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ORIGINAL ARTICLE

Effect of zinc and lead on the physiological and biochemical properties of aquatic plant *Lemna minor*: its potential role in phytoremediation

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Abstract Plants have gained importance in situ bioremediation of heavy metals. In the present study, different concentrations of zinc (Zn^{2+}) (0.5, 5, 10, 15, 20 mg/l) and lead (Pb^{2+}) (1, 2, 4, 6, 8 mg/l) were used to evaluate metal tolerance level of *Lemna minor*. *L.minor* were exposed to metals for 4 days and tested for its dry to fresh weight ratio (DW/FW), photosynthetic pigments production and protein content. The oxidative damage was detected by measuring catalase activity. *L.minor* showed tolerance against Zn^{2+} and Pb^{2+} at a concentration of 10 and 4 mg/l, respectively. Among the metals, Pb^{2+} showed a significant toxicity at 8 mg/l. High concentration (20 mg/l of Zn^{2+} and 8 mg/l of Pb^{2+}) of the metals displayed a considerable negative effect on soluble proteins (13 fold decrease with Zn^{2+} and 4 fold decrease with Pb^{2+}) and photosynthetic pigments (twofold decrease with Zn^{2+} and onefold decrease with Pb^{2+}) and lead to a consequent reduction in number of fronds. Further, the catalase was greatly increased (twofold decrease with Zn^{2+} and sixfold decrease with Pb^{2+}) under metal stress. The results indicate that *L.minor* withstands Zn^{2+} and Pb^{2+} toxicity up to the concentration of 10 and 4 mg/l, respectively. Hence, the metal tolerant property of this plant shall be exploited for bioremediation of Zinc and Lead in polluted water. Further, the detailed and wide range of heavy metal toxicity studies should be done to reveal the possible use of this plant on large scale bioremediation purpose.

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Keywords *Lemna minor* · Zinc · Lead · Tolerance · Phytoremediation

Abbreviations

Zn^{2+}	Zinc
Pb^{2+}	Lead
L. minor	*Lemna minor*
CAT	Catalase
DW/FW	Dry to fresh weight ratio

Introduction

Urbanization and industrialization had triggered extreme water pollution by draining effluents directly into water bodies without prior treatment. Industries such as smelters, tanneries, metal refineries and mining operations are the major sources of metal release into the environment (Gardea et al. 2004; Srivastava and Thakur 2006). These effluents generally contain metals that can be toxic even in trace amounts and it is very difficult to purify these water bodies due to its large volume. Heavy metal pollution is an important environmental problem in the world because, unlike organic materials, heavy metals cannot be transformed by microorganisms and, therefore, accumulates in water, soil, bottom sediments and living organisms (Miretzky et al. 2004). Most of the heavy metals have been found to be carcinogenic in nature and hence it poses a threat to human health too (Shakibaie et al. 2008; Vinodhini and Narayanan 2009). Metals induce deleterious effect on physiology of aquatic plants by effecting some of the essential phenomenon such as photosynthesis, enzymatic activity, etc. (Teisseire and Vernet 2000;

Prasad et al. 2001; Vaillant et al. 2005; Kanoun et al. 2009; Zhou et al. 2009) Hence, some eco-friendly and economic methods shall be considered to treat heavy metal polluted water.

Plants have been found to accumulate and concentrate the heavy metals within. Phytotolerance studies are used to determine metal tolerant property of plants and also to determine the detrimental effect of metals on physiological response of plants (Basile et al. 2012; Radić et al. 2010). *L.minor*, commonly known as Duckweeds are aquatic plants that float on or just beneath the surface of still or slow-moving fresh water bodies and often form dense floating mats in eutrophic ditches and ponds (Driever et al. 2005). It is also used in wastewater treatment to remove mineral and organic contamination and radionuclides (Chaudhary and Sharma 2014; Axtell et al. 2003). The present work deals with the study of the potential of duckweed to grow in different concentrations of metals, viz. Zinc (Zn^{2+}) and lead (Pb^{2+}) and to assess the tolerance level exhibit by the plant. The metal tolerant efficiency of *L. minor* was evaluated with reference to: (1) dry to fresh weight ratio (DW/FW) (2) changes in soluble protein content; (3) changes in contents of chlorophyll a, chlorophyll b, anthocyanin and carotenoid; (4) changes in enzymatic activity of catalase (CAT) activity.

