Arsenic accumulation and speciation in the submerged macrophyte *Ceratophyllum demersum* L.

Peiying Xue · Changzhou Yan · Guoxin Sun ·
Zhuaxi Luo

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Abstract

Introduction *Ceratophyllum demersum* L. is a widespread submerged macrophyte in aquatic environments.

Methods Simulation experiments were conducted in the laboratory to investigate arsenic (As) accumulation, speciation, and efflux of *C. demersum* exposed to arsenate and arsenite solutions.

Results Plant shoots showed a significant accumulation of As with a maximum of 862 and 963 $\mu\text{g As g}^{-1}$ dry weight after 4 days of exposure to 10 μM arsenate and arsenite, respectively. Regardless of whether arsenate or arsenite was supplied to the plants, arsenite was the predominant species in plant shoots. Furthermore, a dramatically higher influx rate of arsenate compared with arsenite was observed in *C. demersum* exposed to As solutions without the addition of phosphate (P). Arsenate uptake was considerably inhibited by P in this study, suggesting that arsenate is taken up by *C. demersum* via the phosphate transporters. However, arsenite uptake was unaffected by P and markedly reduced in the presence of glycerol and antimonite (Sb), indicating arsenite shares the aquaporin transport pathway. In addition, *C. demersum* rapidly reduces arsenate to arsenite in the shoot of the plant and extrudes most of them (>60 %) to the external solutions. The efflux of arsenite was much higher than that of

arsenate; the former is supposed to be both active and passive processes, and the latter through passive leakage.

Conclusion *C. demersum* is a strong As accumulator and an interesting model plant to study As uptake and metabolism due to the lack of a root-to-shoot translocation barrier.

Keywords Arsenic · Arsenic speciation · Submerged macrophytes · Efflux · Uptake · As phytoremediation

1 Introduction

As one of the top carcinogens, inorganic As contamination of water resources poses a serious threat to the environment and human health in many countries of the world, especially in Bangladesh, West Bengal (India), Vietnam, and China, where millions of people are at risk of drinking As-contaminated water (Smedley and Kinniburgh 2002; Bhattacharjee 2007; Zhao et al. 2010). Additionally, people can also suffer from As toxicity through consumption of rice and vegetables irrigated with As-tainted water (Meharg and Hartley-Whitaker 2002; Tripathi et al. 2007; Zhu et al. 2008). Therefore, it is an urgent need to reduce As contamination in water. Phytoremediation has become a promising method for As removal in recent years because it is a cost-effective and eco-friendly way to remove pollutants (Srivastava et al. 2007; Zhu and Rosen 2009). Since the discovery of the brake fern's arsenic hyperaccumulation (Ma et al. 2001; Zhao et al. 2002), much study has been directed towards the accumulation, speciation, and metabolism of As in plants (Zhao et al. 2009).

Hyperaccumulation seems to have the following characteristics: enhanced arsenate uptake, decreased arsenite–thiol complexation and arsenite efflux to the external medium, markedly improved xylem translocation of arsenite, and arsenite sequestration in the vacuole of shoots (Zhao et al.

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P. Xue · C. Yan (✉) · Z. Luo
Key Laboratory of Urban Environment and Health, Institute of
Urban Environment, Chinese Academy of Sciences,
Xiamen 361021, China
e-mail: czyan@iue.ac.cn

G. Sun
Research Center for Eco-Environmental Sciences,
Chinese Academy of Sciences,
Beijing 100085, China

2009). As for non-hyperaccumulating plants, several studies on terrestrial plants suggested that arsenate and arsenite enter plant cells via phosphate transporters and aquaglycerolporin channels, respectively (Abedin et al. 2002; Meharg and Hartley-Whitaker 2002; Meharg and Jardine 2003). In addition, the reduction of arsenate and complexation of arsenite by thiol peptides are considered to be important mechanisms of As detoxification (Srivastava et al. 2007; Zhao et al. 2009). Recent studies by Xu et al. (2007) and Zhang et al. (2008, 2009) also showed that rice and tomato roots as well as some species of duckweed rapidly reduce arsenate to arsenite and extrude arsenite to the external medium. However, little information is available on As accumulation and metabolism in submerged macrophytes. Studies on *Azolla*, *Wolffia globosa*, and the roots of rice and tomato suggest that As (V) in plant cells is rapidly reduced to As (III), followed by rapid efflux from the plant into external solutions (Xu et al. 2007; Zhang et al. 2008, 2009). If this is one of the detoxification mechanisms for As (V) in submerged macrophytes, the macrophytes themselves would become new sources of metal contamination to surrounding areas.

