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Rhizofiltration of Pb by *Azolla pinnata*

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ABSTRACT

Plant based technologies such as phytoremediation, are cost effective and environmentally friendly for the removal of toxic heavy metals from polluted water ways. Aquatic plants are well known for accumulating and concentrating heavy metals. This study shows the potential of *Azolla pinnata* to remove Pb(II) from aqueous environment through rhizofiltration, which is one of the phytoremediation strategies. The effect of nutrient concentration, initial metal concentration and exposure period on lead uptake capacity of *A. pinnata* was studied. A modified Hoagland's nutrient solution was used as the growth medium. Lead uptake capacity in plants increased with decreasing nutrient concentration in growth medium. Increasing concentrations of lead in the growth medium increased the bio concentration factor of *A. pinnata* up to an optimum value of 1220 while the relative growth of the plants was significantly decreased. The results of the time course study showed that the efficiency of lead removal depends on the duration of exposure. The maximum uptake of lead was 1383 mg/kg of *A. pinnata* dry weight after four days of treatment where the lead concentration in the growth medium has reduced by 83%. It is concluded that *A. pinnata* is a potential candidate for the removal of Pb from polluted waterways.

Keywords: Phytoremediation; Lead; Aquatic plants; Bio concentration factor; Nutrients.

1. Introduction

Pollution of aquatic environments by heavy metals is one of the major threats to the water resources of the world today. Heavy metal pollutants are of concern because their non-degradability creates a hazard when discharged into a water body. Lead is a common heavy metal found in industrial effluents, particularly in developing countries, where legislative measures of control are either lacking or are not strictly enforced. The main sources of lead contamination are mining and smelting activities, batteries, paints and automobiles (Kim et al., 2003; Sekhar et al., 2005; Ruley et al., 2006). Over 40 million children worldwide are threatened by lead poisoning and 97% of them are living in developing countries (CEJ 2012). Lead is toxic to many organs and tissues in the human body including heart, kidneys, reproductive and nervous systems (Waranusantigul et al., 2011). Excess lead in plants causes growth reduction, chlorosis, inhibition of photosynthesis, alteration of the mineral nutrition and water balance (Sharma and Dubey, 2005). The limits for lead in water as stipulated by the US-Environmental Protection Agency (EPA) are 0.015 mg L^{-1} and 0.20 mg L^{-1} for drinking water and effluent, respectively. Consequently removal of Pb(II) from industrial wastewater is important.

In Sri Lanka, paints are a major source of Pb pollution. According to the Centre for Environmental Justice (CEJ 2012), lead content in some paints in Sri Lanka is as high as

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137,325 mg L^{-1}. This value is more than 1500 times greater than the US limit of 90 mg $L^{-1}$and more than 200 times greater than the Sri Lankan limit of 600 mg $L^{-1}$for lead content in paint.

Several conventional physico-chemical methods, such as membrane filtration (Yoon et al.,2009), chemical precipitation (Matlock et al., 2002; Ramos et al., 2009), ion exchange (Inglezakis and Loizidou, 2007), chemical oxidation or reduction (Mitra et al., 2011), electrochemical treatment (Rana et al., 2004), solvent extraction (Miretzky et al., 2006) and activated carbon adsorption (Malik, 2003) have been used to remove heavy metals and other contaminants from effluents. However, the application of these methods has been limited especially in developing countries since they are not economical, require expensive equipment and generate secondary waste.

Phytoremediation is a cost effective and environmental friendly technology to clean up the aquatic systems contaminated with heavy metals using plants (Salt et al., 1995). Rhizofiltration is one of the phytoremediation strategies, which uses plant roots to absorb, concentrate and precipitate heavy metals from water (Ensley, 2000). Plants that can accumulate and tolerate high levels of heavy metals are good candidates for phytoremediation (Sekhar et al., 2005; Ruley et al., 2006). The physiological processes and phytoaccumulation efficiency are dependent on the specific composition of polluted streams and the climate regime in the country (Veselý et al., 2011). Many terrestrial and aquatic plants have been screened for their ability to take up heavy metals from contaminated aquatic systems including ground water (Bennicelli et al., 2004; Miretzky et al., 2004; Khellaf and Zerdaoui, 2009; Narain et al., 2011; Veselý et al., 2011).

