plants and the related microbiome may make As transformation at the interfaces between plants and the medium unique but remains largely unknown.

The coexistence of arsenite-oxidizing and arsenate-reducing communities in rice rhizosphere makes the relative abundance of these two functional groups a key determinant to As uptake and accumulation in rice.11 Floating macrophytes, which occupy the surface water in wetlands, may harbor divergent microbiomes compared to those directly contacting with sediments or soils. However, little is known about the As dynamics mediated by floating macrophytes and the associated microorganisms in the surface water. *Wolffia*, in the family of Lemnaceae, is a rootless floating duckweed vastly distributed in static fresh waters.12,13 Although belonging to vascular plants, the small fronds (0.5–2 mm) of *Wolffia* are composed mostly

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of chlorenchymous cells surrounded by a layer of epidermis, without xylem or phloem cells. The reduced structure of this plant facilitates the maintenance of a complete sterile culture, and with other advantages, such as high growth rate and As accumulating ability, making it a potential model plant in studies of As biogeochemical cycling. Previous studies have revealed diverse bacterial communities associated with the phyllosphere of aquatic macrophytes. However, the diversity of As-speciation related bacteria in these habitats still remains unknown. We hypothesize that the phyllosphere of *Wolffia* may harbor a unique community of bacteria capable of As speciation which could have great impact on the uptake and accumulation of As by the plant per se.

Therefore, in the present study, we investigated (1) the role of *Wolffia* and the phyllosphere bacterial community in the dynamics of As(III) and As(V) transformation and (2) the effect of phyllosphere bacterial community on the uptake and accumulation of As by *Wolffia*. In addition, functional genes responsible for As(III) oxidation (*aioA*, originally known as *aobB*, *aroA* or *asoA*) and As(V) reduction (*arsC*) were quantified, and the diversity of the relative genes in the phyllosphere were analyzed to gain further insights into As speciation in this unique habitat.

### MATERIALS AND METHODS

**Culture of *W. australiana***. *W. australiana* was originally provided by Prof. Dr. Elias Landolt (ETH, Zurich) and was first propagated in a sterile culture. The sterile culture and sterilization maintenance were described in the Supporting Information. The sterile *W. australiana* was grown in modified (with some concentration adjustments) Hoagland solution (Table S1, Supporting Information) supplemented with 1% sucrose. To obtain *W. australiana* with phyllosphere bacteria, a subculture of the sterile *W. australiana* was grown in 1/5 Hoagland solution in the open air under sunlight in a greenhouse (temperature: 20–32 °C; relative humidity: 60%–85%) in Xiamen, China. The culture medium was renewed every 2 weeks. *Wolffia* fronds were collected from the greenhouse after 2 months of growth with a sterile nylon net, rinsed with sterile deionized water, and stored at 4 °C (less than 1 day) until further experiments. Fresh fronds of *Wolffia* with phyllosphere bacteria were subjected to preparation for scanning electron microscopy (SEM; S-4800, HITACHI, Japan) with a previously described method, and the examination was conducted with a tension at 5 KV. Collection of the sterile *Wolffia* was conducted in a laminar flow cabinet with a sterile nylon net and rinsed thoroughly with sterile deionized water just before the following experiment. Morphological differences with respect to plant size and color were not observed between the two types of *Wolffia* (with or without phyllosphere bacteria).

