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# Arsenic accumulation by aquatic macrophyte coontail (*Ceratophyllum demersum* L.) exposed to arsenite, and the effect of iron on the uptake of arsenite and arsenate

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ABSTRACT

In this study, the aquatic macrophyte *Ceratophyllum demersum* L. (coontail or hornwort) was tested for its efficiency of arsenic (As) uptake under laboratory conditions. Our results revealed that the solution pH had a significant effect on As accumulation by *C. demersum* ($p < 0.001$). The accumulation was highest at pH 5 and decreased as pH values increased. Plants that were exposed to various concentrations of arsenite (As(III)) for 24 and 48 h, exhibited tolerance and toxic responses, respectively. As accumulation by *C. demersum* depended on the concentrations of As(III) and the duration of exposure ($p < 0.001$). At 40 μM after 24 h, plants accumulated 227.5 μg As $g^{-1}$ dw and showed no visible symptoms of toxicity. However, after 48 h, As level reached 302.4 μg $g^{-1}$ dw and biomass production decreased significantly. Toxic effects were evident by plant necrosis and negative biomass production, leading to a decrease in the amount of accumulated As. Also, the addition of iron (Fe) into the nutrient solutions (0.18 mM) had contrasting effects on the uptake of 2 As species – the uptake of As(III) was enhanced by the presence of Fe, but the uptake of arsenate (As(V)) was considerably inhibited.

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## 1. Introduction

Arsenic (As) is a naturally occurring element in the earth's crust. It ranks 20th in natural abundance, 14th in sea water, and 12th in the human body (Mandal and Suzuki, 2002). Natural As elevation of drinking water supplies has been reported from more than 70 countries, posing a serious health hazard to an estimated 150 million people world-wide (Brammer and Ravenscroft, 2009). As-contaminated groundwater used for irrigation may pose an equally serious health hazard to people eating food from irrigated crops (Williams et al., 2006), and As accumulating in irrigated soils poses a serious threat to sustainable agriculture in affected areas (Heikens, 2006).

Phytoremediation, a plant-based green technology, is a promising technology for environmental pollution caused by unavoidable limitations of traditional technologies (Rahman et al., 2008a). The use of some submerged aquatic macrophytes and floating plants in the process of phytoremediation is more commonly known as phytofiltration. Recently, As accumulation by aquatic plants, such as *Eichhornia crassipes*, *Lemna minor* (Alvarado et al., 2008), *Salvinia natans* (Rahman et al., 2008a), *Spirodela polyrhiza* (Rahman et al., 2008b,c), *Hydrilla verticillata* (Srivastava et al., 2007), and *Wolffia globosa* (Zhang et al., 2009) have been reported in the literature. The promising results of phytoremediation technology for As removal from contaminated water has gained the attention of researchers.

Thus far, 3 mechanisms have been proposed for the uptake of As species in aquatic macrophytes: (i) active uptake through phosphate uptake transporters, (ii) passive uptake through aquaglyceroporins, and (iii) physicochemical adsorption on plant surfaces (Rahman and Hasegawa, 2011). Physicochemical adsorption, an alternative mechanism for As accumulation by aquatic plants, was hypothesized by Robinson et al. (2006). In this mechanism, oxides/hydroxides of iron (Fe plaque) suspended on the aquatic plant surfaces adsorb and accumulate As. In most As-affected areas of South and South East Asia, groundwater is rich in Fe (Brammer and Ravenscroft, 2009). Water soluble $Fe^{2+}$ is oxidized when water is exposed to the air and is then precipitated as Fe hydroxides that adsorb As. If aquatic macrophytes are used for phytoremediation of As, the elevated Fe concentrations in water could be presumed to be a factor affecting the accumulation characteristics.

Coontail (*Ceratophyllum demersum* L.), a completely submerged aquatic macrophyte is reportedly a scavenger of As(V) in contaminated water (Mishra et al., 2008). However, available knowledge of the accumulation characteristics of plants exposed to As(III) is insufficient. Therefore, the present study was focused on

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investigating As uptake efficiency when *C. demersum* is grown in As(III)-containing nutrient solutions. Additionally, the effects of Fe on As adsorption and accumulation properties of *C. demersum* exposed to As(III) and As(V) were also elucidated. These results may be useful when this aquatic plant is used as a phytoremediator for As-contaminated water.