Since submerged macrophytes are completely inundated and have very thin cuticle, plant shoots may have the ability to directly take up metals from the surrounding water (Jackson 1998). In addition, due to their capacity for fast growth and high biomass production even under moderate nutritional conditions, it is possible that submerged macrophytes have tremendous potential to accumulate As from water. *Hydrillia verticillata* (L. f.) Royle and *Ceratophyllum demersum* are reported to be promising candidates for the phytoremediation of As-contaminated water owing to their higher tolerance of As than those normally occurring in contaminated areas and adequate As detoxification system, by stimulating antioxidant enzyme activity and augmenting the synthesis of thiols to overcome As phytotoxicity (Srivastava et al. 2007; Mishra et al. 2008). Moreover, *Myriophyllum propinquum* (A. Cunn) is shown to be a strong As accumulator ($>2 \text{ g kg}^{-1}$) after a field survey of lakes and rivers containing elevated levels of As in the Taupo volcanic zone, New Zealand (Robinson et al. 2006). However, that accumulation may be primarily a consequence of surface adsorption of arsenate, facilitated by co-deposition with other adsorptive species such as hydrated Fe oxides, rather than a direct uptake into plant cells.

C. demersum L. (family Ceratophyllaceae), a worldwide-distributed submerged macrophyte, has been established to accumulate more than $1,000 \text{ } \mu\text{g As g}^{-1}$ dry weight (DW) (Robinson et al. 2006). This plant is considered to be suitable for As phytoremediation because it is effective in capturing As from water and grows rapidly (Moreno-Jiménez et al. 2012). Besides, its rootless physiological feature and no translocation barrier from roots to shoots make it very different from terrestrial plants and an interesting model

for investigating As metabolism in submerged macrophytes. However, the mechanisms of As uptake, accumulation, and efflux in this plant have not been elucidated. Thus, the main objectives of the present study were: (1) to determine the kinetics of arsenate and arsenite uptake, (2) to investigate As accumulation and transformation in *C. demersum* after exposure to arsenate and arsenite, and (3) to explore the possible influx and efflux mechanisms of As.

2 Materials and methods

2.1 Plant growth

C. demersum were obtained from Taihu Lake, Wuxi City, China, and were grown for 1 month in the pond of a greenhouse. Before the experiment, the plants (the tip portion of the shoot about 3 cm) were grown in fresh water solution for 7 days. The composition of the synthetic fresh water was: $22.7 \text{ mg L}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, $30.7 \text{ mg L}^{-1} \text{ MgCl}_2 \cdot 2\text{H}_2\text{O}$, $20.4 \text{ mg L}^{-1} \text{ CaCl}_2 \cdot 2\text{H}_2\text{O}$, $45.7 \text{ mg L}^{-1} \text{ NaCl}$, $26.0 \text{ mg L}^{-1} \text{ NaHCO}_3$, $3.61 \text{ mg L}^{-1} \text{ KCl}$, $1.41 \text{ mg L}^{-1} \text{ FeCl}_3 \cdot 6\text{H}_2\text{O}$, $0.97 \text{ mg L}^{-1} \text{ Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$, $0.19 \text{ mg L}^{-1} \text{ MnCl}_2 \cdot 4\text{H}_2\text{O}$, $3.86 \text{ } \mu\text{g L}^{-1} \text{ ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $2.17 \text{ } \mu\text{g L}^{-1} \text{ CuCl}_2 \cdot 2\text{H}_2\text{O}$ (pH adjusted to 7.0 with NaOH or HCl solutions) (Stauber and Florence 1989). Plants were acclimatized inside a controlled environment growth cabinet under the following conditions: $115 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ light with a 14-h photoperiod at $25 \pm 2 \text{ } ^\circ\text{C}$.

