RESEARCH ARTICLE

Bioaccumulation of Multi-metals and Associated Oxidative Stress in *Salvinia molesta* D.S. Mitchell

Komal Sharma, Priya Saxena and Alka Kumari*

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Abstract

In the present investigation, three sets of hydroponic experiments were designed to explore the bioaccumulation abilities of multi-metals in the aquatic weed plant, *Salvinia molesta*. In these experiments, one set of plants was treated with fly ash, a second set of plants was treated with multi-metal solution (1 mM concentration) which was prepared by mixing salts of different metals in a nutrient solution, and in the third set, plants were kept in a nutrient solution without any exposure for control data. Metal accumulation in plants was observed in all three sets. However, *S. molesta* proved to be highly efficacious for copper (Cu) and chromium (Cr) removal (>50%), and to a lesser extent for zinc (Zn) (34–44%), lead (Pb) (35-54%), nickel (Ni) and cobalt (Co) (below 30%) elimination from both the contaminated waters. The biomass was also reduced by up to 17.8 % in multi-metal solution as compared to the control. A slight increment in biomass of fly ash treatment indicates the availability of essential elements through metal-endowed fly ash. Reduction in chlorophyll contents was also found less in the fly ash solution (7%) in comparison to the synthetic multi-metal solution (10%). A significant increase in MDA content in multi-metal solution indicates oxidative stress generated due to membrane damage. Overall outcomes accrued through this study evinced that *Salvinia molesta* could be exploited in the simultaneous accretion of multiple metals from tainted wastewater. It could also be utilized as an ecological indicator in the assessment of metal-contaminated habitats.

Keywords: Aquatic, Indicator, metals, Phytoremediation, Antioxidants

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Introduction

During the last decades, the expeditious rate of mechanization and uncontrolled metropolitan development became accountable for the rising levels of contamination in aquatic bodies. Increasing growth of the economic sector with elevating needs of the population has resulted in rapid commercial expansion. The release of untreated wastewater from various industries straight into the environment alters the realistic composition of metals in aquatic habitats. Lack of enforcement and improper management of environmental laws has also intensified metal pollution (Eid *et al*., 2020; Akhtar *et al*., 2023). Overconsumption of water, urbanization, population explosion, and water pollution have resulted in the deterioration of water quality (WHO [World Health Organization] & UNICEF, 2006) and contributed to water scarcity faced by 40 % of the world population (Calzadilla *et al*., 2011; Pang *et al*., 2023). Wastewater released through domestic and industrial utilization accommodates elevated concentrations of vociferous metals which muddle the living and natural environment of aquatic habitats (Mendoza *et al*., 2015; Ahmed *et al*., 2017). Metals are toxiferous pollutants that can enter the food chain without being decomposed. The exploitation of aquatic weed plants could be a potential alternative in the decontamination process which can terminate the risk of biomagnification of the food chain. These toxic metals have been proclaimed as the most common noxious and imperishable contaminants for a long time (Mallick and Mohn, 2003). However, they not only persist in the environment but also mass through time which jeopardizes the sustenance of aquatic flora and fauna and human too. Concentrations beyond standard limitations incite their infiltration into the food chain and ultimately lead to bio-magnification (Hassan *et al*., 2022; Singh *et al*., 2023). Elimination of these metals is an extremely challenging process due to their different chemical formations (Gall *et al*., 2015).

Department of Botany, University of Lucknow, Lucknow, India.

***Corresponding author:** Alka Kumari, Department of Botany, University of Lucknow, Lucknow, India. Email: kumarialkasanjay@gmail.com