## 2. Materials and methods

### 2.1. Plant material

*C. demersum* plants were purchased from a local shop and were grown for 2 weeks in a large aquarium. Before treatments, plants (approximately 5 cm tip portion) were acclimatized in 10% Hoagland solution (Hoagland and Arnon, 1950) for one week in a growth chamber with the conditions set to a light intensity of 70 W $m^{-2}$, a 16 h photoperiod at 25 ± 2 °C, and a relative humidity of 70%. After acclimatization, the plants were used for subsequent studies. All experiments were set up in triplicate and each replicate contained approximately 5 plants of equal size (approximately 2 g in total).

### 2.2. pH dependence of plant As accumulation

The acclimatized plants were exposed to 5 μM As(III) ($NaAsO_2$, Wako, Japan) maintained in 10% Hoagland solution in 300 ml conical flasks under the above-mentioned laboratory conditions for 96 h. At the beginning of the experiments, solution pH was adjusted to a range of 5–10 by adding 0.1 M $HNO_3$ or 0.1 M NaOH. pH values were then measured using a pH meter (HM-20J, Analyticon Instruments Corporation, USA) and were re-adjusted every 24 h to maintain the initial values throughout the experiment.

### 2.3. Effects of As(III) concentrations and duration of exposure on plant As accumulation

The acclimatized plants were incubated in 10% Hoagland solutions containing different concentrations of As(III) ($NaAsO_2$, Wako, Japan) (0–40 μM) under the laboratory conditions mentioned above for periods of 24 h and 48 h. Initial solution pH was approximately 6 and was not adjusted throughout this experiment. Flasks not exposed to As were maintained with each experiment group and served as controls.

Plant biomass was measured on a fresh weight basis. Plants were blotted dry gently to remove excess water and were weighed (approximately 2 g in total) prior to As exposure. After harvesting, plants were similarly blotted dry and weighed.

### 2.4. Effects of Fe on plant As adsorption and uptake

Plants were maintained in 10 l of deionized (DI) water (Millipore, USA) for 12 h to minimize any interference with other elements of Fe. Subsequently, plants were collected from stock culture and rinsed 3 times with DI water. They were then separately used for 3 treatments. In the first treatment, plants were grown in 10% Hoagland solution additionally supplied with 0.18 mM Fe ($Fe_2(SO_4)_3$, Wako, Japan). In the second treatment, plants were grown in a 10% Hoagland solution that contained 5 μM Fe. Ten mM MES buffer was used to control the pH of the solutions at approximately 6.0 by adding 0.1 M $HNO_3$ or 0.1 M NaOH. The last treatment in which plants were grown in DI water served as the control. Then, 2 μM As(III) or As(V) was added to the culture solutions and plants were grown for 168 h. All treatments were kept in the growth chamber.

Plant samples were subsequently harvested and washed 3 times with DI water. Adsorbants on the plant surfaces were extracted using a Citrate–Bicarbonate–EDTA (CBE) extraction method (Rahman et al., 2008b).

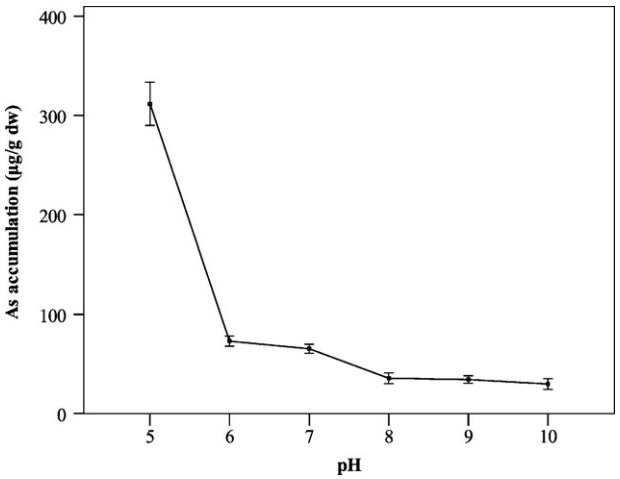



**Fig. 1.** Effects of solution pH on As accumulation of *C. demersum* exposed to 5 μM As(III) for 96 h. Error bars represent means ± SD ($n = 3$).

