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ASSESSING THE PHYTOREMEDIATION POTENTIAL AND THE METABOLIC RESPONSES OF AZOLLA MICROPHYLLA ON LEAD EXPOSURE

Biotechnology

KEYWORDS: Heavy metal, lead, Phytoremediation, Azolla, photosynthetic pigments, proline, bioconcentration factor

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ABSTRACT

Phytoremediation is a novel green technology being applied for removing pollutants from soil and water. Being cost effective and ecofriendly, it is gaining importance in the research areas. The selection of the plant with hyperaccumulating capability depends on type of polluted site and the type of pollutants to be removed. The present work was undertaken to study the metabolic responses of an aquatic fern: Azolla microphylla and its ability to remove heavy metal lead from the waste waters. The changes in the growth pattern and physiological parameters under stress conditions can be used as a factor to determine plant tolerance capacity and the adverse toxic effect of the contaminant. Azolla microphylla was allowed to grow in E&W growth media supplemented with 0.15ppm lead. The physiological and biochemical parameters were assessed after 7, 15 and 30 days. The results suggest that lead induces oxidative stress in Azolla microphylla which shows acclimation as a defense mechanism in response to stress induced injury to plant. This implies that Azolla microphylla can be used as potential candidate for remediation in heavy metal loaded water.

INTRODUCTION:

Industrial growth and population explosion has led to over exploitation of our natural resources and degradation of the environment. Rapid pace of urbanization has increased the demand for finished goods. As a result more and more pollutants are being added to the three compartments of the environment i.e. air, water and soil. Among the various pollutants, heavy metals are of great environmental concern and many of them are toxic even at very low concentrations.

Lead (Pb) is a non essential ubiquitous heavy metal originating from different sources like mining and smelting activities, burning of coal, effluents from storage battery industries, automobile exhausts, pesticides, and from additives in pigments and gasoline as well as from the disposal of municipal sewage sludge enriched with Pb (Eick et al. 1999). Its impact on human health through the food chain and its high persistence in the environment owing to non biodegradable nature has made it a major ecological concern (Piechalak et al. 2003). Its unique properties like softness, high malleability, ductility, corrosion resistance, low melting point etc. have resulted in its increased use automobile industry, paint, ceramics, plastics, etc. Pb can change the enzyme activity and their quantity in different metabolic pathways such as those of the photosynthetic Calvin cycle (Stevens et al. 1997), nitrogen metabolism (Kumar and Dubey 1999), and sugar metabolism (Verma and Dubey 2001). In humans it is known to interfere with various body functions, mainly affecting the central nervous, hematopoietic, hepatic and renal system producing serious disorders (Kalia & Flora, 2005). Chronic Pb toxicity occurs at blood lead levels of about 40–60 ug/dL and is characterized by persistent vomiting, encephalopathy, lethargy, delirium, convulsions and coma (Flora et al., 2006; Pearce, 2007).

Pollution of aquifers has led to reduction in available pure water. The US-Environmental Protection Agency (EPA) has stipulated 0.015 mg L-1 and 0.20 mg L-1 as limits for lead in drinking water and for effluent respectively. Hence removing Pb (II) from industrial wastewater is necessesary for protection and restoration of waterways.

Different conventional physico-chemical methods have been used for heavy metal removal from effluents, such as membrane filtration [Yoon,et.al, 2009], chemical precipitation[Matlock, et al. 2002][Ramos, et al. 2009], ion exchange[Inglezakis et al.,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]. However, their applications have limitations as these are costly, specific and generate secondary waste which again can be a pollutant. So the focus has now shifted to greener and economical environmental friendly technology to clean up the aquatic systems. Phytoremediation is one such cheap, eco-friendly, solar driven technology utilizing plants to remove, reduce, sequester or degrade pollutants into nontoxic or less toxic forms. Rhizofiltration is one of the best known phytoremediation strategies, using plant roots to absorb, concentrate and precipitate heavy metals from contaminated 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 [Veseley et al.,2007].