This paper presents a study of the rhizofiltration potential of aquatic plant species, *Azolla pinnata* to remove Pb(II) from aqueous solutions and further investigations into the effect of nutrients on lead uptake, uptake capacity and removal efficiency.

2. Material and methods

2.1 Instrumentation and chemicals

Analytical grade chemicals and reagents were used in all experiments. Distilled water was used to prepare all aqueous solutions. The pH of solutions was measured using a pH meter (Thermo Russell Model RL060P). A muffle furnace (Lenton Model EF 11/8) was used to ash the plant material. Lead was analysed using atomic absorption spectrophotometer (AAS) (GBC Model 932 AB Plus) at the wavelength of 283.3 nm using air-acetylene flame.

2.2 Multiplication of aquatic plants

A. pinnata was collected from water ways in the Colombo district. The plants were identified at the National Herbarium of the Royal Botanical Garden at Peradeniya, Sri Lanka. Mature healthy plants were selected for all the experiments. Plants were rinsed with tap water to remove any epiphytes and insect larvae growing on the plants. Plants were acclimatised in fresh water for 3 – 7 days in a green house before use in the experiments.

2.3 Composition of 100% modified Hoagland's nutrient stock solution

The 100% nutrient solution consisted of 5.0 mmol L^{-1} KNO_3, 1.0 mmol L^{-1} $NH_4H_2PO_4$, 4.0 mmol L^{-1} $Ca(NO_3)_2$, 2.0 mmol L^{-1} $MgSO_4$, 10.0 µmol L^{-1} $MnSO_4$, 0.7 µmol L^{-1} $ZnSO_4$, 0.3 µmol L^{-1} $CuSO_4$, 50.0 µmol L^{-1} H_3BO_3, 0.1 µmol L^{-1} Na_2MoO_4 and 100.0 µmol L^{-1} Iron(II)

ethylenediaminetetraacetic acid (FeEDTA). All the nutrient solutions of different strengths were prepared in distilled water by appropriate dilution of the stock solution and their pH was maintained at 5.5 using HNO_3 and NaOH.

2.4 Effect of nutrient strength on Lead(II) uptake

A series of hydroponic media of different Pb(II) concentrations with different nutrient concentrations was prepared. The Pb concentration in the test solutions varied from 2 - 10 mg L^{-1} while the nutrient concentration varied from 10% to 75%. For 2.0 L of each test solution with a specific Pb concentration and a specific nutrient concentration, 5.0 g of pre-acclimatized *A. pinnata* was introduced. After eight days of culture, plants were harvested, washed and dried.

2.5 Uptake capacity of Lead(II) by *A. pinnata*

In this experiment, test solutions of different Pb(II) concentrations (2 - 10 mg L^{-1}) were prepared in 10% modified Hoagland's nutrient solution; 10% was selected based on the study of effect of nutrient strength on Lead(II) uptake. Black plastic basins were filled with 2 L of test solutions. Healthy plants of equal size were selected and each plant was carefully blotted on filter paper and their initial wet weight was recorded and introduced into the test solution. The experiment was run in triplicate for 7 days at ambient temperature (28 – 30 °C). An experimental set-up without the metal served as control. At the end of the experiment, plants were harvested and washed thoroughly in running water, followed by 10 mmol L^{-1} solution of disodium salt of ethylenediaminetetraacetic acid (EDTA) and deionised water.

