sustainability

MDPI

Article

Interspecific Variability in Growth Characteristics and Phytoremediation of Cu by Free-Floating *Azolla* Macrophytes

Muhammad Shahbaz Akhtar [1,2,*], Sohaib Aslam [1], Allah Ditta [3,4,*], Bedur Faleh A. Albalawi [5], Yoko Oki [2] and Yoshitaka Nakashima [2]

[1] Department of Environmental Sciences, Forman Christian College University, Lahore 54600, Pakistan
[2] Department of Environmental Management Engineering, Graduate School of Environmental and Life Science, Okayama University, Okayama 700-8530, Japan
[3] Department of Environmental Sciences, Shaheed Benazir Bhutto University, Sheringal, Dir (U) 18000, Pakistan
[4] School of Biological Sciences, University of Western Australia, Perth, WA 6009, Australia
[5] Department of Biology, University of Tabuk, Tabuk 47512, Saudi Arabia
* Correspondence: shahbazakhtar@fccollege.edu.pk (M.S.A.); allah.ditta@sbbu.edu.pk or allah.ditta@uwa.edu.au (A.D.)

Abstract: The phytoremediation potential of aquatic plants, particularly for Cu, is scarcely reported in the pertinent literature. In this regard, differential growth behavior and phytoaccumulation ability of three free-floating *Azolla* species (*A. japonica*, *A. pinnata*, and *A. hybrid*) were evaluated in a climatically controlled (a temperature of 25/20 °C, light/dark 16/8 h, a light intensity of 60 µmol m^{-2} s^{-1}, and a relative humidity of 65%) microcosm study. *Azolla* plants were exposed to solutions having three Cu concentrations (0, 3, and 6 mg L^{-1}) under two incubation periods (4 and 8 days). Different Cu treatments significantly reduced *Azolla* biomass during both incubation periods and *A. pinnata* was the most sensitive species. *Azolla* plants grown in aqueous solutions showed substantial variations in Cu removal capacity. Higher bioconcentration values displayed by *Azolla* plants indicated that these plants can be deployed as potential plants for Cu removal from Cu contaminated water. Nevertheless, the plants exposed to higher Cu concentrations displayed color changes and root detachment due to Cu phytotoxic effects which may also ultimately lead to plant death. Significant correlations between Cu removed from the aqueous solutions and Cu contents of plant biomass indicated that Cu phytoremediation by *Azolla* plants was due to the phytoaccumulation mechanism because the removed Cu from aqueous solutions was accumulated in plant biomass. Introduced *Azolla* species, i.e., *A. hybrid*, displayed comparable Cu removal efficiency with naturally grown *Azolla* species, i.e., *A. japonica* and *A. pinnata*. Tested *Azolla* species proved to be suitable candidates to remediate Cu contaminated water and can be deployed for phytoremediation.

Keywords: *Azolla* biomass; bioconcentration factor; Cu removal efficiency; Cu toxicity; translocation factor

Citation: Akhtar, M.S.; Aslam, S.; Ditta, A.; Albalawi, B.F.A.; Oki, Y.; Nakashima, Y. Interspecific Variability in Growth Characteristics and Phytoremediation of Cu by Free-Floating *Azolla* Macrophytes. *Sustainability* **2023**, *15*, 497. https://doi.org/10.3390/su15010497

Academic Editor: Maria Gavrilesc

Received: 22 November 2022
Revised: 21 December 2022
Accepted: 24 December 2022
Published: 28 December 2022

1. Introduction

Environmental pollution due to the substantial release of toxic metals into all environmental compartments has been accelerated due to abrupt changes in the processes of industrial manufacturing and many other anthropogenic activities [1,2]. Among hazardous inorganic contaminants in the aquatic environment, geologic, as well as anthropogenic discharges of heavy metals, are the real challenge for aquatic biodiversity because of their high mobility and potential toxicity. Some of the potential sources of release of heavy metals include weathering of parent materials/rocks, volcanic eruptions, agricultural activities, erosion, wastewater, electroplating, mining, milling, urban sewage and waste incineration, smelting, tanneries, automobile exhaust, and textile and chemical industries [3–5]. Because of higher persistence and resistance to degradation and biotransformation, pollution caused by these heavy metals is more problematic than that of organic substances [6–8].

Water polluted with heavy metals has serious concerns for aquatic biodiversity and has potential health-related issues for humans, particularly in resource-limited countries [9]. Among heavy metals, the annual average global release of copper (Cu) in different environmental components is about 939,000 metric tons [10]. Copper as a micronutrient is required for plant, animal, and human health; however, the same element can accumulate to high concentrations in living organisms and may potentially cause ecological damage [11,12]. Plant growth is also affected due to its toxic effects on their metabolic and developmental processes mainly due to photosynthetic inhibition, necrosis, chlorosis of leaves, hindrance in nutrient acquisition, and transport mechanisms [13,14]. Therefore, the potentially toxic effects of heavy metals, particularly of Cu on living organisms (animals, plants, and humans), impel us to treat or detoxify them and to purify our water environments. Different methods/protocols such as chemical precipitation and disinfection, exchange of ions, activated carbon adsorption process, nano-filtering, and electrolytic and reverse osmotic processes are available for this purpose; however, most of these processes are costly, less efficient, and not eco-friendly, as reviewed by Xu et al. [15]. Therefore, cost-effective, safe, and eco-friendly methods/approaches are direly needed to remediate our environment contaminated with these toxic metals. Phytoremediation is an attractive solar-driven technique to remediate these toxic metals from the aquatic environment [16].

Phytoremediation is a green technology that serves as an alternative or complementary biological technique for expensive, invasive, and energy-intensive engineering-based protocols to clean up the environment [16]. In this technique, highly persistent metals and contaminants can be remediated from the environment by deploying suitable plants, a few of which are commonly known as 'hyperaccumulators'. Xue et al. [5] and Valderrama et al. [17] reported that these hyperaccumulators possess favorable growth and physiological characteristics, such as better absorption, translocation, and accumulations, as well as the fact that these plants have a high tolerance against elevated levels of toxic metals [15,17]. In the plant kingdom, taxonomically widespread natural hyperaccumulators have >400 known species and constitute <0.2% of all angiosperms [18]. Furthermore, some higher land plants, such as plants of the Brassicaceae family, can produce sufficient biomass when these plants are exposed to higher metal levels [19]. Nevertheless, such plant species display low efficacy for phytoremediation due to their slow growth, less biomass accumulation, and low metal bioaccumulation ability [20,21]. Metal removal is the resultant product of plant biomass accumulation and plant tissue concentration; hence, these are the limiting factors for the phytoremediation of metals, as these factors depend upon the plant growth rate and their phytoaccumulation potential [17,22–24].

Among different types of phytoremediation, phytofiltration is a promising type of phytoremediation in which the sequestration of toxic metals and pollutants from the aquatic environment can be carried out by deploying suitable aquatic plants. These aquatic plants can be merged or submerged in water, or they can freely float on water. Merged and submerged plants clean up the environment by phytoaccumulating the metals on a whole-plant basis, whereas, free-floating types aquatic plants generally remove metals from water by absorbing these metals through their roots [25]. More recently, free-floating macrophytes displayed a substantial capability to deploy these plants as potential phytoremediator plants to remediate metals from contaminated water. Among free-floating macrophytes, the fast growth (capable to double their biomass in 3–9 days) [26], and the hyperaccumulation ability for metals, make the *Azolla* plants the best phytoremediator plants among different aquatic macrophytes [6].

It is generally believed that plant species grown in the natural environment are highly suitable for phytoremediation. However, data about the phytoremediation ability of introduced aquatic plants, particularly for Cu, is scarcely reported in the pertinent literature. In the present study, three *Azolla* ferns, i.e., two naturally growing *Azolla* species (*A. japonica* Fr. et Sav. and *A. pinnata* R. Br.) and one *Azolla* hybrid, i.e., *A. hybrid* (*A. cristata x A. filiculoides*) were exposed to different Cu concentration. We conducted microcosm experiments under climatically controlled environmental conditions to (1) evaluate the

relative growth response and phytofiltration of Cu from aqueous solutions in three *Azolla* species, (2) time the course and Cu concentration effects on plant growth characteristics to estimate the physiological response of *Azolla* species exposed to different levels of Cu, and (3) evaluate the effectiveness of removing Cu along with the potential of plants to accumulate Cu in biomass.