The plant selected for the study, Azolla is a small aquatic floating fern having symbiotic association with cyanobacteria Anabaena azollae(Peter &Meeks,1989). For this reason this has been used as fertilizer in botanical gardens and as green manure in paddy fields in Asia (Carrapico, 2001). Another advantage of this plant is its high biomass production .Owing to its fast growth rate so it can form colonies rapidly giving an appearance of dense mats over water surfaces. It is considered a weed in South Africa and north part of Iran (Ashton, 1976). Studies have shown that Azolla can effectively adsorb hexavalent and trivalent chromium, zinc(II) and nickel(II) from solutions and electroplating effluents (Bennicelli,et al.,2004),(Zhao,et al.1997,1998,1999) and gold from aqueous solutions(Antunes,2001).

This study assesses the ability of aquatic plant species, Azolla microphylla Kauf. to remove Pb (II) from aqueous solutions and further investigation into the effect of lead on its growth and physiological parameters.

MATERIAL AND METHODS:

Azolla microphylla was collected from National facility for culture collection and utilization of blue green algae (NCCBGUA) Indian Agriculture Research Institute, New Delhi and maintained in open ponds and in the laboratory. Before the heavy metal treatment, plants were washed and surface sterilized with a solution of 0.1% mercuric chloride for 20 seconds and immediately dipped in large volume of sterile water. Plants were transferred into plastic trays containing N free Expianase and Watanabe (1992) nutrient solution with pH maintained between 6.7- 7.0. Two sets of three trays were maintained, first set had only nutrient media taken as control;

second set contained nutrient medium along with 0.1ppm Pb. The fresh medium was added every alternate day into the exposure sets, to overcome the losses due to evaporation. The experiment was performed in triplicate. The fronds were given chronic exposure and collected at three control points i.e. after 7 days, 15and 30 days. Plants were blot dried and tested for different metabolites

Estimation of Increase in Biomass:
Fresh biomass was measured after 7, 15 and 30 days in both control and exposed plants. The difference between initial weight and final weight gives the increase in biomass of the plants.

Estimation of Electrolyte Leakage:
Under stress conditions the protective cell membrane looses it capacity to retain the electrolytes present in their cytoplasm and these leak into the exterior. Electrolyte leakage was estimated by method given by Lutts (1996).

Estimation of Photosynthetic pigments:
The chlorophyll was determined by the method given by Litchenthaler & Welburn (1985). For chlorophyll absorbance was read at 645 nm and 670 nm. The values were obtained in µg/gm of the plant.

Estimation of Carotenoid Content:
The carotenoid content was estimated by Litchenthaler & Welburn (1985) method. The absorbance at 470 nm was read and the values were calculated in µg/gm of the plant.

Estimation of Proline Content:
Proline estimation was done using method given by Bates et.al.1973. 500mg of Azolla fronds were homogenized in 10ml 3% sulphosalicyclic acid (w/v), centrifuged at 10,000Xfor 10 min. 2ml of the supernatant was mixed with glacial acetic acid and ninhydrin and heated for 1 hr at 950 C. Samples were cooled and extracted with 4 ml toluene and vortexed for 1 min. Toluene layer was separated with pipette and absorbance at 520 nm was read taking toluene as blank.

Estimation of Flavonoid Content:
Flavonoid was estimated according to method given by Harborne (1973). Flavonoids were extracted from 500mg Azolla fronds in10 ml of 95% ethanol by keeping for 24 hrs at 200 rpm. To 1ml of above extract was added 10% AlCl3 and 1M Potassium acetate. The solution was made 10 ml using distilled water and kept at room temperature for 30 min. and then absorbance was read at 420 nm.

