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Estimation of Phytofiltration Potential for Cu and Zn and Relative Growth Response of *Azolla japonica* and *Azolla Pinnata*

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ABSTRACT

Microcosm experiments were conducted under controlled environmental conditions in order to estimate growth response and phytoremediation ability of *A. japonica* and *A. pinnata.* Plants were exposed to solutions of different Cu-concentrations [Cu] (0, 1, 2, 5 and 7 mg L^{-1}) and Zn-concentrations [Zn] (0, 0.5, 1, 2 and 4 mg L^{-1}) under different incubation periods (0, 3, 6, and 12 days) along with control treatments. Lower metal concentrations [< 2 mg L^{-1} (Cu) and < 1 mg L^{-1} (Zn)] enhanced plant growth; however, growth was significantly inhibited at higher concentrations during Longer Incubation Periods (LIPs). *Azolla* species showed substantial metal removal capacity (on an average, Removal efficiency> 80% for Cu and > 60% for Zn during LIPs). The higher the metal concentrations with LIPs, the higher the metal removal amounts. Plant`s exposure to high (Cu) and (Zn) during LIPs showed changes in color and detachment of the roots that might result in plant death due to phytotoxicity effect. Highly significant relationships (r= 0.91^{**} & 0.82^{**} for Cu and r= 0.93^{**} & 0.92^{**} for Zn in case of *A. pinnata* and *A. japonica,* respectively) between metal removal amounts and metal concentrations in biomass indicated that phytoaccumulation was the possible mechanism for phytoremediation because the metals removed from solutions were actually accumulated into the plant`s biomass. The high value of bioconcentration factor indicated that *Azolla* species were hyperaccumulators, and can be deployed effectively for phytofiltartion of Cu and Zn.

Keywords: Bioconcentration factor, Phytoaccumulation, Phytoremediation, Phytotoxicity.

INTRODUCTION

Heavy Metals (HM) are one of the most hazardous inorganic contaminants that can contaminate entire aquatic ecosystem rapidly and their levels could be highly toxic to aquatic biodiversity due to their high mobility (Zouboulis *et al.,* 2004). Substantial amounts of HM are discharged into aquatic environment due to abrupt changes in industrial manufacturing or breakdown processes and anthropogenic activities (Demim *et al.,* 2013). These metals are released into environment from a variety of sources including electroplating, mining, milling, urban sewage, smelters, tanneries, and textile and chemical industries. On an average, 939,000 metric ton (t) of Cu and 1,350,000 t of Zn are globally released into environment (Singh *et al.,* 2003). Pollution caused by HM is a much more serious and insidious problem than the pollution caused by organic substances (Jain *et al.,* 1990) due to high bioaccumulation, persistence and non-degradable nature of HM in different environmental components (Miretzky *et al.,* 2004; Sood *et al.,* 2012). Polluted water has remarkable issues in natural and agricultural ecosystems. Drinking water contaminated with HM poses severe health hazards in humans. In resource limited countries, toxic metals in water bodies affect the lives of local people

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that rely on these water resources for their daily requirements (Rai *et al.*, 2002). Among HM, Copper (Cu) and Zinc (Zn) can lead to kidney and liver dysfunction, non-fatal metal fume fever, pneumonitis and blocking of functional groups of vital enzymes after entering into food chain as a result of biomagnifications processes (Jain *et al.*, 1990; Lizieri *et al.*, 2012). Plants exposed to environment contaminated with higher/toxic HM levels display depressed growth by affecting chlorophyll fluorescence, photosynthesis, chlorosis, necrosis, leaf yellowing and rendering, root shredding and nutrient acquisition (Mishra and Tripathi, 2009). By considering the toxic effects of HM on humans, animals and plants, there is a dire need to treat HM properly. Nevertheless, various detoxifying methods/processes such as ion-exchange, electrolysis, chemical precipitation and disinfection, adsorption by activated carbons, reverse osmosis and nano-filtration have been employed to clean-up waste effluents; however, most of these methods are quite expensive, energy extensive and inefficient for complete removal of HM. These facts impel us to devise and develop/select cheap, safe and effective strategies for removing HM completely from the aquatic environment. Tailoring the plant to fit the environment (use of suitable plants to clean the environment) is an emerging strategy in this context.

Phytoremediation is an emerging eco-friendly, inexpensive and noninvasive alternative to quite expensive conventional cleanup techniques, or a complementary green technology to replace energy intensive engineering based remediation methods (Pilon-Smits, 2005). Phytoremediation is a biological technique that relies on the use of plants to mitigate concentration of contaminants. This technique can be highly useful to clean up persistent and non-degradable toxic metals from the environment. In *planta*, hyperaccumulators are taxonomically widespread throughout the plant kingdom and can absorb, translocate, accumulate, and tolerate high levels of certain metals compared to other organisms (Xue *et al.*, 2010; Valderrama *et al.*, 2013). Even some higher plant species, e.g. *Brassica napus L.*, are capable to produce more biomass under higher metal (Zn) levels (Belouchrani *et al.*, 2016). To date, more than 400 plant species have been recognized as natural metal hperaccumulators, that is < 0.2 % of all angiosperms (Mcgrath and Zhao, 2003). As slow growth, low plant biomass, and low metal bioavailability are the limiting factors of phytoremediation (Neilson and Rajakaruna, 2015; Li *et al.*, 2018), these slow growing plant species with limited biomass result in low efficacy for phytoremediation. Because the total metal extraction is the product of biomass and tissue concentration (Valderrama *et al.*, 2013; Ebbs *et al.*, 1997), the success of phytoremediation mainly depends on the plant growth rate and high metal accumulation ability (Abhilash *et al.*, 2009; Pandey, 2012).

Phytofiltration is a strategy of phytoremediation in which plants sequester the metals and other contaminants from aquatic environment. Aquatic plants used in phytofiltration can be either merged or submerged in water (accumulate metals by whole plant) or free floating on the surface of water (absorb metals mainly by roots) (Demim *et al.*, 2014). These aquatic plants can offer a promising solution for phytofiltration of HM in an aquatic system. Among aquatic plants, free-floating macrophytes as phytoremediator plants can play an undeniable role in the remediation of the water contaminated with metals. Among macrophytes, *Azolla* might be a potential candidate for phytofiltration because the fern can hyperaccumulate a variety of pollutants and can produce double biomass in 3-9 days depending on the culture conditions (Arora and Singh, 2003). *A. pinnata* has been investigated in different studies; however, the ability of *A. japonica* (naturally growing native species in Japan) for HM removal, especially for Cu and Zn, has been scarcely documented in literature. Furthermore, naturally growing plant species are more suitable phytoremediators in comparison to the introduced plants (Pandey and Singh, 2011). *Azolla* species (*A. japonica* Fr. et Sav. and *A. pinnata* R. Br.) used in the

present study have also been recognized as naturally growing water ferns. The objective of this study was to investigate the relative growth response and phytofiltration ability of *A. pinnata* and *A. japonica* for the removal of Cu and Zn from aqueous solutions. Additionally, we aimed to investigate the effect of HM-concentrations and exposure time on plant growth after plant`s exposure in order to estimate physiological response of both *Azolla* species.

