Capacity of *Salvinia minima* Baker to Tolerate and Accumulate As and Pb*

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The use of *Salvinia minima* Baker for the removal of lead (Pb) and arsenic (As) from aqueous solutions was investigated. In a first approach, the effect of different concentrations of AsO_4^{3-} and Pb(II) on the growth and accumulation of these metals was studied. The plants tolerated concentrations of 20–40 μM Pb(II) and 200 μM of AsO_4^{3-}. Toxic effects occurred when 20 μM of Pb(II) and 100 μM AsO_4^{3-} were used. These effects included growth inhibition (decreased yield of biomass and frond area) as well as an altered frond (leaf-like structure in ferns) appearance and tissue consistence. *S. minima* showed a high uptake of Pb (34 mg/g dry weight) compared to As (0.5 mg/g dry weight). The uptake of As was inhibited by phosphate. Additional kinetic studies revealed a two-stage accumulation of both elements: a rapid first phase within the first 6–12 hours and a slow second phase up to the end of the 96-hour experiment.

1 Introduction

Lead and Arsenic are toxic elements ubiquitously encountered in the environment. Both metals are released into the biosphere by geological and anthropogenic activities. Lead is one of the most common heavy metals on Earth [1]. The use of paints containing lead, old pesticides (lead arsenate), metal processing and lead-containing gasoline have been the main anthropogenic sources of lead [2]. The metalloid arsenic is ranked 20 according to its frequency in the Earth's crust. Arsenic contamination by humans is also caused by metal processing and burning.

Mining and smelting operations in Northern Mexico [3] have caused contamination of waters with arsenic and lead and are therefore a potential danger for humans and the environment. The cleaning of such contaminated waters is an increasing concern. A promising ecological and inexpensive cleaning attempt is the use of plants. This technology, called phytoremediation, is based upon the ability of some plants to accumulate high amounts of toxic metals. *S. minima* is a floating water fern with a wide distribution in tropical and subtropical areas. Due to its close relationship to the well-studied metal-accumulating *Lemna* (duckweed) it is expected to have a high potential for accumulating heavy metals.

2 Materials and Methods

2.1 Test Plant and its Cultivation

The aquatic water fern *Salvinia minima* Baker was collected in Mérida, Yucatán (20.967° N and 89.617° W, altitude: 10 m). The plants were washed in 10 mM chelating EDTA solution followed by double-distilled water to remove metallic elements, if any, from the plant surface. Plants were then transferred to a modified medium according to Hoagland *et al.* [4]. The nutrient solution contained [μM]: KNO_3, 6000; $Ca(NO_3)_2 \cdot H_2O$, 4000; Na_2HPO_4, 200; $MgSO_4 \cdot 7\ H_2O$, 200; H_3BO_3, 45; $MnSO_4 \cdot H_2O$, 20; $CuSO_4 \cdot 5\ H_2O$, 0.4; $ZnSO_4 \cdot 7\ H_2O$, 0.7; $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, 0.2; $FeCl_3$, 15 and $Na_2EDTA \cdot 5\ H_2O$, 10 at pH 5.5. All chemicals used were of analytical grade.

2.2 Experimental Setup

Toxicity experiments were performed in a greenhouse under controlled temperature (25 ± 2 °C) and discontinuous natural light illumination (E_{max} = 100 μmol/m² s; photo-period: 12 hours) during April/May 2002 in Mérida, Yucatán (Mexico). For uptake experiments, *in vitro* plants were used under controlled conditions (25 ± 2° C, E = 165 ± 15 μmol/m² s, photo-period: 14 hours). The tested plants were maintained in plastic vessels each of 65 × 65 × 100 mm (Magenta, SIGMA). An amount of 150 mL of nutrient solution was poured into each vessel. The resulting solution depth was 40 mm. *S. minima* was incubated with various concentrations of $Pb(NO_3)_2$ (0–160 μM) and Na_2HAsO_4 (0–1000 μM). 15 units of *S. minima* (1 unit = 2 leaf-like and 1 root-like frond) per container were used as an inoculum in three replicates. The incubation time was 15 days. Uptake kinetics and phosphate/arsenate uptake experiments were stopped after 4 days.

It was expected that phosphate ions could cause highly insoluble precipitation of $Pb_3(PO_4)_2$, $PbHPO_4$ or $Pb(H_2PO_4)_2$. Therefore, all experiments with $Pb(NO_3)_2$ and all kinetic studies were maintained without Na_2HPO_4 in the medium.