Digestion of Plant Material and Analysis:
Plant samples (1g) were digested by adding tri-acid mixture (HNO3, H2SO4, and HClO4 in 5:1:1 ratio) at 80°C until a transparent solution was obtained (Singh,2011: Allen 1986)After cooling, the digested sample was filtered using Whatman no. 42 filter paper and the filtrate was finally made to 50 ml with distilled water. Pb ion concentrations were determined using flame atomic absorption spectrometer (FAAS) of ECIL Model- AAS4129). All the chemicals and reagents of analytical grade were purchased from Merck, India. Triple distilled analytical grade water was used for the preparation of all standards and solutions.

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
BCF= Conc. of metal in dried plant tissue (mg/g)
Initial conc. of metal in external sol. (mg/l)
Bio-concentration factor is a useful parameter to evaluate the potential of plants for accumulating metals (Lu et al., 2004), (Mun, et al.,2008).

STATISTICAL ANALYSIS:
The data obtained was statistically analyzed using Excel software. T-test was applied to test the significance at 5% level (p 0.05). All values are mean ± S.E. of three replicates.

RESULTS AND DISCUSSION:
Chronic exposure to lead resulted in different physiological and biochemical changes within the plant

Biomass:
Inhibition of growth is a common observed response to heavy metal stress and is also one of the most important agricultural indices of heavy metal tolerance (Mallar, 2014). Lead affected the growth of Azolla microphylla which was observed as reduction in fresh weight (fig 1). There was constant reduction in the biomass produced in Pb exposed plants with time and after 30 days it was 5.27gm as compared to 512.55gm in control.

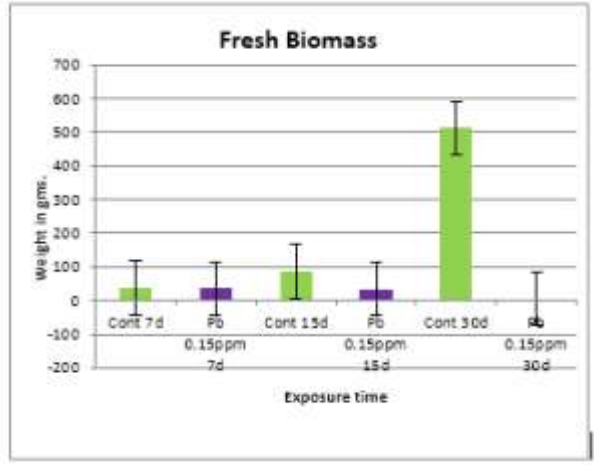


Fig-1 Changes in biomass with increase in exposure time to Lead (Pb)

Electrolyte Leakage:
Membrane stability is an important criterion to assess stress induced changes. The electrolyte leakage is graphically represented in fig 2. Electrolyte leakage was maximum in Pb 30days exposure i.e. 84.31 % increase in electrolyte leakage. Electrolyte leakage increased with increase in exposure time suggesting increase in damage to cell membrane with exposure to metal.

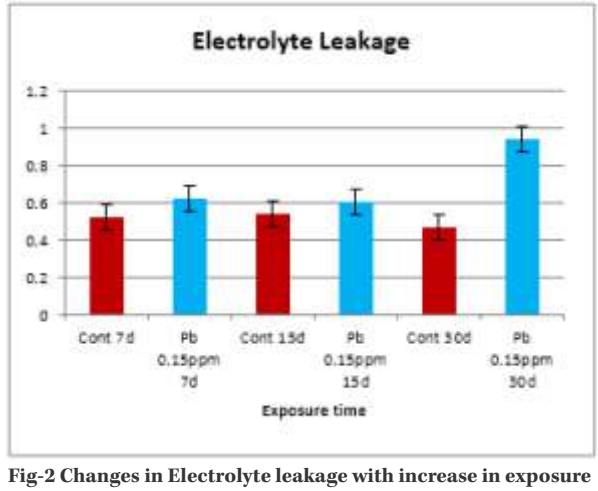


Fig-2 Changes in Electrolyte leakage with increase in exposure time to Lead (Pb)