### 2.5. Sample preparation and chemical analysis

Oven-dried plant material (100 mg) was digested in 3 ml of concentrated $HNO_3$ on a heating block (ALB-121, Scinics, Japan) at 130 °C for 1 h. After cooling to room temperature, 1.5 ml of 30% hydrogen peroxide ($H_2O_2$, Wako, Japan) was added to the samples and they were re-heated at 130 °C for 20 min. Again, the digests were cooled to room temperature and diluted to 10 ml using Milli Q water (Millipore, USA), filtered through a PTFE 0.45 μm filter membrane (Millipore, USA), and stored in 15 ml polypropylene tubes.

Arsenic speciation in the solutions was analyzed as described previously (Huang et al., 2011). As(III) and As(V) were separated using an As speciation cartridge (Metal Soft Center, Highland Park, NJ, USA), which retains As(V) (Meng et al., 2001).

Quantification of As and Fe was performed using inductively coupled plasma mass spectrometry (ICP-MS) (ELAN 9000, Perkin Elmer, SCIEX). A 10 ng/ml indium (In) solution was used as an internal standard. All chemicals were of analytical grade. Glassware was washed with detergent solution, 3% $HNO_3$, and then DI water 10 times before use.

### 2.6. Data analysis

Analysis of variance (ANOVA) was performed using a PASW 18 statistical package (formerly SPSS statistics, IBM) to confirm the variability of the data and the validity of the results. All values were expressed as the mean ± standard deviation of triplicates.

## 3. Results and discussion

### 3.1. As accumulation of C. demersum in response to different values for solution pH

As accumulation of *C. demersum* varied significantly ($p < 0.001$) when the experiment was carried out at different pH values (Fig. 1). Within a pH range of 5–10, maximum As uptake by the plant occurred at pH 5, and decreased with an increase in solution pH. As uptake decreased abruptly as pH was increased from 5 to 6, and was not appreciably affected by changes in pH from 8 to 10. No visual negative effects on plant growth such as necrosis/chlorosis or the weakening of plants was observed.

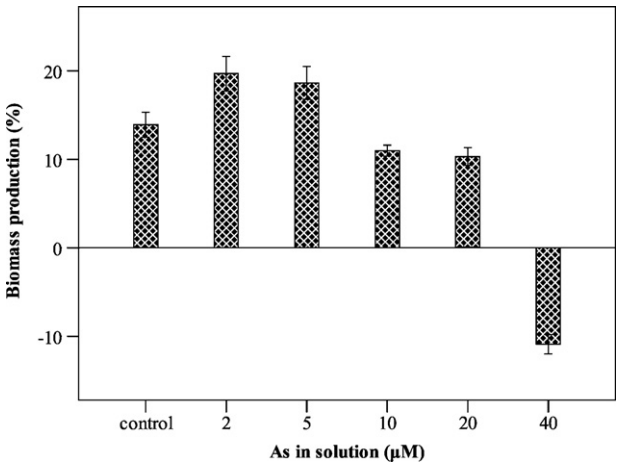



**Fig. 2.** Effects of As(III) treatment on biomass production of *C. demersum* after 48 h. Error bars represent means ± SD ($n = 3$).

The effect of pH on metal(loid)s toxicity is very complex: the effect is primarily dependent on the metal species (Wang, 1987). The results in the present study indicated that slightly acidic pH values accelerated the As accumulation process. Consideration of pH is crucial to phytoremediation strategies since pH varies greatly in As-polluted soils and waters (Tu and Ma, 2003). As uptake properties of *C. demersum* as a function of pH were reported for the first time in the present study in an attempt to investigate appropriate remediation strategies for aquatic environments with As contamination. It was apparent that the increase in pH suppressed As uptake when As was supplied at a concentration of 5 μM As(III). Changing the pH altered the binding affinity of the transport sites for As and affected membrane permeability (Wells and Richardson, 1985). These facts are important because they help determine the conditions for the most efficient As accumulation.

