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Location map of HPCL, Jagir Road dumping site

ENVIRONMENTAL HEALTH | RESEARCH ARTICLE

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Endurance assessment of *Eichhornia crassipes* (Mart.) Solms, in heavy metal-contaminated site—A case study

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Abstract: In this study, the ability to hyper accumulate heavy metals from contaminated site by water hyacinth (*Eichhornia crassipes*) was monitored. The heavy metal-contaminated habitat was validated by X-ray diffraction and energy dispersive X-ray elemental spectrometry analysis of the soil samples. Heavy metal contamination in soil and water; accumulation in foliar, root and bulb tissue samples were determined by atomic absorption spectroscopy and were monitored as a function of accumulation in different tissues. Significant differences were recorded in the bioaccumulation capability of heavy metals by different tissue. Discrete variation in protein profile of leaves and high expression of alcohol dehydrogenase (ADH), peroxidase (POX) and altered regulation of esterase (EST) in root tissue was observed in contaminated site grown *Eichhornia*. The high metal accumulation efficiency of water hyacinth due to the biomass production suggests this species as reliable organic biomarker for heavy metal contamination.

Subjects: Bioscience; Earth Sciences; Environment & Agriculture; Environmental Studies & Management; Food Science & Technology

Keywords: AAS, atomic absorption spectrophotometry; ADH, alcohol dehydrogenase; CAT, catalases; CN, cyanide; EDX, energy dispersive X-ray elemental spectrometry; EST, esterase; HM, heavy metal; HPLC, Hindustan paper corporation limited

1. Introduction

Water hyacinth (*Eichhornia crassipes*), a perennial aquatic macrophyte, is considered to be noxious and extremely invasive for freshwater environments (Plants database, United States Department of Agriculture (USDA), 2004), but has a proven record of potential heavy metal hyperaccumulator (Alvarado et al., 2008; Delgado, Bigeriego, & Guardiola, 1993; Kay, Haller, & Garrard, 1984; Maine, Duarte, & Suñé, 2001; Prasad, Greger, & Landberg, 2001a; Prasad, Malec, Waloszek, Bojko, & Strzałka,

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PUBLIC INTEREST STATEMENT

The study reveals that the unplanned disposal of paper mill wastes leads to irreversible contamination of agricultural land and water with heavy metals, which are toxic to human, animal and natural plant resources. This study also reaffirms the need to use planned disposable system and provide plants as bioaccumulators to use in bioremediation of waste land and water, which can be a beneficial approach for remediation of water and soil.

2001b; Suñe, Sánchez, Caffaratti, & Maine, 2007). Hence, *E. crassipes* always has been a popular candidate for rhizofiltration of metal-polluted aquatic environments with inherent ability of high biomass production, wide distribution and tolerance to cyanide (CN) and metals (Ghabbour, Davies, Lam, & Vozzella, 2004; Jayaweera & Kasturiarachchi, 2004; Singhal & Rai, 2003; Williams, 2002). This reckons the use of *Eichhornia* as model plant to study heavy metal-induced responses. Heavy metals basically are essential for normal plant growth and development, but their elevated concentrations may lead to toxicity symptoms and growth inhibition. Metal ions such as Mg, Cu, Zn, Mn, Ni and Fe are essential for plant growth processes that range from respiration to photosynthesis, but deleterious when exceed limit. Whereas, Cd, Al and Pb have no nutritional value, toxic and when present in excess in soil interferes with the uptake of essential ions, biosynthesis of chlorophyll and nucleic acids, lipid metabolism; thus profoundly affecting plant growth and development (Dykema et al., 1999; Ouariti, Boussama, Zarrouk, Cherif, & Habib Ghorbal, 1997). Therefore, plants have evolved a range of potential cellular mechanisms and proteins that may be involved in detoxification of heavy metals to achieve tolerance by regulating metal ion uptake and metal ion homeostasis (De Vos & Schat, 1991; Dietz, Krämer, & Baier, 1999).

Longer persistence in soil, absorption through plant roots and increased emission of heavy metals with a risk of entry into the food chain has led to great concern (Angelone & Bini, 1992; Galloway, Thornton, Norton, Volchok, & McLean, 1982; Lantzy & Mackenzie, 1979; Tomas, Cery, Lahucky, & Musilova, 2008; Verma, Madan, & Hussain, 2016; Vollmannova, Tomas, Bajcan, & Kovacak, 2008). So, the recovery of degraded soils and the reclamation of industrial sites have become imperative using stress-tolerant hyper accumulator plants. Hence, endeavours are on to improve plant stress tolerance and pollutants accumulating potential with the aim to be used for soil remediation (Salt et al., 1995). As such, a model system is a requisite now to understand the basic principles of toxicity mechanisms at the cellular and biochemical levels.

Plants subjected to heavy metal stress undergo biochemical changes producing ROS such as superoxide anion radical (O^{2-}), hydrogen peroxide and hydrogen radicals (^{-}OH) (Cho & Park, 2001). The expression of antioxidation enzymes like, superoxide dismutase (SOD), aldehyde dehydrogenase (ADH), esterase (EST), peroxidise (POX) and catalases (CAT) have been found fundamental in scavenging ROS and providing sustenance to stressed plants (Gupta, Cuypers, Vangronsveld, & Clijsters, 1999). A significant induction of antioxidant enzymes (POX and CAT) along with an increase in the levels of protein, lipid peroxidation and GSH was noted in response to $CdCl_2$ in rice seedling (Yu, Wang, & He, 2000). Heavy metals may cause alteration either in the expression of stress proteins or enzyme activity. Although, *Eichhornia* as biosorbent of heavy metal ions has been reported (Sabale, Jadhav, Jadhav, Mohite, & Patil, 2010; Schneider, Rubio, Misra, & Smith, 1995) but there are few correlative studies with heavy metal contamination and morphological and biochemical level changes (Mishra & Tripathi, 2009; Paiva et al., 2009). In this article, an effort was made to assess the metal stress-induced morphological indicative changes in various tissues and protein and isozyme level of *Eichhornia* grown in heavy metal-contaminated area with focus to access the potential of *Eichhornia* as organic indicator and model system for heavy metal stress study.

