International Journal of Phytoremediation

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/bijp20

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To cite this article: A. A. El-Khatib , A. K. Hegazy & Amany M. Abo-El-Kassem (2014) Bioaccumulation Potential and Physiological Responses of Aquatic Macrophytes to Pb Pollution, International Journal of Phytoremediation, 16:1, 29-45, DOI: 10.1080/15226514.2012.751355

To link to this article: http://dx.doi.org/10.1080/15226514.2012.751355

International Journal of Phytoremediation, 16:29–45, 2014

ISSN: 1522-6514 print / 1549-7879 online
DOI: 10.1080/15226514.2012.751355

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BIOACCUMULATION POTENTIAL AND PHYSIOLOGICAL RESPONSES OF AQUATIC MACROPHYTES TO PB POLLUTION

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In view of their potential bioaccumulation of heavy metals, Ceratophyllum demersum and Myriophyllum spicatum was studied under hydroponic cultures enriched by different Pb concentrations (25, 50, 75 mg/l) for 1–7 days. Both species exerted remarkable capabilities to concentrate Pb in their tissues as compared to control. The highest accumulation value of Pb (164.26 mg/g.dw) was recorded in C. demersum and the most of metal (91.72 mg/g dw) accumulated after 1 d. Significant reduction in photosynthetic pigments and appearance of morphological symptoms such as chlorosis and fragmentation of leaves were evident after 7d at 75 mg/l. The activity of POX and APX, carotenoids and proline showed induction at lower concentration and duration followed by decline. Major re-shuffle in protein patterns appeared as a tolerant mechanism, which both species developed under Pb toxicity. Results suggest that both species responded positively to Pb concentration and accumulated high amount of metal. Due to metal accumulation coupled with detoxification potential, both species appear to have potential for use as phytoremediators and the developed responses can be used as reliable biomarkers for Pb water pollution.

KEY WORDS: biomarkers, biomonitor, proline, carotenoids, antioxidant defense, *Ceratophyllum demersum*, *Myriophyllum spicatum*, protein pattern

INTRODUCTION

Lead (Pb) is one of the most abundant, ubiquitously distributed toxic metals that can cause damage to biota (Zeng *et al*. 2006). Its contamination results from mining and smelting activities, lead containing paints, paper and pulp, gasoline and explosives as well as from the disposal of municipal sewage sludge enriched with Pb (Sharma and Dubey 2005). The use of aquatic plants for the removal of heavy metals from wastewater has gained many attentions (Keskinkan *et al*. 2004). Submerged plants play important roles in maintaining the health of aquatic ecosystems through the accumulation and/or decomposition of toxins (Rai *et al*. 1995). These plants adopt many defensive mechanisms in response to heavy metals for their survival. With regard to Pb, once entering cytosol, chelators such as proteins (e.g., heat shock proteins), peptides (e.g., metallothioneins), ligands (e.g., glutathione), proline

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and polyamines can bind and reduce its toxicity (Mishra *et al*. 2006; Islam *et al*. 2008). Other mechanisms include production of antioxidant (e.g., ascorbic acid) and enzymes (e.g., peroxidase (POX) and ascorbate peroxidase (APX)), which remove Reactive Oxygen Species (ROS) resulting from oxidative stress caused by Pb and can rapidly attack nucleic acids, protein, lipids and amino acids. (Sharma and Dietz 2009; Gupta *et al*. 2010; Singh *et al*. 2010).

Myriophyllum spicatum L. and *Ceratophyllum demersum* L. are cosmopolitan species, exhibiting similar life forms and colonizing mainly eutrophic stagnant and flowing waters (Martincic *et al*. 1999). They are very common species in drainage channel systems and can be found throughout the year in Upper Egypt, where the dry climate is prevailing. Metal accumulation by these species indicated that the species might be considered as useful vehicles for the removal and recovery of heavy metal ions from aqueous solutions (El-Khatib and El-Sawaf 1998; Kamal *et al*. 2004, Manal *et al*. 2012).

The objective of the present study is to determine the potential of these macrophytes for accumulation of lead (Pb) from the water column by means of laboratory experiences and to ascertain if they can use the resulted biochemical responses as biomarkers for determining the extent of lead pollution in hydrophytes.

MATERIAL AND METHODS

Plant Species Collection and Initial Preparation

Plants of *Ceratophyllum demersum* L. and *Myriophyllum, spicatum* L., were collected from the main stream of the River Nile bank. They transported into the laboratory within water tanks. Before the experimental setup, whole plants of *Ceratophyllum* or *Myriophyllum* have thoroughly cleaned under running tap water to remove debris and other foreign particles, and then gently rinsed with bidistilled water. Plants were analyzed to determine the initial concentration of Pb and then transported into hydroponic cultures placed in growth chamber and kept for 2 weeks to acclimatization, before adding the contaminants (Cowgill *et al*. 1989).

Hydroponic Experimental Apparatus and Growth Conditions

The experiment was carried out within growth chamber equipped with four hold tanks for each species (three for treatments and the other for control); each tank divided into 3 compartments. Each compartment received 10 litter of water and the recommended amount of Hoagland' E-medium nutrient solution (Cleland and Briggs Formulation referenced in Cowgill *et al*. 1989). In the growth chamber, day light lamps provided supplementary light with an irradiance of 1950 lux, at a light: dark cycle of 14:10 h. Temperature readings were recorded every day in order to monitor the change in the water ($21 \pm 2^{\circ}$C during light period and $20 \pm 2^{\circ}$C during dark periods) and the ambient air temperatures ($25 \pm 2^{\circ}$C) during the experimental period. An aeration unit was installed for each compartment to provide oxygen for aquatic plants. The air flows from the main laboratory supply to a manifold with four outlets. Each outlet was connected to the aerator, which located in each compartment. Dissolved oxygen daily monitored to be ranged between 7–7.5 mg/l.