Various workers (Vangronsveld and Clijsters, 1994) suggested oxidation of cross linking of protein thiols, inhibition of plasma membrane ATPase as the mechanism of heavy metal induced membrane damage. Green et al., (1980) have suggested interaction of

heavy metals with membrane phospholipids or the displacement of membrane-bound calcium as a cause of heavy metal-induced changes in membrane structure

Photosynthetic Pigments:
A decrease in the chlorophyll content was observed as the plant was exposed to heavy metal. Decrease in Chlorophyll a was 0%, 34.29%, 32.86 % after 7, 15 and 30 days respectively.

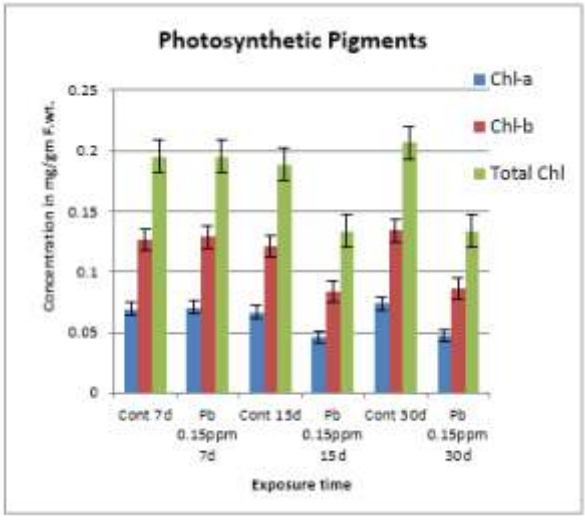


Fig-3 Changes in photosynthetic pigments with increase in exposure time to Lead (Pb)

Chlorophyll b also showed similar trend of decrease. An initial 0.79% increase was seen which again decreased to 33.86%, and 32.28% at 7, 15, and 30 day exposure. Change in total chlorophyll was recorded as decrease by 1.02%, 32.49% and 31.98% for Pb after 7,15and 30 days exposure respectively (fig 3). This suggests that with increase in metal exposure metal interference with pigment metabolism increases. Mukherji and Maitra (1976) in rice observed that Pb toxicity resulted in lowering Chl a/b ratio.

Carotenoid Content:
Carotenoids are accessory photosynthetic pigments which also act as non-enzymatic antioxidants protecting chlorophyll from damages due to oxidative stress. Carotenoid content showed a slight increase as compared to control after 15 days but with the increase in exposure time the carotenoid content showed a decrease (fig 4).

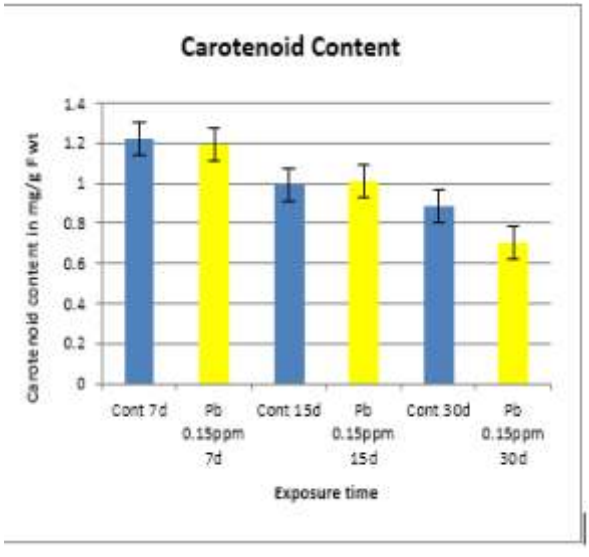


Fig-4 Changes in Carotenoid content with increase in exposure time to Lead (Pb)