As(III) is slowly oxidized to As(V) in water. In the present study, experimental data revealed that 14% of As(III) was oxidized to As(V) after 96 h with a negative control. In other words, two As species co-existed in the solutions at the end of the experiment. As(V) and As(III) is transported into plant cells via phosphate transporters and aquaglyceroporin channels, respectively, in some plants (Catarecha et al., 2007; Bienert et al., 2008). Thus, the assumption was that the *C. demersum* plant would take up both As species in this experiment.

### 3.2. Effects of As(III) on the growth of C. demersum

The present study revealed that the growth of *C. demersum* differed significantly among various As(III) doses ($p < 0.001$). A small dose of As(III) (2 μM) induced an increase in biomass production while higher levels (10–20 μM) inhibited plant growth. A concentration of 40 μM was found to be highly toxic to this aquatic plant. After 48 h exposure, plants treated at this concentration exhibited significant necrosis and negative biomass production (Fig. 2).

Toxic chemicals stimulate plant growth at lower doses and exert a toxic effect at higher doses (Cedergreen, 2008), which is called the "hormesis effect" (Southam and Erlich, 1943). In a similar manner, the present study revealed that a lower dose of As(III) stimulated plant growth. However, higher doses negatively affected plant growth. Biomass production may tend to decrease when a high dose of toxicants are applied based on the fact that the cell walls and the cell membrane may be damaged due to the reactive oxygen species formed as a result of stress depending on an increased concentration of exposure (Srivastava et al., 2009).

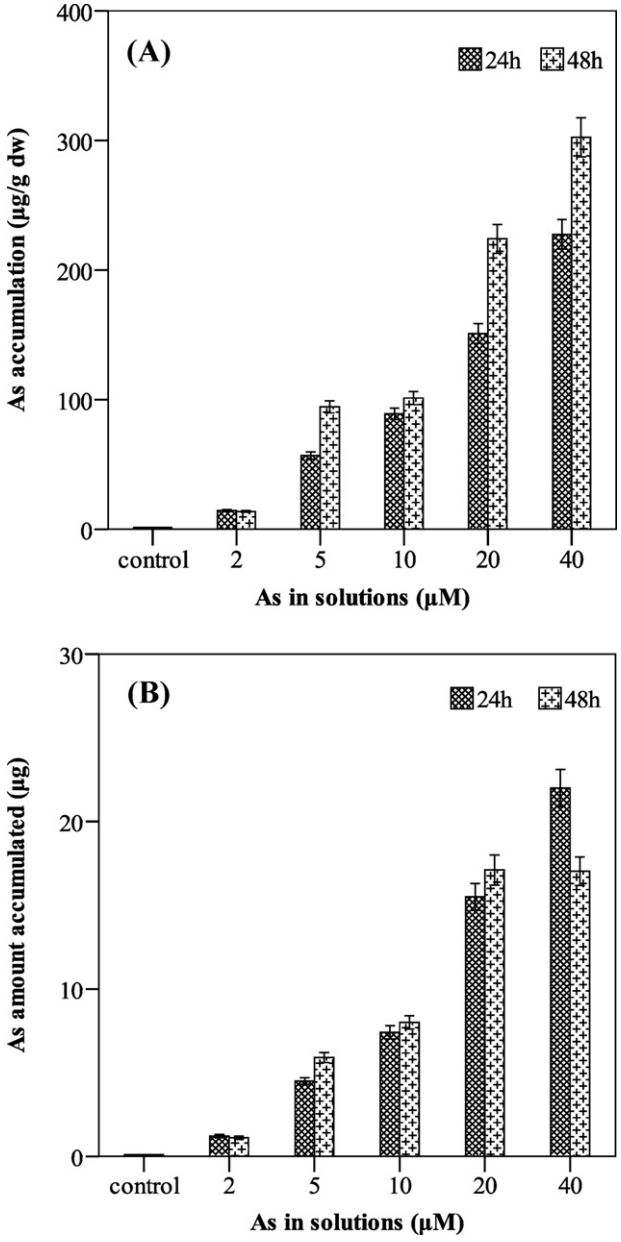



**Fig. 3.** As accumulation of *C. demersum* exposed to different concentrations of As(III) for 24 h and 48 h (A), and As amount removed from solutions by plant biomass after 24 h and 48 h (B). Error bars represent means ± SD ($n = 3$).

