



Alteration in biochemical contents of *Azolla* by heavy metals (Zn and Pb)

¹ Dr. Rimjhim Sheel, ² Dr. Kumari Nisha

¹ Associate Professor & HOD, Department of Botany, Ganga Devi Mahila College, K. Bagh, Patna, Bihar, India

² Department of Biotechnology, College of Commerce Arts & Science, Patna, Bihar, India

Abstract

According to Gill (2014), heavy metals are major environmental pollutants, especially in high anthropogenic areas. These heavy metals can bind with essential cellular components of plant such as proteins, amino acids, chlorophyll and nitrogen and finally influence their functions. Several water ferns are capable of accumulating Heavy Metals. One of which is *Azolla* species. So, it is useful in the detoxification of industrial effluents. Roots of *Azolla* plant absorb pollutants including Heavy Metals Zn and Pb and can be used for phytoremediation. The aim of this study was to assess the influence of heavy metal on some biochemical parameters (Chlorophyll, Free Amino acid, Protein and Nitrogen) of water fern *Azolla*. Plants were grown in nutrient medium (Shive and Robbins medium-I), according to Sheel & Sinha (2003), for 30 days containing heavy metals 2.5%, 5% and 10% of Zn and Pb separately. These Heavy metals transfer from root to the shoot resulting in reducing the concentration of essential nutrients such as K⁺, Na⁺ and Mg⁺⁺ in plants and inhibits normal growth (Lumpkin and Plucknett, 1980). The uptake of heavy metals also affect the concentration of Chlorophyll, Protein, Free Amino acid and Nitrogen. Result of the experiment also shows that high concentration of Zn is more effective on biochemical parameters of *Azolla* sps. than high concentration in Pb.

Keywords: phytoremediation, *Azolla*, biochemical parameters, heavy metals, nutrients

Introduction

Among various water pollutants, heavy metals are the major concern because of their bioaccumulative nature (Lokeshwari and Chandrappa 2007; Yadav *et al.* 2009; Chang *et al.* 2009) [22, 37, 6]. Continuous modernization increases urbanization and industrialization which induces the level of heavy metals in the biosphere (Lu *et al.*, 2004) [23]. Toxic levels of some heavy metals appear as a result of environmental pollution due to removal from mining, automobile traffic, manufacturing of goods and agricultural wastes (Oncel *et al.*, 2000) [27]. Zinc and lead compounds are widely used in industry to make paint, rubber, detergents, dyes, wood preservatives and ointments. Some amount of zinc is also released into the environment by natural processes, but most comes from human activities like mining, steel production, coal burning and waste burning (Andra *et al.*, 2010) [1]. Heavy metals plays an important role in many biochemical reactions within the plants (Lu *et al.*, 2004) [23] as essential micronutrient. Several plants species, such as water lettuce (*Pistia stratiotes*) (Mishra *et al.*, 2008) [26], water lilies (*Nymphaea spontanea*) (Choo *et al.*, 2006) [7], parrot feather (*Myriophyllum aquaticum*), creeping primrose (*Ludwigia palustris*), and watermint (*Mentha aquatic*) (Kamal *et al.*, 2004) [20] have been studied to determine their potential in accumulating heavy metals.

Aquatic water ferns are capable of removing different type of heavy metals from polluted water bodies. Aquatic ferns has an ability to take up heavy metals and makes them interesting research material in the treatment of industrial effluents and sewage waters (Andra *et al.*, 2010) [1]. *Azolla* is an aquatic macrophytes and is derived from Greek word *azo* (to dry) and *alloyo* (to kill) meaning that plant dies when it dries. These