MATERIALS AND METHODS

Treatments

For Heavy Metal (HM) treatments, Cu treatments were prepared by adding Cu in 1% modified Hoagland N-free medium to make Cu-concentrations ([Cu]) of 0, 1, 2, 5 and 7 mg L^{-1} by using analytical grade $CuSO_4 \cdot 5H_2O$ salt. For Zn treatments, Zn-concentrations ([Zn]) of 0, 0.5, 1, 2 and 4 mg L^{-1} were prepared by using $ZnSO_4 \cdot 6H_2O$.

Plant Species and Experimental Set-up

Two naturally growing *Azolla* species (*A. japonica* and *A. pinnata*) were used in the present experiments. *A. japonica* was collected from Toyooka city (35° 33′ N 134° 49 ′E), Hyogo prefecture, Japan, while *A. pinnata* was used from our laboratory stock of Okayama (34° 39′ N 133° 55′ E) University, Okayama, Japan. *Azolla* plants were re-cultured in a glass house of Tsushima campus, Okayama University, Japan. Plants were washed twice with tap water and cultured in 50-L containers having 20-L water and 10-kg soil to get reasonable biomass of plants. Plants were then re-cultured again in nutrition-free distilled water for 3 days in a climatically controlled chamber for acclimatization prior to their exposure to the actual treatments. Plants were then exposed to different HM treatments in order to estimate the concentration and time dependent effects on HM removal ability of *Azolla* plants.

Eight grams of *Azolla* fronds, with approximately uniform size and age, were used to estimate uptake and removal capacity for Cu and Zn. Plants were transferred into a 1.0-L capacity plastic pots containing 800 mL of 1% modified Hoagland N-free medium having initial pH of 6.5 in controlled climate growth chamber (Eyela Eyeltron FLI-1001) and the culture conditions were as follows: light/dark 16/8 hr; temperature 25/20 ^oC, respectively; light intensity 60 µmol m^{-2} s^{-1}; relative humidity 65%. Elemental composition along with salts of N-free solution medium was (in mg L^{-1}): P= [1]-$Na_2HPO_4.12H_2O$, K = [2]-KCl, Ca= [2.06]-$CaCl_2.2H_2O$, Mg= [0.48]-$MgSO_4.7H_2O$, Fe= [0.062]-EDTA-Na-$Fe.H_2O$, Mn = [0.005]-$MnSO_4.5H_2O$, B= [0.005]-H_3BO_3, Mo= [0.005]-$H_2MoO_4.H_2O$. Cu and Zn were added to make concentrations of 0, 1, 2, 5, 7 mg L^{-1} Cu and 0, 0.5, 1, 2 and 4 mg L^{-1} Zn, by using $CuSO_4 \cdot 5H_2O$ and $Zn \cdot SO_4 \cdot 6H_2O$ salts, respectively. Heavy metal solutions without plants were used as the control treatments for valid comparisons. *Azolla* plants were also grown in only distilled water as well as in 1% modified Hoagland N free medium in order to assess the *Azolla* growth between untreated and treated solutions with HM. All the treatments were replicated thrice by using completely randomized design. Experiments were conducted in sequential steps during different incubation periods (3, 6, and 12 days after fronds transfer) in a cultivation chamber and all experimental pots were covered with pierced nylon cover having approximately 200 holes per cover.

Biomass and Chemical Assay

After incubation periods of 3, 6, and 12 days, solution samples were immediately filtered by using filter papers. Plant samples collected at the start of the experiments were used as control plants. Treated *Azolla* plants were collected after different incubation

periods. The plants were washed twice with distilled water, excess water was allowed to drain off and then plants were weighed. Plants were dried in a forced air-driven oven for 24 hours at 80°C, and dry mass was recorded. Oven dried plants were ground into fine powder and 0.3 g powdered samples were taken into porcelain crucibles, which were placed into a cool muffle furnace and temperature was increased gradually to 550°C and continued ashing for 5 hours after attaining temperature of 550°C. Then, the furnace was shut off and opened after cooling. Cooled ash was dissolved in 5 mL of 2N HCl and thoroughly mixed. For complete digestion, solutions containing 5 mL of 2N HCl were evaporated at 80°C until we got pellets, which were dissolved again in 5 mL of 2N HCl and mixed well. Solutions were filtered into 100 mL flask by washing crucibles thrice with demineralized water and made the volume up to the mark. These samples were subsequently used for elemental analysis. An atomic absorption spectrometer (Hitachi Z-6100 Polarized Zeeman AAS) was used for analysis of Cu and Zn in the solution and plant samples.

Parameter Calculations

Amounts of Heavy Metals (HM) removed (mg m^{-2}) from the solutions was estimated as below:

HM removed (mg m^{-2})= [Volume of initial water×Initial HM-concentration (HM)-Volume of water at the termination of experiment×Final (HM)]/Surface area (1)

The HM removal efficiency was calculated by using the expression described by Zabihi *et al*. (2009).

HM removal efficiency (%)= (Ci-Cf /Ci)×100 (2)

Where, *Ci* is the initial HM amount and *Cf* is the remaining HM amount in solutions.

The HM removal rate was calculated by using the following expression:

Removal rate= (Initial HM amount-Final HM amount)/(Surface area×Treatment time) (3)

BioConcentration Factor (BCF) is the ratio of HM accumulated by the plants to that dissolved in the aquatic medium. The *BCF* was computed form [HM] as described by Zayed *et al*. (1998).

$$BCF = [HM]_{plant}/[HM]_{aquatic\ medium} \quad (4)$$

Statistical Analysis

Means of three estimations were presented with their standard deviation and Analysis Of Variance (ANOVA) was used to detect significant differences among means. Treatment means were separated using Duncan's Multiple-Range Test (DMRT). Correlation coefficient (r) values were estimated by using treatment means. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Growth Assessment and Biomass Assay

The effects of Cu and Zn on growth of *A. pinnata* and *A. japonica* at different HM concentrations ([HM]) and exposure times are depicted in Figure 1. For *A. pinnata* and 6 days of exposure, maximum Dry Weights (DWs) (35 g m^{-2}) were obtained at 0 mg L^{-1} Cu and Zn-treatments. Among Cu and Zn treatments, maximum DWs (34.5 and 33.7 g m^{-2}) were obtained at 2 and 0.5 mg L^{-1} Cu and Zn treatments, respectively, after 6 days of exposure. The DWs of *A. pinnata* increased (from 30.4 to 33.7 and 28.5 to 32.1 g m^{-2} at 1 mg L^{-1} Cu and Zn treatments, respectively) when exposed to low [HM] after 12-day incubation periods. However, the DWs of *A. pinnata* exposed to high [HM] fluctuated during three incubation periods. The DWs increased at low [HM], but decreased at high [HM]. The DW of *A. pinnata* decreased from 30.4 g m^{-2} at 1 mg L^{-1} Cu treatment to 24.5 g m^{-2} at 7 mg L^{-1} Cu treatment. Similarly, the highest DW of *A. japonica* was 39 g m^{-2} at 2 mg L^{-1} Cu and 37.1 g m^{-2} at 0.5 mg L^{-1} Zn treatments in 6 days of exposure. When the [HM] increased,