### 3.3. Plant As accumulation as a function of As(III) concentrations and duration

In the present study, As accumulation of *C. demersum* (Fig. 3A) depended on the concentration of As(III) and increased with incubation time increased from 24 to 48 h ($p < 0.001$). At 40 μM, plants accumulated 227.5 μg As $g^{-1}$ dw after 24 h and exhibited no visual symptoms of toxicity. However, after 48 h, As concentration reached 302.4 μg $g^{-1}$ and biomass production decreased significantly to a value that was remarkably lower than the control plants (not exposed to As) (Fig. 2) due to necrosis. Plant debris was deposited at the bottoms of the containers. As a result, biomass production was negative, and the intracellular As uptake was lower than that measured after exposure for 24 h (Fig. 3B).

The capability for As accumulation of the aquatic plant *C. demersum* was previously investigated (Mishra et al., 2008). However, the available data for As accumulation by plants exposed to As(III) was

insufficient. As(III) is more common than As(V) in most groundwater (Henke, 2009). In addition, As(III) is supposed to be more phytotoxic than As(V) (Carbonell-Barrachina et al., 1998). Mishra et al. (2008) reported *C. demersum* efficiently tolerated As toxicity at concentrations as high as 50 μM of As(V) for a duration of up to 4 d with no significant effect on growth and at an approximate accumulation of 76 μg As $g^{-1}$ dw. The present study recorded a much higher accumulation. *C. demersum* accumulated up to 302.4 μg As $g^{-1}$ dw after 2 d of incubation with 40 μM of As(III). This could have been due to the presence of phosphate (approximately 0.1 mM) in the nutrient solution. Phosphate ions compete with As(V), but not with As(III) for absorption sites, and as a result, decrease the As(V) uptake by plants.

Substantial amounts of As(III) may have been oxidized to As(V) during 48 h. Our results did show that As(III) could be oxidized to As(V) in the nutrient solution under these laboratory conditions. However, the amounts of As(V) formed during 48 h were insignificant. The experimental data showed that only 8% of As(III) was oxidized to As(V). Therefore, it was assumed that As(III) was the predominant species in the nutrient solution throughout the experiment.

In the present study, the As accumulation by *C. demersum* exposed to As(III) was considerably higher than that found in other aquatic plants such as *E. crassipes*, *L. minor*, *Myriophyllum proinquum*, and *H. verticillata* (Alvarado et al., 2008; Robinson et al., 2006; Srivastava et al., 2007). Since *C. demersum* showed good potential for the accumulation of As and tolerated concentrations of As that were higher than those normally present in contaminated areas (Smedley and Kinniburgh, 2002), this plant may be a useful phytoremediator in aquatic environments contaminated with As.

### 3.4. Fe adsorption and uptake of C. demersum

The aquatic plant *C. demersum* was grown under the following conditions: (i) 10% Hoagland solution additionally supplied with 0.18 mM Fe; (ii) 10% Hoagland solution that contained 5 μM Fe; and (iii) DI water only (Fe free). For plants incubated in nutrient solutions supplied with 2 μM As(III) or As(V), 0.18 mM Fe had no significant effect on growth. Concentrations of Fe found on plant surfaces and in plant tissues were much higher in plants grown in nutrient solutions supplemented with 0.18 mM Fe (2374 μg $g^{-1}$ and 1586 μg $g^{-1}$, respectively) (Fig. 4). *C. demersum* easily adsorbed high concentrations of Fe (present study) as well as Cu, Zn, and Pb (Keskinkan et al., 2004). A small concentration of Fe was also found in the CBE-extract of plants grown in DI water only (Fe free). There are some possible explanations for these results. First, the plant material was grown in nutrient solution for weeks and was acclimatized in a 10% Hoagland solution containing 5 μM Fe for one week before the experiments. This resulted in the formation of an Fe coating on the plant surfaces. Second, the extraction buffer not only removed coatings on the surfaces, but also extracted some elements accumulated in the apoplast of the plants (Otte et al., 1989; Strasser et al., 1999). Therefore, Fe in the apoplast could be extracted by a CBE solution.

