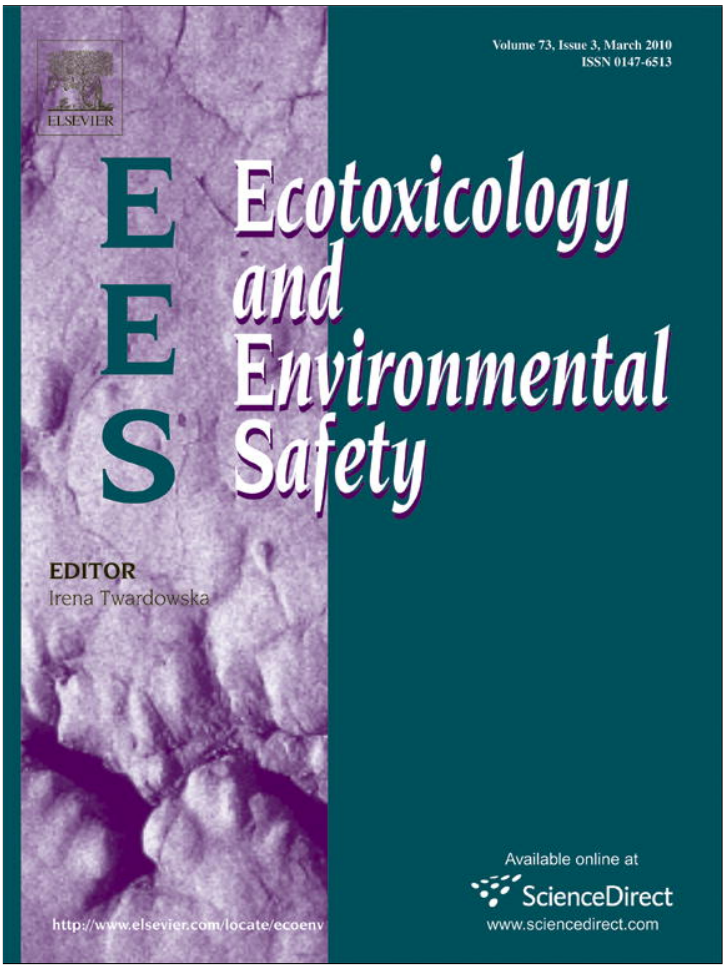

Ecotoxicology and Environmental Safety 73 (2010) 336–342

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Ecotoxicological effects of aluminum and zinc on growth and antioxidants in *Lemna minor* L.

Sandra Radić [a,*], Marija Babić [a], Dragan Škobić [b], Vibor Roje [c], Branka Pevalek-Kozlina [a]

[a] Department of Botany, Faculty of Science, University of Zagreb, Rooseveltov trg 6, HR-10000 Zagreb, Croatia
[b] Department of Biology and Chemistry, Faculty of Education, University of Mostar, Matice hrvatske bb., 88 000, Mostar, Bosnia and Herzegovina
[c] Division for Marine and Environmental Research, Laboratory for Physical Chemistry of Traces, Rudjer Boskovic Institute, P.O. Box 180, Bijenicka 54, HR-10002 Zagreb, Croatia

ARTICLE INFO

Article history:
Received 14 April 2009
Received in revised form
20 October 2009
Accepted 25 October 2009
Available online 14 November 2009

Keywords:
Duckweed
Metal toxicity
Oxidative stress
Antioxidative enzymes
Lipid peroxidation
Hydrogen peroxide

ABSTRACT

The present study aimed at investigating effects of zinc and aluminum (0.15 and 0.3 mM) in duckweed (*Lemna minor* L.) over a 15-day period. High bioaccumulation of both metals was accompanied by an increase in dry weight under higher metal treatments. Antioxidant response was observed under both metal stresses, with large increases in superoxide dismutase and peroxidases. Catalase activity declined only in duckweed exposed to Zn while lipid peroxidation as well as H_2O_2, proline and ascorbate levels increased. The results suggest induction of oxidative stress under both aluminum and zinc toxicity, and also demonstrate duckweed's capacity to upregulate its antioxidative defense. Additionally, Zn was found to be more toxic than Al to duckweed for the concentrations applied. Due to its high bioaccumulation potential and tolerance via increased antioxidant capacity, duckweed has a potential for phytoremediation of water bodies polluted by low levels of zinc and aluminum.

1. Introduction

Metals have received particular attention among non-degradable toxic chemicals because of their adverse effects on aquatic life forms. Accumulation of metals and their toxic effects throughout the food chain can lead to serious ecological and health problems (Malik, 2004). Zinc and aluminum are released into the environment in significant amounts by both natural processes and anthropogenic activities (Gensemer and Playle, 1999; Shikazono et al., 2008). The main source of these metals in waterways of Croatia and Bosnia and Herzegovina originates from mining and mining-related activities (zinc and aluminum smelters) and various industrial processes such as domestic appliances industry and phosphate fertilizers plants (Englar, 2007). Although some heavy metals like zinc are required as micronutrients in biological systems, all are toxic at higher levels. Zn acts as a constituent or a cofactor for several enzymes; it is involved in protein synthesis, as well as carbohydrate, nucleic acid and lipid metabolism. It forms complexes with DNA and RNA and affects their stabilities (Rout and Das, 2003). Aluminum, in contrast, is a nonessential element for metabolic processes (Fodor, 2002). So far, Al has been shown to interfere with uptake and transport of some essential nutrients as well as with cell division in roots, to increase cell wall rigidity (cross-linking pectins), to alter plasma membrane, and to alter activities of many enzymes and metabolic pathway involved in repair mechanisms (Rout et al., 2001). A significant correlation between low pH and high aluminum concentration has been reported in fresh water, where this metal may reach levels of 0.3–1.6 mM (Dickson, 1978) and cause serious metabolic disruption in food chain (Pettersson et al., 1985; Gensemer and Playle, 1999).

Along with free aluminum (Al^{3+}), free zinc (Zn^{2+}) is found to be one of the most common phytotoxic elements under acidic conditions (Stephan et al., 2008). Zinc contamination in freshwater bodies has been reported to exceed the environmental limit by up to 100 times (Srikanth et al., 1993; Pistelok and Galas, 1999; Shikazono et al., 2008).

