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Influence of epiphytic bacteria on arsenic metabolism in *Hydrilla verticillata*☆

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ABSTRACT

Microbial assemblages such as biofilms around aquatic plants play a major role in arsenic (As) cycling, which has often been overlooked in previous studies. In this study, arsenite (As(III))-oxidizing, arsenate (As(V))-reducing and As(III)-methylating bacteria were found to coexist in the phyllosphere of *Hydrilla verticillata*, and their relative activities were shown to determine As speciation, accumulation and efflux. When exposed to As(III), As(III) oxidation was not observed in treatment H(III)-B, whereas treatment H(III)+B showed a significant As(III) oxidation ability, thereby indicating that epiphytic bacteria displayed a substantial As(III) oxidation ability. When exposed to As(V), the medium only contained 5.89% As(III) after 48 h of treatment H(V)-B, while an As(III) content of 86.72% was observed after treatment H(V)+B, thereby indicating that the elevated As(III) in the medium probably originated from As(V) reduction by epiphytic bacteria. Our data also indicated that oxidizing bacteria decreased the As accumulation (by approximately 64.44% compared with that of treatment H(III)-B) in plants, while reducing bacteria played a critical role in increasing As accumulation (by approximately 3.31-fold compared with that of treatment H(V)-B) in plants. Regardless of whether As(III) or As(V) was supplied, As(III) was dominant in the plant tissue (over 75%). Furthermore, the presence of epiphytic bacteria enhanced As efflux by approximately 9-fold. Metagenomic analysis revealed highly diverse As metabolism genes in epiphytic bacterial community, particularly those related to energetic metabolism (*aio*AB), and As resistance (*ars*ABCR, *acr*3, *ars*M). Phylogenetic analysis of As metabolism genes revealed evidence of both vertical inheritance and horizontal gene transfer, which might have contributed to the evolution of the As metabolism genes. Taken together, our research suggested that the diversity of As metabolism genes in epiphytic bacterial community is associated with aquatic submerged macrophytes which may play an important role in As biogeochemistry in aquatic environments.

1. Introduction

Arsenic (As) is a class one, nonthreshold human carcinogen that is ubiquitous in the natural environment and is considered to be a global health risk factor (Abdul et al., 2015; Wang et al., 2014; Zhu et al., 2008b). Millions of people are estimated to be at risk of consuming drinking water with As concentrations above the baseline standard, especially in Southeast Asia (Zhu et al., 2014). Arsenic can exist in four oxidation states: arsenate (As(V)), arsenite (As(III)), arsenic (As(0)), and arsine (As(-III)) (Oremland and Stolz, 2003), whereas As(0) and As(-III) are rare in aquatic environments (Mandal and Suzuki, 2002). The speciation of As determines its toxicity, mobility, behavior in the environment, and As transformation are driven, at least to some extent, by biological processes (Zhu et al., 2014). In aquatic environments, inorganic As(V) and As(III) are predominant, which are among the most toxic As species (Smedley and Kinniburgh, 2002). However, the dominant inorganic arsenicals can be converted to methylated organoarsenicals such as monomethylarsenate ($MMAs^V$), dimethylarsenate ($DMAs^V$), and trimethylarsine oxide (TMAsO) owing to microbial-mediated biomethylation (Oremland and Stolz, 2003; Zhang et al., 2017). These organic As species are less toxic than their inorganic As counterparts and are also commonly detected in freshwater environments (Zhang et al., 2017).

Previous studies have confirmed that submerged macrophytes play an important role in heavy metal phytoremediation (Favas et al., 2012; Human et al., 2015; Xing et al., 2013). In addition,

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submerged macrophytes offer a vast surface area for the attachment of large amounts of bacteria (epibiotic bacteria) (He et al., 2014; Hempel et al., 2009), and As metabolism in microorganisms is much more diverse than that in plants (Paez-Espino et al., 2009). Over the last few decades, numerous studies have led to great leaps in our understanding of microbial-mediated As biotransformation, including As(III) oxidation (*aio* genes), As(V) respiration (*arr* genes), As(V) reduction (*ars* genes), and As(III) methylation (*ars*M genes) (Yang and Rosen, 2016; Zhang et al., 2017). These genes are frequently used as molecular markers for As-related studies. Most studies have focused on highly contaminated As environments such as soil (Luo et al., 2014), sediment (Chauhan et al., 2017), wetlands (Zhang et al., 2017), and groundwater (Das et al., 2017; Li et al., 2017). Few studies have investigated the role of epiphytic bacteria in As metabolism in plant-microbe systems. Thus, little is known about the As dynamics mediated by submerged macrophytes and the associated epiphytic bacteria. Moreover, the composition of epiphytic bacterial communities is complex. Therefore, it is necessary to fully investigate the functional genes of epiphytic bacteria that are related to As metabolism in aquatic environments.

Hydrilla verticillata is a widely distributed invasive submerged perennial plant that has the ability to accumulate, immobilize and detoxify As (Srivastava and D'souza, 2010; Srivastava et al., 2007; Xue and Yan, 2011). Previous studies have demonstrated that endophytic or rhizospheric bacteria could mediate As transformation, including oxidation and reduction, which influenced As uptake and speciation in rice plants (Jia et al., 2014) and As-hyperaccumulator *Pteris vittata* (Han et al., 2016). Therefore, some previous studies might have overestimated the capacity of As uptake and efflux by macrophytes due to no consideration of the role of epiphytic bacteria. Although the impact of epiphytic bacteria associated with floating macrophytes on As transformation has been discussed in previous studies (Xie et al., 2014), it is difficult to illustrate the influence of epiphytic bacteria on As biochemistry because of its complexity and limited data in the literature. The present study proposed a hypothesis that epiphytic bacteria attached to *H. verticillata* have a potential impact on the transformation, accumulation and efflux of As. In the present study, this hypothesis was tested by comparing As uptake, accumulation and efflux in *H. verticillata* with or without epiphytic bacteria during exposure to As(V) or As(III). To fully understand the phylogenetic composition and expression of As metabolism genes, functional genes related to the metabolism of As were further identified using the metagenomic method. To our knowledge, reports on the diversity of epiphytic bacteria and their functions have mainly focused on energy metabolism and nutrient cycling using metagenomic methods (Sanli et al., 2015; Comba-Gonzalez et al., 2016). However, until now, little research has been conducted on metal resistance genes using this method.

