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PHYTOREMEDIATION OF MERCURY USING *AQUATIC PLANT Azolla microphylla*

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Abstract: Heavy metals are among the most important sorts of contaminant in the environment. Heavy metals are responsible for various health hazards in human beings, pertaining to their property of persistence and bio-magnification. Levels of mercury in the environment are increasing due to discharge from hydroelectric, mining, pulp, and paper industries. The most harmful forms are inorganic compounds of Hg, which easily accumulates in organisms through water. Phytoremediationis an effective and affordable technological solution used to extract or remove inactive metals and metal pollutants from contaminated water. *Azolla Microphylla* bind metals like Zn, Pb, Cu, Cd, Au, Ni, Sr, Cr and Hg has also described the potential of Azolla Microphylla for the removal of Hg from wastewaters. The present review highlights the phytoaccumulation potential of macrophytes with emphasis on utilization of *Azolla Microphylla* as a promising candidate for phytoremediation. *Azolla Microphylla* might bio concentrate Hg from contaminated water. However, in natural or real conditions, which may be synergistic or antagonistic with respect to metal uptake in a plant system. High metals content in the biomass suggests the tremendous potential of *Azolla microphylla* to take up heavy metals, i.e., Hg and may be used as a bio accumulator to polish heavy metals in industrial effluent.

INTRODUCTION

Heavy metals are among the most important sorts of contaminant in the environment. Pollution of the biosphere with toxic metals has accelerated dramatically since the beginning of the Industrial Revolution (Nriagu,1979; Rai,2007a). Heavy metals are responsible for various health hazards in human beings, pertaining to their property of persistence and bio-magnification (Rai,2007a). The use of heavy metals contaminated sewage water for irrigation leads to the accumulation of heavy metals in vegetables and other crops which poses a serious threat to human health (Rai and Tripathi,2007c).

Therefore, the removal of these contaminants is essential prior to their disposal into aquatic bodies and agro-ecosystems.Levels of mercury in the environment are increasing due to discharge from hydroelectric, mining, pulp, and paper industries. Incineration of municipal and medical waste and emissions from coal-using power plants also contribute to high levels of mercury. Bacteria in lake, stream, and ocean

sediments then convert elemental mercury into organic mercury compounds such as methyl mercury. The most harmful forms are inorganic compounds of Hg, which easily accumulates in organisms through water. Inorganic forms of Hg are weakly mobile but biological and chemical processes can transform it into toxic methyl mercury (MeHg) (Rugh, Wang, and Meagher, 1998). Plants and animals are very sensitive to the presence of this element (Raskin and Ensley, 2000).

In humans, Methyl mercury are toxic to the central and peripheral nervous system. The inhalation of mercury can produce harmful effects on the nervous, digestive and immune systems, lungs, kidney and may be affect the fatal brain damage. Further organic mercury, mostly methyl mercury (MeHg) the most toxic species is bio accumulating in the biota and subsequently biomagnified in the aquatic food chain, especially in fish. Given the human health concern, it is critical and important that awareness programme is launched to educate the populations to the risk and impact of mercury exposure in humans especially potentially vulnerable population viz pregnant women, breast feeding women, the fetus new born and young children residing in the hot spot areas of the country and also consequences of MeHg exposure through fish consumption. Several methods already used to clean up the environment from these kinds of contamination, but most of them are closely and difficult to get optimum results. Currently, Phytoremediationis an effective and affordable technological solution used to extract or remove inactive metals and metal pollutants from contaminated water. This technology is environmental friendly and potentially cost effective. Aquatic macrophytes play an important role in the structural and functional aspects of aquatic ecosystems by altering water movement regimes, providing shelter to fish and aquatic invertebrates, serving as a food source and maintaining water quality by regulating oxygen balance, nutrient cycles and accumulating heavy metals.