2. Materials and Methods

2.1. Experimental Protocol and Plant Culture Environment

2.1.1. Preparation of Plant Material and Cu Treatments

Freely floating aquatic *Azolla* plants, commonly known as mosquito ferns, belong to the monotypic genus, the *Azollaceae* family, salviniales order, the Polypodiopsida class, and and pteridophyta phylum [27]. Three *Azolla* ferns, i.e., naturally growing *Azolla* species (i.e., *A. japonica* Fr. et Sav. and *A. pinnata* R. Br.), and *Azolla* hybrid, i.e., *A. hybrid* (*A. cristata x A. filiculoides*) were tested in the present study. *Azolla* japonica was collected from the Hyogo prefecture (Toyooka city) (35°33′ N 134°49′ E), Japan. *Azolla* pinnata was taken from re-cultured laboratory stock of Okayama University, Okayama, (34°39′ N 133°55′ E), Japan. *Azolla* hybrid was collected from Sasagase river near Kojima Lake (the biggest artificial lake), Okayama, (34°28′ N 133°33′ E), Japan, where this fern forms mats in surrounding water bodies, as well as in Kojima Lake. *A. japonica* is characterized by stems having a thick pinnately branched pattern and leaves with lined tile patterns. It is commonly known as an endemic fern that is indigenously found in Japan. This fern displays a triangular pattern (approximately 1.5–7 cm long), is greenish in the summer season, and shows a pink appearance in the winter season. *A. pinnata* is adapted in most tropical and subtropical regions. *A. pinnata* is characterized by a roughly triangular shape (approximately 1.5–2.5 cm long with 1–2 mm long leaves) with side branches arranged with a pinnate pattern and it is longer toward the base.

These three *Azolla* species were exposed to different copper (Cu) treatments in the present study. The treatments were prepared in N-free modified 1% Hoagland medium by using analytical grade $CuSO_4 \cdot 5H_2O$ salt to make the Cu concentrations of 0, 3, and 6 mg L^{-1}. These *Azolla* species were exposed to different Cu solutions during two incubation periods, i.e., 4 and 8 days after transferring *Azolla* fronds. Photographic representations of plant materials, culture conditions, and experimental setups are represented in Figure 1.

2.1.2. Experimental Protocol and Culture Conditions

After collection, *Azolla* plants were re-cultured in a glass house located at the Tsushima campus, Okayama University, (34°39′ N 133°55′ E), Japan. To obtain reasonable biomass of plants, the collected plants were washed twice with tap water and re-cultured in 50 L capacity containers filled with 20 L of water and 10 kg of soil. To acclimatize the plants to a controlled environment before their exposure to the actual treatments, *Azolla* plants were re-cultured again in nutrition-free distilled water in a climatically controlled growth chamber for 3 days. Then, experimental treatments were applied to the plants to estimate the concentration and time course effects on the Cu removal ability of plants of three *Azolla* species.

Azolla fronds of uniform size and age were selected and weighed. Approximately eight gram fronds were exposed to different Cu concentrations during two different incubation periods to estimate Cu uptake and removal capacity. *Azolla* fronds were transferred in 1 L capacity plastic pots. Approximately, 800 mL of 1% modified Hoagland N-free medium (pH of 6.5 ± 0.2) was used for the growth of plants under a climatically controlled growth chamber (Eyela Eyeltron FLI-1001). Plants were grown in light/dark periods of 16/8 h, respectively, day and night temperatures of 25/20 °C, respectively, a light intensity of 60 ± 3 μmol m^{-2} s^{-1}, and a relative humidity of 65%. The elemental composition of 1% N-free modified Hoagland nutrient medium is represented in Table 1. Cu was added as three Cu concentrations, i.e., 0 (control), 3, and 6 mg L^{-1}, respectively, using analytical grade $CuSO_4 \cdot 5H_2O$ salt. Pots containing metal-contaminated solutions without plants

were considered blank treatments for comparison. To estimate growth characteristics and the comparison between metal-treated and metal-untreated solutions, *Azolla* plants were also grown in demineralized water and 1% modified Hoagland N-free medium. Three replications of each treatment were arranged in a completely randomized design (CRD). Plants were exposed to Cu treatments in the growth chamber for two incubation periods, i.e., 4 and 8 days after exposure to copper solutions. Pierced nylon covers with 200 holes per cover were used to cover all the experimental pots, including control treatments.

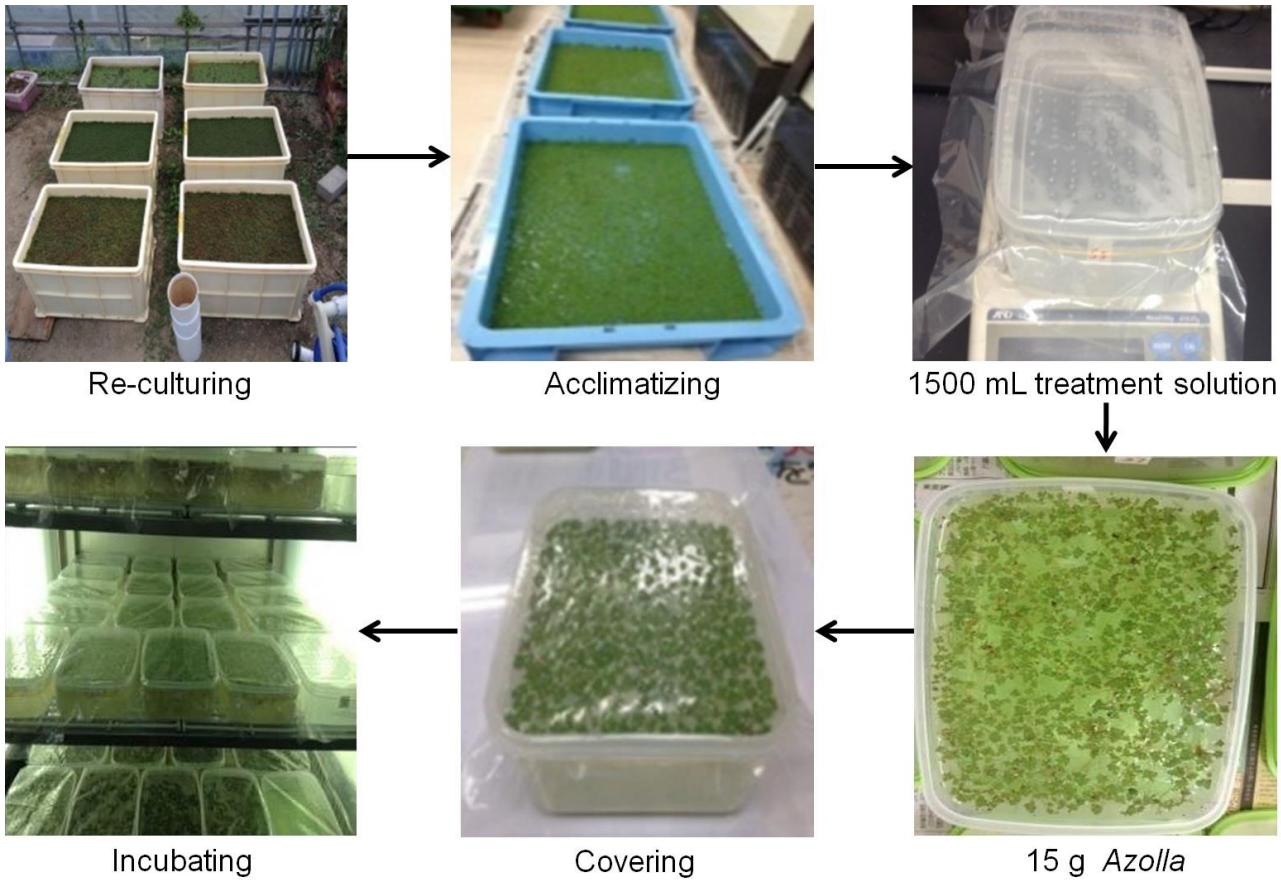

Figure 1. Experimental steps for phytoremediation of Cu by *Azolla* species exposed to different Cu solutions during two incubation periods.

Table 1. Composition of N-free modified (1%) Hoagland medium used in the experiment.