Experimental Design and Protocol

For exposure, healthy acclimatized plants of both species (100 g fresh weight, each) were left to grow in nutrient solution of compartments containing different concentrations

of Pb (25, 50, 75 mg/l; prepared from Pb $(NO_3)_2$ - Sigma, St. Louis, MO) for consecutive 1, 3, 5 or 7 days along with one set without Pb that served as control. To prevent the media to get concentrated day after day as a result of evaporation and transpiration, bidistilled water was continuously added to aquaria. For analysis, one sample from each of the water and plants were taken from each compartment resulted in triplicate for each Pb treatment (12 samples / interval / species). This resulted in a total of 48 samples for water or plant for each during the whole experimental period.-.Before study of various parameters, the harvested plants were washed by ultra-pure water.

Lead Quantification

Water (5 ml) and plant shoot (5 g fresh weigh) samples were collected in the same time as in the experimental design described above at intervals of 1, 3, 5, and 7 days. The collected water samples were acidified by super pure nitric acid (0.1 ml/15 ml sample).

After washing, Plant samples were dried in a convection oven for 24 h at 48°C. Dried material was powdered. For analysis, dry plant material was wet digested according to the method of Campbell and Plank (1998). Ten ml concentrated nitric acid (HNO_3) and 0.5 ml hydrofluoric acid (HF) was added to 0.5 g dry plant sample in a closed Teflon vessel designed for the purpose at a temperature of 130 ºC for 24 hrs. Digestion in solution continues until clear. The resultant liquid diluted up to 25 ml with ultra-pure water; then stored for analysis. Pb was determined by the same analytical methods used for water samples.

The Pb concentration was determined by Atomic absorption spectrophotometric (AAS) Model 210 VGP Buck Scientific. The concentration is expressed as mg/l (ppm), for water samples, and mg/g dw for plant samples.

Photosynthetic Pigment Content

Chlorophylls a and b, and total carotenoids (xantophylls + β carotene) concentrations are quantified during the experimental period. The pigments are extracted from 0.5 g of fresh samples. The samples are grinded at 4°C in a mortar with 1 g Fontainebleau sand and 5 mg MgCO3 to neutralize tissue acidity. When pulverulent, the samples are grinded once again with pure acetone and 1.25 g Na2SO4 (anhydrous) to fix tissue water, centrifuged 10 min at 5,000 rpm at 4 °C and the supernatant is diluted with bidistilled H_2O to a final concentration of 80% acetonic extract. The absorbance of extracts is measured at 470, 646.6, and 663.6 nm using Unico ® 1200 spectrophotometer and the concentrations of pigments are calculated with the equation of Lichtenthaler (1987). Concentrations of pigments were calculated on a fresh weight basis and expressed as mg/g.

Proline Content

Free proline content was estimated following the method of Bates *et al*. (1973). Dry leaves (0.5 g) were extracted in 3% sulphosalicylic acid and the homogenates were centrifuged at 10,000 g for 10 min. A 2 ml of the supernatant was reacted with 2 ml of acid ninhydrin reagent and 2 ml of glacial acetic acid in a test tube for 1 h at 100°C and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml of toluene and mixed vigorously with a vortex mixture for 15–20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance measured at 520 nm using toluene as blank. Proline concentration was

calculated from a standard curve using 0–100 μg/l proline (Sigma) and expressed on the dry weight basis as mg/g.

Antioxidant Enzymes (APX & POD)

Activities of the tested antioxidant enzymes were assayed using the method of Nakano and Asada, (1981) (*EC 1.11.1.11*) for APX, and those of Wakamatsu and Takahama (1993) (*EC 1.11.1.7*) for POD. In reference to control, the relative activity of the two enzymes calculated using the extinction coefficient 26.6 mM^{-1} cm^{-1} and 2.8 mM^{-1} cm^{-1} for APX and POD, respectively.

Protein Electrophoresis (SDS-PAGE)

Protein profiles of control and treated plant samples of both species were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) following the procedure of Laemmli (1970). A 10% separating gel was prepared and 40 μg of protein solubilized with sample buffer [62.5 mM Tris–HCl, pH 6.8, 20% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2- mercaptoethanol and 0.01% (w/v) bromophenol blue] was loaded in each lane of the gel. Electrophoresis was accomplished at 35 mA for 3 h using Bio-Rad, Protein II electrophoresis system. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma) in 50% (v/v) methanol and 10% (v/v) acetic acid for 2 h and destained with 50% (v/v) methanol and 10% (v/v) acetic acid until the background was clear. The gels were photographed and scanned using Olympus camera (model No C-7070). Protein bands were assigned in reference to protein marker (Fermentas, Page Ruler ^{TM} Unstained protein Ladder #SM0661, 10 kDa to 200 kDa) and analyzed to determine their molecular weight using BioDoc Analyze, Biometra 2006 software Version 2.49.8.1.

Statistical Analysis

All measures, because dependent from the equipment standard error, are carried out in triplicate. SPSS.15. software is used for statistical analysis. Normality of the variables is tested with K-S and Liliefors test for normality, which indicates that the results follow a normal distribution. Thus parametric tests are used: the Spearman's correlation coefficient to estimate a possible link between normally correlated biological parameters and Pb concentration; all the data were analyzed by Analysis Of Variance (Two-Way ANOVA) and significant differences among treatments were identified using Turkey's post hoc tests and were assumed when $P < .01$; respond of biological parameters to exposure concentration and duration were analyzed by using $Y = A + \beta x$ regression model.