2. Materials and methods

2.1. Sampling

The *Eichhornia* root and shoot samples were collected on site from the Hindustan Paper Corporation Limited (HPLC) Paper Mill dumping area situated beside National Highway No. 37, Morigaon, Assam, India (26 7′43.60 N and 92 14′29.38 E) and stored in liquid nitrogen. The soil samples were collected with the help of auger to depth ranging from 20 to 100 cm. A total of 18 soil samples with 3 replicates for each sample of varying depth and 3 replicates per sample were collected from site in randomized block experimental design. The water samples were also collected from contaminated site for preliminary analysis of heavy metals. The clod soil was first homogenized and then incubated in hot air oven at 80°C overnight, grounded and sieve analysed with cut-offs, 425, 300, 150 and 75 µm, respectively. The 75-µm sieve passed was used for further downstream analysis of heavy metal

composition. The sieve retained and passed soil samples were analysed for its physical characteristics and aggregation.

2.2. Mineralogical characterization

The metal composition of the soil sample was characterized using non-destructive AXS D8 ADVANCE fully automatic powder X-ray Diffractometer (XRD). The crushed and homogenized 75-µm sieve passed soil sample was baked overnight in hot air oven at 80 C and analysed by the XRD device for the reflection angle (2θ) from 10 to 70 . To identify the crystalline phases, the results were compared with the Joint Committee on Powder Diffraction Standards. Furthermore, 75-µm sieve passed flat surface soil sample was selected, cured overnight in hot air oven and treated with acetone–alcohol solution to stop hydration reactions (Asavapisit, Fowler, & Cheeseman, 1997) and coated with gold as conducting medium under 30-min vacuum time. The soil sample was then characterized using a scanning electron microscope (SEM, Leo 1430vp), equipped with energy dispersive X-ray elemental spectrometry (EDX).

2.3. AAS analysis

The root and shoot samples were repeatedly washed with bidistilled water, oven-dried at 65°C for 4 days and digested by USEPA Method (USEPA, 1997). The water samples were filtered prior to use and the 75-µm sieve passed soil samples were extracted with 20 mL of 0.01 M ethylene diamine tetraacetic acid (EDTA) solution by repeated shaking for 15–20 min, centrifuged and the supernatant was decanted and filtered into fresh test tubes for analysis. The leachate pH was maintained to less than 2.0 by the addition of nitric acid to avoid metal precipitation before the metallic analysis. Metal concentration was determined by flame atomic absorption spectrometer using Air-Acetylene or Nitrous oxide-Acetylene Burner. Concentration of the metal ion standard solutions were adjusted to cover a range of 0 to 25 ppm and quality control metal standard reference were used.

2.4. Morphological imprinting

The growth of the *Eichhornia* plants in the industrial dumping site and control areas was monitored and compared in terms of shoot and root length, root hair proliferation, bulb girth and discolouration of the bulb and apical parts. Observation for evident heavy metal stress symptoms like leaf rolling, chlorosis and discolouration of bulb were also recorded. Triplicate readings for each parameter were taken and the average mean value was considered for comparisons between the morphological changes of stressed and control plants.

2.5. Protein and isozyme analysis

Protein was extracted from homogenized tissue sample in a buffer consisting of 30 mM Tris-HCl (pH 8.5), 1 mM ascorbic acid, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Zivy, Thiellement, de Vienne, & Hofmann, 1983). Proteins in the supernatant were precipitated by adding 8 volumes of chilled acetone containing 10 mM of 2-mercaptoethanol at −20°C, centrifuged (15,000 rpm, 10 min, 4°C) and the precipitate was dissolved in Laemmli buffer (Laemmli, 1970). An aliquot of the supernatant was used for estimation of protein content by Bradford method (Bradford, 1976), 20 µg of the protein electrophoresed at 50 mA constant current, stained and documented.

The samples were ground in prechilled pestle and mortar in 0.1 M Tris-HCl, pH 7.4 containing 10 mM DTT, 50 mM Tris-Cl buffer (pH 7.6) containing 5 mM β-ME and 5 mM EDTA in the ratio of 1:2 (w/v) and in 50 mM Tris-Cl buffer (pH 7.6) for alcohol dehydrogenase (ADH) assay, esterase (EST) and peroxidase (POX), respectively. The ground mixture was centrifuged at 15,000 rpm for 20 min at 4°C and the supernatant obtained was immediately electrophoresed in anionic polyacrylamide gel (Davis, 1964). The staining of the ADH was carried out in Tris-Cl buffer (50 mM, pH 8.0) containing 0.02% NAD^+, 0.03% NBT, 0.004% PMS and 5% absolute alcohol (Dongre, 1988). Whereas, the staining of EST and POX isozyme were carried out by modified Brewer's method (Reddy & Garber, 1971) and in solution containing 2.08% Benzidine, 18% acetic acid, 3% H_2O_2 (Cotter-Howells, 1996),

respectively. After stopping the enzymatic reactions, the gels were fixed in 7% acetic acid solution and documented.