The main purpose of this study was to investigate (1) the role of *H. verticillata* and epiphytic bacterial community in the dynamics of As transformation, (2) the effect of epiphytic bacterial community on the uptake, accumulation and efflux of As by *H. verticillata*, and (3) the diversity and abundance of As metabolism genes in epiphytic bacterial community. The findings of this study will contribute to further insights into As speciation in the phyllosphere of aquatic macrophytes and provide a better understanding of microbially mediated As biogeochemistry in aquatic environments.

2. Materials and methods

2.1. Culture of H. verticillata

H. verticillata was collected from Taihu Lake, the third largest shallow freshwater lake (2338 km^2, average depth 1.9 m) in China, and grown in greenhouse ponds for months. The plants were acclimatized for a week in modified Hoagland solution (Hoagland and Arnon, 1950) (Table S1). The procedures for plant sterilization and maintenance are described in the Supplementary Methods and Materials.

2.2. Arsenic uptake and accumulation mediated by H. verticillata with or without epiphytic bacteria

The As treatments were performed in Hoagland solution prepared with $Na_3AsO_4 \cdot 12H_2O$ (As(V)) and $NaAsO_2$ (As(III)). Conical flasks (250 mL) filled with 150 mL of modified Hoagland solution (Table S1) were autoclaved at 121 °C for 30 min. After one week of preincubation in the nutrient solutions, three replicates of *H. verticillata* with or without epiphytic bacteria (1 g total weight) were exposed to As(V) or As(III) as described in Table 1. A control treatment without *H. verticillata* was also included. Notably, As(V) uptake is inhibited by phosphate, therefore, *H. verticillata* was subjected to phosphate starvation for 24 h before exposure to As(V) in order to completely consume the phosphate stored in plant cells. $Na_3AsO_4 \cdot 12H_2O$ or $NaAsO_2$ (300 μL of 1 mM) was added to each flask to a final As(V) or As(III) concentration of 2 μM, which was similar to that measured in natural aquatic environment under intermediate pollution levels (Mandal and Suzuki, 2002; Smedley and Kinniburgh, 2002). All the flasks were placed in a controlled environment growth chamber (14 h/10 h light/dark cycle with a light intensity of 115 μmol m^{-2} s^{-1}, 28/23 °C day/night temperature). Samples of the solutions (1 mL) were collected at 0, 1, 6, 12, 24, and 48 h after the addition of As(III) or As(V). The nutrient solutions were diluted with a phosphate buffer solution (PBS, containing 2 mM NaH_2PO_4 and 0.2 mM Na_2-EDTA, pH 6.0) and passed through a 0.45 μm filter for As species analysis. All procedures were conducted on a clean bench.

After 48 h of exposure, all the plants were rinsed in ice-cold phosphate solution (1 mM K_2HPO_4, 5 mM 2-(N-morpholino) ethanesulfonic acid and 0.5 mM $CaCl_2$, pH 6.0) for 10 min to remove apoplastic As and then immediately frozen in liquid nitrogen until analysis for As speciation (Xue and Yan, 2011). The process of As species extraction has been described in previous studies (Rubio et al., 2010; Xu et al., 2007). Briefly, frozen plants were ground to a fine powder before extracting As species. Aliquots (0.5 g) of the ground plants were extracted with 20 mL of PBS (diluent solution) for 1 h under sonication at 4 °C and centrifugation at 4000 rpm (1680 G) for 10 min. The extracts were filtered through 0.45-μm filters before the analysis of As speciation.

2.3. Arsenite efflux assay in H. verticillata with or without epiphytic bacteria

The objective of this experiment was to investigate the role of epiphytic bacteria in the As(III) efflux of *H. verticillata* to the external medium. In order to ensure that the As excreted from the

Table 1
Treatments applied in the experiments examining the uptake and accumulation or efflux of arsenic.

Treatment	Arsenic exposure	States of *H. verticillata*
H(V)-B	$Na_3AsO_4 \cdot 12H_2O$ (As(V))	Sterile, without epiphytic bacteria
H(V)+B	$Na_3AsO_4 \cdot 12H_2O$ (As(V))	Nonsterile, with epiphytic bacteria
H(III)-B	$NaAsO_2$ (As(III))	Sterile, without epiphytic bacteria
H(III)+B	$NaAsO_2$ (As(III))	Nonsterile, with epiphytic bacteria
Control	$Na_3AsO_4 \cdot 12H_2O$/$NaAsO_2$	—

In the control treatment no *H. verticillata* was supplied.

plants could be detected in the medium, *H. verticillata* were exposed to a high concentration of As(III) or As(V) (5 μM) for 24 h as indicated in Table 1. Afterwards, the plants were rinsed briefly in deionized water, followed by 10 min of desorption of apoplastic As with ice-cold phosphate solution as described previously (section 2.2), and then transferred to 150 mL of As free medium (0.1 mM KH_2PO_4 was added to suppress the uptake of As(V)). At 1, 3, 5, 8, 12, 24 and 48 h, 0.5 mL aliquots of the nutrient solution were removed from each flask and diluted with PBS for As speciation analysis. All operations were conducted on a clean bench and all treatments were performed in triplicate.

