Arsenic accumulation by the aquatic fern *Azolla*: Comparison of arsenate uptake, speciation and efflux by *A. caroliniana* and *A. filiculoides*

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Arsenic accumulation and efflux differ between strains of the aquatic fern Azolla.

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ABSTRACT

This study investigates As accumulation and tolerance of the aquatic fern *Azolla*. Fifty strains of *Azolla* showed a large variation in As accumulation. The highest- and lowest-accumulating ferns among the 50 strains were chosen for further investigations. *Azolla caroliniana* accumulated two times more As than *Azolla filiculoides* owing to a higher influx velocity for arsenate. *A. filiculoides* was more resistant to external arsenate due to a lower uptake. Both strains showed a similar degree of tolerance to internal As. Arsenate and arsenite were the dominant As species in both *Azolla* strains, with methylated As species accounting for <5% of the total As. *A. filiculoides* had a higher proportion of arsenite than *A. caroliniana*. Both strains effluxed more arsenate than arsenite, and the amount of As efflux was proportional to the amount of As accumulation. The potential of growing *Azolla* in paddy fields to reduce As transfer from soil and water to rice should be further evaluated.

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1. Introduction

Arsenic (As) contamination has received widespread attention owing to its high toxicity to plants, animals and humans. In particular, As contamination in groundwater has become a crucial environmental and health problem in some parts of the world, especially in Bangladesh, Vietnam, India and China (Berg et al., 2001; Smith et al., 2000; Milton et al., 2005). The WHO guideline for As in drinking water has been changed from 50 to 10 µg/L (Smith et al., 2002; Mohan and Charles, 2007). However, millions of people in the developing countries are at risk of having drinking water with As concentration above the guideline. Water contamination with arsenic is an urgent and extensive problem that requires immediate actions (Rahman, 2002). In addition to the problem of As in drinking water, groundwater containing elevated

levels of As has been used to irrigate rice crops during the dry season in some parts of south and southeast Asia. This practice has resulted in accumulation of As in paddy soils and elevated transfer of As to the food chain (Meharg and Rahman, 2003).

Some conventional methods have been applied for removing As from contaminated water, but found to be high-cost or low-efficiency (Mondal et al., 2006). Phytoremediation, a plant-based green technology, may provide an alternative and cost-effective method for As removal. For example, Huang et al. (2004) showed that the As hyperaccumulating ferns *Pteris vittata* and *Pteris cretica* were very effective in removing As from the water in a hydroponic system to below the 10 µg/L guideline value. Other studies have investigated the As accumulation potential of aquatic macrophytes (Robinson et al., 2006; Rahman et al., 2007). Robinson et al. (2006) conducted a survey of aquatic plants growing in lakes and rivers containing elevated levels of As as a result of the influence of geothermal activity in the Taupo volcanic zone in New Zealand. They reported a geometric mean As concentration in 28 species of aquatic macrophytes of 125 mg kg⁻¹ dry weight (DW), with some species containing more than 1000 mg As kg⁻¹ DW. However, Robinson et al. (2006) concluded that As accumulation by aquatic plants occurs primarily via physicochemical adsorption to the

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plant's surface, facilitated by co-deposition of other adsorptive species such as hydrated Fe-oxides, which are similar to the iron plaque formed on rice roots (Chen et al., 2005). Rahman et al. (2007) tested the potential of the aquatic macrophyte *Spirodela polyrrhiza* for phytofiltration of arsenate and DMA (dimethylarsinic acid). They found that *S. polyrrhiza* accumulates As by both a physicochemical adsorption and the phosphate uptake pathway.

Azolla is a floating aquatic fern, which is widely distributed in paddy fields, rivers, ponds and lakes. It can fix nitrogen by its symbiotic partnership with *Anabaena* which resides in the dorsal cavity of *Azolla* fronds; for this reason *Azolla* has been used as a green manure to improve soil fertility and rice production (Wagner, 1997). It has been reported that *Azolla* has a high capacity to accumulate heavy metals such as Cd, Cr, Cu, Ni and Zn (Sela et al., 1989), and can be used to remove heavy metals from wastewater (Bennicelli et al., 2004; Arora and Saxena, 2005; Rakhshae et al., 2006). However, the ability of *Azolla* to accumulate As from water has not been evaluated in detail. If this aquatic fern can accumulate As from water substantially, it may provide an effective way to reduce As transfer from soil and irrigation water to paddy rice.

