Phytofiltration of arsenic and cadmium by using an aquatic plant, *Micranthemum umbrosum*: Phytotoxicity, uptake kinetics, and mechanism

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Abstract

Arsenic (As) and cadmium (Cd) are noxious and carcinogenic pollutants that can be removed from water by using emerging, ecofriendly, phytofiltration technology that employs *Micranthemum umbrosum*. After culturing *M. umbrosum* for 7 days in a hydroponic experiment, accumulation of 1219±44.11 µg As g^{-1} and 799.40±30.95 µg Cd g^{-1} were observed in the leaves, from 1000 µg As L^{-1} and 1000 µg Cd L^{-1} of water, respectively. Plant and water samples were analyzed for assessing the As and Cd accumulations, translocations, phytotoxic effects, uptake mechanisms and kinetics, and for evaluating the potential of *M. umbrosum* in As and Cd phytofiltration. The uptake pattern was leaf > stem > root for both pollutants. The plant showed higher resistance to As than to that to Cd. Uptake of inorganic As species was much greater than that of organic As and was found at above the substrate concentration. However, Cd showed similar uptake pattern to that of inorganic As species, and the data was better fit to a non-linear than a linear model. Low molecular weight substances that have thiol group(s) may be responsible for the binding of As in plants whereas Cd showed a different mechanism to that of As. *M. umbrosum* showed good As phytofiltration capabilities without any phytotoxic effects, but it was found to be a moderate accumulator of Cd with some phytotoxic effect compare to some other previously studied plant.

Key words: arsenic, cadmium, *Micranthemum umbrosum*, phytotoxic, phytofiltration, uptake.

1. Introduction

Arsenic (As) and cadmium (Cd) are classified as group 1 carcinogenic compounds to human (IARC, 2012). In some areas of Bangladesh and India, As concentration in groundwater has exceeded 2000 µg L^{-1} (British Geological Survey, 2000; Hossain, 2006). According to the British Geological survey (2000), 51% of tube well water samples collected from 41 out of 64 districts contained more than 10 µg L^{-1} of As, 35% were above 50 µg L^{-1}, 25% were above 100 µg L^{-1}, 8.4% were above 300 µg L^{-1} and 0.1% were above 1000 µg L^{-1}, whereas the permissible limit of World Health Organization (WHO) for As in drinking water is 10 µg L^{-1} (WHO, 2011), and the national standard for drinking water in Bangladesh is 50 µg L^{-1} (World Bank, 2005). There are different forms of As that exist in the environment e.g., inorganic (arsine, arsenious acid, arsenite, arsenic acids or arsenate), organic (monomethyl arsonic acid [MMAA] and dimethylarsinic acid [DMAA]), biological, and other forms (Rahman and Hasegawa, 2011). Arsenic toxicity depends on the As species; and generally inorganic As species (arsenite and arsenate) are more toxic as compared with organic As species (Meharg and Hartley-Whiteker, 2002; Ng, 2005). The toxicity level of the various As species is As (III)>As(V)>DMAA>MMAA (Patrick et al., 2000).

Cadmium is a widespread toxic heavy metal that is mainly released into the environment by paints and pigments, plastic stabilizers, electroplating, incineration of cadmium-coated plastics, by-products of cement, and phosphate fertilizer factories (Sanita di Toppi and Gabbrielli, 1999; Salem et al., 2000; Pulford and Watson, 2003). It causes carcinogenic, mutagenesis, interferes with calcium regulation in biological systems, renal failure, and chronic anemia (Degraeve, 1981; Salem et al., 2000). More than 30 µg L^{-1} Cd was recorded in the drinking water, though the recommended Cd

level in drinking water is only 3 µg L^{-1} (WHO, 2011).

Therefore, the removal of As and Cd from contaminated water has been of the utmost importance in order to minimize their impacts on ecosystems. Although different physical, chemical, and biological approaches have been employed for this purpose, one novel approach is phytofiltration, which has been proposed as a promising, environment friendly, esthetically pleasant technology by using live plants to remove As and Cd from contaminated water (Ali et al., 2013). There are some plants that can accumulate As and Cd in their harvestable parts. *Pteris vittata* L. (Ma et al., 2001), *Wolffia globosa* (Zhang et al., 2009), *Spirodela polyrhiza* L. (Rahman et al., 2007a), *Lemna gibba* L. (Mkandawire and Dudel, 2005), *Polygonum hydropiper* (Robinson et al., 2005), and *Azolla caroliniana* (Zhang et al., 2008) were identified for As accumulators. *Limnocharis flava* L. (Abhilash et al., 2009), *Nymphae aurora* (Schor-Fumbarov et al., 2003), *Solanum nigrum* L. (Sun et al., 2007), *Thlaspi caerulescens* (Zhao et al., 2003), and *Arabidopsis halleri* (Küpper et al., 2000) were found to be Cd accumulators.

However, only few aquatic plants were known to be applied removal of heavy metals from water body, although aquatic plants could be expected to remedy heavy metals from surface water. *M. umbrosum*, commonly called the Water fern or Baby`s tears, is one of them that has been identified as an As hyperaccumulator because of its high bio-concentration factors (>1000) and translocation factor (>1.0) and also a moderate Cd accumulator at low concentrations, and it can remove 79.3–89.5% As and 60.0–73.1% Cd from 0 to 1.0 µg As mL^{-1} and 0.3 to 30.0 µg Cd mL^{-1} solutions, respectively (Islam et al., 2013). The precise mechanism of heavy metal removal in *M. umbrosum* could not be clarified yet. Plants exposed to heavy metals showed tolerance and hyper accumulation by adjusting and/or altering some physiological mechanism