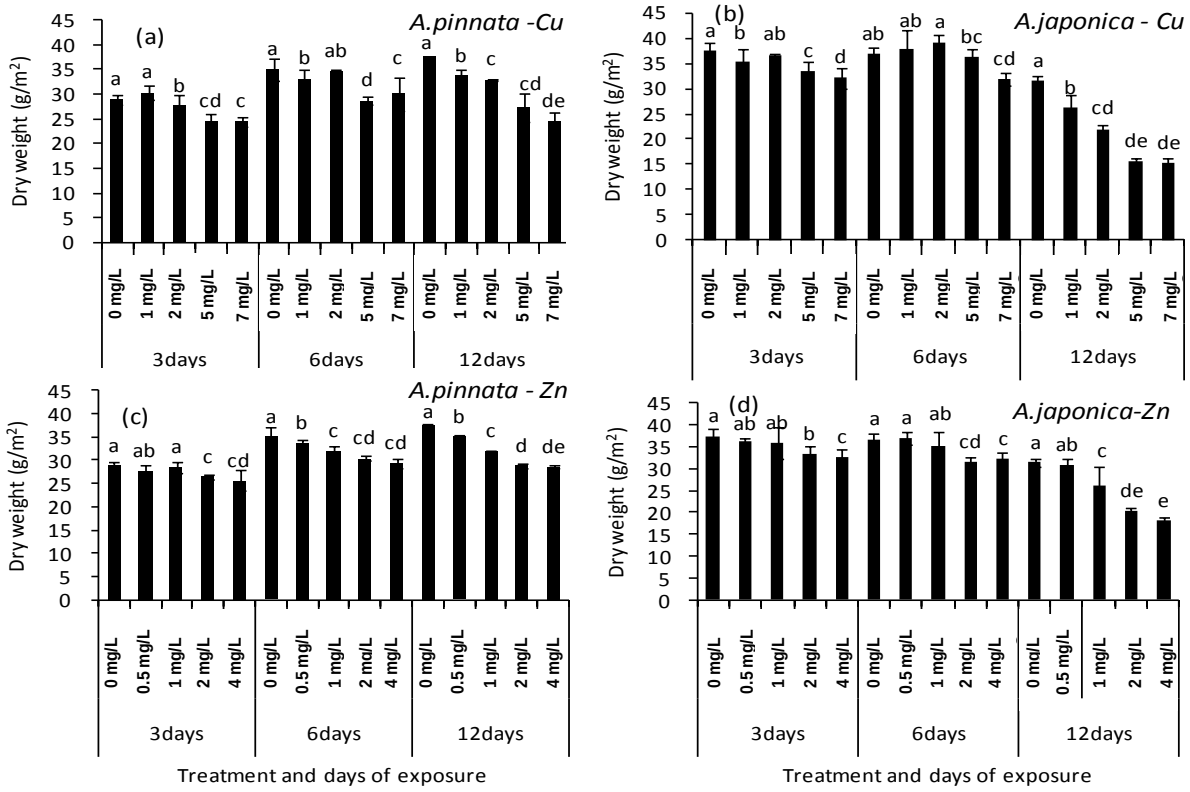

Figure 1. Dry weight of *A. pinnata* and *A. japonica* exposed to different Cu and Zn treatments during different incubation periods. Error bars show ±*SD* (n= 3). Values designated over the bars sharing different letters are significantly different at *P*= 0.05 level by DMRT.

the DWs of *A. japonica* decreased. Compared to *A. pinnata*, the DWs of *A. japonica* decreased in all [HM] from 6th to 12th days of exposure. The DWs of *A. japonica* decreased from 37.7 to 26.1 g m^{-2} after 6 to 12 days of exposure to 1 mg L^{-1} Cu treatment.

Biomass accumulation is always considered an important plant trait in growth analysis. The *Azolla* species used in the present study showed substantial growth response to Cu and Zn contaminated aquatic environment, indicating that these naturally grown ferns can be utilized as a biofilter for Cu and Zn removal. This is in agreement with Zhao *et al.* (1999) who also reported substantial growth of *Azolla* plants exposed to metal contaminated solutions. Growth changes are often the first and most obvious responses of plants exposed to HM stress (Hagemeyer, 1999). Cu and Zn concentrations had different effects on the growth of *Azolla* species in the present study. The growth of both plant species was stimulated at low [HM] but plant growth was inhibited at higher [HM]. These results are in agreement with Singh *et al.* (2010) who reported that at low concentrations, Cu, Zn, Mo, Mn and Fe showed significant enhancement in growth activities of *A.microphylla* and *A.filiculoides*, and displayed an inhibitory effect at higher concentrations. Hasan *et al.* (2007) also reported that at higher [HM], growth of aquatic plants was significantly inhibited. The decreased growth was probably due to HM induced stressed environmental conditions that influenced the plant's biomass yield. The favorable effect of low [Cu] and [Zn]-treatments on *Azolla* plants can be attributed to the fact that plants utilized Cu and Zn as micronutrients for

their growth and productivity (Jain *et al.*, 1990) because they act as activators/co-factors for different essential physiological and metabolic plant functions. The *A. pinnata* was able to grow slightly better with Longer Incubation Periods (LIPs) when compared to *A. japonica*. This can be ascribed to the fact that the frond morphology of *Azolla* species is different. The *Azolla* species have distinct differential morphological features; i.e. (i) *A. japonica*- frond elongate, 1.5-7 cm long, irregularly branched, triangular form, and root length is 2.5-7.0 cm, (ii) *A. pinnata*- fronds typically triangular, 1.0-1.5 cm long, and root length is 1.5 cm (Pereira *et al.*, 2011; Madeira *et al.*, 2013). Because fronds of *A. japonica* are larger than that of *A. pinnata* (Wagner, 1997; Bozzini *et al.*, 1982), therefore, metal uptake rate by *A. japonica* might be faster than *A. pinnata* during the first few days of the treatments. Thus, the toxic effect of HM had more impact on the fronds of *A. japonica* due to higher sensitivity of *A. japonica* exposed to [HM] during LIPS.