Nutrient	Salt/Compound Formula	Salt/Compound Names	Concentration (mg L^{-1})
Ca	$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate	2.06
K	KCl	Potassium chloride	2.00
Mg	$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate	0.48
Mn	$MnSO_4 \cdot 5H_2O$	Manganese sulfate pentahydrate	0.005
Fe	EDTA-Na-Fe·H_2O	Ethylenediaminetetraacetic acid disodium	0.062
Zn	$ZnSO_4 \cdot 6H_2O$	Zinc sulfate hexahydrate	0.007
Mo	$H_2MoO_4 \cdot H_2O$	Molybdic acid monohydrate	0.005
B	H_3BO_3	Boric acid	0.005

2.2. Plant Biomass Measurements and Metal Assay

After exposure of plants to Cu treatments for two incubation durations, i.e., 4 and 8 days, filtration of solution samples was performed with filter paper. Plants without exposure were considered control plants. All plants of the *Azolla* species were collected for further analysis. Plant fresh weights were recorded after washing samples twice with demineralized water followed by the draining of the excess amount of water. Then, the

dry mass of plants was recorded after drying the plants at 80 °C for one day by using a forced air-driven oven. After oven drying, these plants were ground into fine powder. Approximately 0.3 g of finely ground *Azolla* plant samples were taken into porcelain crucibles for ashing in a muffle furnace at 550 °C for 5 h. Crucibles with samples were taken out after the cooling process. Then, 5 mL of 2 N hydrochloric acid (HCl) was added to crucibles containing cooled ash samples and mixing was performed for the dissolution of plant samples in 2 N HCl. For those samples where digestion was incomplete, evaporation of solutions containing 5 mL of 2 N HCl was performed at 80 °C until the formation of pellets. Dissolution of obtained pellets was performed by dissolving them again in 5 mL of 2 N HCl followed by mixing. Filtration of sample solutions was performed into a 100 mL flask followed by three washings of crucibles with demineralized water, and flasks were filled by making the volume up to the mark. Elemental analysis was performed by using these filtered samples. An atomic absorption spectrometer (Hitachi Z-6100 Polarized Zeeman AAS) was used for Cu analysis in solution as well as in plant samples.

2.3. Tested Parameters

Different parameters to determine the potential of macrophytes for copper removal were estimated by following expressions. The amount of Cu removed was calculated by the following equation:

$$\text{Amount of Cu removed } \left(\text{mg m}^{-2}\right) = \frac{(V_i \times Cu_i) - (V_f \times Cu_f)}{\text{Surface area}}$$

where V_i = initial volume of water, Cu_i = initial concentration of Cu, V_f = volume of water at the end of the experiment, and Cu_f = final concentration of Cu.

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

$$\text{Cu removal rate } \left(\text{mg m}^{-2}\text{ day}^{-1}\right) = \frac{Cu_i - Cu_f}{\text{Surface area} \times \text{Treatment time}}$$

where Cu_i is the initial Cu amount and Cu_f is the Cu amount in solution at end of the experiment.

Cu removal efficiency was estimated by using the expression described by Zabihi et al. [28]:

$$\text{Cu removal efficiency } (\%) = \left[Cu_i - \frac{Cu_f}{Cu_i}\right] \times 100$$

where Cu_i is the initial Cu amount and Cu_f is the Cu amount in solution at end of the experiment.

The bioconcentration factor (BCF) is the ratio of Cu accumulated by the plants to that dissolved in the aquatic medium. The BCF was computed from [Cu], as described by Zayed et al. [29]:

$$\text{BCF} = \text{Cu concentration in plant biomass } \left(\text{mg kg}^{-1}\text{ DW}\right) / \text{Cu conc. in water } \left(\text{mg L}^{-1}\right)$$

The translocation factor (TF) was estimated by using the following expression:

$$\text{TF} = \text{Cu in plant tissue (parts)} / \text{Cu in the corresponding medium or root}$$

2.4. Quality Control and Quality Assurance

The present study was conducted under strict quality control and quality assurance by following standard protocols to ensure the reliability and accuracy of the laboratory tests and the results obtained. First of all, the experimental design included a blank containing contaminated water treatments without any plant and control with plants in the absence of Cu treatment to nullify any contamination or environmental effect. Each treatment was tested in three biological replicates. For different copper treatments and other experiment

work, the salts used were of analytical grade purchased from Sigma-Aldrich, St. Louis, MO, USA. During Cu analysis using an atomic absorption spectrometer (Hitachi Z-6100 Polarized Zeeman AAS), standard solutions were run before the sample analysis. Each sample was analyzed in triplicates and the mean values were accepted if the difference was within ±10%. To prevent cross-contamination, all laboratory glassware and plastics, including polypropylene digestion tubes and centrifuge tubes, were soaked in an HNO_3 bath (10% v/v) overnight, followed by rinsing three times with deionized water.

2.5. Statistical Analysis

All treatments were carried out in triplicates. Experimental data were subjected to statistical analysis using MS Excel and SPSS. All data presented in the figures show averages of three repetitions ± standard deviations. To see the statistical difference among the treatment, a p-value of 0.05 was set. The difference among the treatment means was based on the least significant difference (LSD) test. A simple Pearson correlation was also applied to determine the relationship between different variables.

3. Results and Discussion

3.1. Removed Amounts and Removal Rates of Cu from Solutions

Removed amounts of Cu by three *Azolla* species exposed to different Cu solutions (0, 3, and 6 mg L^{-1}) for two incubation periods (4 and 8 days) are represented in Figure 2A.

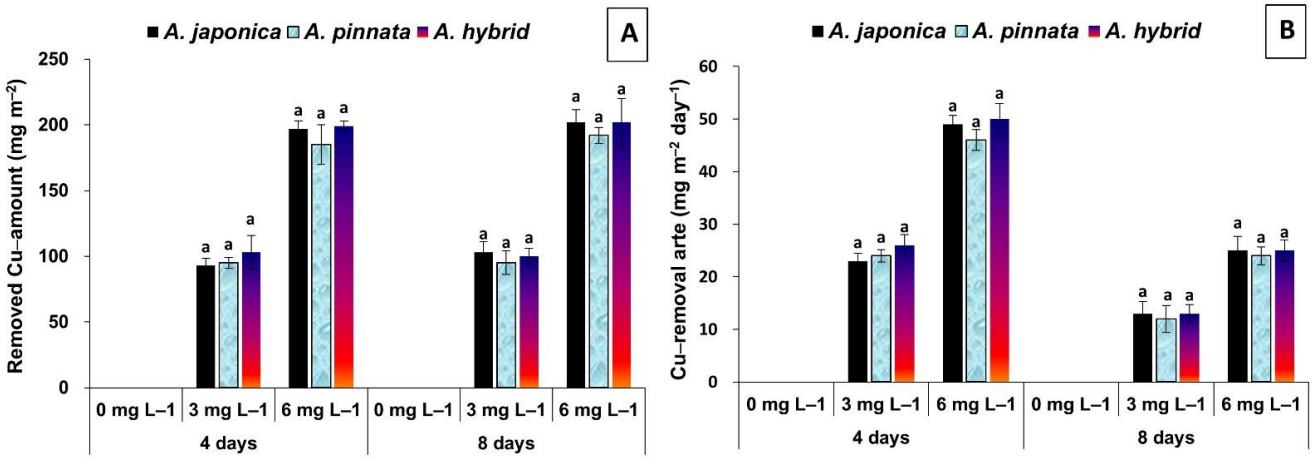

Figure 2. Removed amounts (**A**) and removal rates (**B**) of Cu by three *Azolla* species exposed to different Cu solutions for two incubation periods. Error bars show ±SE where n = 3. The letters on each column represent the statistical difference ($p \leq 0.05$) among different *Azolla* species at each Cu solution concentration.

Plants of all *Azolla* species displayed a similar trend in Cu removal rates, i.e., the higher the solution Cu concentrations, the higher the Cu removal rates from the aqueous medium. The highest removed amount of Cu (202 mg m^{-2}) was shown by *A. japonica* and *A. hybrid* from an aqueous medium after their exposure to the highest Cu concentration (6 mg L^{-1}) after 8 days of plant exposure. Cu removal amounts from solutions of the highest Cu concentrations were slightly but non-significantly increased ($p \leq 0.05$) when the plant's exposure time was increased from 4 days to 8 days. The Cu removal amount was increased from 93 mg m^{-2} on the fourth day to 103 mg m^{-2} on the eighth day when plants of *A. japonica* were exposed to 3 mg L^{-1} Cu treatment. Similarly, the Cu removal amount was decreased without any significant difference from 103 mg m^{-2} on the fourth day to 100 mg m^{-2} on the eighth day when plants of *A. hybrid* were exposed to 3 mg L^{-1} Cu treatment. However, the Cu removal amount was found to be the same in *A. pinnata* for both incubation times at 3 mg L^{-1} Cu treatment. At 6 mg L^{-1} Cu treatment, the Cu removal amount was increased from 197 mg m^{-2} on the fourth day to 202 mg m^{-2} on

the eighth day in *A. japonica*, 185 mg m^{-2} on the fourth day to 192 mg m^{-2} on the eighth day in *A. pinnata*, and 199 mg m^{-2} on the fourth day to 202 mg m^{-2} on the eighth day in *A. hybrid*, respectively. Obtained results showed that Cu treatments, incubation time, and *Azolla* species all affected the removed amounts of Cu from the aqueous medium. However, the removed amounts and removal rates of Cu by three *Azolla* species exposed to different Cu solutions for two incubation periods showed no significant difference. Therefore, based on the above results, the *Azolla* species might not affect the Cu removal. Previously, Mishra and Tripathi [13] and Mishra et al. [27] have reported a positive relationship between metal removal and increasing metal concentration in aquatic plants. Similarly, the *Azolla* species tested in our study can be potentially used to remove Cu from Cu contaminated solutions with varying concentrations.