2.6 Removal efficiency- time course study on Lead(II) uptake by *A. pinnata*

In this experiment, the initial fresh weight of *A. pinnata* was 4.6 g and the best concentration of Pb(II) (4.0mg L^{-1}) from uptake capacity experiment was used. The plants were allowed to grow for 5 days at ambient temperature (28 – 30 °C) in 500.0 mL of 10% modified Hoagland's solution containing 4.0 mg L^{-1} Pb(II). An experimental set up with distilled water served as control. Deionised water was added occasionally to compensate for water loss through plant transpiration and evaporation. The experiment was run in triplicate. Plant biomass and test solution in each treatment were withdrawn for analysis at 24 hours intervals for 5 days.

2.7 Digestion and analysis of plant material

After recording the fresh weights of harvested plants after washing, they were dried at 60 °C for three days and subsequently the dry weights were determined. Plant biomass was digested by dry ashing according to Hoenig et al., (1998). Dried, powdered plant sample in a crucible was placed in a cold muffle furnace and the temperature was progressively elevated to 450 °C over two hours and held for four hours. After cooling, a drop of distilled water was added, and then 5.0 mL conc. HNO_3 was added to the ash. The sample was slowly heated on a sand bath for 30 minutes at 120-130 °C. To this, 5.0 mL of hydrogen peroxide was added with care in small amounts to avoid strong foaming. The heating was continued at that temperature until a clear solution was obtained. The solution was cooled and its volume made up to 50.00 mL by adding distilled water. The samples were analysed by AAS to determine their lead content.

2.8 Relative growth and bio-concentration factor

Relative growth (Lu et al., 2004) of the plants during the experiment and the bio-concentration factor (BCF) were calculated as follows.

Relative growth = Final fresh weight / Initial fresh weight (1)

$$BCF = \frac{\text{Concentration of metal in dried plant tissue } (\mu g\ g^{-1})}{\text{Initial concentration of metal in external solution } (mg\ L^{-1})} \quad (2)$$

Bio-concentration factor is a useful parameter to evaluate the potential of plants for accumulating metals (Lu et al., 2004; Mun et al., 2008).

2.9 Statistical analysis

The data were statistically analysed by one-way analysis of variance (ANOVA) using the computer software MiniTab to determine the significance of differences between the pairs of means. The treatment means were compared using Tukey's 95% simultaneous confidence intervals test. The differences were statistically significant when $P < 0.05$.

3. Result and conclusion

3.1 Effect of nutrient strength on lead uptake

The growth of *A. pinnata* was normal up to 6.0 mg L^{-1} of lead concentration at all levels of nutrient solution. Toxic effects on *A. pinnata* grown under increasing external Pb concentrations appeared at lower nutrient strength rather than at higher nutrient strength. *A. pinnata* treated at 10 mg L^{-1} in 10% nutrient strength showed toxic symptoms on the fifth day of the experiment while the species exposed to the same concentration but at 75% nutrient strength were normal and fresh up to five days. Similar observation was made in water spinach by Gothberg et al., (2004).

