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Effect of Heavy Metals on Biochemical Profile of *Azolla filiculoides*

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A B S T R A C T

The species of *Azolla* (Water fern) are small, delicate, moss- like plants with fragile rhizomes covered by crowded overlapping leaves. They are highly productive plants that double their biomass in 3–10 days. They form an endo symbiotic relationship with the diazotrophic cyanobacterium *Anabaena azollae*, which fixes atmospheric nitrogen, giving the plant access to the essential nutrient. They seem to be an excellent candidate for removal, disposal, and recovery of heavy metals from the polluted aquatic ecosystems. In the present investigation the effect of four different heavy metals viz. Lead, Cadmium, Mercury and Zinc on the biochemical profile of *Azolla filiculoides* was studied. It was observed that the heavy metals viz. Pb, Cd, Hg and Zn greatly influenced the total sugar and protein contents of *Azolla filiculoides*. However, the biochemical profile of *A. filiculoides* depended upon the nature and concentration of heavy metals. All the four heavy metals caused a great reduction in soluble sugar, reducing sugar, amylolytic activity, α amylase activity, total nitrogen content, protein content and increased free amino acids.

Introduction

The species of *Azolla*, belonging to family Salviniaceae (Water fern) are small, delicate, moss- like plants with fragile rhizomes covered by crowded overlapping leaves. Branching is free, and dense, fern- frond- like plants are formed. On the lower side simple roots, solitary or in clusters, extend a short distance downward in the water. The leaves are alternate and stand on the dorsal surface of the rhizomes in two rows. Each leaf is divided into an upper aerial and a lower submerged lobe. The upper lobe, which stands oblique and touches the water only on one edge, is several cells thick in the central region and is photosynthetic, with palisade tissue below and stomata on both surfaces. In its lower surface are large cavities where mucilage is secreted and where colonies of *Anabaena* live. This lobe is said to be inverted, its dorsal surface being uppermost. The submerged lobe is one cell layer thick through most of its extent; its function appears to be, in part, water absorption.

Plants of *Azolla* retain their floating position because they are not readily wet. The prevention of wetting is secured in *Azolla* by papillae on the exposed leaf surfaces and by the abundant small spaces between the many close- packed leaf lobes.

Azolla is a highly productive plant. It doubles its biomass in 3–10 days, depending on conditions, and yield can reach 8–10 tonnes fresh matter/ha in Asian rice fields. 37.8 t fresh weight/ha (2.78 t DM/ha dry weight) has been reported for *Azolla pinnata* in India (Hasan *et al.,* 2009).

They form an endo symbiotic relationship with the diazotrophic cyanobacterium *Anabaena azollae*, which fixes atmospheric nitrogen, giving the plant access to the essential nutrient. This has led to the plant being dubbed a "super-plant", as it can readily colonise areas of freshwater, and grow at great speed - doubling its biomass every two to three days. The only known limiting factor on its growth is phosphorus, another essential mineral. An abundance of phosphorus due to eutrophication or chemical runoff often leads to *Azolla* blooms.

Rapid industrialization, urbanization, and population in the last few decades have added huge loads of pollutants in the water resources (CPCB 2008). Such unprecedented pollution in aquatic ecosystems needs eco-friendly cost-effective remediation technology. A large number of industries including textile, paper and pulp, printing, iron–steel, electroplating, coke, petroleum, pesticide, paint, solvent, and pharmaceutical etc. consume large volumes of water and organic chemicals which differ in their composition and toxicity. The discharge of effluents from these industrial units to various water bodies (rivers, canals, and lakes etc.) leading to water pollution is a matter of great concern, especially for developing countries like India. Developed countries have water pollution problems mainly due to industrial proliferation and modern agricultural technologies, which are mainly addressed through improving wastewater treatment techniques. However, the lack of technical knowhow, weak implementation of environmental policies, and limited financial resources has given rise to serious challenges.

Among various water pollutants, heavy metals are of major concern because of their persistent and bio-accumulative nature (Rai *et al.,* 1981; Lokeshwari and Chandrappa 2007; Chang *et al.,* 2009; Yadav *et al.,* 2009). Water is an indispensable part for the sustenance of mankind and the increasing awareness about the environment; especially aquatic ecosystems have attracted the attention of researchers worldwide. A definite need exists to develop a low cost and eco-friendly technology to remove pollutants particularly heavy metals, thereby improving water quality. Phytoremediation offers an attractive alternative. Among these, *Azolla*, a free-floating, fast growing, and nitrogen fixing pteridophyte seems to be an excellent candidate for removal, disposal, and recovery of heavy metals from the polluted aquatic ecosystems (Arora *et al.,* 2006; Umali *et al.,* 2006).

In India, where most of the developmental activities are still dependent upon water bodies, heavy metal pollution is posing serious environmental and health problems (Sánchez-Chardi *et al.,* 2009; Siwela *et al.,* 2009). Heavy metals are metallic chemical elements with a high atomic weight and density much greater (at least five times) than water. They are highly toxic and cause ill effects at very low concentrations e.g. mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), thallium (Tl), and lead (Pb). They are added to the aquatic system, either naturally by slow leaching from soil/rock to water or through anthropogenic sources. In recent times, anthropogenic inputs, such as discharge of untreated effluent (waste water), have contributed to the predominant causation. A survey carried out by Central Pollution Control Board (2008) reported that ground water in 40 districts from 13 states of India i.e. Andhra Pradesh, Assam, Bihar, Haryana, Himachal Pradesh, Karnataka, Madhya Pradesh, Orissa, Punjab, Rajasthan, Tamil Nadu, Uttar Pradesh, West Bengal, and

five blocks of Delhi is contaminated with heavy metals. Lokeshwari and Chandrappa (2007) reported the bioavailability of heavy metals in Dasarahalli tank located in Bangalore (India) as Zn > Cd > Ni > Fe > Cu > Pb > Cr and warned the high health risks to human beings, due to the ability of these metals to enter the food chain.

Increasing environmental awareness and concern has attracted scientific community to extend its exploitation more vigorously in the area of phytoremediation because the fern can hyperaccumulate variety of pollutants such as heavy metals, radionuclides, dyes, and pesticides etc. from aquatic ecosystems along with other macrophytes (Padmesh *et al.,* 2006; Rai and Tripathi 2009; Mashkani and Ghazvini 2009; Sood *et al.,* 2011). This fern has many features that prove it as a better plant system than many other macrophytes, which include:

Fast Growth Rate: The ability of *Azolla* to grow rapidly, doubling its biomass in 2–4 days, is the most important attribute, along with its free-floating nature (Arora and Saxena 2005; Kathiresan 2007; Sood *et al.,* 2008a). Both these qualities help in its easy harvest for disposal or recovery of heavy metals from biomass.

Nitrogen-fixing ability

Nitrogen is among the most important macronutrient required for the synthesis of nucleic acids, proteins, phospholipids, and many secondary metabolites which play an important role in the overall growth of the plants (Amtmann and Armenguad 2009). The ability of *Azolla* to fix atmospheric nitrogen allows this fern to grow successfully in aquatic habitats lacking or having low levels of nitrogen (Pabby *et al.,* 2001, 2003b; Sood *et al.,* 2007). This may help *Azolla* to proliferate in polluted waters as well.

Biomass disposal

The removal of heavy metal contaminants by aquatic macrophytes presents the problem of plant biomass disposal so as to prevent recycling of accumulated metals (Rai 2009). The water content in *Azolla* fronds varies between 90 and 94% (Serag *et al.,* 2000). Therefore after drying, its volume reduces drastically, hence solving the problem of disposal to a much greater extent. The dry *Azolla* biomass can easily be transported for recovery of the heavy metal. Both living and dead biomass of *Azolla* have been exploited for the removal of heavy metals from industrial effluents and sewage water (Bennicelli *et al.,* 2004; Upadhyay *et al.,* 2007; Rai 2008; Mashkani and Ghazvini 2009).

Heavy metals have significant biological roles as metallo-enzyme and are required as micronutrients by all organs. Some heavy metals at low doses are essential micronutrients for plants but in higher doses they cause metabolic disorders and growth inhibition for most of the plant species with the ongoing technological advancement in industrialization and urbanization process, release of toxic contaminants like heavy metals in natural resources has become a serious problem worldwide (Sethy *et al.,* 2013).

Sucrose and starch are the major products of carbon assimilation pathways in most of the plants. Sugars are considered important metabolites because it is the first complex organic compounds formed in the plant as a result of photosynthesis, and it also provides a major source of respiratory energy. Sugars play a number of ecological roles in plant protection against wounds and infection as well as in the detoxification of foreign substances (Sativir *et al.,* 2000). Enzymes contributing to starch breakdown- α- amylase, β- amylase, α- glucosidase, oligo-1, 6-

glucosidase and starch phosphorylase has been studied particularly in seeds where they are responsible for the breakdown of polysaccharides reserve. Heavy metals have inhibitory effect on α and β-amylase during germination. The moderate levels of heavy metals generally play an important role in plant growth and productivity. They act as activators or co-factors in all vital processes, but relatively elevated level of heavy metals induced harmful effects on all physiological processes of plants. According to Deef (2007) low concentration of copper treatment exhibit an increase in the total carbohydrates content in growing axis and reverse is true for the high concentrations. Amylase activity showed a lower level in plants with the increase in concentration of heavy metals. So, it results in accumulation of soluble saccharides and polysaccharides content in seeds due to their negative effect on amylase activity.

Heavy metal stress affects the enzyme activity by reducing the antioxidant glutathione pool and affecting the iron mediated defense processes. Heavy metal toxicity greatly impaired not only the breakdown of the polysaccharides, but also the translocation of soluble sugars to the growing embryonic axis (Bhushan and Gupta, 2008). Heavy metal cause significant restriction in reserve mobilization such as soluble sugars and amino acids in pea seeds.