2.4. Arsenic speciation and total arsenic detection

Arsenic speciation in nutrient solutions and plant extracts was assayed by high-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC—ICP-MS, Agilent LC1200 series and Agilent ICP-MS 7700cx; Agilent Technologies) as described by Zhu et al. (2008a) and Yan et al. (2016). Total As levels in Hoagland solutions were measured by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500cx) as described in a previous study (Yan et al., 2016). Fig. S1 showed the chromatogram of four standard As species, As(III), MMA, DMA and As(V), respectively. The detailed protocols for As detection are available in the Supplementary Methods and Materials.

2.5. Detachment of epiphytic bacteria

Epiphytic bacteria attached to submerged macrophytes were collected following the method described by Hempel et al. (2009). Briefly, 2 g of *H. verticillata* was selected and rinsed with sterile deionized water three times to remove large particles adhering to the plant surface. The macrophyte was then transferred to a sterile 50 mL polyethylene tube containing 40 mL of sodium pyrophosphate (0.1 M $Na_4P_2O_7 \cdot 10H_2O$, NaPPi). The epiphytic bacteria were detached by ultrasonication for 3 min, followed by 30 min of shaking (225 rpm) and then 3 min of ultrasonication. The suspensions were filtered through 0.22 μm polycarbonate membranes (Millipore, USA) to collect the detached epiphytic bacteria, which were then stored at −20 °C for DNA extraction.

2.6. DNA extraction

DNA was extracted using the FastDNA ® SPIN Kit for Soil (MP Bio, USA). The filters with epiphytic bacteria were cut into small pieces with sterile scissors and then loaded into Lysing Matrix tubes. The further steps followed the manufacturer's protocol. The extracted DNA samples were stored at −20 °C for further molecular analyses.

2.7. Metagenomic analysis

Metagenomic analysis was conducted to identify the composition and diversity of the microbial community and the As metabolism genes of the epiphytic bacteria. DNA samples of the epiphytic bacteria were sent to Guangdong Magigene Biotechnology Co., Ltd. (Guangzhou, China) for sequencing. The strategy yielded 5.42 Gb of raw metagenomic data. The raw data were then filtered to eliminate reads with low quality or ambiguous sequence reads. Clean data (Q20% = 100, Q30% = 99.63) were obtained, and the quality-filtered sequences were de novo assembled with k-mers by MEGAHIT (https://github.com/voutcn/megahit) to generate high-quality scaffold fragments. Next, scaffolds with a length over 500bp were extracted and broken into contigs, which were used for further predictions and annotations. The open reading frames were predicted from the samples using Prodigal (https://github.com/hyattpd/Prodigal). After removing "redundant" sequences, the nonredundant sequences (Unigenes) were searched (BLAST, e-value ≤ 0.0001) against the NCBI-NR database for species annotation and then classified into taxonomic groups using MEGAN.

Since no As metabolism genes or protein databases have been reported, protein sequences related to As resistance were retrieved from the BacMet database (Antibacterial Biocide&Metal Resistance Genes Database).

The metagenome sequencing data are available at the NCBI Sequence Read Archive (SRA) under accession number SAMN12206523.

3. Results and discussion

3.1. Effect of epiphytic bacteria on As transformation

After growing in a medium amended with 2 μM As(III) for 48 h, epiphytic bacteria of *H. verticillata* showed oxidation and methylation abilities, while plants without epiphytic bacteria did not exhibit these abilities. Specifically, in treatment H(III)-B, a substantial decrease in As(III) (83.24%) was observed in the medium after 48 h, but it was not accompanied by an increase in As(V), suggesting that sterile *H. verticillata* showed no ability for internal As(III) oxidation (Fig. 1A). More specifically, compared to the

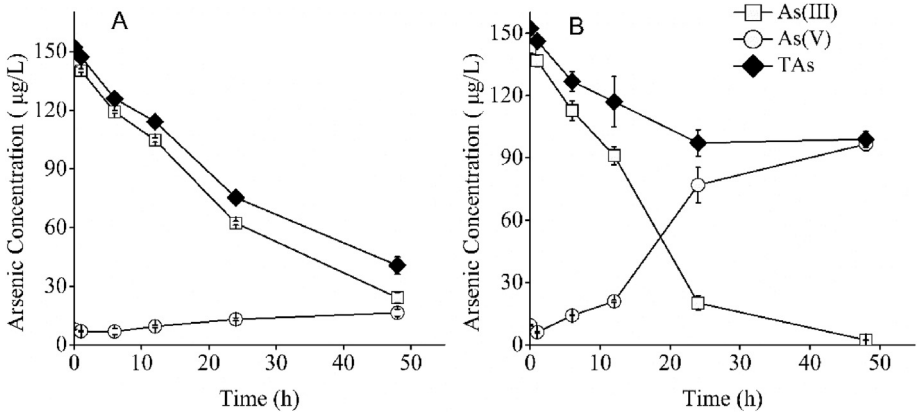

Fig. 1. Arsenic speciation in the nutrient solution during 48 h of exposure of *H. verticillata* to arsenite as influenced by epiphytic bacteria: (A) *H. verticillata* without epiphytic bacteria; (B) *H. verticillata* with epiphytic bacteria. Data are shown as the mean ± SE (n = 3).

control experiment, the As(V) concentration in the H(III)-B solution decreased slightly owing to As(V) uptake by macrophytes (Fig. S2 and Table S2). Previous studies have indicated that the oxidation process is largely mediated by microbial activities, and direct evidence of As(III) oxidation in higher plants is still lacking (Amend et al., 2014; Xie et al., 2014; Zhu et al., 2014). However, a significant As(III) oxidation ability was observed in the medium in treatment H(III)+B, with As(V) accounting for 97.66% of total As in the medium after 48 h (Fig. 1B). Since sterile *H. verticillata* showed no ability for internal As(III) oxidation, the significant As(III) oxidation in treatment H(III)+B was owing to the epiphytic bacterial community attached to the plant (Fig. 1B). It is worth noting that a small amount of organic As (DMA) was observed at 24 and 48 h in the medium with epiphytic bacteria, 1.43 ± 0.26 and 1.62 ± 0.37 μg L^{-1}, respectively. The pathway and enzymology of As methylation in plants have not yet been demonstrated (Zhao et al., 2009). In contrast, a fair number of studies have demonstrated the roles of As(III) oxidation and methylation in microbe detoxification because the products of oxidation and methylation are less toxic than As(III) (Farooq et al., 2016; Muller et al., 2003). Because epiphytic bacteria are widely distributed on the surface of plants (Baker and Orr, 1986), considerable As(III) oxidation and methylation can be mediated by epiphytic bacteria.