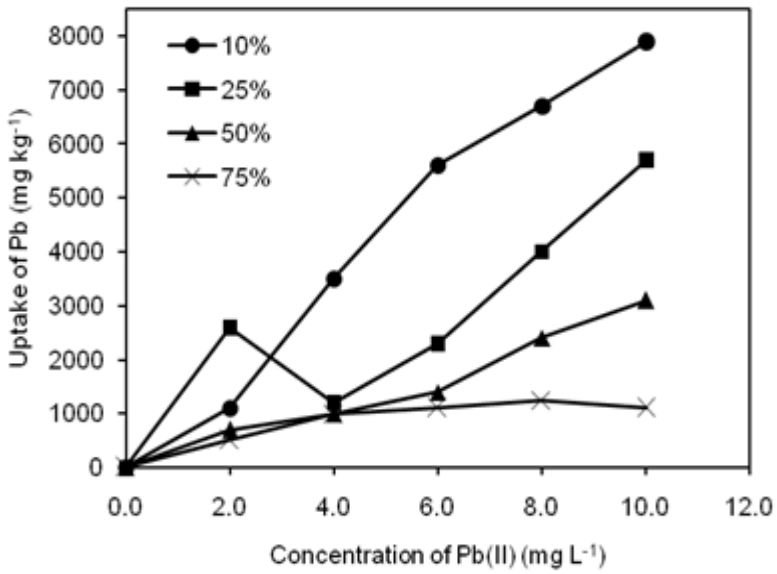

Figure 1: Uptake of lead by *A. pinnata* in different strengths of modified Hoagland's nutrient solution with varying concentration of lead after 8 days of hydroponic culture (n = 3).

Uptake of lead by *A. pinnata* in different strengths of modified Hoagland's nutrient solution with varying concentration of lead is given in Figure 1. The highest absorption of Pb was observed at 10% nutrient level and the absorption decreased with increasing concentrations of Hoagland's solution. The control plants did not show any Pb in its tissues. Previous studies also showed similar results of the influence of nutrient level in the growth medium on the uptake of toxic metals by plants. The net uptake of Cd in the roots of sugar beet (*Beta vulgaris* L.*)* was greater when the nutrient concentration was minimal, rather than optimal (Greger et al., 1991**)**. The Cd uptake rate in *Eichhornia crassipes* was much higher in deionized water than in 50% Hoagland's nutrient solution (O'Keeffe et al., 1984). The strength of the external nutrient solution was important for the accumulation and toxicity of heavy metals in water spinach (Gothberg et al., 2004).

In a nutrient enriched environment, the bioavailable fraction of metals may be reduced as a result of binding to nutrient anions. The uptake of heavy metals in plants may also be affected by competition, since nutrient cations compete with the metal for uptake sites (Greger, 1999). Thus, the uptake of the metal under investigation decreases with increasing concentration of nutrients. However, a generous availability of nutrients promotes plant growth, which in turn creates an increasing number of uptake sites for metal in the plants. This may increase the uptake, and metal concentrations in plants may be expected to increase, decrease or stay constant, depending on the relative responses of metal uptake and growth rate.

3.2 Lead uptake capacity of *A. pinnata*

The relative growth of *A. pinnata* exposed to Pb at each concentration decreased significantly ($P \leq 0.05$) with respect to the control (Figure 2). At high Pb concentrations, *Azolla* growth was reduced (59%) after 7 days. In a similar study Lamai et al., (2005) reported that the relative growth of green alga *Cladophora fructa* exposed to lead was significantly reduced when metal concentrations were increased.

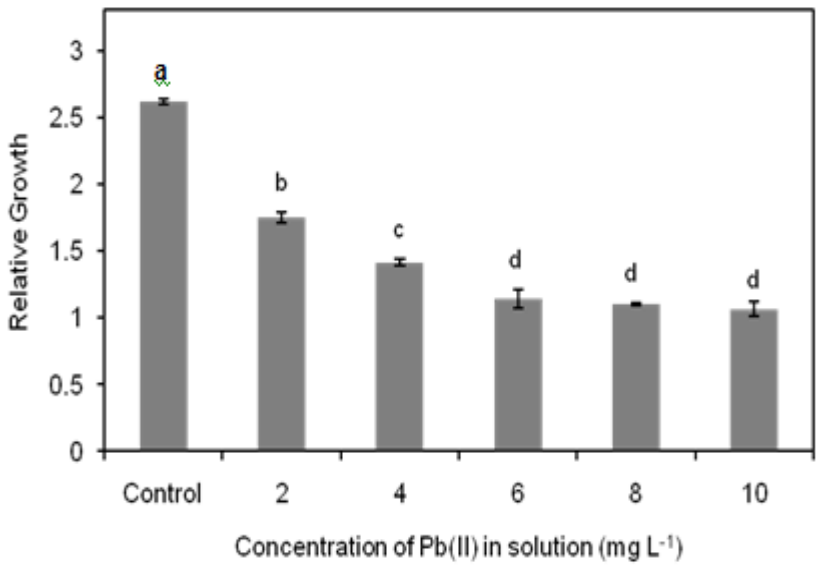

Figure 2: Average relative growth of *A. pinnata* in different concentrations of lead compared to the control after 7 days of growth in 10% modified Hoagland's medium. Bars indicate mean ± SD, where n = 3. Different letters indicate statistically significant differences between treatments ($P < 0.05$) based on Tukey's 95% simultaneous confidence intervals test.