RESULTS AND DISCUSSION

Removal and Accumulation

The studied species exerted high remarkable abilities to concentrate Pb in their tissues (Figure 1a–c). On the seventh day exposure, maximum removal efficiencies were 87.69% by *M. spicatum* and 92.8% by *C. demersum*, when plant species exposed to 50 and 75 mg/l Pb, respectively. The rate of Pb bioaccumulation decreased with the increasing of time of exposure. High bioaccumulation values recorded in the studied species after the first

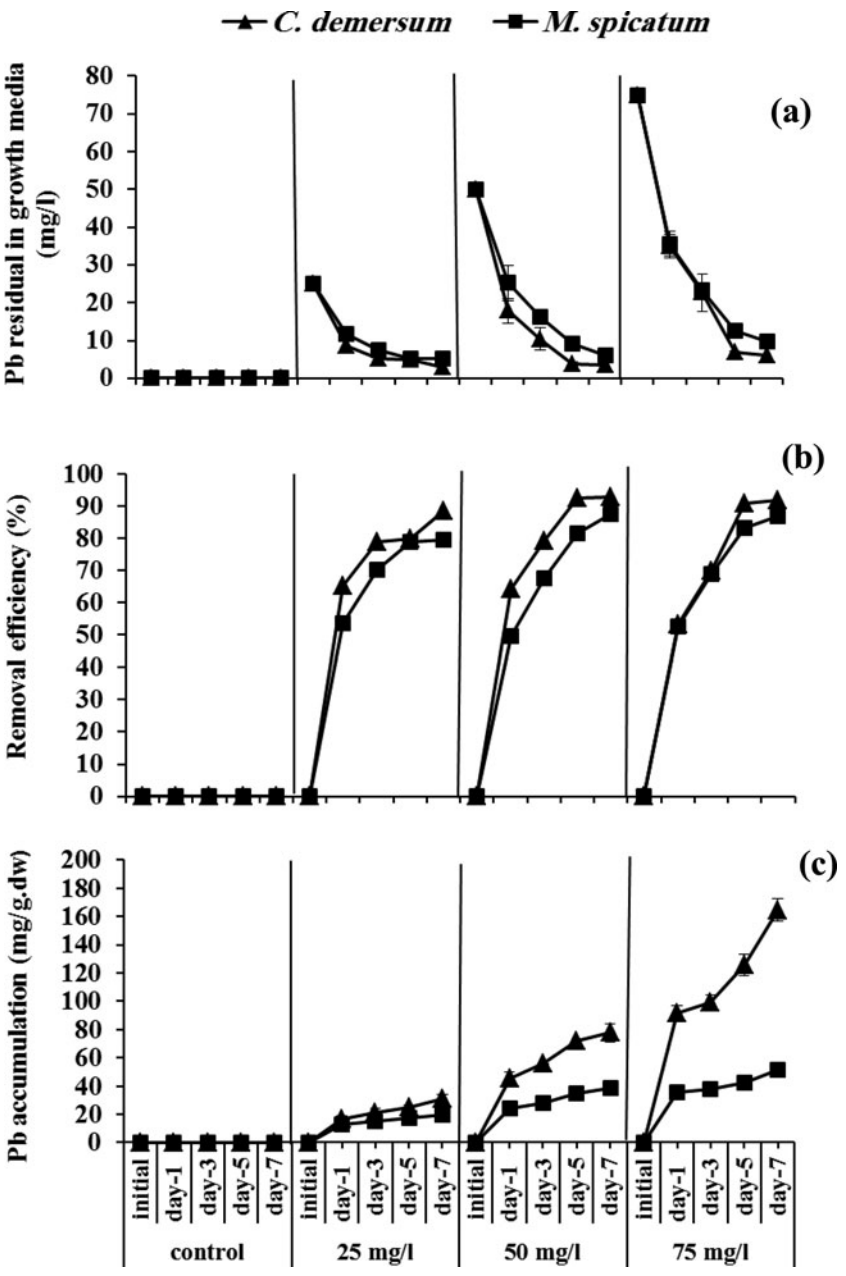

Figure 1 Lead residual in the growth media enriched by Pb (a), Removal efficiency of lead (b) and lead accumulation (c) by *c. demersum* and *M. spicatum* exposed to different Pb concentrations during the experiment duration.

day exposure, under the different Pb treatments. The highest accumulation value of Pb (164.26 mg/g.dw) was recorded in *C. demersum*. However the most of metal (91.72 mg/g dw) accumulated after 1 d. This may be attributed to occupation of the majority of the active sites by the captured Pb from the ambient media. In comparison, *C. demersum*

accumulated pb in quantity three folds higher than those of *M. spicatum*. The floating wide leaves and thin cuticle *of C. demersus* provide higher surface for Pb uptake, making it as a good Pb phytoremediator. Determinant coefficient (R^2) values imply the effects of exposure time and Pb concentration on both species accumulation capacities, where there is a linear relationship in between (Table 1). Spearman's coefficient values (r = –0.38 and – 0.51 for *C. demersum* and *M. spicatum*, respectively) confirm the significant correlation between amount of metal removed from growth media to the amount of metal accumulated by each one of the studied macrophytes. It was noticed that the trend to raise metal removal and accumulation is observed as metal concentration in solution increased in a time-dependent manner. Maryam (2011) reported high capability of *C. demersum* to remove trace elements directly from the contaminated water. The studied species reported by other authors (Mazej and Germ 2010; Manal *et al*. 2012) as Pb tolerant plants and can withstand the extreme conditions.