Phytoremediation involves growing plants in a contaminated matrix, for a required growth period, to remove contaminants from the matrix, or facilitate immobilisation (binding/containment) or degradation (detoxification) of the pollutants. The use of aquatic macrophytes, such as AzollaMicrophylla with hyper accumulating ability is known as environmentally friendly option to restore polluted aquatic resources. *AzollaMicrophylla*is a small free floating aquatic fern that has a symbiotic association with *cyanobacteriumAnabaenaAzollae*of the family *Nostocacceae*, which was selected for this investigation due to its high abundance and easy and luxuriant growth, is common in many parts of the world, especially in tropical environments (Watanbe et al., 1992). *Azolla Microphylla* bind metals like Zn, Pb, Cu, Cd, Au, Ni, Sr, Cr and Hg (Gaur and Noraho, 1995; Sanyahumbi et al., 1998; Antunes et al., 2001; Cohen-Shoel et al., 2002; Bennicelli et al., 2004). Z.Stepniewska, et al 2005) has also described the potential of Azolla Microphylla for the removal of Hg from wastewaters. The present review highlights the phytoaccumulation potential of macrophytes with emphasis on utilization of *Azolla Microphylla* as a promising candidate for phytoremediation.

The ability of *Azolla Microphylla* to purify waters polluted by heavy metals as Hg was also observed to find its suitability and the impact of uptake of heavy metals on the effect of Hg in the various concentration on the metabolic parameters such as starch, Total soluble sugar, Reducing sugar and non-reducing sugar of *Azolla Microphylla* have been discussed for a better understanding and utilization of this symbiotic association in the field of phytoremediation.

MATERIALS AND METHODS

Sample collection

Azolla Microphylla were collected from Azolla Park of *Tamil Nadu Agricultural University* (*TNAU*)at Coimbatore, Tamil Nadu. The Azolla pond was provided with fresh water without additional fertilizers. Azolla Microphylla were maintained and cultured in the Dr.N.G.P Arts and science at Coimbatore, Tamil Nadu, India.

Growth in presence of mercury (Hg)

There was taken a concentration of 2 mg, 4mg and 6mg of mercury as mercury chloride was poured in 1 litre of distilled water in 60×41×10 cm plastic trays with various concentration of mercury (Hg) solution. Azolla Microphylla without metal solution was used as control. At the start of the experiment 120.3g, 122.5g and 124.2g (fresh mass) of Azolla Microphylla was added to each trays containing the mercury solution. The experiment was conducted to grown for 7 days (15- 22 February 2019) under the control conditions of temperature (27`C in day and 22`C in night) and constant day length (8 hours in light and 16 hours in dark).

Table 1: Experimental scheme to test the efficiency of Azolla microphylla in heavy metal phytoremediation

s.no	Plant	Heavy metals	Initial doses of heavy metals	Number of trays with heavy metals and test plant	Duration of investigation and plant biomass
1)	Azolla Microphylla	Hg (as HgCl2)	2mg/l	1	7 days with 120.3g biomass
2)	Azolla Microphylla	Hg (as HgCl2)	4mg/l	1	7 days with 122.5g biomass
3)	Azolla Microphylla	Hg (as HgCl2)	6mg/l	1	7 days with 124.2g biomass

Analysis of mercury content in water & Azolla dry mass

After 7 days of cultivation , Azolla biomass harvested from each treatment was dried in an oven at 80`C till constant weights were obtained.

The dried matter was powdered and each plant sample taken an 800mg were digested in 20ml of conc.HNO3 with heating 580Watts for 10 mins in a microwave. After cooling, the digested plant sample was diluted to 200ml with distilled water in the standard flask. Hg content in the solution and biomass was determined by the Atomic Absorption Spectrophotometer (AAS) model of APHA 23rd Edition 2017 AAS/HVG instrument.

Biochemical Estimation of Azolla Microphylla

Preparation of reagents

Anthrone reagent

Dissolve 200mg anthrone in 100ml of ice cold 95% conc. H2SO4 (Prepare freshly).

Harding reagent

12gm Sodium potassium tarterate dissolved in 1000ml of distilled water.

Nelson reagent

Prepared by dissolving 50gm ammonium molybdate in 900ml of distilled water. To this 42ml of H2SO4 were added slowly. 6gm hydrated Sodium Arsenate were dissolved in 50 ml of distilled water and was added to the ammonium molybdate and H2So4 solution.

Iodine Solution

For preparing 100ml of 0.1M solution, 3gm of potassium iodide (KI) was moisten with few drops of water in a beaker. Measured out 2.54gm of iodine and add to the moisten potassium iodide. Add a small volume of water and stir. Pour the solution in a graduated cylinder and add distilled water to make the 1000ml. of it.