Removed Amounts of Cu and Zn from Solutions

Removed Cu and Zn amounts from the solutions are depicted in Figure 2. The higher the Cu and Zn concentrations in the solutions, the higher the removal amount of these metals in all incubation periods. Highest amounts of Cu (278 mg m^{-2}) and Zn (131 mg m^{-2}) were removed from solutions by *A. pinnata* during 12 days of exposure to the highest [Cu] and [Zn]-treatments, respectively. Removed amounts of metals from solutions exposed to *A. pinnata* were significantly increased when the exposure time increased. Zn removal amount was increased from 60 mg m^{-2} on 3rd day to 80 mg m^{-2} on 12th day at 2 mg L^{-1} Zn-treatment. In case of *A. japonica*, the maximum Cu removal amounts (> 300 mg m^{-2}) at 7 mg L^{-1} Cu and 220 mg m^{-2} at 5 mg L^{-1} Cu-treatments were observed during 12 days of exposure, whereas the maximum Zn removal amounts (80 mg m^{-2}) at 2 mg L^{-1} Zn and > 120 mg m^{-2} at 4 mg L^{-1} Zn-treatments were observed during 6 days of exposure. After 6th day of treatment, Cu removal amount was increased in high [Cu]-treatment, however, Zn removal amount slightly decreased in high [Zn]-treatment. Cu removal amount was increased from 200 mg m^{-2} during 6 days of exposure to 225 mg m^{-2} on 12th day of exposure to 5 mg L^{-1} Cu-treatment, however, Zn removal amount was decreased from 80 mg m^{-2} on 6th day to 73 mg m^{-2} on 12th day of exposure to 2 mg L^{-1} Zn-treatment. The results obtained suggested that different HM treatments, exposure time, and *Azolla* species have significant effect on removed amounts of HM from the solutions. Mishra *et al.* (2008) and Mishra and Tripathi (2009) reported that with increasing [HM], aquatic plants were able to remove and accumulate high amounts of metals. *Azolla* species also removed substantial amounts of these metals, indicating that this species can be used effectively to remove Cu and Zn.

Cu and Zn Removal Rates

Cu and Zn removal rates from the aqueous solutions during different incubation periods after exposure to *Azolla* plants are depicted in Figure 3. The results obtained in the present study indicated that removal rates of the metals were significantly different between incubation periods and the metal concentrations. The removal rates were highest on the 3rd day of exposure to the treatments for all [HM]. Metal removal rates decreased with increasing the exposure time for *Azolla* species. Cu and Zn removal rates of *A. pinnata* decreased from 68 mg m^{-2} d^{-1} on 3rd day to 23 mg m^{-2} d^{-1} on 12th day at 7 mg L^{-1} Cu-treatment, and 28 mg m^{-2} d^{-1} on 3rd day to 11 mg m^{-2} d^{-1} on 12th day at 4 mg L^{-1} Zn-treatments, respectively. Treatments with higher [HM] showed higher removal rates. Cu removal rates of *A. pinnata* ranged between 4 mg m^{-2} d^{-1} at 1 mg L^{-1} [Cu] to 45 mg m^{-2} d^{-1} at 7 mg L^{-1} [Cu]-treatments, and Zn removal rates ranged from 3 mg m^{-2} d^{-1} at

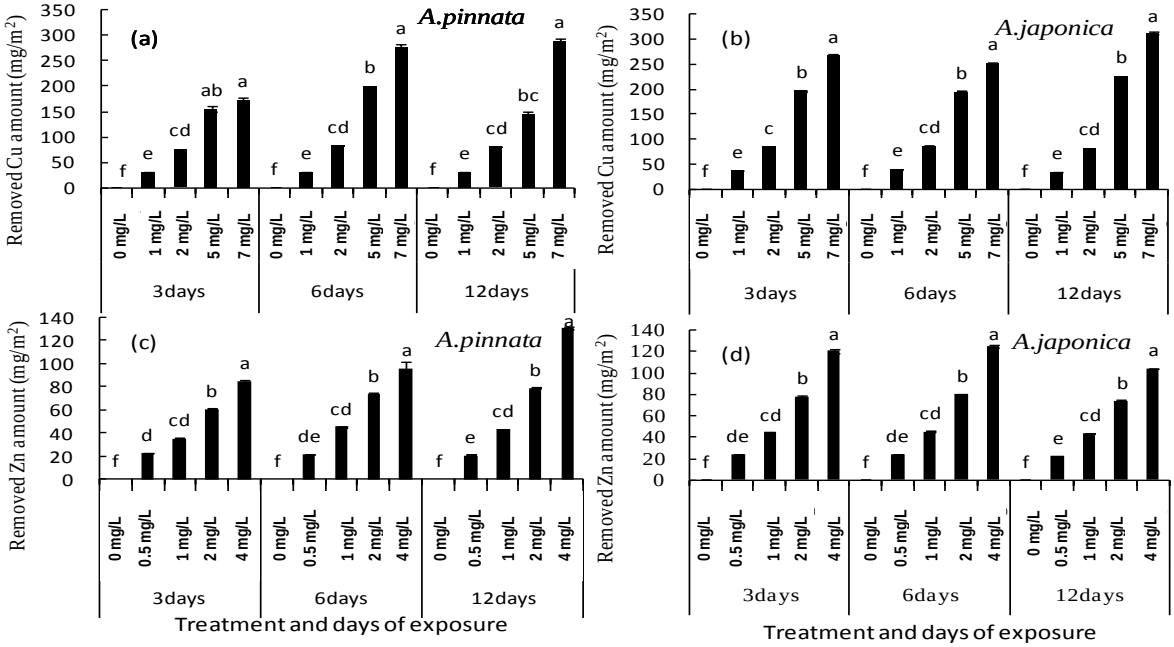

Figure 2. Amounts of Cu and Zn removed from solutions during different incubation periods after exposure to *A. pinnata* and *A. japonica.* Error bars show ±*SD* (n= 3). Values designated over the bars sharing different letters are significantly different at *P*= 0.05 level by DMRT.

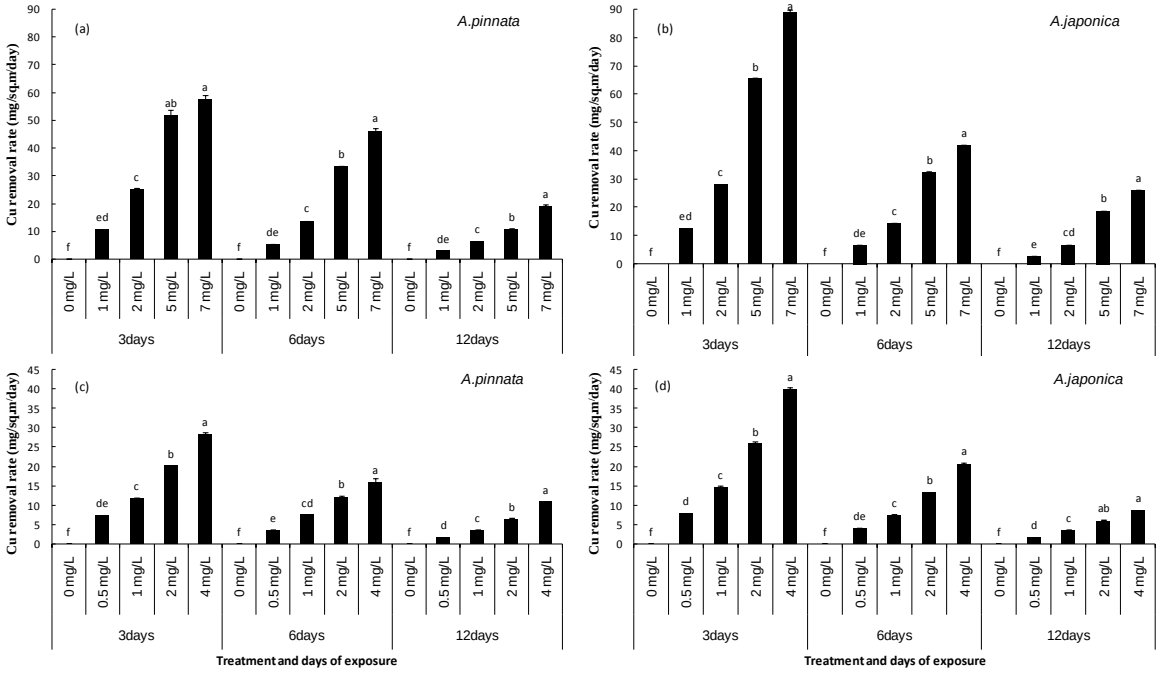

Figure 3. Removal rates of Cu and Zn during different incubation periods after exposure to *A. pinnata* and *A. japonica*. Error bars show ±*SD* (n= 3). Values designated over the bars sharing different letters are significantly different at *P*= 0.05 level by DMRT.