The mobilization of storage protein is one of the most important post-germinative events in the growth and development of seedling. During the germination period, the storage proteins are degraded by a variety of proteases, which convert the insoluble storage proteins into soluble peptides and free amino acids. These are mobilized to the embryonic axis to support its growth and also provide energy by oxidation of the carbon skeleton after deamination. These protein reserves are depleted, and nitrogen accumulates in the developing axis. The transfer of nitrogen is apparently achieved by the release of amino acids from the reserve protein by hydrolysis and their subsequent translocation to the developing axis. Protein breakdown and its recycling are essential for several developmental processes such as germination, morphogenesis, senescence, etc. (Palma *et al.*, 2002). Plant proteolytic system includes proteases mainly localized inside the organelles and the ubiquitin proteosome pathway in both the cytoplasm and the nucleus. Proteases are expressed in germinating seeds and are required for the degradation of storage proteins to mobilize amino acids for the growth of embryonic axis.

Amino acid transport is thought to be of primary importance in organic nitrogen acquisition, and might be involved in processes such as direct uptake of amino acids from the soil, phloem and xylem loading, phloem to xylem exchange, and retrieval of amino acids that “leak” from the cells.

In addition to amino acid transport, peptide transport seems to play an important role in periods of rapid protein mobilization such as redistribution of nitrogen during leaf senescence, protein deposition during seed development and storage protein hydrolysis during germination because during these stages when the efficiency of nitrogen transport may be increased by direct uptake of peptides.

Proline an imino amino acid, is known to get accumulated in wide variety of organisms ranging from bacteria to higher plants on exposure to abiotic stress. Proline accumulation in plants can serve as biomarker of heavy metal stress. Plants are known to accumulate high concentration of proline when treated with toxic heavy metals Heavy metals causes the decline in protein content and the corresponding rise in the activity of hydrolytic enzymes such as protease due to heavy metal stress strongly suggests the

catabolic activities. In the present investigation an effort has been made to study the effect of four different heavy metals viz. Lead, Cadmium, Mercury and Zinc on the biochemical profile of *Azolla filiculoides.*

Materials and Methods

Plants of *Azolla filiculoides* were grown separately in mineral salt solution in laboratory water tank (aquarium) exposed to six different concentrations viz. 100, 200, 400, 600, 800 and 1000mg/L of each of the four heavy metals. The sample size in each treatment was ten. The tank not exposed to heavy metal was treated as control. After ten days of growth total carbohydrate, total soluble sugar, reducing and non-reducing sugar, starch, soluble nitrogen, free amino acids, proline and protein estimated in leaf tissues. 500.0 mg of fresh leaves was homogenized with 10.0 ml of 80.0 percent ethanol and centrifuged at 2000 rpm for 20 minutes. The supernatant was collected and the carbohydrate content (reducing sugar) was estimated by Anthrone (Hedge, 1962) method. To one ml sample 4ml of Anthrone reagent was added and absorbance was recorded at 620nm using D- glucose as standard.

The phenol sulphuric acid reagent method of Dubois *et al.,* (1956) was followed for estimating the amount of total soluble sugars. 500.0 mg of fresh leaf tissue was homogenized with 10.0 ml of 80.0 percent ethanol and centrifuged at 2000 rpm for 20 minutes. The supernatant was collected. To one ml of the alcoholic extract, one ml of 5.0% phenol reagent was added. To this 5 ml of concentrated (96%) H2SO4 was added rapidly. It was gently agitated during addition of sulphuric acid and then allowed to stand in water bath at 26-30°C for 20 minutes. Optical density (O.D.) of the characteristic yellow orange colour thus developed was measured at 490 nm in a spectrophotometer (Electronic Corporation of India Limited, (GS 570IV) after setting for 100% transmission against the blank (by substituting distilled water for test solution). The standard curve was prepared by using known concentration of glucose. The quantity of sugar was expressed as mg/g fresh weight of tissue.

In order to estimate the amount of starch in the leaf tissue of *Azolla* plants of each of the four species of uniform size and colour were selected and surface sterilized with 0.2 percent (w/v) mercuric chloride solution for one minute, washed thoroughly several times with glass distilled water and kept for growth in water tank (aquarium).Plants were grown separately in six different concentration of each of the four heavy metals viz. lead, cadmium, mercury and zinc.

Plants were withdrawn after ten days and thoroughly with distilled water, their leaves were separated, dried in an oven at 70ºC until a constant weight was observed, ground to a fine powder and stored in a desiccators over anhydrous calcium chloride. Total, α and β amylase activity were assayed (Swain-Dekker, 1966) and liberate maltose was estimated (Yomo-Varner, 1973) in the dialyzed extract. The quantity starch was estimated by taking a constant weight which is known as the remaining sludge which was dried to 0.01 g and then 0.2 ml of enzyme Aldeastaz (0.1%) + (0.1 ml) of acetate solution organizer was added. 3 ml of distilled water was added and the mixture was left at 28°C for 24 h and 1 ml of toluene was added and then the amount of starch in a given volume was estimated by taking the same steps as for the estimation of reducing sugars.

Amino acids (AA) are the precursors to proteins and also their constituents and they play an important role in metabolism and development. Plants that were exposed to

toxic metals have also been shown to accumulate specific AA, which may have beneficial functions. The AA which are accumulated under heavy metal stress, play various roles in plants, including acting as signaling molecules, and osmolytes, regulating ion transport and facilitating detoxification (Xu *et al.,* 2012). Reports of the role of AA in the hyper accumulation of metals by plants are limited. Therefore the present investigation has been undertaken to determine the changes and differences in accumulation of selected free AA. Asparagine and glutamic acid as well aspartic acid and glutamine are involved in N-assimilation, transport and transamination processes of vascular plants, therefore the changes of these amino acids were investigated in detail. In the present study the total free AA compounds were determined using an EZ-faast amino acid analysis procedure (Phenomenex, Santa Clara, USA). Samples were analyzed for AA contents by the gas chromatography coupled with mass spectrometry detection using a HP 6890N/5975 instrument.

Similarly protein was estimated by Lowery (1951) method. 500.0 mg leaves were extracted with 5.0 ml of 5% TCA (Trichloroacetic acid). The homogenate was centrifuged at 2000 rpm. for 20 minutes and the supernatant discarded. The residue was dissolved in 10.0 ml of 0.1 N NaOH. 0.1 ml of this solution was made up to 1.0 ml by adding distilled water.

The following reagents were prepared:

Alkaline sodium carbonate solution (50 ml of 2% Na2CO3 in 0.1 N NaOH).

Copper sulphate sodium tartarate solution (0.5 ml of 0.5% CuSO4.5H2O in 0.5 ml of 1.0% sodium potassium tartarate, prepared fresh).

50 ml of reagent 'a' was mixed with 1.0 ml of reagent 'b'. This was mixed just before use.

Folin-ciocalteau reagent (diluted with equal volume of distilled water i.e. in the ratio of 1:1.

Stock standard solution – 100 mg of bovine serum albumin dissolved in 100 ml of 1N NaOH (1 mg/ml). 3.5 ml of alkaline copper reagent or Lowery reagent was added, to the dissolved residue and allow standing for 10 minutes. 0.5 ml of folin-ciocalteau reagent was added in last, and O.D. was measured at 720 nm in spe3.5 ml of alkaline copper reagent or Lowery reagent was added, to the dissolved residue and allows standing for 10 minutes. 0.5 ml of folin-ciocalteau reagent was added in last, and O.D. was measured at 720 nm in spectrophotometer. The reference curve was prepared by using known concentration of BSA (bovine serum albumin) in 0.1 N NaOH. The quantity of protein was expressed as mg/g fresh weight of tissue. The results obtained have been presented in Tables- 1- 14 and Figures- 1- 14.

Results and Discussion

In the present investigation it was observed that the heavy metals viz. Pb, Cd, Hg and Zn greatly influenced the total sugar and protein contents of *Azolla filiculoides*. However, the biochemical profile of *A. filiculoides* depended upon the nature and concentration of heavy metals. In control plants the total soluble sugar content in *A. filiculoides* was 0.065mg/g of fresh weight. Up to 200mg/L Pb, Cd and Zn heavy metals caused an increase in the content of soluble sugar of about 0.067mg/L and 0.076mg/L respectively.

Hg caused an increase in the sugar content to about 0.067mg/L upto concentration of 100mg/L and the showed a decreasing trend. Above 400mg/L all the four heavy metals caused a decline in the content of soluble sugar. All the four heavy metals viz. Lead, Cadmium, Mercury and Zinc caused a great reduction in soluble sugar to about 0.032-

0.038mg/gFW at concentration of 1000mg/L (Table- 1; Fig- 1).

The reducing sugar in control plants of *Azolla filiculoides* was about 0.035mg/gFW. Chemical analysis of carbohydrates showed significant increases in the contents of reducing sugars in response to lead, cadmium, mercury and zinc stress, which were decreased by liming treatments (Table- 2; Fig- 2). The contents of total soluble sugars also increased in all heavy metal-treated plants expect for zinc (Figure- 1). At 1000mg/L of Lead the concentration of reducing sugar slightly increased to 0.038mg/GFW whereas Cadmium, Mercury and Zinc caused a decline in the value of reducing sugar to 0.025- 0.028mg/gFW. Moreover, the contents of polysaccharides increased under all heavy metal stress, and then decreased by liming treatment in all heavy metal-treated plants except for lead-treated plants.

The contents of non-reducing sugars decreased in lead, cadmium, mercury and Zinc-treated plants (Table- 3; Fig- 3). In the present investigation it was observed that the content of non- reducing sugar decreased with increasing concentration of heavy metals. At concentration of 1000mg/L all the four heavy metals caused a decline in the content of reducing sugar to 0.008mg/gFW (by Pb), 0.010mg/gFW (by Cd), 0.011mg/gFW (by Hg) and 0.013mg/gFW (by Zn) (Table- 3; Fig- 3).

The total starch content of *Azolla filiculoides* also showed a decreasing trend on increasing the concentration of heavy metals (Table- 4; Fig- 4). The total starch content in control plants of *A. filiculoides* was found to be 2.25mg/gFW. This value showed a rapid declined on treating different concentrations of heavy metals. At 1000mg/L concentration Pb, Cd, Hg and Zn caused a decline in the total starch content to about 1.18mg/gFW, 1.16mg/gFW, 1.15mg/gFW and 1.26mg/gFW respectively (Table- 4; Fig- 4).