2.2 Kinetics of arsenic uptake

After being pre-cultured for 1 week, *C. demersum* were washed with de-ionized water and blotted dry. Plant shoots (about 0.3 g) were incubated in conical flasks (150 mL) containing 5.0 mM MES (2-(*N*-morpholino) ethanesulfonic acid hydrate) and 0.5 mM CaCl_2 (pH 6.0), with different concentrations (0, 20, 40, 80, and 160 μM) of arsenate ($\text{Na}_3\text{AsO}_4 \cdot 12\text{H}_2\text{O}$) and arsenite (NaAsO_2). Experiments were set up in triplicate at each As concentration. The flasks were shaken at 60 rpm for 20 min, then plants were collected and rinsed in an ice-cold phosphate solution (1 mM K_2HPO_4 , 5 mM MES, and 0.5 mM CaCl_2 , pH 6.0) for 10 min to remove the adsorbed As from the shoot-free space (Abedin et al. 2002). After desorption, plants were blotted dry and oven-dried to constant weight at $70 \text{ } ^\circ\text{C}$. Subsamples were digested by microwave, and As concentrations were determined.

2.3 Arsenic accumulation and speciation

C. demersum (1-g shoots of plants in each container) were transferred to vessels (10 cm in diameter and 10 cm in depth) containing 500 mL freshwater with different concentrations of arsenate or arsenite (0, 1.25, 5, 10, 20, 40 μM),

each with three replicates. After exposure at 10 μM arsenate or arsenite for 1 and 4 days, 1 mL solution was taken from each pot, diluted with phosphate buffer solution (PBS) containing 2 mM NaH_2PO_4 and 0.2 mM $\text{Na}_2\text{-EDTA}$ (pH 6.0), which can effectively prevent arsenite oxidation (Xu et al. 2007), and kept on ice before analysis of As speciation. Plants were harvested after 4 days of exposure and immersed for 10 min in an ice-cold desorption solution as described before to remove apoplastic As (Abedin et al. 2002). Half of the plant samples were blotted dry and ground in a mortar and pestle with liquid N_2 to fine powder. Subsamples (0.3–0.5 g) of the ground materials were extracted with 10 mL PBS solution for 1 h under sonication at 4 °C. The extract was filtered through 0.45- μm filters before analysis of As speciation (Xu et al. 2007), while the other half of the plant samples were oven-dried and used to determine the total As concentrations (see Section 2.7).

2.4 Effect of microorganisms on arsenic transformation

In order to test the effects of microbes living in solution or exudates of *C. demersum* on transformation of As, the solution used to cultivate *C. demersum* for 4 days was collected and divided into two portions according to the method reported by Xu et al. (2007). A portion was unfiltered (solution B) and another filtered through a sterilized 0.2- μm filter into a sanitized vial to remove microorganisms living in the solution (solution C). The freshwater solution that had not been used for growing plants was used as a control (solution A). Arsenate or arsenite (10 μM) was added to the three solutions, followed by incubation in darkness for 1 day inside the same incubator as used for experiment 2.3. Each treatment was replicated threefold. Arsenic speciation was determined.

2.5 Arsenic influx and efflux mechanisms

This experiment was set up to examine if arsenate and arsenite are taken up via the P and aquaporin transporters, respectively, in *C. demersum*, as have been shown for other non-hyperaccumulator species. The effects of P ($\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$); glycerol, which is transported by aquaporins; and an arsenite analog Sb ($\text{C}_4\text{H}_4\text{KO}_7\text{Sb} \cdot 1/2\text{H}_2\text{O}$) on arsenic influx were investigated. Shoots of plants (about 0.3 g) were subjected to four treatments: control (20 μM arsenate or arsenite solutions containing 5.0 mM MES and 0.5 mM CaCl_2 , pH 6.0), + 100 μM P, + 20 mM glycerol, and + 0.5 mM Sb. Each treatment was replicated threefold. Plants were harvested after 20 min of exposure and oven-dried to constant weight at 70 °C after desorption of the apoplastic As. Arsenic concentrations of plant shoots were determined (see Section 2.7).