2.6. Statistical analysis

Data for the control and heavy metal-contaminated samples were pooled together andanalysed statistically for the control of normality and equality of the variance assumption (Levene's test *p*-val 0.0029 < 0.05) (Glantz & Slinker, 2001) and when necessary, the data were transformed to Log_{10} values of the metal accumulation in soil and water and Posthoc test: Bonferroni-Holm analysis (Holm, 1979). Whenever the parametric analysis failed, we opted for Kruskal–Wallis test with Dunn's post-test (Dunn, 1964) for nonparametric analysis to compare three or more unmatched groups using GraphPad Prism (version 5.03 for Windows; GraphPad Software, San Diego, CA, U.S.A.). For all statistical analyses, significance was set to * = $0.01 \leq p \leq 0.05$, ** = $0.001 \leq p \leq 0.01$, and ***/ ΔΔΔ = $p \leq 0.001$ throughout the experiments and accordingly plotted in the graphs for clear understanding.

3. Results and discussion

3.1. Mineralogical characterization

The *Eichhornia* plants were collected from HPLC industrial dumping site and monitored for morphological imprinting. It was followed by the confirmation of heavy metal contamination of site and plant sample. State of the *Eichhornia* plant's growth in the contaminated dumping site is well documented as scribed in the legend to Figure 1. The soil sample was primarily checked for pH and was found to be acidic around pH 4.8, which has been reported to be associated with areas of heavy metal contamination (Zhao, Xia, Fan, Zhao, & Shen, 2012). The low pH of the soil may have contributed to higher solubility of heavy metals (Chuan, Shu, & Liu, 1996). The soil physical structure and texture were analysed by passing through different sieves (Figure 2(A)) and microscopic analysis of the contaminated dumping site soils by SEM (Figure 2(B)). Solid matrix of heavy metal-contaminated soils is a very complex system and is still not well understood. The micrographs and sieving analysis of contaminated site soil showed the formation of aggregates, which generally occurs to entrap the metal adsorbed on the surface of two overlapping particles (Montinaro, Concas, Pisu, & Cao, 2012; Tica, Udovic, & Lestan, 2011).

The metal detection in the soil samples was confirmed by XRD analysis (Figure 3(A)). Data for control soil is not shown. A distinctive peak at 36.10 A in the contaminated soil sample corresponds to Cd and Co presence in the tested soil. Various peaks at 48.7A, 28.85A, 23.05A, 33.0A suggested *in situ* formation of a $ZrSiO_4$, CaF_2, Cristoballite and Platternite-like mineral in the heavy metal-contaminated soil. The characteristic weak peak at 66.1A and Zincite ascertained the presence of Hg-like mineral in the contaminated soil. This corroborates with previously reported studies where heavy metal-contaminated soil tends to form soil aggregate of Calcite ($CaCO_3$), β Quartz (SiO_2), χ Goethite FeO(OH), δ Hemimorphite $Zn_4Si_2O_7(OH)_2(H_2O)$ (Montinaro et al., 2012; Tica et al., 2011; Xu & Lu, 2012).

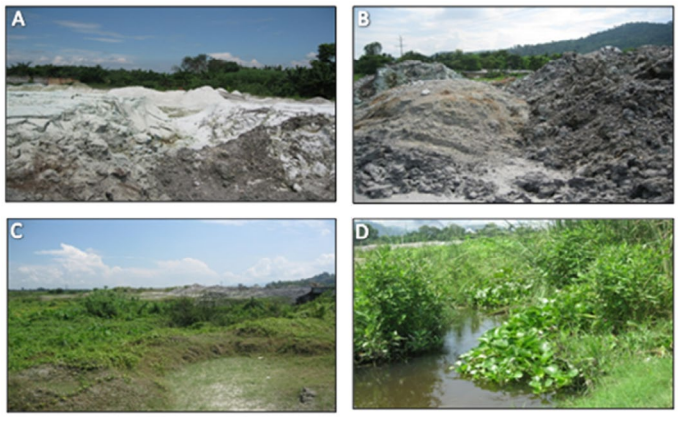

Figure 1. (A, B) Industrial waste dumping area; (C, D) Eichhornia crasssipes sample collection site.

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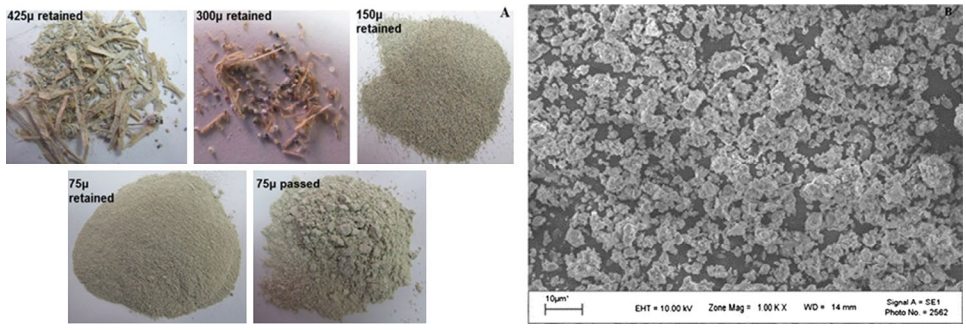

Figure 2. (A) Micrograph of industrial dumping site soil after passing various sieve; (B) SEM micrograph of industrial dumping site soil 200X micrograph after physical changes and passing sieve number 75µm.