Stunted growth, chlorosis and necrosis are some of the visible symptoms indicating severe metal phytotoxicity. The phytotoxicity of metals arises partly from the generation of reactive oxygen species (ROS), which cause direct damage to lipids, proteins and DNA. ROS mainly originate from the dissipation of electrons across chloroplastic and mitochondrial membranes. Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and peroxidases (APX, EC 1.11.1.11; POX, EC 1.11.1.7), together with low-molecular mass scavengers such as ascorbate, glutathione and proline, act as the main defense against ROS produced in various parts of plant cells (Apel and Hirt, 2004). At the cellular level, enzymatic activity is altered, resulting

* Corresponding author. Fax: +385 14826260.
E-mail addresses: sandra@zg.biol.pmf.hr (S. Radić), mbabic@zg.biol.pmf.hr (M. Babić), dragan.skobic@gmail.com (D. Škobić), vroje@irb.hr (V. Roje), bpevalek@zg.biol.pmf.hr (B. Pevalek-Kozlina).

0147-6513/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2009.10.014

in enzyme activation or inhibition (Dazy et al., 2009). Antioxidant enzyme induction has been proposed to play an important metabolic role under conditions of metal stress (Van Assche and Clijsters, 1990). Although both aluminum and zinc are not transition metals and cannot catalyze redox reactions, they can result in oxidative damage to major biomolecules (DNA, lipids, proteins) as well as induction of antioxidative defense mechanisms (Weckx and Clijsters, 1997; Exley, 2003; Lin et al., 2005). Several studies have proposed that the pro-oxidant activity of aluminum might be explained by the formation of an Al superoxide semireduced radical cation (Exley, 2003; Khan et al., 2006). Involvement of the mitochondrial electron transport chain has been inferred in both Zn- and Al-induced ROS generation (Yamamoto et al., 2002; Chang et al., 2005).

Unlike organic pollutants, heavy metals are not degraded via biological processes. Therefore, they require removal for water decontamination (Cheng et al., 2002). Common duckweed (*Lemna minor* L.) is a cosmopolitan monocot found year-round in fresh and brackish water ecosystems. Small size, high multiplication rate and vegetative propagation by which a population of genetically homogeneous plants is produced make duckweed species ideal organisms for toxicity testing (Wang, 1990). Due to tremendous metal accumulation capacity and ease of harvest *L. minor* is also considered as a suitable candidate for phytoremediation of certain heavy metal-contaminated water bodies (Jain et al., 1990; Axtell et al., 2003; Kanoun-Boulé et al., 2009). The ability of duckweed to both tolerate and accumulate copper and cadmium has been associated with induction of several antioxidative enzymes (Teisseire and Guy, 2000; Hou et al., 2007; Kanoun-Boulé et al., 2009). However, to date scarce information is available on the involvement of oxidative stress under zinc and, in particular, aluminum toxicity in duckweed or other freshwater macrophytes (Severi, 1997; Artetxe et al., 2002). Hence, the aim of the present study was to assess the roles of major antioxidative enzymes and some non-enzymatic antioxidant molecules (ascorbate, proline and carotenoids) as protection from oxidative stress produced by accumulation of the tested heavy metals. Additionally, H_2O_2 and malondialdehyde (MDA) contents were investigated as indicators of oxidative stress and lipid peroxidation.

2. Materials and methods

2.1. Plant material and culture

L. minor L. was chosen as the object of this study as it is convenient for experimental handling and cost effective to maintain. The plant material was collected from the Botanical Garden, Faculty of Science, University of Zagreb. Collected plants were sterilized and maintained as stock cultures in Pirson–Seidel nutrient medium for over a year (Pirson and Seidel, 1950); pH value of the nutrient medium was adjusted to 4.55 with 0.1 M KOH. The medium was autoclaved at 118 kPa and 120 °C for 20 min.

Individual healthy colonies with 2–3 fronds (growth experiments) or 5–10 healthy colonies with 2–3 fronds (antioxidant analysis) from stock cultures were transferred to Erlenmeyer flasks containing Pirson–Seidel nutrient medium, and each was supplemented with $ZnSO_4 \cdot 7H_2O$ (Sigma Aldrich) or $Al_2(SO_4)_3 \cdot 16H_2O$ (Sigma Aldrich) at two concentrations (0.15 or 0.3 mM), except in the case of control. The culture flasks were manually shaken once a day. The cultures were placed in a growth chamber at 24 ± 2 °C, under illumination provided by cool fluorescent light with a light intensity of 80 μmol m^{-2} s^{-1} and a light:dark cycle of 16:8 h.

2.2. Analytical methods

Frond numbers were monitored over a 15-day period on days 0, 3, 5, 8, 10, 12 and 15 (*n*). Relative frond number (RFN) was calculated using the equation: RFN=(frond no. at day *n*–frond no. at day 0)/frond no. at day 0. Relative growth rate (RGR) was based on the increase in fresh weight (FW) determined after 15 days of experimentation and calculated using the following formula: RGR=[ln(final weight)–ln(initial weight)]/weeks.

Two-week old duckweed plants were weighed and oven dried at 80 °C for 30 h, at which time constant dry weights (DWs) were obtained. The Al and Zn contents of all samples were determined after a 15-day period by graphite furnace atomic absorption spectrophotometry (Perkin Elmer AA 600) after microwave wet digestion (Anton Paar Multiwave 3000) of the dried and powdered material in 10 ml of supra-pure concentrated HNO_3 at 230 °C. Al and Zn contents are expressed as μg g^{-1} DW.

Photosynthetic pigments were measured according to the method described by Arnon (1949). In short, 30 mg fresh samples were homogenized with 80% (w/v) cold acetone, centrifuged at 5000*g* for 10 min and absorbances of the supernatant at 663, 646 and 470 nm read. Chlorophyll *a*, *b* and total carotenoid content were determined according to Lichtenthaler (1987).

Free proline content was measured by the method of Bates et al. (1973). Fresh tissue (100 mg) was homogenized in 3% (w/v) sulphosalycylic acid and centrifuged at 700*g* for 3 min. After addition of ninhydrin reagent, mixtures were heated at 100 °C for 1 h and cooled in an ice bath. The chromophore obtained was extracted from liquid phase with toluene and the absorbance of organic layer was read at 520 nm. Proline concentration was determined from calibration curve using L-proline as standard and expressed as nmol g^{-1} FW.