Additionally, in order to investigate whether the efflux of As by *C. demersum* takes the same transport pathway as the

influx, the effects of three inhibitors on As efflux were determined: metabolic inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), P-type ATPase inhibitor sodium vanadate, and glycerol. After 24 h of treatment with 20 μM arsenate, plants were desorbed of the apoplastic As as described before, then exposed to freshwater solutions (50 mL) with different treatments: control (freshwater), + 1 μM CCCP, + 200 μM sodium vanadate, and + 10 mM glycerol. All the solutions were buffered at pH 6.0 with 1 mM MES. There were three replicates in each treatment. Solutions were sampled after 2 h of treatments, preserved in PBS, and stored on ice to determine As speciation.

2.6 Analysis of arsenic speciation

Arsenic speciation in solutions and plant extracts was determined by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) (Agilent LC1100 series and Agilent ICP-MS 7500a; Agilent Technologies) as reported by Zhang et al. (2009). Arsenic species (arsenite, arsenate, dimethylarsinic acid, and monomethylarsonic acid) were separated by an anion-exchange column (Hamilton PRP-X100) with a mobile phase of 7.5 mM ammonium di-hydrogen phosphate and 7.5 mM ammonium nitrate (pH 6.2). Arsenic species in the samples were identified by comparisons with the retention times of standard compounds and quantified by external calibration curves with peak areas. For each batch of samples, the analysis was completed within 12 h of collection; no changes in As speciation were observed during this period. The recovery for the sum of the As species compared to the total As concentrations in plant tissue was about 60 %.

2.7 Digestion and analysis of total As

For the estimation of total As, samples were prepared and analyzed according to that reported by Zhang et al. (2009). Dried plant shoots (0.02 g) were digested in 5 mL of high-purity nitric acid overnight and heated in a microwave-accelerated reaction system (CEM Microwave Technology Ltd, USA). The temperature was raised gently (over a 5-min period), first to 55 °C and then to 75 °C with holding times of 10 min. Finally, the digest was taken up to 95 °C for 30 min. After cooling, the digests were diluted to 25 mL with Millipore ultrapure water. Arsenic concentration was determined by atomic fluorescence spectrometry (AF-610A, Beijing Haiguang Analytical Instrument Co., Beijing, China). Quality assurance of As analysis was included using a reagent blank and a standard reference material (bush twigs and leaves, GBW07602 from the National Research Center for Standard Materials in China) which was within 100 $\pm$ 20 % of the expected quality control checks.

2.8 Data analysis

Data are analyzed by multivariate analysis of variance using windows based SPSS 13.0, followed by post hoc testing using ANOVA.

3 Results

3.1 Uptake kinetics of arsenate and arsenite

Both arsenate and arsenite uptake followed a hyperbolic pattern in relation to the external concentration (Fig. 1). Arsenic influx kinetics can be well described by the Michaelis–Menten equation with R^2 values of 0.986 and 0.998 for arsenate and arsenite, respectively (data not shown). From the fitted model, $V_{\max}$ for arsenate uptake ($214 \pm 13 \text{ nmol g}^{-1} \text{ DW min}^{-1}$) was almost two times of the arsenite uptake ($128 \pm 8 \text{ nmol g}^{-1} \text{ DW min}^{-1}$), whereas the K_m for arsenate uptake ($18 \pm 4.5 \text{ }\mu\text{M}$) was about one tenth of the arsenite uptake ($120 \pm 14 \text{ }\mu\text{M}$).

3.2 Accumulation of As inorganic species

In the control treatment, the concentration of As in the shoot of *C. demersum* was only $1.7 \text{ }\mu\text{g g}^{-1} \text{ DW}$, indicating that the original plant was not contaminated by As. After 4 days of incubation, As accumulation was higher in plant shoots exposed to lower concentrations of arsenate compared to arsenite (1.25 and $5 \text{ }\mu\text{M}$). The accumulation of As increased significantly ($P < 0.05$) with increasing concentrations of As in external solutions until it reached maximums at $10 \text{ }\mu\text{M}$ As treatments, with arsenite accumulation ($963 \text{ }\mu\text{g g}^{-1} \text{ DW}$) markedly exceeding arsenate accumulation ($862 \text{ }\mu\text{g g}^{-1} \text{ DW}$) (Fig. 2). Then sharp decreases of As accumulation in