Materials and methods

Sample collection

Duckweed (*L.minor*) was collected from natural pond water of Vellore Institute of Technology, Vellore.

Plant sample preparation

Lemna minor fronds were prepared and disinfected in 1 % of sodium hypo chloride solution and 2 g fronds were then inoculated in synthetic media, i.e., Quarter Coic and Lessaint solution (Khellaf and Zerdaoui 2009) along with various concentrations of lead (1, 2, 4, 6 and 8 mg/l) and zinc (0.5, 5, 10, 15, and 20 mg/l) to induce metal stress. Aeration was provided, and fronds were allowed to grow at 25 °C in an incubator with 16 h illumination per day provided from fluorescent tubes for 4 days before proceeding with sampling methods. After 4 days of exposure with heavy metals, fronds were collected and stored in the freezer for further analysis like determining biomass, soluble protein, photosynthetic pigments and enzyme assay. A control with untreated fronds was also maintained.

Measurement of dry to fresh weight ratio (DW/FW)

The number of fronds, fresh weight, and dry weight was calculated as per the ISO/DIS 20079 protocol (2004). Dry to fresh weight ratio (DW/FW) was calculated using the formula dry weight (g)/fresh weight (g).

Determination of photosynthetic pigments

The contents of chlorophyll a, chlorophyll b, and carotenoid content of both control and metal treated fronds were determined as described earlier (Lichtenthaler 1987). Briefly, 150 mg of *L. minor* frond was homogenized with 80 % cold acetone. The homogenate was centrifuged, and the absorbance of the supernatant was measured at 470, 537, 647, 663 and 730 nm with a spectrophotometer (Wenhua et al. 2007). Anthocyanin content of both control and metal treated fronds were determined spectrophotometrically as explained by Suzuki (1995).

Catalase assay

Catalase activity in fronds was measured as described by Wenhua et al. (2007). Approximately 500 grams of *L. minor* fronds treated with lead and zinc of different concentrations were homogenized in 5 ml of cold potassium phosphate buffer, pH 7.8. The homogenate was centrifuged at 9000 rpm for 15 min with a temperature of 4 °C and supernatant were stored at 4 °C for analysis. The reaction mixture (1 ml) containing potassium phosphate buffer (50 mM, pH 7.5, 750 µl), H_2O_2 (200 mM, 100 µl) and enzyme extract (150 µl) was evaluated for catalase activity by measuring the consumption of H_2O_2 spectrophotometrically at 240 nm (Wenhua et al. 2007).

Protein estimation

Approximately 500 grams of *L. minor* fronds were crushed in pestle and mortar with 5 ml of potassium phosphate buffer, collected into 15 ml centrifuge tube and centrifuged at 12,000 rpm for 20 min. The clear supernatant was taken and used for estimation of protein by Lowry's Method (Lowry et al. 1951).

Results and discussion

Effect on dry to fresh weight ratio (DW/FW)

After 4 days of exposure to zinc and lead the physiological and morphological conditions were analyzed. The frond samples with green color and with no signs of necrosis

were taken for further analysis. Dry to fresh weight ratio (DW/FW) was measured after 4 days. Comparatively, the biomass yield in fronds treated with a lower concentration (0.5 mg/l) of Zn^{2+} was higher (30 %) than the control. 10 mg/l had little effect on growth while 20 mg/l retarded the growth after 4 days. The zinc at lower concentration promotes the growth of duckweeds, but it becomes toxic to the duckweed at higher concentration. Similarly, the lead induced stress also had a negative impact on the growth of *L. minor*. At 6 mg/l concentration, the biomass was drastically reduced by 80 %. Both the metals at higher concentration are known to interfere with the photosystem alteration and resultantly induce the chlorosis.