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) using the thiobarbituric acid method (Heath and Packer, 1968). Fresh tissues (100 mg) were homogenized in 1.5 ml of 0.25% thiobarbituric acid (TBA) in 10% trichloracetic acid (TCA). The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10 000*g* for 10 min the absorbance of the supernatant was read at 532 nm and correction for unspecific turbidity was done by subtracting the absorbance at 600 nm. A total of 0.25% TBA in 10% TCA served as blank. The MDA content was calculated according to its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} FW.

H_2O_2 was extracted from 100 mg fresh tissue in ice-cold acetone and estimated according to the method of Mukherjee and Choudhuri (1983). After addition of titanyl-sulfate and concentrated NH_4OH solution, the formed peroxide-titanium precipitate was dissolved in 2 M H_2SO_4 and absorbance of the mixtures read at 415 nm. The H_2O_2 content was calculated from a standard curve and expressed as μmol g^{-1} FW.

Ascorbate was isolated by extraction with 6% trichloroacetic acid from 100 mg fresh plant tissue, following the method of Mukherjee and Choudhuri (1983). Extracts were mixed with 2% dinitrophenyl hydrazyne followed by addition of 1 drop of 10% thiourea solution and boiled for 15 min in a water bath. After adding 80% (w/v) H_2SO_4 (in an ice bath), absorbance of the mixture containing the hydrazone complex was read at 530 nm. The ascorbate content was determined using calibration curve and expressed as μmol g^{-1} FW.

For enzyme analysis, fresh plant tissue was homogenized in a 100 mM potassium phosphate (K_2HPO_4/KH_2PO_4) buffer pH 7.0 solution that included 1 mM EDTA using a pre-chilled mortar and pestle. The homogenates were centrifuged at 22 000*g* for 30 min at 4 °C. The supernatant was used for enzyme activity and protein content assays. Total soluble protein contents of the enzyme extracts were estimated according to Bradford (1976) using bovine serum albumin (BSA, Sigma Aldrich) as a standard.

The activity of SOD was assayed by the method of Beauchamp and Fridovich (1971) by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT). The reaction mixture contained 50 mM phosphate (KPO_4) buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and a suitable aliquot of enzyme extract. The test tubes were shaken and placed 30 cm below a light source consisting of a 15 W fluorescent lamp. The absorbance was taken at 560 nm. The activity of SOD was expressed as unit mg^{-1} protein. One unit of SOD was taken as the volume of the enzyme extract causing 50% inhibition of NBT reduction under light.

Catalase (CAT) activity was determined by decomposition of H_2O_2 and was measured spectrophotometrically by assessing the decrease in absorbance at 240 nm (Aebi, 1984). The reaction mixture contained 50 mM KPO_4 buffer (pH 7.0), 10 mM H_2O_2 and enzyme extract. Activity was calculated using the extinction coefficient (ε=0.04 mM^{-1} cm^{-1}) and μmol H_2O_2 decomposed g^{-1} FW min^{-1} was defined as a unit of CAT.

Ascorbate peroxidase activity was done according to Nakano and Asada (1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM sodium ascorbate, 0.1 mM EDTA and a suitable aliquot of enzyme extract. The ascorbate oxidation was followed at 290 nm and its concentration calculated using the molar extinction coefficient (ε=2.8 mM^{-1} cm^{-1}). Corrections were done for low, non-enzymatic oxidation of ascorbate by H_2O_2. One enzyme unit was defined as μmol ascorbate oxidized g^{-1} FW min^{-1}.

The activity of non-specific peroxidase was measured following the method of Chance and Maehly (1955). The reaction mixture contained 50 mM KPO_4 buffer (pH 7.0), 1 mM H_2O_2, 20 mM pyrogallol and enzyme extract. The formation of purpurogallin was followed at 430 nm and was quantified, taking into account its molar extinction coefficient (ε=2.47 mM^{-1} cm^{-1}).

The specific enzyme activity for all enzymes was expressed as unit mg^{-1} protein.

2.3. Statistical analysis

Statistical analysis was performed using STATISTICA 7.1 (StatSoft, Inc., USA) software package. For each analysis, data were compared by analysis of variance

(ANOVA) to confirm the variability of data and validity of results, and Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments ($p<0.05$). Regarding growth parameters each data point is the average of twelve replicates (n=12). Estimation for metal analysis was carried out in triplicate (n=3) while for all other measurements each data point is the average of six replicates (n=6). Each replicate represents the sample from a new Erlenmeyer flask.

3. Results

3.1. Uptake of Zn and Al and their effects on duckweed growth

Uptake of metals increased 27- to 66-fold and 25- to 43-fold following duckweed exposure to zinc (Zn) and aluminum (Al), respectively (Fig. 1). The amounts of tested metals detected in control duckweed were 75 and 59 $\mu g\,g^{-1}$ DW for Zn and Al, respectively. The Zn contents ranged from 2041 ± 72 to $4945\pm240\ \mu g\,g^{-1}$ DW at 0.15 and 0.3 mM solution concentrations, respectively. The Al contents ranged from 1505 ± 110 to $2554\pm180\ \mu g\,g^{-1}$ DW at the supplied concentrations after 15 days of exposure.

Growth parameters, in terms of relative frond number and relative growth rate (based on FW), were affected more strongly by Zn than by Al. Both Zn treatments had negative influences on relative growth rate (20% and 35% decreases with 0.15 and 0.3 mM Zn, respectively, compared to the control) after the 15-day period (Fig. 2A). A statistically significant decrease of relative frond number was observed only under the higher Zn treatment starting from day 12, at 25%, and ending after a 15-d period with a 44% decrease (Table 1). In the case of Al, both observed growth parameters were affected only by its higher concentration (18%), while duckweed multiplication was mildly stimulated at the lower applied concentrations. Lower metal treatments (0.15 mM) had no significant effect on dry matter yield, while higher ones (0.3 mM) caused marked increases in DW (80% and 38% in response to Zn and Al, respectively; Fig. 2A).

3.2. Zn and Al effects on chlorophyll and carotenoid contents

Duckweed leaves started to show signs of chlorosis after a 2-week exposure to Zn treatments. Accordingly, there were significant decreases in chlorophyll *a* and *b* and carotenoid contents (approximately 30% each) for the plants exposed to Zn treatments compared with the control (Table 2). However, the observed pigment contents were not affected by Al treatment, and no visible symptoms of toxicity were noticed following Al treatments during the 15-d period of exposure.