dichotomously branched free floating fast growing aquatic ferns are naturally available on moist soils and marshy ponds. The shape of Indian species is typically triangular measuring about 1.5 to 3.0 cm in length, 1 to 2 cm in breadth. In the dorsal leaf lobe there is an ellipsoidal cavity filled with gases is lined with mucilage (Lumpkin. *et al.*, 1980) [24, 32] which contains the cyanobiont *Anabaena azollae* (Peters *et al.*, 1980) [12] and a gram positive non-nitrogen fixing bacteria (Hates *et al.*, 1980) [2] identified as *Arthrobacter* species (Grilli Caiola *et al.*, 1988) [25]. *Azolla* sps. is worldwide distributed and have been intensively studied due to their high growth rates combined with high bioremediation efficiency rich in proteins, essential amino acids, vitamins A, B12, beta-carotene, growth promoter intermediates and minerals like calcium, phosphorus, potassium, iron, copper and magnesium. Being rich in Phosphorus and Nitrogen, *Azolla* has been successfully used as a green manure in rice fields in Asia and Africa and as a feed supplement for aquatic and terrestrial animals (Marwaha. *et al.*, 1992 and Teckle-Haimanot *et al.*, 1995) [33, 11]. Zinc deficiency in plants affect photosynthesis due to altered chloroplast pigments (and Kösesakal, and Ünal, 2009) [21]. The most visible zinc deficiency symptoms are short internodes and a decrease in leaf size and delay in maturity (Brown *et al.*, 1993) [5].

The present investigation demonstrates that the Heavy Metals (Zinc and Lead) affect biochemical parameters (Chlorophylls, Free amino acids, Proteins and Nitrogen.) of *Azolla* plant during phytoremediation.

Material and Methods

Plants were collected from natural pond of Rajendra

Agriculture University, Pusa, Bihar. The pond was provided with fresh water without additional fertilizers. *Azolla filliculoid* plants were maintained and cultured in the Ganga Devi Mahila Mahavidyalaya, Kankarbagh, Bihar, India under control condition of temperature (27°C in Day and 22°C in night) and constant day length (16 hrs light and 8 hrs dark).

Biochemical Tests

Seven containers were taken and 500ml of Shive and Robbins medium-I was poured in each container. Three container contained 2.5%, 5% & 10% Zinc, whereas the other three contained the above mentioned percentage of Lead and one container with only nutrient medium, was considered as control. After 30 days of culture, the plant materials were taken from Control and also from each container containing different percentage of Zinc and Lead for detection of Chlorophyll – a, Chlorophyll-b, Total Chlorophyll and some plants were air dried for Amino acid, Protein and Nitrogen detection.

Test for Total Chlorophyll

(www.jenway.com/adminimages/A09_001A_Spectrophotometry): 0.5gm of fresh plant leaves were taken, pulverized with 5ml of 90% Acetone, left for 2-3 minutes. The extract was filtered through Whatman No. 1 filter paper into a volumetric flask. Spectrophotometer was set up in Spectrum mode to scan between 665nm for total Chlorophyll, 662nm For Chlorophyll a and Chlorophyll b at 646nm.

Test for free amino acid (Ninhydrin test): 2gm of crushed plant tissue powder was weighed and ground in a pastel and mortar with a small quantity of washed sand. To these homogenate 5 to 10ml of ethanol (80%) was added, filtered and centrifuged. The extract was used for quantitative estimation of total free amino acids. 1ml of extract and 1ml of ninhydrin and 2ml of D.W was added in a fresh test tube and heated in a boiling water bath for 20 minutes. To which 5ml of 90% ethanol was added and content were mixed. After 15 minutes the intensity of the purple colour against a reagent blank on spectrophotometer at 570 nm was recorded.

Crude protein test (Biuret test): Fresh tissue was homogenized in chilled TCA (Trichloro acetic acid) and centrifuged at 5000rpm. Residue was treated with NaOH and filtered. 4 or 5 drops of Biuret reagent was added and incubated for 10 minutes at room temperature. Absorbency

was measured at 540 nm.

Test for nitrogen: Dried sample was sent to CDRI Lucknow for detection of Nitrogen percentage.