Similarly, when *H. verticillata* (sterile or unsterile) was exposed to 2 μM As(V), As species in the medium were also investigated. Specifically, in treatment H(V)-B, the As(V) concentration decreased from 150.69 ± 3.68 to 119.95 ± 12.88 μg L^{-1} within 48 h (Fig. 2), with 24.43% of the depleted As(V) reduced to As(III). After 48 h, 5.89% and 94.11% of the remaining As was present as As(III) and As(V) species in the medium, respectively. These results suggested that *H. verticillata* can absorb As(V) and reduce it to As(III). Once As(V) enters a plant cell, it can probably move easily from one cellular compartment to another through the various Pi transporters, and rapid equilibrium of As throughout the cell can result in the exposure of all components of the cellular metabolism to the toxicant (Finnegan and Chen, 2012). As part of As detoxification, most plants exhibit a high capacity for As(V) reduction, after which As(III) either undergoes efflux from the cells through As(III) transporters or sequestering into the vacuoles (Tripathi et al., 2007; Zhao et al., 2009). Our results showed that internal As(V) reduction occurred in plants. Low concentration of As(III) observed in the medium might have been due to low uptake of As(V) and sequestration of As(III) in the vacuoles of plants. In treatment H(V)+B, by contrast, 94.33% of the initial As(V) was depleted with a substantial increase of As(III) in the medium (Fig. 2B). After 48 h, 86.72% of the remaining As was present as As(III) in the medium. In treatment H(V)+B, a higher content of As(III) was detected in the medium compared with that in treatment H(V)-B, indicating that it might have mainly originated from As(V) reduction by epiphytic bacteria. In addition, organic As, such as MMA or DMA, was not observed in treatments H(V)+B or H(V)-B. Furthermore, in all the treatments, the net decrease in the concentration of As in the medium reflects absorbed or adsorbed by plant (Fig. S3).

To further verify the potential role of epiphytic bacteria in As transformation, the effect of detached bacteria on As transformation in the medium was tested. The results showed that epiphytic bacteria exhibited a significant As(V) reduction ability and As(III) oxidation ability (Fig. S4). Under As(III) stress, epiphytic bacteria showed a strong As oxidation ability, and converted almost all the As(III) to As(V) within 48 h (Fig. S4A). Under As(V) stress, 84.32% of the total As was found as As(III) in the medium after 48 h, indicating As(V) reduction occurred (Fig. S4B). Our results depicted that, besides interacting with the host plant, epiphytic bacteria play an imperative role in As biogeochemistry in the aquatic environment by affecting As reduction and oxidation.

3.2. Effects of epiphytic bacteria on As accumulation in *H. verticillata*

Arsenite was the predominant speciation in sterile or nonsterile *H. verticillata*, regardless of whether the plants were exposed to As(V) or As(III). However, the As speciation distribution in the plants showed a significant difference between sterile and nonsterile treatments (Fig. 3).

After exposure of 2 μM As(III) for 48 h, the concentrations of As(III) and As(V) in sterile *H. verticillata* were 8.33 ± 0.59 and 0.87 ± 0.08 mg kg^{-1} FW (fresh weight), respectively, whereas 2.28 ± 0.66 and 0.97 ± 0.01 mg kg^{-1} FW in nonsterile *H. verticillata*, respectively (Fig. 3A). In other words, total As in nonsterile *H. verticillata* decreased by 64.44% compared with that in sterile *H. verticillata*. As mentioned above (section 3.1), the existence of epiphytic bacteria, specifically As(III)-oxidizing microbes, could significantly elevate the concentration of As(V) in the medium. Previous studies demonstrated that the rate of As(V) uptake by plants is slower than that of As(III) uptake (Zhang et al., 2009; Abedin et al., 2002). Therefore, microbial-mediated As(III) oxidation may result in less influx of As, and thus, less As accumulation in plants. This finding was similar to the results of a study on rice in which the author demonstrated that microbial oxidation of As(III) in the rhizosphere reduced As bioavailability and uptake in rice

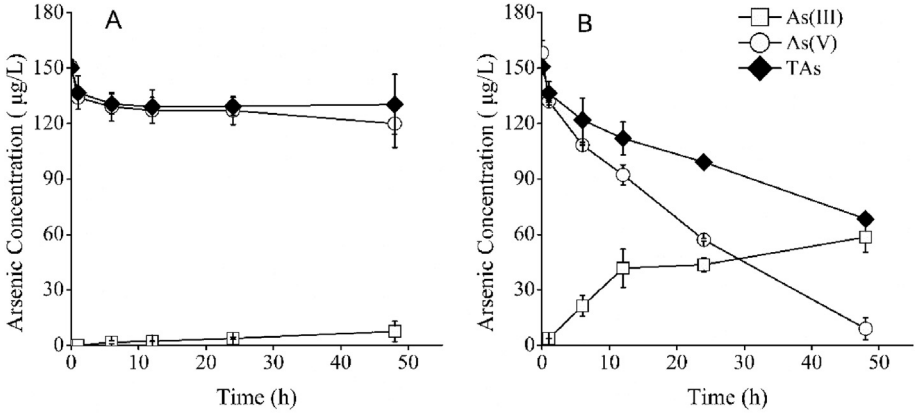

Fig. 2. Arsenic speciation in the nutrient solution during 48 h of exposure of *H. verticillata* to arsenate as influenced by epiphytic bacteria: (A) *H. verticillata* without epiphytic bacteria; (B) *H. verticillata* with epiphytic bacteria. Data are shown as the mean ± SE (n = 3).