2.3 Sampling and Analysis

After harvesting, the plants were washed in distilled water. The fresh weight of the plants was determined by removing excess liquid on filter paper for 15 minutes. In order to measure the dry weight, the plants were freeze-dried. The leaf-like fronds were put between two glass plates and scanned at 300 dpi. Afterwards the frond area was calculated by using the Scion Image Beta 4.0.2 software.

20 mg of freeze-dried plant material were digested with 200 μL HNO_3 (69 %) for 3 h at 80 °C in a sand bath in tightly closed 2-mL Eppendorf reaction tubes [5]. The completely digested biomass was brought up to 2 mL with deionized water. The element quantification in plant biomass and solution samples were performed against standard calibration curves prepared with a known concentration of $Pb(NO_3)_2$ and Na_2HAsO_4 using an ICP-OES (ES Plasma 400, PERKIN ELMER) at 220.353 nm and 193.696 nm, respectively. The detected concentrations were expressed in mg per g dried biomass.

The bioconcentration factor *BCF* was calculated as the quotient of the measured concentration of metal in the plant material [mg × kg/L dry weight]) per initial metal concentration in solution [mg/L]) [6,7].

3 Results

3.1 Toxic Effect of Pb(II) and AsO_4^{3-}

The effects of different $Pb(II)$ and AsO_4^{3-} concentrations on the appearance of the plants, the relative yield of biomass and frond area are illustrated in Figs. 1 and 2. The toxic action of both $Pb(II)$ and AsO_4^{3-} was evident above 20 μM $Pb(II)$ (4.14 mg/L) and 100 μM AsO_4^{3-} (7.50 mg/L). Above 100 μM $Pb(II)$ and 600 μM AsO_4^{3-} no or only a slight yield of frond area or biomass was detectable (Figs. 1A and C as well as 2B and D). At equal concentrations, $Pb(II)$ was about 4 to 6 times more toxic than AsO_4^{3-}. More than 80 % and 90 % of all leaves were damaged above 60 μM Pb and 300 μM AsO_4^{3-} after 7 and 11 days, respectively.

Furthermore, biomass and frond area were reduced to about 10 % at high concentrations in comparison to the initial values (Figs. 1C and D). $Pb(II)$ caused visible symptoms after 1 or 2 days of incubation and AsO_4^{3-} after 3 or 4 days. The observed symptoms caused by $Pb(II)$ differed from those caused by AsO_4^{3-}. $Pb(II)$ brought about a dark brown spot in the centre of the fronds. Later the whole frond became brown. In contrast, the leaves treated with AsO_4^{3-} showed a bleached appearance and centred brown to grey spots and sometimes brown edges. In both cases, the fronds became very brittle.

3.2 Uptake of Pb(II) and AsO_4^{3-}

The $Pb(II)$ and AsO_4^{3-} uptake showed no linear correlation with the initial concentrations in solution (Fig. 1E). It seems that there was a limited capacity for Pb (34 ± 5 mg/g d.w.) and As accumulation (0.5 ± 0.1 mg/g d.w.) (Fig. 1F). However these values were estimated at concentrations where the plants showed a high degree of damage. At non-toxic 20 μM $Pb(II)$ and 100 μM AsO_4^{3-}, *S. minima* accumulated 10.62 ± 0.74 mg Pb/g d.w. and 0.14 ± 0.04 mg As/g d.w., respectively.

The treatment of *S. minima* with increasing phosphate concentrations ranging from 0.0–0.4 mM in the presence of 0.1 mM AsO_4^{3-} caused an enhanced As uptake (Fig. 2). Without phosphate, 0.28 ± 0.17 mg/g d.w. (*BCF* = 37.3) of As were measured. After the treatment with 0.4 mM phosphate almost no As (0.013 ± 0.010 mg/g d.w., *BCF* = 1.7) was detectable.

Additional uptake kinetics revealed a two-stage accumulation (Figs. 3 and 4). In the first 12 hours, a very rapid accumulation of Pb and As occurred. Afterwards the increase of Pb and As continued at a slower rate up to a maximum concentration. At an initial concentration of 5 μM and 10 μM of $Pb(II)$, the final concentration reached 2.77 ± 0.23 mg Pb per g d.w. (*BCF* = 2677) and 4.47 ± 0.27 mg Pb/g d.w. (*BCF* = 2159), respectively. With 200 μM of AsO_4^{3-}, 0.90 ± 0.04 mg As/g d.w. (*BCF* = 60) and with 400 μM of AsO_4^{3-}, 1.05 ± 0.06 mg As/g d.w. (*BCF* = 35) were detected.