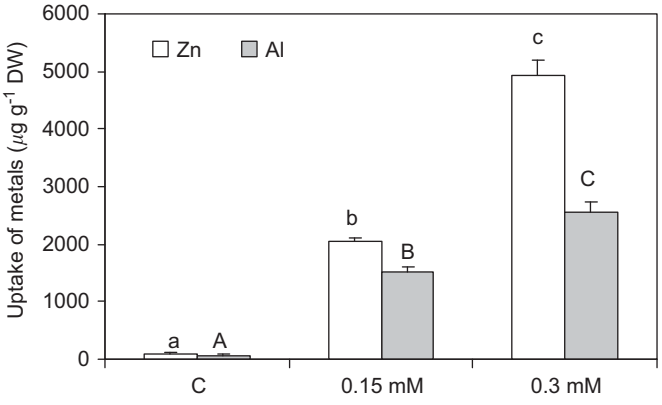

Fig. 1. Al and Zn uptake by duckweed (*Lemna minor* L.) following a 15-d period of exposure. Values are mean ± SD based on three replicates. Different letters indicate significantly different values (DMRT, $p<0.05$).

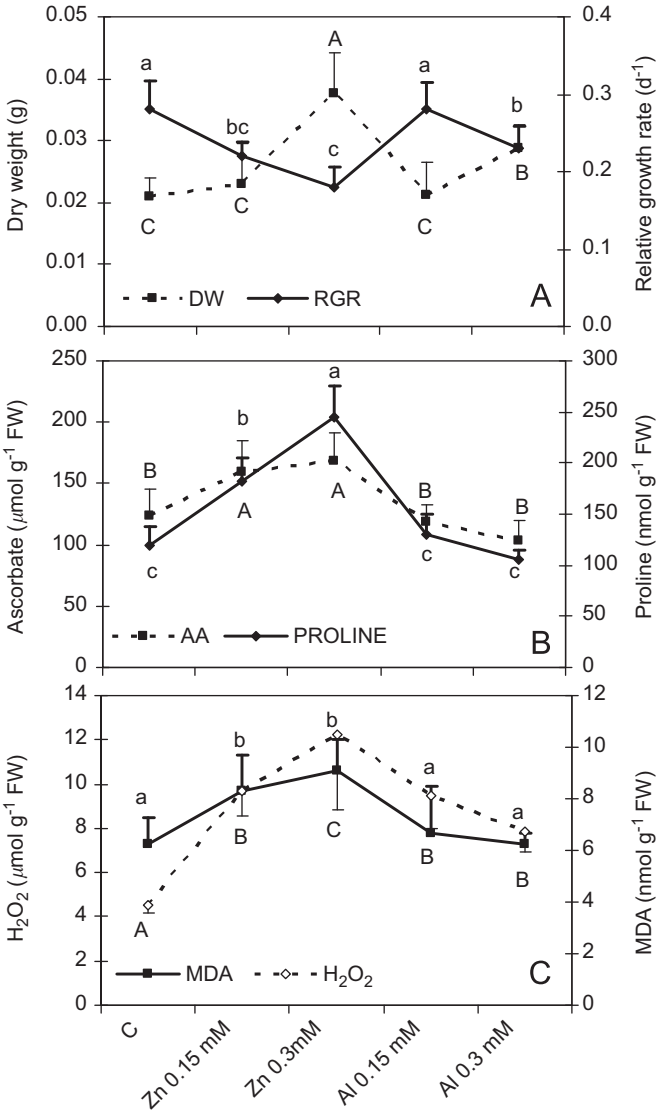

Fig. 2. (A) Relative growth rate (bold line) and dry weight (dashed line), (B) proline (bold line) and asorbate (dashed line) contents, (C) MDA (bold line) and H_2O_2 (dashed line) contents in control duckweed plants (C) and plants exposed to Zn or Al sulfate (0.15 and 0.3 mM). Values are mean ± SD based on six replicates. Different letters indicate significantly different values (DMRT, $p<0.05$).

3.3. Zn and Al effects on oxidant and antioxidant metabolite contents

To understand contributions of non-enzymatic antioxidants in the response of duckweed to Zn and Al toxicity, their proline and ascorbate contents were examined. Zn treatments increased proline content in *L. minor* plants by 53% (0.15 mM) and 105% (0.3 mM) compared with the control, whereas under both Al concentrations proline values were similar to those in the control (Fig. 2B). The ascorbate pool exhibited a significant (30–36%) increase under Zn (Fig. 2B) and no change under Al treatments compared to the control.

The changes in MDA and H_2O_2 contents with tested solutions are shown in Fig. 2C. Both observed parameters significantly increased due to Zn toxicity—MDA formation by 33% (0.15 mM) and 46% (0.3 mM), and H_2O_2 levels by 115% (0.15 mM) and 170% (0.3 mM), in comparison with the control. Malondialdehyde content showed no change with Al treatment in comparison to the control, although H_2O_2 levels increased by 110% and 74% with 0.15 and 0.3 mM Al treatments, respectively.

Table 1
Effect of zinc and aluminum (relative frond number) on growth of *L. minor* plants cultivated on PS medium.

Concentration (mM)	3rd day	5th day	7th day	10th day	12th day	15th day
Control	0.63 ± 0.034	2.53 ± 0.27	4.53 ± 0.27	7.80 ± 0.55ab	16.47 ± 0.51b	24.20 ± 2.28ab
Zn 0.15	0.63 ± 0.034	2.07 ± 0.42	3.97 ± 0.41	6.93 ± 1.40ab	13.47 ± 2.21bc	19.10 ± 2.77bc
Zn 0.3	0.40 ± 0.042	1.90 ± 0.22	3.50 ± 0.35	6.50 ± 0.71b	12.20 ± 1.75c	13.60 ± 2.53c
Al 0.15	0.67 ± 0.047	2.53 ± 0.74	4.57 ± 1.18	8.83 ± 2.00a	20.30 ± 4.47a	28.03 ± 7.14a
Al 0.3	0.70 ± 0.045	2.50 ± 0.35	4.10 ± 1.24	7.10 ± 1.52ab	14.70 ± 3.38bc	19.90 ± 5.49b

Values represent means ± SD (n=12). Means in each columns followed by different letters are significantly different ($p < 0.05$).

Table 2
Contents of chlorophyll *a*, *b*, total chlorophylls and carotenoids (mg g^{-1} FW) in *L. minor* plants subjected to Al or Zn stress for 15-day period.