Pb accumulation in *A. pinnata* at different concentrations of Pb is shown in Figure 3. Plants treated with 10.0 mg L^{-1} of Pb, accumulated 10,590 mg of Pb/kg of dry weight (DW) of plants in 7 days while control plants showed an accumulation of only 20 mg/kg of DW. The uptake of Pb in plants increased significantly ($P \leq 0.05$) when Pb concentration was increased and the differences between the pairs of mean absorption were also significant. Plants of the families Brassicaceae, Euphorbiaceae, Asteraceae and Lamiaceae have also been identified as having a good potential for extraction of Pb from soil (Romeiro et al., 2006).

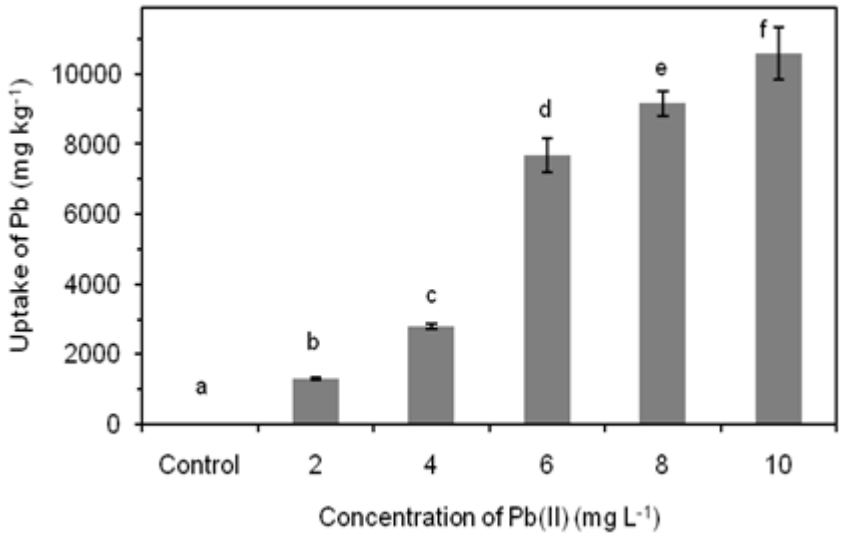

Figure 3: Uptake of lead by *A. pinnata* grown separately in 10% modified Hoagland's medium treated with different concentrations of Pb^{2+} for 7 days. Bars indicate mean ± SD, where n = 3. Different letters indicate statistically significant differences between treatments ($P < 0.05$) based on Tukey's 95% simultaneous confidence intervals test.

Bio concentration factors (BCF) for *A. pinnata* grown in different concentrations of Pb are given in Table 1.The potential of a plant for phytoremediation process is often judged by its bio-concentration factor. The BCF values over 1000 are considered as evidence of a useful plant for phytoremediation (Zayed et al., 1998). In our study we found that BCF was greater than 1000 for initial Pb concentrations in excess of 4.0 mg L^{-1} (Table 1).

Table 1: Variation of mean bio concentration factor of *A. pinnata* for Pb under different initial Pb concentrations

Initial Pb^{2+} concentration in the medium (mg L^{-1})		Mean Bio Concentration Factor
2.0		781^{a}
4.0		809^{a}
6.0		1171^{b}
8.0		1220^{b}
10.0		1147^{b}

Means of BCF values followed by different superscript letters are significantly different at $P < 0.05$ (based on Tukey's 95% simultaneous confidence intervals test).