Proline Estimation:
Accumulation of proline is considered as an important physiological response to stress. It is an non-enzymatic anti-oxidant compound. Proline content is graphically represented in fig 5. An increase of 15.79%, 105.26% and 131.58% was observed for 7, 15 and 30 days respectively. Proline behaves as a free radical scavenger to overcome the oxidative stress (Reddy,et al., 2004). Proline accumulation can be correlated to detoxification against any stress induced

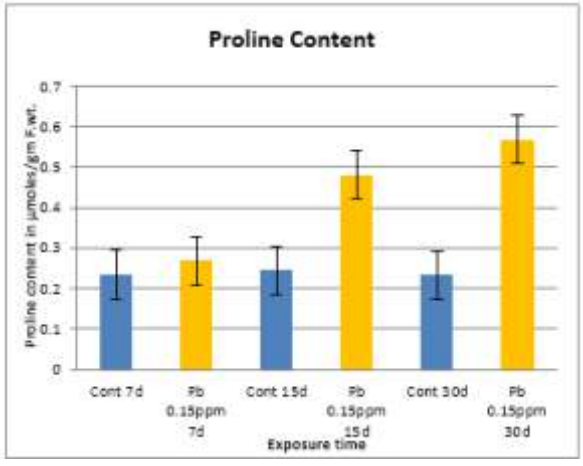


Fig-5 Changes in proline content with increase in exposure time to Lead (Pb)

Flavonoid Estimation:
Flavonoids are another non-enzymatic anti-oxidants produced in response to stress conditions. It was observed that initially there was decrease in the flavonoid content, but after 30 days the flavonoid content increased by 9.1% in case of Pb exposed Azolla microphylla plants as evident from fig 5.

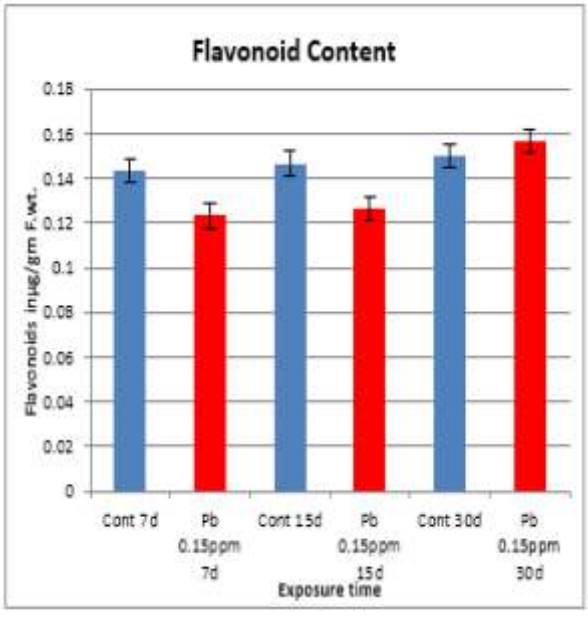


Fig-6 Changes in flavonoid content with increase in exposure time to Lead (Pb)

Relative Growth:
With increase in time duration of Lead exposure, relative growth reduced. After 30 days it was reduced to 0.21 as compared to control which was 20.5.Thayaparan et al. (2013) has reported decrease in the relative growth of Azolla pinnata exposed to lead when metal concentrations were

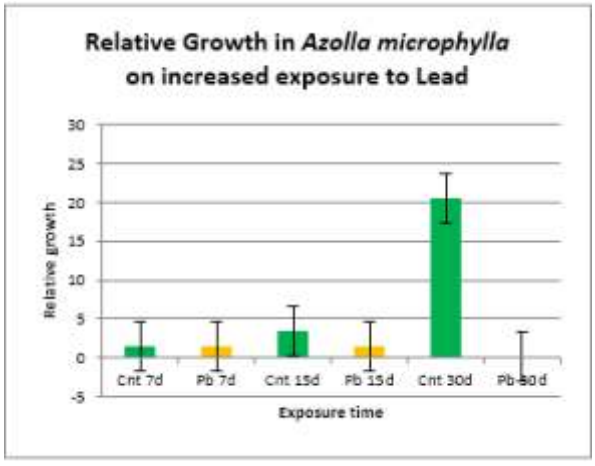


Fig-7 Relative growth in Azolla microphylla with increase in exposure time to Lead (Pb)