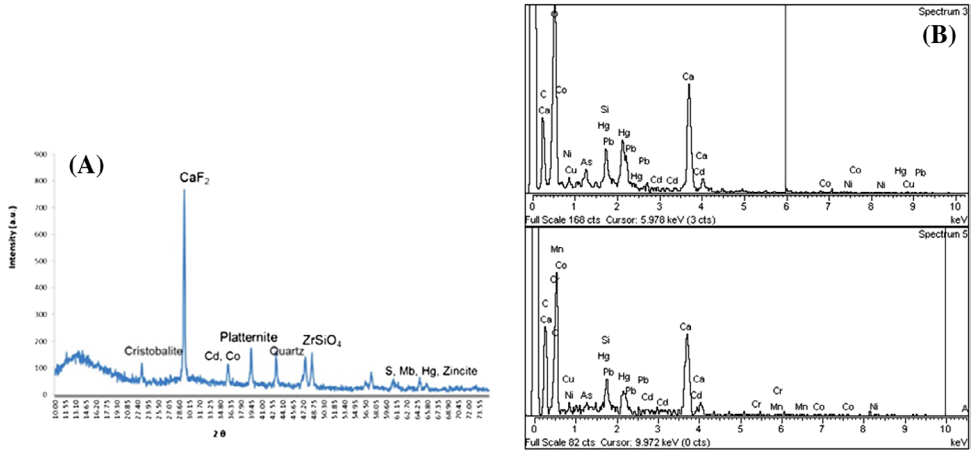

Figure 3. (A) *X*-ray Diffractogram of industrial dumping site soil sample; (B) EDX spectrum of industrial dumping site soil sample.

The presence of substantial amount of such minerals also has been reported in contaminated garden, and urban and motorway roadsides (Cotter-Howells, 1996).

Discrete presence of heavy metals in the dumping site soil was further documented by EDX analysis of 75-µm sieve passed soil sample (Figure 3(B)). The EDX analysis revealed the presence of heavy metals such as Hg, Pb, Ni, Cd, Co, Cu and As in the dumping site soil with decreasing Co > Pb > Hg > Cd > As > Cu intensity order. Heavy metal contamination following such Pb > Cd > Zn > Cr > Cu order has also been reported in Lead-acid factory dumping site of Nigeria (Adejumo, Adediran, & Ogundiran, 2011). The AAS analysis of the soil and water sample also revealed significant difference in the heavy metal concentration ($p < 0.05$, Figure 4). The degree of heavy metal contamination in the dumping site soil was found to be in Pb > Cr > Cu > Cd > Ni order (Figure 4). Whereas, the water analysis shows the heavy metals get accumulated in water bodies in following order Pb > Cr > Ni > Cu > Cd (Figure 4). The Cd, Cr, Ni concentration in soil and water were significantly different between control and contaminated sites (>1 mg/L, $p < 0.001$). The Cu contamination level was found to be on the higher side (>1 mg/L, $p < 0.05$) and significantly different between control and contaminated sites. The Ni concentration level was higher in water than soil but only Pb concentration was not found to be significant between soil and water. The high level existence of these heavy metals in the site soil may be because of usage of these metals in paper mill facilitated by the low pH of the soil providing increased solubility in acidic conditions (Muramoto & Oki, 1983; Ogundiran, 2007).

3.2. Metal phytoaccumulation

The metal accumulation *in vivo* in the naturally growing *Eichhornia* in the contaminated site was analysed for its accumulation efficiency. Although in all plants, the metal concentration in the tissues increased with the external metal concentration, and metal concentrations were generally higher in roots than in shoots, but there was tissue specific variation in the metal accumulation for different plant parts (Figure 5). Cd and Cu concentrations were significantly higher in contaminated