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Maintenance expenses and operational load in different conventional techniques have endorsed the involvement of natural resources in the abatement of metal contamination (Ali *et al*., 2020). The introduction of plants in the remediation process has evolved as a more environmental-friendly and sustainable alternative in which plants mainly accumulate metals through their root system and then translocate them into shoot or aboveground parts (Singh and Pant, 2023). These Plants are usually classified as excluders, accumulators, or indicators according to their metal tolerance and accumulation abilities (Dhir and Srivastava, 2011). Although, hyper-accumulator plants can easily survive at normal growth rates in metalliferous conditions without manifesting any toxicity symptoms (Saraswat and Rai, 2018). Various remediation contrivances such as phytoaccumulation, phytodegradation, phytovolatilization, and rhizofiltration have been utilized by plants for the accrual of toxic metals. However, in the case of aquatic macrophytes, the sole viable depuration method is phytofiltration or rhizofiltration of contaminants from wastewater (Rezania *et al*., 2016). The exploitation of aquatic macrophytes in metal exclusion has become the most economical and efficient technique in view of their natural absorbing capacity (Pratas *et al*., 2014). So far, numerous researchers have documented and verified the importance of aquatic plants in the decontamination of aquatic ecosystems both through hydroponic and field experimentation (Miretzky *et al*., 2004; Dhir *et al*., 2009; Ansari *et al*., 2020).

Aquatic macrophytes usually possess attributes like a pervasive root system, high multiplication rates, and biomass, varied habituation, high tolerance, and exorbitant accumulative efficacy to cumulate metals in above and below-ground parts which contribute to the accumulation of metals in their tissues through rhizofiltration (Khellaf *et al*., 2022; Yuliasni *et al*., 2023). However, the exorbitant efficiency of accumulation shown by plant species depends on various aspects such as the abundance, multiplication rate, tolerance, and absorption capacity and is monitored by some environmental aspects such as pH, temperature, salinity, redox potential, and chemical speciation of the metal (Dhir *et al*., 2009). However, the prime downside of phytoremediation is the extended removal time which can be eliminated by employing multiple remediation methods simultaneously (Rezania *et al*., 2016).

Salvinia molesta is a free-floating aquatic pteridophyte belonging to the order Salviniales. It displays varied structures in different habitats according to the area and nutrients. It has a rootless crawling stem with cylindrical hydrophobic hairs present on leaf surfaces that repels water. It spreads rapidly through vegetative fragmentation and forms thick mats on the surface of the water which results in oxygen reduction and degraded water quality. It possesses an exorbitant accumulation capacity restricted to some toxic metals (Rezania *et al*., 2016; Ng *et al*., 2017; Silva *et al*., 2018; Sitarska *et al*., 2023). Till now, numerous studies have been performed by environmentalists which reported *S. molesta* as a hyperaccumulator of toxic metals. WHO has ascertained some metals such as copper (Cu), cadmium (Cd), chromium (Cr), nickel (Ni), mercury (Hg), lead (Pb), zinc (Zn) and cobalt (Co) to give specific attentiveness in view of quality standards of drinking water (WHO, 1984). So, considering the toxicity of these metals, simultaneous assessment of toxicity and bio-concentration of six toxic metals (Cr, Co, Cu, Ni, Pb, and Zn) was determined in *Salvinia molesta* from both fly ash and synthetic multi-metal conditions. Synthetic multi-metal solutions cannot mimic the natural concentrations of metals in a realistic environment. For that, we have also incorporated the fly ash solution in this study for comparison. Furthermore, photosynthetic pigments and antioxidant enzymes were also evaluated in *S. molesta* for assessing the toxicity induced by the combination of six toxic metals and comparing the symptoms with plants growing in fly ash solution which encompasses various macro-and micronutrients.

Methods and Methodology

Experimental Design

Fresh and young plants of *Salvinia molesta* were collected from an unpolluted pond in Lucknow. These plants were acclimatized in natural conditions after proper washing. For the hydroponic experimental setup, three sets of plants of uniform size and weight (2.50-3.50 gm) were placed separately in plastic containers of 1-liter capacity. For the preparation of fly ash (1 gm) and synthetic multi-metal (1 mM) solutions, 1 gm of fly ash and salts of each metal (Cu, Cr, Co, Ni, Pb and Zn) were dissolved in 1 liter of Hoagland solution respectively. Hoagland's solution was prepared using double distilled water in sterilized glassware. Metal salts utilized were Cobalt (II) chloride ($CoCl_2$), Potassium dichromate ($K_2Cr_2O_7$), Copper (II) sulphate ($CuSO_4$), Nickel (II) chloride hexahydrate ($NiCl_2.6H_2O$), Lead (II) acetate trihydrate [$Pb(C_2H_3O_2)_2.3H_2O$] and Zinc sulphate ($ZnSO_4$). Plant set treated with only 5% of Hoagland solution without any treatment served as control. After the exposure period, plants were collected and analyzed for metal uptake.

