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Effects of Lead on Chlorophyll Content, Total Nitrogen, and Antioxidant Enzyme Activities in Duckweed (*Lemna minor*)

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Abstract

The aim of this study was to investigate the effects of lead on the biochemical and physiological processes in duckweed (*Lemna minor* L.), a free-floating aquatic macrophyte known for its phytoremediating properties. Duckweed was treated with 1, 10 and 100 mg L^{-1} of lead for five days and Pb effects were compared to control. Lead accumulation in duckweed fronds increased with metal concentrations; at 1, 10 and 100 mg L^{-1} it was 6.5, 42.6 and 136.3 times greater than in control plants, respectively. In turn, its bioconcentration factor decreased as the applied Pb concentrations increased. Lead exposure decreased the level of chlorophylls and total nitrogen in a dose-dependent manner. Activity of antioxidant enzymes was exchanged by Pb supply in *L. minor* fronds. These results showed that ascorbate peroxidase and glutathione reductase were significantly correlated with the applied Pb concentration, while catalase showed a negative correlation. Therefore, it can be concluded that Pb-toxicity provoked oxidative stress, which is confirmed by altered enzyme activities.

Key Words: *Lemna minor*; Lead toxicity; Oxidative stress; Antioxidant enzymes

Introductıon

Heavy metals are natural elements that found at various concentrations at diffrent places of the world. Heavy metals are persistent and can not be deleted from environment. Thus, a problem arises when their availability is high due to high background levels or human activities (Greger, 2004). Accumulation of heavy metals in plants may cause physiological and biochemical changes. Heavy metals may impair the amount of chlorophyll in plants through directly or indirectly inhibiting chlorophyll synthesis (Van Assche and Clijsters, 1990). Heavy metals may also cause deficiency of essential elements and even change the concentrations of basic nutrients such as nitrogen and phosphorus in plant tissues (Siedlecka, 1995).

Lead (Pb) is a hazardous heavy metal and known environmental pollutant. Much of the contamination with Pb stems from human activities such as mining and ore smelting, coal burning, automobile exhausts, and industrial production (Eick *et al.,* 1999). Pb impairs biological systems by inactivating several enzymes, especially by interacting with their SH-groups (Rauser, 1995). In addition, Pb ions can stimulate the production of reactive oxygen species (ROS) (Prasad *et al.,* 1999). Oxidative stress can impair cell structure and metabolism. An imbalance between the production of toxic reactive intermediates and the ability of the cell to detoxify them eventually lowers oxidation-reduction enzyme activity (Stroiński and Kozłowska, 1997; Foyer and Shigeoka, 2011). Plants effectively counteract the oxidative damage using a defence system that consists of antioxidant enzymes catalase (EC 1.11.1.6), peroxidases (EC 1.11.1.7), and superoxide dismutases (EC 1.15.1.1). In addition, several non-enzymatic compounds such as α-tocopherol, ascorbate, and reduced glutathione help to eliminate, deactivate, and scavenge the ROS (Foyer *et al.,* 1997; Shah *et al.,* 2001). Ascorbate peroxidase (EC 1.11.1.11), monodehydro ascorbate reductase (MDAR, EC 1.6.5.4), dedydroascorbate-reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2) also play a significant role in scavenging H_2O_2, mainly in chloroplasts. Their interactions are important to maintain the redox environment in the cell and proper glutathione (GSH) to glutathione disulphide (GSSG) concentration ratio in cells (Asada, 1994).

Duckweed (*Lemna minor* L.) is convenient for ecotoxicological investigations, both in the laboratory and field *in situ* studies. It has also been used to remove potentially toxic heavy metals from contaminated lakes, ponds and wastewaters. The results of previous studies have shown that duckweed can bio-concentrate different heavy metals and trace elements (Jain *et al.,* 1988; Khellaf and Zerdaoui, 2009).

The aim of this study was to estimate how the exposure of duckweed to different concentrations of Pb affects the levels of photosynthetic pigments, total nitrogen and antioxidant enzyme activities.