depending on the type of pollutant, dose intensity and plant species; for example, Cd caused increased synthesis of phytochelatins (PCs), but reduced the synthesis of ascorbate and antioxidant enzymes in *Pistia stratiotes* L. than in *Eichhornia crassipes* (Mart.) Solms (Sanita di Toppi et al., 2007), whereas Cd compartmentalization occurred in the epidermal vacuoles of *Thlaspi caerulescens* leaves (Küpper et al., 2004). In the case of As, main route of As (V) uptake within plant is through phosphate transporter (Asher and Reay, 1979; Meharg et al., 1994) and As (III) is through aquaglyceroporins (Meharg and Jardine, 2003; Isayenkov and Maathuis, 2008; Ma et al., 2008). Arsenic substantially increases the synthesis of glutathione (GSH) and PCs (Schat et al., 2002; Grill et al., 2006). Raab et al. (2005) identified 14 different As complexes, including PCs, in *Helianthus annuus* L., but As appears to be present in its unbound inorganic form in *P. vittata* hyperaccumulator (Rabb et al., 2004; Pickering et al., 2006). These phenomena suggest that As and Cd hyper accumulation occurred through different mechanisms in various plant species. *M. umbrosum* plant have the tendency to absorb As and Cd (Islam et al., 2013). However, interestingly, the current study showed that this plant has different uptake mechanisms with different As and Cd doses exposures and different response to growth with respect to photopigment production and macro and micronutrient uptake.

Thus, in the present study, we evaluated the potential of *M. umbrosum* for the phytofiltration of As and Cd from contaminated water by investigating the phytotoxic effects, effects on macro and micro nutrient uptake, uptake kinetics, and possible uptake mechanisms that these two carcinogenic elements showed in this plant.

2. Materials and Methods

2.1 Plant culture

Micranthemum umbrosum was obtained from Aqua Friend Hokusui (Hokkaido, Japan). Initially, the plants were grown in hydroponic cultures in laboratory conditions for seven days to allow for adaptation. Then about 3.5 g (fresh weight) of *M. umbrosum* were grown in glass pots in Milli-Q water (Millipore-Gradient A10, Milli-Q Gradient ZMQG) containing 0, 200, 500, 1000 µg As L^{-1} [from sodium (meta) arsenite, $NaAsO_2$], and 0, 300, 1000 µg Cd L^{-1} from $CdCl_2.2.5H_2O$ with 500 mL Hoagland nutrient solution (Hoagland and Arnon, 1950) as a nutrient source. The pH of the solution was adjusted to 6.0 by adding KOH or HCl. The experiments were carried out for 7 days in a growth chamber under a controlled environment at the following conditions: 14L:10D light/dark cycle, 100-125 µmol.m^{-2}.s^{-1} light intensity, 75% humidity and 21 ± 1ºC temperature. Three replications were done in all cases and a control was maintained both for the metal and the plant.

2.2 Sampling and photopigment analysis

Leaf sampling were done at 0, 4, and 7 day intervals and immediately used for the estimation of chlorophyll a, chlorophyll b, total chlorophyll, carotenoids and anthocyanine contents. Chlorophyll and carotenoid contents in leaves were extracted using 80% chilled acetone, contents of these were estimated using the equation given by Lichtenthaler and Wellburn (1983). Anthocyanine content was also estimated in leaves as described by Sims and Gamon (2002). Two milliliters water samples were collected from each pot at 24 h intervals to measure the As and Cd status in the water. After 7 days, whole plants were harvested and rinsed with Milli-Q water three times to remove any apoplastic As and Cd, then kept in clean absorbent paper to remove the remaining water from the surface. Final fresh weight was taken on a digital balance (A&D Co. Ltd,

Japan, HF-200, Max 210 g, d = 0.001 g). Then, the whole plants were separated into leaves, stems and roots for analysis of As, Cd, potassium (K), calcium (Ca), magnesium (Mg), manganese (Mn), and zinc (Zn) contents.

2.3 Sample preparation and chemical analysis

Separated leaf, stem, and root samples were kept for 24 h for air drying at room temperature on absorbent paper. Then, most of the samples (some of 0 and 1000 µg L^{-1} As and Cd treated leaf were kept fresh for amino acid and thiol content analyses) were oven dried at 65ºC (Constant Temperature Oven, DKN602, Yamato Scientific Co. Ltd., Japan) for at least 48 h until they reached a constant weight. After grinding the samples, 25-40 mg samples of roots, stems, or leaves were separately placed into 15 mL polyethylene tubes (Thermo Fisher Scientific, NY, USA). Two milliliters of 65% HNO_3 (Wako Pure Chemical Ind. Ltd., Japan) were added, and the samples were kept under the fume hood for 12 h. Then, the samples were covered and heated on a heating block (TAH-2G, Dry Thermo Unit, Japan) at 95ºC for 2 h to digest. After cooling, 1.0 mL of 30% H_2O_2 (Wako Pure Chemical Ind. Ltd., Japan) was added and the samples were covered and heated again at 105ºC for 20 min (Rahman et al., 2007a). Digested samples were diluted up to 10 mL with Milli-Q water using 10 mL volumetric flasks (Pyrex, IWAKI Glass), as described by Cai et al. (2000) and Islam et al. (2013). The diluted samples were then filtered using a 0.45-µm syringe-driven filter unit (Millipore, Billerica, USA) and stored in 15 mL polyethylene bottles. As, Mg, Mn and Zn contents were measured using an inductively coupled plasma-mass spectrophotometer (ICP-MS; Agilent G1820 Model), whereas Cd, K and Ca contents were measured by a flame-type atomic absorption spectrophotometer (AAS; Model 180–80, Hitachi, Japan). The accuracy of the analysis was checked using certified standard reference materials for As

(013–15481, Lot ALK 9912, 1000 mg L^{-1}) and Cd (036–16171, Lot TSP9842, 1000 mg L^{-1}) obtained from Wako Pure Chemical Ind. Ltd., Japan.