### 3.5. Effects of Fe on As adsorption and uptake of C. demersum

#### 3.5.1. As concentrations in CBE-extracts

As concentrations in CBE-extracts of Fe-supplemented treatments were significantly higher than those in control treatments when As(III) or As(V) was added (Fig. 5A). CBE–As concentrations were increased approximately 300% by the addition of Fe. Thus, it could be assumed that Fe oxides/hydroxides coating the plant surfaces might facilitate the co-precipitation of As. The As species supplied to the plants grown in nutrient solution or in DI water only had little effect on CBE–As concentrations.

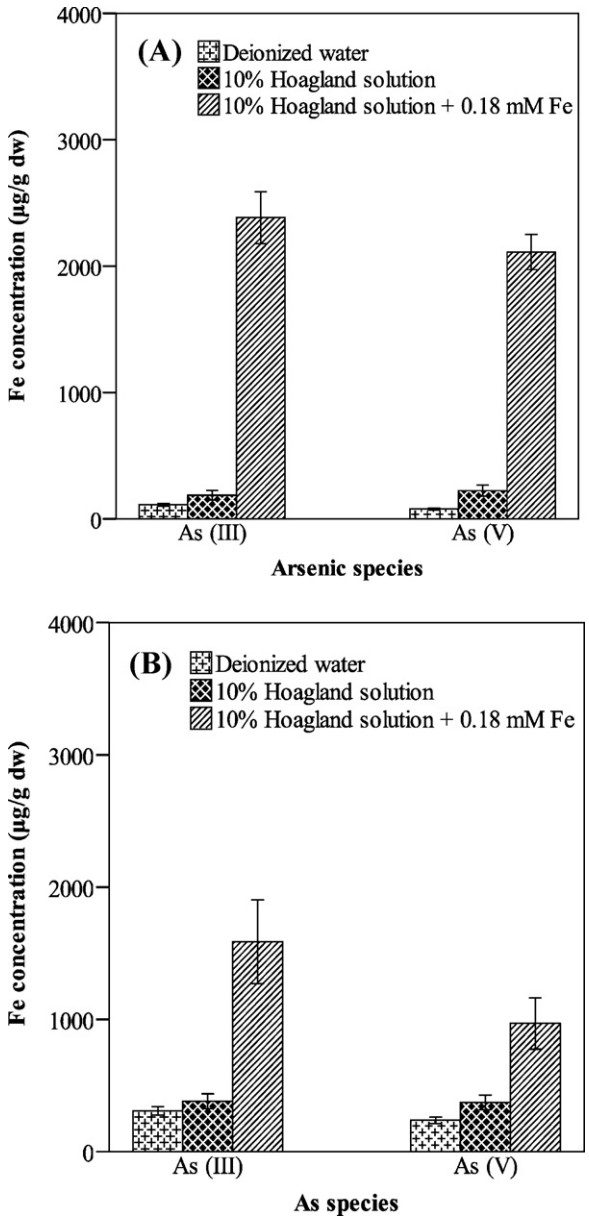



**Fig. 4.** Fe concentrations in CBE-extracts (A) and plant tissues (B) of *C. demersum* exposed to solutions containing different levels of Fe for 168 h. Error bars represent means ± SD ($n = 3$).

Though the As adsorption on the Fe plaque of plant roots has been reported and discussed in many studies (Blute et al., 2004; Liu et al., 2005; Otte et al., 1995; Rahman et al., 2008a,b), neither the formation of Fe plaque on the surfaces of rootless submerged plants nor its influences on the accumulation of As has been widely investigated. Robinson et al. (2006) described the physicochemical adsorption as an alternative mechanism of As accumulation in aquatic plants. In this mechanism, Fe oxides/hydroxides deposited on the plant surfaces adsorb and accumulate As. The precipitation of Fe oxides/hydroxides and formation of plaque on the plant surfaces was likely the result of radial oxygen diffusion and the subsequent oxidation of ferrous ion (Hansel et al., 2002). The present study has elucidated the role of Fe in As accumulation by the aquatic macrophyte *C. demersum*.