The results obtained indicate important role of both species in aquatic ecosystems, and confirm presumption that chemical analysis of test-species can give very important picture of ecological status of the investigated aquatic ecosystem. Their potential of remediation could be enhanced by combination with several different species of macrophytes to develop a cleaner, more economic and efficient way in removing pollutant from the environment.

Photosynthetic Pigments

Photosynthetic pigments (chlorophyll and carotenoids) are the central part of the photosynthetic system in green plants, and any significant alteration in their levels is likely to cause a marked effect on the entire metabolism of the plant (Mishra and Agrawal 2006). In the present study, chlorophylls exhibited similar trends of reduction in both species since the first day of exposure (Fig. 3a–c). In comparison to control, the exposed plants showed significant decreases ($P < 0.01$) in their chlorophylls with increasing Pb concentration and duration. This chlorophyll loss associated with the elevation of Pb concentration (Table 1) and might be the reason behind the observed little area of chlorosis in the studied species. Yan-Hua *et al*. (2008) reported that the elevated levels of most metals in plants will interfere with chlorophyll content and induce chlorosis.

On 7d exposure, both species exerted their minimum contents of chlorophylls (Fig. 2a,b). The interaction effects between concentration and species appeared to be highly significant ($P < 0.01$), where the minimum contents of chlorophylls in *C. demersum* (29.5% and 42.7% of control for *Chl* (*a*) and (*b*), respectively) were smaller than those in *M. spicatum* (43.04% and 46.07% of control for *Chl* (*a*) and (*b*), respectively). In this concern, many authors (Padmaja *et al*. 1990; Van Assche and Clijsters 1990; Prasad 1998; Sandalio *et al*. 2001) reported the induced role of Pb for inhibition of chlorophyll biosynthesis and structure, which leads to decline in the level of photosynthetic pigments. Based on the above mentioned interpretation and calculated values of determinant coefficients (Table 2) chlorophyll responses to Pb toxicity may possibly be use as biomarkers in a biomonitoring program. In this concern, chlorophyll a and chlorophyll b fluorescence were considered by many authors (Popovic *et al*. 2003, Bragato *et al*. 2006) as sensitive biomarkers when the plant cellular system was exposed to heavy metals.

With regard to carotenoids, at lower concentration and duration (Fig. 3c), their contents in the treated plants were higher than those of control by 165.11% in *C. demersum* and 173.11% in *M. spicatum*. Toppi and Gabbrielli (1999) attributed the increase in carotenoid level to the ability of plant to counteract the toxic effect of free radicals generated under

Table 1 Person's correlation matrix of the studied Parameters

Plant species	POD	APX	proline	carotenoids	Chl b	Chla	Pb residual	Pb accumulated	parameters
Myriophyllum spicatum	.644 (**)	.706 (**)	.826 (**)	−.371 (*)	−.834 (**)	−.918 (**)	−.512 (**)	1	*Pbaccumulated*
	−.520 (**)	−.580 (**)	−.629 (**)	−.259	.603 (**)	.434 (**)	1		*Pbresidual*
	−.489 (**)	−.584 (**)	−.768 (**)	.459 (**)	.827 (**)	1			*Chla*
	−.479 (**)	−.655 (**)	−.817 (**)	.224	1				Chl b
	.081	.023	−.165	1					Carotenoids
	.634 (**)	.838 (**)	1						Proline
	.816 (**)	1							APX
	1								POD
Ceratophyllum demersum								1	Pb accumulated
							1	−.387 (**)	Pb residual
						1	.760 (**)	−.621 (**)	Chl a
					1	.918 (**)	.754 (**)	−.671 (**)	Chl b
				1	.384 (**)	.538 (**)	.390 (**)	−.081	Carotenoids
			1	.166	−.458 (**)	−.363 (*)	−.520 (**)	.470 (**)	Proline
		1	.751 (**)	.215	−.470 (**)	−.294	−.465 (**)	.291	APX
	1	.468 (**)	.491 (**)	−.332 (*)	−.811 (**)	−.737 (**)	−.658 (**)	.581 (**)	POD

**Correlation is significant at the 0.01 level (2-tailed)., * Correlation is significant at the 0.05 level (2-tailed).

Table 2 Regression analysis of the studied parameters under the effects of exposure time (t) and Pb concentration (c).