2.4 Uptake kinetics of inorganic and organic As species and Cd

Approximately 3.5 g of fresh plant (whole) were cultured in 0, 200, 500, 1000 µg As L^{-1} [each from sodium (meta) arsenite ($NaAsO_2$, Sigma-Aldrich, India), DMAA ($C_2H_7AsO_2$, TCI, Tokyo, Japan), and MMAA (CH_5AsO_3, Wako Pure Chemical Ind. Ltd., Japan)], and 0, 300, 1000 µg Cd L^{-1} (from $CdCl_2.2.5H_2O$, Wako Pure Chemical Ind. Ltd., Japan) contaminated water with Hoagland nutrient solution (Hoagland and Arnon, 1950) for 24 h. Then plant samples were collected and washed with ice-cold deionized water to remove As and Cd from plant`s surface (Irtelli and Navari-Izzo, 2008). Water samples were also collected for each pot to determine the As and Cd absorption by the plants after the first 24 h. Kinetics parameters were measured by fitting data to the Michaelis-Menten function. MMAA and DMAA were only used for the uptake kinetics study, so no data for MMAA and DMAA, other than uptake kinetics, are shown in this manuscript.

2.5 Separation and quantification of thiol containing peptides

Arsenic- and Cd-treated (1000 µg L^{-1}) leaf samples (500 mg fresh wt) were homogenized in a stirrer (Yamato Labo-Stirrer, L-35, Japan) in 5 mL of ice cold 50 mM Tris-Cl (Promega, USA and Wako Pure Chemical Ind. Ltd., Japan) buffer, pH 7.4, containing 0.1% sodium dodecyl sulfate (SDS; Sigma, USA). Homogenized samples were then centrifuged (Kubota 6200 Centrifuger, Japan) at 10000 × g for 30 min at 4ºC. The obtained supernatants were filtered through a 0.45-µm syringe-driven filter unit (Millipore, Billerica, USA) and immediately 3.0 mL of supernatant was on a Gel filtration column chromatography equipped with a column (1.1 × 110 cm^2) containing

Sephadex G-50 (Pharmacia, Sweden). The chromatography was carried out in the presence of 50 mM Tris-Cl buffer pH 7.4 (Schmoger et al., 2000) and eluted at a flow rate of 2.5 mL/min. Sixty fractions of the eluate (2 mL fraction size) were measured for As (using ICP-MS) or Cd (by AAS) and absorbance at 280 nm of each fraction using a UV/VIS spectrophotometer (Beckman, DU-65 Spectrophotometer, USA) for protein quantification (Walker, 1996). Thiol-containing peptides (GSH, PCs and related peptides) were detected at 412 nm after post column derivatization with Ellman`s reagent (5-5´dithio(2-nitrobenzoic acid); Sanita di Toppi et al., 2007). For Cd-treated leaves, we again concentrated the Cd containing fractions and applied them to a Gel filtration column chromatography equipped with a column (1.1 × 110 cm^2) containing Sephadex G-15 (Pharmacia, Sweden). The chromatography was carried out in the presence of 50 mM Tris-Cl solution and eluted at a flow rate of 0.8 mL/min (Schmoger et al., 2000). Sixty fractions of the eluate (2 mL fraction size) were tested for Cd (by AAS), and Cd containing fractions were separated out. After drying these samples using a SpeedVac Concentrator SVC100H (Savant, USA), performic acid oxidation (Walker, 1996) was performed using 1.5 mL performic acid (HCOOH:H_2O_2 = 9:1) at 6ºC for 24 h. Again, samples were dried and analyzed for cysteine or cysteic acid and other amino acids using a High Performance Amino Acid Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

2.6 Statistical analysis

Results were expressed as the means ± standard error of mean (SEM) of three replicates. Significance degree was calculated using a *t*-test and curve fitting was done using the computer package Microsoft Excel program (Microsoft Office 2007 Professional).

3. Results

3.1 Arsenic and Cd contents in each parts of the plant and growth medium

The concentrations of the metals studied in the different parts of *M. umbrosum* and the water are depicted in Figs. 1, 2. As and Cd concentrations in the plant increased significantly with increasing added As and Cd levels in hydroponic solution (Figs. 1a, 1b). The maximum accumulation of As (about 1220 µg g^{-1}) and Cd (800 µg g^{-1}) were found in leaves at 1000 µg L^{-1} treatment (Figs. 1a, 1b). Arsenic accumulation pattern was root < stem < leaf, and translocations of As from contaminated water to root, root to stem and stem to leaf were significant ($p < 0.01$ and 0.05) in almost all cases (Fig. 1a). Cd uptake occurred significantly from Cd-tainted water but translocation was not significant from root to stem to leaf (Fig. 1b).

Arsenic and Cd concentration remaining in the solution at each day after culturing *M. umbrosum* for 7 days are shown in Fig. 2. It was indicated that As and Cd concentrations decreased significantly up to the 5^{th} day (Fig. 2a) and 4^{th} day (Fig. 2b), respectively. After 7 days, total As and Cd concentration remaining in the solution was below 50 µg L^{-1} (Fig. 2a) and 100 µg L^{-1} (Fig. 2b) from about 500 and 310 µg L^{-1}, respectively, and the accumulation pattern was leaf > stem > root for both pollutants.

3.2 Phytotoxicity of As and Cd on *M. umbrosum*

Phytotoxicity of As and Cd on *M. umbrosum* was evaluated in response to measuring the final fresh weight and contents of the photosynthetic pigments like chlorophyll a, chlorophyll b, total chlorophyll, carotenoids and anthocyanin. As shown in Supplementary Fig. 1a, the plant growth increased significantly ($p < 0.01$) with increasing As concentration in the growth medium (up to 500 µg As L^{-1}). Similarly total chlorophyll (Supplementary Fig. 2c) and anthocyanin (Supplementary Fig. 2e) content in leaves increased significantly up to 500 µg As L^{-1}. Contents of chlorophyll a

(Supplementary Fig. 2a) and carotenoids (Supplementary Fig. 2d) were observed to increase for the entire sampling period, as compared with their controls. However, in the case of chlorophyll b, it increased significantly up to 4 days but later increases were not significant as compared with the controls (Supplementary Fig. 2b).