Fig. 1 Concentration-dependent kinetics for arsenate (closed circles) and arsenite (open circles) uptake by a shoot of *C. demersum* exposed to different concentrations of As for 20 min. The graph shows Michaelis–Menten curves for each As species. Data are mean $\pm$ SD ($n=3$)

Fig. 2 Arsenic concentrations in shoots of *C. demersum* exposed to different concentrations of arsenate (closed circles) and arsenite (open circles) for 4 days. Data are mean $\pm$ SD ($n=3$)

plant shoots exposed to $20 \text{ }\mu\text{M}$ arsenate and arsenite were observed, probably due to the chlorosis of the leaf caused by As toxicity. Plants died after being exposed to $40 \text{ }\mu\text{M}$ As for 4 days, so data of these treatments were not shown in Figs. 2 and 3.

3.3 Arsenic speciation in *C. demersum*

In the control plant, arsenite concentration was only $0.01 \text{ }\mu\text{g g}^{-1} \text{ FW}$, about one third of the arsenate concentration ($0.03 \text{ }\mu\text{g g}^{-1} \text{ FW}$). Regardless of the As species supplied, arsenite was the dominant species of As in *C. demersum*, accounting for 76–94 and 89–98 % of the total extractable As in plants exposed to arsenate and arsenite, respectively (Fig. 3). Both arsenite and arsenate concentrations in plants increased with increasing solution concentration of As ranging from 1.25 to $10 \text{ }\mu\text{M}$, with no significant differences between different As speciation treatments ($P > 0.05$). Dramatic decreases of arsenite concentrations in plants were observed in the $20 \text{ }\mu\text{M}$ As treatments. No methylated As species were detected in the plants.

3.4 Arsenic speciation in external solutions

At days 1 and 4, arsenate concentrations in the solutions of the arsenate treatments decreased by 49.1 and 95.5 %, respectively. However, arsenite was observed in the solution, accounting for 84 and 67 % of arsenate removal by plants at days 1 and 4, respectively. At the end of the experiment (4 days), 94 % of the As remaining in the solution was present as arsenite (Fig. 4). By contrast, arsenite remained relatively stable in the freshwater solutions of the arsenite treatments and comprised about 97 % of the As in the solution at both days 1 and 4. No methylated As species was found in the solutions (Fig. 4).

Fig. 3 Speciation of As (arsenate, closed circles; arsenite, open circles) in a shoot of *C. demersum* after exposure to different arsenate (A) or arsenite (B) concentrations for 4 days. Data are mean $\pm$ SD ($n=3$)

After 24 h of incubation, arsenite remained stable in all three solutions, occupying 99 % of the initial concentration (Fig. 5). As for the arsenate treatments, there was only 0.83 % of arsenite in solution A. About 11 and 6.0 % of the arsenate were reduced to arsenite in solutions B and C, respectively. It was far less than the extent of arsenate reduction observed in the freshwater solution growing the plant shoot (Figs. 4 and 5).

3.5 Effects of inhibitors to influx and efflux of As

In the control treatment, the influx of arsenate was about twice that of arsenite (Fig. 6). Arsenate transport was markedly inhibited by P but was unaffected by other inhibitors (glycerol and Sb). Compared to the control treatment, arsenite uptake was unaffected in the presence of P, whereas it was reduced by 85 and 44 % in the presence of 20 mM glycerol and 0.5 mM Sb, respectively ($P<0.05$).

Both arsenate and arsenite efflux to the external solution were observed, and the concentration of arsenite was about

twofold higher than that of arsenate in the control treatment (Fig. 7). Vanadate significantly enhanced arsenate efflux but exerted no effect on arsenite efflux ($P>0.05$). In addition, CCCP and glycerol had no effect on arsenate efflux but markedly decreased about 65 and 49 % arsenite efflux, respectively, compared to the control ($P>0.05$).