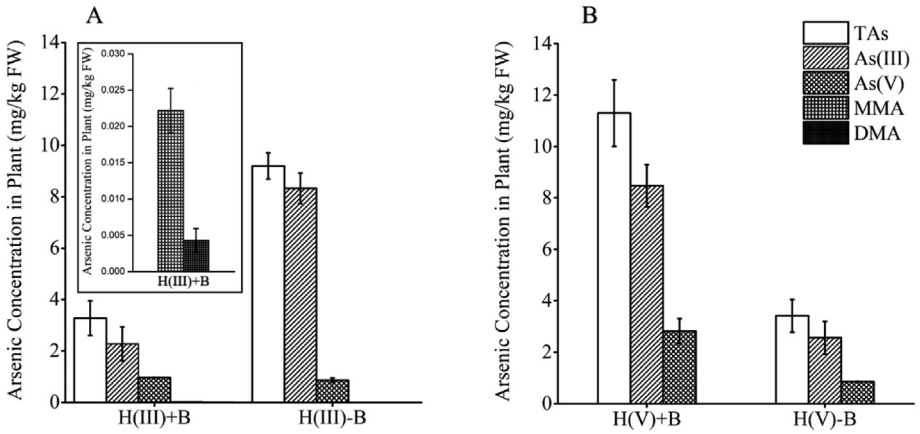

Fig. 3. Arsenic concentrations in *H. verticillata* with or without epiphytic bacteria after a 48 h of exposure to initial 2 μM (A) As(III) and (B) As(V). Data are shown as the mean ± SE (n = 3). (H(III)+B and H(V)+B: *H. verticillata* with epiphytic bacteria. H(III)−B and H(V)−B: *H. verticillata* without epiphytic bacteria. The concentrations of MMA and DMA in nonsterile *H. verticillata* treated with As(III) were shown in the small figure correspondingly.).

plants (Jia et al., 2014). Notably, very small amounts of DMA and MMA, accounting for only 0.81% of the total As, were detected in the plants with epiphytic bacteria (Fig. 3A). Several reports have demonstrated that the uptake of organoarsenic species in plants is lower than that of inorganic species, however, the mechanisms for the cellular uptake of DMA or MMA are still unclear (Chen et al., 2017; Raab et al., 2007; Rahman et al., 2011).

After *H. verticillata* was exposed to 2 μM As(V) for 48 h, only As(III) and As(V) were detected in both treatments (Fig. 3B). The concentrations of As(III) and As(V) in nonsterile *H. verticillata* were 8.47 ± 0.82 mg kg^{-1} FW and 2.82 ± 0.48 mg kg^{-1} FW, respectively, while 2.56 ± 0.64 mg kg^{-1} FW and 0.85 ± 0.01 mg kg^{-1} FW in sterile plants, respectively. Interestingly, unlike exposure to As(III), the total As concentration in nonsterile *H. verticillata* was higher than that in sterile *H. verticillata* by approximately 3.31-fold. The results obtained here revealed that the presence of epiphytic bacteria may favor the reduction of As(V) and increase the accumulation of As in plants. Microbially increased uptake of As has been reported in various plants, and all the microbes involved were identified as As(V)-reducing bacteria (Huang et al., 2010; Mukherjee et al., 2018; Yang et al., 2012). Arsenate-reducing bacteria took up As(V) and reduced it to As(III), which elevated As(III) in the medium significantly. Moreover, As(III) uptake by plants was faster than As(V) uptake, which is consistent with the aquaporin-mediated fast flux of neutral solutes (Zhang et al., 2009). As a result, epiphytic bacteria, specifically As(V)-reducing bacteria, are effective in increasing As accumulation in plants. Based on the general pathway of As(V) metabolism, the production of organic As requires the reduction of As(V) to As(III) and subsequent methylation (Meharg and Hartley-Whitaker, 2002). The process of As methylation was time and concentration dependent, therefore, DMA and MMA were not detected when nonsterile *H. verticillata* was exposed to As(V) for 48 h.

There are inconsistent viewpoints regarding the role of bacteria in As accumulation by plants. Our data indicated that oxidizing bacteria contribute to the oxidation of As(III) to less toxic and less mobile As(V) and decrease As accumulation in the plant. Conversely, reducing bacteria play a critical role in increasing As accumulation in plants. Therefore, it is crucial to elucidate the functional roles of diverse epiphytic bacteria in As biogeochemistry in aquatic environments and wetlands.

3.3. Effects of epiphytic bacteria on As(III) efflux in H. verticillata

As shown in Fig. 4 and Fig. 5, the pattern of As efflux was almost unaffected by As(III) or As(V) treatments, except that As efflux from the As(III)-treated plants was greater than that from the As(V)-treated plants. Because As(III) uptake was faster than As(V)

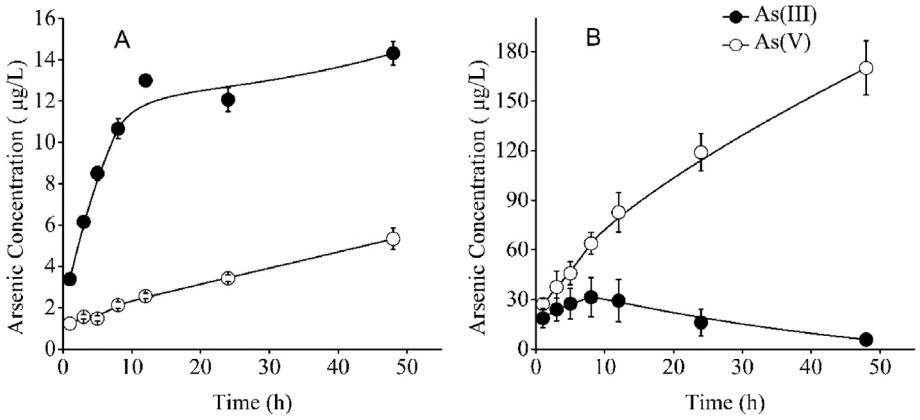

Fig. 4. Efflux of arsenate and arsenite after *H. verticillata* (with or without epiphytic bacteria) had been exposed to 5 μM arsenite for 24 h. (A) *H. verticillata* without epiphytic bacteria; (B) *H. verticillata* with epiphytic bacteria. Data are the mean ± SE (n = 3).