0.5 mg L^{-1} [Zn] to 16 mg m^{-2} d^{-1} at 4 mg L^{-1} [Zn]-treatments during 6 days of treatment exposure. The trend of heavy metal removal rates from solutions treated with *A. japonica* was similar to that of *A. pinnata*. Cu and Zn removal rates of *A. japonica* decreased from 88 mg m^{-2} d^{-1} on 3rd day to 26 mg m^{-2} d^{-1} on 12th day at 7 mg L^{-1} [Cu]-treatment, and 40 mg m^{-2} d^{-1} on 3rd day to 8 mg m^{-2} d^{-1} on 12th day at 4 mg L^{-1} [Zn]-treatments, respectively. Cu and Zn removal rates of *A. japonica* were also increased with increasing [HM]. Cu removal rate of *A. japonica* increased from 6 mg m^{-2} d^{-1} at 1 mg L^{-1} to 42 mg m^{-2} d^{-1} at 7 mg L^{-1} [Cu]-treatments, and Zn removal rate was increased from 4 mg $m^{-2}d^{-1}$ at 0.5 mg L^{-1} to 21 mg m^{-2} d^{-1} at 4 mg L^{-1} [Zn]-treatments during 6 days of exposure. Maximum removal rate of *A. japonica* was 88 mg m^{-2} d^{-1} at 7 mg L^{-1} [Cu]-treatment during 3 days of exposure. *Azolla* species showed substantial variability in HM removal rates and higher removal rates were observed in higher HM concentrated solutions exposed to *A. japonica* than *A. pinnata*, during three days of exposure time. Among treatments, higher Cu than Zn removal rates by *Azolla* species indicated that these species have better removal ability for Cu than Zn from the aqueous solutions. Upadhyay *et al.* (2007) also reported that sequence order of removal of HM by five plant species (*Eichorium crassipes*, *Pistia stratiotes*, *Lemna minor*, *Azolla pinnata* and *Spirodela polyrhizha*) was Fe> Cr> Cu> Cd> Zn> Ni. The *Azolla* species showed substantial removal rates of HM indicating that these species can be deployed effectively to remove Cu and Zn from contaminated aqueous solutions. Among treatments, removal rate decreased with increased exposure time, indicating that the absorption sites in *Azolla* plant roots become saturated with increasing time.

Cu and Zn Removal Efficiencies by *Azolla*

Removal efficiencies (%) of Cu and Zn from solutions exposed to *Azolla* species at different [HM] and exposure times are depicted in Figure 4. Data showed that the removal

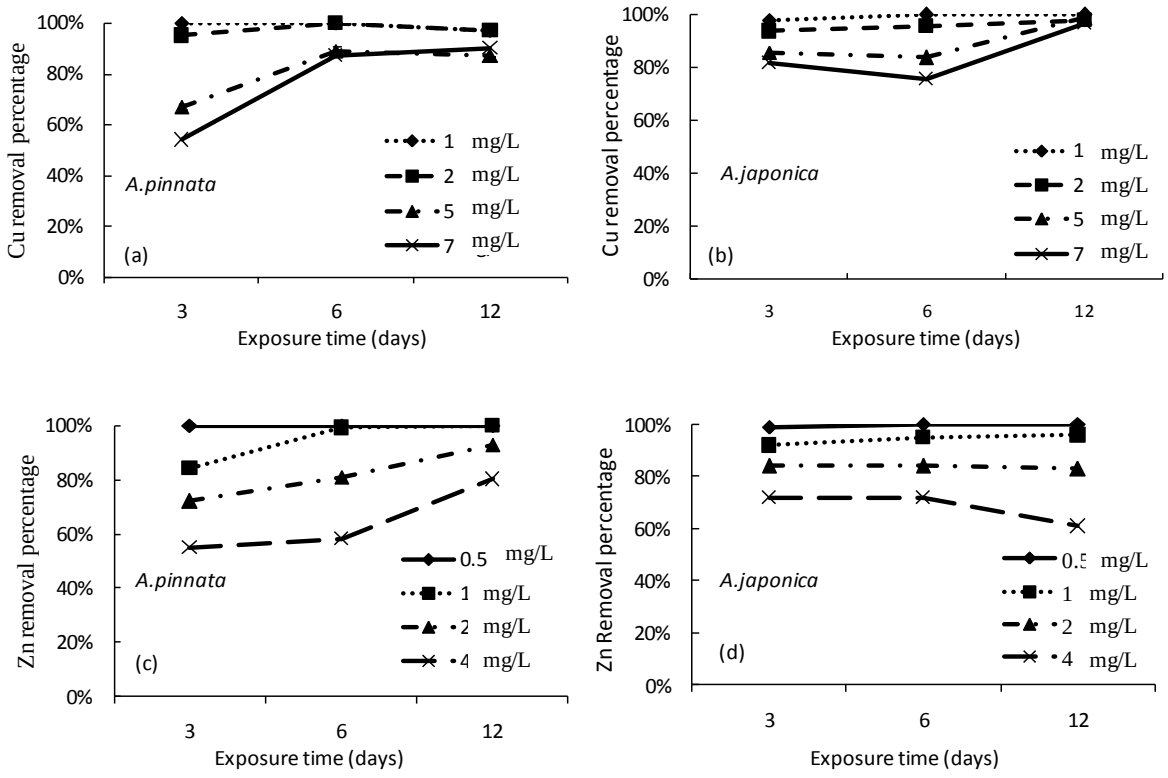

Figure 4. Removal percentage of Cu and Zn by *A. pinnata* and *A. japonica* exposed to metals.