Concentration (mM)	Chlorophyll *a*	Chlorophyll *b*	Total chlorophylls	Carotenoids
C	0.560 ± 0.059a	0.287 ± 0.034a	0.892 ± 0.082a	0.263 ± 0.028a
Zn 0.15	0.369 ± 0.058b	0.204 ± 0.039b	0.573 ± 0.093b	0.191 ± 0.027b
Zn 0.3	0.407 ± 0.027b	0.221 ± 0.028ab	0.633 ± 0.042b	0.223 ± 0.013b
Al 0.15	0.618 ± 0.067a	0.257 ± 0.037a	0.902 ± 0.097a	0.286 ± 0.036a
Al 0.3	0.559 ± 0.080a	0.268 ± 0.038a	0.817 ± 0.078a	0.264 ± 0.033a

Values are means of six replicates ± SD per treatment. Means in each column followed by different letters are significantly different ($p < 0.05$).

Soluble protein content in duckweed plants declined as a result of Zn treatments, especially at the 0.15 mM concentration, and showed no change in response to Al treatments (Fig. 3A).

Significant differences in enzyme capacity were observed between the treatments. Both Zn and Al treatments significantly increased SOD activity in *L. minor* plants, but induction of the enzyme was most conspicuous under the higher Zn concentration—a 104% increase in comparison with the control (Fig. 3B). Hydrogen peroxide, a product of SOD, is detoxified by CAT, POX or APX. The activity of CAT in duckweed markedly declined (up to 30%) as a result of Zn toxicity and was not affected by Al treatments (Fig. 3C).

The lower Zn treatment did not affect APX activity, while the higher Zn treatment increased activity of the enzyme by 52%. The activity of APX showed 52% and 72% increases with 0.15 and 0.3 mM Al treatments in comparison with the control, respectively (Fig. 3D). Both Zn and the lower Al concentration induced POX activity. However, the higher Al concentration caused only a slight increase in enzyme activity (Fig. 3D).

4. Discussion

The results presented here indicate that both zinc and aluminum ions were toxic to *L. minor*, although zinc to a much greater extent. The greater phytotoxic effect of Zn compared with Al appears to be related to a higher Zn uptake, and thus its more effective bioaccumulation. Bioconcentration factors (BCFs; estimated ratios of accumulated metal content in dry weight to initial metal concentration in the culture medium) were 245 and 252 for Zn and 186 and 158 for Al at 0.15 and 0.3 mM, respectively. In the study of Mishra and Tripathi (2008) the BCF value of 300 was estimated in duckweed *Spirodela polyrrhiza* after 15 d of exposure to 5 mg Zn l^{-1}.

The greater accumulation of Zn than Al in *L. minor* found in our study could reflect different cellular mechanisms for the bioconcentration of essential and nonessential trace metals in plants. It is documented that solubility of Al increases greatly with decreasing pH, and below 5.5 most Al occurs in soluble forms (Rout et al., 2001). However, availability and bioaccumulation of Zn and Al could also be influenced by chemical speciation of the metal, organic chelators, the presence of other metals and anions, ionic strength, light intensity, temperature and oxygen level (Greger, 1999).

The declines in total chlorophyll and carotenoid contents as well as the growth inhibition can be regarded as general responses associated with metal toxicity (Rout et al., 2001; Artetxe et al., 2002; Rout and Das, 2003). In the present study these responses were noticed mostly in the case of Zn, as common duckweed could tolerate Al at the applied concentrations without a notable effect on photosynthetic pigment contents or significant multiplication inhibition. Relative frond number was even slightly stimulated under the lower Al treatment, but a higher number of single fronds and small colonies were also observed (data not shown); these are considered to be indicative of environmental stress (Fodor, 2002; Li and Xiong, 2004). In addition, the relative growth rate decreased with 0.3 mM Al. In the present study, dry matter content significantly increased in response to both higher metal concentrations, especially 0.3 mM Zn, probably reflecting high amounts of these metals in duckweed plants.

Overall, the destruction of photosynthetic pigments by metals could be due to: impairment of the electron transport chain, replacement of Mg^{2+} ions associated with the tetrapyrrole ring of chlorophyll molecules, inhibition of important enzymes (Van Assche and Clijsters, 1990) associated with chlorophyll biosynthesis or peroxidation processes in chloroplast membrane lipids by the reactive oxygen species (Sandalio et al., 2001). It has previously been shown that Zn preferentially accumulates in the chloroplast (Van Assche and Clijsters, 1986), where it can directly interact with thylakoid membranes (Szalontai et al., 1999). The increased lipid peroxidation observed under both Zn treatments used in this study supports this hypothesis.

Concomitant increases in proline as a function of metal accumulation have been observed in response to heavy metals, including zinc (Bassi and Sharma, 1993; Alia et al., 1995). Here, only Zn treatments induced accumulation of the proline, regardless of the fact that soluble protein content declined in response to Zn. Aside from acting as a metal chelator and osmolyte, proline has been reported to scavenge hydroxyl radicals and singlet oxygen thus providing protection against ROS-induced cell damage (Matysik et al., 2002). Increases in both proline and lipid peroxidation levels with increasing Zn concentration are indicative of a correlation between ROS generation (hydroxyl radicals mostly) and ROS scavenging by proline. Moreover, duckweed