Little information is available on As accumulation and tolerance mechanisms in the aquatic fern *Azolla*. In this study, we screened a range of *Azolla* strains for As accumulation. This was followed by a more detailed investigation on the uptake kinetics of arsenate, As speciation and tolerance in two *Azolla* strains with contrasting abilities in As accumulation.

2. Materials and methods

2.1. Plant growth

Fifty strains of *Azolla* were obtained from the Institute of Agricultural Ecology, Fujian Academy of Agricultural Sciences, Fuzhou, China or collected from ponds in Nanchang, Jiangxi province of China. The plants were grown hydroponically inside a growth chamber with the following climatic conditions: 14 h light period per day with a light intensity of around $280 \mu\text{mol m}^{-2} \text{s}^{-1}$, $25/20^\circ\text{C}$ day/night temperatures, and 70% relative humidity. The composition of the nutrient solution was as follows: 1 mM CaSO_4 , 1.6 mM MgSO_4 , 0.3 mM KH_2PO_4 , 0.3 mM KCl, 0.7 mM NaNO_3 , 10 μM $\text{FeNa}_2\text{-EDTA}$, 20 μM H_3BO_3 , and 7.67 μM Na_2MoO_4 (pH adjusted to 6.0 with KOH or HCl solutions). Nutrient solution was renewed twice every week.

2.2. Screening of *Azolla* strains

Initially, each of the 50 strains of *Azolla* was exposed to $50 \mu\text{M}$ arsenate for 10 days, with a single replicate for each strain. Based on As tolerance and accumulation from the initial screening experiment, eight strains were selected for a second screening experiment, with three replicates for each strain. Arsenate was applied as $\text{Na}_3\text{AsO}_4 \cdot 12\text{H}_2\text{O}$. The nutrient solution was changed every 2 days. After 10 days, the fronds were harvested, washed with de-ionized water, blotted and oven-dried at 70°C for 48 h. Sub-samples were digested and total As in the fronds were determined by atomic fluorescence spectrometry after the samples were digested with 5 ml concentrated HNO_3 (see below). The highest- and lowest-accumulating strains of *Azolla* were chosen for further study.

2.3. Kinetics of arsenate uptake

After being pre-cultured for 1 week, the two selected strains of *Azolla* (*Azolla caroliniana* and *Azolla filiculoides*) were washed with de-ionized water and blotted dry. One gram of each strain was incubated in a test solution (500 ml) containing 5.0 mM MES and 0.5 mM $\text{Ca}(\text{NO}_3)_2$ (pH 5), with different concentrations of arsenate (0, 20, 40, 80, 160 and $320 \mu\text{M}$). Four replicates were included for each *Azolla* strain at each arsenate concentration. The solutions were shaken gently at 60 rpm. After 20 min, *Azolla* was collected and rinsed in an ice-cold phosphate buffer solution (1 mM K_2HPO_4 , 5 mM MES and 0.5 mM $\text{Ca}(\text{NO}_3)_2$) for 10 min to remove As adsorbed on the cell walls (Abedin et al., 2002). The fronds were then oven-dried at 70°C for 48 h, sub-samples were digested and As concentrations were determined.

2.4. One-week arsenic exposure experiment

The two selected strains of *Azolla* (*A. caroliniana* and *A. filiculoides*) were pre-cultured for 1 week. Fronds (3 g) of each strain were cultured in a bowl (12 cm in diameter and 4 cm in depth) containing 200 ml nutrient solution. Six concentrations of arsenate were used in this study: 0, 5, 10, 20, 40 and $80 \mu\text{M}$, each with four replicates. The bowls were arranged randomly inside the growth chamber and re-

arranged every day. The nutrient solution was renewed every 2 days. After 7 days the plants were harvested, washed carefully with de-ionized water, blotted dry and fresh weight (FW) recorded. The samples were frozen in liquid nitrogen and freeze-dried. The concentrations of total As, P and As species were determined.

2.5. Arsenic efflux from *Azolla*

Three grams of *A. caroliniana* and *A. filiculoides* were incubated in 200 ml nutrient solution containing $40 \mu\text{M}$ arsenate. Twenty-four hours later the fronds were rinsed briefly in de-ionized water and then incubated in the solution containing 1 mM K_2HPO_4 , 5 mM MES and 0.5 mM $\text{Ca}(\text{NO}_3)_2$ for 10 min to remove the As in the apoplast of the fronds. One gram of each strain was then placed in a plastic vial with 25 ml nutrient solution. After 1, 3, 5, 8 or 24 h the frond was transferred to a new vial containing the same nutrient solution. The efflux solution was filtered through a $0.45 \mu\text{m}$ nylon filter and used for the analysis of total As and As species.