efficiency of *A. pinnata* varied with different [HM] and exposure time. In Cu-treatments, *A. pinnata* removed 100% at 1 mg L^{-1}, 94 to 100% at 2 mg L^{-1}, 67 to 89% at 5 mg L^{-1}, and 54 to 89% of Cu at 7 mg L^{-1} [Cu]-treatments, respectively, during 12 days of exposure. In Zn treatments, *A. pinnata* removed 100% at 0.5 mg L^{-1}, 84 to 100 % at 1 mg L^{-1}, 72 to 93% at 2 mg L^{-1}, and 55 to 80% of Zn at 4 mg L^{-1} [Zn]-treatments, respectively. This differential behavior for removal percentage can be attributed to the decreasing capability of the plants to accumulate Cu with increasing [Cu] in the solutions because the selective sites for Cu in the plants can also become saturated with time. As evident in Figure 4, the Cu removal percentages at all [HM] were maximized on 12th day. Removal efficiencies of *A. japonica* were the highest (100%) at 1 mg L^{-1} [Cu] and 0.5 mg L^{-1} [Zn]-treatments. The Cu removal efficiencies increased with increasing the incubation time. *A. japonica* removed Cu from 94 to 98%, 84 to 99%, and 76 to 97% of Cu at 2, 5, and 7 mg L^{-1} [Cu]-treatments, respectively, during 12 days of exposure. Zn removal percentage fluctuated slightly between 0.5, 1, and 2 mg L^{-1} [Zn]-treatments; however, it decreased at 4 mg L^{-1} [Zn]-treatment when exposure time was increased. At higher [HM] during LIPS, the ion selectivity for species was Cu> Zn. The results are in agreement with Pandey (2012) and Valderrama *et al.* (2013) who reported that *A.caroliniana* and *A. filliculoides* showed significant removal efficiencies when exposed to Cu and Zn. Although species showed significant differences in HM removal, particularly during six days of exposure, higher values of removal efficiency indicated that *Azolla* species are efficient candidates for Cu and Zn removal; this might be attributed to their better absorption and translocation of these metals.

Cu and Zn Concentrations in *Azolla* Plants

Cu and Zn-concentrations in *Azolla* plants are presented in Table 1. [HM] in solutions and exposure time had significant main and interactive effects on [HM] in *Azolla* plants. On an average, a comparison between initial and final [HM] within the plant showed that the final [HM] were 10 and 4 times more than the initial [HM] in Cu and Zn treatments, respectively, for *Azolla* species. The [Zn] and [Cu] in *Azolla* species increased with increasing solution [HM] and incubation periods. The highest [HM] (13.56 mg/g DW) in *A. pinnata* was observed at 7 mg L^{-1} Cu during 12 days of exposure. In *A. pinnata*, [Cu] was 3.93 times more at 7 mg L^{-1} [Cu]-treatment than at 2 mg L^{-1} [Cu]-treatment during 6 days of exposure, and 1.4 times more at 7 mg L^{-1} Cu in 12 days than the amount observed at 7 mg L^{-1} Cu-treatment in 6 days. The highest amount of Cu was 16.85 mg g^{-1} DW of the fronds in *A. japonica* at 7 mg L^{-1} [Cu]-treatment during 12 days of exposure. [Cu] was 3.47 times more at 7 mg L^{-1} [Cu]-treatment than at 2 mg L^{-1} [Cu]-treatment during 6 days of exposure, and 2.2 times more at 7 mg L^{-1} Cu in 12 days than the amount observed at 7 mg L^{-1} Cu in 6 days. Abhilash *et al.* (2009) reported that macrophytes have suitable attributes such as high biomass, fast growth, and high tolerance and HM phytoaccumulation ability. Higher HM removal efficiencies (Figure 4) by *Azolla* species can be ascribed to better growth, and better uptake and translocation of HM in plants. This is also confirmed by high values of bioconcentration factor (Figure 5) of *Azolla* species. The results are in agreement with Pandey (2012) and Sela *et al.* (1989) who reported that *A.caroliniana* and *A. filliculoides* exposed to HM also accumulated substantial amounts of Cu and Zn.

Biconcentration Factor

BioConcentration Factor (BCF) values for Cu and Zn at different [HM] and exposure times are shown in Figure 5. The *BCF* was calculated on dry weight basis. Plants showed differential responses for *BCF* at

Table 1. Cu and Zn concentrations in *A. pinnata* and *A. japonica* after exposure to different Cu and Zn treatments during different incubation periods.[a]

Concentration (mg g^{-1} DW)	Day	Initial	Treatment								
			DI water	0 (mg L^{-1})	Cu (mg L^{-1})				Zn (mg L^{-1})			
					1	2	5	7	0.5	1	2	4
Cu-concentrations in *A. pinnata*												
Cu	3	0.00	0.00	0.00	1.10 c	2.42 c	5.72 c	6.81 c	0.00	0.00	0.00	0.00
	6	0.00	0.00	0.00	1.33 b	2.55 bc	6.98 b	10.02 b	0.00	0.00	0.00	0.00
	12	0.00 ns	0.02 ns	0.01 ns	1.41 a	2.85 a	9.06 a	13.56 a	0.04 ns	0.00 ns	0.00 ns	0.01 ns
Zn	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.80	1.27 b	2.1 b	3.62 c
	6	0.08	0.06	0.07	0.07	0.07	0.07	0.08	0.80	1.47 a	2.58 a	4.33 a
	12	0.03 ns	0.00 ns	0.02 ns	0.00 ns	0.02 ns	0.02 ns	0.00 ns	0.60	1.12 cd	2.48 ab	3.99 b
Cu-concentrations in *A. japonica*												
Cu	3	0.05	0.02	0.04	1.09 c	2.08 cd	5.11 bc	7.23 c	0.03	0.06	0.05	0.07
	6	0.05	0.04	0.04	1.17 b	2.25 b	5.39 b	7.82 b	0.06	0.02	0.04	0.03
	12	0.07 ns	0.00 ns	0.00 ns	1.48 a	3.51 a	12.22 a	16.85 a	0.05 ns	0.00 ns	0.00 ns	0.00 ns
Zn	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.56 b	1.11 bc	2.21 b	3.76 bc
	6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.51 c	1.07 c	2.20 bc	3.87 b
	12	0.01 ns	0.00 ns	0.00 ns	0.00 ns	0.00	0.00	0.00	0.60 a	1.34 a	2.78 a	4.67 a

[a] Means with different letter(s) differ significantly according to Duncan's Multiple Range Test (P= 0.05); DI= Distilled Water.