On the other hand, growth was significantly inhibited ($p < 0.05$) with elevated levels of Cd concentration in the hydroponic medium (Supplementary Fig. 1b) because of decreases in the photosynthetic pigments (Supplementary Figs. 3a-e).

3.3 Macro and micro elemental compositions of *M. umbrosum*

The concentrations of essential macro-(K, Ca and Mg) and micro-(Mn and Zn) nutrient elements in the plant parts were examined after 7 days to find out the effect of As and Cd on the uptake process of these mineral nutrient elements and determine the implications for water management (fertilization in particular) of *M. umbrosum* in As and Cd phytofiltration practices.

The present study showed that treatment of *M. umbrosum* with As supply increased K and Mg concentrations in the stems and leaves, respectively, up to 500 µg As L^{-1} treatment, later on, decreases were seen compared to the control (Supplementary Tables 1, 2); however As accumulation negatively influenced Ca accumulation in the leaves (Supplementary Table 1). Enhanced Cd treatment decreased the Ca and Mg contents in the roots and leaves whereas K content increased in roots (Supplementary Table 2).

Mn showed negative correlation with As accumulation (Supplementary Table 1), but significant positive correlation (Supplementary Table 2) on Cd uptake within *M. umbrosum* as compared with the control. Zn concentration in leaves, ranging from around 50 to 70 µg g^{-1} for As and 65 to 70 µg g^{-1} for Cd treatment, was significantly decreased by As and Cd treatment.

3.4 Arsenic and Cd uptake kinetics

The long term (24 h) concentration-dependent As (Inorganic arsenite, organic MMAA and DMAA) and Cd influx isotherm exhibited a hyperbolic pattern in relation to the external concentration (Figs. 3a, 3b) and the data fit moderately well for arsenite and Cd, but not for MMAA and DMAA, using *Michaelis-Menten* equation for linear regression model (Supplementary Table 3). However, uptake kinetics data were described satisfactorily by the *Michaelis-Menten* equation using non-linear curve fitting by applying the natural logarithm of As and Cd influx within *M. umbrosum* (Fig. 3c, 3d) rather than the linear one (Table 1 and Supplementary Table 3). The V_{max} for inorganic arsenite (403.43 µg g^{-1} DW 24 h^{-1}) was about 5 to 9 times greater than that of MMAA and DMAA (Table 1), and marginally more than Cd (365.04 µg g^{-1} DW 24 h^{-1}). However V_{max} values were almost similar using non-linear and linear regression models but the K_m values were quite different from each other.

3.5 Arsenic and Cd uptake mechanism

After Sephadex G-50 gel filtration of leaf extract from the plant treated with 1000 µg L^{-1} As and Cd, the maximum amounts of As (Fig. 4a) and Cd (Fig. 5a) were found at 90-108 and 80-104 mL of eluents, respectively. Absorbances at 280 nm were also counted for each fraction because proteins give the maximum absorbance at this wavelength. According to Fig. 4a, small amounts of As were scarcely observed in the high molecular weight fraction, whereas Cd-binding substance(s) might be those other than proteins, as they gave different peaks (Fig. 5a) in the G-50 column. Later each fraction was treated with Ellman's reagent and SH contents were measured by recording the absorbance at 480 nm (Ellman, 1959). Data represented in Fig. 4b show that the maximum amount of thiol (9 µM) was found in the fraction having the maximum

amount of As. On the contrary, control leaf eluent samples have lower thiol content (2.8 µM) when treated with the 0 mg As L^{-1} solution (Fig. 4c). In the case of Cd, no thiol formation occurred in high Cd-containing eluents (Fig. 5b). For confirmation of whether Cd formed any substances having thiol compounds or not, we again purified the eluents containing high levels of Cd through a Sephadex G-15 gel filtration column and after performic acid treatment we measured the cysteic acid/cysteine (thiol containing peptides) and other amino acids. The data in Supplementary Table 4 indicate that only 0.95 nmol of cysteic acid which is 2.13% of the total amino acids were present in the eluents containing high levels of Cd. This means that Cd might follow different uptake mechanism than that for As.

3.6 Phytofiltration potential

M. umbrosum shows one of the effective phytofiltrator of As and moderate accumulator for Cd compare to other previously studied plant species (Supplementary Table 5), and it can decrease the total As concentration from about 500 and 200 to 40 and 25 µg L^{-1} (Fig 2a), respectively, without showing any phytotoxic effects. This are ranges below the national standard for drinking water in Bangladesh and China, which is 50 µg L^{-1} (World Bank, 2005). However, while Cd concentration in the solution could be lowered from around 1040 and 310 to 415 and 80 µg L^{-1} (Fig 2b), respectively, evident phytotoxic effects were seen. As and Cd concentration remained stable (205-220 and 1000-1017 µg L^{-1} for As and Cd, respectively) in the control treatment without plants for the entire experimental period. As and Cd uptake kinetic data also showed that *M. umbrosum* is a better accumulator of inorganic As than Cd and organic As at lower substrate concentration. Arsenic uptake involved the thiol formation mechanism, which was different from the Cd uptake mechanism in *M. umbrosum*.

4. Discussions

In this study, *M. umbrosum* was shown to be a strong accumulator of As, because it accumulated more than 1000 µg As g^{-1} in stem and leaf biomasses and also reduced the As concentration in the solution of about 10-fold, whereas other plants used for phytofiltration, such as *W. globosa*, decreased the total As concentration in the solution from 200 to 116 µg L^{-1} within the first 48 h, but no further decrease in As concentration was noted (Zhang et al., 2009). However, *M. umbrosum* was shown to be a moderate accumulator of Cd based on the <1000 µg Cd g^{-1} root and shoot biomass uptake that was observed. The As accumulation capacity of *M. umbrosum* is much higher than any non-hyper accumulator species, which suffer from As phytotoxicity when tissue As concentration exceeds 10-100 µg g^{-1} (Kabata-Pendias and Pendias, 1992). Cadmium-treated plant roots contained relatively higher concentrations of Cd than the As concentrations in the roots of As-treated plants; however, aerial part (stem and leaf combinedly) contained higher amount of Cd than root. These results were consistent with the studies of Abhilash et al. (2009), who reported that *Lemna flava* roots contained higher amount of Cd than the peduncle and leaf separately, but that the total Cd concentration in the aerial parts was higher than in the roots.