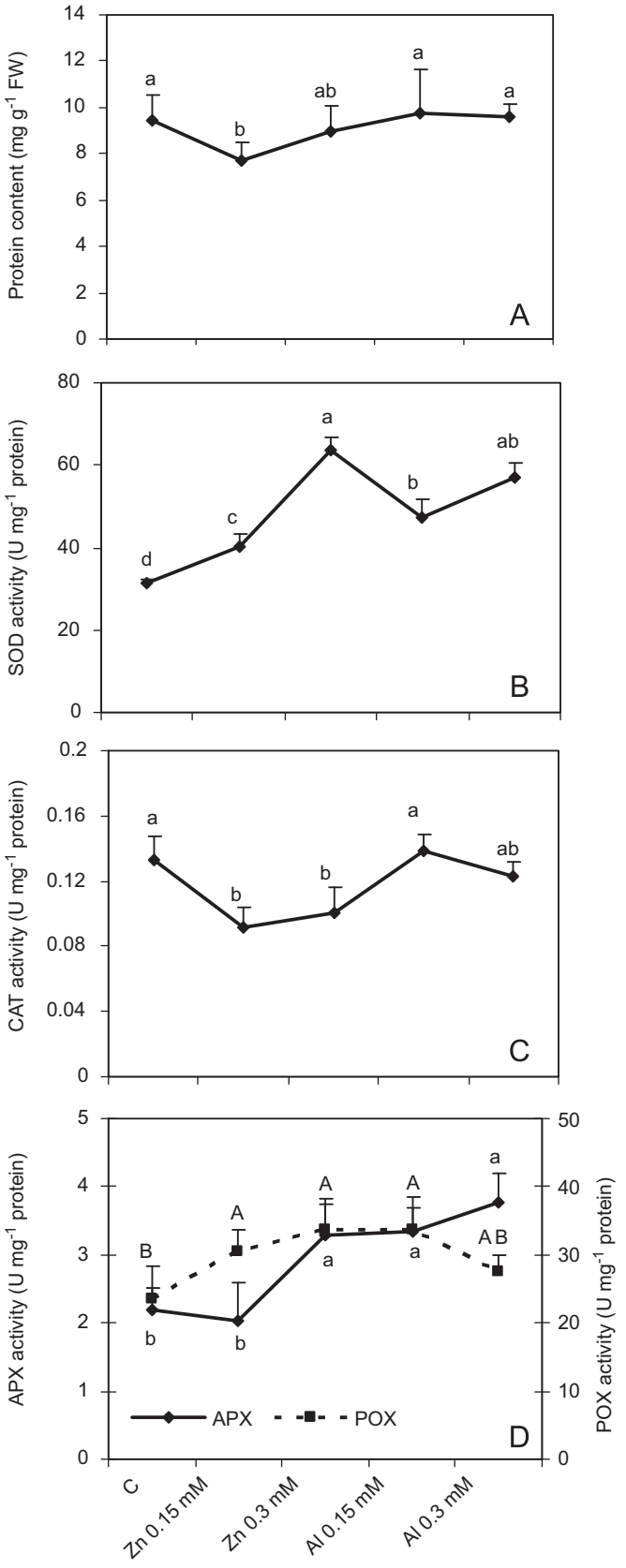

Fig. 3. (A) Protein content, (B) SOD activity, (C) CAT activity, (D) APX (bold line) and POX (dashed line) activity in control duckweed plants (C) and plants exposed to Zn or Al sulfate (0.15 and 0.3 mM). Values are mean ± SD based on six replicates. Different letters indicate significantly different values (DMRT, $p < 0.05$).

exposure to Zn caused a remarkable increase of H_2O_2 as well, indicating that Zn promoted accumulation of ROS. Lin et al. (2005) demonstrated that exposure of rice cells to Zn can enhance the intracellular level of ROS. On the other hand, both Al concentrations prompted H_2O_2, but not MDA, accumulation in duckweed cells. An elevated amount of H_2O_2 coupled with the absence of lipid peroxidation suggests that under experimental conditions, Al induces oxidative stress in duckweed plants, although to a much lesser extent than Zn.

Contrary to our results, in the study of Severi (1997), Al sulfate increased MDA content in duckweed over the same time of exposure, but Al was applied at a much higher concentration (1 mM). Yamamoto et al. (2002) demonstrated that Al cannot catalyze the peroxidation reaction by itself, but enhances the Fe (II or III)-mediated nonenzymatic peroxidation of phospholipids. Absence of lipid peroxidation has also been reported in in maize (Boscolo et al., 2003). The authors suggested that the possible reason for the absence of lipid peroxidation in maize is that the Fe^{2+} bound to the membrane is not exposed to the attack of H_2O_2. A similar mechanism could be responsible for the absence of lipid peroxidation in duckweed.

There have also been reports on changes in the activities of antioxidant enzymes in response to metal stress, including both Al and Zn (Weckx and Clijsters, 1997; Prasad et al., 1999; Aravind and Prasad, 2003; Rama Devi et al., 2003). Regulation of these enzymes is essential to keep the contents of superoxide and hydrogen peroxide under tight control. Superoxide dismutase, the first enzyme in the detoxifying process, converts superoxide radicals to H_2O_2 at a very fast rate. The enhanced SOD activity observed in the present study is consistent with studies in which other plant species were treated with Zn and Al (Prasad et al., 1999; Bonnet et al., 2000; Sharma and Dubey, 2007). Due to the action of SOD, H_2O_2 concentration is expected to increase inside the cell; this was found in duckweed plants under both Al and Zn toxicity (Sharma and Dubey, 2007). Hydrogen peroxide is reduced by CAT, POX or APX to water, of which peroxidases use ascorbate and/or phenolic molecules as electron donors (Asada, 1992). Catalase does not require reducing power, and has a high reaction rate but a low affinity for H_2O_2, thereby only removing the bulk of H_2O_2. In the present work, CAT activity was inhibited under Zn toxicity, whereas Al did not affect the activity of the enzyme. The decline in CAT activity in Zn-treated duckweed plants might be due to inhibition of enzyme synthesis or due to a change in the assembly of enzyme subunits under such conditions (Hou et al., 2007). Boscolo et al. (2003) also reported no change in CAT activity under Al toxicity in maize, while in some other plants a decline (soybean, rice) or enhancement (tobacco, wheat) of CAT activity has been found (Rama Devi et al., 2003; Darkó et al., 2004; Sharma and Dubey, 2007). Thus, in our experiments, CAT did not appear to be an efficient scavenger of H_2O_2. These inconsistent results regarding CAT activity might be due to differences in the plant organs studied, the durations and concentrations of metals utilized, and the plant species under investigation.