4 Discussion

In the present study, a higher accumulation of As was observed in plant shoots exposed to arsenate compared to arsenite (1.25 and 5 μM) (Fig. 2). This could result from the higher influx rate of arsenate in contrast to that of arsenite in solutions without P (Fig. 1). Compared with other aquatic plants, such as *Azolla caroliniana*, *Azolla filiculoides*, and *W. globosa*, *C. demersum* has a much higher arsenate influx rate (about three- to tenfold) and a lower K_m (about half to one tenth), indicating the higher affinity of transporters for arsenate. For the arsenite influx, the V_{max} and K_m values

Fig. 4 Concentrations (mean $\pm$ SD, $n=3$) of arsenate (closed bars) and arsenite (open bars) in the synthetic freshwater solutions after *C. demersum* were supplied with 10 μM arsenate and arsenite for 1 and 4 days

Fig. 5 Transformation of arsenate (closed bars) and arsenite (open bars) in freshwater solutions with three different treatments: solution A (control that had not been used to grow plants), solution B (had been used to grow *C. demersum* for 4 days), and solution C (solution B after being further sterilized). Data are mean $\pm$ SD ($n=3$)

Fig. 6 Inhibition of arsenate (closed bars) and arsenite uptake (open bars) by P (100 μ M), glycerol (20 mM), and Sb (0.5 mM). Plants were exposed to 20 μ M arsenate and arsenite solutions with different treatments for 20 min. Data are mean $\pm$ SD ($n=3$). Different letters indicate significant difference between treatments for arsenate and arsenite influx ($P<0.05$)

were comparable to those for *W. globosa* (Zhang et al. 2008, 2009). However, a maximum accumulation of As (963 μ g g $^{-1}$ DW) in plants exposed to 10 μ M arsenite after 4 days was higher than that (862 μ g g $^{-1}$ DW) in plants exposed to 10 μ M arsenate ($P<0.05$) (Fig. 2). In the 20- μ M As treatment, the dramatic drop of total As and arsenite concentrations in plants possibly indicated a decrease of arsenate reduction capacity caused by As toxicity, with the chlorosis of the leaves being observed. These findings also clearly indicated *C. demersum* as a strong accumulator of

Fig. 7 Effects of CCCP (1 μ M), sodium vanadate (200 μ M), and glycerol (10 mM) on the efflux of arsenate (closed bars) and arsenite (open bars) from plant shoots to external solutions. Plants were incubated in 20 μ M arsenate solution for 24 h before being used in the efflux experiment. Results are normalized by shoot FW. Data are mean $\pm$ SD ($n=3$). Different letters indicate significant difference between treatments for arsenate and arsenite efflux ($P<0.05$)

As. It accumulated As in 4 days, twice more than other aquatic plants in ≥ 7 days, such as *Azolla*, *H. verticillata*, and *M. propinquum*, all of which accumulated <400 μ g As g $^{-1}$ DW after exposure to >20 - μ M arsenate solutions (Robinson et al. 2006; Srivastava et al. 2007; Zhang et al. 2008). Its accumulation of As was similar to that of *W. globosa*, which has recently been reported as a strong accumulator of As (1,057 and 1,070 μ g As g $^{-1}$ DW accumulated after 7 days of exposure to 15 μ M arsenite and 30 μ M arsenate, respectively). The authors attributed the strong As accumulation to the absence of roots and direct uptake by shoots (Zhang et al. 2009), which could also apply to *C. demersum*. However, according to that reported by Mishra et al. (2008), *C. demersum* only accumulated 525 mg As kg $^{-1}$ after being grown for 7 days in 250 μ M arsenate, which may have been due to the competition of arsenate with P (9.6 mg L $^{-1}$) in their nutrient solution. This speculation can be supported by the fact that arsenate uptake by *C. demersum* was considerably inhibited by P in this study (Fig. 6), which confirmed that arsenate shares the P pathway to plant cells. Like arsenite ($pK_a=9.2$), Sb ($pK_a=11.8$) exists predominantly as an undissociated neutral molecule at pH 6.0, and it can be transported via aquaporins in some plants (Zhao et al. 2009). The competition of arsenite uptake by Sb and glycerol in *C. demersum* (Fig. 6) may indicate that arsenite is transported across the plasma membrane via major intrinsic proteins/aquaporins as found in *Saccharomyces cerevisiae* and rice (Wysocki et al. 2001; Meharg and Jardine 2003).