Dry Weight Calculation

The biomass of the plant samples was calculated by drying the weighed samples at 80°C in a hot air oven for 48 hours followed by desiccation and reweighing. Growth was measured according to the following formula:

Growth (%) = (Final Biomass – Initial Biomass)/Initial Biomass *100

Heavy Metal Analysis

Freshly harvested samples were cleaned with distilled water by washing at least three times. 500 mg of leaf samples were dehydrated in an oven for 48 hours at 80°C and then macerated in HNO_3: $HClO_4$ (3:1, v/v) (Kumari et al., 2011; 2016). The digested product was used for the analysis of metal content by Flame Atomic Absorption Spectrophotometer (Perkin Elmer 2380).

Biochemical Analysis

Chlorophyll estimation

About 500 mg of fresh leaves of *S. molesta* were macerated in 80% acetone in a mortar. Centrifugation of macerated samples was done at 10,000 rpm for 10 minutes at 4°C and obtained aliquots were transferred in test tubes. A UV-Vis spectrophotometer was utilized to estimate the absorbance of supernatants which was recorded at 470, 510, 645, and 663 nm. Chlorophyll estimation was ascertained by following the Arnon methodology (1949) and Duxbury and Yentsch (1956) method was adopted to analyse carotenoids.

$$\text{Chlorophyll a (mg g}^{-1}\text{ fw)} = [12.7(A_{663})-2.69(A_{645})] \times V/1000xW$$
$$\text{Chlorophyll b (mg g}^{-1}\text{ fw)} = [22.9(A_{645})-4.68(A_{663})] \times V/1000xW$$
$$\text{Total Chlorophyll (mg g}^{-1}\text{ fw)} = [20.2(A_{645})-8.02(A_{663})] \times V/1000xW$$
$$\text{Carotenoid (mg g}^{-1}\text{ fw)} = [7.6(A_{480})-1.49(A_{510})] \times V/Dx1000xW$$

Malondialdehyde Estimation

Lipid peroxidation in plant samples was evaluated by quantifying malonaldehyde (MDA) content following thiobarbituric acid (TBA) (Heath and Packer, 1968). Approximately 500 mg of leaf samples of the plant were crushed in 1% (w/v) of trichloroacetic acid and centrifuged at 10,000 rpm for 10 minutes at 4°C. Obtained aliquots were added to 1 ml of 0.5% (w/v) thiobarbituric acid in 20 % TCA and put in a water bath at 94°C. After half an hour, samples were cooled down immediately and absorbance was measured at 532 and 600 nm (Extinction sufficient 155 Mm^{-1} cm^{-1}).

Statistical Analysis

All the observations were conducted in triplicates (n = 3) and data were presented as mean ± SD. The statistical analysis was performed by one-way ANOVA analysis at P ≤ 0.05.

Result and Discussion

The data represented in Table 1 illustrates the regulatory standards for toxic metals in drinking water suggested by leading environmental organizations. Fly ash utilized in the experimentation exhibits metals beyond the standard limits in which Chromium (1.65) and lead (0.032) are way beyond the maximum limitations and Copper (2.06) and Nickel (0.075) are slightly higher.

The accrual of multi-metals in *S. molesta* is represented in Table 2, which illustrates the better performance of *Salvinia* in fly ash solution with a higher accretion percentage than in synthetic multi-metal solution. However, being an accumulator of heavy metals, *Salvinia* cumulates all six metals examined in this study through both fly ash solution and synthetic multi-metal solution. In fly ash treatment, approximately 50 % accumulation percentage was seen in Cu (50%) and Cr (47%). 45 % removal was seen in zinc and 30 % removal of lead, nickel, and cobalt was recorded (Fig. 1). However, in synthetic multi-metal solution, the percentage accumulation was slightly lower. Above 40 % removal was seen only in chromium and copper. Approximately 30% accumulation percentage was seen in nickel and zinc and below 30% cumulation was recorded in lead and cobalt. Our results were substantiated by an earlier study in which *S. natans* cumulated the highest accumulation of chromium followed by Ni, Co, Pb, and Zn from different multi-metal combinations suggesting its highly tolerant nature against chromium (Dhir and Srivastava, 2013).