The Amylolytic activity of *Azolla filiculoides* decreased with increasing the concentration of each of the four heavy metals viz. Pb, Cd, Hg and Zn ind incubation time. In control plants of *A. filiculoides* the amylolytic activity was high i. e. untreated plants produced 80.35mg of maltose per hour after ten days of growth in hydroponic system. At 1000mg/L concentration all the four heavy metals caused maximum inhibition of amylolytic activity. At this concentration Pb, Cd, Hg and Zn caused decreased production of maltose to 61.45mg/hr, 62.15mg/hr, 60.45mg/hr and 63.25mg/hr respectively after ten days of growth (Table- 5; Fig- 5). The present findings gain support from the work of Bhushan and Gupta (2008) who also reported the decreased amylolytic activity in Oat seed germination in presence of heavy metal Lead. A similar trend in amylolytic activity was reported during seed germination in presence of Lead by Thimmaiah, 1989; Mukherji and Mukherji, 1990; Deep, 2002; Bansal, 2001; Dua and Sawhney, 1991; Bindu and Bera, 2002.

In control plants of *A. filiculoides* the α amylase activity was high i. e. the plants produced 42.75mg of maltose per hour ten days after growth in hydroponic condition. The α amylase activity in heavy metal treated plants decreased with increasing concentration of heavy metals. At 1000mg/L concentration Pb, Cd, Hg and Zn caused reduction in maltose production to 35.65mg, 36.35, 30.50mg and 35.75mg of maltose per hour after ten days of growth (Table- 6; Fig- 6). The present findings are also in agreement with the work of Bhushan and Gupta (2008) who also reported the decreased α amylolytic activity in Oat seed germination in presence of heavy metal Lead. A similar trend in α

amylolytic activity was reported during seed germination in presence of Lead by Thimmaiah, 1989; Mukherji and Mukherji, 1990; Deep, 2002; Bansal, 2001; Dua and Sawhney, 1991; Bindu and Bera, 2002. All the four heavy metals selected for present investigation caused deplition in total nitrogen Table- 7; Fig- 7).

In control plants of *A. filiculoides* the total nitrogen was 11.5mg/10 plants. The concentration of total nitrogen decreased with increasing the concentration of heavy metals. At 1000mg/L concentration each of the four heavy metals caused depletion in total nitrogen content to 1.3mg to 2.5mg/10 plants ten days after growth (Table- 7; Fig- 7). Mercury caused maximum depletion in total nitrogen (1.3mg) followed by Lead (1.5mg), Cadmium (1.7mg) and Zinc (2.5mg) per ten plants. Similarly the soluble nitrogen in *A. filiculoides* also decreased on increasing the concentration of each of the four heavy metals. At 1000mg/L Pb, Cd, Hg and Zn caused a decline in the soluble nitrogen to 0.6mg, 0.8mg, 0.3mg and 0.7mg per ten plants ten days after growth (Table- 8; Fig- 8).

An appreciable reduction in total nitrogen and soluble nitrogen was observed in *Vigna radiata* (mungbean) treated with various concentrations of Zn and Hg by Pratima Nag *et al.,* (1989). The heavy metal treated plants of *A. filiculoides* did not significantly modify the total contents of free amino acids.

Table.1 Effect of Heavy Metals on soluble sugar of *Azolla filiculoides* in mg/gFW

Heavy Metals	Concentration of Heavy Metals in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Pb	0.065±0.02	0.066±0.01	0.067±0.02	0.063±0.02	0.052±0.02	0.045±0.03	0.038±0.05
Cd	0.065±0.02	0.066±0.03	0.068±0.04	0.061±0.03	0.051±0.01	0.043±0.01	0.037±0.01
Hg	0.065±0.02	0.067±0.04	0.063±0.01	0.055±0.03	0.048±0.04	0.039±0.02	0.032±0.03
Zn	0.065±0.02	0.081±0.01	0.076±0.02	0.061±0.01	0.054±0.01	0.042±0.05	0.035±0.02

CD at P <0.05

Table.2 Effect of Heavy Metals on reducing sugar of *Azolla filiculoides* in mg/gFW

Heavy Metals	Concentration of Heavy Metals in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Pb	0.035±0.02	0.046±0.01	0.047±0.02	0.043±0.01	0.042±0.04	0.040±0.03	0.038±0.04
Cd	0.035±0.02	0.042±0.02	0.046±0.04	0.041±0.03	0.040±0.01	0.040±0.02	0.028±0.02
Hg	0.035±0.02	0.043±0.04	0.046±0.02	0.040±0.02	0.038±0.04	0.032±0.02	0.027±0.03
Zn	0.035±0.02	0.041±0.01	0.046±0.02	0.040±0.01	0.036±0.03	0.034±0.03	0.025±0.02

CD at P <0.05

Table.3 Effect of Heavy Metals on non- reducing sugar of *Azolla filiculoides* in mg/gFW

Heavy Metals	Concentration of Heavy Metals in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Pb	0.030±0.02	0.020±0.01	0.020±0.02	0.022±0.01	0.010±0.04	0.005±0.001	0.008±0.002
Cd	0.030±0.02	0.024±0.02	0.021±0.04	0.022±0.03	0.012±0.01	0.005±0.002	0.010±0.002
Hg	0.030±0.02	0.023±0.04	0.021±0.02	0.023±0.02	0.014±0.04	0.013±0.002	0.011±0.003
Zn	0.035±0.02	0.025±0.01	0.021±0.02	0.023±0.01	0.016±0.03	0.011±0.003	0.013±0.002

CD at P <0.05

Table.4 Effect of Heavy Metals on starch content of *Azolla filiculoides* in mg/gFW

Heavy Metals	Concentration of Heavy Metals in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Pb	2.25±0.003	1.75±0.01	1.55±0.02	1.46±0.01	1.40±0.04	1.35±0.002	1.18±0.002
Cd	2.25±0.003	1.78±0.02	1.57±0.04	1.44±0.03	1.38±0.03	1.30±0.002	1.16±0.002
Hg	2.25±0.003	1.71±0.04	1.48±0.02	1.38±0.02	1.32±0.04	1.26±0.002	1.15±0.003
Zn	2.25±0.003	1.85±0.01	1.70±0.02	1.56±0.01	1.45±0.03	1.37±0.003	1.26±0.002

CD at P <0.05

Table.5 Effect of Heavy Metals on total amylase activity in *Azolla filiculoides* in terms of mg of maltose produced/hr ten days after growth

Heavy Metals	Concentration of Heavy Metals in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Pb	80.35±0.15	78.25±0.11	75.35±0.22	71.25±0.11	67.35±0.14	62.25±0.12	61.45±0.12
Cd	80.35±0.15	79.25±0.12	77.25±0.34	73.45±0.023	69.50±0.13	65.35±0.22	62.15±0.12
Hg	80.35±0.15	77.35±0.14	71.25±0.12	70.15±0.22	67.45±0.14	61.35±0.12	60.45±0.23
Zn	80.35±0.15	79.35±0.21	76.25±0.32	72.50±0.11	70.15±0.13	65.30±13	63.25±0.12

CD at P <0.05

Table.6 Effect of Heavy Metals on total α- amylase activity in *Azolla filiculoides* in terms of mg of maltose produced/hr ten days after growth

Heavy Metals	Concentration of Heavy Metals in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Pb	42.75±0.15	42.65±0.15	42.30±0.22	41.85±0.14	40.75±0.14	38.75±0.12	35.65±0.12
Cd	42.75±0.15	42.55±0.13	42.32±0.34	41.65±0.23	40.630.13	38.55±0.22	36.35±0.12
Hg	42.75±0.15	40.25±0.14	38.35±0.12	36.55±0.22	34.65±0.14	32.75±0.14	30.50±0.23
Zn	42.75±0.15	42.66±0.21	42.37±0.32	41.80±0.11	40.75±0.15	39.76±13	35.75±0.12

CD at P <0.05

Table.7 Effect of Heavy Metals on total nitrogen in *Azolla filiculoides* in terms of mg/ 10 plants ten days after growth

Heavy Metals	Concentration of Heavy Metals in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Pb	11.5±0.35	10.5±0.15	8.8±0.22	7.5±0.14	6.0±0.14	4.2±0.12	1.5±0.13
Cd	11.5±0.35	10.7±0.11	9.5±0.34	7.6±0.24	6.2±0.13	4.5±0.22	1.7±0.12
Hg	11.5±0.35	9.5±0.12	7.6±0.12	6.5±0.20	5.7±0.14	3.5±0.14	1.3±0.23
Zn	11.5±0.35	10.80.21	8.7±0.32	7.6±0.11	7.0±0.15	3.5±13	2.5±0.12

CD at P <0.05

Table.8 Effect of Heavy Metals on soluble nitrogen in *Azolla filiculoides* in terms of mg/ 10 plants ten days after growth

Heavy Metals	Concentration of Heavy Metals in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Pb	2.9±0.16	2.5±0.05	2.1±0.22	1.7±0.14	1.5±0.14	1.2±0.12	0.6±0.13
Cd	2.9±0.16	2.6±0.11	2.3±0.14	1.8±0.14	1.7±0.13	1.5±0.22	0.8±0.12
Hg	2.9±0.16	2.4±0.10	2.0±0.12	1.6±0.10	1.3±0.14	0.8±0.14	0.3±0.13
Zn	2.9±0.16	2.7±0.12	2.5±0.12	1.9±0.11	1.8±0.15	1.6±13	0.7±0.12

CD at *P* <0.05

Table.9 Effect of Heavy Metals on free amino acids in *Azolla filiculoides* in terms of µmole/g FW ten days after growth

Heavy Metals	Concentration of Heavy Metals in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Pb	40.50±1.10	39.35±1.15	38.75±1.22	37.65±1.14	36.55±1.14	35.45±1.12	34.35±1.13
Cd	40.50±1.10	39.65±1.11	38.65±1.14	37.70±1.14	36.50±1.13	35.50±1.22	34.45±1.12
Hg	40.50±1.10	38.75±1.10	38.75±1.12	34.50±1.10	31.65±1.14	27.70±1.14	24.50±1.13
Zn	40.50±1.10	39.50±1.12	38.65±1.12	37.50±1.11	36.55±1.15	35.25±1.32	34.25±1.12

CD at *P* <0.05

Table.10 Effect of Heavy Metal Lead (Pb) on some free amino acids in *Azolla filiculoides* in terms of µmole/g FW ten days after growth