Accumulations of As and Cd within *M. umbrosum* were significant up to the 5^{th} and the 4^{th} day, respectively, since *M. umbrosum* can grown in submerged conditions and the whole plant can act as an active site for As and Cd absorption as there is no evidence of Fe-oxide deposition or physiochemical adsorption of these pollutants by this plants or glass pot as described by Robinson et al. (2006).

There is a co-relation between plant growth and photosynthetic pigments. The final fresh weight of *M. umbrosum* showed that As initially enhanced the growth significantly

($p < 0.01$) up to the concentration of 500 µg L^{-1} in hydroponic culture due to the increase in photosynthetic pigments, but at higher concentration, the growth of the plant was reduced. Therefore it appeared that low-level As concentration stimulated plant growth. Mirza et al. (2010) also reported that growth and total chlorophyll contents of *Arundo donax* L. increased in solutions up to the 600 µg As L^{-1}, with subsequent decreases at 1000 µg L^{-1}, and Rahman et al. (2007b) found that chlorophyll contents and rice plant growth decreased with increasing As concentration in the soil. On the other hand, the final fresh weight was inversely characterized by Cd treatment due to the significant reduction in chlorophylls a and b and other pigments, which resulted in the development of some toxic symptoms, such as the yellowing of leaves and necrotic leaf margin. Stobart et al. (1985) also concluded that Cd inhibited the formation of chlorophylls by interfering with protochlorophyllide reduction and the synthesis of aminoevulinic acid in barley. Heavy metals generate reactive oxygen species, which damage photosynthetic pigments, in the plants grown under stress conditions (Romero-Puertas et al., 2002). Here in Cd treated leaves, though chlorophyll a and b ratio increases but total chlorophyll contents decreases compare to control indicated the plant showing Cd stress. Delfine et al. (1999) also found that chlorophyll a and b ratio increases and decrease in total chlorophyll contents in salt stressed spinach leaves after certain periods. Thus, the increased chlorophyll a and carotenoid levels in the As-treated leaves is probably a part of a strategy adopted by the plant to counteract the toxic effects of the free radicals generated under As stress; a finding that agrees with other reports on other aquatic plants (Aslan et al., 2003).

Arsenic supply significantly increased K and Mg contents in shoot parts. K in plants is preferentially transported to young meristematic tissues and has close relationships with

protein synthesis, cytokinin supply and plant growth (Mengel and Kirkby, 1987) whereas Mg serves as a core element of the chlorophyll molecule (Jones, 1998). Therefore K and Mg concentration in the shoots of *M. umbrosum* had a significant relationship with plant biomass production up to 500 µg As L^{-1} treatment. Carbonell et al. (1998) also reported that inorganic As increases K concentration in *Spartina alterniflora* Loisel shoots and a reduction in total dry biomass at a high level of As. On the other hand, K also serves as a dominant cation for counter balancing anions in plants (Marschner, 1995). Therefore, enhanced As uptake in *M. umbrosum* results in an increase in K concentration to balance the excessive anion presence caused by As hyperaccumulation. Tu and Ma (2005) also found that K might function as a counter cation for As hyperaccumulation in *P. vittata*. Increasing Mg concentration in *M. umbrosum* might indicate the increasing chlorophyll contents and enhanced plant growth in As treatment and vice versa for Cd. Ca is an essential macronutrient element for plants (Jones, 1998), and its concentration decreases with increased As or Cd concentration in the growth medium of *M. umbrosum*. This may suggest that Ca has a limited role in the defense mechanism of the plant against As and Cd toxicity. Tu and Ma (2005) also reported that Ca concentration decreased with increasing As in the fronds of *P. vittata*, an As hyperaccumulator. Micronutrient (Mn and Zn) concentrations were higher in Cd-treated leaves than As treated leaves. Mn and Zn concentrations significantly decrease with increased As level compare to respective measurement in the control. Carbonell-Barrachina et al. (1997) showed that As caused a reduction in micronutrient content (B, Cu, Mn, and Zn) in tomato plants (*Lycopersicum esculentum* Mill). Increasing micronutrients concentration in the Cd-stressed plants may be related to a “concentration effect”, since biomass decreased with elevated doses of Cd in the

hydroponic solution.

The long term concentration-dependent arsenite and Cd uptake influx were linear up to the 500 µg L^{-1} treatment. Decreased As and Cd influx after this level is probably due to toxicological inhibition. Meharg and Jardine (2003) reported that the time dependent uptake of 0.1 mM arsenite in excised rice roots showed linear influx up to 30 min, and no further influx thereafter due to toxicological inhibition. V_{max} and K_m (Michaelis-Menten parameter) were calculated from these concentration-dependent experiments. The V_{max} value of arsenite, MMAA, DMAA and Cd were almost similar in order of magnitude by using linear and non-linear models, but the data fitted better to the non-linear model than linear model. Different K_m values resulted from the different calculation methods, for example using natural logarithm (*ln*) to fit the data in the case of the non-linear model. However, the higher maximum K_m value for Cd (around 480 µg L^{-1}) than As (around 455 µg L^{-1}) indicated that *M. umbrosum* has higher affinity to uptake As (V_{max} = 420 µg g^{-1} DW 24 h^{-1}) than Cd (V_{max} = 390 µg g^{-1} DW 24 h^{-1}). At a slow rate, MMAA uptake showed hyperbolic curve and the limited uptake of DMAA occurred because to aerial organ contain a smaller concentration of this species and its translocation from root to shoot is restricted (Odanaka et al., 1987; Carbonell-Barrachina et al., 1998). Abedin et al. (2002) also found the similar uptake kinetics of MMAA and DMAA in rice plants.