Results and Discussion

Excess micro-nutrients (Zinc and Lead) transfer from root to the shoot that reduces the concentration of essential nutrients of plant and affect (Lumpkin and Plucknett, 1980)^[24, 32] their metabolic parameters. Zinc and Lead are mobile and bio-accessible metal, which accumulate in soil and penetrate the food chain easily through plants. Pb uptake in the root of water fern was higher than in the stem and leaves (Erzsebet Buta *et al.* 2011)^[10]. The accumulation of Pb is higher in the case of a low concentration (below 4%). In the stem and leaves the translocation of Pb was lower than root (Erzsebet Buta *et al.*, 2011)^[10] So, it showed less effect then Zn. The high dose (10%) of lead and Zinc negatively influenced the process of growth length and metabolic parameters of plants. Resulted data of biochemical test shows that the total Chlorophyll (Chlorophyll C₅₅H₇₂O₅N₄Mg, Chlorophyll a – C₅₅H₇₂O₆MgN₄, Chlorophyll b- C₅₅H₇₀O₆MgN₄) concentration increases in plants simultaneously with increase of Zn and Pb level in growth medium. The concentration of nitrogen also increased with increase of Zn and Pb concentration in growth medium. Plants uptake nitrogen in the form of ammonia or nitrate.

Chlorophyll a, b and total Chlorophyll concentration was slightly affected in 2.5% concentration of Zn and Pb, in the growth medium, because these heavy metals are micronutrients and all micronutrients are beneficial for plants. According to Deval *et. al.*, 2012^[9]. *Azolla* showed maximum efficiency of accumulation of Zn up to 5% of concentration. Due to this reason, plants were severely affected by 10% concentration of Zn and Pb. According to Khalil *et. al.*, in 1998^[19], Zinc deficient leaves appeared light green due to the low concentration of chlorophyll. Hisamitsu *et al.*, 2001^[18], investigated that Zinc deficiency disrupted the chlorophyll synthesis in some plants. The accumulation of Zinc increased chlorophyll contents as it acts as a structural and catalytic components of proteins, enzymes and co-factors for normal development of pigment biosynthesis (Balashouri, 1995)^[28]. Chlorophyll and nitrogen concentration slightly increase in 2.5% and 5% concentration of Zn and Pb in growth medium but greatly increase in 10% concentrations.

The results are presented in Tables, Figure and Figure.

Table 1

Chlorophyll	Control	Pb Supplemented			Zn Supplemented		
		2.5%	5%	10%	2.5%	5%	10%
Chlorophyll a C ₅₅ H ₇₂ O ₆ MgN ₄	0.18	0.18	0.19	0.24	0.23	0.26	0.30
Chlorophyll b C ₅₅ H ₇₀ O ₆ MgN ₄	0.10	0.11	0.14	0.16	0.16	0.19	0.24
Total Chlorophyll C ₅₅ H ₇₂ O ₅ N ₄ Mg	0.35	0.38	0.42	0.49	0.48	0.51	0.56
Nitrogen (in µg/gm dried leaves)	2.400	2.402	2.450	2.530	2.440	2.500	2.765

Absorbency of Chlorophyll a at 662nm, Chlorophyll b at 646nm, Total Chlorophyll at 665nm & CDRI Lucknow for detection of Nitrogen percentage.

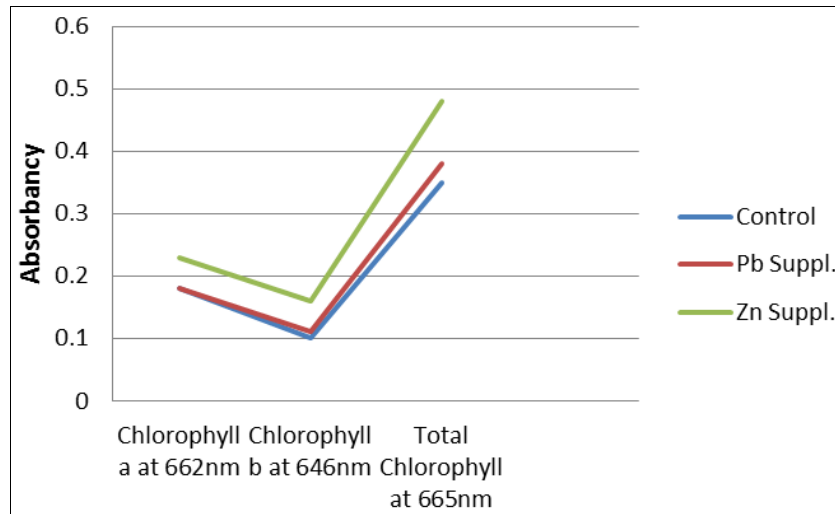


Fig 1: Chlorophyll content at 2.5% concentration of Zinc and Lead in growth medium after 30 days of harvesting.