Different species of *Salvinia* has been documented as hyperaccumulator of various toxic metals such as Ni in *Salvinia minima* (Fuentes *et al*., 2014), Cu in *Salvinia cucullata* (Das and Goswami, 2016) and Co, Zn, Cu, Cr, Fe, and Ni in *Salvinia natans* (Dhir and Srivastava, 2011). Apart from it, different species of *Salvinia* have been found tolerant against toxic metals as *S. minima* have been documented to cumulate high concentrations of Pb (Sánchez-Galván *et al*., 2008), As and Cd (Hoffmann *et al*., 2004), *S. biloba* can accumulate Hg (Casagrande *et al*., 2018), Pb (Loria *et al*., 2019) and *S. molesta* can remove As (III) (Silva *et al*., 2018). *S. molesta* has also been used in the biological treatment of domestic wastewater and has proven to be an ecologically sustainable way (Mustafa *et al*., 2021). Apart from it, another species of *Salvinia, S. natans* has been found to accumulate more than one metal in different combinations of multi-metal solutions (Dhir and Srivastava, 2011). Varied accumulation capacity in different combinations can be elucidated on account of affinity and aversion amidst different metals in the uptake process.

Table 3 represents the alterations in the biomass of *Salvinia molesta* after 24 and 96 h of exposure. The biomass was reduced significantly and was recorded 8.86 grams in the synthetic solution after the completion of the experiment. Approximately, 10.67 % and 7.13 % decrease was noticed in the biomass of *Salvinia* after 24 and 96 h respectively which demonstrated the toxicity effects of multi-metals in different biochemical processes. However, in fly ash solution, after 24 h, a slight decrease in the dry weights was recorded but after 96 h it was elevated. The increase in dry biomass of *Salvinia* can be substantiated by an earlier study which suggested that a combination of multi-metals containing Fe metal had endorsed the growth and chlorophyll synthesis in *S. natans* (Dhir and Srivastava, 2013). A significant increase in dry biomass of *S. molesta* after 96 h of exposure suggested that augmentation of biomass production could be attributed to the better growth performance of *S. molesta* in fly ash treatment. Fly ash encompasses toxic heavy metals and other metals such as Fe, Mn, and Zn which plants require for augmented growth and productivity also seen in terrestrial plants grown on contaminated soil (Abhilash *et al*., 2016). Enhanced plant growth and less toxicity induced in fly ash treatment substantiated that the metal composition of fly ash had a positive effect on *S. molesta* however, in synthetic multi-metal solution, the existence of all toxic metals (Cu, Cr, Co, Ni, Pb, and Zn) was detrimental for plant's growth and photosynthetic activity.

Table 1: Permissible limits of heavy metals in drinking water suggested by different organizations

Heavy Metals	WHO 2022 In mg/l	USEPA 2018	BIS (ISO: 10500, 2012)
Cr	0.05	0.1	0.05
Cu	2	1.3	0.05
Ni	0.07	NGL	0.02
Pb	0.01	0.015	0.01
Zn	NGL	NGL	5

Table 2: Heavy metal concentrations in Salvinia molesta in control, fly ash and multi-metal solution after experiment

Heavy metals	Control	Concentration (mg/g) Fly ash solution	Multi-metal solution
Cr	0.006 ± 0.01	0.520 ± 0.12	0.035 ± 0.01
Pb	0.045 ± 0.03	0.019 ± 0.01	0.052 ± 0.12
Ni	0.005 ± 0.01	0.024 ± 0.02	0.017 ± 0.03
Cu	0.005 ± 0.01	1.045 ± 0.32	0.029 ± 0.04
Zn	0.086 ± 0.04	0.087 ± 0.01	0.023 ± 0.03
Co	0.009 ± 0.02	0.520 ± 0.13	0.013 ± 0.01