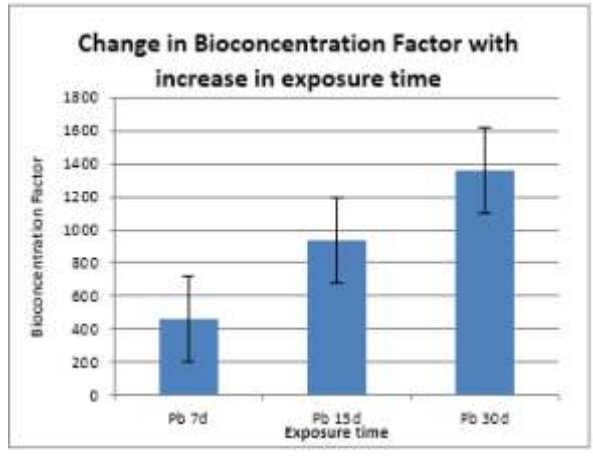


Fig-8 BCF of Azolla microphylla with increase in exposure time to Lead (Pb)CONCLUSION:

Bioconcentration Factor(BCF):

The phytoremediation potential of a plant is judged by its bio-concentration factor. The BCF values over 1000 indicate that plant can be used for phytoremediation (Zayed, 1998). In this study it was observed that BCF of A. microphylla for Pb increased significantly with increasing exposure time and the highest BCF was observed after 30 days.

CONCLUSION:

Above results show that though changes are induced in Azolla microphylla in response to stress produced by Lead exposure, it is quite capable in resisting stress. This study showed that the uptake ability and the BCF of A. microphylla for lead increased with the increase of Pb exposure time. Thus A. microphylla is a potential candidate for removal of Pb from waterways polluted with effluents containing Pb.