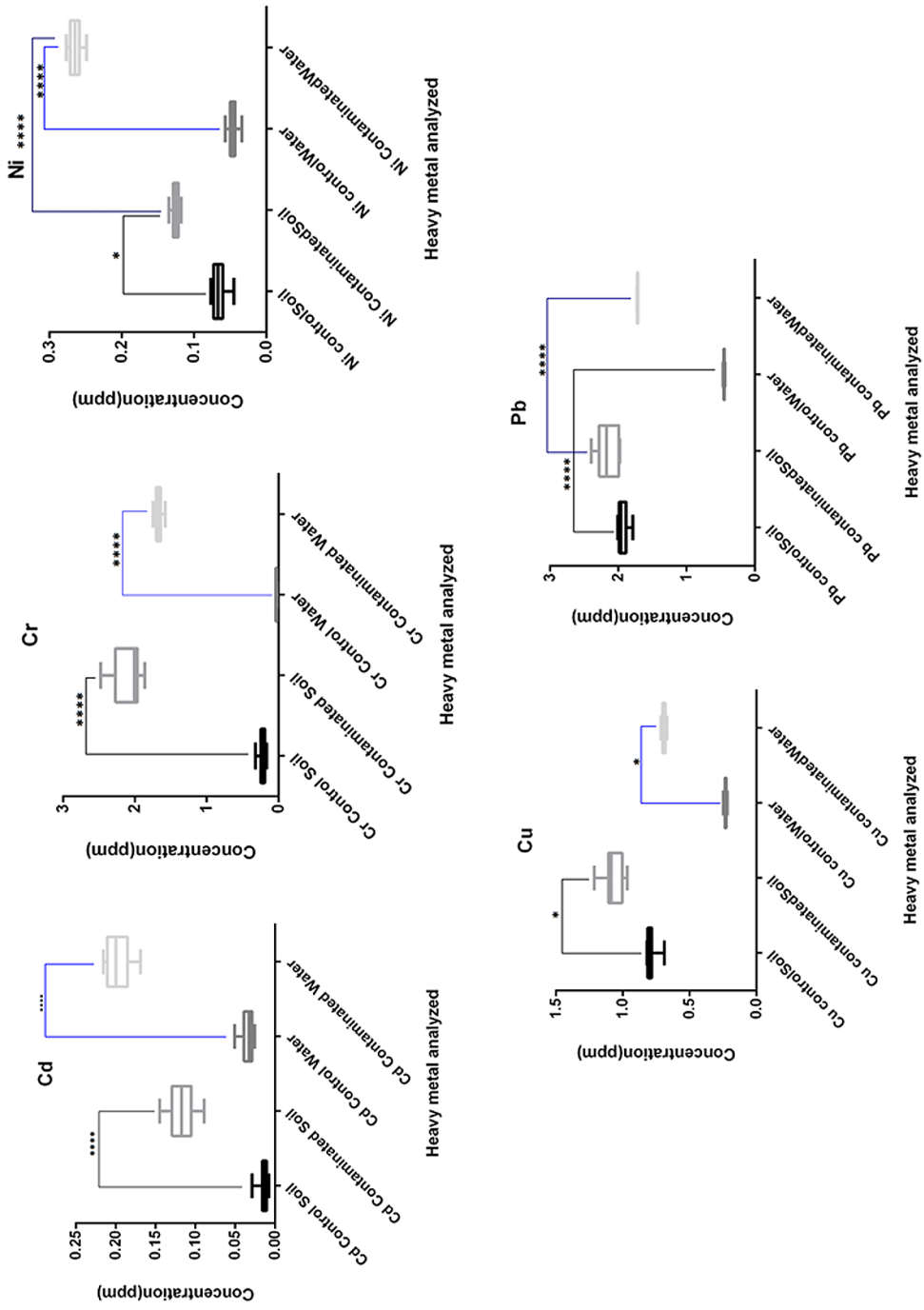

Figure 4. Analysis of heavy metals contamination in soil and water samples through AAS. For all statistical analyses, significance was set to * = $0.01 \leq p \leq 0.05$, ** = $0.001 \leq p \leq 0.01$, and */ΔΔΔ = $p \leq 0.001$ throughout the experiments and accordingly plotted in the graphs for clear understanding.**

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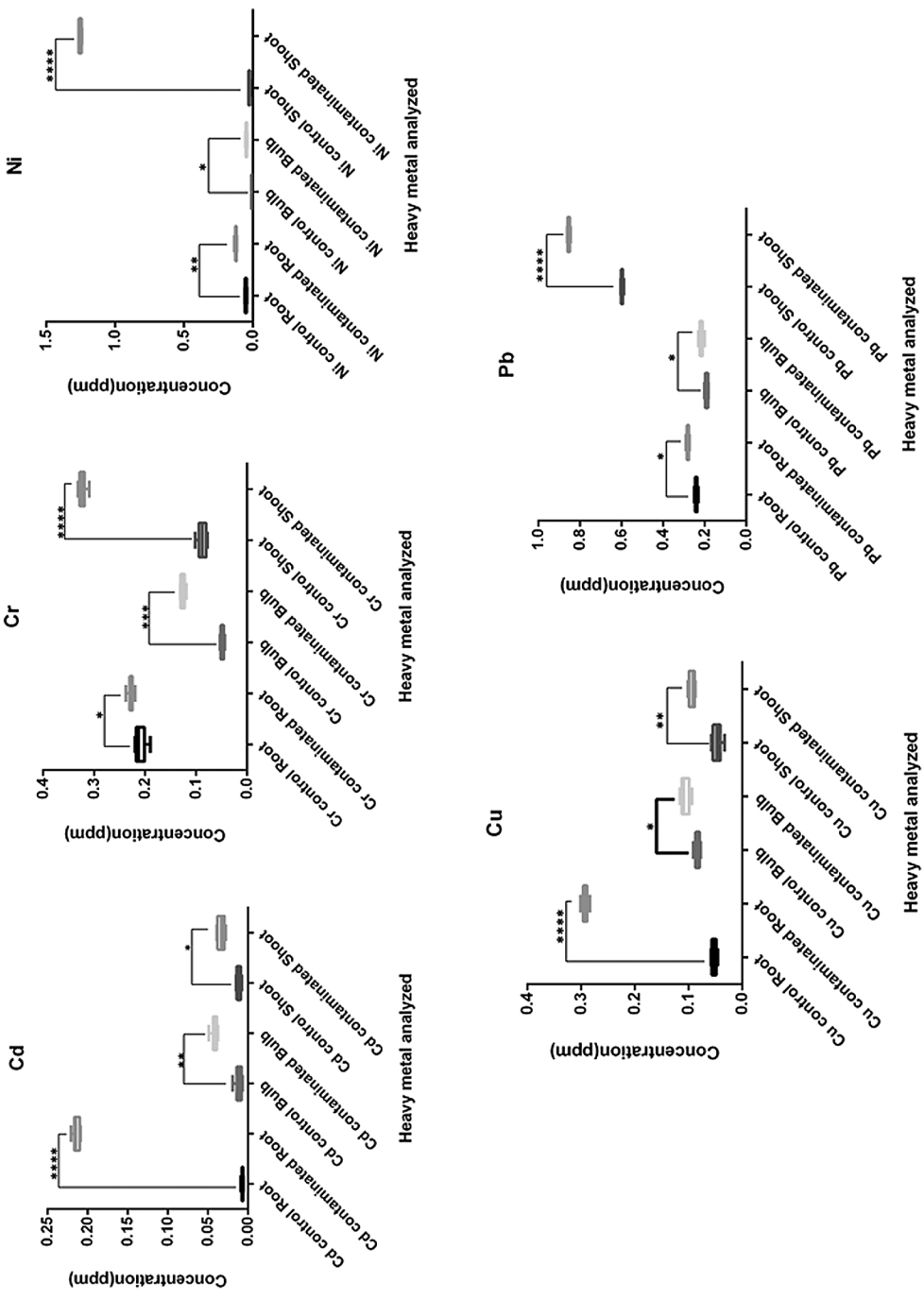