Copper reagent

Prepared by dissolving 20g of sodium carbonate in 260ml of distilled water, then 0.4g of cupric sulphate in 20ml of water and 0.2g of sodium potassium tarterate in 20ml of distilled water and finally mixed well.

Test for biochemical estimation

Preparation of extraction solution

100mg of dried sample was taken in a test tube, 6-7 ml of 80% ethanol was added. The sample was heated in a water bath at 800C for 30 minutes and centrifuged at 3000 rpm for 5 minutes. The supernatant was collected in a flask and 80% ethanol was added to a final volume of 50ml.The residue was used for starch estimation.

Test for Total soluble sugar

1ml of extraction solution was taken and 1.5ml of water was added, followed by 6.5ml of anthrone reagent. The sample was mixed and incubated at room temperature for 15 minutes to allow colour development. Absorbance at 620nm was read on spectrophotometer.

Test for Non- reducing sugar

For the test of Non Reducing Sugar 1ml of extraction solution was taken in a clean test tube and heated in a boiling water bath until it condenses to 0.05-0.1ml. Now, 0.1ml of 30% KOH was added and incubate in a boiling water bath for 10 mins. The solution was cooled down to room temperature, 3ml of

anthrone reagent was added and incubated at room temperature for 10-15 mins. The absorbance was read at 620nm.

Test for reducing sugar

2gm of dried sample were taken in a test tube 10ml of 2% oxalic acid added and covered with aluminium foil to reduce evaporation and heated in a water bath at 800C for 25 minutes. After cooling, 0.2ml of extraction solution taken in a clean test tube and made final volume 1ml. 1ml of copper reagent and 1ml of Harding's reagent was added and mixed well. Test tube was heated in a boiling water bath for 10 minutes. Then, 1ml of Nelson's reagent was added, and left for few minutes for colour development. Absorbance was read at 600nm.

Test for Starch

The residual sugar was rinsed with 2030ml D.W in 50ml flask. The solution was gelatinized in a boiling water bath for 15 minutes. 0.1ml of Iodine solution was added and absorbance was taken at 610nm. Same protocol was followed for the detection of total soluble sugar, non-reducing sugar, reducing sugar and starch in the plants grown in three different concentrations of Hg and control.

RESULTS AND DISCUSSION

Hg accumulation in *Azolla Microphylla*

Mercury accumulation occurred in all treatment, excluding the control which contained no addition of Hg. As Hg treatment increased, plants were accumulated with higher concentrations of the metal. It was treated plants in the 2mg, 4mg and 6mg/ L treatment
groups showed the appearance of smaller and slightly brown color as compared to the control under the conditions of 7 days incubation period.

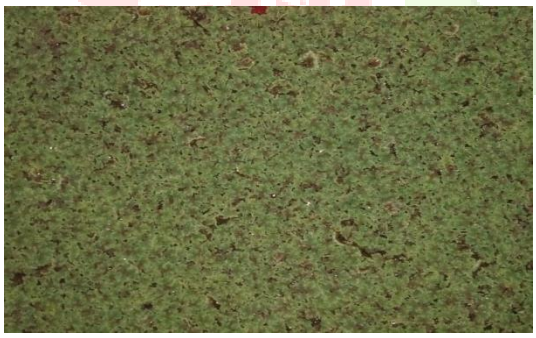

Figure 4: After 7 days, control with *Azolla microphylla*

Figure 5: After 7 days, 2mg of Hg with *Azolla microphylla*

Figure 7: After 7 days, 6mg of Hg with *Azolla microphylla*

The Hg concentration was reduced to 0.02, 0.07 and 0.07 mg/L Hg treatment respectively. Therefore, after 7days the removal of Hg from the solution was 99%, 98.25% and 98.83% from the selected initial concentration.