2.6. Digestion and analysis of plant tissues

Approximately 0.2 g oven-dried plant sample was weighed into digestion tubes, to which 5 ml concentrated HNO_3 was added. The sample was digested first at 100°C for 1 h and then at 120°C for 2 days. A reagent blank and a standard reference plant material (bush twigs and leaves, GBW07603 from the National Research Center for Standard Materials in China) were included to verify the accuracy of the analytical procedure. After digestion, the solutions were cooled, diluted to 25 ml with ultra-pure water and filtered through filter paper. The concentrations of Fe and P in the digests were determined by inductively coupled plasma-optical emission spectrometer (ICP-OES, Optima 2000 DV, Perkin-Elmer, USA). Arsenic concentration was determined by an atomic fluorescence spectrometry (AF-610A, Beijing Haiguang Analytical Instrument Co., Beijing, China). The mean As concentration of the standard reference material was $1.10 \pm 0.01 \text{ mg kg}^{-1} \text{ DW}$, which agrees well with the certified value $1.25 \pm 0.15 \text{ mg kg}^{-1} \text{ DW}$.

2.7. Determination of As species

Freeze-dried frond samples were extracted with 10 ml of 1% nitric acid in a microwave accelerated reaction system (CEM Microwave Technology Ltd, USA). The NIST Certified Reference Material (CRM) 1568a long grain rice flour and GBW CRM 10010 Chinese rice flour were used to validate the analytical procedure. Spikes (0.1 ml of $1000 \mu\text{g As ml}^{-1}$) of both As(III) and As(V) and blanks were run with each extraction batch. The extract solutions were centrifuged and passed through a $0.45 \mu\text{m}$ nylon filter. To minimize potential transformation of As species, samples were kept on ice and in the dark and analyzed within a few hours after extraction. Arsenic speciation was assayed by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) (7500a Agilent Technologies). Chromatographic columns consisted of a Hamilton precolumn (11.2 mm, 12–20 mm) and a Hamilton PRP-X100 $10\text{-}\mu\text{m}$ anion-exchange column ($240 \times 4.1 \text{ mm}$). The mobile phase consisted of 8 mM ammonium di-hydrogen phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$) and 8 mM ammonium nitrate (NH_4NO_3), adjusted to pH 6.2 using ammonia. Arsenic species in the samples were identified by comparing their retention times with those of the standards including arsenite (As(III)), arsenate (As(V)), dimethylarsinic acid (DMA^V) and monomethylarsonic acid (MMA^V), and quantified by external calibration curves with peak areas.

2.8. Data analysis

All data were subjected to the analysis of variance (ANOVA) using windows-based SPSS 13.0.

3. Results

3.1. Screening experiments

In the initial screening experiment, As concentrations in the fronds varied by 13.5-fold, from 29 to $397 \text{ mg kg}^{-1} \text{ DW}$ among the 50 strains of *Azolla* (data not shown). Thirty strains did not survive 10-day exposure to $50 \mu\text{M}$ arsenate. In the second screening experiment, As concentration in the frond tissue varied by approximately 5-fold among the eight strains of *Azolla* tested (Fig. 1). *A. caroliniana* had the highest As concentration in the frond ($284 \text{ mg kg}^{-1} \text{ DW}$) and the lowest As concentration was found in *A. filiculoides* ($54 \text{ mg kg}^{-1} \text{ DW}$). These two strains were therefore selected for further experiments.

Fig. 1. Arsenic concentration in different strains of *Azolla* grown in nutrient solutions with 50 μM arsenate for 10 days. Data are mean $\pm$ SE ($n = 3$).

3.2. Arsenate influx kinetics

Arsenate influx into both *Azolla* strains followed a hyperbolic pattern in relation to the increasing concentration of As in the incubation solutions (Fig. 2). Arsenate influx kinetics were adequately described by the Michaelis–Menten equation with R^2 values of 0.978 and 0.996 for *A. caroliniana* and *A. filiculoides*, respectively. There was little difference between the K_m values of the two strains (Table 1). In contrast, the $V_{\max}$ value of the high-As accumulating strain *A. caroliniana* was significantly ($p < 0.001$) higher (by 43%) than that of the low-As accumulating strain *A. filiculoides* (Table 1).