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Materials and Methods

Test organism used in this study was duckweed (*Lemna minor* L.), which was collected from the local streams of the Cukurova area around the town of Adana, Turkey, where it is abundantly found. The plants were acclimatized in a 10% Arnon and Hoagland nutrient solution (Arnon and Hoagland, 1940) at 25-27°C and a 16-h light (120 µmol m^{-2} s^{-1}) and 8-h dark cycle. Three groups of samples were exposed to Pb in the form of lead (II) acetate [$Pb(CH_3COO)_2.3H_2O$ (Merck, KGaA, Darmstadt Germany]. Lead concentrations in the polluted waters are in the range of 1-100 mg L^{-1}. These concentrations are, however, frequently reduced during treatment, prior to discharge to the receiving waters (Harrison and Laxen, 1981). For this reason, Pb acetate was dissolved in 500 mL of nutrient solution (adjusted to pH 7.0) in beakers (V=1000 mL) to achieve the concentrations of 1, 10, and 100 mg L^{-1}. The treatment lasted for five days. The nutrient solutions supplemented with Pb (II) acetate were replaced with freshly prepared solutions every 48 h. Duckweed kept in nutrient solution without Pb served as negative control. After five days of exposure, the duckweed samples were washed three times with distilled water and immediately frozen in liquid nitrogen and stored at -80°C for enzyme activity. All chemicals used in the experiment were of the analytical grade.

To extract photosynthetic pigment from duckweed fronds, 0.1 g of fresh fronds was homogenized with 10 mL of 100% acetone in a porcelain mortar and filtered mixture through Whatman No. 1 filter paper. The absorbance of extracts was measured spectrophotometricaly (Shimadzu UV/VIS 1240, Kyoto, Japan) at 662 nm and 645 nm and the corresponding level of chlorophyll a and chlorophyll b calculated using formula of Lichtenthaler and Wellburn (1985).

We prepared fresh leaf samples in the amount of 0.5 g according to procedure of Cakmak *et al.* (1993). Briefly, the samples were homogenised in a mixture of 8 mL of 50 mmol L^{-1} phosphate buffer (pH 7.6) and 0.1 mmol L^{-1} Na-EDTA and centrifuged at 15000 g and 4 C for 15 min. Catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (APX) activity were measured as described by (24). The CAT assay was based on the degradation of H_2O_2 at 240 nm (ε=39.4 mmol L^{-1} cm^{-1}), GR assay on the decrease in absorbance at 340 nm due to NADPH oxidation (ε=6.2 mmol L^{-1} cm^{-1}), and APX on the rate of H_2O_2-dependent oxidation of ascorbate at 290 nm (ε=2.8 mmol L^{-1} cm^{-1}). We made corrections for NADPH oxidation in the absence of oxidized glutathione (GSSG).

Harvested fronds were washed three times with distilled water and weighed. Four replicates for each treated and the control group were dried in an oven at 80°C to a constant weight and pulverised in a mortar with a pestle. The samples were then dissolved in a mixture of 10 mol L^{-1} HNO_3 and 10 mol L^{-1} HCl. After mineralisation, Pb was determined using an atomic absorption spectrometer (Perkin Elmer Model 3100, Norwalk, CT, USA). The bioconcentration factor (BCF) was calculated as the ratio between the measured Pb in duckweed and the applied Pb concentration (Zayed *et al.,* 1998). To determine total nitrogen (N), we used ammonia distillation following the Micro-Kjeldahl method (Kacar, 1972).

Lead, total N and chlorophyll levels in duckweed fronds are the means of four replicate measurements. The results were analysed with the analysis of variance (ANOVA) using SPSS 11.0 for Windows®. The significance of differences were determined with the least significant difference (LSD) test. Pearson's correlation was analysed between Pb concentration and other parameters.