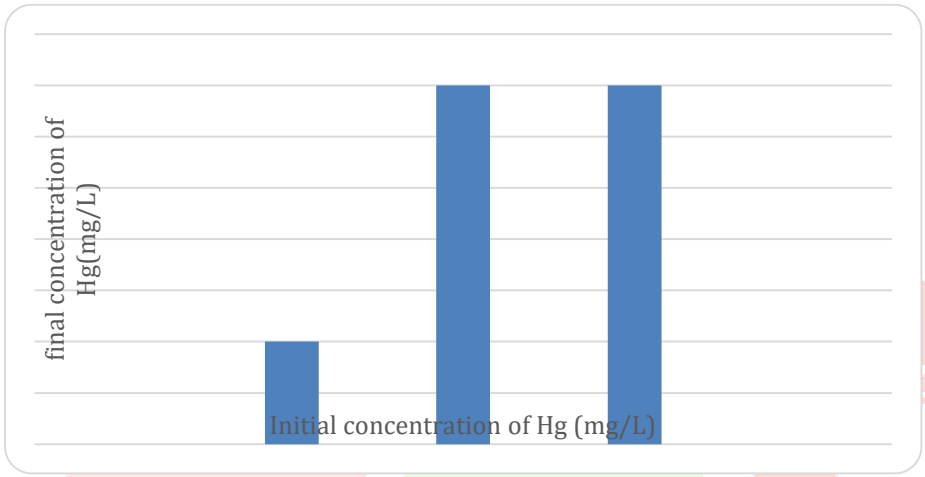

Figure 8: Mercury removed after the 7 days growth of *Azolla Microphylla*

The data of heavy metals concentration in the Phytoremediation process were collected and the removal efficiency percentage was calculated by the following equation

$$\text{Removal Efficiency (\%)} = 100 \times \text{Co-Ct / Co}$$

Where Ct is the concentration (mg/L) at the end of adsorption and C0 is the initial concentration (mg/L) of heavy metals.

They found that a water fern, *Azolla Microphylla* could remove up to 98.6% of Hg contamination in water within 7 days period. Likewise, parrot feather, creeping primrose and water mint removed up to 99.8% of Hg from contaminated water, but after 21 days (Kamal et al., 2004). Rai et al. (2007) showed that *Vallisneria spiralis*, a submerged aquatic macrophyte, removed Hg (70–84%) from the 0.1, 0.5, 1.0, and 3.0 mgL−1 initial metal concentrations. Further, four aquatic plants, including water hyacinth (*Eichornia crassipes*), water lettuce (*Pistia stratiotes*), zebra rush (*Scirpus tabernaemontani*), and taro (*Colocasia esculenta*), were evaluated for their capabilities in removing mercury from water at 0-, 0.5-, or 2-mg/L concentrations of Hg in 30 days (Skinner, Wright, and Goff, 2007).

Analysis confirmed an increase in Hg within the plant root tissue and a corresponding decrease of Hg in the water. It was found that all plant species appeared to reduce the Hg concentration, but among four plants water lettuce and water hyacinth proved to be the most effective (Skinner et al., 2007). In the control, the percentage reduction in the heavy metal concentration was in the range of 4−7% only, which may be due to sedimentation or microbial decomposition. The average value of metal reduction in the control was deducted from the average values of the plant–metal system before calculating the percentage removal from Azolla. The contents of metals under examination in the dry mass of Azolla microphylla. Hg content in the biomass of *Azolla microphylla* was 0.49, 0.76, and 0.49 mg L−1 for the 2, 4, and 6 mgL−1 treatments.

Table 2: *Azolla microphylla* biomass and concentration of heavy metals after 7 days of cultivation

Treatment	Concentration (mg /L)	Fresh mass (g)	Concentration in solution (mg/ L)	Concentration in biomass (mg/ L dry mass)
Control		120.8g		
Hg	2mg	120.3g	0.02	0.49
Hg	4mg	122.5g	0.07	0.76
Hg	6mg	124.2g	0.07	0.76

From the present investigation, it is clear that the *Azolla microphylla* were sensitive and severely affected by mercury in nutrient medium and biochemical datas also confirms that the effect on plant is directly proportional to the concentration of heavy metals which might be due to higher uptake of heavy metals and its harmful nature. The uptake ability and the Bio concentration factor of *Azolla microphylla* for Hg increased with the increase of concentration in the growth medium.As mercury is mobile and bioaccessible metal,

its accumulation in plants can reach the food chain easily. Pb uptake in the roots of water fern was higher than in the stem and leaves. In stem and leaves the translocation of Hg was lower than roots. So, it showed less effect then Hg. The high dose of mercury negatively influenced the metabolic process of plant.