Arsenite within *M. umbrosum* appeared to involve an induction of thiol synthesis or binding with protein –SH groups, and the importance of these thiol groups to counteract the biotic and abiotic stresses, including the stress imposed by As (Hartley-Whitaker et al., 2001; Zhao et al., 2003; Mishra et al., 2008; Srivastava and D'Souza 2009; Srivastava et al., 2010) at a specific level (500 µg As L^{-1}) compared to the respective

control. It was suggested that As induced low molecular thiol compound(s) such as PCs in *M. umbrosum* for detoxification of As. Recent studies also support these current findings, and showed that eight species were identified as thiol-bound As species (PCs, GSH, and cysteine), including three newly identified complexes: Cys-As(III)-PC_2, Cys-As-$(GS)_2$, and GS-As(III)-desgly-PC_2 in C*eratophyllum demersum* macrophytes (Mishra et al., 2013). By contrast, Cd is likely to be taken up by a mechanism other than thiol formation. Further confirmation by measuring the content of amino acids concluded that only 2.13% of the amino acids present in the Cd treated leaf samples were thiol containing cysteic acid. Cosio et al. (2004) found that Cd accumulation increases in *T. caerulescens* 'Ganges' and decreases in *A. halleri* protoplasts indicating that Cd-permeable transport proteins are differentially regulated. They also concluded that Cd could be transported by a Zn and Ca pathway in *Thlaspi caerulescens* 'Prayon', whereas in 'Ganges', Cd was transported by other pathways (Cosio et al., 2004). Thus, Cd uptake mechanisms might vary from one species of plant to another. More intensive research, such as vacuole sequestration and other mechanism will be conducted in the future to determine the Cd uptake mechanism in *M. umbrosum.*

Finally, if we compare with other aquatic plants used for As and Cd phytoremediator then we found that *M. umbrosum* is one of the effective aquatic plant species used for the remediation As and Cd from contaminated water.

4. Conclusions

M. umbrosum, has the potential to be an As and Cd phytofiltration species in drinking water contaminated with low levels of As and Cd (500 µg L^{-1}) without any phytotoxic effect, in addition to its beautification potential (from the aesthetic view point of phytoremediation) by culturing in an aquarium. This plant has high affinity to uptake

inorganic As rather than Cd and organic As species, and the intensity of uptake order is Arsenite > Cd > MMAA > DMAA. Arsenic induced low molecular thiol containing compound(s) within *M. umbrosum* for detoxification or enhanced the accumulation of As from the water environment but Cd follow different uptake mechanism than that for As. Furthermore, it may play a significant role in the understanding of the As and Cd mobility and detoxification mechanisms within other aquatic plant systems. More intensive biochemical and physiological parameters will be analyzed to find out the Cd uptake mechanism within this plant in near future.

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Figure Title

Fig. 1 Arsenic (a) and Cd (b) uptake pattern in root, stem and leaf of *M. umbrosum* seven days after exposure to 0, 200, 500, 1000 μg As L^{-1} and 0, 300, 1000 μg Cd L^{-1} water. Error bars indicate mean ± SEM (n =3). ** and * denote significant differences at $P < 0.01$ and 0.05, respectively, compared to As from water to root, root to stem, and stem to leaf.

Fig. 2 Arsenic (a) and Cd (b) remaining (μg L^{-1}) in water in which *M. umbrosum* was grown with 0, 200, 500, 1000 μg As L^{-1} and 0, 300, 1000 μg Cd L^{-1} water. Error bars indicate mean ± SEM (n =3). ** and * denote significant differences at $P < 0.01$ and

0.05, respectively, compared to previous days.

Fig. 3 Concentration dependent kinetics for arsenite, MMAA, DMAA (a, c) and Cd (b, d) within *M. umbrosum*, curve fitting with Michaelis Menten linear (a, b) and non-linear (c, d) model.

Fig. 4 Arsenic concentration (µg L^{-1}), absorbance at 280 nm (a), and SH content (b) of each 2 mL eluent obtained from Sephadex G-50 gel filtration column using *M. umbrosum* leaf treated with 1000 and 0 (c) µg As L^{-1} solution.

Fig. 5 Cd concentration (µg L^{-1}), absorbance at 280 nm (a), and SH content (b) of each 2 mL eluent obtained from Sephadex G-50 gel filtration column using *M. umbrosum* leaf treated with 1000 µg Cd L^{-1} solution.

Table 1 Non-linear model for uptake kinetic parameters of inorganic and organic As species; and Cd influx into *Micranthemum umbrosum*

Species	Non-linear model		
	V_{max} (μg g^{-1} DW $24h^{-1}$)	K_m (μg L^{-1})	R^2
Arsenite	403.43	141.68	0.9616
MMAA	81.45	120.24	0.9129
DMAA	44.70	94.21	0.8468
Cadmium	365.04	120.1	1.0