Results and Discussion

We found that Pb accumulation in duckweed fronds increased with increasing metal concentration (Table I). Control plants had a low Pb content that reflected its uptake from the water and atmosphere. The concentration of Pb in duckweed exposed to 1 mg L^{-1} was 6.5 times greater than in control ($p>0.05$), 10 mg L^{-1} 42.6 times ($p<0.05$) and 100 mg L^{-1} 136.3 times ($p<0.05$). Metal concentrations in plant tissue are generally a function of the metal concentration in the growth solution. Duckweed as a free-floating macrophyte floats on water surface and is not attached to the sediment. For this reason, it takes up metals directly from water (Saygideger and Dogan, 2004; Drost *et al.,* 2007). The bioconcentration factor (BCF) measures the potential of a plant to accumulate a pollutant (Zhu *et al.,* 1999). In general, when metal concentration in water increases, the amount of metal accumulation in plant increases, whereas the BCF values decrease (Saygideger and Dogan, 2004; Dogan *et al.,* 2009). In our study, the BCF of duckweed dropped as Pb concentrations rose (Table I). In plants treated with 1 mg of Pb per litre, the maximum BCF was 23.3, while the minimum BCF of 4.9 was calculated at 100 mg of Pb per litre. Our results showed that duckweed has a high potential to accumulate Pb. Accumulation of Pb in duckweed was also investigated in a study by Zayed *et al.* (1998), who tested several metals in the range 0.1 mg L^{-1} to 10 mg L^{-1}. They also observed that duckweed accumulated Pb, but to a lesser extent than cadmium, copper, selenium, and chromium. Recent, Kaur *et al.* (2010) tested the accumulation of Pb in the concentration range of 1 mg L^{-1} to 20 mg L^{-1} and found that duckweed can effectively accumulated Pb, which depended on exposure time, pH, and the concentration of the Pb in solution.

In the present study, chl a, chl b, and chl a+b were negatively correlated with Pb accumulation by duckweed fronds (r=-0.707, $p<0.05$; r=-0.731, $p<0.05$; and r=-0.762; $p<0.01$, respectively; Table II). This decrease in chlorophyll content has been attributed to the inhibition of chlorophyll synthesis by Pb (Saygideger and Dogan, 2004; Saygideger *et al.,* 2004).

As an earlier study, Pb lowered the N content in *T.*

Table 1: Pb concentrations and bioconcentration factors (BCFs) in unexposed (control) and Pb-exposed duckweed fronds

Pb exposure levels (mg L^{-1})	Pb concentrations measured in duckweed fronds (µg g^{-1} dry weight)	BCF
Control	3.6±0.1 ^{a}	-
1	23.3±4.2 ^{a}	23.3±4.2 ^{a}
10	153.3±15.0 ^{b}	15.3±1.5 ^{b}
100	490.5±49.5 ^{c}	4.9±0.5 ^{c}

Values represent the mean of four replicate measurements ± standard deviation. Different superscript letters denote significant difference in respective values according to the LSD test ($p<0.05$)

Table 2: Chlorophyll (Chl) content (mg g^{-1} fresh weight) in control and Pb-exposed duckweed fronds

Pb exposure levels (mg L^{-1})	Chl a	Chl b	Chl a+b
Control	2.23±0.23 ^{a}	0.82±0.10 ^{a}	3.05±0.33 ^{a}
1	2.16±0.14 ^{ab}	0.82±0.16 ^{a}	2.98±0.26 ^{ab}
10	2.06±0.43 ^{ab}	0.77±0.06 ^{ab}	2.83±0.44 ^{ab}
100	1.71±0.11 ^{b}	0.62±0.02 ^{b}	2.33±0.13 ^{b}

Values represent the mean of four replicate measurements ± standard deviation. Different letters in the same column are significantly different from one another according to the LSD test ($p<0.05$)

latifolia and *Ceratophyllum demersum* (Saygideger *et al.*, 2004), now it lowered total N content in duckweed fronds in a dose-dependent manner (Fig. 1). This reduction was insignificant at Pb concentrations of 1 mg L^{-1} and 10 mg L^{-1} ($p>0.05$). The lowest total N content was found in the fronds exposed to Pb concentration of 100 mg L^{-1} ($p<0.05$) (16.6 % lower than in control). Accumulated Pb and total N content showed a negative correlation (r=-0.568; $p>0.05$). Several authors established a close relationship between chlorophyll and N content (Field and Mooney, 1986; Amaliotis *et al.*, 2004). Nitrogen is a structural element of chlorophyll and protein molecules, and its deficiency affects the formation of chloroplasts and accumulation of chlorophyll in leaf (Tucker, 2004).