Table 3: Shows O.D of TSS and NRS at 620nm, RS at 600nm and Starch at 610nm

Biochemical test	Control	2mg/L concentration of Hg	4mg/L concentration of Hg	6mg/L concentration of Hg
Total soluble sugar	0.235	0.170	0.157	0.046
Non-reducing sugar	0.214	0.110	0.061	0.043
Reducing sugar	4.000	0.507	0.338	0.272
Starch	0.146	0.132	0.084	0.058

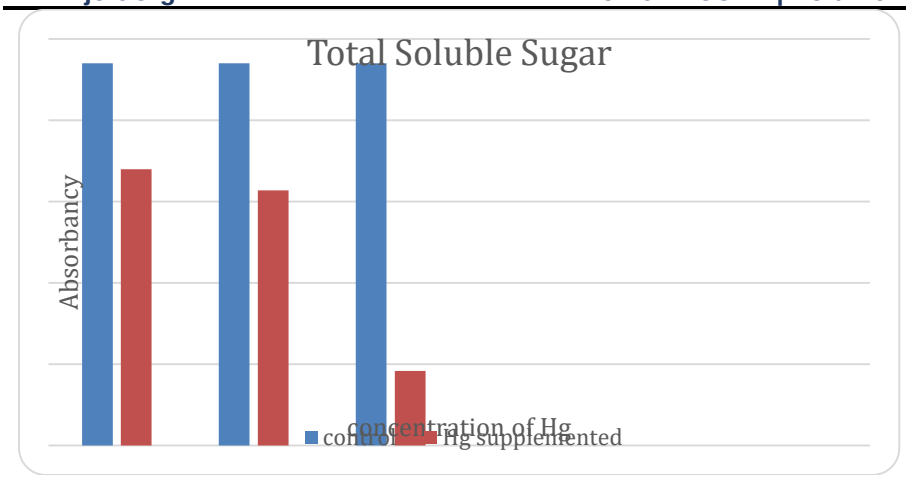

Figure 9: Graph of Total Soluble Sugar at 620nm

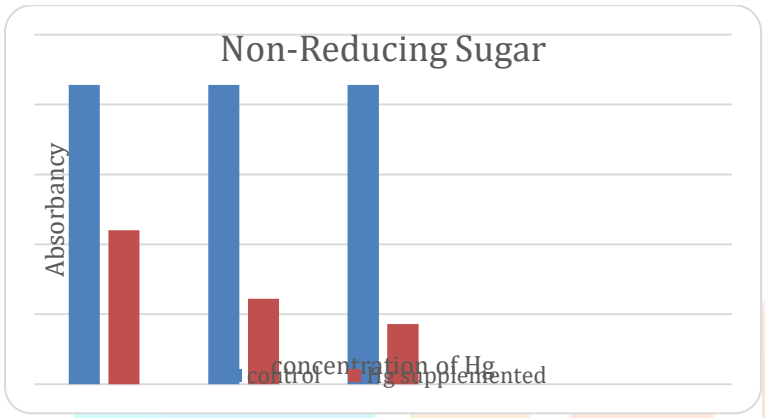

Figure10: Graph of Non Reducing Sugar at 620nm

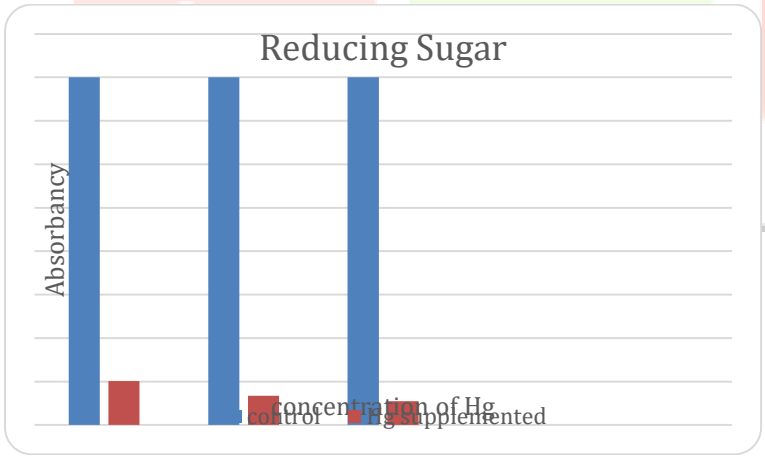

Figure 11: Graph of Reducing Sugar concentration at 600nm

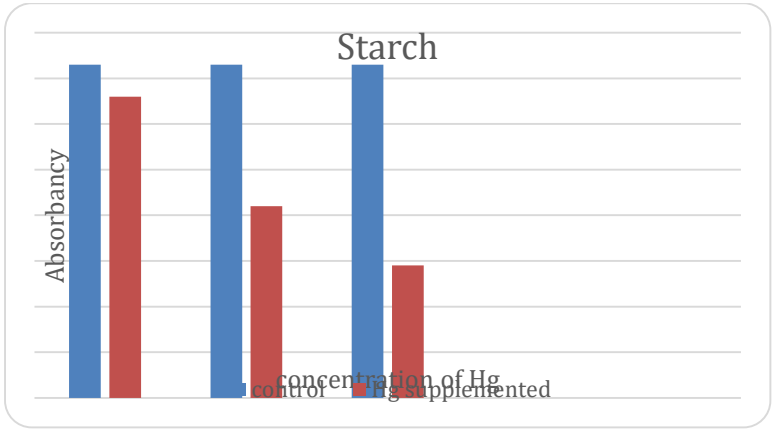

Figure 12: Graph of Starch concentration at 610nm

In his review on heavy metal phytoremediation, discussed the issues related to the harvesting and disposal of wetland plants' biomass in natural/field conditions (Khan et al. 2000) noted concerns regarding the fate of the plants after they had reached their capacity to accumulate heavy metals. "Phytomining," the recovery of accumulated trace metals, might be side benefits of phytoremediation; however, concerns about plant matter getting into the food chain by direct consumption or by decomposition pathways include considerable ecological and human health problems (Khan et al.,2000).Also, there covery of metals is very costly. Henceforth, harvesting and disposing of plant biomass is essential to prevent recycling of accumulated metals when the aquatic plants decompose. Further, the biomass of aquatic macrophytes may also be used for biogas production.

SUMMARY AND CONCLUSION