Removal rates of Cu by three *Azolla* species exposed to different Cu solutions (0, 3, and 6 mg L^{-1}) are presented in Figure 2B. Cu removal rates were significantly different at two incubation times and different Cu concentrations. The fastest Cu removal rates were observed during the first four days of the plant's exposure to all Cu treatments. Cu removal rates were decreased after the fourth day of the plant's exposure. At 3 mg L^{-1} Cu concentration, removal rates decreased from 23 mg m^{-2} day^{-1} on the fourth day to 13 mg m^{-2} day^{-1} on the eighth day for *A. japonica*, from 24 mg m^{-2} day^{-1} on the fourth day to 12 mg m^{-2} day^{-1} on the eighth day for *A. pinnata*, and from 26 mg m^{-2} day^{-1} on the fourth day to 13 mg m^{-2} day^{-1} on the eighth day for *A. hybrid*. At 6 mg L^{-1} Cu concentration, removal rates decreased from 49 mg m^{-2} day^{-1} on the fourth day to 25 mg m^{-2} day^{-1} on the eighth day for *A. japonica*, from 46 mg m^{-2} day^{-1} on the fourth day to 24 mg m^{-2} day^{-1} on the eighth day for *A. pinnata*, and from 50 mg m^{-2} day^{-1} on the fourth day to 25 mg m^{-2} day^{-1} on the eighth day for *A. hybrid*. Higher removal rates were observed when plants were exposed to treatments with higher Cu concentrations. The Cu removal rate was increased from 23 mg m^{-2} day^{-1} at 3 mg L^{-1} to 49 mg m^{-2} day^{-1} at 6 mg L^{-1} Cu concentration during 4 days of exposure for *A. japonica*, from 24 mg m^{-2} day^{-1} at 3 mg L^{-1} to 46 mg m^{-2} day^{-1} at 6 mg L^{-1} Cu concentration during 4 days of exposure for *A. pinnata*, and from 26 mg m^{-2} day^{-1} at 3 mg L^{-1} to 50 mg m^{-2} day^{-1} at 6 mg L^{-1} Cu concentration during 4 days of exposure for *A. hybrid*. Similarly, the removal rate increased from 13 mg m^{-2} day^{-1} at 3 mg L^{-1} to 25 mg m^{-2} day^{-1} at 6 mg L^{-1} Cu concentration during 8 days of exposure for *A. japonica*, from 12 mg m^{-2} day^{-1} at 3 mg L^{-1} to 24 mg m^{-2} day^{-1} at 6 mg L^{-1} Cu concentration during 8 days of exposure for *A. pinnata*, and from 13 mg m^{-2} day^{-1} at 3 mg L^{-1} to 25 mg m^{-2} day^{-1} at 6 mg L^{-1} Cu concentration during 8 days of exposure for *A. hybrid*. The maximum removal rate was shown by *A. japonica* and *A. hybrid* species at 6 mg L^{-1} Cu concentration during 8 days of exposure compared to *A. pinnata*. Upadhyay et al. [28] tested five plant species (*Pistia stratiotes*, *Azolla pinnata*, *Eichorium crassipes*, *Spirodela polyrhizha*, and *Lemna minor*) and reported that these plant species removed Cu in the metal removal sequence of Fe > Cr > Cu > Cd > Zn > Ni. In the present study, substantial removal rates by *Azolla* species exposed to different Cu concentrations indicated that these plant species have sufficient potential to remove Cu from aqueous media. The inverse relationship between removal rate and exposure time for all treatments showed the time-dependent saturation of absorption sites in the roots of *Azolla* plants. Many other floating plants have been reported in the literature to remove Cu from water, e.g., *Lemna minor* [29], *Carex pseudocyperus*, *Carex riparia* [30], *Eichhornia crassipes*, *Pistia stratiotes* [5], and *Nasturtium officinale* [31].

3.2. Cu Concentration and Content in Azolla Plants

Cu concentrations (mg/g) in three *Azolla* species exposed to different Cu solutions are presented in Figure 3A. Incubation time and Cu concentrations in solution had significant effects on *Azolla* Cu content. Averaged over all treatments, final Cu concentrations in *Azolla* were approximately 15 times higher than initial Cu contents in *Azolla* plants. Cu concentrations in *Azolla* plants were increased with an increase in solution Cu concentrations, as well as incubation times. The highest Cu concentration (14.61 mg g^{-1} dry biomass of *Azolla*

fronds) was observed in *A. japonica* after 8 days of incubation when exposed to 6 mg L^{-1} Cu solution. At 3 mg L^{-1} Cu, *A. japonica*, *A. pinnata*, and *A. hybrid* showed 2.5, 1.9, and 2.3 times more Cu concentration after 8 days of exposure than after 4 days of exposure. Similarly, at 6 mg L^{-1} Cu, *A. japonica*, *A. pinnata*, and *A. hybrid* showed 2.9, 2.1, and 2.2 times more Cu concentration after 8 days of exposure compared to Cu concentration after 4 days of exposure.

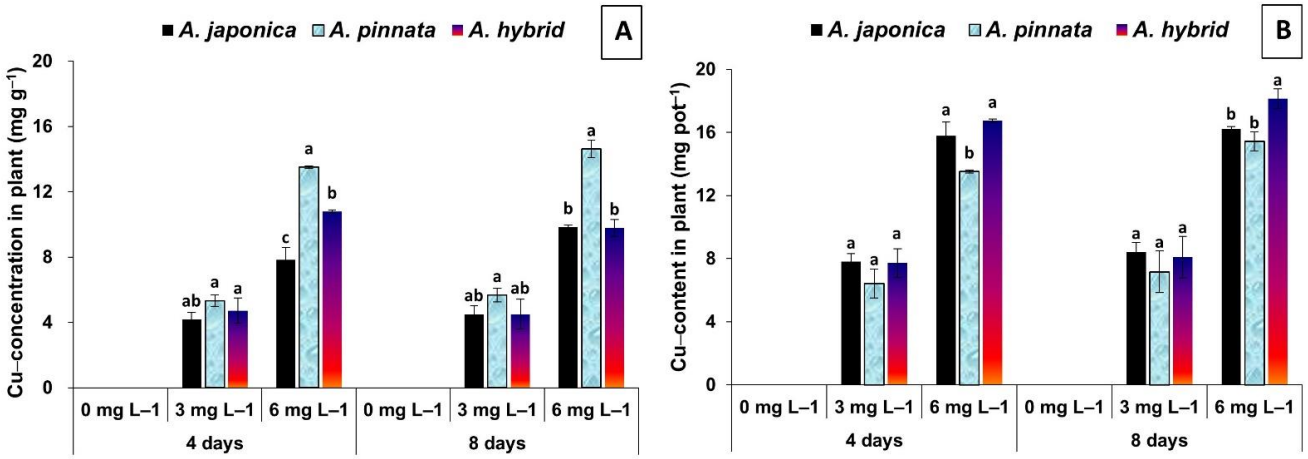

Figure 3. Cu concentration (**A**) and contents (**B**) of three *Azolla* species exposed to different Cu solutions for two incubation periods. Error bars show ±SE where n = 3. The letters on each column represent the statistical difference ($p \leq 0.05$) among different *Azolla* species at each Cu solution concentration.