Figure 5. Analysis of soil and water samples through AAS for heavy metals accumulation. Error bars indicate 95% confidence intervals. For all statistical analyses, significance was set to * = 0.01 ≤ $p \leq 0.05$, ** = $0.001 \leq p \leq 0.01$, and */ΔΔΔ = $p \leq 0.001$ throughout the experiments and accordingly plotted in the graphs for clear understanding.**

root than control root ($p < 0.001$) as validated for *Eichhornia* being good accumulator of Cd (Muramoto & Oki, 1983). The higher concentration of Cu can be attributed to being as active constituent of plastocyanins, ascorbic acid oxidase, tyrosinase and phenol oxidase (Odjegba & Fasidi, 2007). The lowest Cr values were observed in both roots ($p < 0.05$) and bulb ($p < 0.05$) and highest accumulation of (0.323 mg/L) was found in shoot as reported earlier in *Eichhornia* (Lytle et al., 1996). The Ni, Pb, Cr concentration in control and contaminated shoot was significantly different with highest Ni accumulation in contaminated shoot ($p < 0.001$). Whereas, the Pb accumulation in control and contaminated root and bulb parts was more homogeneous with significant accumulation in the contaminated shoot ($p < 0.01$). All the metals were accumulated in substantially higher concentration in roots than in shoots with significanyly higher accumulation of all heavy metals in contaminated bulb. As the plant parts' metal concentration ratio does not seem to change in an exposure-dependent way, there was significant accumulation of all the heavy metals in root and shoot under contaminated conditions ($p < 0.001$).

3.3. Morphological changes of Eichhornia in contaminated site

The growth of the *Eichhornia* plants in dumping site and control areas was monitored and the real-time changes in the various observable parameters have been recorded. The shoots of contaminated site *Eichhornia* showed evidence of chlorosis, leaf rolling and loss of chlorophyll, whereas underground root and bulb showed increment in length, girth and number as compared to control plants (Figure 6). Though *Eichhornia* has been reported to substantially accumulate Cu at 0.5 mg/L Cu^{2+} concentration without marked changes in its physiology, loss of chlorophyll and increase in protein level has been reported (Hu et al., 2007). The observed soil and water Cu concentration was 1.078 and 0.679 mg/L respectively towards extreme higher side of contamination level and hence, the appearance of heavy metal stress symptoms was obvious. The decline in chlorophyll content in plants exposed to heavy metal contamination such us Cu is believed to be due to enzymes inhibition associated with chlorophyll biosynthesis (John, Ahmad, Gadgil, & Sharma, 2009). The decrease in chlorophyll content was also reported in sunflower (Zengin & Munzuroglu, 2006) and in almond (Elloumi et al., 2007). These observed symptoms corroborate with Cd toxicity as leaf rolling, chlorosis and stomatal closure in plants (Clemens, 2006) and may be attributed to the negative effects of the heavy metals on mineral nutrition and homeostasis in plant shoot, root growth and development

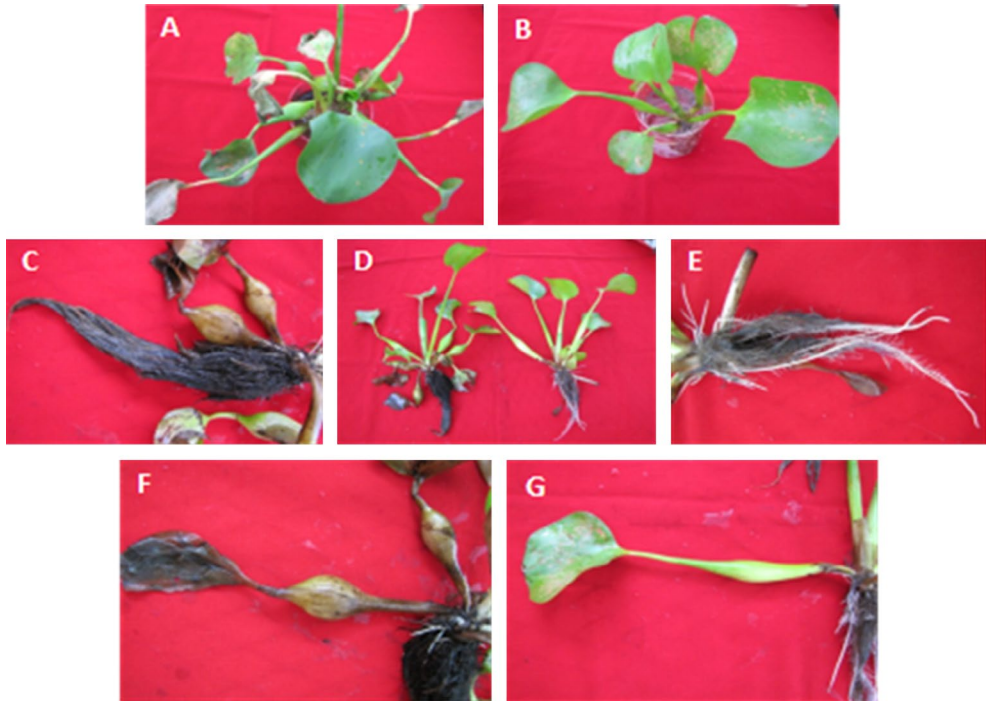

Figure 6. Morphological changes of Eichhornia induced by heavy metals contamination. (A) contaminated plant; (B) Control plant; (C) Contaminated roots; (D) Shoot and root of contaminated and control plants; (E) Control roots; (F) Contaminated bulb; (G) Control bulb.