#### 3.5.2. As concentrations in plant tissues

The addition of Fe and As species significantly affected As uptake by *C. demersum* (Fig. 5B). As concentrations in the tissues of plants

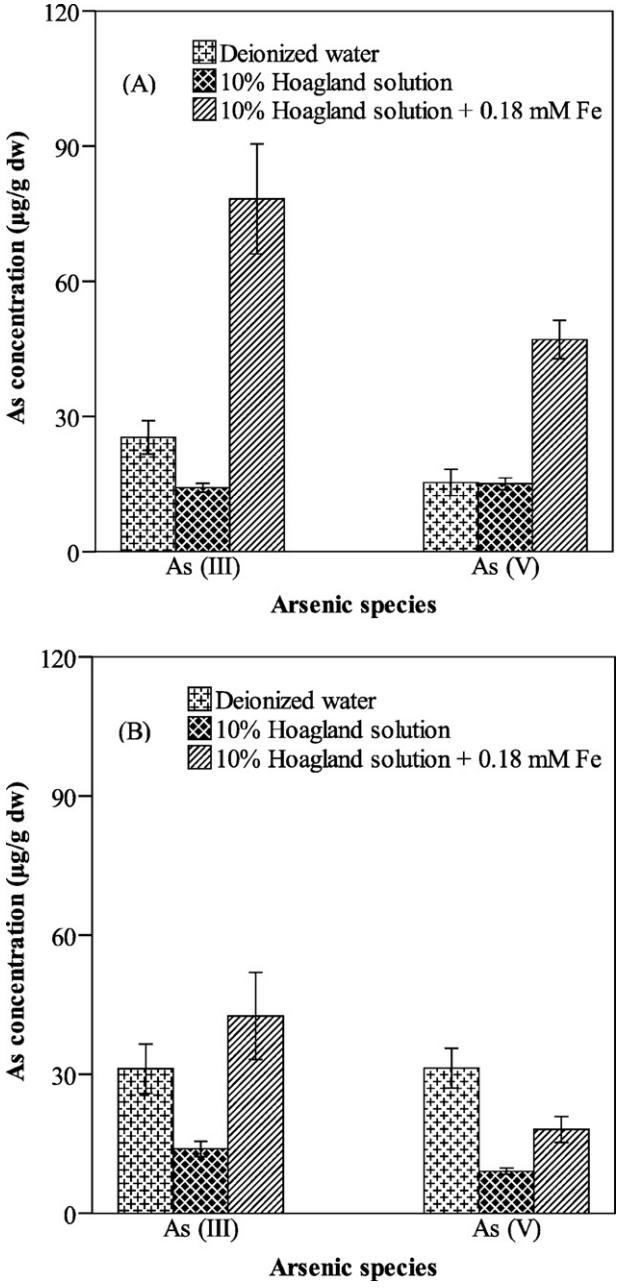



**Fig. 5.** As concentrations in CBE-extracts (A) and plant tissues (B) of *C. demersum* exposed to 2 μM As(III) or As(V) for 168 h in solutions containing different levels of Fe. Error bars represent means ± SD ($n = 3$).