Cu contents (mg/pot) of three *Azolla* species exposed to different Cu treatments are shown in Figure 3B. Averaged over all treatments, Cu contents in *Azolla* were approximately 18 times higher than initial Cu contents in *Azolla* plants. Cu contents in *Azolla* plants were increased with an increase in solution Cu concentration, as well as with incubation time. The highest Cu content (18.1 mg pot^{-1} of *Azolla* fronds) was observed in *A. hybrid* after 8 days of incubation period when exposed to 6 mg L^{-1} Cu solution. When comparing the incubation times, *A. japonica*, *A. pinnata*, and *A. hybrid*, respectively, showed 2, 2.1, and 2.2 times more Cu content at 8 days of exposure to 3 mg L^{-1} Cu solution and 1.9, 2.1, and 2.3 times more Cu content at 8 days of exposure to 6 mg L^{-1} Cu solution. On average, *A. hybrid* retained more amounts of Cu when exposed to higher Cu concentration, which can be attributed to its larger biomass accumulation. Metal tolerance and better metal accumulation by *Azolla* can be ascribed to suitable attributes, such as fast growth and better biomass accumulation ability of these floating ferns [22]. A plant's metal uptake efficiency is affected by metal concentration in the environment and that ultimately affects the plant's phytoremediation ability [32]. Results obtained in the present study are in agreement with Sela et al. [33] and Pandey [23] who exposed two *Azolla* species, *A.caroliniana* and *A. filliculoides*, to Zn and Cu solutions and reported that both plants substantially accumulated metal amounts in their biomass.

3.3. The Bioconcentration Factor (BCF) and the Translocation Factor (TF)

The bioconcentration factor (BCF) is a relative indicator determining the metal accumulation ability of plants to the metal concentration in the surrounding environment. Values of bioconcentration factors (BCFs) of three *Azolla* species exposed to different Cu solutions are presented in Figure 4A. Dry biomass was used to estimate BCF values. Interspecific variations were observed when plants were exposed to different Cu concentrations at two incubation periods. The highest BCF values 2250 and 2435 were found for *A. pinnata* when plants were exposed to 3 and 6 mg L^{-1} Cu solutions, respectively, for 8 days. During 4 days of incubation, BCF values of *A. hybrid* were higher than *A. japonica*. *A. pinnata* displayed the highest BCF values when exposed to Cu solutions at both concentration levels and both

incubation times (i.e., 4 days and 8 days), indicating its potential to accumulate metals. Nevertheless, a differential response of plants was observed in BCF values after their exposure to Cu.

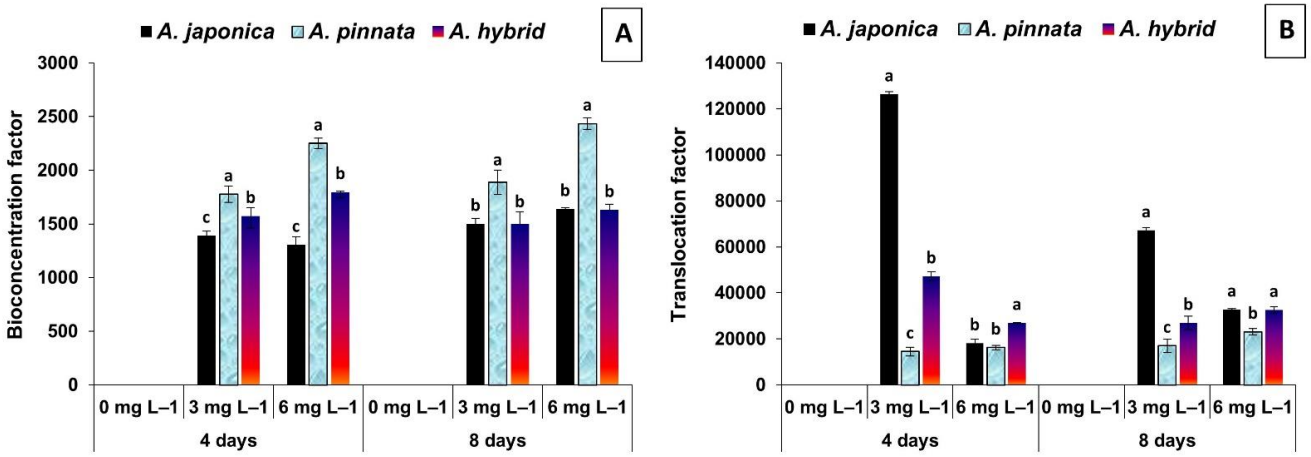

Figure 4. The bioconcentration (**A**) and translocation factors (**B**) in three different *Azolla* species exposed to different Cu solutions during two incubation periods. Error bars show ± SE where $n = 3$. The letters on each column represent the statistical difference ($p \leq 0.05$) among different *Azolla* species at each Cu solution concentration.

The BCF is an indicative parameter to estimate the suitability of plants for the phytoremediation of metals [23]. Higher BCF values (>1000) are indicating better suitability of plants to remediate contaminants from the environment [34,35]. The results of the present study are in agreement with Pandey (2012) who investigated that *A. caroliniana* plants showed better performance for phytoremediation of Cu and Zn because of their high BCF values. Plants displaying high BCF values are hyperaccumulators that can remediate even small amounts of contaminants by extracting these contaminants. The BCF values in the present study showed that plants of tested species can be deployed effectively for metal phytoremediation because of their high BCF values.

Translocation factor (TF) values of three different *Azolla* species exposed to different Cu solutions are shown in Figure 4B. The translocation factor is an indicator of metal translocation from rooting media to plant parts. Dry biomass was used to estimate TF values. Interspecific variations between plant species were observed when plants were exposed to different Cu solutions during two incubation periods. The highest TF values of 126,363 and 67,164 were observed in *A. japonica* when plants were exposed to 3 mg L^{-1} for 4 days and to 6 mg L^{-1} Cu treatments for 8 days, respectively. The TF values of 47,200 and 269,461 were observed in *A. hybrid* when plants were exposed to 3 mg L^{-1} Cu solution for 4 days and 6 mg L^{-1} Cu treatments for 8 days, respectively. Among Cu treatments and incubation periods, *A. pinnata* showed lower translocation values compared to the other two species. Results of the present study showed that different TF values are indicating the existence of substantial interspecific variations among tested *Azolla* species.

3.4. Biomass Assay

The effects of Cu concentrations and incubation times on the growth characteristics of three *Azolla* species (*A. pinnata*, *A. japonica*, and *A. hybrid*) are presented in Figure 5A. Maximum dry biomass was accumulated by three species under the control treatment (0 mg L^{-1} Cu treatment) at both incubation times. There was a decreasing trend in the biomass of plants with increasing Cu concentrations at both incubation times, except for *A. japonica* and *A. hybrid* at 6 mg L^{-1} Cu treatments at 8 days of incubation. *A. japonica* and *A. hybrid* accumulated more biomass than *A. pinnata* when plants were exposed to Cu solution. At 6 mg L^{-1} Cu concentration, *A. japonica* accumulated maximum biomass (47 mg m^{-2}) and *A. hybrid* accumulated maximum biomass (43 mg m^{-2}) compared to other species.

Biomass is an allosteric parameter and an important trait in growth and biomass analysis. Substantial growth response after exposure to Cu solutions indicated that these free-floating ferns have substantial potential for phytoremediation of contaminated aquatic medium. Our results agree with Zhao et al. [36] and Akhtar et al. [37], who reported that these aquatic ferns showed significant differences in their growth characteristic after exposure to metals. Cu treatments affected the growth behavior of tested *Azolla* species because changes in growth characteristics are the first responses of plants after their exposure to metals [38]. More biomass of plants at low Cu concentration compared to high Cu concentration might be due to the fact that Cu is also a micronutrient that can favor growth and can inhibit growth at higher metal concentrations due to metal stress. This also agrees with Singh et al. [39] and Hasan et al. [40], who reported similar results when *A. microphylla* and *A. filiculoides* were exposed to metals and plant biomass was compromised at higher concentrations. Interspecific variations in growth characteristics of *Azolla* species may also be attributed to the differential morphology of *Azolla* fronds. Fronds of *A. pinnata* are elongated to a root length of 2.5–7 cm with irregular branching and triangular shapes, while fronds of *A. japonica* and *A. hybrid* are comparatively larger than *A. pinnata* [41–43] and might be useful in efficient metal uptake. Fronds of *A. pinnata* are more impacted due to their sensitive nature to metals during long incubation periods at high Cu concentrations.