High metals content in the biomass suggests the tremendous potential of *Azolla microphylla* to take up heavy metals, i.e., Hg of (98.25- 99%) and may be used as a bio accumulator to polish heavy metals in industrial effluent. The concentrations of metals in the *Azolla microphylla* were directly treated plants in the 2mg, 4mg and 6mg/ L treatment groups showed the appearance of smaller and slightly brown color as compared to the control under the conditions of 7 days incubation period. The Hg concentration was reduced to 0.02, 0.07 and 0.07 mg/L Hg treatment respectively. Therefore, after 7days the removal of Hg from the solution was 99%, 98.25% and 98.83% from the selected initial concentration. In India, Azolla microphylla is widely used as green manure, biofertilizer, and animal feed. Therefore, proper precaution before their application must be taken, because *Azolla microphylla* cultured on polluted waters may contain heavy metals, e.g. Hg. Reduces the concentration of Total Soluble Sugar, Non Reducing Sugar, Reducing sugar and Starch content of the plant Therefore in summary, this article addresses whether or not, and to what degree, *Azolla Microphylla* might bio concentrate Hg from contaminated water. However, in natural or real conditions, which may be synergistic or antagonistic with respect to metal uptake in a plant system.

Further , some other issues such as heavy metals release overtime, growth of *Azolla microphylla* over time, disposal of harvested Azolla pinnata from natural system, comparative assessment with other potent aquatic macrophytes, and cost–benefit analysis encourage further studies in this direction before suggesting the present phytoremediation technology as an eco-friendly and cost-effective one. Henceforth, harvesting and disposing of plant biomass is essential to prevent recycling of accumulated metals when the aquatic plants decompose. Further, the biomass of aquatic macrophytes may also be used for biogas production.

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