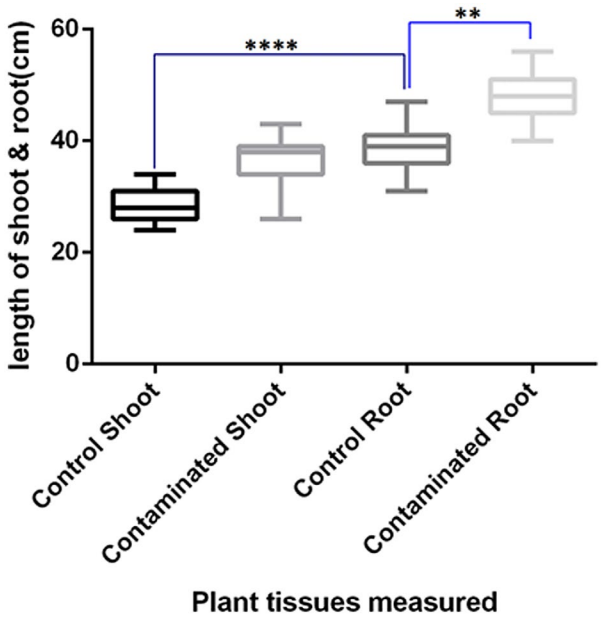

Figure 7. Effect of heavy metals contaminated industrial waste on growth of shoot and root of Eichhornia plants. For all statistical analyses, significance was set to * = 0.01 ≤ p ≤ 0.05, ** = 0.001 ≤ p ≤ 0.01, and */ ΔΔΔ = p ≤ 0.001 throughout the experiments and accordingly plotted in the graphs for clear understanding.**

(Farinati, DalCorso, Varotto, & Furini, 2010; Hassanein, Hashem, El-Deep, & Shouman, 2013; Metwally, Safronova, Belimov, & Dietz, 2005). Such differences in micromorphological characteristics of leaves and roots due to HM content have been reported recently in dandelion (Maleci, Buffa, Wahsha, & Bini, 2014). There has been report of Cd damaging photosynthetic apparatus and causing a decrease in chlorophyll content in contaminated plants (Sanità di Toppi & Gabbrielli, 1999). There seem to be clear symptoms of abscission in the root and leaves of contaminated *Eichhornia* plants. Such negative correlation of HM with root–shoot length, leaf area and biomass of plant has been also reported in *Albizia procera* (Pandey & Tripathi, 2011). It has been reported that exposure to heavy metals can induce increase in abscissic acid (ABA) and jasmonate level in plants (DalCorso, Farinati, Maistri, & Furini, 2008) and this enhanced ABA level may be responsible for the abscission and browning of contaminated root and leaves.

Eichhornia grown in contaminated site showed relative increased shoot and root growth as compared to the control (Figure 7). There was significant increased root growth in contaminated site than control site ($p < 0.001$) suggesting increased accumulation of metals. Though there has been report of increased shoot growth of *Eichhornia* with time and only became constant in the final harvest when grown with continuous water (Henry-Silva & Camargo, 2005; Martins, de Fátima Vitória de Moura, & Bezerra Loiola, 2011). Such increased growth might be the first line of acclimatization to sequester HMs to different cellular compartments of the plant. These observed changes in shoot, root length and bulb girth increment under contaminated condition may also be attributed to alterations in cell cycles and division and chromosomal aberrations due to Cd (Benavides, Gallego, & Tomaro, 2005). Furthermore, Cd has been reported to inactivate DNA mismatch repair in yeast and human cells (Jin et al., 2003) and the same mechanisms may be applicable in plants leading to abnormal growth. A chromatin-remodelling factor OXS3 isolated from *Brassica juncea* was found to protect DNA or alter its transcriptional selectivity under heavy metal stress (Blanvillain, Kim, Wu, Lima, & Ow, 2008). This may be the first general mechanism of *Eichhornia* plants to accumulate the heavy metals and sequester it to various organelles to survive.

3.4. Protein profile changes in Eichhornia

When a plant is exposed to hostile condition, protein synthesis is one of the most negatively affected anabolic processes along with photosynthesis (loss of chlorophyll, ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO), transport of metabolites, and uptake and translocation of ions

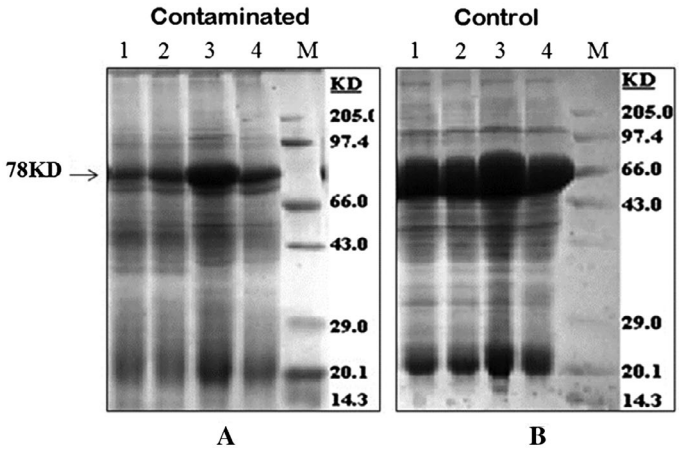

Figure 8. Leaf protein profiles of Eichhornia grown in heavy metals contaminated and control habitat. (A) Lane: 1, 2, 3, 4 Proteins from different individual contaminated samples; Lane M: Molecular Weight Marker (MWM); (B) Lane: 1, 2, 3, 4 Proteins from different individual control samples; Lane M: Molecular Weight Marker.

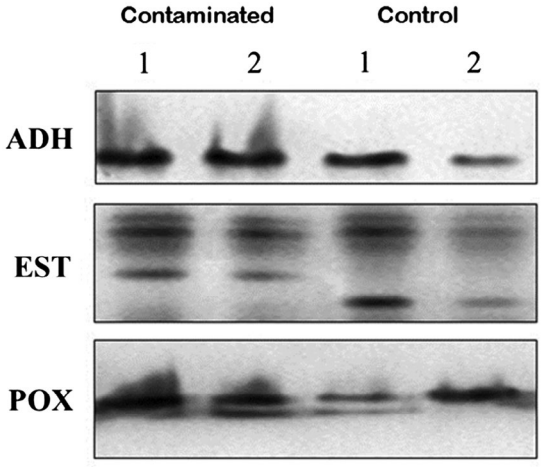

Figure 9. Activity staining of isozymes of Alcohol dehydrogenase (ADH), esterase (EST) and peroxidase (POX) on anionic PAGE.