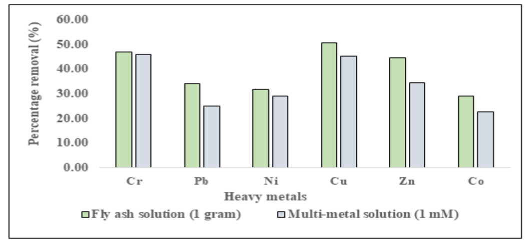

Fig. 1: Percentage removal of heavy metals by Salvinia molesta

Table 3: Effect of multi-metal on dry biomass of *S. molesta* after 24 and 96 hours

Concentration (mM)	Dry biomass (g) Initial	24 h	96 h	% Decrease 24 h	% Decrease 96 h
Control	10.87	10.93	10.82	-0.55	1.01
1 gram	10.45	10.23	10.67	2.1	-4.31
1.0 mM	10.68	9.54	8.86	10.67	7.13

Table 4: Chlorophyll and carotenoid (mg g^{-1} fw) of *S. molesta* after 24 and 96 hours of multi-metal exposure

Dura-tion	Concentration (mM)	Chlorophyll a	Chlorophyll b	Chl a+ b	Chl a: Chl b	Carotenoid
	Control	0.083 ± 0.02	0.057 ± 0.02	0.140 ± 0.03	1.447 ± 0.02	0.123 ± 0.04
24 h	1 gm FA	0.053 ± 0.03	0.041 ± 0.02	0.094 ± 0.04	1.293 ± 0.02	0.074 ± 0.03
	1 mM	0.041 ± 0.02	0.032 ± 0.03	0.073 ± 0.02	1.281 ± 0.03	0.065 ± 0.02
	Control	0.075 ± 0.07	0.049 ± 0.03	0.124 ± 0.02	1.531 ± 0.04	0.121 ± 0.02
96 h	1 gm FA	0.049 ± 0.03	0.038 ± 0.02	0.068 ± 0.03	1.925 ± 0.05	0.067 ± 0.02
	1 mM	0.035 ± 0.03	0.027 ± 0.02	0.062 ± 0.03	1.631 ± 0.03	0.059 ± 0.03

Besides, a decline in biomass, the synthetic multi-metal solution also prompted phytotoxicity in *S. molesta*. Reduced root length and the appearance of chlorosis with necrotized areas on the surface of the leaves demonstrate the phenotypic alterations caused by multi-metal solution. Fig. 2 shows the pictorial representation of necrotic areas as discolouration or dark brown pigmentation from the edge to the centre of the leaves in *S. molesta* exposed to multi-metal toxicity after 96 h of exposure. These phenotypic modifications display metal existence in a contaminated environment that evinced its characteristic of the ecological indicator.

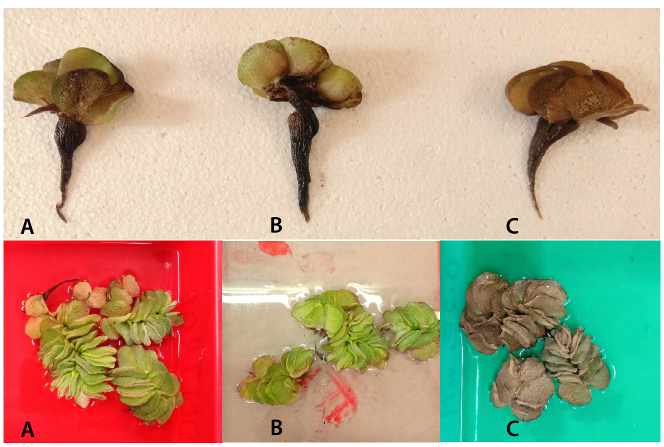

Fig. 2: Phenotypic changes detected in ***S. molesta*** after 96 h A. Control plant, B. Fly ash treated plant, C. Synthetic multi-metal solution treated plant. Chlorosis and Necrosis signs are mainly observed in plants treated with synthetic multi-metal solution (C).