It has been found that duckweed shows wide range of tolerance against several factors that generally leads to necrosis and hence causes death in many plants. These macrophytes can tolerate a wide pH range but survive best between pH of 4.5–8.3. It is also used in wastewater treatment to remove mineral and organic chemicals and radionuclides (Susarla et al. 2002). Results obtained in the present study shows the tolerance of *L. minor* to the highest concentration of metals to which it was exposed; i.e.; 10 and 4 mg/l of the concentration of Zn^{2+} and Pb^{2+}, respectively. It has already been shown in some studies that the metals like Pb and Zn were tolerated by *L. minor* at 0.4, 0.4, 3 and 15 mg/l, respectively (Khellaf and Zerdaoui 2009; Sasmaz et al. 2015). In our observations, it is evident that the duckweeds tolerate the zinc up to 10 mg/l.

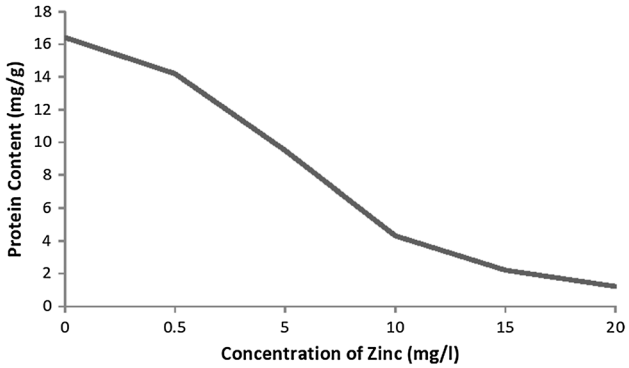

Fig. 1 Effect of various concentrations of Zinc on the soluble protein of *Lemna minor*

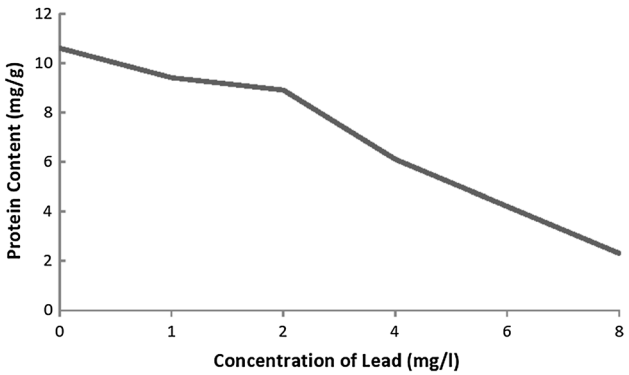

Fig. 2 Effect of various concentrations of Lead on the soluble protein of. *Lemna minor*

Effect of Zinc and Pb on soluble protein in *Lemna minor*

Variations in soluble protein content can be considered as a sign of some physiological changes in plant metabolism (Singh and Tewari 2003). Changes in the soluble protein content of *L. minor*, when treated with different concentrations of Zn^{2+} and Pb^{2+}, were shown (Figs. 1, 2). The soluble protein content in *L. minor* decreased by an increase in the concentration of Zn^{2+}. Exposure to 0.5 mg/l of Zn^{2+} showed only a slight decrease in soluble protein when compared to the control content after 4 days of treatment. However, exposure to 10 mg/l of Zn^{2+} showed a 75 % protein content reduction in *L. minor*. Beyond 10 mg/l of Zn^{2+} indicated more than 90 % decrease in protein content (Fig. 1). The soluble protein content of *L. minor* fronds exposed to different concentrations of lead was also found to decrease on the consequent increase in the concentration of lead. From 0 to 4 mg/l concentration, there was a gradual reduction in the protein content. At a concentration of 8 mg/l, the plant showed almost same level of protein content as compared to the frond exposed to 6 mg/l (Fig. 2).