**Time-Dependent As Speciation by *W. australiana***. The uptake and transformation of As species by different *Wolffia* systems were studied with 3 treatments (6 replicates each) (Table 1). Conical flasks (100 mL) filled with 50 mL of modified Hoagland solution (pH = 5.5; phosphate concentration of 1.0 mM; Table S1, Supporting Information) and covered with breathable sealing film were autoclaved at 121 °C for 30 min. The high concentration of phosphate was employed to inhibit As(V) uptake by *Wolffia*. Transplantation of *Wolffia* was conducted in a laminar flow cabinet. In each replicate 2.5 g (fresh weight) of *Wolffia* was sufficient to cover the whole water surface in the flask. The parts of the conical flasks beneath the water level were covered with aluminum foil to avoid algal growth. The flasks were then placed randomly in a ray radiation incubator (14 h light period with a light intensity of 50 μmol photons m⁻² s⁻¹, 30:25 °C day:night temperature and 70% relative humidity) for preincubation for 1 day. NaAsO₂ (100 μL of 500 mg L⁻¹) was added into each flask to a final As(III) concentration of 100 μg L⁻¹. Solutions (1 mL) in 3 of the 6 replicates were taken out at 0, 10, 24, and 72 h after the addition of As(III) and passed through 0.22-μm filters for As species determination. At 72 h, the fronds were collected in sterile nylon bags (with known weights), rinsed 3 times with sterile water, blotted dry with sterile filter papers, and weighed. The differences between the two sets of weights were recorded as *Wolffia* biomass. The solutions and *Wolffia* fronds in the remaining 3 replicates were sampled in the same manner at 168 h. The fresh fronds were used directly for As species extraction and determination of As(V), As(III), MMA (CH₃AsO(OH)₂), and DMA ((CH₃)₂AsO(OH)) as previously described. Detailed protocol of the species extraction and determination is available in the Supporting Information. The As extraction rate was 95.8% ± 2.0% using rice flour as the certified reference material (GBW 10010, National Research Center for Standard Materials, China). In the treatments of control and *Wolffia* without bacteria (W–B), sterility checks were performed by plating the solutions on solid LB media at 72 and 168 h. No bacterial growth was observed after 7 days incubation in the ray radiation incubator.

**Real-Time PCR of the *aioA*, *arsC*, and 16S rRNA Genes in the Phyllosphere**. Phyllosphere bacteria and the total DNA were extracted from 10 g of nonsterile *W. australiana* (3 replicates) using the method in the Supporting Information. Real-time PCR was carried out using Roche LightCycler 480 (Roche). The 10-μL reaction system contained 5 μL of SYBR Premix Ex TaqII (2x) (Takara Bio, Dalian, China), 0.5 μL of each primers (10 nmol L⁻¹), 0.5 μL of bovine serum albumin (20 mg mL⁻¹), and around 5 ng of DNA template. Three technical replicates were included for each DNA sample. Gene encoding the large subunit of arsenite oxidase (*aioA* gene) was quantified with the primer set AroAdeg1F (5′-GG TGATTGTYGTTTAACTGC-3′) and AroAdeg1R (5′-TTGTASGCGTGGCCTT-3′). The *arsC* gene was quantified with amlt-42-f (5′-TCCGCTATATCGCTTGGG-3′) and amlt-376-r (5′-ACTTTTCTCGCCGTCTTCC-3′). The 16S rRNA gene was quantified with 341f

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### Table 1. Treatment Description and Biomass Record of *Wolffia* for the Experiment of Time-Dependent As Speciation

<table>
<thead>
<tr>
<th>treatment</th>
<th><em>Wolffia</em> biomass at 0 h (g)</th>
<th><em>Wolffia</em> biomass at 72 h (g)</th>
<th><em>Wolffia</em> biomass at 168 h (g)</th>
<th>sterility of <em>Wolffia</em> before the experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>W–B</td>
<td>2.50 ± 0.00 aC</td>
<td>2.99 ± 0.02 ab</td>
<td>3.13 ± 0.04 aA</td>
<td>sterile, without bacteria</td>
</tr>
<tr>
<td>W+B</td>
<td>2.50 ± 0.00 aC</td>
<td>2.52 ± 0.01 bb</td>
<td>2.63 ± 0.01 ba</td>
<td>nonsterile, with phyllosphere bacteria</td>
</tr>
</tbody>
</table>

“Data are average values ± SD (n = 3). Different letters mean significant differences (P < 0.05, LSD test) between treatments. Lower letters and upper letters are results from vertical and horizontal comparisons in the table, respectively.”
(5′-CCTACGGGAGGCAGCAG-3′) and 517r (5′-ATTA-
CCGGGCTGCTGG-3′). The real-time PCR conditions
for the aiaA gene and the arsC gene were as follows: 95 °C for
5 min; followed by 45 cycles of 95 °C for 10 s, 56 °C for 30 s,
72 °C for 30 s, and 15 s for the fluorescence detection at 84 °C.
For the 16S rRNA gene, annealing was at 55 °C, and the
processes of elongation and fluorescence detection were
combined at 72 °C for 30 s. For the standard curve preparation,
the target gene fragments were ligated with pMD 19-T vector
(Takara Bio, Dalian, China) and transformed in competent
Escherichia coli DH5α. The plasmids containing the inset genes
were sequenced and identified by blasting in NCBI. Plasmids
with target genes were extracted with TIANpre Mini Plasmid
kit (Tiangen, China), and the concentrations were detected by
Nanodrop 1000 spectrophotometer. Copy numbers of the
plasmids were calculated according to Fierer et al.26