In contrast to CAT and POX, APX has a higher affinity for H_2O_2, allowing for the scavenging of small amounts of H_2O_2 in more specific locations (Asada, 1992). Both types of peroxidases (APX and POX) have been shown to be inducible on exposure to Al and Zn in different aquatic and terrestrial plant species (Severi, 1997; Prasad et al., 1999; Cuypers et al., 2001; Boscolo et al., 2003; Darkó et al., 2004; Sharma and Dubey, 2007). In our experiments, Zn and Al treatments induced APX (Al in particular) and POX (Zn in particular) activities in duckweeds, but some differences in the activities of these antioxidative enzymes in response to the tested metals were observed. Namely, lower Zn and higher Al treatments caused no significant changes in the activities of APX and POX, respectively. Additionally, ascorbate content was enhanced only

following exposure to Zn, indicating that ascorbate is involved in the antioxidant response of duckweed to Zn toxicity. Similar to our results, Artetxe et al. (2002) have found increased ascorbate levels in Zn-stressed duckweed. Of the antioxidants found in plants, ascorbate is the most abundant and has diverse physiological roles as a substrate for APX in addition to directly scavenging superoxide, hydroxyl radicals and singlet oxygen (Asada, 1992). Enhanced levels of ascorbate in duckweed under zinc toxicity suggest direct (non-enzymatic) active participation of the antioxidant in detoxification of oxygen species/free radicals, while the unchanged ascorbate content and simultaneous induction of APX activity under Al toxicity indicate enzymatic H_2O_2 detoxification, suggesting ascorbate consumption as an electron donor for APX.

5. Conclusion

The results suggest the involvement of oxidative stress in the toxicity of Zn and Al, but also somewhat different defense/adaptive strategies of duckweed in response to the tested heavy metals. Since both Zn and Al induced ROS production, as seen by accumulation of H_2O_2, a significant component of their phytotoxicity can be explained by oxidative stress—the generation of oxidative damage. With regard to metal (growth, chlorophyll and carotenoid contents) and oxidative stress (levels of H_2O_2, MDA and of antioxidants) indicators, Zn was found to be more toxic to duckweed than Al in the observed concentration ranges, which is probably related to the higher level of Zn uptake. Among the antioxidative enzymes SOD, POX and APX appear to play key roles in the antioxidative defense mechanism under Zn and Al toxicity. The ability of duckweed to both accumulate and tolerate Al and Zn at the concentrations used here could be partly derived from detoxification of ROS through an efficient antioxidant system. The results of the study suggest that, combined with advisable harvesting, *L. minor* can be used for the phytoremediation of low-level Zn and Al contaminated waterways. Since many environmental factors can have an impact on the bioavailability of the metals, in situ studies over a prolonged period of time are being conducted in order to estimate the efficiency of *L. minor* in treating effluents from mining operations.

Acknowledgment

This study has been funded by the Croatian Ministry of Science, Education and Sport, as part of Project no. 119-1191196-1202.

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol 105, 121–126.
Alia, Prasad, K.V.S.K., Pardha Saradhi, P., 1995. Effect of zinc on free radicals and proline in *Brassica and Cajanus*. Phytochemistry 39, 45–47.
Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373–399.
Aravind, P., Prasad, M.N.V., 2003. Zinc alleviates cadmium-induced oxidative stress in Ceratophyllum demersum L.: a free floating freshwater macrophyte. Plant Physiol.Biochem. 41, 391–397.
Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24, 1–15.
Artetxe, U., García-Plazaola, J.I., Hernández, A., Becerril, J.M., 2002. Low light grown duckweed plants are more protected against the toxicity induced by Zn and Cd. Plant Physiol. Biochem. 40, 859–863.
Asada, K., 1992. Ascorbate peroxidase: a hydrogen peroxide-scavenging enzyme in plants. Physiol. Plant. 85, 235–241.
Axtell, N.R., Sternberg, S.P.K., Claussen, K., 2003. Lead and nickel removal using *Microspora* and *Lemna minor*. Bioresour. Technol. 89, 41–48.
Bassi, R., Sharma, S.S., 1993. Proline accumulation in wheat seedlings exposed to zinc and copper. Phytochemistry 33, 1339–1342.
Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water stress studies. Plant Soil 39, 205–207.
Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assay and an assay applicable to PAGE. Anal. Biochem. 44, 276–287.
Bonnet, M., Camares, O., Veisseire, P., 2000. Effects of zinc and influence of *Acremonium lolii* on growth parameters, chlorophyll *a* fluorescence and antioxidant enzyme activities of ryegrass (*Lolium perenne* L. cv Apollo). J. Exp. Bot 51, 945–953.
Boscolo, P.R.S., Menossi, M., Jorge, R.A., 2003. Aluminum-induced oxidative stress in maize. Phytochemistry 62, 181–189.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Chance, B., Maehly, A.C., 1955. Assay of catalases and peroxidases. In: Colowick, S.P., Kaplan, N.O. (Eds.), Methods in Enzymology. Academic Press, New York, pp. 764–775.
Chang, H.B., Lin, C.W., Huang, H.J., 2005. Zinc-induced cell death in rice (*Oryza sativa* L.) roots. Plant Growth Regul. 46, 261–266.
Cheng, S., Grosse, W., Karrenbrock, F., Thoennessen, M., 2002. Efficiency of constructed wetlands in decontamination of water polluted by heavy metals. Ecol. Eng. 18, 317–325.
Cuypers, A., Vangronsveld, J., Clijsters, H., 2001. The redox status of plant cells (AsA and GSH) is sensitive to zinc imposed oxidative stress in roots and primary leaves of *Phaseolus vulgaris*. Plant Physiol. Biochem. 39, 657–664.
Darkó, É., Ambrusa, H., Stefanovits-Bányai, É., Fodor, J., Bakos, F., Barnabás, B., 2004. Aluminum toxicity, Al tolerance and oxidative stress in an Al-sensitive wheat genotype and in Al-tolerant lines developed by in vitro microspore selection. Plant Sci. 166, 583–591.
Dazy, M., Masfaraud, J.F., Férard, J.F., 2009. Induction of oxidative stress markers associated with heavy metal stress in *Fontinalis antipyretica* Hedw. Chemosphere 75, 297–302.
Dickson, W., 1978. Some effects of the acidification of Swedish lakes. Verh. Int. Verein. Limnol. 20, 851–856.
Englar, M., 2007. In: Bosnia–Herzegovina in Pictures second ed. Twenty-First Century Books, Minneapolis, USA.
Exley, C., 2003. The pro-oxidant activity of aluminum. Free Radical Biol. Med 36, 380–387.
Fodor, F., 2002. Physiological responses of vascular plants to heavy metals. In: Prasad, M.N.V., Strzatka, K. (Eds.), Physiology and Biochemistry of Metal Toxicity and Tolerance in Plants. Kluwer Academic Publisher, Netherlands, pp. 149–177.
Gensemer, R.W., Playle, R.C., 1999. The bioavailability and toxicity of aluminum in aquatic environments. Crit. Rev. Environ. Sci. Technol. 29, 315–450.
Greger, M., 1999. Metal availability and bioconcentration in plants. In: Prasad, M.N.V., Hagemeyer, J. (Eds.), Heavy Metal Stress in Plants—From Molecules to Ecosystems. Springer Press, Berlin, pp. 1–27.
Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. I-kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.
Hou, W., Chen, X., Song, G., Wang, Q., Chang, C.C., 2007. Effects of copper and cadmium on heavy metal polluted waterbody restoration by duckweed (*Lemna minor*). Plant Physiol. Biochem. 45, 62–69.
Jain, S.K., Vasudevan, P., Jha, N.K., 1990. *Azolla pinnata* R.Br. and *Lemna minor* for removal of lead and zinc from polluted water. Water Resour. 24, 177–183.
Kanoun-Boulé, M., Vicente, J.A.F., Nabais, C., Prasad, M.N.V., Freitas, H., 2009. Ecophysiological tolerance of duckweeds exposed to copper. Aquat. Toxicol. 91, 1–9.
Khan, A., Dobson, J.P., Exley, C., 2006. Redox cycling of iron by $A\beta_{42}$. Free Radical Biol. Med. 40, 557–569.
Li, T.Y., Xiong, Z.T., 2004. Cadmium-induced colony disintegration of duckweed (*Lemna paucicostata* Hegelm.) and as biomarker of phytotoxicity. Ecotoxicol. Environ. Saf 59, 174–179.
Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic membranes. Methods Enzymol. 148, 350–382.
Lin, C.W., Chang, H.-B., Huang, H.-J., 2005. Zinc induces mitogen-activated protein kinase activation mediated by reactive oxygen species in rice roots. Plant Physiol. Biochem. 43, 963–968.
Malik, A., 2004. Metal bioremediation through growing cells. Chemosphere 30, 261–278.
Matysik, J., Alia, Bhalu, B., Mohanty, P., 2002. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Curr. Sci. 82, 525–532.
Mishra, K., Tripathi, B.D., 2008. Concurrent removal and accumulation of heavy metals by the three aquatic macrophytes. Bioresource Technol. 99, 7091–7097.
Mukherjee, S.P., Choudhuri, M.A., 1983. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. Physiol. Plant 58, 166–170.
Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880.
Pettersson, A., Hallbom, L., Bergman, B., 1985. Physiological and structural responses of the cyanobacterium *Anabaena cylindrica* to aluminum. Physiol. Plant. 63, 153–158.
Pirson, A., Seidel, F., 1950. Zell- und stoffwechselphysologische Untersuchungen an der Wurzel von *Lemna minor* unter besonderer berücksichtigung von Kalium- und Kalziummangel. Planta 38, 431–473.