(Bonjoch & Tamayo, 2001). Hence, the leaf protein profile of *Eichhornia* from contaminated site and control areas was analysed. Significant difference between the control and contaminated leaf protein profiles of *Eichhornia* (Figure 8) was found. The abundant band of 66KD believed to be RubisCO protein in control is missing in contaminated protein gel, where in fact, a low-intensity band of 78KD was observed. The reduction of photosynthetic protein in contaminated plants may be due to increase of ABA level which is believed to down regulate enzymes needed for photosynthesis (Chandler & Robertson, 1994). Similarly, the intense 20.1KD band seen in control protein profile was less intense in contaminated sample, suggesting significant variation in other protein bands also. The difference in protein profile may be due to the negative effect of the heavy metals such as Cd and Cu on the photosynthetic machinery, chlorophyll content and enzyme inhibition related to carbon assimilation (John et al., 2009; Perfus-Barbeoch, Leonhardt, Vavasseur, & Forestier, 2002; Sanità di Toppi & Gabbrielli, 1999). The decrease in protein band intensity observed in contaminated condition may be due to excess Cu that would have caused cellular damage at the DNA level and organelles such as mitochondria or lysosomes (Lee & Wei, 2001). It may suggest plant activating its stress-coping mechanisms like acclimation of metabolic fluxes, activation of repair processes and long-term metabolic and morphological adaptations, which conform to the named general adaptation syndrome (Lichtenthaler, 1996). Such mechanisms include *de novo* synthesis of proteins with specific adaptive functions, osmotic adjustment, antioxidative defence, among others. The altered protein profile in HM contamination may be due to synthesis of stress-responsive proteins or due to accumulation of free amino acids as histidine, proline and cysteine in tissues, this has been reported in stress metal such as Cd (Nedjimi & Daoud, 2009).

3.5. Isozymic profile in heavy metal stress

The activity and expression of genes encoding antioxidant enzymes during heavy metal-induced oxidative stress have provided variable and controversial results (Gomes-Junior et al., 2006). There has been report of high soil Cd concentration drastically altering antioxidant system such as SOD, CAT and POX in wheat seedlings (Lin et al., 2007). The growth of pea plants in 50 μM $CdCl_2$ has been affected to have reduction in SOD activity (Rodriguez-Serrano et al., 2009). Increasing levels of 0–500 μM Cd_2 alone and/or heat stress has showed increased activities of SOD, guaiacol POX, ascorbate POX and GSH reductase enzymes associated with induced oxidative stress and altered enzyme activities (Nahakpam & Shah, 2011). Plant ADH enzymes were considered to be dimers and the two subunits of ADH are encoded by two unlinked genes (Gottlieb, 1982; Sachs & Ho, 1986). In this study, appearance of comparative high-intensity ADH isozyme band during contaminated than control condition indicates the increase in ADH activity leading to increased enzyme synthesis (Figure 9). Increase in ADH enzyme activity has been shown by a change in isozymic profiles of the ADH protein in rice during abiotic stress (Rivoal, Ricard, & Pradet, 1989; Xie & Wu, 1989). The EST isozymes of contaminated and control conditions were found to be significantly different in terms of mobility, whereas POX isozymes were variable with respect to intensity. EST and POX patterns were found to be polymorphic and able to differentiate contaminated and control *Eichhornia* plants under study. Such differences of EST and POX have been previously reported to be a useful induction of abiotic stress tolerance in rice (Mandal, Mandal, Hazra, Mukherjee, & Das, 2004; Zhang, Zheng, Gao, & Cao, 1988).

These results showed a strategy of defence of *Eichhornia* against oxidative stress induced by heavy metal stress leading to variation in the expression of the antioxidant enzymes. Similar results are reported in plants stressed by Cu (Demirevska-Kepova, Simova-Stoilova, Stoyanova, Hölzer, & Feller, 2004), or other metals such us Mn, Pb, Ni and Cd (Demirevska-Kepova et al., 2004; Gomes-Junior et al., 2006; Kopyra & Gwóźdź, 2003; Sobkowiak, Rymer, Rucińska, & Deckert, 2004). Stress that disrupts the cellular homeostasis, including heavy metal toxicity, can enhance the production of ROS and increase the steady-state level of H_2O_2 up to 30-fold (Mittler, 2002). Although we have not measured H_2O_2 or other ROS in this study, alterations are likely to have occurred based on the responses of the antioxidant enzymes that were measured (Gratão, Monteiro, Antunes, Peres, & Azevedo, 2008). Hence, the significant differences of EST and POX isozymes observed during contaminated condition in *Eichhornia* may hint to induction of HM stress tolerance and survival.

4. Conclusions

The high level of Cd, Cu bioaccumulation in soil and roots respectively was revealed by AAS study. This may lead to future study of finding out the genetic mechanisms and regulations especially in roots. The shoot and root showed enhanced growth in *Eichhronia* growing in dumping site than control, which may indicate better acclimatization and sequestration of heavy metals. This may advocate the applicability of *Eichhornia* as agent for phytoremediation of contaminated site. Significant difference in the protein profile of the *Eichhornia* and altered expression of EST and POX suggests the increase in antioxidant enzymes' activities to limit the cellular damage to cope with heavy metal stress. The various morphological changes along with altered profile of protein and isozymes may be another strategy of *Eichhornia* to cope with the heavy metal stress by the synthesis of proteins with specific adaptive functions, which remain to be identified. The stress-adaptive potential of *Eichhornia* may be harnessed and it can be further tried as model system to study heavy metal stress tolerance in plants.

Supplementary material
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