**Library Construction and Sequencing of the aiaA Gene in the Phyllosphere.** The aiaA gene was amplified from the DNA extracted from the phyllosphere of *W. australiana* with the primer set AroAdeg1F and AroAdeg1R using the following protocol: 95 °C for 5 min; followed by 35 cycles of 95 °C for 30 s, 56 °C for 40 s, 72 °C for 40 s; and a final extension time of 10 min. The 25-μL PCR reaction system contained 12.5 μL of Dream Taq Green PCR Master Mix (2×) (Thermo Scientific), 1 μL of each primer, and 40 ng DNA templates. The combined amplicons from the triplicate reactions were ligated with pMD18-T vectors and transformed in competent *E. coli* DH5α, and the positive clones were sequenced with ABI automatic DNA sequencer (3730XL, ABI, America) by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. The sequences were blasted to exclude the ones with low similarity to the existing genes in NCBI. Representative sequences were selected from each operating taxonomic units (OTU) determined with a cutoff value of 97%11 using Mothur.27 Rarefaction analysis was performed using Analytic Rarefaction 1.3 (http://strata.uga.edu/software/anRareReadme.html) in terms of the unique OTU numbers. The Representative sequences were transferred into amino acid sequences (http://web.expasy.org/translate) and aligned with the AioA sequences retrieved from NCBI using MAGA 5.2. A neighbor-joining phylogenetic tree was constructed using a bootstrap value of 1000. All the representative gene sequences have been deposited in NCBI under the accession numbers of KJS86502-KJS86515.

**Statistical Analysis.** The means and standard deviations (SD) were calculated by Microsoft Office Excel 2010. Analysis of variance (ANOVA) was conducted using SPSS 16.0, and *P* < 0.05 was considered as statistically significant.

### RESULTS

**Arsenic Speciation in the Medium.** Although freshly prepared, a small portion (3%) of As in the solution was in the form of As(V) at the beginning of the experiment (Figure 1). In the control without biological activities, a small increase of As(V) concentration was observed (from 3.23 ± 0.04 μg L⁻¹ at the beginning to 5.70 ± 0.15 μg L⁻¹ at 168 h) in the solution, probably due to chemical oxidation of As(III). *Wolfia* with bacteria (*W+B*) showed the highest As(III) oxidation ability. Within 24 h, 97.8% of As(III) in the solution was depleted, of which 84.7% was oxidized into As(V), and the rest was absorbed by the plant (Figure 1; Figure S1, Supporting Information). Arsenate was the dominant species thereafter, accounting for 99.0% of the total As in the solution at 168 h.

*Wolfia* without bacteria (*W−B*) did not show oxidation ability to the ambient As(III). Arsenate concentration in *W−B* solution decreased slightly (Figure 1B), due to the uptake by the fronds. The As(III) concentration in the culture medium of *W−B* gradually decreased, mainly because of the uptake by the *Wolfia* fronds, which resulted in the decrease of the total As as well (Figure 1A; Figure S1, Supporting Information). In the treatment of *W+B*, the total As concentration in the solution decreased marginally after 24 h due to the absorption by *Wolfia* (Figure S1, Supporting Information). Organic As such as MMA or DMA was not observed in all the treatments.

**Arsenic Accumulation in the Fronds of *Wolfia.* During the one-week incubation period, the *Wolfia* biomass increased significantly (*P* < 0.05; Table 1). Biomass of *Wolfia* without bacteria increased faster than that of *Wolfia* with bacteria (Table 1). In the *Wolfia* fronds sampled at 72 and 168 h, the total As concentrations showed significant (*P* < 0.01) differences between the treatments (Figure 2). Arsenic concentration in *Wolfia* without bacteria was 840.4 ± 17.4 μg kg⁻¹ fresh weight (FW) and 1028.8 ± 55.3 μg kg⁻¹ FW at 72 and 168 h, respectively. *Wolfia* with bacteria contained a much lower level of As (176.6 ± 2.1 and 209.6 ± 6.4 μg kg⁻¹ FW at 72 and 168 h, respectively), accounting for only 20.7% of that in *W−B*.