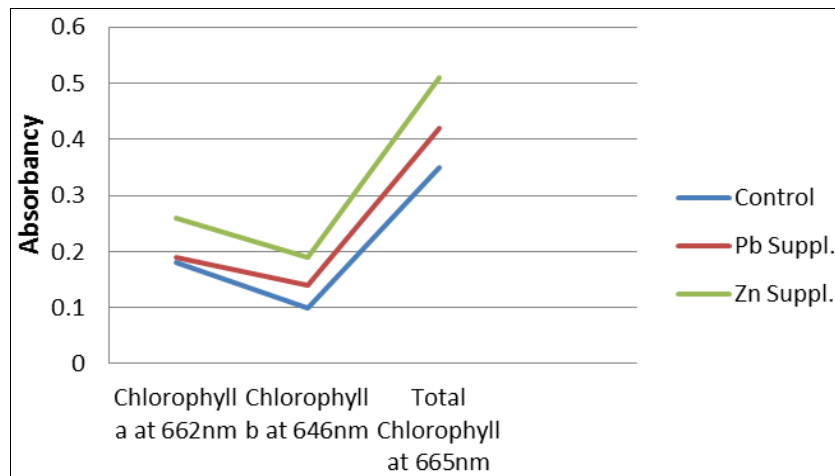


Fig 2: Chlorophyll content at 5% concentration of Zinc and Lead in growth medium after 30 days of harvesting.

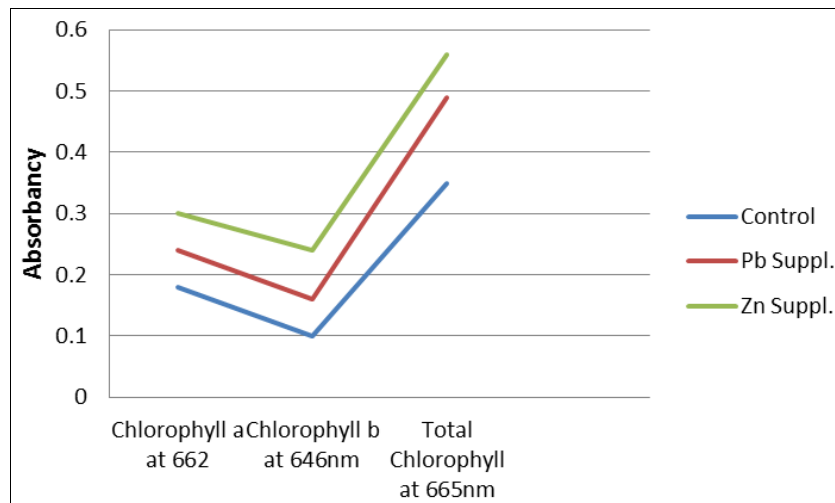


Fig 3: Chlorophyll content at 10% concentration of Zinc and Lead in growth medium after 30 days of harvesting.

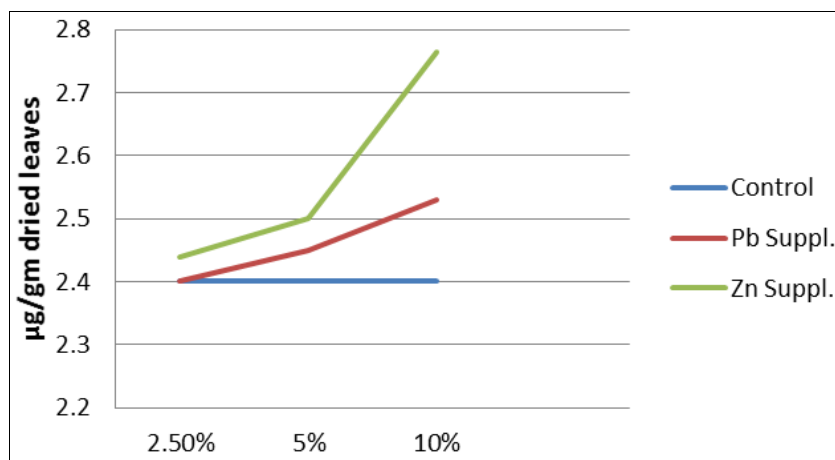


Fig 4: Nitrogen content after 30 days of harvesting.