4 Discussion

The observed symptoms after the treatment with $Pb(II)$ or AsO_4^{3-} can be described as chlorosis (loss of green pigments) and necrosis (local death of tissue). A weaker consistence, a loss of integrity and an increased respiration rate could lead to the observed loss of biomass and frond area at higher concentrations of $Pb(II)$ and AsO_4^{3-}. In *Salvinia rotundifolia* Willd., a loss of biomass in response to $Pb(II)$ was also revealed [8].

S. minima is able to accumulate very high amounts of lead. Our findings (10.62 mg Pb/g d.w. at 20 μM initial $Pb(II)$ concentration, *BCF* = 2560) confirm the results reported earlier (9.780 mg Pb/g d.w. at 14.47 μM initial $Pb(II)$ concentration, *BCF* = 3260) [9]. Comparable accumulation values were mentioned before in roots of terrestrial plants (*Phaseolus vulgaris*, 75 mg/g d.w. [10]; *Zea mays*, 9.389 mg/g d.w. [11]; *Agropyron elongatum*, roots: 92.754 mg/g d.w., shoots: 11.683 mg/g d.w.) [12]). For *Lemna gibba*, another related aquatic plant, the reported uptake of Pb was much lower at about 0.02–0.07 mg/g d.w. On the other hand, *L. gibba* is able to tolerate much higher concentrations of $Pb(II)$ (up to 50–100 mg/L (241–482 μM)) [13] than *S. minima*. Banerjee and Sarker [8] reported a maximum uptake of 0.2344 mg/g d.w. in *Salvinia rotundifolia*. However, in these experiments there was a limitation of available $Pb(II)$ since the metal was removed to 100 % and as a result the plants could not take up more $Pb(II)$.