Decrease in total soluble protein content after exposure to metals could be due to changes in metabolism caused due to metal stress. Exposure of plants with heavy metals could also induce synthesis of some proteins related to stress to overcome the effects of heavy metal stress which show an increase in protein content (Doganlar et al. 2012). The above results clearly indicate that zinc and lead considerably affect the physiology of plant *L. minor*. Zinc and lead have a significant toxic effect on soluble proteins present in *L. minor*, and hence, leads to a consequent decrease in soluble protein with an increase in the concentration of metal and stress. Heavy metals had been found to have a strong inhibition effects on soluble protein and photosynthetic pigments of *L. minor* (Wenhua et al. 2007). Proteins represent the reversible and irreversible changes in the physiological state of the biological system and hence from above results we may infer that metal stress highly disturbs the homeostasis and normal metabolism in the plant *L. minor* (Singh and Tewari 2003).

Effect on photosynthetic pigments

Different trends were observed for different pigments in the heavy metal exposed *L.minor* fronds. The pigment contents in *L.minor* fronds treated with different

Table 1 Effect of various concentrations of zinc on photosynthetic pigment of *Lemna minor*

Pigments	Concentrations (mgl^{-1})					
	C	0.5	5	10	15	20
Chlorophyll a	0.19	0.23	1.82	1.29	0.77	0.66
Chlorophyll b	0.60	0.74	0.94	0.20	0.10	0.11
Total chlorophyll	0.79	0.68	0.89	0.56	0.32	0.30
Anthocyanin	0.0078	0.0097	0.069	0.035	0.021	0.013
Carotenoid	0.10	0.66	0.75	0.43	0.38	0.21

Table 2 Effect of various concentrations of lead on photosynthetic pigments of *Lemna minor*

Pigments	Concentrations (mgl^{-1})					
	C	1	2	4	6	8
Chlorophyll a	1.75	1.97	1.65	1.24	1.21	0.99
Chlorophyll b	0.71	0.87	0.61	0.45	0.33	0.31
Total chlorophyll	3.17	3.46	3.27	3.05	3.01	2.46
Anthocyanin	0.024	0.051	0.036	0.026	0.020	0.012
Carotenoid	0.79	0.50	0.39	0.38	0.33	0.32

concentrations of Zn^{2+} and Pb^{2+} are shown in Table 1 and 2. The Zn^{2+} concentrations used were 0.5, 5, 10, 15 and 20 mg/l. The amount of Chlorophyll a was found highest 5 mg/l of Zn^{2+} (1.82) whereas in control, it was found to be 0.19. A gradual decrease in chlorophyll a was seen when Zn^{2+} concentration was further increased. In the case of chlorophyll b 5 mg/l concentration of Zn^{2+} yielded highest (0.94) as compared to that of control (0.60). The chlorophyll b was gradually decreased with as increasing concentration of Zn^{2+}. A similar pattern was obtained in total chlorophyll, higher (0.89) at 5 mg/l concentration of Zn^{2+} and decreased as the concentration was elevated. Anthocyanin was found to be in its maximum (0.0097) at a concentration of 0.5 mg/l while control showed 0.0078 and a steady decrease was seen when the concentration was increased. Carotenoid showed maximum (0.75) at 5 mg/l of Zn^{2+} concentration, and it was decreased beyond that concentration.

The effect of Pb^{2+} on photosynthetic pigments of *L. minor* showed a variance in the pigment production with increased concentration of Pb^{2+}. All pigments such as chlorophyll a, chlorophyll b, total chlorophyll and anthocyanin exhibited initial increase from the control and gradual decrease as the concentration of the Pb^{2+} was increased. Chlorophyll a had highest (1.97) at a concentration of 1 mg/l. Higher amount was observed with 1 mg/l concentration for Chlorophyll b (0.87), total chlorophyll (3.46) and anthocyanin (0.051) as well. Whereas carotenoid showed a gradual decrease from the control (0.79) when varying the concentration of Pb^{2+} was administered.