	C. demersum				M. spicatum			
Parameters	R^2 (C) (%)	R^2 (t) (%)	Equation Model (c)	Equation Model (t)	R^2 (C) (%)	R^2 (t) (%)	Equation Model (c)	Equation Model (t)
Pb residual	16.1	50.4	$Y = -1.21 + 0.39 \times P < 0.01$	$Y = 36.3–5.56 \times P < 0.0001$	19.3	51.0	$Y = 0.77 + 0.4 \times P < 0.01$	$Y = 38.06–5.28 \times P < 0.0001$
Pb accumulation	43.4	33.4	$Y = -22.43 + 10.54 \times p < 0.0001$	$Y = 20.36 + 10.8 \times p < 0.0001$	27.5	45.8	$Y = 3.48 + 0.4 \times p < 0.0001$	$Y = 10.46 + 4.17 \times p < 0.0001$
Chlorophyll (a)	38.7	28.9	$Y = 17.33 - 1.08 \times p < 0.001$	$Y = 177.4 - 11.72 \times p < 0.001$	73.3	10.7	$Y = 350.5 -2.4 \times p < 0.0001$	$Y = 305.98 -11.5 \times p < 0.05$
Chlorophyll (b)	47.7	25.2	$Y = 139.39 - 0.087 \times p < 0.001$	$Y = 138.38 - 7.9 \times p < 0.001$	46.8	41.1	$Y = 220.86 - 1.4 \times p < 0.0001$	$Y = 13.37 + 2.9 \times p < 0.0001$
Carotenoids	19	18.4	$Y = 90.9 + 0.23 \times p < 0.01$	$Y = 61.23 - 2.88 \times p < 0.01$	30.5	2.0	$Y = 108 - 0.48 \times p < 0.0001$	$Y = 94.69 - 1.5 \times p < 0.05$
Proline	52.5	8.9	$Y = 0.7 + 0.025 \times p < 0.0001$	$Y = 2.18 - 0.13 \times, p < 0.05$	60.5	5.8	$Y = 0.8 + 0.02 \times p < 0.0001$	$Y = 1.29 + 0.08 \times p < 0.05$
APX	6.9	10.2	$Y = 357.07 + 1.95 \times p < 0.05$	$Y = 541.19 - 21.6 \times p < 0.05$	16.5	14.8	$Y = 972.68 + 7.45 \times p < 0.05$	$Y = 1604 - 64.3 \times P < 0.05$
POD	5.1	17.1	$Y = 265.5 + 1.25 \times P < 0.05$	$Y = 244.2 + 21.01 \times P < 0.05$	13.3	46.6	$Y = 824.9 + 4.45 \times P < 0.05$	$Y = 1352.29 - 76.1 \times P < 0.0001$
Prot in pattern	34	4.6	$Y = 60.3+0.71 \times P > 0.05$	$Y = 78.34–2.73 \times P > 0.05$	26.9	19.8	$Y = 45.1+0.36 \times P > 0.05$	$Y = 60.75–4.29 \times P > 0.05$

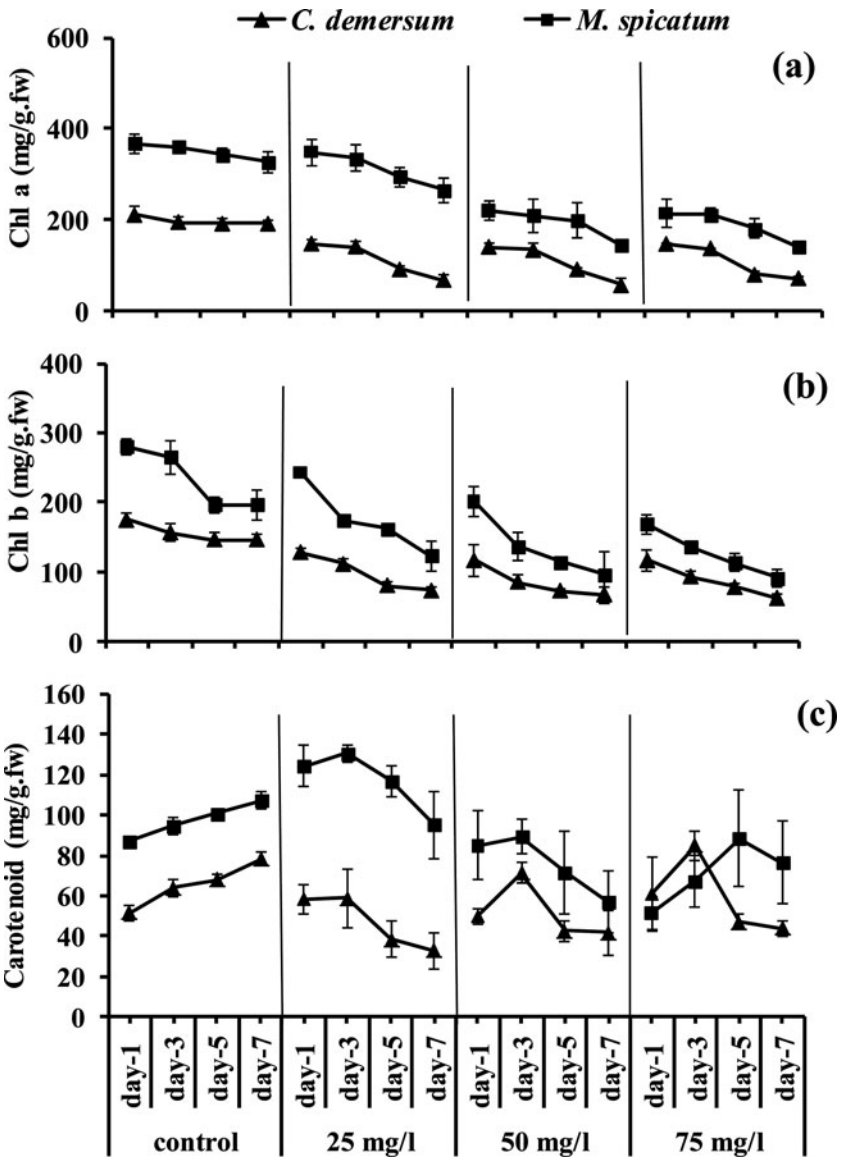

Figure 2 Photosynthetic pigments, chlorophyll a (a), chlorophyll b (b), and carotenoid (c) contents of *C. demersum* and *M. spicatum* growing in the growth media enriched by different concentrations of Pb during the experiment duration.

stress, where carotenoids serve as antioxidants to quench or scavenge the free radicals in chloroplast (Piotrowska *et al*. 2009). Unfortunately, the elevation of Pb concentration and prolonged time of exposure appeared to inhibit the carotenoids contents. In agreement with our results, Mishra *et al*. (2006) reported such decrease of carotenoids content in *C. demersem* growing under toxic metal stress. Dietz *et al*. (1999) stated that heavy metals cause a reduction for chlorophyll, which is accompanied by an increase in peroxidase activity and the depletion of other antioxidants, such as carotenoids, as in the present case. It is known