Lead Supplemented

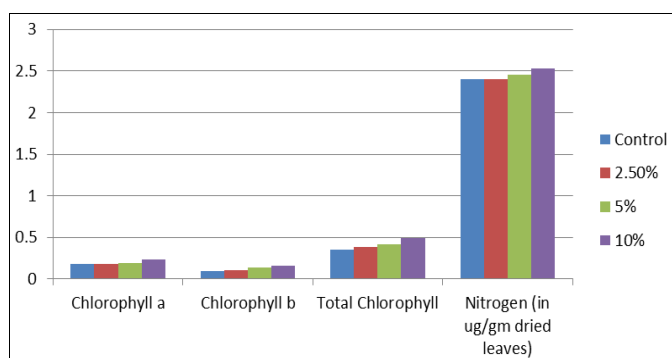


Fig 5: Absorbance of Chlorophyll a at 662nm, Chlorophyll b at 646nm, and Total Chlorophyll at 665nm

Zinc Supplemented

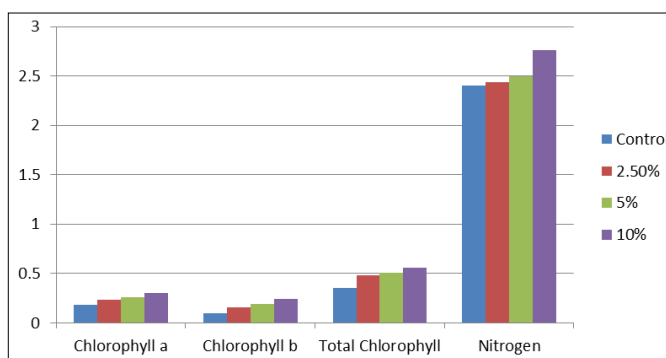


Fig 6: Absorbance of Chlorophyll a at 662nm, Chlorophyll b at 646nm and total Chlorophyll at 665nm

Table 2

	Control	Pb Supplemented			Zn Supplemented		
		2.5%	5%	10%	2.5%	5%	10%
Amino acid	0.20	0.17	0.15	0.14	0.14	0.11	0.09
Total Protein	0.19	0.19	0.186	0.12	0.19	0.16	0.10

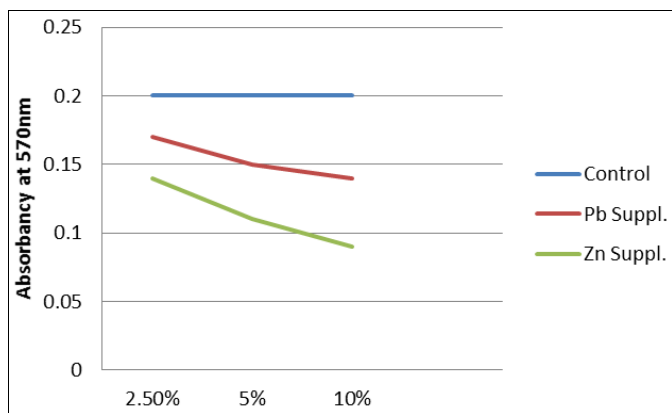


Fig 7: Amino acid concentration in Zinc and Pb Supplemented medium after 30 days of harvesting.