Further, the results obtained also indicate that metal stress, on an average, decreases the expression of photosynthetic pigments. From Tables 1 and 2, it could be inferred that the rate of degradation of Chlorophyll b under heavy metal stress of zinc and lead was slower than that of Chlorophyll a and carotenoid, respectively. Hence, the effect of heavy metals on Chlorophyll a is greater than that of Chlorophyll b. As it is evident that Chlorophyll a is one of the most important center pigments in photosynthesis, therefore, reduced amount of Chlorophyll a can inhibit the photosynthesis greatly. Carotenoid, which usually plays an important role in protecting chlorophyll, also serves as an antioxidant to quench or scavenge the free radicals and prevents the damage of cell, cell membrane, and its main genetic composition (Han 1999). In the present study where the concentration of carotenoid is found to decrease on its exposure to Zinc and Lead, therefore, the susceptibility of plant tissue of *L. minor* towards damage also increases significantly. The mechanism of effect of heavy metals on plant level of photosynthetic pigments may be owed to three reasons. First, heavy metals enter frond chloroplast (Sandalio et al. 2001) and may get over-accumulated locally causing oxidative stress that will cause damages like peroxidation of chloroplast membranes (Puertas et al. 2004). They can also directly destroy the structure and function of chloroplast by binding with –SH group of the enzyme and may also inhibit the overall chlorophyll biosynthesis by targeting Mg^{2+} and Fe^{2+}. Second, heavy metal ions inhibit uptake and transportation of other metal elements such as Manganese and Iron by

antagonistic effects and therefore, the fronds lose their capacity to synthesize pigments (Das et al. 1997). Third, Heavy metals may activate pigment enzyme and accelerate the decomposition of pigment (Wenhua et al. 2007). On comparing the results of the responses produced by *L. minor* under the metal stress of Pb^{2+} and Zn^{2+}, this can be clearly observed that Pb^{2+} induces more uniform and significant alteration in the physiological processes of *L. minor* as compared to Zn^{2+}. As it is known, that Zn application increases plant chlorophyll and protein contents; thereby plant can utilize and tolerate higher levels of Zinc concentration (Samreen et al. 2013; Lin and Wu 1994). Hence, Pb^{2+} is more potent and toxic towards *L. minor* than Zn^{2+}.

Effect of Zn and Pb on CAT activity of *L.minor*

Increased production of reactive oxygen species occurs in plants under environmental stresses, such as heavy metals, salt stress, ultraviolet radiation, air pollution such as ozone and SO_2 (Mittler et al. 2002). Under these stress conditions antioxidant enzymes like CAT, SOD will be activated by plants as a mode of detoxification to scavenge excess reactive oxygen species. This study shows a significant increase in the CAT activity in the presence of Pb^{2+} and Zn^{2+} (Figs. 3, 4). The increased activity of CAT in the presence of Pb^{2+} and Zn^{2+} indicates the increased amounts of hydrogen peroxide and additionally effective scavenging of H_2O_2. The maximum value for CAT activity under zinc stress was observed at the concentration of 5 mg/l (8.2 U mg/protein/min). However, the activity was found to be gradually decreased upon increased concentration. At the concentration of 20 mg/l, the activity was found to be 4.9 U mg/protein/min. In the *L. minor* fronds under Pb^{2+} stress, an exponential increase in CAT activity was observed. Figure 4 shows the dose dependent CAT activity observed in *L. minor* fronds. Maximum activity (28.0 U mg/protein/min) was found at the concentration of 8 mg/l of lead. A continuous increase in the CAT activity was observed with increasing concentration of Pb^{2+}. At a concentration of 1 mg/l of Pb^{2+}, the catalase activity was seen to be less (5U mg/protein/min) while it increased at higher concentrations of Pb^{2+}. The results show similar findings with earlier reports showing the effects of Mn and Ni on CAT activity in this plant (Fridovich 1978). When *Lemna* was faced with low-level metal stress, fronds could activate CAT activities, which led to a strengthening of fronds, to scavenge ROIs responsible for lipid peroxidation (Fridovich 1978). While the activities decreased distinctly under too acute stress that overloaded cellular defense system of fronds (Guecheva et al. 2003; Lichtenthaler 1996). Reduction in the activities of CAT might be due to the formation of a protein complex with metals that resulted in the structural integrity of proteins