Kinetic studies for the uptake of Pb showed a two-stage accumulation (Fig. 3). Similar findings were described for
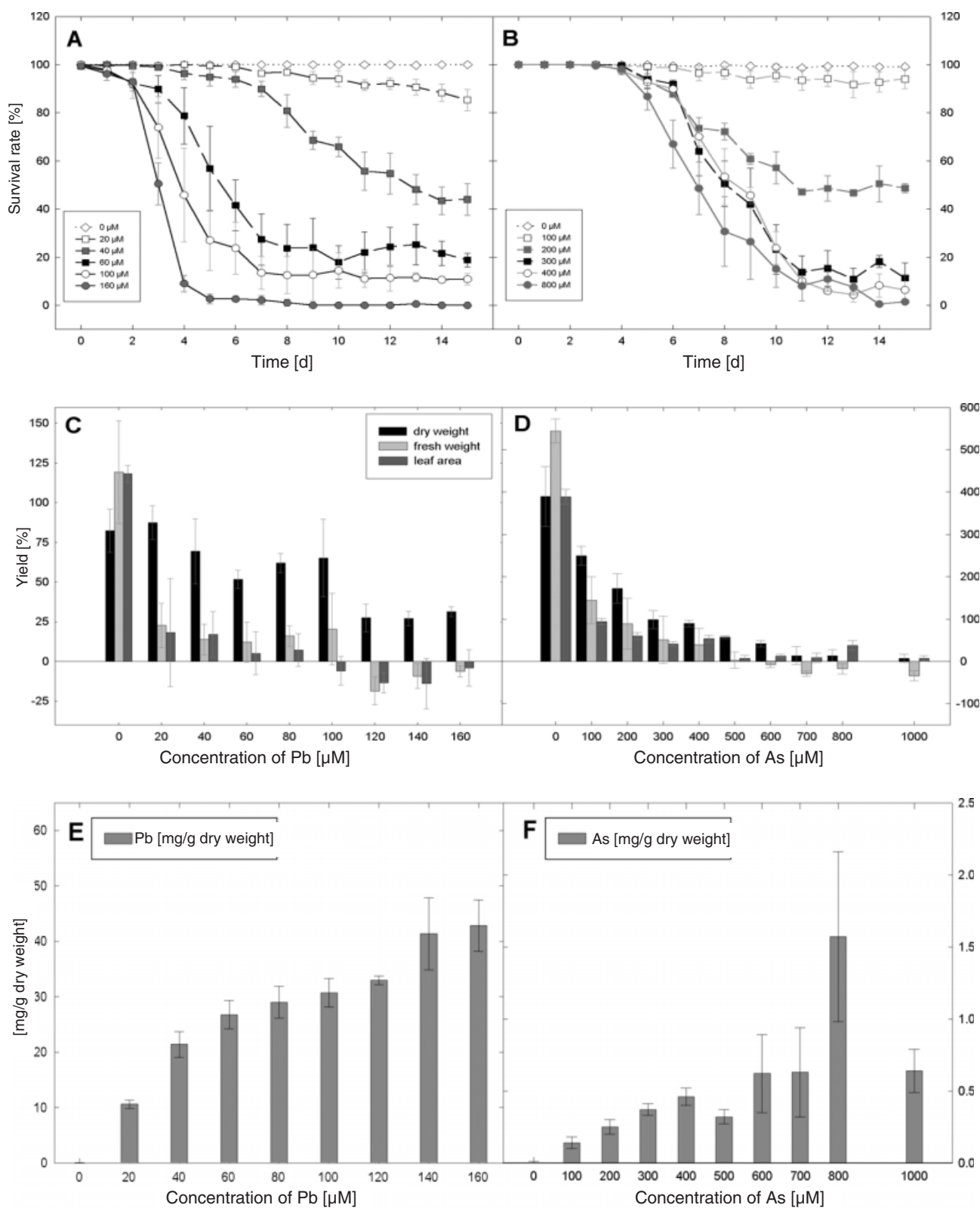
Figure 1. Effects of different concentrations of Pb (left) and As (right) on frond survival (A, B), yield of biomass and frond area (C, D) as well as accumulation of metal (E, F).
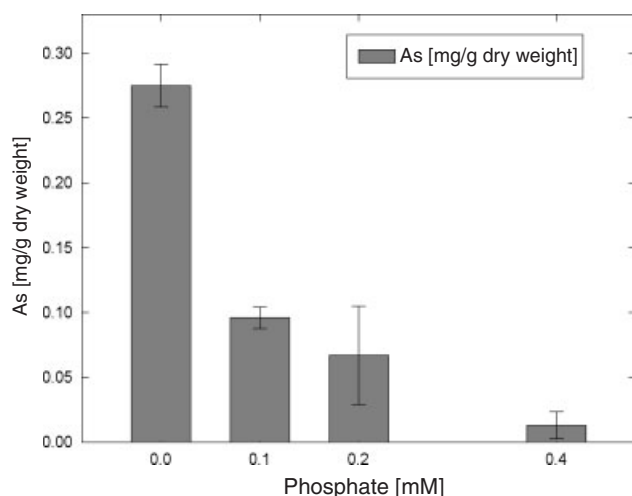
Figure 2. As accumulation in *S. minima* exposed to different concentrations of phosphate.
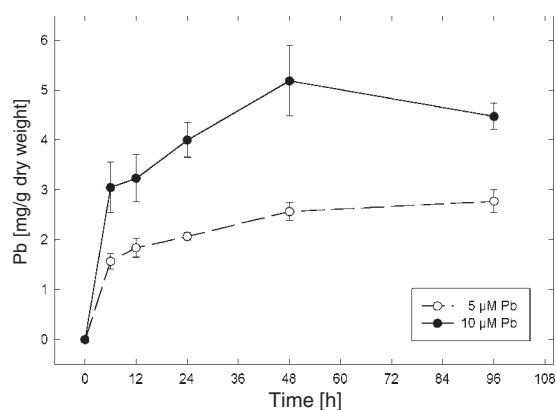

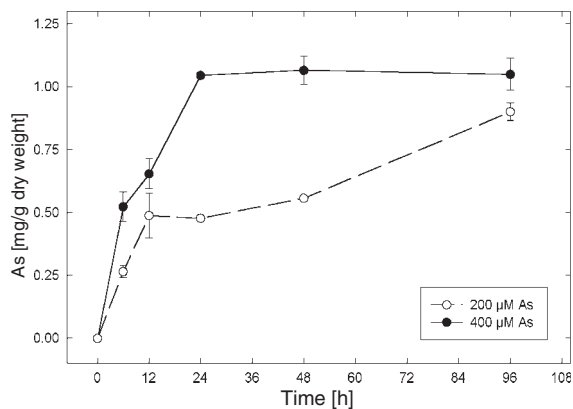**Figure 3.** Kinetics of Pb accumulation; *S. minima* was cultivated in the presence of 5 μM and 10 μM Pb(II).