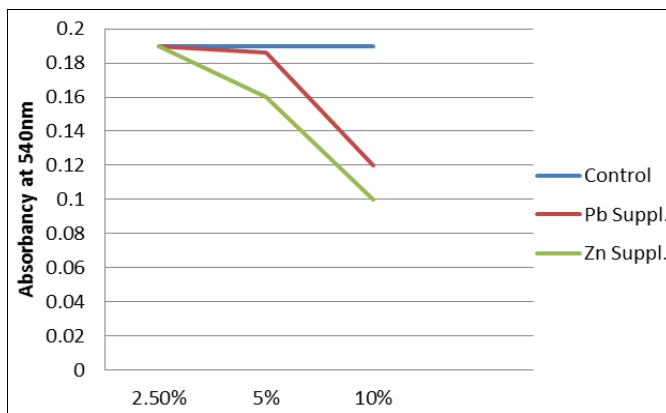


Fig 8: Protein content in Zinc and Lead supplemented medium after 30 days of harvesting.

Lead & Zinc supplemented plants

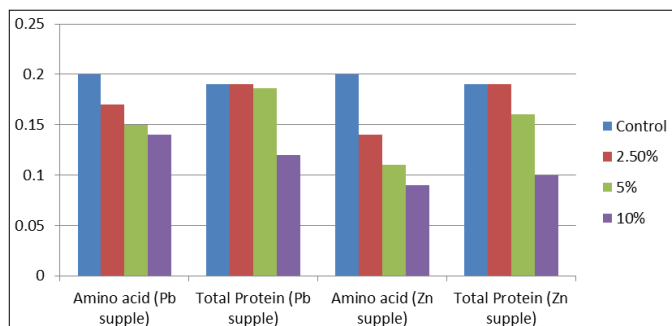


Fig 9: O.D of Amino Acid at 570nm and Protein at 540nm.

Excess Zinc and Lead causes the decline in protein and amino acid content. Decline in protein level are due to decrease in level of Nitrate reductase activity that affects total protein content in plant (Beevers & Hageman, 1969) [3]. Lead inhibit total amylase activity in *Avena sativa L.* (Bhushan and Gupta, 2008) and Nitrate reductase *Vigna radiate* (Gautam *et al.*, 2008) [16].

Excess heavy metals concentration in growth medium suppress the metabolic pathway of some free amino acids. Proteins are made up of amino acids so, both are affected simultaneously. Zinc and lead are an essential and beneficial element for human bodies and plants because they are essential microelement, but when they are present in excess quantity they induces toxic environmental factor (Xiaomei *et al.*, 2004) [36]. Zinc and Lead reduced the protein content in *Vicia faba.L.* (Singh 2007) [31]. Toxicity may result from the binding of metals to sulphhydryl group in protein leading to an inhibition of activity or disruption of structure or by the displacement of an essential element (Van Assche and Clijsters, 1990) [35]. Decreases in protein level might be due to the breakdown of the protein synthesis mechanism at the toxic concentration level of heavy metal or due to reduced incorporation of free amino acid into protein. Protein showed decreasing trend with increase in zinc concentration in *V. mungo* (Pavada *et al.*, 2004) [15]. Protein degradation might contribute to amino acid accumulation in metal stress plant (Chen *et al.*, 2001) [8]. Protein get damage due to reactive oxygen species, which cause oxidation of protein (Reinhackal *et al.*, 1998). Some amino acids are modified by heavy metals and such modification affect proteins (Ian, 2006).

From the present investigation, it is clear that the biochemical data confirms the alteration of Chlorophyll, Free amino acid, Protein and Nitrogen. Which might be due to higher uptake of heavy metals and indicates the harmful nature of effluent.

Conclusion

It can be concluded that *Azolla* plant absorbs high quantity of Zn and Pb which affect their growth and biochemical parameters. Zinc accumulate in large quantity in shoot and leaves where as Pb concentration is high in root (Lumpkin and Plucknett, 1980) [24, 32]. Excess concentration of Zn and Pb affect concentration of Chlorophyll, Free amino acid, Protein and Nitrogen. The Chlorophyll and Nitrogen content increases with increase in concentration of Pb and Zinc in growth medium, The Chlorophyll contains nitrogen, so, concentration

of nitrogen also increases with increase of chlorophyll content. But Proteins made up of amino acids so, amino acid reduces the concentration of protein, whereas the concentration of proteins and amino acids decreases with the increase in uptake of Zinc and Lead.

Acknowledgement

We are thankful to Central Drug Research Institute, Lucknow (India) for Nitrogen detection of the samples.