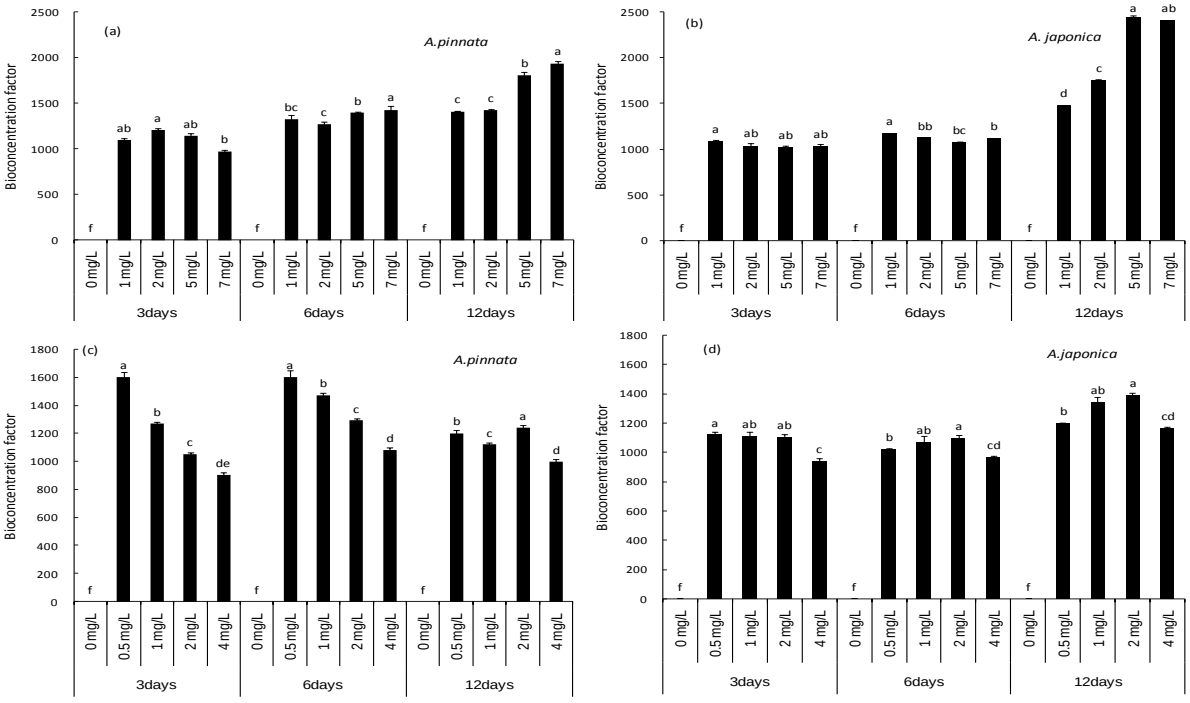

Figure 5. Bioconcentration factor of metals in fronds of *Azolla*. Error bars show ±*SD* (n = 3). Values designated over the bars sharing different letters are significantl y different at *P*= 0.05 level by DMRT.

different [HM] and exposure times. For plants of *Azolla* species treated with Cu, the maximum *BCF* values were obtained at 5 and 7 mg L^{-1} [Cu]-treatments and *A. japonica* showed higher *BCF* values than *A. pinnata* during 12 days of exposure. The *BCF* values of *A. pinnata* were slightly higher than *A. japonica* during 3 and 6 days of exposure to Cu-treatments. This indicated that *A. pinnata* had slightly higher Cu accumulation potential during 3 and 6 days of exposure, whereas *A. japonica* had higher Cu accumulation potential during 12 days of exposure. For plants of both *Azolla* species treated with Zn, the *BCF* values were higher at 0.5 than 4 mg L^{-1} [Zn]-treatments during all incubation periods. For *A. pinnata*, the maximum *BCF* value was observed at lowest [Zn]-treatment during 3 and 6 days of exposure, whereas for *A. japonica*, the maximum *BCF* was obtained at 2 mg L^{-1} [Zn]-treatment during 12 days of exposure. This indicated the differential Zn accumulation potential of *Azolla* plants during different exposure times.

The fitness of plants for phytoremediation can be judged by *BCF*, which is a relative index of the ability of plant to accumulate the metal with respect to the metal concentration in an ambient environment. Phytoaccumulation of metals by macrophytes can be affected by metals concentrations in environment by influencing the metal uptake efficiency (Rai and Chandra, 1992). The *BCF* is an important index to estimate the feasibility of any plant species for phytoremediation of heavy metals (Pandey, 2012), and the *BCF* values over 1000 are generally considered an indicator of the useful plants for phytoremediation (Zayed *et al.*, 1998; Zhu *et al.*, 1999). Pandey (2012) also reported that *Azolla caroliniana* plants are effective phytoremediators for Cu and Zn due to high *BCF* values. Hyperaccumulators are plants that can absorb and extract extremely excess amounts of contaminants. The higher *BCF* values showed that A*zolla* species were hyperaccumulators of metals and could be used effectively for phytoremediation of Cu and Zn in the aqueous solutions.

Relationships between Metal Removal Amount and Metal Concentrations in Plant Biomass

Cu and Zn removal amounts from the solutions by *Azolla* species were significantly correlated with [Cu] and [Zn] in plant biomass (Figure 6). Highly significant correlations between these two parameters ($r= 0.91^{**}$ and $r= 0.93^{**}$ in case of *A. pinnata*, and $r= 0.82^{**}$ and $r= 0.92^{**}$ in case of *A. japonica* for Cu and Zn, respectively) indicated that Cu and Zn amounts removed from the aqueous solutions by *Azolla* plants were mostly accumulated in plant biomass. This type of phytoremediation is termed as phytoextraction or phytoaccumulation, where contaminants, particularly inorganics, are removed by the plant parts. Thus, this type of phytofiltration is highly indispensable to clean up non-degradable HM from environment. *Azolla* species in the present study showed substantial potential for phytofiltration of Cu and Zn as evident from highly significant relationships between HM removed from the solutions and HM accumulated in plants.

Effect on Physiochemical Characteristics of *Azolla:* Color Changes and Falling Roots

Temporal changes in color (green to brown) of *A. pinnata* and *A. japonica* exposed to the highest [Zn] and [Cu]-treatments are depicted in Figure 7. Changes in color of *Azolla* fronds in response to Cu-treatments were first observed on the 2nd day of plants exposure to 5 and 7 mg L^{-1} Cu-treatments, and on the 3rd day to 2 and 4 mg L^{-1} Zn-treatments, respectively. Similar changes were also observed on the 5th day at 2 mg L^{-1} Cu and 1 mg L^{-1} Zn-treatments. These changes were very clear at the termination of

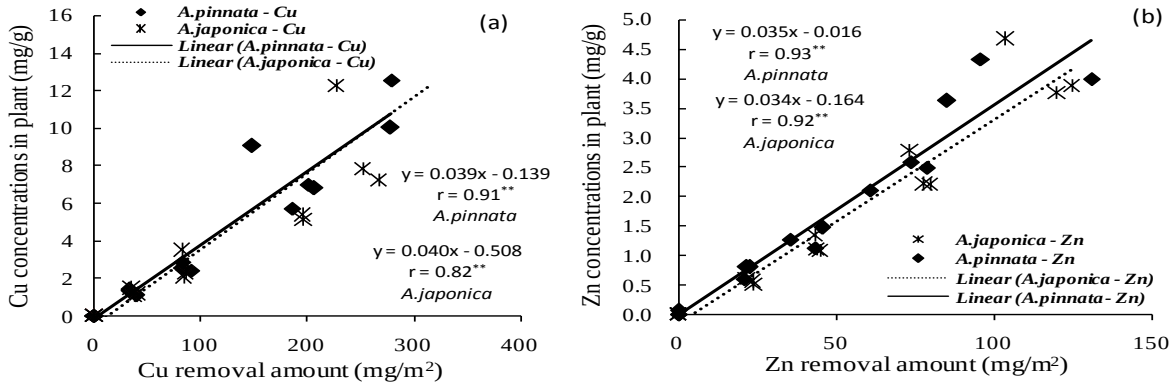

Figure 6. Relationships between removal amounts and metal concentrations in *Azolla* plants exposed to Cu and Zn treatments during different periods.