Only As(V) and As(III) were detected in the fronds (Figure 2), with As(III) as the predominant form (88.4%–94.4%). The amounts of As(III) in the plant increased significantly (*P* < 0.05) during the incubation period (Figure 2). Arsenate uptake...
by the plant was observed, given the concentration increase in the plant tissue (Table 2) and the concomitant concentration decline in the solution (Figure 1). The percentages of As(V) in W+B fronds were about 2 times of that in the W–B fronds, showing no significant difference between the two sampling times ($P > 0.05$) (Table 2). The higher As(V) in W+B solution did not result in higher As(V) concentration in the plant tissue compared to that in W–B treatment (Figure 1, Table 2). In the fronds of sterile Wolffia at 72 and 168 h, the amounts of As(V) were almost equal ($P < 0.05$) to the decreases of As(V) in the solution, considering the chemical oxidation of As(III) in the solution (Table 2).

## Discussion

Our results demonstrate that the phyllosphere bacterial community of *W. australiana* had substantial ability to oxidize As(III) in water environments, while the plant alone did not exhibit this ability. Chemical oxidation of As(III) was relatively slow as shown in the control treatment without biological activities (Figure 1), suggesting that the prevalence of As(V) in the surface water is most likely due to microbial activities of As oxidation. Arsenate is taken up by phosphate transporters in higher plants; therefore, its influx can be suppressed by phosphate. In *W. globosa*, the influx rates of As(III) and As(V) were comparable without phosphate in the solution, while the absorption of As(V) was considerably inhibited at a phosphate concentration of 0.1 mM. In the present study, the successful observation of As(V) accumulation in the W+B solution generated from As(III) oxidation (Figure 1) attributed as well to the effective suppression of As(V) uptake in Wolffia by phosphate at an initial concentration of 1 mM. The oxidation in the solution was conducted by the phyllosphere bacteria, and/or the bacteria in the medium originating from the phyllosphere, given the observation of no oxidation by the Wolffia without bacteria (Figure 1). With most of the As(III) being oxidized into As(V) before being absorbed by the Wolffia with bacteria (W+B), phyllosphere bacterial community showed rapid As(III) oxidation.

Although confronted by a strong competition from the high level of phosphate, the uptake of As(V) by the plant was still observed, through the phosphate transporters which were not totally blocked by the phosphate. The amounts of total As(V) accumulated in the sterile fronds were no more than the decreases of As(V) in the solution at the two sampling times, which implied that essentially all the As(V) in the plant was due to the direct uptake from the solution. This suggests that there was no internal As(III) oxidation in Wolffia tissue. The redox conditions in plant cells (with Eh much lower than 0 mM) make As(V) reduction prevail over As(III) oxidation, and no enzymatic pathway of As(III) oxidation has been reported in

### Table 2. As(V) Balance in W–B and Percentages of As(V) in Wolffia with or without Phyllosphere Bacteria

<table>
<thead>
<tr>
<th>treatments</th>
<th>As(V) balance in W–B</th>
<th>As(V) concentration (µg kg$^{-1}$ FW)</th>
<th>As(V) percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>72 h</td>
<td>168 h</td>
</tr>
<tr>
<td>W–B</td>
<td>As(V) content in Wolffia$^a$ (10$^{-3}$ µg)</td>
<td>141 ± 5.14 aB</td>
<td>188 ± 14.4 aA</td>
</tr>
<tr>
<td></td>
<td>decrease of As(V) in the solution$^b$ (10$^{-3}$ µg)</td>
<td>144 ± 18.1 aB</td>
<td>190 ± 22.1 aA</td>
</tr>
<tr>
<td>W+B</td>
<td>18.6 ± 3.67 bB</td>
<td>24.3 ± 0.74 bA</td>
<td>11.6 ± 0.69 aA</td>
</tr>
</tbody>
</table>