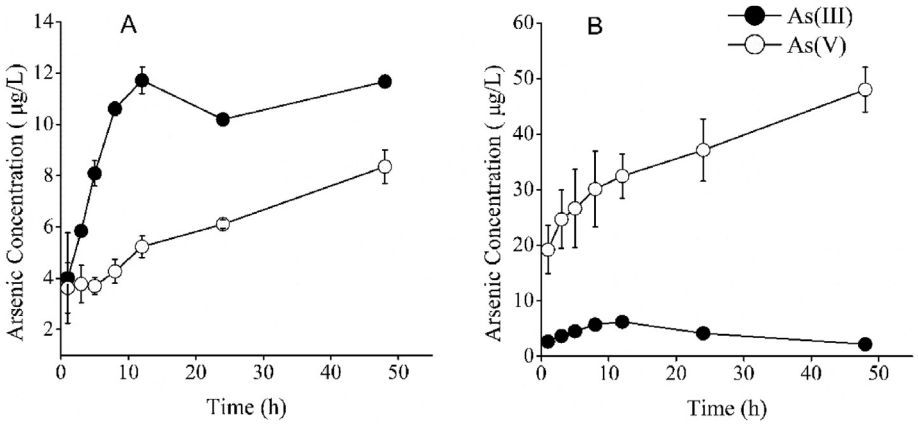

Fig. 5. Arsenate and arsenite efflux in *H. verticillata* after exposure to 5 μM arsenate for 24 h. (A) *H. verticillata* without epiphytic bacteria; (B) *H. verticillata* with epiphytic bacteria. Data are shown as the mean ± SE (n = 3).

uptake (Zhang et al., 2009), it resulted in more As accumulation in plants (Fig. S5) and consequently promoted As efflux from plants. Here, As efflux under the supply of 5 μM As(III) was presented as an example (Fig. 4).

The question specifically addressed by this experiment was whether epiphytic bacteria increased As(III) efflux. The answer was affirmative because As(III) efflux was significantly affected by epiphytic bacteria ($P < 0.001$). Nonsterile *H. verticillata* released approximately 9 times more As to the solution than sterile *H. verticillata*, indicating that the presence of epiphytic bacteria stimulated As efflux from the plant (Fig. 4). As a basal detoxification and tolerance mechanism, As efflux will logically lead to a decreased As burden in plant cells (Logoteta et al., 2009). As shown in Fig. 4A and B, when epiphytic bacteria were present, both species of inorganic As were detected in the solution, but the amount of As(V) was approximately 29 times higher than that of As(III), which was similar to the results of a study on As efflux by *A. caroliniana* and *A. filiculoides* (Zhang et al., 2008). The authors proposed that the reason for this phenomenon was that most of the As(III) inside the cells was complexed with thiol compounds (Zhang et al., 2008). However, our results cannot be explained by this theory. In the present study, epiphytic bacteria induced substantial As(III) oxidation to As(V) in the solution, which subsequently facilitated As(III) efflux from the plant. It is reasonable to assume that the plant and the epiphytic bacterial symbionts coevolved to exclude As. The mechanism responsible for As(III) efflux from the plants is not clear. One possible mechanism involves aquaporin channels, which is a passive mechanism, with the flux direction depending on the concentration gradient (Chen et al., 2017; Zhao et al., 2009). Another possible mechanism might be mediated by Acr3p (Arsenic Compounds Resistance protein 3), which would rely on the proton-motive force for energy (Chen et al., 2017; Zhao et al., 2009). In the present study, epiphytic bacteria mediated substantial As(III) oxidation to As(V), and significantly decreased As(III) concentration in the medium, and consequently facilitated As(III) efflux from plant cells to the external medium. Our results showed that As(III) efflux from the plants depended on the concentration gradient. Therefore, we presumed that aquaporin channels might play an important role in As(III) efflux from plant cells.

3.4. Diversity and abundance of arsenic metabolism-related genes

To provide an overview of the As biogeochemical cycle in this specific community, functional marker genes for all known As metabolism and resistance pathways in the BacMet predicted database were selected. The abundance and affiliation of As metabolism genes are shown in Fig. 6. The most common As metabolism genes were associated with As transportation, including *pst*B, *glp*F, *ars*B, *ars*A, *pst*(S, A and C), and *arc*3. Predicted genes related to As transformation, such as *ars*T, *aio*AB, *ars*C and *ars*M, and As resistance, such as *ars*R, were also detected. As shown in Fig. 6, the predicted genes with the highest abundances were *ars*T, *pst*B and *glp*F (with 88.9, 55.7 and 37.6 RPM (reads per million mapped reads), respectively). Moreover, the *ars*M, *ars*C and *acr*3 genes showed the highest diversity. *Gammaproteobacteria* was the most frequent class within this metagenome. Among all the As metabolism-like genes, *ars*M, *ars*C and *aio*AB mediate As methylation and redox processes, which are the most indispensable parts of the As biogeochemical cycle (Zhu et al., 2017). In the present study, these genes mediated As species changes in the solution (Figs. 1 and 2). Thereafter, microbial As transformation could significantly affect the phytoextraction efficiency (Fig. 3). Among the 26 detected *aio*AB sequences, the majority were from *Alphaproteobacteria*. The most abundant *ars*C gene was from *Actinobacteria*, accounting for 27.64% of the total *ars*C gene sequences detected. Approximately 37.25% of the *ars*M genes present were from *Candidatus Rokubacteria* (Fig. 6). Research has demonstrated that As(III) can be extruded from cells via the existing As(III) efflux systems (Zhao, 2016), which may help plants to alleviate As toxicity. There are three different routes by which intracellular As(III) can be pumped out: (1) ArsB, (2) ACR3 and (3) the ArsA/ArsB complex with the interaction of ArsD (Cai et al., 2013). In the present study, three genes involved in As(III) efflux were identified: *ars*A, *ars*B and *acr*3. These As detoxification mechanisms in bacteria have the benefit of eliminating As.