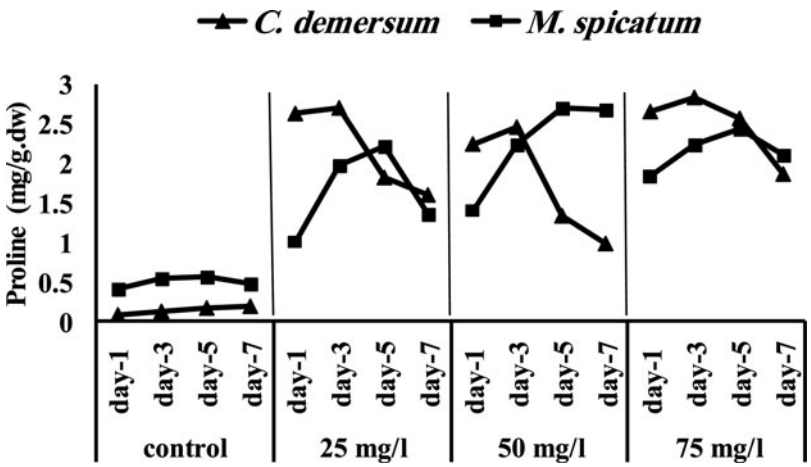

Figure 3 Proline contents in *C. demersum* and *M. spicatum* growing in the growth media enriched by different concentrations of Pb during the experiment duration.

that carotenoids protect photosystems from oxidative stress as they act as ROS scavengers. The imbalance between carotenoid production and carotenoid oxidation in case of intense metal stress (i.e. strong production of ROS) lead to a decrease in carotenoid content. It was noticed that exposure time has slightly determinant effect on carotenoids content of *M. spicatum* ($R^2 = 2\%$) than those of C. *demersum* ($R^2 = 18.4\%$). In contrast, elevation of Pb concentration appears to have more effect on the carotenoids content of *M. spicatum* than those of *C. demersum*, where the determinant coefficient values were 30.5% and 19%, respectively (Table 2).

Proline Content

Proline content of the treated species exhibited significant variations ($P < 0.01$) in comparison to control (Fig. 3). Three days exposure and 75 mg/l found to induce the maximum proline contents in *C. demersum* (∼15 folds of control), while five days and 50 mg/l stimulated *M. spicatum* to exhibit its proline maximum value (∼5.35 folds of control). This increase was transient followed by decrease to reach the minimum values 0.97 mg/g dw *(C. demersum*) at 50 mg/l and 1.35 mg/g dw (*M. spicatum)* at 25 mg/l, after7 d. Although the observed reduction, proline level is still higher in treated plants when compared to control.. Our results are in agreement with those reported by Wang *et al*. (2010) who stated the great accumulation of proline in *Paulownia fortunei* as an effective defense mechanism to Pb treatments. Also, with those of Delmail *et al*. (2011) who stated the accumulation of proline in *Myriophyllum alterniflorum* under Cd stress. The free proline generally accumulated in the cytosol, is involved in the cellular osmatic adjustment and acts as free-radical scavenger (Farago and Mullen 1979; Alia and Saradhi 1991; Kaul *et al*. 2008). Anyhow, at high level and long exposure time, Pb may act as either stimulation of proline degradation or inhibited its synthesis (Chen *et al*. 2001; 2004; Megateli *et al*. 2009; Azooz *et al*. 2011). Regression analysis of proline data (Table 2) exerted higher Pb sensitivity of *C. demersum* ($\beta = 0.025$) as compared to those of *M. spicatum* ($\beta = 0.020$). Hence, *M. spicatum* appears to be more tolerant than *C. demersum*, the finding that recommend the use of both species as bioindicators of Pb water pollution.