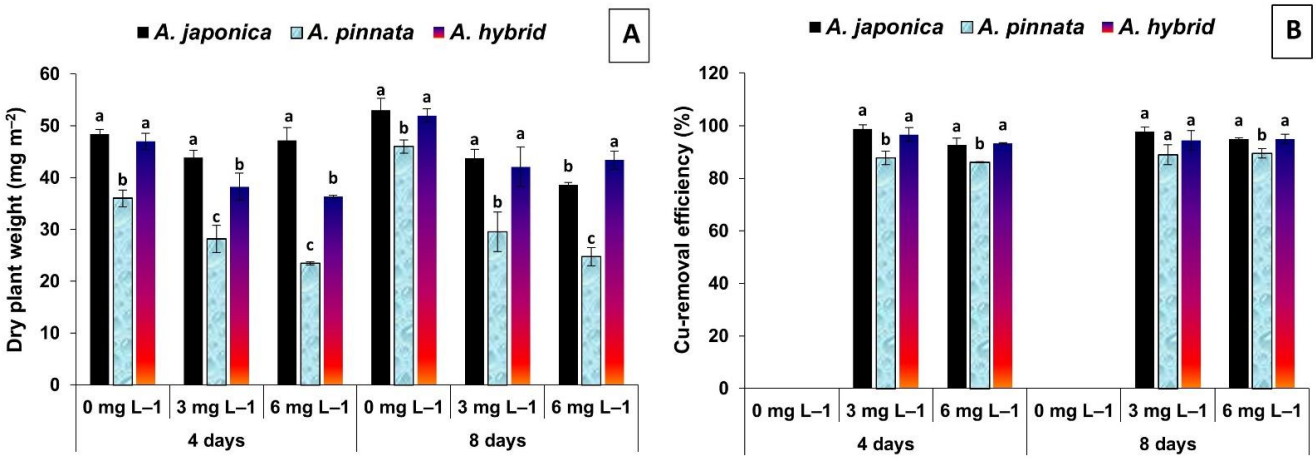

Figure 5. Dry plant biomass (**A**) and Cu removal efficiency (**B**) of three different *Azolla* species exposed to different Cu solutions during two incubation periods. Error bars show ±SE where $n = 3$. The letters on each column represent the statistical difference ($p \leq 0.05$) among different *Azolla* species at each Cu solution concentration.

3.5. Cu Removal Efficiency of Azolla Plants

The removal efficiency (%) of Cu by three *Azolla* species exposed to two Cu concentrations at two incubation times is depicted in Figure 5B. *A. japonica* and *A. hybrid* displayed higher Cu removal efficiency than *A. pinnata* after their exposure to both Cu concentrations at two incubation times. The maximum removal efficiency (98.9%) was depicted by *A. japonica* during 4 days of incubation at 3 mg L^{-1} Cu concentration and removal efficiency of 97.8% at 6 mg L^{-1} Cu concentration. *A. hybrid* showed 96.7% removal efficiency at 3 mg L^{-1} and 95% removal efficiency at 6 mg L^{-1} Cu concentration during 8 days of incubation.

The decrease in removal efficiencies at higher metal concentrations can be attributed to the time-dependent saturation of the plant's active sites with metals that can ultimately decrease the plant's capability for metal accumulation [44,45]. Results are in agreement with Valderma et al. [17] and Pandey [23], who documented that *A. caroliniana* and *A. filliculoides* showed significant variations in metal removal efficiencies. Because of higher Cu removal efficiencies in the present study, tested *Azolla* species can be considered better candidates for metal phytoremediation, and that can be ascribed to their better ability to absorb and translocate copper.

Cu concentrations in plant biomass of *Azolla* species have significant correlations with Cu removal amounts from solutions (Figure 6). It is plausible to conclude that the amount of Cu removed from aqueous solutions was accumulated into *Azolla* biomass because of highly significant relationships between these parameters. These two parameters were significantly correlated (R^2 = 0.98 ** ($p \leq 0.01$) in the case of *A. pinnata* and R^2 = 0.99 ** ($p \leq 0.01$) for both *A. japonica* and *A. hybrid*) for Cu removal and Cu accumulation in plant biomass in different Cu treatments during two incubation periods. This relationship indicates that this type of phytoremediation is phytoaccumulation, in which different inorganics are accumulated in plant biomass. Tested *Azolla* species displayed significant potential for phytoremediation of Cu which was evident from highly significant relations between these two parameters.

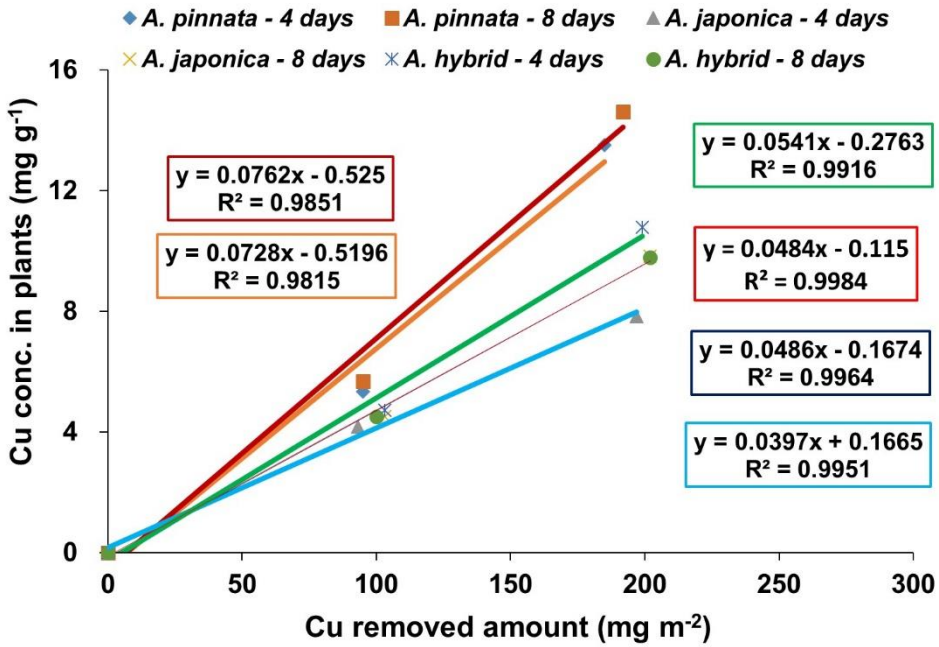

Figure 6. Relationship between Cu removed amounts from solutions and Cu concentrations in plant biomass.

Cu concentration in aquatic media had a significant impact on growth, which is evident from physicochemical changes in the growth characteristics of *Azolla* when exposed to high Cu concentrations in longer incubation periods. The color of the *Azolla* fronds changed from green to brown after exposure to two Cu treatments (Figure 7).

Color changes appeared after 4 days of exposure and were more pronounced when plants were exposed to higher Cu concentration (6 mg L^{-1} Cu) for a longer period. Nevertheless, changes in frond color were not observed in the plants grown in the control treatment (0 mg L^{-1} Cu). Similarly, detaching of plant roots was also pronounced during a longer incubation period for higher Cu treatment. Detaching of roots and changes in color were more pronounced in *Azolla pinnata* compared to other species, which might be due to the strength of Cu toxicity for plant morphological and physiological growth. Growth and growth-related parameters (e.g., altered nitrogen metabolism due to a reduced amount of total nitrogen) of macrophytes are impacted due to metal-induced physicobiochemical responses, particularly when these plants are exposed to higher metal concentrations for longer incubation times [7,27,46]. Similar findings were noted in our study where growth was inhibited during a longer incubation period that might be due to changes in plant biochemical processes/mechanisms due to excessive Cu accumulation in plant tissues.