Amino acids	Concentration of Heavy Metal Lead (Pb) in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Glu	0.244±0.10	0.305±1.15	0.625±0.25	0.935±0.34	1.235±1.14	1.375±0.12	1.475±0.13
Gln	9.550±1.15	11.450±1.21	12.750±1.16	14.850±2.14	16.756±1.13	18.650±1.22	20.750±2.12
Pro	6.955±1.75	7.765±1.15	9.975±1.15	11.725±2.12	13.456+±1.14	16.365±1.14	19.250±2.13
Hyp	ND	ND	ND	ND	ND	ND	ND
Asp	2.635±0.25	3.675±0.13	5.675±0.53	9.275±1.18	12.350±1.21	14.375±1.36	16.256±2.12
Asn	2.840±0.75	3.736±0.71	5.750±0.23	8.325±1.25	11.565±1.25	14.675±1.26	16.375±2.15

CD at *P* <0.05

Table.11 Effect of Heavy Metal Cadmium (Cd) on some free amino acids in *Azolla filiculoides* in terms of µmole/g FW ten days after growth

Amino acids	Concentration of Heavy Metal Lead (Pb) in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Glu	0.244±0.10	0.375±1.14	0.635±0.24	0.975±0.35	1.250±1.14	1.575±0.12	1.565±0.15
Gln	9.550±1.15	11.575±1.21	13.675±1.16	15.350±2.14	17.253±1.15	18.655±1.22	20.635±2.15
Pro	6.955±1.75	7.875±1.16	10.235±1.15	13.450±2.15	15.375±1.14	18.565±1.14	19.775±2.13
Hyp	ND	ND	ND	ND	ND	ND	ND
Asp	2.635±0.25	3.775±0.12	5.465±0.54	9.385±1.15	12.535±1.25	14.535±1.35	16.365±2.14
Asn	2.840±0.75	3.575±0.73	5.585±0.25	9.545±1.25	13.763±1.25	15.275±1.25	17.485±2.15

CD at *P* <0.05

Table.12 Effect of Heavy Metal Mercury (Hg) on some free amino acids in *Azolla filiculoides* in terms of µmole/g FW ten days after growth

Amino acids	Concentration of Heavy Metal Lead (Pb) in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Glu	0.244±0.10	0.385±1.14	0.675±0.25	1.635±0.25	2.235±1.16	2.350±0.17	2.375±0.18
Gln	9.550±1.15	11.665±1.22	14.535±1.16	16.375±2.16	17.355±1.15	18.735±1.22	21.355±2.15
Pro	6.955±1.75	7.735±1.17	11.335±1.15	14.550±2.15	16.275±1.15	18.635±1.15	20.350±2.13
Hyp	ND	ND	ND	ND	ND	ND	ND
Asp	2.635±0.25	3.785±0.14	5.575±0.15	10.450±1.15	12.765±1.25	15.545±1.35	16.605±2.11
Asn	2.840±0.75	3.685±0.75	5.775±0.25	10.305±1.25	13.853±1.25	16.455±1.25	19.565±2.12

CD at P <0.05

Table.13 Effect of Heavy Metal Zinc (Zn) on some free amino acids in *Azolla filiculoides* in terms of µmole/g FW ten days after growth

Amino acids	Concentration of Heavy Metal Lead (Pb) in mg/L						
	Control±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Glu	0.244±0.10	0.345±1.14	0.665±0.25	1.525±0.25	1.650±1.16	1..660±0.17	1.675±0.16
Gln	9.550±1.15	11.450±1.22	12.575±1.16	13.350±2.16	15.250±1.15	17.565±1.22	19.375±2.12
Pro	6.955±1.75	7.775±1.17	9.125±1.15	11.457±2.15	12.375±1.15	14.575±1.15	15.850±2.13
Hyp	ND	ND	ND	ND	ND	ND	ND
Asp	2.635±0.25	3.575±0.14	5.565±0.15	8.375±1.15	11.435±1.25	16.545±1.35	15.475±2.12
Asn	2.840±0.75	3.665±0.75	5.750±0.25	8.640±1.25	10.265±1.25	11.315±1.25	13.255±2.12

CD at P <0.05

Table.14 Effect of different concentration of heavy metals on the total protein contents in mg/g of fresh weight in *A. filiculoides*

Heavy metals	Concentration of Heavy metals in mg/L						
	Control ±SD	100±SD	200±SD	400±SD	600±SD	800±SD	1000±SD
Pb	0.235 ±0.002	0.220 ±0.001	0.214 ±0.002	0.207 ±0.003	0.130 ±0.003	0.053 ±0.002	0.000
Cd	0.235 ±0.002	0.215 ±0.002	0.201 ±0.001	0.135 ±0.003	0.121 ±0.004	0.061 ±0.002	0.000
Hg	0.235 ±0.002	0.213 ±0.001	0.200 ±0.002	0.133 ±0.003	0.122 ±0.002	0.064 ±0.001	0.000
Zn	0.235 ±0.002	0.218 ±0.002	0.245 ±0.001	0.257 ±0.003	0.261 ±0.001	0.218 ±0.004	0.210 ±0.002