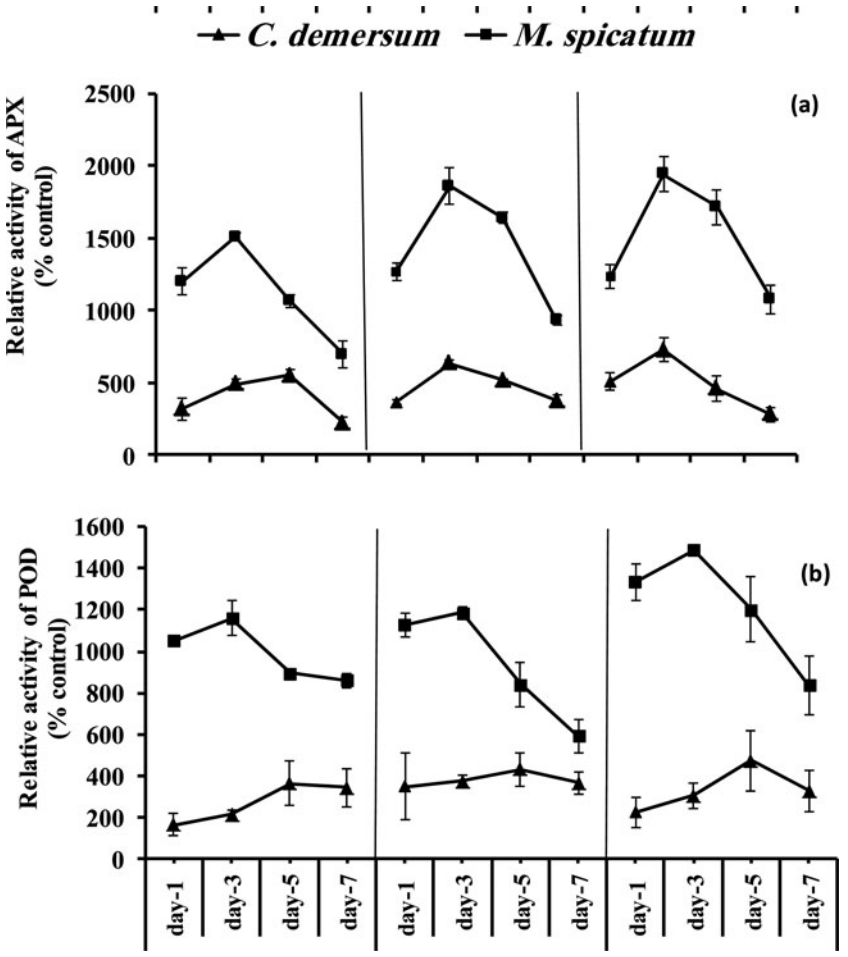

Figure 4 Changes in relative activities of antioxidant enzymes, APX (a), and POD (b) in *C. demersum* and *M. spicatum* growing in the growth media enriched by different concentrations of Pb during the experiment duration.

It is clear that Pearson's correlation coefficients data (Table 1) support our recommendation described immediately.

Antioxidant Enzymes Relative Activity

In the present study, with increase in exposure concentration, the induction maxima were limited to short durations only followed by decline. In the treatments, the maximum activity of APX was observed after 3 d at 75 mg/l, which was higher (729.7% in *C. demersum* and 1943.03 in *M. spicatum*) than control. After 5 d, significant decrease in activity was observed at all concentration in comparison to control. With longer duration (7 d), APX minimum activity (220.6%) was noticed when plants of *C. demersum* exposed to 25 mg/l (Fig. 4a). With regard to POD, the studied species showed different trends. At 75 mg/l, POD in *C. demersum* exhibited its maximum activity (474.5%) after 5 d and its minimum one (327.5%) after 7 d. Meanwhile, in *M. spicatum,* the maximum value

(1490.1%) was observed at 75 mg/l after 3 d and its minimum value (592.56%) at 50 mg/l, after 7 d (Fig. 4b). Wang *et al*. (2010) reported similar finding when they investigated physiological responses and detoxific mechanisms to Pb in young seedling of *Paulownia fortune*. In addition, Sharma and Dietz (2009) reported that antioxidant enzyme activities in metal-stressed plants are highly variable, depending on the plant species, metal ion, concentration and exposure duration.

As reported by many authors (Groppa *et al*. 2007; Khatun *et al*. 2008; Singh *et al*. 2010; Wang *et al*. 2011), plants possess defensive system that constitutes various antioxidant enzymes to combat increased production of ROS caused by heavy metals.-Therefore, we considered the increase of POD and APX activities as an adopted strategy whereby studied species can overcome the toxicity resulting from lead toxicity. The current results showed that prolonged exposure and increase concentration reduce the antioxidant enzymes activities. This decrease can be attributed to the over-production of ROS that cause the enzyme inactivation (Verma and Dubey 2003; Dazy *et al*. 2008) and consequently increase ROS accumulation. The observed associated changes in the levels of proline, carotenoids and relative activities of antioxidant enzymes with increasing Pb concentration (Table 1) can be an indicator of a correlation between ROS generation and ROS scavenging by antioxidant enzymes. Sandalio *et al*. (2001) stated that the accumulation of these reactive oxygen species cause a severe damage to thylakoid membranes. Based on this, we could explain the recorded reduction in chlorophylls. Data of table (2) explain the sensitivity of antioxidant enzymes activities in the studied species to Pb toxicity. This is of prime importance when their use as reliable biomarkers for Pb water pollution is considered. Earlier studies by many authors stated the use of these enzymes as biomarker for herbicides in *Hydrilla verticillata* (Byl *et al*. 1994) for aromatic hydrocarbons in *Eichhornia crassipes* (Roy and Hanninen 1993) as well as for heavy metals in *Myriophyllum quitense* (Nimptsch *et al*. 2005)

Protein Patterns

In comparison to control, the treated plants exhibited protein patterns of their own. It was found that Pb-exposure affected the synthesis and/or disappearance of a considerable number of proteins in the studied species (Fig. 5a-b). The density and number of the developed bands in the studied species protein profiles appeared to decrease with the increase of Pb exposure concentration and duration. Induction in protein content at shorter duration is possible due to induction of stress proteins (Srivastava *et al*. 2005) under metal exposure. These stress proteins may constitute various antioxidant enzymes and other enzymes involved in GSH and PC biosynthesis and also some heat shock proteins. Reduction in protein content at higher concentration of Pb and Cd has been reported earlier in lupine roots and *C*. *demersum*, respectively (Pryzmusinski *et al*. 1991; Arvind and Prasad 2005). This reduction may be due to degradation by proteases. Pb induced ROS can directly affect proteins by oxidation of amino acid side chains and cause oxidation of proteins, where oxidized proteins can be degraded more effectively by proteases.