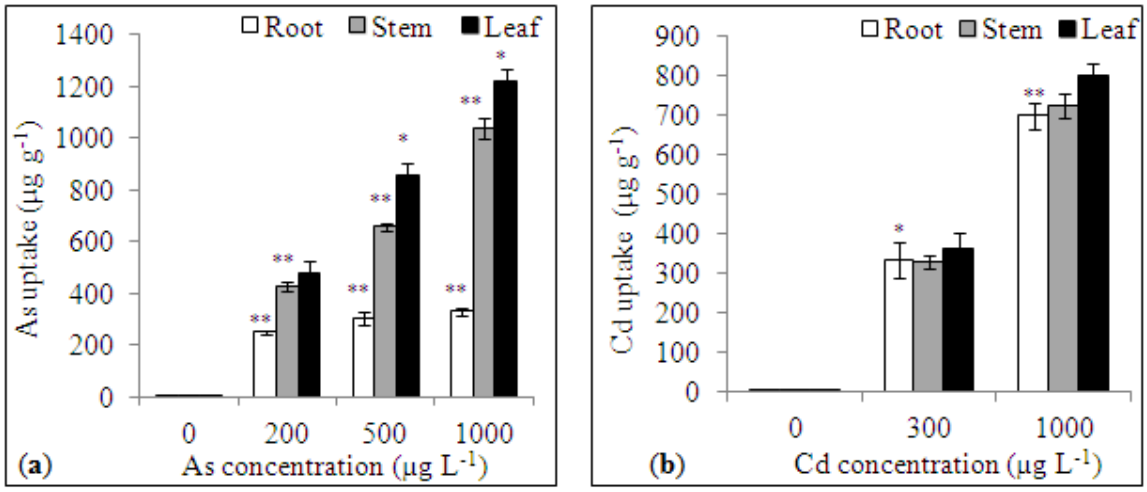

Fig. 1

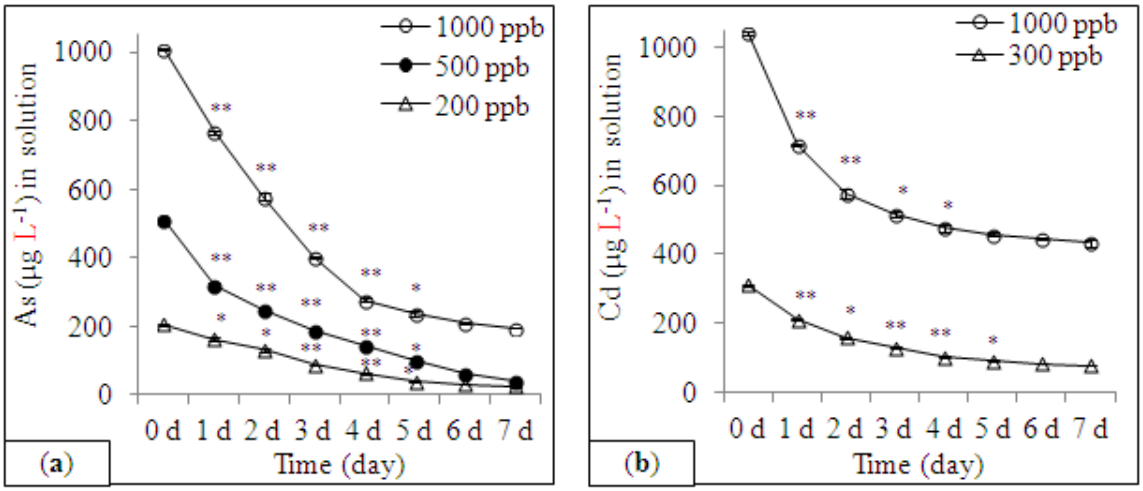
1000 ppb
500 ppb
200 ppb
As (μg L-1) in solution
1000
800
600
400
200
0
0 d 1 d 2 d 3 d 4 d 5 d 6 d 7 d
Time (day)
(a)
1000 ppb
300 ppb
Cd (μg L-1) in solution
1000
800
600
400
200
0
0 d 1 d 2 d 3 d 4 d 5 d 6 d 7 d
Time (day)
(b)

Fig. 2

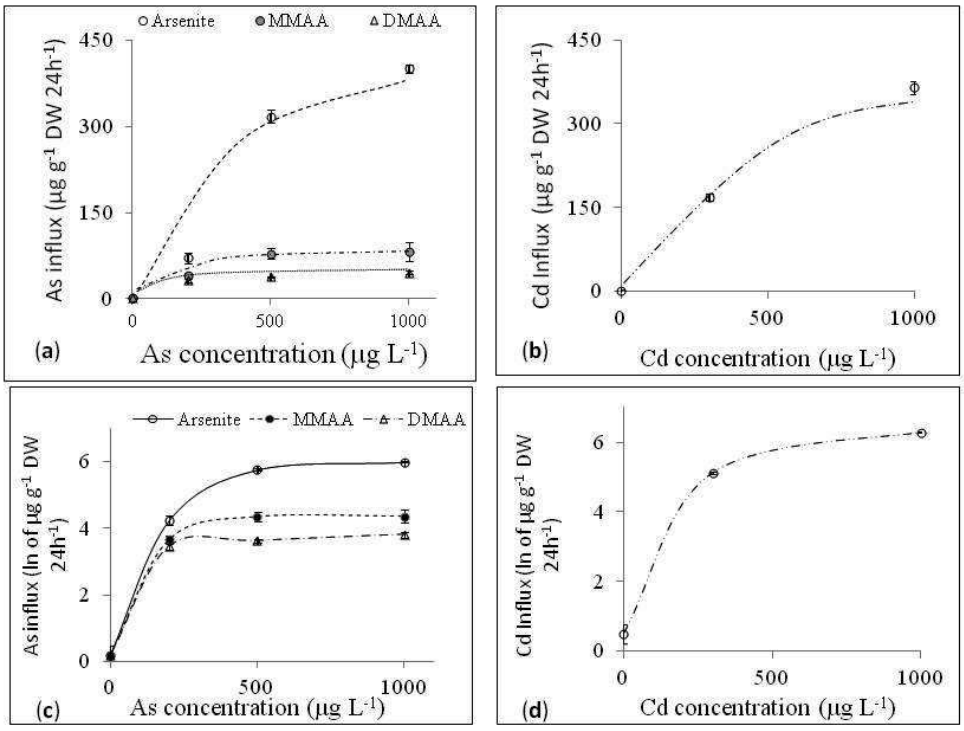
Arsenite
MMAA
DMAA
As influx (μg g-1 DW 24h-1)
450
300
150
0
0
500
1000
As concentration (μg L-1)
(a)
Cd influx (μg g-1 DW 24h-1)
450
300
150
0
0
500
1000
Cd concentration (μg L-1)
(b)
Arsenite
MMAA
DMAA
Asinflux (ln of μg g-1 DW 24h-1)
6
4
2
0
0
500
1000
As concentration (μg L-1)
(c)
Cd Influx (ln of μg g-1 DW 24h-1)
6
4
2
0
0
500
1000
Cd concentration (μg L-1)
(d)