REFERENCE

1)Allen SE, Grimshaw HM, Rowland AP. [1986] Chemical analysis. In: Moore, PD, Chapman, SB. (Eds.), Methods in Plant Ecology, Blackwell Scientific Publication, Oxford, London, pp. 285–344.|2)Ashton, P. J. and Walmsley, R. D.(1976)The aquatic fern Azolla and Anabaena symbiot. Endeavour,35: 39-45, 1976.|3)Antunes, P. M.,Watkins, G.M and Duncan, J. R.(2001) Batch studies on the removal of gold(III) from aqueous solution by Azolla filiculoides. Biotechnol. Lett., 23,pp 249-251.|4)Bates, L.S., Waldren, R.P. and Teare, I.D. (1973). Rapid determination of free proline for water stress studies. Plant and Soil, 39,pp 205-207.|5)Bennicelli, R., Z. Stepniewska, A. Banach, K. szajnocha and J. Ostrowski,(2004) The ability of Azolla Caroliniana to remove heavy metals (Hg(II), Cr(III), Cr(VI)) from municipal waste water. Chemosphere, 55,pp 141-146.|6)Carrapiço F. (2001). The Azolla-Anabaena-bacteria system as a natural micro-cosm. Proc. SPIE, 4495,pp 261-265.|7)Eick,M.J., Peak, J.D., Brady, P.V. and Pesek, J.D. (1999) Kinetics of Pb absorption/desorption on goethite: residence time effect. Soil Sci. 164, pp28–39.|8)Flora,S.J.S., Flora, G. and Saxena, G. (2006). Environmental occurrence, health effects and management of lead poisoning. (In: Jose, S. C, Jose, S., eds. Lead. Amsterdam: Elsevier Science B.V.). pp. 158–228.\9)Green, D.E., Fry, M., Blondin, G.(1980) Phospholipids the molecular instruments of ions solute transport in biological membranes. Proc Nac Acad Sci USA 77,pp 257-261.|10)Harborne JB (1973). Phytochemical methods, London. Chapman and Hall, Ltd. pp. 49-188.|11)Kalia, K. and Flora, S.J. (2005). Strategies for safe and eff ective therapeutic measures for chronic arsenic and lead poisoning. J. Occup. Health 47,pp1–21.|12)Kumar, R.G. and Dubey, R.S. (1999) Glutamine synthetase isoforms from rice seedlings: effects of stress on enzyme activity and the protective roles of osmolytes. J. Plant Physiol 155,pp 118–121.\13) Inglezakis, V.J. and Loizidou, M.D.(2007) Ion exchange of some heavy metal ions from polar organic solvents into zeolite, Desalination, 211, pp 238-248. \14)Lu,X., Kruatrachue,M., Pokethiyook,P. and HomyokK.,(2004) Removal of cadmium and zinc by water hayacinth, Eichhornia crassipes, Science Asia, 30, pp 93- 103. |15)Lutts, S., Kinet, J.M. and Bouharmont, J. (1996). NaCl-induced senescence in leaves of rice (Oryza sativa L.) cultivars differing in salinity resistance. Ann. Bot. 78, pp 389–398.|16)Malik,P.K.(2003) Use of activated carbons prepared from sawdust and rice husk for adsorption of acid dyes: a case study of Acid Yellow 36, Dyes and pigments, 56, pp 239-249. \17)Matlock, M.M., Howerton, B.S., and Atwood, D.A (2002) Chemical precipitation of heavy metals from acid mine drainage, Water research, 36, pp 4757- 4764. |18)Miretzky,P., Saralegui,A., and Cirelli, A.F.(2006) Simultaneous heavy metal removal mechanism by dead macrophytes, Chemosphere, 62, pp 247-254. |19) Mitra, P., Sarkar, D., Chakrabarti, S. and Dutta, B.K. (2011) Reduction of hexa-valent chromium with zero-valent iron: Batch kinetic studies and rate model, Chemical engineering journal (Amsterdam, Netherlands), 171, pp 54-60. |20)Momodu M.A. and. Anyakora C.A.(2010) Heavy Metal Contamination of Ground Water: The Surulere Case Study, Research Journal Environmental and Earth Sciences 2(1),pp 39-43|21)Mukherji, S. and Maitra, P.(1976) Toxic effects of lead on growth and Hg2+ on germinating rice (Oryza sativa L.) seeds and on mitosis of onion (Allium cepa L.) root tip cells. Indian J. Exp Biol.,14,pp 519-521.|22)Mun, H.W. Hoe, A.L. and Koo, L.D.(2008) Assessment of Pb uptake, translocation and immobilization in kenaf (Hibiscus cannabinus L.) for phytoremediation of sand tailings, Journal of environmental sciences, 20, pp 1341-1347. |23)Peters, G. A.and Meeks, J. C.