The phylogenetic analyses also supported these results and provided more information (Fig. 7). Specifically, among the 31 detected *aio*AB unigenes, the majority originated from *Rhodobacterales* at the order level. Nevertheless, the *ars*C and *ars*M unigenes showed branching in diverse phyla. Arsenic metabolism genes generally showed a similar phylogeny to their 16S rDNA sequences, indicating an ancient origin of these genes (Rensing, 2013). Nevertheless, the branching of several strains was not strictly in accord with the phylogenetic tree based on 16S rDNA sequences, suggesting the occurrence of horizontal gene transfer (HGT), which plays a role in the spreading of As metabolism genes in bacterial communities (Arsène-Ploetze et al., 2010; Heinrich-Salmeron et al., 2011; Suhadolnik et al., 2017). For example, the *ars*C of *Nitrospirae* bacteria (*Nitrospirae*) clustered together with the *ars*C of the *Planctomycetes* strains *Planctomyces* sp. SH-PL14 and

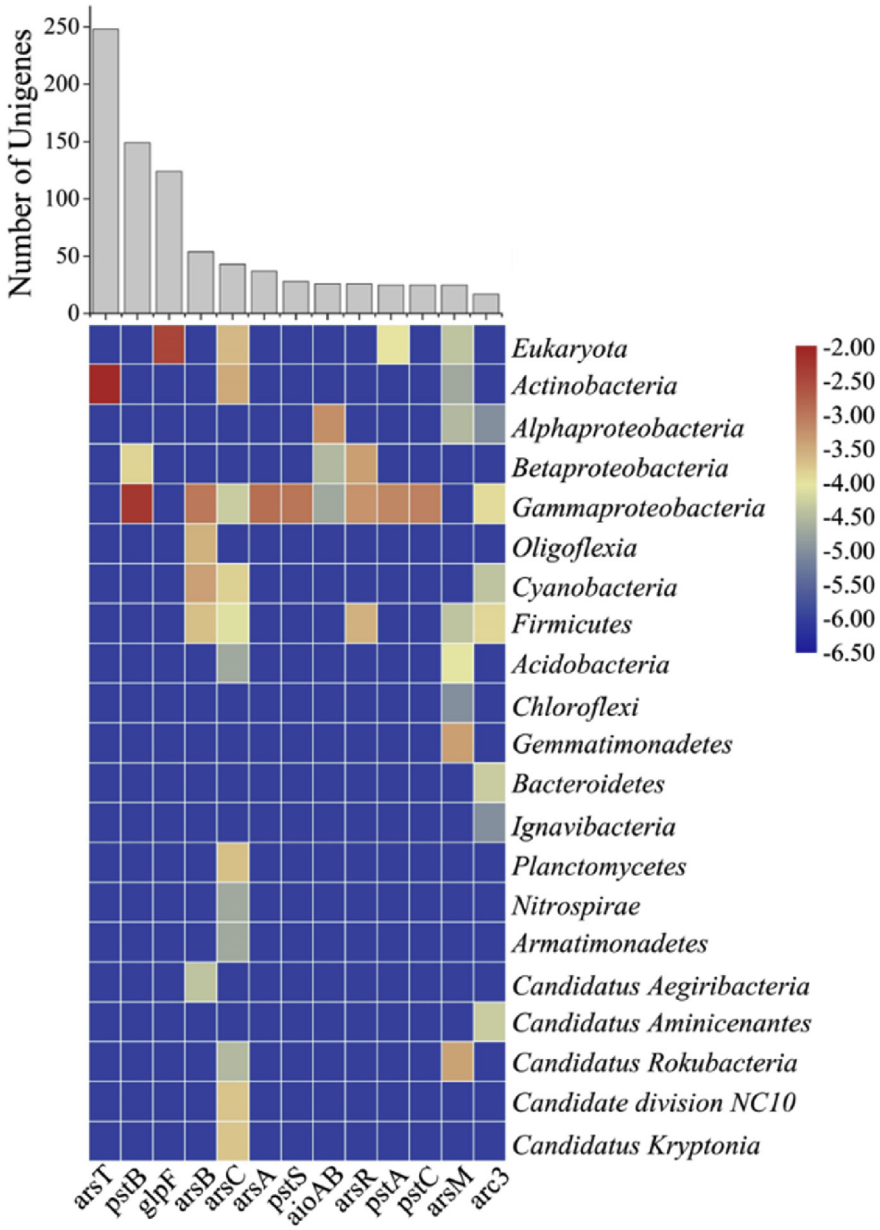

Fig. 6. Arsenic metabolism gene abundance and affiliation. Counts were log 10 transformed and drawn as a heatmap.

Fimbriiglobus ruber. In the present study, *ars*M and *ars*C (especially *ars*C) showed putative HGT events, as previously observed in other studies (Jackson and Dugas, 2003; Rensing, 2013; Suhadolnik et al., 2017). The elucidation of the phylogeny and distribution of As metabolism genes suggested that the As islands might have been formed independently by acquisition of functionally related genes or operons in respective strains.