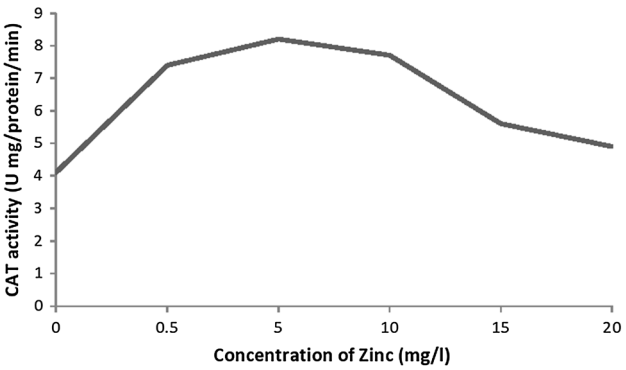

Fig. 3 Effect of various concentrations of Zinc on CAT activity of *Lemna minor*

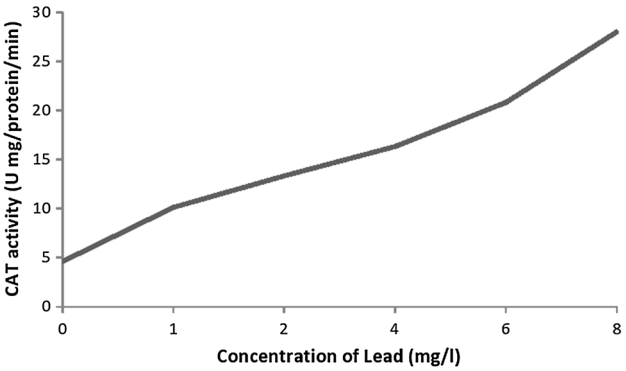

Fig. 4 Effect of various concentrations of Lead on CAT activity of *Lemna minor*

(Florence 1986; Mohan et al. 1997). The results suggest that Pb^{2+} was more toxic to *L. minor* than Zn^{2+} for the reason that at relatively lower concentrations, antioxidant system appeared to be in disorder.

The present study indicates that under metal stress the CAT activity was increased in an exponential order, in the case of lead and showed a biphasic response in case of Zinc. CAT participates in the main defense system against accumulation and toxicity of hydrogen peroxide and can play the role in controlling H_2O_2 level in cells. It acts on H_2O_2 and converts it to water and oxygen (Mstte's 2000). The present study shows that enzymatic activity of *L. minor* increases with increase in metal concentration.

Conclusion

L. minor shows tolerance against the high metal concentration of 10 and 4 mg/l of Zn^{2+} and Pb^{2+}, respectively. Among these, Pb^{2+} was found to be more toxic and potent against *L. minor*. Hence, *L. minor* can be used for the bioremediation of low-level polluted water body by Zinc and Lead. However, large scale studies should be done on

the tolerance level against a wide range of heavy metals and also on other biochemical and physiological factors. This will imply the *L. minor* for recommended usage on a large scale for treating polluted water resources by cheap and eco-friendly approach. The present work will provide a greater insight to the study of *L. minor* species and its relevance to be used for in situ bioremediation on a large scale that puts forward a cheap and convenient method for treating polluted water.

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Author's contributions MAJ and KS have designed the study and performed the statistical analysis. MAJ performed the experiments, collected the data and analyzed. KS drafted the manuscript, analyzed the data critically and validated. Both the authors approve the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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