Figure 4. Kinetics of As accumulation; *S. minima* was cultivated in the presence of 200 μM and 400 μM AsO_4^{3-}.

nonliving biomass of brown algae where most of the metal was adsorbed in about 30 minutes [14]. In the white-rot fungus an equilibrium was reached after 3 hours [15]. It can be assumed that the first phase of this fast accumulation is mainly caused by the adsorption of Pb(II) ions to the plant surface. Once

equilibrium is reached, any further observable increase of Pb is probably produced as a result of uptake processes. Nevertheless, at low concentrations there seems to exist a limited capacity for Pb accumulation which depends on the initial concentration of this element in the medium.

Plants, which are able to accumulate metals to more than 1 mg/g d.w., are considered to be hyperaccumulators (exceptions: 10 mg/g d.w. for Mn and Zn) [16]. Based on this definition and the results reported in this work and earlier articles [9] *S. minima* might be called a hyperaccumulator for lead. The plant seems to fulfil the expectations for use in phytoremediation, especially when considering the reported Pb concentrations found in drinking water in Mexico. Wyatt and Fimbres [17] determined, for example, a concentration of 0.05–0.12 mg/L Pb in Hermosillo, Guaymas, which is clearly below the acceptable level of Pb for *S. minima*.

According to the concentrations of AsO_4^{3-} tolerated by *S. minima* it should be able to grow in contaminated drinking waters encountered in Mexico (0.117 mg/L Hermosillo, Sonora, [17]; 0.014 mg/L in Nazareno, 0.100 mg/L in Santa Ana and 0.300 mg/L in Benito Juarez, Region Lagunera, [18]). Arsenate uptake *via* a phosphate uptake system has already been reported for other species [19,20]. It is known that arsenate competes with phosphate for the same uptake system in the root plasma membrane [21,22]. Furthermore it is described that the arsenate tolerance found in grasses is caused by a suppression of this high affinity phosphate/arsenate uptake system [23]. If this mechanism also exists in *S. minima*, it could explain the low uptake (0.14 $\pm$ 0.04 up to 0.5 $\pm$ 0.1 mg/g d.w.) compared to the high concentrations of AsO_4^{3-} in the nutrient solution.

We were able to improve the accumulation of AsO_4^{3-} by decreasing the amount of phosphate. Without phosphate, the accumulation of As was about four times higher (Fig. 2). These results support the idea that in *S. minima* arsenate is also taken up by a phosphate uptake system. The findings open the possibility to improve the phytoremediation of As in contaminated water.

Even in plants with high arsenate tolerance there is a certain uptake of arsenate [23]. It could be that *S. minima* can accumulate As in higher concentrations after a prolonged exposure. To investigate this possibility it was necessary to determine the internal As concentration at different times in order to evaluate if the accumulation follows a linear function or if there exists a limit. A limited capacity cannot be concluded from the results shown in Fig. 2 F since these data only showed a limited accumulation at higher "outside" AsO_4^{3-} concentrations. This limit could be caused by the disruption of transport processes and phytochelatin production due to a higher initial uptake of AsO_4^{3-}.

At lower, non-toxic concentrations, the plants might be able to keep control over the AsO_4^{3-} uptake and the detoxifying mechanisms such as phytochelatin and glutathione synthesis [24], and therefore they might be able to deal with a higher accumulation of As. Kinetic studies could not confirm these presumptions. Similar to the results of Pb uptake, *S. minima*

was able to accumulate As very fast in the first 6–12 hours (Fig. 4). But then it slowly approached an accumulation equilibrium that was dependent on the initial concentrations of AsO_4^{3-}. One possible explanation for the observed two-stage accumulation could be that almost immediately AsO_4^{3-} ions are bound to adsorption sites on the plant surface. This process ends, once equilibrium is reached. The slower accumulation of As after 12 hours is probably caused by the uptake of As into the plant tissue.

5 Conclusions

It can be concluded that *S. minima* Baker possess a very good capacity for the phytoremediation of Pb. Its ability to accumulate As is mainly limited by high phosphate concentrations. In all probability, the observed accumulation is the combination of two mechanisms: the adsorption of metal ions on the plant surface and the uptake of metal ions inside the plant tissue. Further research will be focused on the differentiation of metal adsorption and uptake, the distribution of Pb and As in root-like and leaf-like fronds as well as in rhizomes. Moreover, the formation of heavy metal binding peptides will be investigated.

Acknowledgements

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