The study also showed that BCF of *A. pinnata* for Pb increased significantly ($P<0.05$) with increasing Pb concentration in the growth medium, up to 6.0 mg L^{-1} and the highest BCF was

observed at 8.0 mg L^{-1} of Pb. Hence *A. pinnata* is a potential candidate for removal of Pb at low concentrations from waterways polluted with effluents containing Pb.

3.3 Efficiency of Pb uptake by *A. pinnata*- Time course experiment

The growth of *A. pinnata* was normal during the experimental period. The effect of lead on the relative growth of *A. pinnata* with exposure time is shown in Figure 4. The relative growth of *A. pinnata* increased significantly ($P \leq 0.05$), when exposure time was increased. The relative growth of control plant also increased with exposure time.

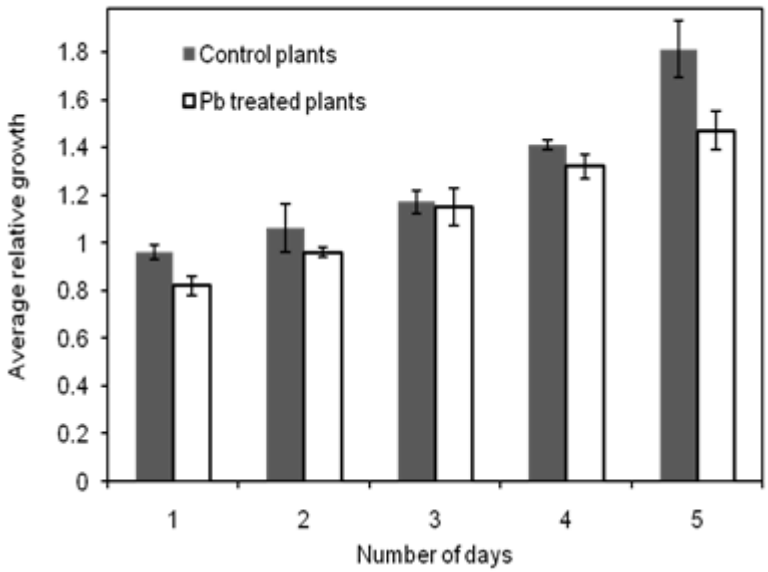

Figure 4: Variation of average relative growth of *A. pinnata* with time. Initial Pb(II) concentration in growth medium = 4.0 mg L^{-1}. Bars indicate mean ± SD, where n = 3.

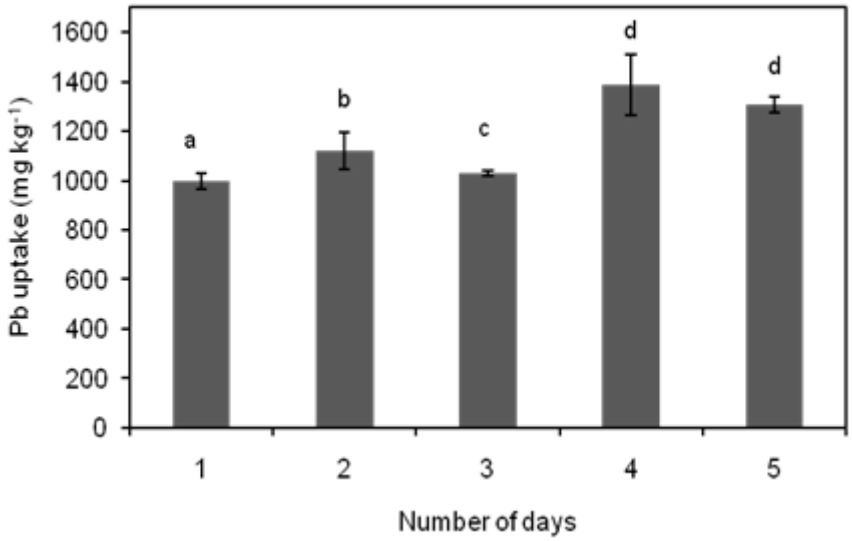

Figure 5: Variation of Pb uptake in *A. pinnata* against the exposure time. Initial Pb(II) concentration in growth medium = 4.0 mg L^{-1}.Bars indicate mean ± SD, where n = 3. Different letters indicate statistically significant differences between treatments ($P < 0.05$) based on Tukey's 95% simultaneous confidence intervals test.