Protein synthesis in *C*. *demersum* appears to be more sensitive to Pb exposure than in *M*. *spicatum*, where R^2 reached 34% and 26.9% for *C*. *demersum* and *M*. *spicatum*, respectively (Table 2).The disappearance of some proteins (13 and 23 kDa in *C*. *demersum* and 108 kDa in *M*. *spicatum*) and the *de novo* synthesis of others (138, 118, 58, 39, 32, 30, 16, and 15 kDa in *C*. *demersum* and 47, 43, 23,12, 200, 181, and 164 kDa in *M*. *spicatum*). This indicates that Pb pollution is highly effective in causing a major re-shuffle

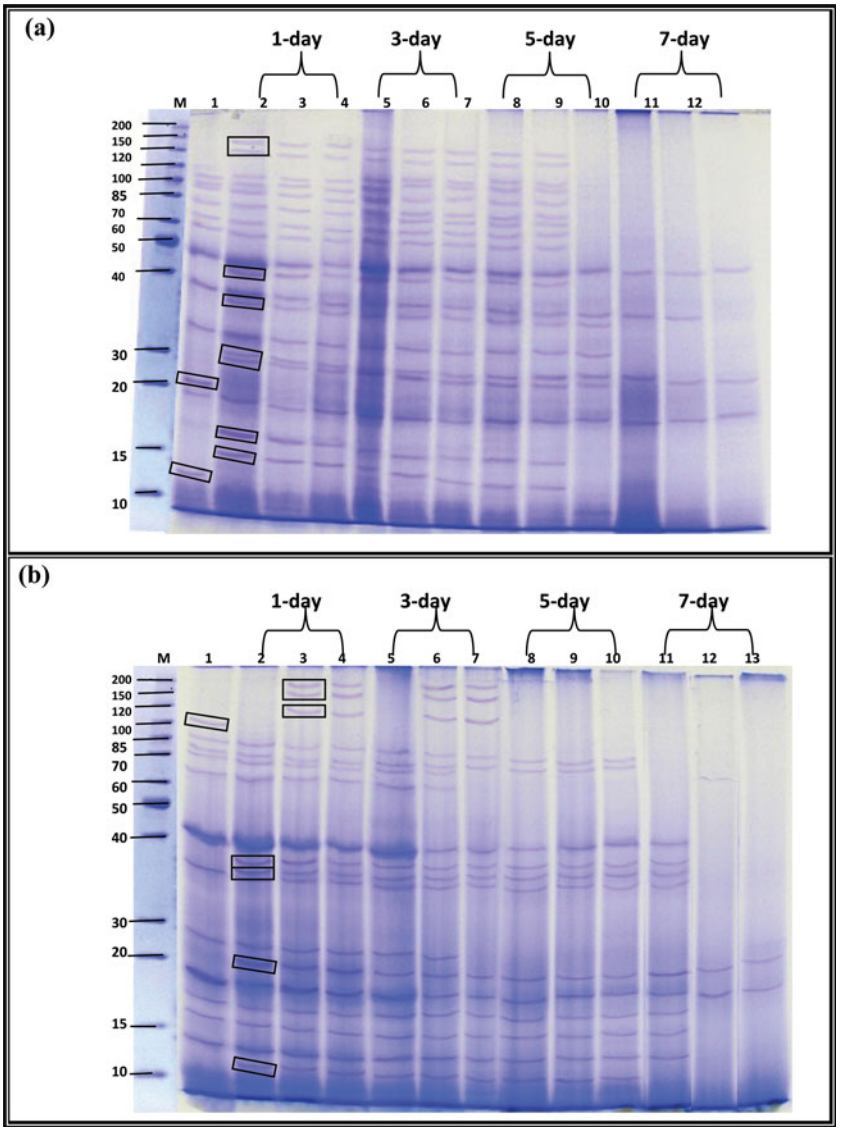

Figure 5 Protein patterns of *C. demersum* a) and *M. spicatum* b) growing in the growth media enriched by different concentrations of Pb during the experiment duration. M = markers, Lane 1: control. Lanes 2, 5, 8, and 11 represent protein pattern of 25 mg/l Pb treatment. Lanes 3, 6, 9, and12 represent protein pattern of 50 mg/l Pb treatment. Lanes 4, 7, 10, and13 represent protein pattern of 75 mg/l Pb treatment, respectively. The boxes indicate appearance of new bands. (Color figure available online).

of protein patterns in the studied species, which appear to us as a tolerant mechanism against the excessive Pb concentration. The majority of the synthesized proteins are reported as metal-binding proteins that act as heavy metal chelates to cope the toxicity resulted from oxidative stress due to heavy metal pollution. On the other side, the disappeared proteins are mostly belonging to the ribosomal protein families, which services as one of the primary rRNA binding proteins and they also related to NDH shuttling electrons from NAD(P)H:

plastoquinone, via iron-sulfur (Fe-S) centers, to quinones in the photosynthetic chain and possibly in a chloroplast respiratory chain (UniProtKB/Swiss-Prot database (2011). Their disappearance in protein profiles may explain the observed reduction in chlorophylls and the appearance of morphological symptoms include chlorosis and leaves failing of the studied species. Rebechini and Hanzely (1974) stated that, *C. demersum* plants growing in aquatic medium containing Pb $(NO_3)_2$ showed distinct changes in chloroplast fine structure.

Biomonitoring Prospective

The use of aquatic plants in water quality assessment has been in practice for years as in-situ biomonitors and bioremediators (Boyod 1974; Kamal *et al*. 2004). In the present study, plants accumulated high amount of Pb and thus showed potential to be used as phytotoremediator species in aquatic bodies having moderate pollution of Pb. This is of prime importance, especially when the in situ ecological survey by both species is considered. Since their immobile nature makes them effective bioindicators of Pb water pollution, as they represent real level of metals present at that site and consequently smooth using in different technologies of the phytoremediation. From the biomarker point of view, the recorded physiological and morphological changes in the studied species can measure their exposure to the elevated levels of Pb. Such biological responses may be useful in the monitoring of Pb pollution in aquatic ecosystems. Such a biological approach makes it possible to determine whether the natural ecosystem is being altered by pollutants without relying on expensive techniques and conducting long term field experiments.

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