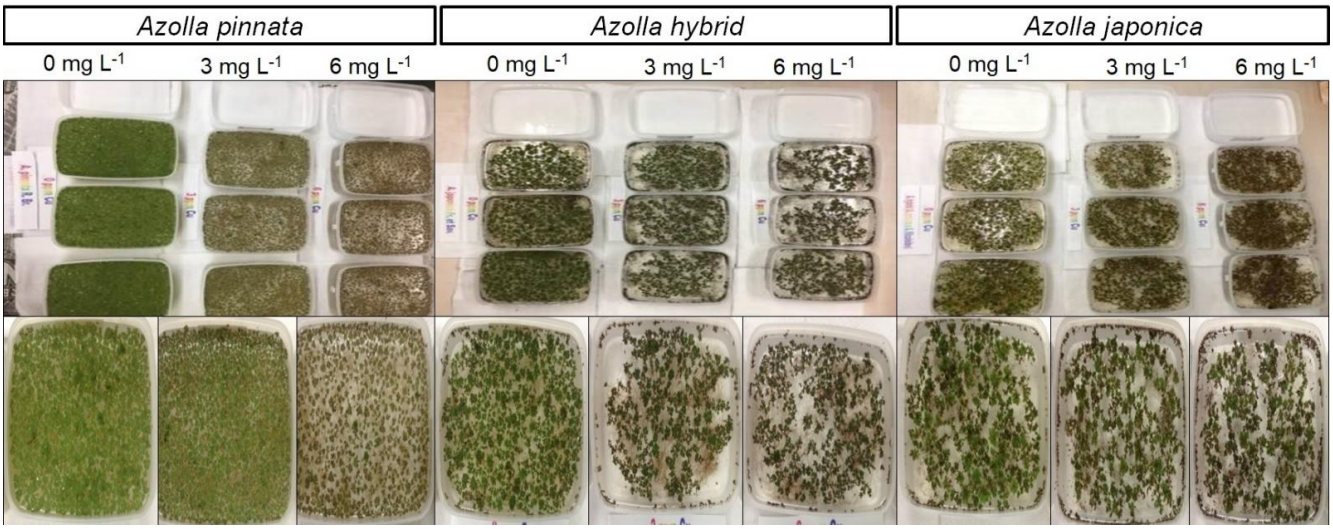

Figure 7. Three *Azolla* species were exposed to different Cu solutions during a longer incubation period.

4. Conclusions

Azolla species displayed substantial interspecific variability in growth characteristics and Cu removal efficiency after the plant's exposure to different Cu concentrations in a climatically controlled environment. The higher the Cu concentration in the solutions, the higher the Cu removal amount by the *Azolla* plants at both incubation periods. The Cu removal rate was higher at 4 days of the incubation period. *Azolla* plants exposed to higher Cu treatment exhibited higher Cu content in plants. *Azolla* plants showed a sufficient capacity for Cu removal due to their high bioconcentration (>1000) values and can be deployed effectively to remove Cu from Cu contaminated solutions. *A. hybrid* was very similar to *A. japonica*, being superior to *A. pinnata*, especially in terms of Cu removal efficiency. Nevertheless, compared with control plants, Cu concentration inhibited the growth of *Azolla* plants, particularly, of *A. pinnata.* Phytoaccumulation was the mechanism for phytoremediation because the removed Cu amounts were significantly correlated with plant Cu contents, indicating that the Cu removed from the solution was accumulated into plant biomass. Changes in color and detachment of the roots were more obvious in *Azolla* plants exposed to high Cu concentrations that can induce phytotoxicity. Further field trials are needed to validate the phytoremediation potential of these *Azolla* species.

Author Contributions: Conceptualization, M.S.A., S.A., A.D., B.F.A.A., Y.O. and Y.N.; Data curation, M.S.A. and Y.O.; Formal analysis, M.S.A., S.A., A.D., B.F.A.A., Y.O. and Y.N.; Funding acquisition, A.D. and B.F.A.A.; Investigation, M.S.A.; Methodology, M.S.A., Y.O. and Y.N.; Project administration, A.D., Y.O. and Y.N.; Resources, M.S.A., S.A., A.D., B.F.A.A., Y.O. and Y.N.; Software, M.S.A., S.A., A.D., B.F.A.A., Y.O. and Y.N.; Supervision, Y.O. and Y.N.; Validation, M.S.A., S.A., A.D., B.F.A.A., Y.O. and Y.N.; Visualization, M.S.A., S.A., B.F.A.A. and Y.N.; Writing—original draft, M.S.A. and S.A.; Writing—review and editing, M.S.A., S.A., A.D., B.F.A.A., Y.O. and Y.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors greatly acknowledge the technical support of the Environmental Center of Okayama University, Japan for the safe disposal of plants after phytoremediation.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Demim, S.; Drouiche, N.; Aouabed, A.; Semsari, S. CCD Study on the Ecophysiological Effects of Heavy Metals on Lemna Gibba. *Ecol. Eng.* **2013**, *57*, 302–313. [CrossRef]
2. Rai, P.K. Heavy Metal Pollution in Aquatic Ecosystems and Its Phytoremediation Using Wetland Plants: An Ecosustainable Approach. *Int. J. Phytoremed.* **2008**, *10*, 133–160. [CrossRef] [PubMed]
3. Zouboulis, A.I.; Loukidou, M.X.; Matis, K.A. Biosorption of Toxic Metals from Aqueous Solutions by Bacteria Strains Isolated from Metal-Polluted Soils. *Process Biochem.* **2004**, *39*, 909–916. [CrossRef]
4. Hargreaves, A.J.; Constantino, C.; Dotro, G.; Cartmell, E.; Campo, P. Fate and Removal of Metals in Municipal Wastewater Treatment: A Review. *Environ. Technol. Rev.* **2018**, *7*, 1–18. [CrossRef]
5. Ali, S.; Abbas, Z.; Rizwan, M.; Zaheer, I.E.; Yavaş, İ.; Ünay, A.; Abdel-DAIM, M.M.; Bin-Jumah, M.; Hasanuzzaman, M.; Kalderis, D. Application of Floating Aquatic Plants in Phytoremediation of Heavy Metals Polluted Water: A Review. *Sustainability* **2020**, *12*, 1927. [CrossRef]
6. Sood, A.; Uniyal, P.L.; Prasanna, R.; Ahluwalia, A.S. Phytoremediation Potential of Aquatic Macrophyte, Azolla. *Ambio* **2012**, *41*, 122–137. [CrossRef]
7. Miretzky, P.; Saralegui, A.; Cirelli, A.F. Aquatic Macrophytes Potential for the Simultaneous Removal of Heavy Metals (Buenos Aires, Argentina). *Chemosphere* **2004**, *57*, 997–1005. [CrossRef]
8. Briffa, J.; Sinagra, E.; Blundell, R. Heavy Metal Pollution in the Environment and Their Toxicological Effects on Humans. *Heliyon* **2020**, *6*, e04691. [CrossRef]
9. Rai, U.N.; Tripathi, R.D.; Vajpayee, P.; Jha, V.; Ali, M.B. Bioaccumulation of Toxic Metals (Cr, Cd, Pb, and Cu) by Seeds of Euryale Ferox Salisb. (Makhana). *Chemosphere* **2002**, *46*, 267–272. [CrossRef]
10. Singh, O.V.; Labana, S.; Pandey, G.; Budhiraja, R.; Jain, R.K. Phytoremediation: An Overview of Metallic Ion Decontamination from Soil. *Appl. Microbiol. Biotechnol.* **2003**, *61*, 405–412. [CrossRef]
11. Jain, S.K.; Vasudevan, P.; Jha, N.K. Azolla Pinnata r.Br. and Lemna Minor l. for Removal of Lead and Zinc from Polluted Water. *Water Res.* **1990**, *24*, 177–183. [CrossRef]
12. Lizieri, C.; Kuki, K.N.; Aguiar, R. The Morphophysiological Responses of Free-Floating Aquatic Macrophytes to a Supra-Optimal Supply of Manganese. *Water Air Soil Pollut.* **2012**, *223*, 2807–2820. [CrossRef]
13. Mishra, V.K.; Tripathi, B.D. Accumulation of Chromium and Zinc from Aqueous Solutions Using Water Hyacinth (*Eichhornia crassipes*). *J. Hazard. Mater.* **2009**, *164*, 1059–1063. [CrossRef]
14. Nagajyoti, P.C.; Lee, K.D.; Sreekanth, T.V.M. Heavy Metals, Occurrence and Toxicity for Plants: A Review. *Environ. Chem. Lett.* **2010**, *8*, 199–216. [CrossRef]
15. Xue, P.; Li, G.; Liu, W.; Yan, C. Copper Uptake and Translocation in a Submerged Aquatic Plant *Hydrilla verticillata* (L.f.) Royle. *Chemosphere* **2010**, *81*, 1098–1103. [CrossRef] [PubMed]
16. Pilon-Smits, E. Phytoremediation. *Annu. Rev. Plant Biol.* **2005**, *56*, 15–39. [CrossRef] [PubMed]
17. Valderrama, A.; Tapia, J.; Peñailillo, P.; Carvajal, D.E. Water Phytoremediation of Cadmium and Copper Using *Azolla filiculoides* Lam. in a Hydroponic System. *Water Environ. J.* **2013**, *27*, 293–300. [CrossRef]
18. McGrath, S.P.; Zhao, F.-J. Phytoextraction of Metals and Metalloids from Contaminated Soils. *Curr. Opin. Biotechnol.* **2003**, *14*, 277–282. [CrossRef]
19. Belouchrani, A.S.; Mameri, N.; Abdi, N.; Grib, H.; Lounici, H.; Drouiche, N. Phytoremediation of Soil Contaminated with Zn Using Canola (*Brassica napus* L.). *Ecol. Eng.* **2016**, *95*, 43–49. [CrossRef]
20. Neilson, S.; Rajakaruna, N. *Phytoremediation of Agricultural Soils: Using Plants to Clean Metal-Contaminated Arable Land BT—Phytoremediation: Management of Environmental Contaminants, Volume 1*; Ansari, A.A., Gill, S.S., Gill, R., Lanza, G.R., Newman, L., Eds.; Springer International Publishing: Cham, Switzerland, 2015; pp. 159–168, ISBN 978-3-319-10395-2.
21. Li, Y.; Luo, J.; Yu, J.; Xia, L.; Zhou, C.; Cai, L.; Ma, X. Improvement of the Phytoremediation Efficiency of Neyraudia Reynaudiana for Lead-Zinc Mine-Contaminated Soil under the Interactive Effect of Earthworms and EDTA. *Sci. Rep.* **2018**, *8*, 6417. [CrossRef]
22. Abhilash, P.C.; Pandey, V.C.; Srivastava, P.; Rakesh, P.S.; Chandran, S.; Singh, N.; Thomas, A.P. Phytofiltration of Cadmium from Water by *Limnocharis Flava* (L.) Buchenau Grown in Free-Floating Culture System. *J. Hazard. Mater.* **2009**, *170*, 791–797. [CrossRef] [PubMed]
23. Pandey, V.C. Phytoremediation of Heavy Metals from Fly Ash Pond by Azolla Caroliniana. *Ecotoxicol. Environ. Saf.* **2012**, *82*, 8–12. [CrossRef] [PubMed]
24. Ebbs, S.D.; Lasat, M.M.; Brady, D.J.; Cornish, J.; Gordon, R.; Kochian, L. V Phytoextraction of Cadmium and Zinc from a Contaminated Soil. *J. Environ. Qual.* **1997**, *26*, 1424–1430. [CrossRef]
25. Demim, S.; Drouiche, N.; Aouabed, A.; Benayad, T.; Couderchet, M.; Semsari, S. Study of Heavy Metal Removal from Heavy Metal Mixture Using the CCD Method. *J. Ind. Eng. Chem.* **2014**, *20*, 512–520. [CrossRef]
26. Arora, A.; Singh, P.K. Comparison of Biomass Productivity and Nitrogen Fixing Potential of *Azolla* spp. *Biomass and Bioenergy* **2003**, *24*, 175–178. [CrossRef]
27. Mishra, V.K.; Upadhyaya, A.R.; Pandey, S.K.; Tripathi, B.D. Heavy Metal Pollution Induced Due to Coal Mining Effluent on Surrounding Aquatic Ecosystem and Its Management through Naturally Occurring Aquatic Macrophytes. *Bioresour. Technol.* **2008**, *99*, 930–936. [CrossRef]