CD at P < 0.05

Fig.1 Effect of heavy metals on soluble sugar of *A. filiculoides*

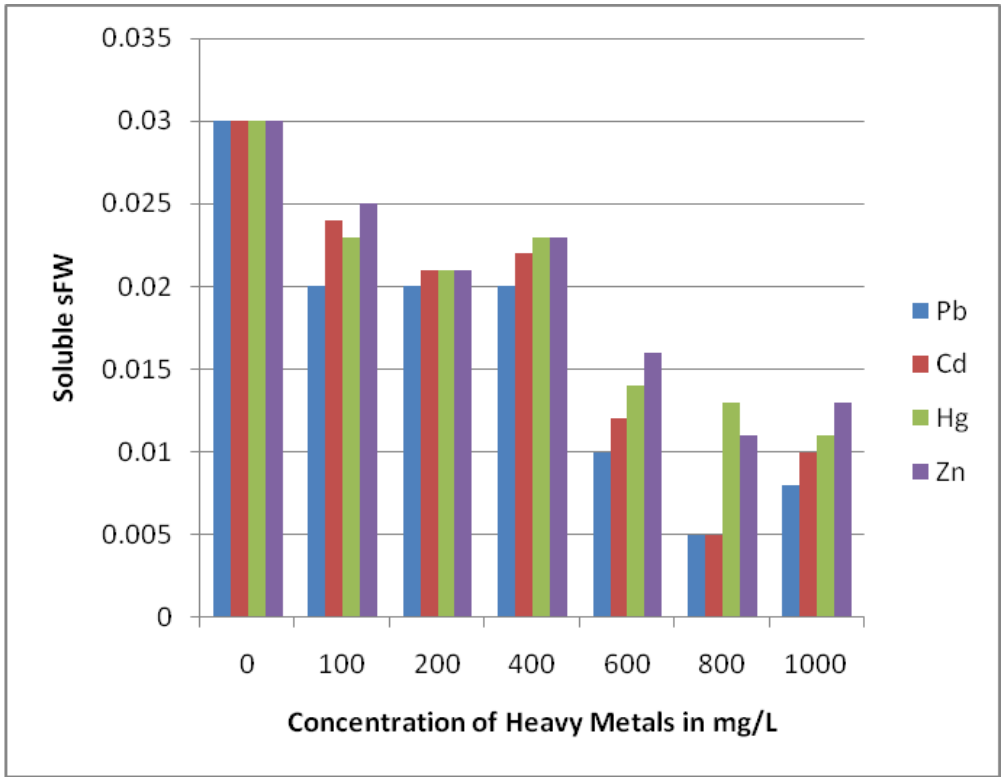


Fig.2 Effect of Heavy metals on Reducing sugar in *Azolla filiculoides*

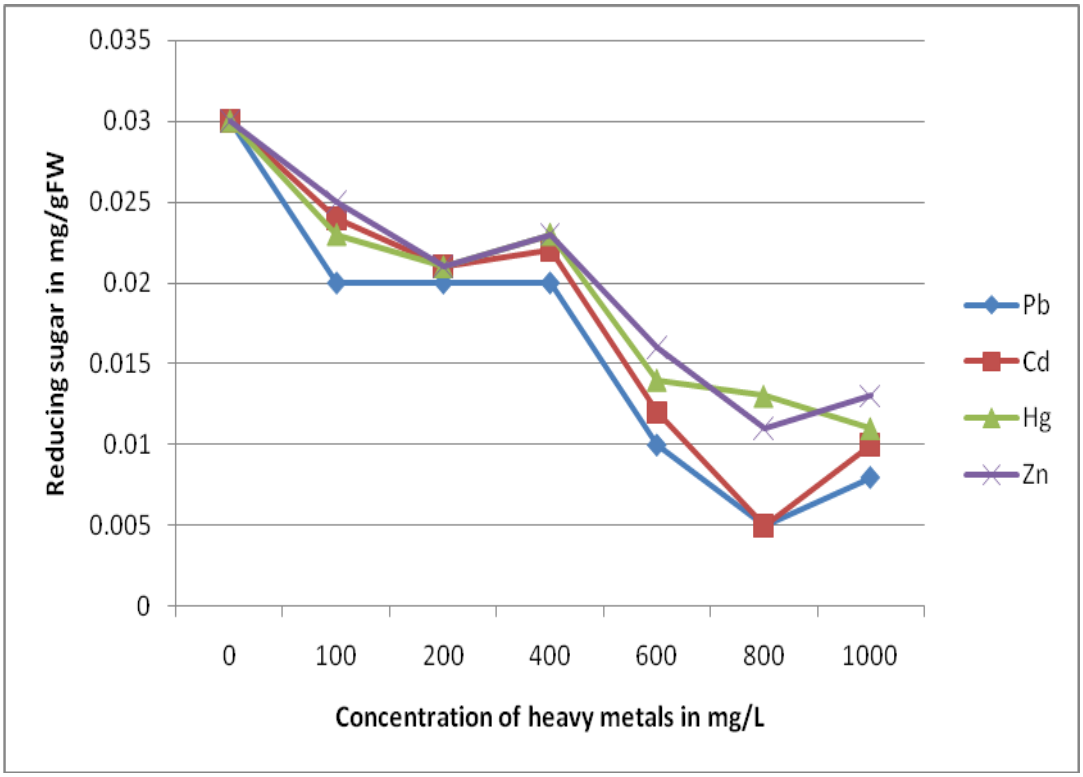


Fig.3 Effect of heavy metals on non- reducing sugar in *Azolla filiculoides*

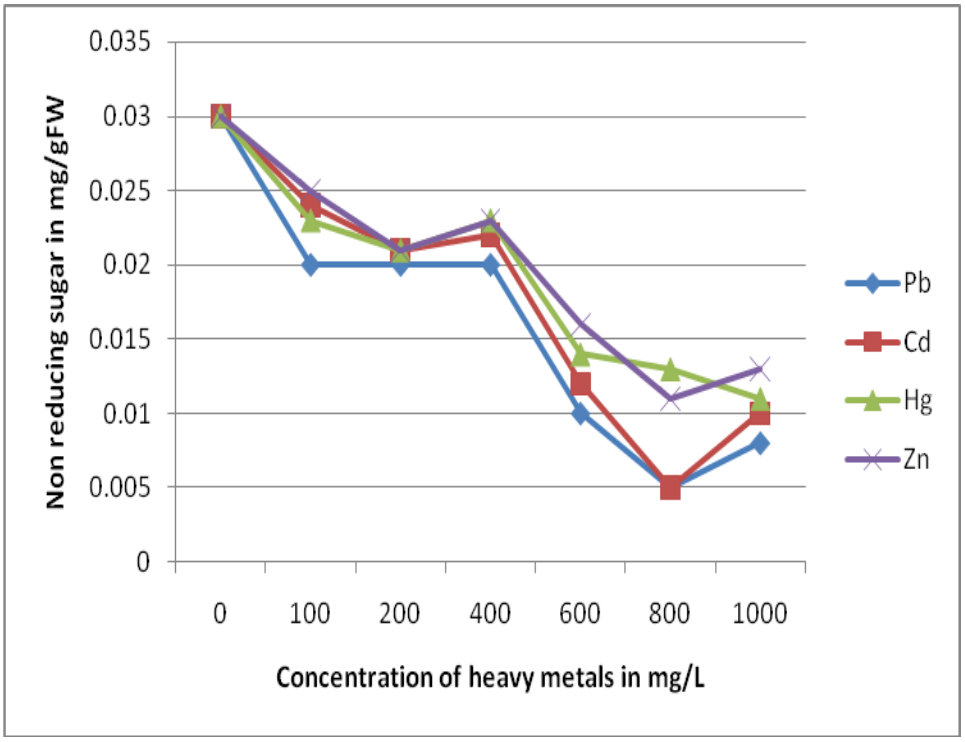


Fig.4 Effect of heavy metals on starch content of *A. filiculoides*

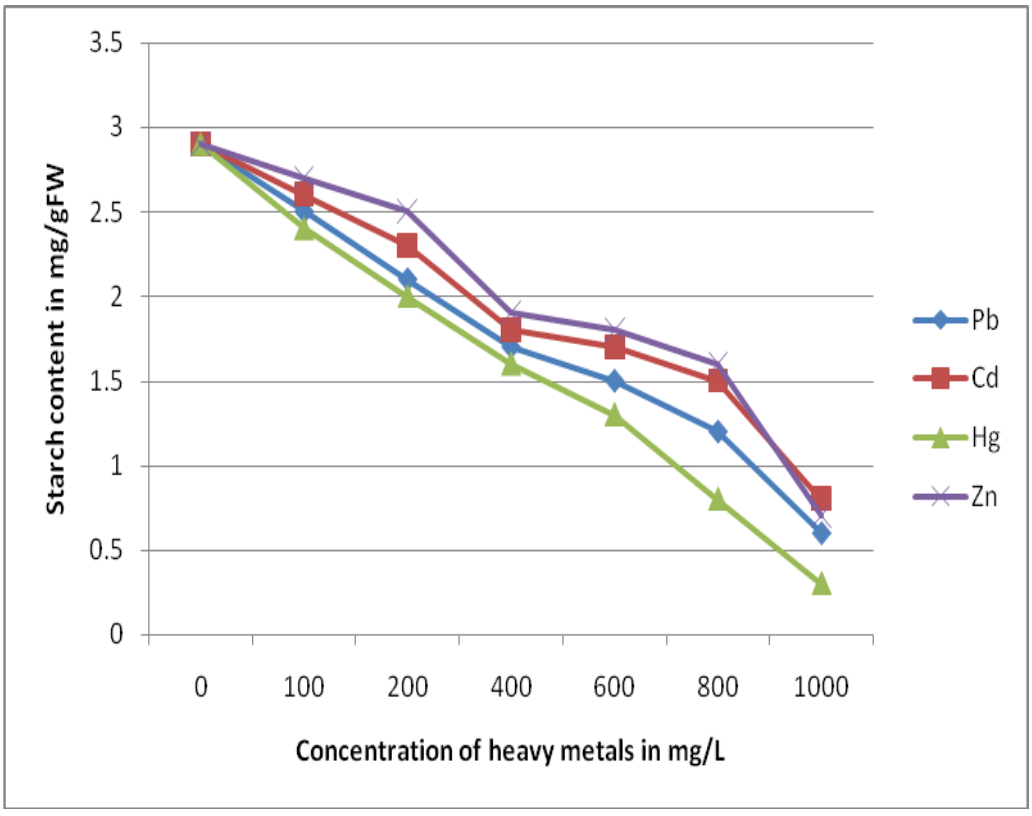


Fig.5 Effect of heavy metals on total amylase activity in *A. filiculoides* in terms of mg of maltose produced/hr ten days after growth

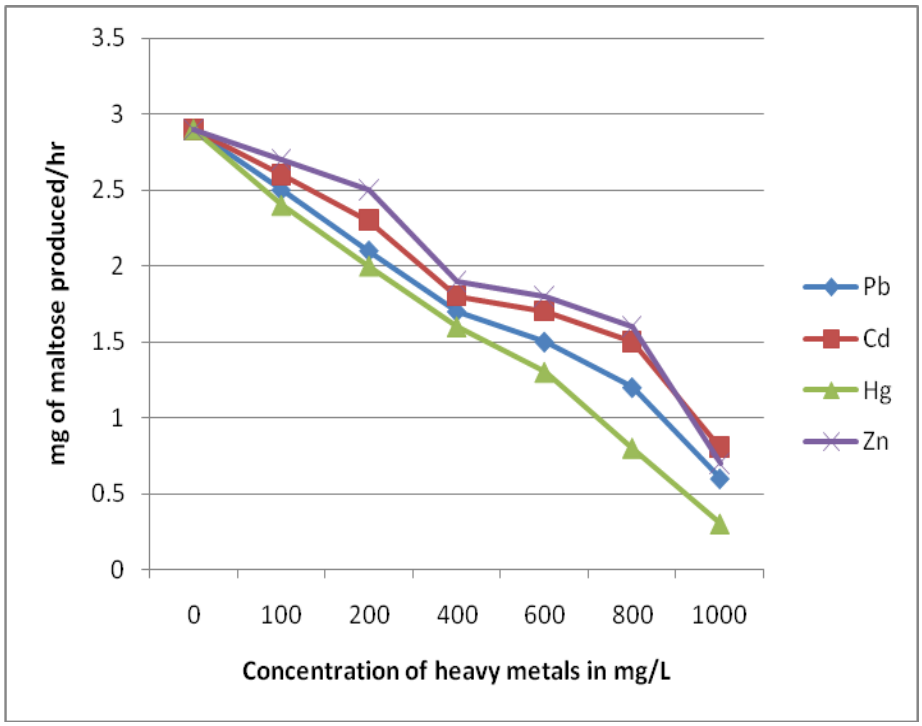


Fig.6 Effect of heavy metals on α- amylase activity of *A. filiculoides* in terms of mg maltose produced/hr

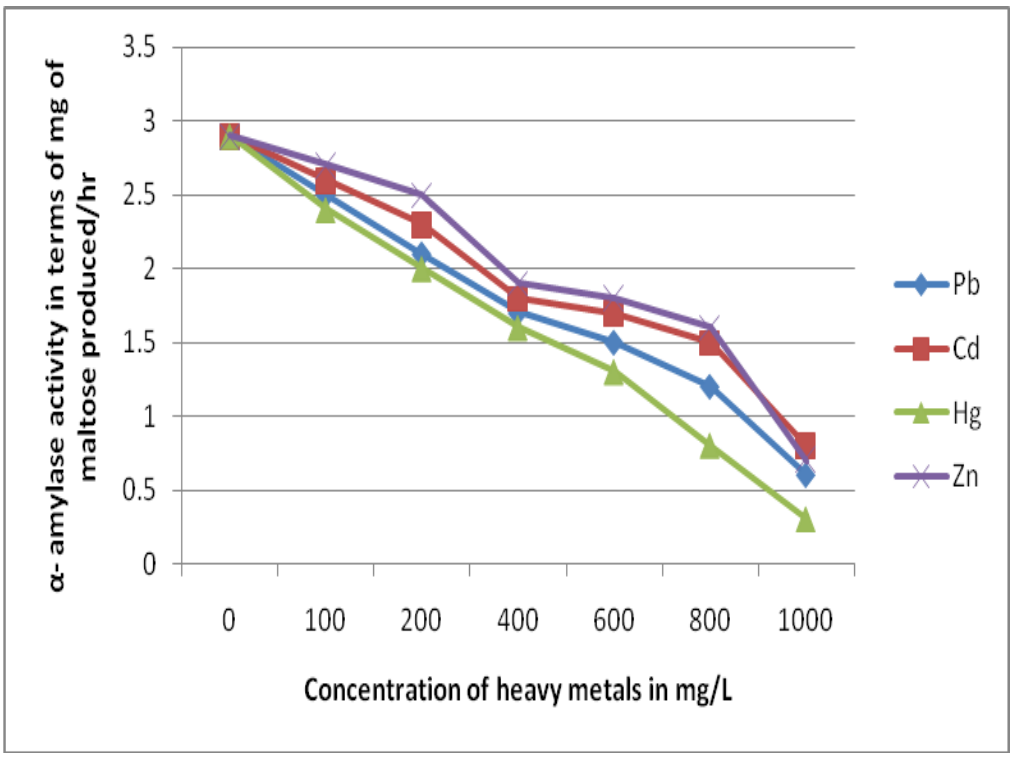


Fig.7 Effect of heavy metals on total nitrogen in *A. filiculoides* in mg/10 plants ten days after growth