References

1. Andra SS, Sarkar D, Makris KC, Mullens CP, Sahi SV, Bach SBH. Synthesis of phytochelatins in vetiver grass upon lead exposure in the presence of phosphorus. *Plant Soil*. 2010, 171-185.
2. Hates B, Frank O, Angells BD, Feingold S. Plasma tocopherol in man at various times after ingesting free or acetylated tocopherol. *Nutr. Rep. Int.* 1980; 21:531-536.
3. Beevers L, Hageman RH. Nitrate reduction in higher plants. *Annu. Rev. Plant Physiol.* [University Illinois, Urbana IL.]. 1969; 20:495-522.
4. Bharat Bhushan, Ajay Pal, Satish Kumar Yadav, Archana Singh. Evaluation of post-germinative lipid peroxidation and enzymatic antioxidant potential in lead absorbing oat (*Avena sativa*) seedlings. *Journal of Environmental Biology*. 2015; 36(1):279-288. with 17 Reads.
5. Brown *et al.*, Brown PH, Cakmak I, Zhang Q. Form and Function of Zinc in Plants Kluwer academic publishers, Dordrecht. 1993, 90-106.
6. Chang JS, Yoon IH, Kim K-W. Heavy metal and arsenic accumulating fern species as potential ecological indicators in As-contaminated abandoned mines. *Ecological Indicators*. 2009; 9:1275-1279.
7. Choo TP, Lee CK, Low KS, Hishamuddin O. Accumulation of chromium (VI) from aqueous solutions using water lilies (*Nymphaea spontanea*). *Chemosphere*. 2006; 62:961-967.
8. Chen J, Rappsilber J, Chiang YC, Russell P, Mann M, Denis. Purification and Characterization of the 1.0 MDA CCR4-NOT complex identifies two novel component of the complex. *J Mol. Bio.* 2001; 314(4):683-94.
9. Deval CG, Mane AV, Joshi NP, Saratale GD. Phytoremediation potential of aquatic macrophyte *Azolla caroliniana* with references to zinc plating effluent. 2012; 24(3):208-223.
10. Erzsebet Buta, Laura Paulette, Tania Mihaiescu, Mihai Buta, Maria Cantor. The Influence of Heavy Metals on Growth and Development of *Eichhornia crassipes* Species, Cultivated in Contaminated Water. 2011; 39(2):135-141.
11. Teckle-Haimanot EVD. Comparison of *Azolla mexicana* and N and P Fertilization on Paddy taro (*Colocasia esculenta*) yield, *Trop. Agric. (Trinidad)*. 1995; 72:70-72.
12. Peters GA, Evans WR, Crist DR, Mayne BC, Poole RE. Characterization and comparison of five nitrogen-fixing *Azolla-Anabaena* association I. optimization of growth conditions for biomass increase and N- content in controlled environment, *Plant Cell Environ*. 1980; 3:261-269.
13. Gill Mukti. Heavy metal stress in plants: a review