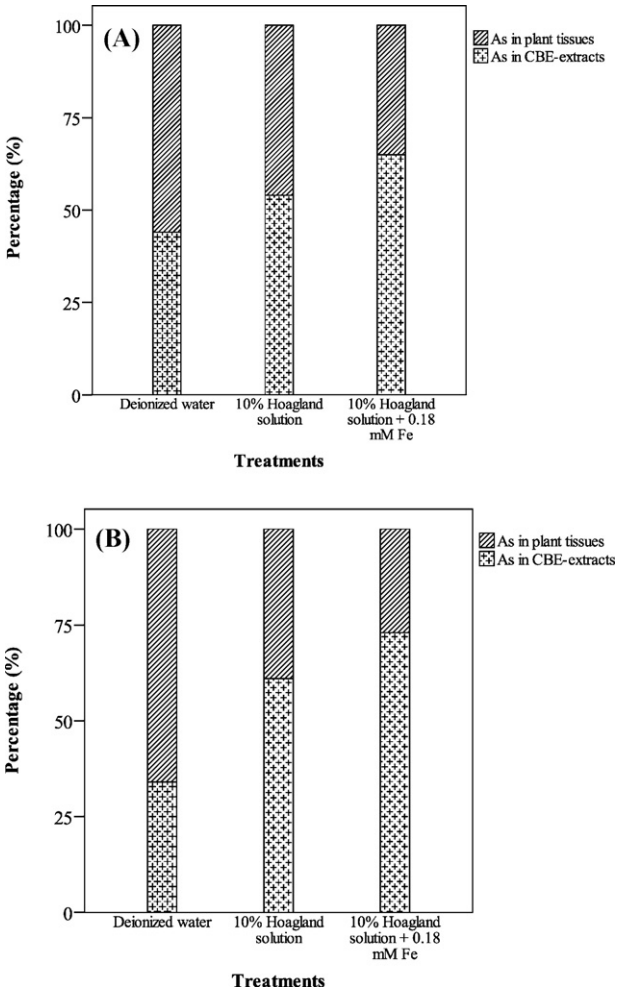



**Fig. 6.** Proportions of As in CBE-extracts to As in plant tissues of *C. demersum* exposed to 2 μM As(III) or As(V) for 168 h. (A): As(III)-exposed plants; and (B): As(V)-exposed plants.

grown in nutrient solutions were lower when As(V) was supplied than when As(III) was supplied, particularly with the addition of Fe into the nutrient solutions ($p < 0.01$). There were no differences in concentrations of tissue-As between As(III) and As(V) treatments in control plants (DI water) ($p > 0.05$). Upon exposure to both forms of the metalloid, As uptake by *C. demersum* was significantly higher in DI water than that in nutrient solution ($p < 0.001$). Furthermore, our results revealed that for plants with Fe added, both As(III) and As(V) accumulation were different from those plants in control treatments-the uptake of As(III) was enhanced by the presence of Fe, but that of As(V) was considerably inhibited.

The present study also demonstrated that an Fe coating on the plant surfaces of *C. demersum* lowers the As accumulation in plant cells. Fe-supplemented treatments resulted in the lowest proportions of As accumulated in plant tissues-35 and 27% for As(III) and As(V), respectively. These results indicated that most of the As was adsorbed onto the plant surfaces when 2 species of the metalloid were supplied. In Fe-free incubation solutions, a larger proportion of the As uptake (approximately 66%) was intracellular (Fig. 6A and B).

Fe plaque played different roles in As(III) and As(V) uptake, which was probably caused by the different affinities of As(III) and As(V) adsorption by Fe plaque (Chen et al., 2005). Fe hydroxides reportedly have a much higher affinity for As(V) than As(III) (Meng et al., 2002). Therefore, As(V) associated with Fe plaque may be largely "locked up". In other words, Fe plaque may act as a "barrier" for As(V), leading to a lower influx into plant tissues. Besides, Fe plaque not only adsorbs As(V) but also sequesters As(III) (Dixit and Hering, 2003; Hansel et al., 2002). However, Fe plaque is not a "barrier" to As(III) uptake (Liu et al., 2005). In our experiment, the pH of the nutrient solutions was 6.0. Thus, the predominant species of As(III) would be $H_3AsO_3$ (uncharged). The transport of this As species into plant tissues might be via aquaglyceroporin channels. In this case, the effects of Fe plaque on As(III) uptake would be small compared to that on As(V) uptake.

Nutrients were found to affect As accumulation by As hyperaccumulator *Pteris vittata* L. (Fayiga et al., 2008). The present study also suggested that dissolved elements in a nutrient solution may have substantial effects on As uptake by *C. demersum*. This was shown by the significant decrease in As uptake when the plants were grown in nutrient solutions, compared to those grown in DI

water only (Fig. 5B). Obviously, other dissolved ions in the nutrient solution posed certain effects on As accumulation by this aquatic plant. This could be explained by the competition for adsorption and transport sites between As and other elements in a given solution.

## 4. Conclusion

Our results demonstrated that As accumulation by the aquatic macrophyte *C. demersum* was decreased with an increase in solution pH. The plant tolerated higher concentrations of As than those normally present in contaminated areas. In addition, an Fe coating on the plant surfaces and other dissolved elements in the nutrient solution were found to pose significant effects on plant As uptake. This information is important when defining strategies for the most efficient As removal in a real treatment situation. In view of their fast growth, high biomass production, and considerable As accumulation efficiency, *C. demersum* has potential for use in the phytoremediation of aquatic environments that are contaminated with As.

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