Fig. 3

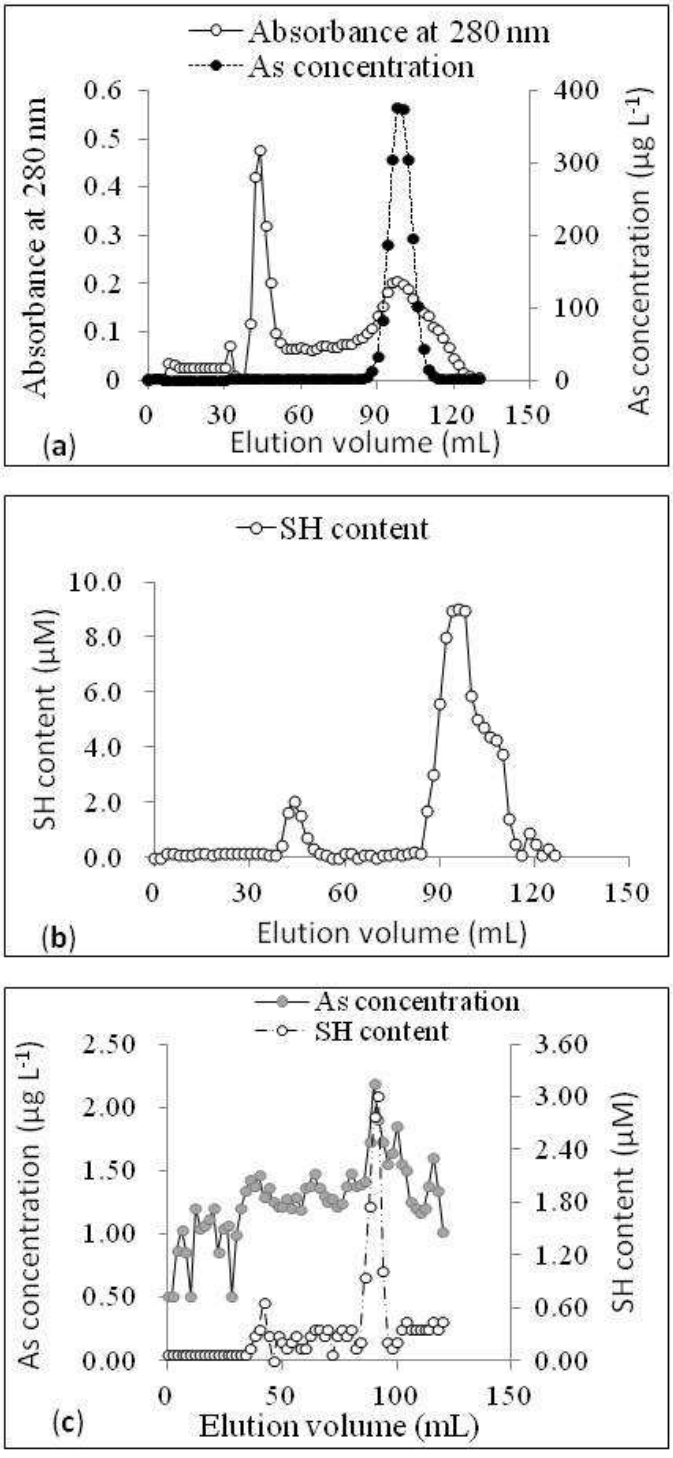
Absorbance at 280 nm
As concentration
Absorbance at 280 nm
As concentration (µg L-1)
Elution volume (mL)
(a)
SH content
SH content (µM)
Elution volume (mL)
(b)
As concentration
SH content
As concentration (µg L-1)
SH content (µM)
Elution volume (mL)
(c)

Fig. 4

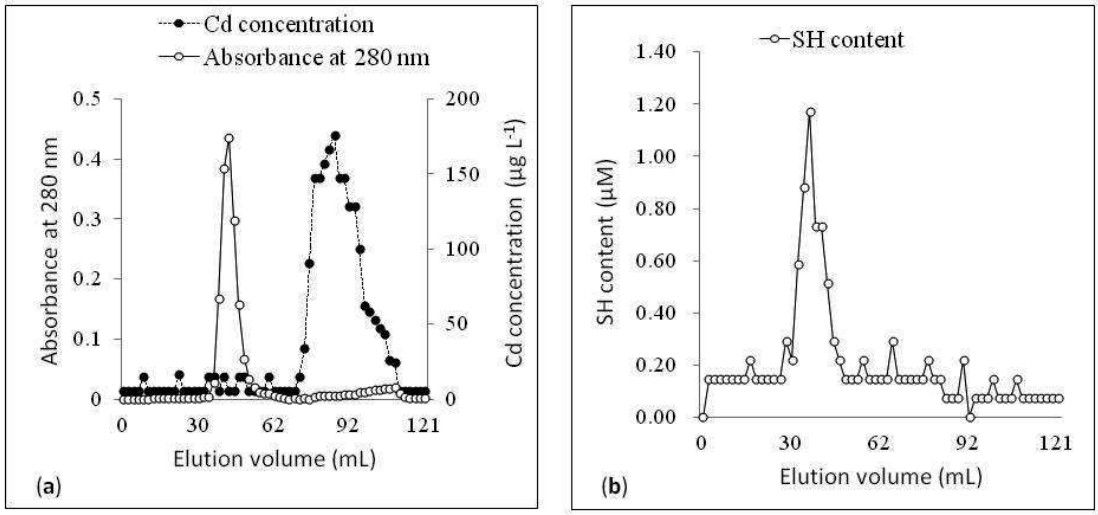
Cd concentration
Absorbance at 280 nm
Absorbance at 280 nm
Cd concentration (μg L-1)
Elution volume (mL)
(a)
SH content
SH content (μM)
Elution volume (mL)
(b)

Fig. 5