- International Journal of Advanced Research. 2014, 2(6). 1043-1055 ISSN 2320-5407, Review Article homepage: <http://www.journalijar.com>
14. Hutchby, Ion. Media Talk; Conversation Analysis and the study of Broadcasting. A Review Glasgow: open university Press. Pp.xiii, 185. SKY Journal of Linguistics. 2006; 19:255-264.
 15. Pavadai P, Dhanavel D, Seethraman N, Selaraju M. The Effect of Zinc on germination and yield parameters in blackgram [Vigna Munga (L.) Hepper Var. Co-3] Plant Achieves. 2004; 4(2):479-481.
 16. Rakesh Singh Sengar, Madhu Gautam, Sanjay Kumar Garg, Reshu Chaudhary, Kalpana Sengar. Effect of Lead on Seed Germination, Seedling Growth, Chlorophyll Content and Nitrate Reductase Activity in Mung Bean (*Vigna radiata*). Research Journal of Phytochemistry. 2008; 2:61-68.
 17. Sheel R, Sinha KS. Studies on the effect of agro chemicals used in paddy field as weedicide on biochemical profile of *Salvinia molesta* (Thesis). 2003.
 18. Hisamitsu TO, Ryuichi O, Hidenobu Y. Effect of Zinc concentration in the solution culture on the growth and contact of Chlorophyll, Zinc and Nitrogen in corn plant (*Zea mays*). J. Trop. Agric. 2001; 36(1):58-66.
 19. Khalil IA, Varonini Z, Pinton R. Chloroplast pigment in bean seedling as influence of Zinc deficiency. J. Sci. Tech. Pesawar. 1998, 49-51.
 20. Kamal A, Ghaly AE, Mahmoud N, Cote R. Phytoaccumulation of heavy metals by aquatic plants. Env. Int. 2004; 29:1029-1039.
 21. Kösesakal T, Muammer Ünal. Role of Zinc Deficiency in Photosynthetic Pigments and Peroxidase Activity of Tomato Seedlings IUFJ J Biol. 2009; 68(2):113-120.
 22. Lokeshwari H, Chandrappa GT. Effects of heavy metal contamination from anthropogenic sources on Dasarahalli tank, India. Lakes and Reservoirs Research Management. j. 2007, 1440-1770. 00337.x
 23. Lu X, Kruatrachue M, Pokethitiyook P, Homyok K. Env. Sci. Tech. Manage. Res. 2004; 30:93-103.
 24. Lumpkin TA, Plucknett DL. *Azolla*: botany, physiology and use as green manure. Econ. Bot. 1980; 34:111-153.
 25. Lutts S, Majerus V, Grilli Caiola JMM, Fornic C, Castagnola M. Bacteria in the *Azolla* – *Anabaena* association Symbiosis, C. Van Hove, *Azolla*. 1988; 2:185-198.
 26. Mishra KK, Rai UN, Prakash O. Bioconcentration and phytotoxicity of Cd in *Eichhornia crassipes*. Env. Mon. Asses. 2008; 130:237-243.
 27. Oncel I, Kele Y, Ustun AS. Interactive effects of temperature and heavy metal stress on the growth and some biochemical compounds in wheat seedlings. Env. Poll. 2000; 107:315-320.
 28. Balashwari P. Effect of Zinc on germination, growth and pigment content and phytomass of *Vigna radiata* and sorghum biocolor. J. Ecobiol. 1995; 7:109-114.
 29. Reinheckel T, Noack H, Lorenz S, Wiswedel I, Augustin W. Free Radical Research. 1998; 29:297-305.
 30. Sheel Rimjhim, Moni Kumari, Kumari Nisha. Impact : International Journal of Research in Applied, Natural and Social Sciences (IMPACT: IJRANSS) ISSN(P): 2347-4580; ISSN(E): 2321-8851 Impact Journals. 2016; 4(9):201-206.
 31. Singh MR. Impurities-heavy metals: IR Prespective. 2007. <http://www.usp.org/pdf/EN/meetings/asMeetingIndia/2008Session4track1>
 32. Lumpkin TA, Plucknett DL. *Azolla*- Botany, Physiology and use as a green manure, Econ. Bot. 1980; 34:111-153.
 33. Marwaha TS, Singh BV, Goyal SK. Effects of incorporation of *Azolla* on wheat (*Triticum aestivum* var. HD-2329), Acta. Bot. Indica. 1992; 20:218-220.
 34. Kosesakal T, Unal M. Role of Zinc deficiency in Photosynthetic pigments and peroxidase activity of tomato seedling, IUFJ J. Biol. 2009; 68(2):113-20.
 35. VanAssche F, Clijsters H. Effects of metals on enzyme activity in plants. Plant Cell Environ. 1990; 13:195-206.
 36. Xiaomei L, Kruatrachue M, Pokethitiyook P, Homyokb K. Removal of cadmium and zinc by water hyacinth, *Eichhornia crassipes*. J Sci Asia. 2004; 30:93-103.
 37. Yadav SK, Juwarkar AA, Kumar GP, Thawale PR, Singh SK, Chakrabarti T. Bioaccumulation and phyto-translocation of arsenic, chromium and zinc by *Jatropha curcas* L.: Impact of dairy sludge and biofertilizer. Bioresource Technology. 2009; 100:4616-4622.