28. Upadhyay, A.R.; Mishra, V.K.; Pandey, S.K.; Tripathi, B.D. Biofiltration of Secondary Treated Municipal Wastewater in a Tropical City. *Ecol. Eng.* **2007**, *30*, 9–15. [CrossRef]
29. Al-Baldawi, I.A.; Yasin, S.R.; Jasim, S.S.; Abdullah, S.R.S.; Almansoory, A.F.; 'Izzati Ismail, N. Removal of Copper by Azolla Filiculoides and Lemna Minor: Phytoremediation Potential, Adsorption Kinetics, and Isotherms. *Heliyon* **2022**, *8*, e11456. [CrossRef]
30. Schück, M.; Greger, M. Screening the Capacity of 34 Wetland Plant Species to Remove Heavy Metals from Water. *Int. J. Environ. Res. Public Health* **2020**, *17*, 4623. [CrossRef] [PubMed]
31. Khalid, K.M.; Ganjo, D.G.A. Native Aquatic Plants for Phytoremediation of Metals in Outdoor Experiments: Implications of Metal Accumulation Mechanisms, Soran City-Erbil, Iraq. *Int. J. Phytoremed.* **2021**, *23*, 374–386. [CrossRef]
32. Rai, U.N.; Chandra, P. Accumulation of Copper, Lead, Manganese and Iron by Field Populations of Hydrodictyon Reticulatum (Linn.) Lagerheim. *Sci. Total Environ.* **1992**, *116*, 203–211. [CrossRef] [PubMed]
33. SELA, M.; GARTY, J.; TEL-OR, E. The Accumulation and the Effect of Heavy Metals on the Water Fern Azolla Filiculoides. *New Phytol.* **1989**, *112*, 7–12. [CrossRef]
34. Zhu, Y.L.; Zayed, A.M.; Qian, J.-H.; de Souza, M.; Terry, N. Phytoaccumulation of Trace Elements by Wetland Plants: II. Water Hyacinth. *J. Environ. Qual.* **1999**, *28*, 339–344. [CrossRef]
35. Zayed, A.; Gowthaman, S.; Terry, N. Phytoaccumulation of Trace Elements by Wetland Plants: I. Duckweed. *J. Environ. Qual.* **1998**, *27*, 715–721. [CrossRef]
36. Zhao, M.; Duncan, J.R.; van Hille, R.P. Removal and Recovery of Zinc from Solution and Electroplating Effluent Using Azolla Filiculoides. *Water Res.* **1999**, *33*, 1516–1522. [CrossRef]
37. Akhtar, M.S.; Oki, Y.; Bich, B.T.N.; Nakashima, Y. Estimation of Phytofiltration Potential for Cu and Zn and Relative Growth Response of Azolla Japonica and Azolla Pinnata TT—برآورد توانایی Azolla Japonica و Azolla Pinnata برای گیاه پالایی Cu و Zn و واکنش رشد نسبی آنها. *Mdrsjrns* **2019**, *21*, 895–909.
38. Hagemeyer, J. *Ecophysiology of Plant Growth under Heavy Metal Stress BT—Heavy Metal Stress in Plants: From Biomolecules to Ecosystems*; Prasad, M.N.V., Ed.; Springer: Berlin/Heidelberg, Germany, 2004; pp. 201–222.
39. Singh, S.S.; Mishra, A.K.; Upadhyay, R.S. Potentiality of Azolla as a Suitable P-Biofertilizer under Salinity through Acid Phosphatase Activity. *Ecol. Eng.* **2010**, *36*, 1076–1082. [CrossRef]
40. Hasan, S.H.; Talat, M.; Rai, S. Sorption of Cadmium and Zinc from Aqueous Solutions by Water Hyacinth (*Eichchornia crassipes*). *Bioresour. Technol.* **2007**, *98*, 918–928. [CrossRef]
41. Madeira, P.T.; Center, T.D.; Coetzee, J.A.; Pemberton, R.W.; Purcell, M.F.; Hill, M.P. Identity and Origins of Introduced and Native Azolla Species in Florida. *Aquat. Bot.* **2013**, *111*, 9–15. [CrossRef]
42. Pereira, A.L.; Martins, M.; Oliveira, M.M.; Carrapiço, F. Morphological and Genetic Diversity of the Family Azollaceae Inferred from Vegetative Characters and RAPD Markers. *Plant Syst. Evol.* **2011**, *297*, 213–226. [CrossRef]
43. Wagner, G.M. Azolla: A Review of Its Biology and Utilization. *Bot. Rev.* **1997**, *63*, 1–26. [CrossRef]
44. Haghnazar, H.; Hudson-Edwards, K.A.; Kumar, V.; Pourakbar, M.; Mahdavianpour, M.; Aghayani, E. Potentially toxic elements contamination in surface sediment and indigenous aquatic macrophytes of the Bahmanshir River, Iran: Appraisal of phytoremediation capability. *Chemosphere* **2021**, *285*, 131446. [CrossRef] [PubMed]
45. Haghnazar, H.; Belmont, P.; Johannesson, K.H.; Aghayani, E.; Mehraein, M. Human-induced pollution, and toxicity of river sediment by potentially toxic elements (PTEs) and accumulation in a paddy soil-rice system: A comprehensive watershed-scale assessment. *Chemosphere* **2023**, *311*, 136842. [CrossRef] [PubMed]
46. Llorens, N.; Arola, L.; Bladé, C.; Mas, A. Effects of Copper Exposure upon Nitrogen Metabolism in Tissue Cultured Vitis Vinifera. *Plant Sci.* **2000**, *160*, 159–163. [CrossRef] [PubMed]