Pistelok, F., Galas, W., 1999. Zinc pollution of the Przemsza river and its tributaries. Pol. J. Environ. Stud. 8, 47–53.
Prasad, K.V.S.K., Paradha Saradhi, P., Sharmila, P., 1999. Concerted action of antioxidant enzymes and curtailed growth under zinc toxicity in *Brassica juncea*. Environ. Exp. Bot. 42, 1–10.
Rama Devi, S., Yamamoto, Y., Matsumoto, H., 2003. An intracellular mechanism of aluminum tolerance associated with high antioxidant status in cultured tobacco cells. J. Inorg. Biochem. 97, 59–68.
Rout, G.R., Das, P., 2003. Effect of metal toxicity on plant growth and metabolism. I. Zinc. Agronomie 23, 3–11.
Rout, G.R., Samantaray, S., Das, P., 2001. Aluminum toxicity in plants: a review. Agronomie 21, 3–21.
Sandalio, L.M., Dalurzo, H.C., Gómez, M., Romero-Puertas, M.C., del Río, L.A., 2001. Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J. Exp. Bot. 52, 2115–2126.
Severi, A., 1997. Aluminum toxicity in *Lemna minor* L.: effects of citrate and kinetin. Environ. Exp. Bot. 37, 53–61.
Sharma, P., Dubey, R.S., 2007. Involvement of oxidative stress and role of antioxidative defense system in growing rice seedlings exposed to toxic concentrations of aluminum. Plant Cell Rep. 26, 2027–2038.
Shikazono, N., Zakir, H.M., Sudo, Y., 2008. Zinc contamination in river water and sediments at Taisyu Zn–Pb mine area, Tsushima Island, Japan. J. Geochem. Explor. 98, 80–88.
Srikanth, R., Madhumohan Rao, A., Shravan Kumar, C.H., Khanum, A., 1993. Lead, cadmium, nickel and zinc contamination of ground water around Hussain Sagar Lake, Hyderabad, India. Bull. Environ. Contam. Toxicol. 50, 138–143.
Stephan, C.H., Courchesne, F., Hendershot, W.H., McGrath, S.P., Chaudri, A.M., Sappin-Didier, V., Sauve, S., 2008. Speciation of zinc in contaminated soils. Environ. Pollut. 155, 208–216.
Szalontai, B., Horváth, L.I., Debreczeny, M., Droppa, M., Horváth, G., 1999. Molecular rearragements of thylakoids after heavy metal poisoning, as seen by Fourier transform infrarred (FTIR) and electron spin resonance (ESR) spectroscopy. Photosynth. Res. 61, 241–252.
Teisseire, H., Guy, V., 2000. Copper-induced changes in antioxidant enzymes activities in fronds of duckweed (*Lemna minor*). Plant Sci. 153, 65–72.
Van Assche, F., Clijsters, H., 1986. Inhibition of photosynthesis in *Phaseolus vulgaris* by treatment with toxic concentration of zinc: effects on electron transport and photophosphorylation. Physiol. Plant 66, 717–721.
Van Assche, F., Clijsters, H., 1990. Effects of metals on enzyme activity in plants. Plant Cell Environ. 13, 195–206.
Wang, W., 1990. Literature review on duckweed toxicity testing. Environ. Res. 52, 7–22.
Weckx, J.E.J., Clijsters, H.M.M., 1997. Zn phytotoxicity induces oxidative stress in primary leaves of *Phaseolus vulgaris*. Plant Physiol. Biochem. 35, 405–410.
Yamamoto, Y., Kobayashi, Y., Rama Devi, S., Rikiishi, S., Matsumoto, H., 2002. Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. Plant Physiol. 128, 63–72.