$^a$Data are average values ± SD ($n = 3$). Different letters mean significant differences ($P < 0.05$, LSD test) between treatments. Lower letters and upper letters are results from vertical and horizontal comparisons in the table, respectively. $^b$As(V) concentration in Wolffia × Wolffia biomass.
higher plants. *Wolffia* with or without phyllosphere bacteria contained different percentages of As(V) and As(III) (Table 2), most likely due to the different As redox conditions in the medium. In *Wolffia* tissues, apart from the major part of As(III) complexed with PCs, a portion of As(III) was in the unbound state, which would have a high tendency to diffuse back into the medium through aquaporin channels when the medium As(III) concentration was low. As a result, the high As(III) in the *Wolffia* without bacteria (*W−B*) contributed a higher percentage of As(III) (lower percentage of As(V)) in the plant tissue. When it comes to the different amounts of As(V) accumulation in the fronds (Table 2), the possible impact of the previous culture conditions on the As(V) absorption could not be ruled out. Before the experiment of As speciation, *W−B* and *W+B* were cultured in Hoagland solution supplemented with 1% sucrose and in 1/5 Hoagland solution, respectively. *Wolffia* in the *W+B* system was probably less adaptive to the incubation condition (in Hoagland solution) during the experiment of As speciation and consequently showed lower growth rate (Table 1). Accordingly, the lower As(V) concentration in the *W+B* fronds (Table 2) with higher medium As(V) concentrations was probably due, at least partially, to a higher solution phosphate concentration resulting from the lower phosphate consumption by the slower plant growth (Table 1).

*W. australiana* was highly efficient in As accumulation, as found with *W. globosa*, in contrast to many other aquatic plants. Arsenite in *Wolffia* fronds, either generated via direct absorption, or via intracellular As(V) reduction, can form As(III)-PC complexes and be stored in vacuoles, contributing greatly to the As accumulation and tolerance in *Wolffia*. Duckweeds, including *Wolffia*, have been suggested as good options for phytoremediation of As-tainted aquatic environments. However, As(III) oxidation by bacterial communities inhabiting in the duckweed phyllosphere and in the adjacent water would significantly decrease the phytoextraction efficiency under phosphate sufficient conditions.

Arsenic reduction was not observed by the phyllosphere microbes when As(V) was the predominant species in the solution. The dominance of arsenite-oxidizing bacteria in the *Wolffia* phyllosphere was confirmed by the fact that the *aioA* gene was much more abundant than the *arsC* gene. The relative abundance of the *aioA* gene (ratio of the *aioA* gene to the 16S rRNA gene) in the phyllosphere was comparable to that found in rice rhizosphere. AioA is the large subunit of As(III) oxidases located in the periplasm, involved in aerobic As(III) oxidation. Detoxification and producing energy are the main purposes of As(III) oxidation in heterotrophic bacteria and chemoheterotrophic/chemolithoautotrophic bacteria, respectively. Arsenite-oxidizing bacteria have been frequently isolated from As-impacted environments. However, the detection of the *aioA* gene in the *Wolffia* phyllosphere which had not been exposed to As implied the ubiquity of this gene. Synthesis of arsenite oxidase is regulated by As(III) in most cases, and the fast oxidation of As(III) by the phyllosphere bacterial community indicated the enzyme activity. The *Aio* sequences in the present study affiliated most closely to Rhizobiales, Burkholderiaceae, and Comamonadaceae (Figure 3), which were common aquatic inhabitants associated with As(III) oxidation. Most of the arsenite-oxidizing bacteria in the *Wolffia* phyllosphere were found to be
typical strains in paddy environments. However, the identification of these bacteria remains unknown. In addition, a considerable number of AioA sequences showed low similarity to known bacteria. The unsaturated rarefaction curve further suggested an underestimated diversity of arsenite-oxidizing bacteria in the phyllosphere. These findings demand more investigations on the arsenite-oxidizing strains and communities in fresh water environments.

W. australiana and the associated phyllosphere bacterial community played different roles in As speciation. The phyllospheric bacterial community had a significant ability in As(III) oxidation, while As(V) reduction by the phyllospheric bacteria was not observed. On the contrary, W. australiana showed no capacities of internal or external As(III) oxidation, which may constitute as an important part of the overall As cycling in wetlands.

ASSOCIATED CONTENT

Supporting Information
Supporting Materials and Methods: Sterile culture of W. australiana; Arsenic species extraction and determination; Extraction of the phyllosphere bacteria and the total DNA. Figure S1: Changes of the total arsenic concentration in the culture medium. Figure S2: Representative image of bacteria in the phyllosphere of W. australiana. Figure S3: Rarefaction curve for the aioA gene library constructed with DNA extracted from the Wolflia phyllosphere. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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