Plants have an effective enzymatic defense system against oxidative stress that includes APX and GR, which are involved in scavenging H_2O_2 (produced mainly in the chloroplasts and other cell organelles) and in maintaining the redox state of the cell (Asada, 1982). In this study, APX activity showed an increase with applied Pb concentration ($p<0.05$) and a strong correlation with Pb accumulation (r=0.834; $p<0.01$). GR activity also increased with Pb concentration; at 100 mg L^{-1} it was 4.7 times greater than in control ($p<0.05$). Again the correlation between GR activity and Pb accumulation was strong (r=0.977; $p<0.01$). According to Verma and Dubey (2003), GR plays a pivotal role in countering Pb-induced oxidative injury in rice plants, while rice seedlings also showed increased activities of APX and GR. Mishra *et al.* (2006) also reported that in Pb-treated *C. demersum* and watercress, and in our earlier study of *Nasturtium officinale* stems (Keser and Saygideger, 2010).

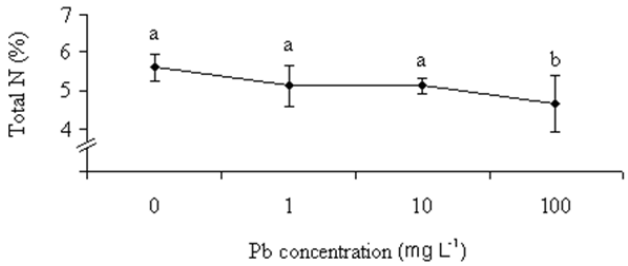
We also found that CAT activity significantly decreased over control as the Pb concentrations increased (Table III) ($p<0.05$). The negative correlation between CAT activity and

Table 3: Activity of antioxidant enzymes in control and Pb-exposed duckweed fronds

Pb exposure levels (mg L^{-1})	APX (µmol g^{-1} min^{-1} fw)	GR (nmol g^{-1} min^{-1} FW)	CAT (µmol g^{-1} min^{-1} FW)
Control	34.2±3.4 ^{a}	0.61±0.03 ^{a}	35.4±4.6 ^{a}
1	49.1±6.8 ^{b}	0.67±0.12 ^{a}	28.9±3.5 ^{bc}
10	83.2±6.4 ^{c}	1.56±0.20 ^{b}	26.1±2.6 ^{c}
100	91.5±8.1 ^{c}	2.84±0.12 ^{c}	15.4±2.3 ^{d}

FW - fresh weight. Values represent the mean of four replicate measurements ± standard deviation. Different superscript letters denote significant difference in respective values according to the LSD test ($p<0.05$)

Fig. 1: Total nitrogen (N) content in control and Pb-exposed duckweed fronds. Different letters denote significant differences according to the LSD test ($p<0.05$)



Pb accumulation in the fronds was also strong (r=-0.855; $p<0.01$). A decrease in CAT activity was reported by Somashekaraiah *et al.* (1992) in the seedlings of *Phaseolus vulgaris* treated with Cd for 3 to 6 days, and by us in the stem of *N. officinale* treated with Pb for 14 days (Keser and Saygideger, 2010). The decline observed at higher Pb concentrations might be attributed to the inactivation of the enzyme by ROS, decrease in its synthesis or change in the assembly of its subunits (Verma and Dubey, 2003).

Conclusions

In conclusion, Pb uptake by duckweed and its toxic effects are concentration-dependent. Induction of APX and GR or reduction of CAT activities confirmed that Pb produces oxidative stress. Increases in the activity of APX and GR may serve as early stress indicators of Pb exposure. In addition, duckweed can be utilized as a good phytoaccumulator of Pb, when taking into consideration Pb contents in duckweed fronds.

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