3.3. One-week arsenate exposure experiment

Increasing arsenate concentration in the nutrient solution significantly ($p < 0.001$) decreased the growth of both strains (Fig. 3). In the treatments with no As or low levels of As, *A. caroliniana* produced more biomass than *A. filiculoides*, whereas the difference was reversed at high arsenate concentrations. The dose–response

Fig. 2. Concentration-dependent kinetics for arsenate uptake by *A. caroliniana* and *A. filiculoides*. Each point is represented as mean $\pm$ SE ($n = 4$).

Table 1

Kinetic parameters for arsenate influx into *A. caroliniana* and *A. filiculoides*

Strains of <i>Azolla</i>	$V_{\max}$ (nmol g ⁻¹ DW min ⁻¹)	K_m (μM)	R^2
<i>A. caroliniana</i>	$68.0 \pm 4.5^*$	34.9 ± 8.2	0.978
<i>A. filiculoides</i>	$47.6 \pm 1.3^*$	35.4 ± 3.5	0.996

*Significant at $P < 0.001$.

data could be described satisfactorily by a log-logistic equation ($R^2 = 0.89$ and 0.94 for *A. caroliniana* and *A. filiculoides*, respectively). From the fitted equations, the effective concentration of arsenate in the nutrient solution that caused a 50% inhibition on biomass growth (EC_{50}) could be estimated. The EC_{50} values ($\pm\text{SE}$) were 60.7 ± 6.9 and 125.8 ± 10.8 μM arsenate for *A. caroliniana* and *A. filiculoides*, respectively. The estimated EC_{50} for *A. filiculoides* exceeded the highest arsenate concentration used in the experiment and may therefore be less reliable. For this reason, we also estimated EC_{25} (i.e. the arsenate concentration associated with a 25% growth inhibition) for the two *Azolla* strains and the values were 22.5 ± 5.9 and 53.1 ± 5.7 μM arsenate for *A. caroliniana* and *A. filiculoides*, respectively. These results indicate that *A. filiculoides* was more resistant to arsenate in the external solution than *A. caroliniana*.

3.4. Arsenic and phosphorus accumulation

In both strains, frond As concentration increased significantly with increasing arsenate concentration in the nutrient solution. Irrespective of external As concentrations, there was a significant ($p < 0.001$) difference in As accumulation between the two strains (Fig. 4); tissue As concentration of *A. caroliniana* was approximately two times higher than that of *A. filiculoides* at every arsenate concentration in the medium.

To determine the EC_{50} values based on tissue As concentration, the growth data were plotted against frond As concentration and the relationship was fitted with a log-logistic equation (Fig. 5). The EC_{50} values ($\pm\text{SE}$) were 98.9 ± 17.4 and 109.4 ± 9.1 mg kg^{-1} DW for *A. caroliniana* and *A. filiculoides*, respectively.

In the treatment without As addition, P concentration in the frond tissue of *A. filiculoides* (low-As accumulating) was higher than that in *A. caroliniana*. The addition of As significantly ($p < 0.001$) and linearly decreased the P concentration in *A. filiculoides* (Fig. 6a).

Fig. 3. Effect of arsenate exposure for 1 week on growth of *A. filiculoides* and *A. caroliniana*. Data are mean $\pm$ SE ($n = 4$). Lines are the fitted log-logistic curves. To allow log transformation, a small value (0.1) was added to the zero As concentration in the control treatment.

Fig. 4. Changes in frond arsenic concentration in two strains of *Azolla* grown in nutrient solutions with different arsenate concentrations for a week. Each point is represented as mean $\pm$ SE ($n = 4$).

In contrast, As addition had no significant effect on the P concentration in *A. caroliniana* except at the highest As concentration of 80 μ M arsenate (Fig. 6a). Regression analysis showed different patterns between the two strains in the relationship between frond As and P concentrations (Fig. 6b); the slope of regression equation for *A. filiculoides* was much more negative than that for *A. caroliniana*.

3.5. Arsenic species in frond tissues

Four As species, namely arsenite, arsenate, DMA^V and MMA^V, were found in *A. caroliniana* (Fig. 7), with the two methylated species accounting for only 1–5% of the total As. The largest proportion (50–60%) of total As was present as arsenate, with the remaining proportion (25–40%) as arsenite. In contrast, *A. filiculoides* contained arsenite, arsenate and DMA, with arsenite accounting for 55–69% of the total As. The relationship between the percentage of arsenite in total As and the arsenate concentration in the external solution showed different patterns in the two strains of

Fig. 6. (a) Changes in phosphate concentration in two strains of *Azolla* under different arsenate concentrations after plants had been supplied with arsenate for a week. Each point is represented as mean $\pm$ SE ($n = 4$), and (b) relationship between frond As and P concentrations in two strains of *Azolla* after 1-week exposure to different arsenate concentrations. Regression equations are shown on the graph.