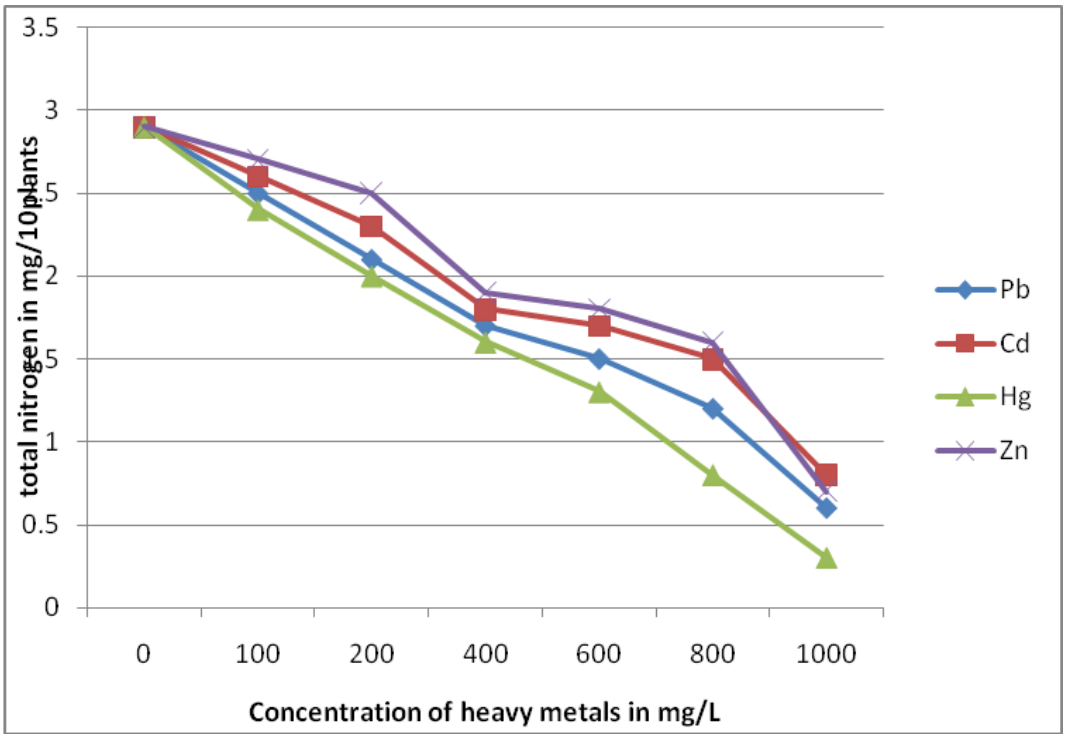


Fig.8 Effect of heavy metals on soluble nitrogen in A. filiculoides in mg/ 10 plants ten days after growth

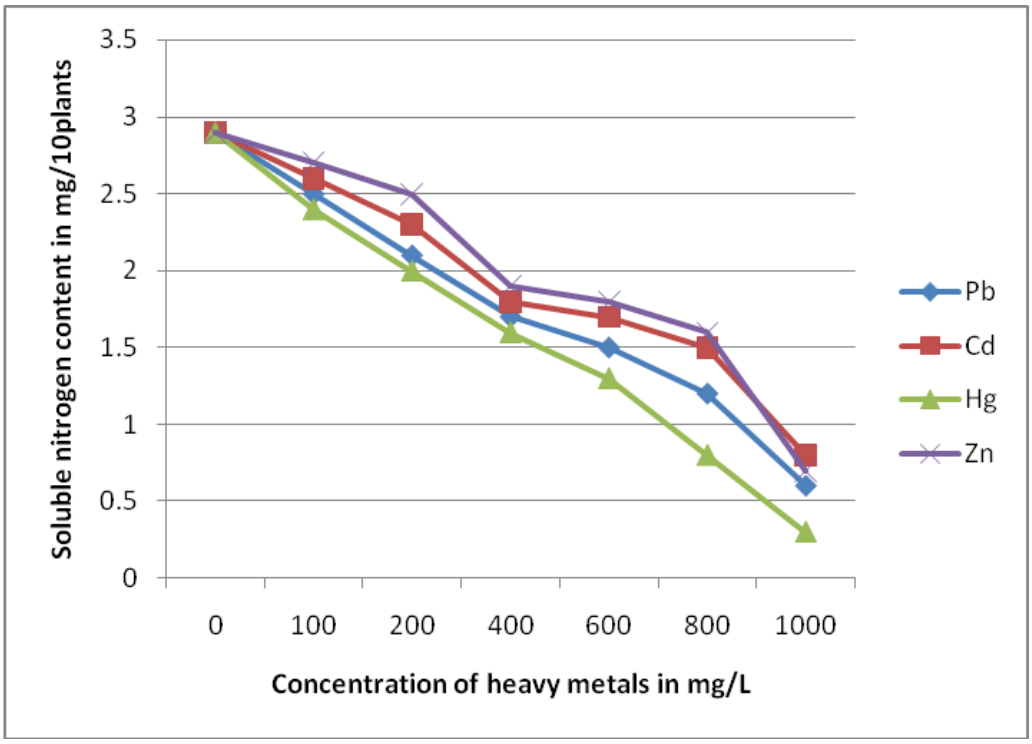


Fig.9 Effect of heavy metals on free amino acids of *Azolla filiculoides* in µmole/gFW after ten days of growth

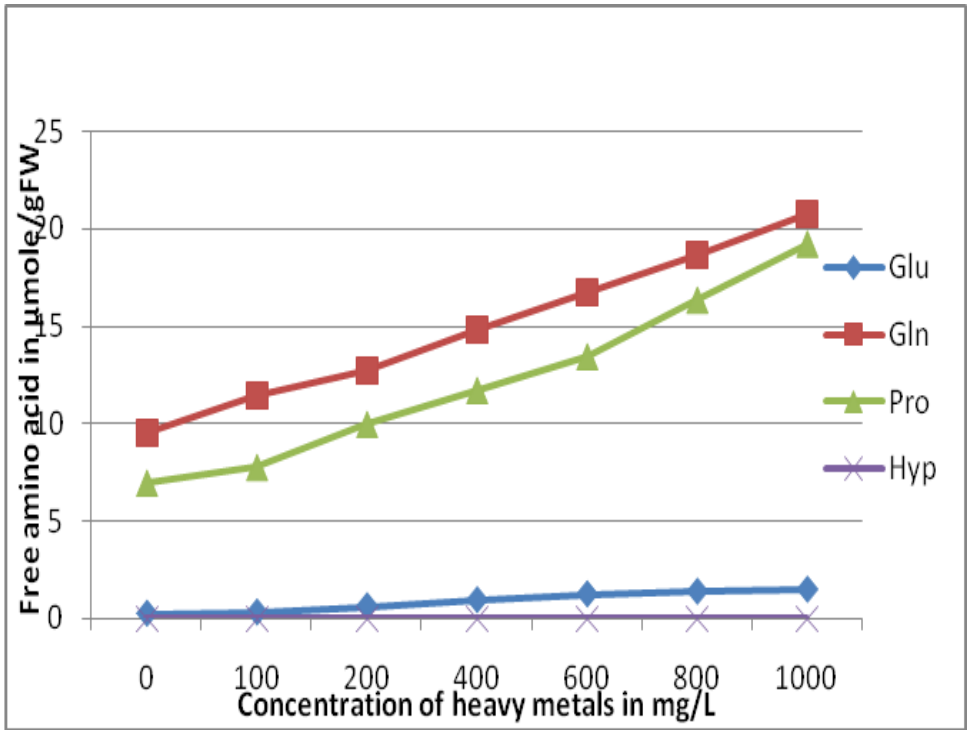


Fig.10 Effect of heavy metal Lead (Pb) on some free amino acids in *Azolla filiculoides* in µmole/gFW