(1989) The Azolla-Anabaena symbiosis: basic biology. Ann. Rev. Plant Physiol. Plant Mol. Biol., 40,pp 193-210.|24)Piechalak, A., Tomaszewska. B., Baralkiewicz, D. (2003) Enhancing phytoremediative ability of Pisum sativum by EDTAapplication.Phytochemistry 64,pp1239–1251.|25Ramos,W.D.S., Poznyak,T., Chairez, I. and C ó rdova, I. (2009) Remediation of lignin and its derivatives from pulp and paper industry wastewater by the combination of chemical precipitation and ozonation, Journal of hazardous materials, 169, pp 428- 434. Rana, P., Mohan, N. and Rajagopal, C. (2004) Electrochemical removal of chromium from wastewater by using carbon aerogel electrodes, Water research, 38, pp 2811-2820. |26)Reddy, A. R., Chaiyanya, K. V., Vivekanandan,, M. (2004). Drought induced responses of photosynthesis and antioxidant metabolism in higher plants. J. Plant Physiol. 133,pp 56-61. |27)Ruley,A.T., Sharma,N.C. Sahi,S.V. Singh,S.R. and Sajwan,K.S.(2006) Effects of lead and chelators on growth, photosynthetic activity and Pb uptake in Sesbania drummondii grown in soil, Environmental pollution, 144, pp 11-18. |28) Sekhar,K.C., Kamala,C.T. Chary,N.S. Balaram,V. and Garcia,G.(2005) Potential of Hemidesmus indicus for phytoextraction of lead from industrially contaminated soils, Chemosphere, 58, pp 507-514. |29)Singh.A and Agrawal. M (2011): Management Of Heavy Metal Contaminated Soil By Using Organic And Inorganic Fertilizers: Effect On Plant Performance, The IIOAB Journal Special Issue On Environmental Management For Sustainable Development Issn: 0976–3104 ©Iioab-India Open Access Vol. 2; Issue 1; pp 22-30 22.|30)Srinivasan,M., Sahi,V., Favas,S., Paula, J.C., and Venkatachalam,P.(2014) Lead heavy metal toxicity induced changes on growth and antioxidative enzymes level in water hyacinths [Eichhornia crassipes(Mart.)]., Botanical studies, 1: pp54-55. |31)Stevens, R.G., Creissen,G.P and Mullineaux, P.M. (1997) Cloning and characterisation of a cytosolic glutathione reductase cDNA from pea (Pisum sativum L.) and its expression in response to stress. Plant Mol. Biol. 35,pp 641–654.|32)Thayaparan,M. Iqbal, S.S. Chathuranga, P.K.D. and Iqbal, M.C.M.(2013) Rhizofiltration of Pb by Azolla pinnata, International Journal of Environmental Sciences 3(6), pp 1811-1821. |33)Verma, S. and Dubey, R.S. (2001) Effect of cadmium on soluble sugars and enzymes of their metabolism in rice. Biol. Plant 44,pp117–123.|34)Verma, S. and Dubey, R.S. (2003) Pb toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci. 64,pp 645–655|35)18Veselý,T., Tlustoš,P. and Száková,J.(2011) The use of lettuce (Pistia stratiotes L.) for rhizofiltration of a highly polluted solution by cadmium and lead, International journal of phytoremediation, 13,pp 859-872. 36)Vangronsveld ,J.and Clijsters, H.(1994) Toxic effect ofmetals; in Plant and chemical elements (ed.) ME Farago (Germany, VCH Verlagsgesclschaft) pp149-177.|37)Watanabe I., Roger P.A., Ladha J.K., and Van Hove C.(1992) Biofertilizer Germplasm Collections at IRRI. IRRI, 8.|38)Yoon, J., Amy, G., Chung, J., Sohn, J. and Yoon, Y.(2009) Removal of toxic ions (chromate, arsenate and perchlorate) using reverse osmosis, nanofiltration and ultrafiltration membranes, Chemosphere, 77, pp 228-235.|39)Zayed,A., Gowthaman,S. and Terry,N,(1998) Phytoaccumulation of trace elements by wetland plants: I. Duckweed, Journal of environmental quality, 27, pp 715-721. |40)Zhao,M., Duncan,J. R. and Van Hille, R. P. (1999) Removal and recovery of zinc from solution and electroplating effluent using Azolla Filiculoides. Wat. Res., 33 (6),pp 1516-1522.|41)Zhao, M. and Duncan J. R.(1997), Batch removal of hexavalent chromium by Azolla filiculoides. Appl. Biochem. Biotechnol., 26,pp 179-183.|42)Zhao, M. and Duncan J. R.(1998) Removal and recovery of nickel from solution and electroplating rinse effluent using Azolla filiculoides. Process Biochem., 33 (3),pp 249-255.