Azolla (Fig. 8). In *A. caroliniana*, the percentage of arsenite in the total As showed a decreasing trend with increasing arsenate concentration in the solution, whereas in *A. filiculoides*, this percentage increased with external arsenate concentration up to 20 μ M.

3.6. Arsenic efflux

Fig. 9 shows the cumulative amount of As efflux from the two strains of *Azolla* after they had been preloaded with As for 24 h. Both arsenate and arsenite were detected in the efflux solution, but the amount of the former was about 10-fold higher than the latter in both strains. During the 24 h efflux period, the cumulative amount of As efflux showed a hyperbolic pattern, suggesting that the efflux rate slowed with time. However, this pattern could also be partly due to re-absorption of As from the efflux solution. The high-As accumulating strain *A. caroliniana* released more, by approximately 2-fold, arsenate and arsenite to the efflux solution than the low-As accumulating strain *A. filiculoides* (Fig. 9, $p < 0.001$).

Fig. 5. Relationship between growth of *A. filiculoides* and *A. caroliniana* and frond As concentration after 1-week exposure to different concentrations of arsenate. Data are individual replicates. Lines are the fitted log-logistic curves. To allow log transformation, a small value (0.1) was added to the zero As concentration in the control treatment.

4. Discussion

Different strains of *Azolla* varied substantially in As accumulation and resistance. This variation may be exploited in selecting

Fig. 7. Arsenic speciation in two strains of *Azolla* after exposure to different arsenate concentrations for 1 week. Data are means of four replicates.

strains that are more effective in removing As from water. The two strains (*A. caroliniana* and *A. filiculoides*) that were selected for detailed investigations of As uptake, tolerance and speciation also showed a considerable difference in As accumulation, by approximately 5-fold in the screening experiment (Fig. 1) and 2-fold in the 1-week exposure experiment (Fig. 4). The screening experiment had a larger volume of nutrient solution per gram of *Azolla* biomass and a longer exposure period (10 days) than the 1-week exposure experiment, resulting in a larger difference between *Azolla* strains in As accumulation. In the short-term influx kinetic study, both strains followed the Michaelis–Menten equation. With regard to the kinetic parameters, similar K_m values for both strains suggest a similar affinity of membrane transporters for arsenate in the absence of phosphate. In contrast, the higher value of V_{max} for *A. caroliniana* was in accordance with its higher ability of As accumulation, indicating that *A. caroliniana* may have a higher density of transporters on the plasma membranes that can transport arsenate.

It has been well documented that arsenate is taken up through the phosphate transporters (Ullrich-Eberius et al., 1989; Abedin et al., 2002; Esteban et al., 2003). The relationship between As and P in the two strains of *Azolla* (Fig. 6a, b) is also consistent with a common transport mechanism for arsenate and phosphate. However, there were differences between the two *Azolla* strains in

the As–P relationship; *A. filiculoides* had a higher uptake of P in the absence of arsenate than *A. caroliniana*, but its P uptake was much more affected by arsenate exposure than the latter. This difference may be attributed to different selectivity for phosphate or arsenate in the two *Azolla* strains.

Unlike some of the strains that could not survive when exposed to 50 µM arsenate for 10 days, both *A. caroliniana* and *A. filiculoides* were reasonably tolerant to As. The EC_{50} and EC_{25} values of the low-As accumulating strain *A. filiculoides*, based on the external arsenate concentration, were about double of those in the high-As accumulating strain *A. caroliniana*. This means that *A. filiculoides* was more resistant to external arsenate than *A. caroliniana*. However, there was no significant difference in the EC_{50} value based on frond As concentration, suggesting that the two strains had a similar level of tolerance to internal As. Therefore, *A. filiculoides* was more resistant to As than *A. caroliniana* because the former had a lower uptake of arsenate. A reduced uptake of arsenate has been shown to be the main adaptive mechanism of arsenate resistance in higher plants such as *Holcus lanatus*, *Deschampsia cespitosa*, *Agrostis capillaries* and *Cytisus striatus* (Meharg and Macnair, 1991a,b; Bleeker et al., 2003). The results from this study indicate that a suppression of arsenate uptake is also likely to be an important mechanism through which the aquatic fern *Azolla* achieves an enhanced resistance to external arsenate. In the As-resistant

Fig. 8. Percentage of As(III) in total arsenic in two strains of *Azolla* after plants had been supplied with different concentrations of arsenate for 1 week. Each point is represented as mean $\pm$ SE ($n = 4$).