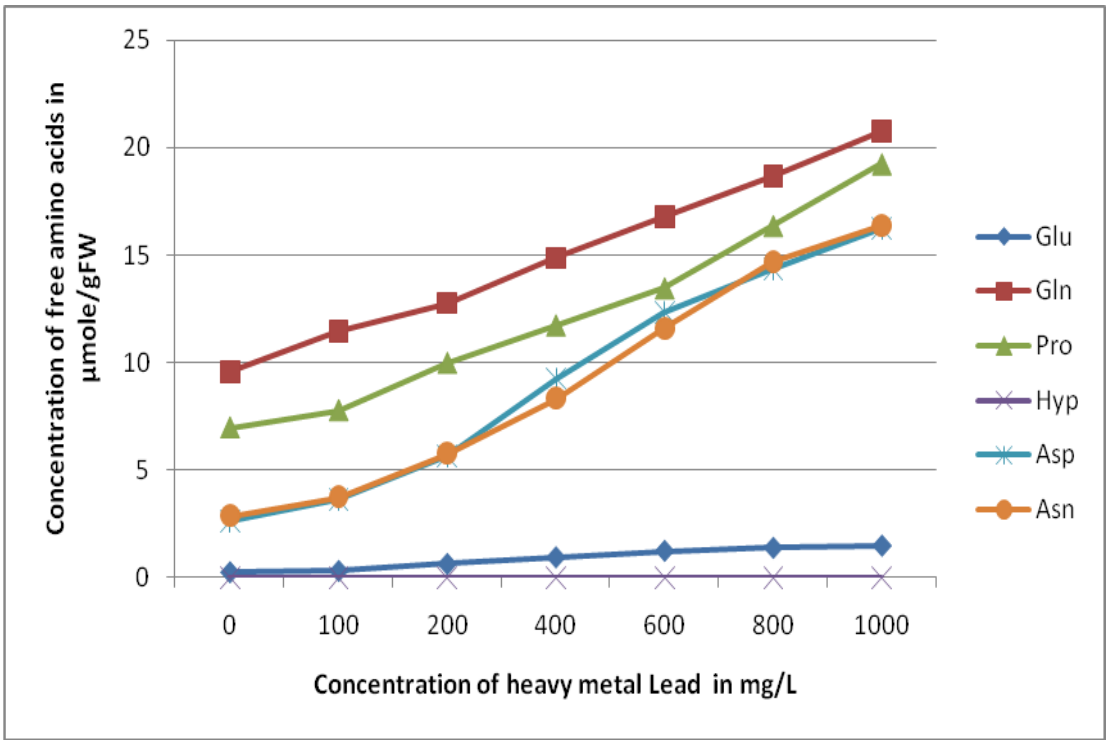


Fig.11 Effect of heavy metals on free aminoacids in *A. filiculoides*

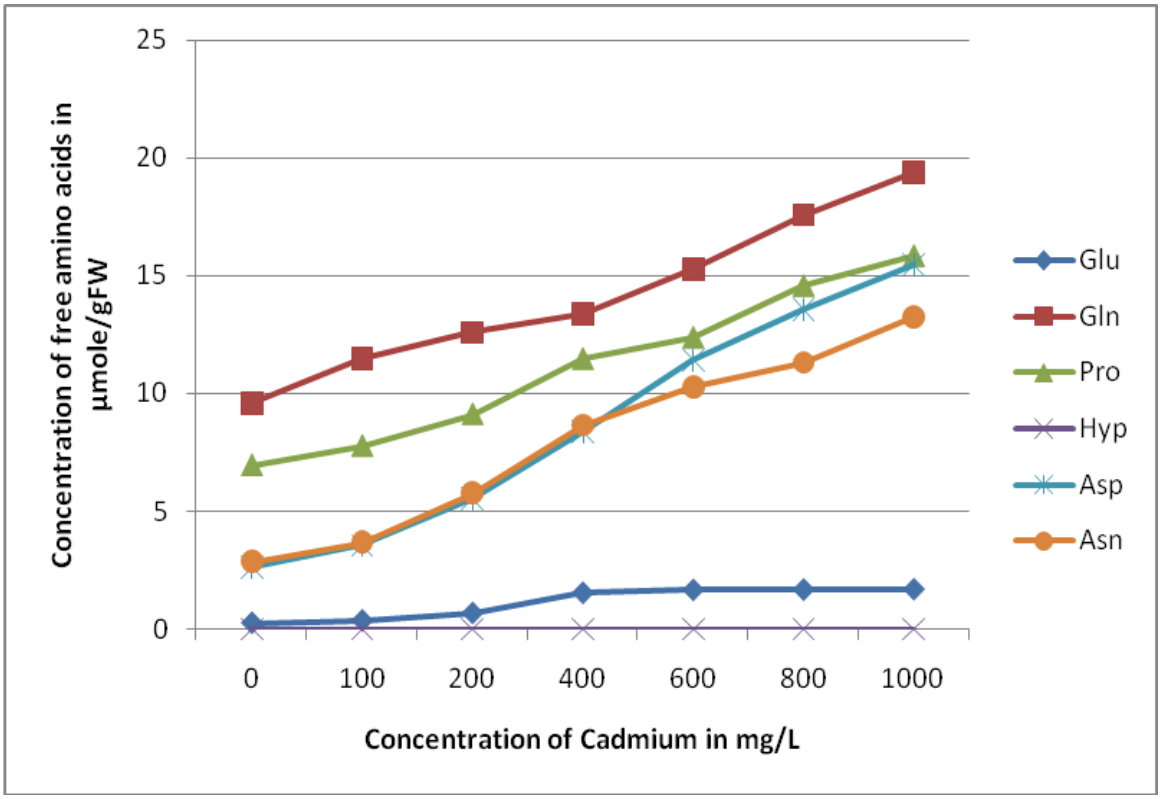


Fig.12 Effect of Mercury on free amino acids in *A. filiculoides* in µmole/gFW

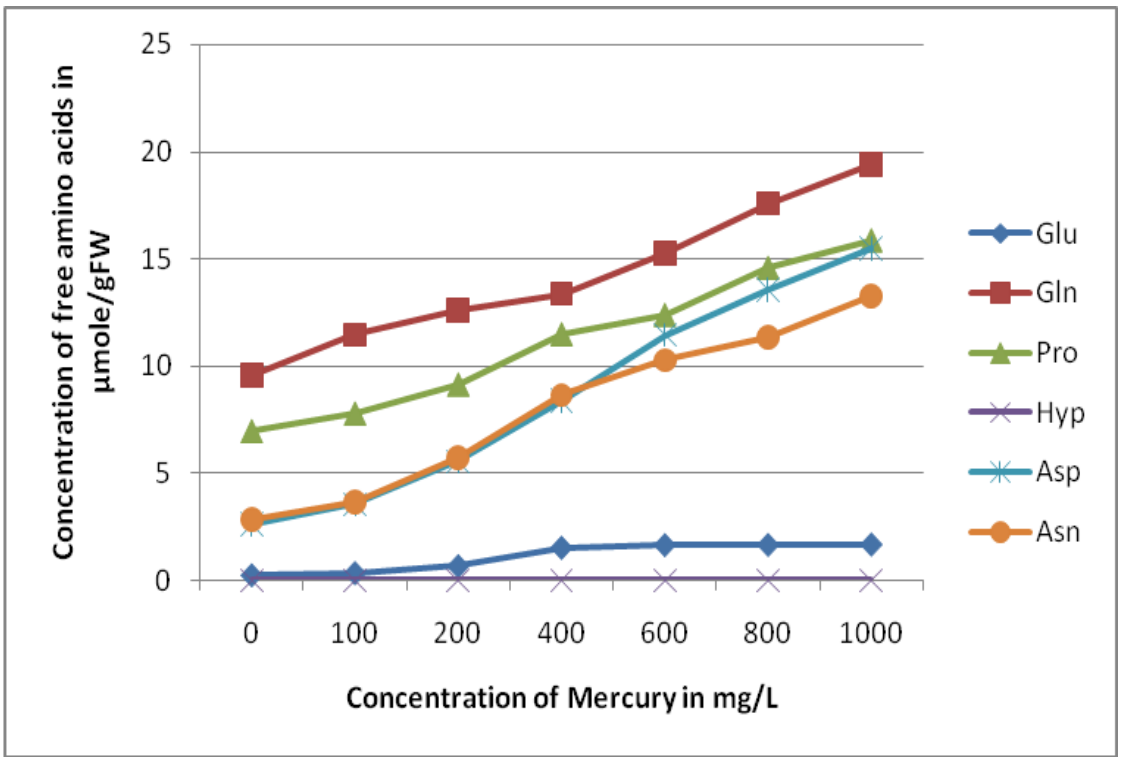


Fig.13 Effect of Zinc on free amino acids in *A. filiculoides* in µmole/gFW

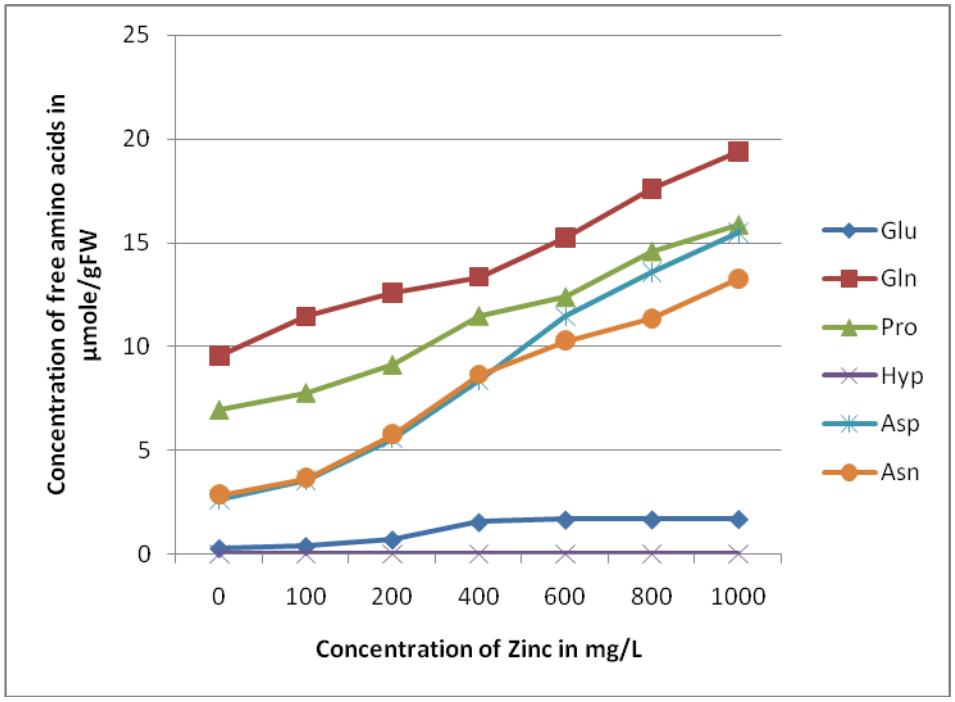
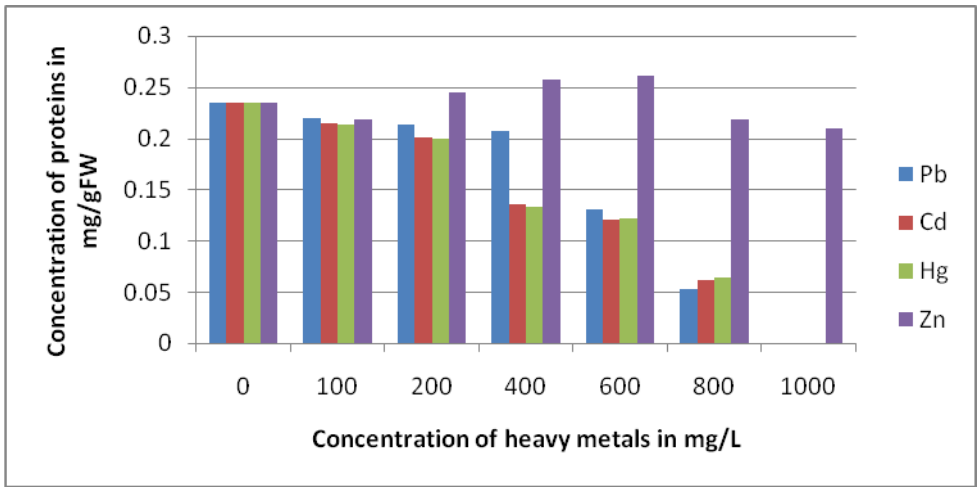


Fig.14 Effect of heavy metals on total proteins in mg/gFW of *A. filiculoides*



Nevertheless, contents of total free amino acids were decreased in *A. filiculoides* in comparison to control40.50µmole/gFW of free AA ten days after growth (Table- 9; Fig- 9).