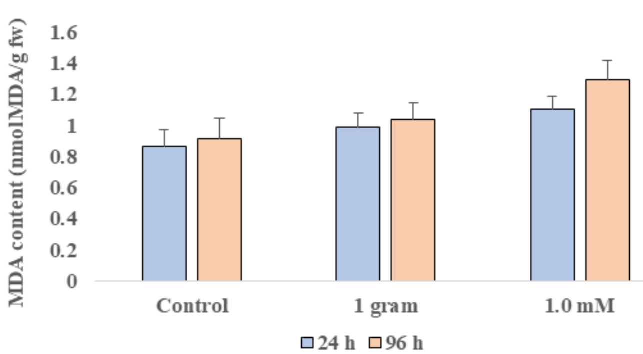

Fig. 3: Effect of multi-metal on MDA content (mmol g-1 fw) of Salvinia molesta

The disastrous consequences of toxic metals in plant growth can be visually detectable through chlorophyll estimation (Liu *et al*., 2019). Total chlorophyll content also represents the consolidated parameter reflecting specific interferences. As illustrated in Table 4, chlorophyll contents were significantly reduced in fly ash as well as in multi-metal conditions by up to 15 % in synthetic multi-metal solution and 7 % in fly ash solution after 96 h. The carotenoid content was also reduced by up to 9 % in both 24 and 96-h harvesting. The loss in pigment contents might be due to either enzymatic inefficiency caused by damaged chloroplast, impairments in chloroplast membranes because of oxidative disruption of phospholipids, or substitution of metal (Mg) in chloroplast molecule by another metal (Dhir and Srivastava, 2013; Prado *et al*., 2016).

Aquatic plants contain an intrinsic ability to combat redundant ROS generated due to metal stress through an antioxidant detoxification system (Das and Goswami, 2016). The effect of multi-metals on malondialdehyde content in *Salvinia molesta* was displayed in Fig. 3. The MDA content of the control plant was not significantly altered in the experiment. However, in fly ash solution treatment, the MDA content was increased slightly up to 5 % in comparison to synthetic multi-metal solution in which the MDA content was raised about 15 % after the completion of the experiment indicating membrane damage which resulted due to oxidative damage (Perreault *et al*., 2014; Das and Goswami, 2016). Elevated MDA content in response to multiple metals has also been displayed by plants which suggested increased lipid peroxidation (Taqueer *et al*., 2019). Their utilization in energy generation after the accumulation of metals has valorized their involvement in remediation procedures (Kumar *et al*., 2022; Khellaf *et al*., 2022; Zhou *et al*., 2023). The exploitation of aquatic weed plants can also eliminate the threat of biomagnification in the food chain. Many aquatic plants have been identified as accumulators of more than one metal in multi-metal conditions as *Pistia stratiotes* (Miretzky *et al*., 2004), *Lemna minor* (Horvat *et al*., 2007), and *Salvinia natans* (Dhir and Srivastava, 2011). Despite unknown metal interactions, studies conducted on multi-metal accretion by aquatic macrophytes suggest that these plants could be exploited more often in the multi-metallic toxicity assessment studies. Their cooperative effects on growth and other parameters suggest their effectiveness also in the natural environment.

Conclusion

Water acts as a fundamental compound that enacts a pivotal role in the sustenance of life forms. However, continual anthropogenic activities have tremendously deteriorated the quality of water resources. The exploitation of aquatic macrophytes with the phytoremediation technique has surfaced as an ideal approach for the purification of polluted aquatic habitats. The obtained results have shown that *S. molesta* exhibits both tolerant and sensitive approach against toxicity of Co, Cr, Cu, Ni, Pb, and Zn in water and display various phenotypic signs and physiological responses to negate metal toxicity which recommends its exploitation as an ecological indicator in metal contaminated environments. Therefore, the present study substantiates that *Salvinia molesta* could be endorsed more frequently in the purification of multi-metal contamination aquatic sites.

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Author Contribution

Komal Sharma: Experimental setup, data cumulation and analysis, formulation of table and graph, statistical analysis of data, and manuscript composition. Priya Saxena: data cumulation and analysis, analytical interpretation, statistical analysis on data. Alka Kumari: experimental design, hypothesis, final editing, and validate the submission of the manuscript.

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