The removal efficiency of lead with exposure time is shown in Figure 5. The removal efficiency increased significantly ($P \leq 0.05$) when exposure time was increased. The maximum uptake of Pb was observed on Day 4 (1383 mg/kg of DW). Control plants showed very low concentration of Pb (20 mg/kg of DW) in its biomass. Though the relative growth increased significantly throughout the test period, the removal efficiency increased up to Day 4. Tukey's 95% simultaneous confidence intervals test shows that there is no difference between the means of removal efficiency on fourth and fifth days. BCF of *A. pinnata* for lead increased significantly ($P \leq 0.05$) when the exposure time was increased (Table 2). The maximum BCF for lead was 331 also on day 4.

Table 2: Variation of mean bio concentration factor of *A. pinnata* for Pb with time

Number of days	Mean BCF
1	238 [a]
2	267 [b]
3	245 [a]
4	331 [c]
5	312 [c]

Means of BCF values followed by different superscript letters are significantly different at $P < 0.05$ (based on Tukey's 95% simultaneous confidence intervals test).

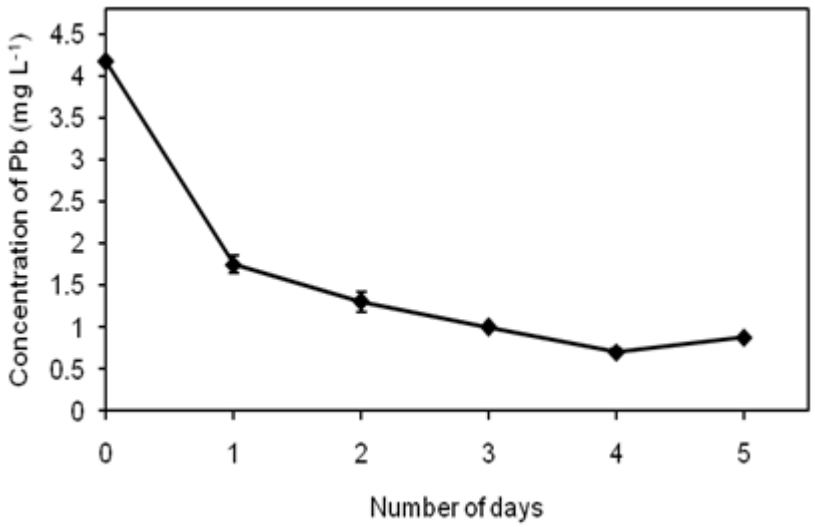

Figure 6: Variation of concentration of Pb(II) in the remaining solution with time. Initial Pb(II) concentration in the solution = 4.0 mg L^{-1}. Bars indicate mean ± SD, where n = 3.

The variation in the residual Pb(II) concentration in the solution with exposure time is shown in Figure 6. The Pb(II) concentration in the test solution reduced from 4.17 mg L^{-1} to nearly 0.88 mg L^{-1} at the end of the Day 5. However, by the end of the fourth day 83% of lead in the solution was absorbed by *A. pinnata*.

3.4 Conclusions

This study showed that the uptake ability and the BCF of *A. pinnata* for lead increased with the increase of Pb(II) concentration in the growth medium. It was also observed that the lower the nutrient strength, the higher the concentration of metal that accumulated in the tissue. Lead uptake capacity significantly increased with the increase of exposure time. *A.*

pinnata is a good accumulator for Pb and is a potential candidate for the removal of Pb from contaminated water.

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