Fig. 9. Cumulative efflux of arsenic species after two strains of *Azolla* had been supplied with 40 µM arsenate for 24 h. Each point is represented as mean $\pm$ SE ($n = 4$).

ecotype of *H. lanatus*, the activity of arsenate reductase and synthesis of phytochelatin were found to be higher than those of the As-non-resistant ecotype, although these biochemical differences are only relatively minor components of the overall As-resistance phenotype (Hartley-Whitaker et al., 2001; Bleeker et al., 2006). It remains to be investigated whether the two contrasting strains of *Azolla* used in our study differ in the capacity of As detoxification through the pathway of arsenate reduction and complexation with phytochelatin.

Arsenic non-hyperaccumulating species of higher plants generally have a toxicity threshold concentration of tissue As of between 5 and 100 mg kg⁻¹ DW (Kabata-Pendias and Pendias, 1992). In contrast, the As hyperaccumulator *P. vittata* can accumulate several thousand milligram As per kilogram DW in fronds without suffering from As toxicity (Tu and Ma, 2002; Caille et al., 2005). The EC₅₀ of tissue As concentration of around 100 mg kg⁻¹ DW observed in the two *Azolla* strains is within the range of As non-hyperaccumulating plants, indicating that the *Azolla* species tested in the present study are not As hyperaccumulators. The level of As accumulation in *Azolla* is similar to the geometric mean As concentration of 125 mg kg⁻¹ DW in 28 species of aquatic macrophytes surveyed by Robinson et al. (2006).

In plants and microorganisms, arsenate is detoxified by reducing arsenate to arsenite, followed by complexation with thiol compounds, storage in vacuoles or efflux to the external medium (Meharg and Hartley-Whitaker, 2002; Rosen, 2002; Duan et al., 2007). A small percentage of As may also be transformed into organic species such as methylated As compounds and arsenosugars (Koch et al., 2000). Similar to other studies on higher plants (e.g. Koch et al., 2000; Quaghebeur and Rengel, 2003; Raab et al., 2005; Xu et al., 2007), we found arsenite and arsenate to be the dominant forms of As in the two strains of *Azolla*, with methylated As compounds accounting for <5% of the total As. However, the two *Azolla* strains showed a significant difference in the proportions of arsenite versus arsenate, with *A. filiculoides* having a higher percentage of arsenite (55–70%) than *A. caroliniana* (25–40%) (Fig. 8). This difference could be explained by two possible reasons. First, *A. caroliniana* had a higher rate of arsenate uptake than *A. filiculoides* (Figs. 2 and 4), leading to a higher accumulation of arsenate inside the cells. Second, *A. filiculoides* may have a higher activity of arsenate reductase than *A. caroliniana*, and therefore is able to reduce arsenate to arsenite more efficiently. The second possibility remains to be investigated.

Arsenite efflux is an important mechanism employed by microorganisms to tolerate As (Rosen, 2002). Recently, Xu et al. (2007) showed that roots of higher plants (tomato and rice) can also mediate a rapid reduction of arsenate to arsenite in nutrient solution by the process of arsenate uptake, reduction and efflux. We investigated if As efflux also occurred in *Azolla*. Similar to the study of Xu et al. (2007), we found that both arsenate and arsenite were released to the efflux solution by *A. caroliniana* and *A. filiculoides*. However, efflux of arsenate was much higher (by about 9-fold) than that of arsenite (Fig. 9). This may be because most of arsenite inside the cells was complexed with thiol compounds. Furthermore, the high-As accumulating *Azolla* (*A. caroliniana*) released approximately two times more As than the low-As accumulating *Azolla* (*A. filiculoides*). It thus appears that the amount of As efflux was proportional to the amount of As accumulation in the two strains of *Azolla*. When *Azolla* fronds preloaded with arsenate were placed in an efflux solution that was initially free of arsenate, arsenate may leak into the external solution passively.

In conclusion, this study shed light on the variation and the underlying mechanisms in As accumulation and resistance in different strains of *Azolla*. The potential of growing *Azolla* to reduce As transfer from soil and water to paddy rice should be further evaluated.

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