Different from the study by Zheng et al. (2003) on *C. demersum*, no matter what arsenic species are initially supplied to the plants, most of the As (>86 %) in plant shoots was in the form of arsenite rather than arsenate (Fig. 3). The possible explanations were that plant samples obtained by Zheng et al. (2003) were air-dried before As speciation analysis, which may cause oxidation of arsenite as it is thermodynamically unstable in oxygenated environments. Further, the extraction efficiency of the methanol–water method used in their study was very low (6.3–16.1 %). Whereas, in this study, plants were preserved in liquid N $_2$ after sampling and extracted by PBS solution with EDTA in it to prevent arsenite oxidation (Bednar et al. 2002), followed by immediate analysis of As speciation using HPLC-ICP-MS. Also, the extraction rate was much higher (about 60 %) in our study. The same phenomenon in which arsenite was the dominant species of As in *C. demersum* when supplied with inorganic As was also observed in other higher plants, such as rice (*Oryza sativa*), tomato (*Lycopersicon esculentum*), *A. caroliniana*, *A. filiculoides*, and *W. globosa*, which were also preserved in liquid N $_2$ before analysis of As speciation with immediate testing using HPLC-ICP-MS after extraction (Xu et al. 2007; Zhang et al. 2008, 2009).

Arsenate added to the solution was rapidly converted to arsenite (Fig. 4). More than 60 % of arsenate taken up by plants was reduced to arsenite and leaked to external solutions after 4 days of exposure. It can be concluded that microbes living in the synthetic solution or shoot exudates contributed little to arsenate reduction (Fig. 5). All these indicated that arsenite in solution is extruded by the plant shoot following arsenate reduction inside the shoot cells. This can also be supported by the fact that most of the As in shoots of plants was in the form of arsenite (Fig. 3). Arsenite efflux has also been observed in other plant species (non-hyperaccumulators), including *W. globosa*, *Azolla*, *Arabidopsis thaliana*, *Holcus lanatus*, rice, tomato, wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and maize (*Zea mays*), but not in the As hyperaccumulator *Pteris vittata* (Xu et al. 2007; Zhang et al. 2008, 2009; Logoteta et al. 2009; Zhao et al. 2009). Accordingly, arsenate reduction followed by arsenite efflux is probably a general phenomenon in higher plants except in hyperaccumulators and may act as an important mechanism of As detoxification as has been shown for microbes (Bhattacharjee and Rosen 2007).

Clear evidence for efflux of both arsenite and arsenate from shoots of *C. demersum* was shown (Figs. 4 and 7), with arsenite efflux being higher than arsenate efflux under freshwater solutions for 2 h. Furthermore, coinciding with the study on tomato root (Xu et al. 2007), arsenite efflux was not affected by vanadate but markedly suppressed by CCCP (66 %) (Fig. 7), suggesting that the efflux is not mediated by a P-type ATPase, but relies on the proton gradient across the plasma membranes for energy. Interestingly, due to the dramatic inhibition of arsenite efflux by glycerol which had no effect on arsenate efflux (Fig. 7), it can be postulated that the efflux of arsenite from plant shoots through aquaporin channels is like arsenite influx, some of which allow bidirectional passage of solutes (Mitani et al. 2008). Also, the fast efflux of arsenite (Figs. 4 and 7) is consistent with an aquaporin-mediated efflux which occurs through diffusion when the internal arsenite concentration exceeds that in the external medium. As a result, arsenite can be extruded from the plant shoot both through passive diffusion and active transport. Moreover, greatly enhanced efflux of arsenate by vanadate may indicate this to be a passive process as suggested in tomato roots (Xu et al. 2007). Because the uptake of arsenate through P transporters probably needs P-type ATPase, diminished ATPase would be expected to decrease arsenate uptake and to increase the release of arsenate to the external solutions, especially when shoots were placed in a solution without As.

In conclusion, owing to the substantial capacity of *C. demersum* for As accumulation, this plant appears to have potential for remediating As moderately contaminated waters. But the rapid leakage of arsenite into the external solution after the reduction of arsenate may limit its application in As phytofiltration. Furthermore, *C. demersum* is

considered as an invasive species, therefore, its use in phytofiltration should be conducted very carefully. However, *C. demersum* can serve as an ideal model plant to study As uptake and metabolism in a submerged macrophyte due to its special morphology without roots.

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