4. Conclusion

In the present study, the effects of epiphytic bacteria on As accumulation, transformation and efflux by *H. verticillata* were investigated. Under 2 μM As stress, epiphytic bacteria promoted efficient As(III) oxidation or As(V) reduction in the growth medium, which subsequently decreased or increased As accumulation (by approximately 64.44% and 3.31-fold, respectively) in plants. Furthermore, the presence of epiphytic bacteria also stimulated As efflux from the plant. Our research indicated that diverse species of epiphytic bacteria played different roles in As speciation and accumulation in plants. Moreover, our study provides an overall picture of the genes related to As metabolism, such as genes associated with As transportation and transformation, which provides a direct and reliable reference regarding the diversity of functional genes in the phyllosphere of aquatic macrophytes. The investigation of As resistance genes helps us to understand the role of epiphytic bacteria in As speciation and toxicity in aquatic environments. In summary, this study suggests a previously overlooked diversity of epiphytic bacteria in the phyllosphere of submerged

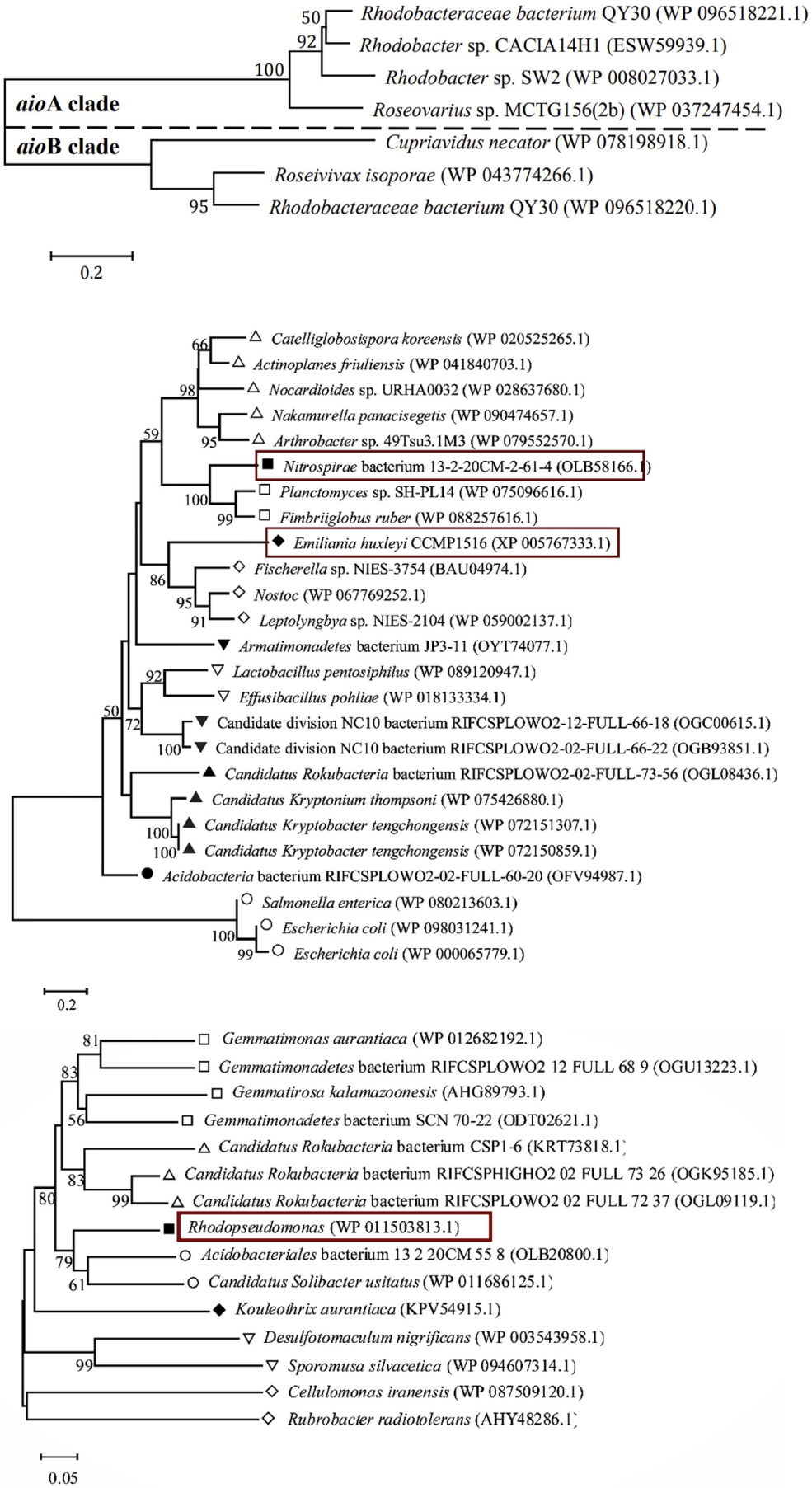

Fig. 7. Phylogenetic analyses of respiratory arsenite oxidases (AioA), arsenate reductase (ArsC) and arsenite methyltransferase (ArsM), respectively. The phylogenetic trees were constructed via the neighbor-joining method with MEGA version 7.0 software. Bootstrapping support was calculated on the basis of 1000 repetitions. The strains in a frame represent putative horizontal gene transfer (HGT) events. Different symbols represent different phyla.

macrophytes, which may have a substantial impact on As phytoremediation and As biogeochemistry in aquatic environments, warranting further research and exploration.

Declaration of competing interest

None declared.

CRediT authorship contribution statement

Zhuo Zhen: Software, Methodology, Writing - original draft, Writing - review & editing, Visualization. **Changzhou Yan:** Conceptualization, Supervision, Project administration, Funding acquisition. **Yuan Zhao:** Validation, Resources.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114232.

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