The major free amino acids determined in *A. filiculoides* were Glutamic acid (Glu), Glutamine (Gln), Aspartine (Asn) and Aspartic acid (Asp) (Table- 10; Fig- 10). In all higher plants, inorganic nitrogen is at first reduced to ammonia prior to its incorporation into organic form. Ammonia is assimilated into organic form as Glu and Gln, which serve as the nitrogen donors in the biosynthesis of essentially all amino acids and other nitrogen containing compounds (Sanchez Pardo *et al.*, 2013). The free Glu, Gln, Pro, Asp and Asn

content in the *A. filiculoides* was stimulated under treatment of heavy metals at all concentration from 100mg/L to 1000mg/L. In control plants the content Glu, Gln, Pro, Asp and Asn was found to be 0.244, 9.550, 6.955, 2.635 and 2.840 µmole/g FW ten days after growth. The amino acid contents increased with increased concentration of heavy metals. Hydroxy proline was not detected neither in control plants nor in plants treated with heavy metals. At 1000mg/L Pb caused an increase in amino acid contents to 1.475µmole/gFW (Glu), 20.750µmole/g FW (Gln), 19.250 µmole/g FW (Pro), 16.256 µmole/g FW (Asp) and 16.375 µmole/gFW (Asn) (Table- 10; Fig- 10). A more or less similar results in increase in amino acid contents was also observed in plants treated with Cadmium (Table- 11; Fig- 11), Mercury (Table- 12; Fig- 12) and Zinc (Table- 13; Fig- 13). The present findings gain support from the work of Sanchez Pardo *et al.,* 2013; Sharma and Dietz, 2006; V. Zemanova *et al.,* 2013; Rimjhim Sheel *et al.,* 2013, 2016; Sweta *et al.,* 2016; 2017 etc.

The heavy metals selected for present investigation caused significant effect on the protein profile of *A. filiculoides*. In control plants the total protein content was recorded as 0.235mg/gFW. A decline in the protein content was noticed on increasing the concentration of all the heavy metals. Lead, Cadmium and Mercury caused a great reduction in protein content to 0.053-0.064mg/gFW at concentration of 800mg/L. At 1000mg/L these three heavy metals caused a complete disruption of protein as its concentration was declined to almost nil (Table- 14; Fig- 14).

Being an essential component of many enzymes and proteins heavy metals are essential and important for normal growth and development of plants. On the other hand, it has been found that increasing heavy metals concentrations have led to the emergence of symptoms of poisoning such as inhibiting plant growth (Hall, 2002). Plants vary in their ability to absorb and accumulate minerals from the soil solution. Several studies demonstrated that heavy metals can function as stressor, causing some physiological constrains that decrease plant vigor and inhibit plant growth. The above mentioned data agreed with those of Kiekens (1983) who found that, the presence of some cations (positive ions) in the soil solution such as Ca2 + and Mg2 + compete with cations of heavy metals efficiently and prevent it from adhering with plasma of plant tissues and subsequently their accumulation decrease. Soluble sugar is an important constituent manufactured during photosynthesis and breakdown during respiration by plants. All metals caused decrease in the content of soluble sugar with increasing concentration as reported in agricultural crops (Hemalatha *et al.,* 1997; Rascio and Navari-Izzo, 2011). Such inhibition of photosynthesis in plants by heavy metals has been reported (Bazzaz *et al.,* 1975). The low sugar levels may be due to lowered synthesis or diversion of the metabolites to other synthesis processes. Proteins are important constituents of the cell that are easily damaged in environmental stress condition (Prasad, 1996; Wu *et al.,* 2010). Hence, any change in these compounds can be considered as an important indicator of oxidative stress in plants. In the present investigation it was observed that the heavy metals declined the protein content of *A. filiculoides*. At 1000mg/L Pb, Cd and Hg caused complete destruction of protein. The present findings gain support from the work of Sweta *et al.*,(2016; 2017) Costa and Spitz (1997); Mohan and Hosetti (1997); Palma *et al.,* 2002; Davies *et al.,* (1987); Jin *et al.,* (2008) who also found a more or less similar results in *Lemna polyrrhiza*. Aldoobie and Beltagi (2013) also found a more or less similar effect of heavy metals on soluble

sugar and protein contents in *Phaseolus vulgaris*. Monalisa *et al.,* (2015) have also found a similar trend on sugar and protein contents in *Phaseolus aconitifolius.*

Vineeth *et al.,* (2015) have studied the effect of Cd, Cr, and Ni on biochemical parameter in *Vigna radiata* (green gram) and found a significant decrease in reducing sugar content in plants treated with heavy metals. Similarly non reducing sugar and protein content were also significantly decreased in plant treated with heavy metals. Effect of mercuric chloride and cadmium chloride on the biochemical parameters of the seedling of pigeon pea (*Cajanus cajan*) (L) Millsp.) Were studied and found that sugar and Protein content decreased with increase in concentration of heavy metals (Patanik and Mohanty, 2013). The total sugar content of green gram seedlings were found to be more in control (0.0912mg/g/Fr.wt.) and a gradual decrease in sugar content (0.0690 mg/g/fr.wt.) found as the concentration of lead and copper increases (Sajid, 2008). Verma and Dubey (2004) studied effect of cadmium on soluble sugar and enzymes of metabolism of rice using two cultivars namely Ratna and Jaya and found similar results with the present study. During 5 to 20 days exposure at 100 µ M or 500 µ M Cd (NO3)2 in growth medium increase total soluble sugars and reducing sugars and decrease in the content of non-reducing sugars. Deef (2007) observed application of Cu at rates of 0, 50 and 200 ppm were gradually increased the dry matters accumulation and sugar fraction. However, high rate up to 3200 ppm, the dry matter gradually diminished as well as sugar fraction in *Rosmarinus officinalis*.

Heavy metals have been shown to increase soluble proteins in leaves of soybean treated with lead and cadmium (Lee *et al.,* 1976) and rice treated with lead (Mukherjee and Maitra, 1976). Effect of copper and cadmium on duckweed (*Lemna minor*) was studied by Hou *et al.,* (2007). Their results demonstrated that exposure to high concentration heavy metals could result the disintegration of antioxidant system in duckweed and the significant decrease of content of soluble protein and photosynthetic pigment was observed to high level metal stress. Additionally Lead, Cadmium and Mercury were found to be more toxic than Zinc heavy metals as observed in present studies. Inhibition of soybean metabolism by cadmium and lead was studied by Huang *et al.,* (1974). They concluded that Pb and Cd inhibited plant metabolism generally, as shown by their reduction of shoot and root growth and pod's fresh weight. The reduction of pod fresh weight was correlated with the effect on shoot, root, leaf, nodule dry weight, carbohydrate and protein content. They also concluded that lead was less effective than Cd. The chloride salts of Cd or Ni were also examined in relation to biomass, seed production and metabolic pattern in soybean (*Glycine max*) by Malan and Farrant (1978). Both metals markedly reduced plant biomass and seed production. Ni was found to be more mobile than Cd, reaching higher levels in all plant parts, especially in seeds. Cadmium reduced mature seed mass. This effect was mostly due to decreased yield of lipids, protein and carbohydrates. Copper tolerance in *Chlorella vulgaris* has been studied by comparing physiological properties (Fathi *et al.,* 2005), and copper uptake in a wild type strain and a copper tolerant one. A concentration dependent reduction in growth rate, dry mass and content of chlorophyll, protein, sugar and amino acids was noticed in both strains at 1.0 and 400 mgl–1 copper. The reduction in all parameters was higher in wild type strain than in the tolerant one. Beside copper, chromium accumulation also reduces chlorophyll biosynthesis, nitrate reductase activity and protein content in *Nymphaea alba* L. (Vajpayee *et al.,* 1999).

The effect of Zn, Cd, Cu and Hg on the soluble protein during germination of lentil seeds was investigated by Ayaz and Kadioglu (1997) and found contradictory results with the presence study. Manios *et al.,* (2002) studied the effect of the heavy metals (Cd, Cu, Ni, Pb and Zn) on the total protein concentration of *Typha latifolia* plants growing in a substrate containing sewage sludge compost and watered with metaliferous waste water and found that higher concentrations of soluble protein was observed at lower concentration of heavy metals and inhibition occurred in case of stronger solutions. Protein synthesis were also significantly reduced with the treatment of Cu+2 in pea plants (Angelov *et al.,* 1993), treatment of Cd in wheat seedling (Lesko *et al.,* 2002), treatment of Pb in maize (Jana and Choudhary, 1984). Changes in some important protein involved in CO2 fixation (Rubisco, Rubisico activase, Rubisco binding protein, NH4+ assimilation and glutamate synthase, as a result of excess in barley leaves (*Hordeum vulgare* L. cv. Obzer) was determined by Kepova *et al.,* (2004). Excess of Cu affected mainly the non-protein SH groups, while Mn influenced the ascorbic acid content. Oxidative stress under Cu or Mn toxicity was most probably the consequence of depletion of low molecular antioxidant as a result of their involvement in detoxification processes and misbalanced in antioxidation enzymes.

In conclusion, the present results indicate that the exposition of *Azolla filiculoides* to different concentrations of heavy metals results in decrease in sugar and protein content. The effect Pb, Cd and Hg was more significant in hampering plant growth and development. Phytoremediation may contribute in the treatment of various sites contaminated with toxic heavy metals. *Azolla filiculoides* can be used to reclaim the water bodies polluted with heavy metals.

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