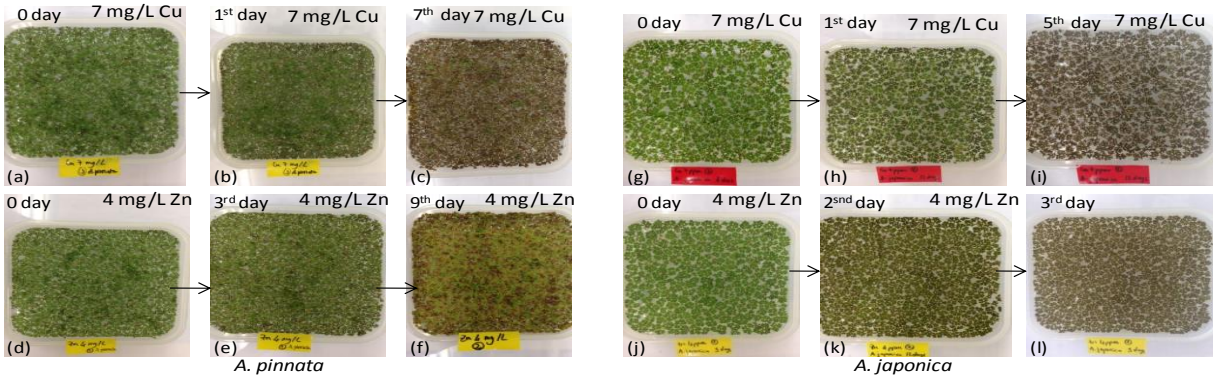

Figure 7. Color changes in *Azolla* fronds exposed to Cu and Zn treatments during different periods.

the experiments. During the same time, the fronds grown in the control and dilute metal treatments (1 mg L^{-1} Cu and 0.5 mg L^{-1} Zn-treatments) did not show any significant changes in the color of fronds. Roots of the fronds started to detach during 2nd day of exposure in 2 and 4 mg L^{-1} Zn-treatments. Moreover, detachment of the roots exposed to the high [Zn]-treatments was more when compared to the high [Cu]-treatments, suggesting the greater toxic strength of Zn for root shredding compared to Cu-treatments. *A. japonica* was more sensitive to high [Zn] than *A. pinnata.* Accumulation of HM produces certain physiobiochemical responses affecting the growth and growth related characteristics of aquatic macrophytes (Miretzky *et al.*, 2004; Mishra *et al.*, 2008). Toxic Cu levels can bring changes in N metabolism with a reduction of total N (Llorens *et al.*, 2000). In the present study, excessive [Cu] in solutions also inhibited the growth of *Azolla* species. Excessive Cu accumulated in plant tissues might be toxic to plants, affecting growth and plant biochemical processes. Exposure to excessive Zn normally leads to the oxidative damage and can change metalloenzymes of the plant by displacement or replacement of metal ions (Mishra and Tripathi, 2009). The strong reducing ability and high solubility of Zn could also cause more phytotoxicity to the *Azolla* plants in the present experiments.

CONCLUSIONS

Conclusively, *Azolla* species showed differential growth response and substantial metal removal efficiency when exposed to the solutions with different [Cu] and [Zn]-treatments during different incubation periods under controlled environmental conditions.

Lower [Cu] and [Zn] enhanced the plant growth and biomass, which can be attributed to the fact that plants utilized Cu and Zn as micronutrients for their growth; however, growth was significantly inhibited at higher metal concentrations during longer incubation periods. The higher the metal concentrations with longer incubation periods, the higher the metal removal amounts. Tested *Azolla* plants proved to be highly effective for metal removal. Plants exposure to high [Cu] and [Zn] during longer incubation periods might result in plants death due to phytotoxicity effect. In this study, fronds of *A. japonica* were more sensitive to toxicity compared to *A. pinnata*, which can be attributed to different frond morphology and differential metal uptake and translocation in plants. Highly significant correlations between metal removal amounts and metal concentrations in plant biomass indicated that phytoaccumulation was the possible mechanism for phytoremediation. This type of phytoremediation is highly indispensable to clean up non-degradable heavy metals from environment. The high values of bioconcentration factor obtained in the present study showed that A*zolla* species were hyperaccumulators of metals and could be deployed effectively for phytofiltration of Cu and Zn from the aqueous solutions. Results obtained in the present study will not only provide useful information for environmental managers but will also provide the necessary database for the scientists in their future ventures.

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برآورد توانایی *Azolla japonica* و*Azolla Pinnata* برای گیاه پالایی Cu و Zn و واکنش رشد نسبی آنها

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چکیده

در این پژوهش، برای برآورد واکنش رشد و توانایی گیاه پالایی (phytoremediation) آزولا A. japonica و A. pinnata، از آزمایش های میکروکاسم (microcosm) در محیط کنترل شده استفاده شد. به این منظور، گیاهان در معرض غلظت های مختلف Cu با نماد [Cu] (۰، ۱، ۲، ۵، و ۷ میلیگرم درلیتر) و Zn با نماد [Zn] (۰، ۰/۵، ۱، ۲، و ۴ میلی گرم در لیتر) همراه با تیمارهای شاهد در دوره های مختلف خواباندن (۰، ۳، ۶، و ۱۲ روز) قرار داده شدند. غلظت های کم [Cu] (کمتر از ۲ میلی گرم در لیتر) و[Zn] (کمتر از ۱ میلی گرم در لیتر) موجب ارتقای رشد گیاه شد، اما غلظت های بالاتر به طور موثری از رشد در دوره های خواباندن طولانی جلوگیری کرد. گونه های آزولا ظرفیت بالایی برای خارج سازی فلزات از محلول نشان دادند(به طور میانگین، راندمان خارج سازی در طی دوره های طولانی خواباندن برای Cu بیش ا ز۸۰٪ و برای Zn بیش از ۶۰٪ بود). در چنین دروه هایی،هرچه غلظت فلز بیشتر بود مقدار خارج سازی فلز هم بیشتر بود. قرار گرفتن گیاه درمعرض غلظت های بالای[Cu] و [Zn] در دوره های طولانی موجب تغییراتی در رنگ و جدا شدن (قطع) ریشه از گیاه شد که می تواند به مرگ گیاه به علت مسمومیت گیاهی (phytotoxicity) منجر شود.وجود رابطه های بسیار معنادار بین مقدار خارج سازی فلز و غلظت فلز در بیوماس (به ترتیب برای A. pinnata و A. japonica، $r = 0.91^{**}$ و 0.82^{**} در مورد Cu و $r =0.93^{**}$ و 0.92^{**} برای Zn) حاکی از آن است که انباشت گیاهی (phytoaccumulation) سازو کار ممکن برای گیاه پالایی است زیرا فلزات خارج شده از محلول در واقع در بیوماس گیاه انباشته شده بود. ارزش بالای فاکتور تغلیظ زیستی (bioconcentration) چنین اشاره داشت که گونه آزولا انباشتگر بوده و می توان از آن برای تصفیه گیاهی (گیاه پالایی) Cu و Zn استفاده کرد.