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# Ecophysiological tolerance of duckweeds exposed to copper

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ABSTRACT

Although essential for plants, copper can be toxic when present in supra-optimal concentrations. Metal polluted sites, due to their extreme conditions, can harbour tolerant species and/or ecotypes. In this work we aimed to compare the physiological responses to copper exposure and the uptake capacities of two species of duckweed, *Lemna minor* (Lm(EC1)) and *Spirodela polyrrhiza* (SP), from an abandoned uranium mine with an ecotype of *L. minor* (Lm(EC2)) from a non-contaminated pond. From the lowest Cu concentration exposure (25 μM) to the highest (100 μM), Lm(EC2) accumulated higher amounts of copper than Lm(EC1) and SP. Dose–response curves showed that Cu content accumulated by Lm(EC2) increases linearly with Cu treatment concentrations ($r^2 = 0.998$) whereas quadratic models were more suitable for Lm(EC1) and SP ($r^2 = 0.999$ and $r^2 = 0.998$ for Lm(EC1) and SP, respectively). A significant concentration-dependent decline of chlorophyll a (chl a) and carotenoid occurred as a consequence of Cu exposure. These declines were significant for Lm(EC2) exposed to the lowest Cu concentration (25 μM) whereas for Lm(EC1) and SP a significant decrease in chl a and carotenoids was observed only at 50 and 100 μM-Cu. Electric conductivity (EC) and malondialdehyde (MDA) content increased after Cu exposure, indicating oxidative stress. Significant increase of EC was observed in Lm(EC2) for all Cu concentrations whereas the increase for Lm(EC1) and SP became significant only after an exposure to 50 μM-Cu. On the contrary, for Lm(EC1), SP, and Lm(EC2), MDA content significantly increased even at the lowest concentration. Protein content and catalase (CAT) activity showed a decrease with an increase in Cu concentration. For the species Lm(EC1) and SP, a significant effect of copper on CAT activity was observed only at the highest concentration (100 μM-Cu) whereas, for Lm(EC2), this effect started to be significant after an exposure to 50 μM-Cu. Superoxide dismutase (SOD) activity increased with increasing concentrations of Cu, with a very similar trend between the three populations of duckweed. However, due to the facts that enzyme activity is expressed as units of activity per gram of protein and that protein content decreased with Cu exposure, the increase in SOD activity might partly result from a relative increase of this enzyme inside the pool of proteins. Consequently, the results obtained in our experimental conditions strongly suggest that duckweed species from the uranium-polluted area have developed mechanisms to cope with metal toxicity and that this tolerance is based on the existence of protective mechanism to limit the metal uptake rather than on an enhancement of the antioxidative metabolism.



## 1. Introduction

Environmental exposure to toxic trace metals is one of the main critical issues on environmental and public health. Aquatic ecosystems are particularly susceptible as they often act as a final receptor for these contaminants. Copper is one of the most commonly used metals and its progressive increase in the aquatic ecosystems arises from various anthropogenic sources including copper mine drainage, copper-based pesticides, industrial and domestic wastes and antifouling paints (Ma et al., 2003; Andrade et al., 2004).

Copper is an essential nutrient for plant growth and development, and is normally present in plant tissue at concentrations around 10 $\mu g\, g^{-1}$ dry plant tissue (Baker and Brooks, 1989; Greger, 1999). The uptake of copper by higher plants is mainly in the form of $Cu^{2+}$ (Welch et al., 1993). Due to its redox properties, copper is a structural and catalytic component of many proteins and enzymes involved in a variety of metabolic pathways (Marschner, 1995; Maksymiec, 1997; Andrade et al., 2004).

Toxicity of copper is mainly due to the existence of two readily interconvertible oxidation states making it highly reactive, and it can catalyze the formation of free radicals through Haber–Weiss reaction. Copper is known to be the most effective metal causing oxidative stress and Cu ions themselves can directly initiate oxidative breakdown of polyunsaturated lipids (De Vos et al., 1993). The

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reactive oxygen species generated by Cu induce severe lipid peroxidation due to the removal of hydrogen from unsaturated fatty acids leading to the formation of lipid radicals and reactive aldehydes. As a consequence, the induced reactions will cause distortion of the lipid bilayer and membrane proteins (Logani and Davies, 1980; Reinheckel et al., 1998). This process generates large quantities of MDA (malondialdehyde), which is commonly used as an indicator of lipid peroxidation. Additionally, these free radicals damage photosynthetic apparatus (Rama Devi and Prasad, 1998; Dewez et al., 2005; Vajpayee et al., 2005) and may also catalyze degradation of proteins through oxidative modification and increased proteolytic activity (Romero-Puertas et al., 2002). Copper disturbs the integrity of thylakoid membranes and changes their fatty acid composition (De Vos et al., 1991), interferes with the biosynthesis of photosynthetic machinery and decreases net photosynthetic rate (Cook et al., 1997; Yruela, 2005). In *Arabidopsis thaliana*, Herbette et al. (2006) demonstrated that cadmium down regulates the genes encoding the enzymes of chlorophyll biosynthesis, proteins of PSI and PSII, electron transporters, $H^+$-ATPases as well as enzymes of the Calvin cycle. It appears that copper may also affect the photosynthesis through its effects at genetic level.

The protective mechanisms of plants to scavenge free radicals and peroxides include several antioxidant enzymes and metabolites (Scandalios, 1993; Clijsters et al., 1999; Scandalios, 2002; Mittler, 2002). The fact that the activity of one or more of these enzymes is generally increased in plants when exposed to stressful conditions, and that this higher activity is correlated to increased stress tolerance clearly demonstrate that the induction of antioxidant enzymes is an important protective mechanism to minimize oxidative damage to cells (Aust et al., 1985; Mazhoudi et al., 1997; Mittler, 2002).

Duckweeds (*Lemna* sp.) are ubiquitous floating freshwater monocotyledons and among the world's smallest flowering plants, providing food and habitat for a variety of herbivorous fish, macroinvertebrates and aquatic fowls (Lewis, 1995). The genetic diversity of different duckweed populations, the rapid vegetative reproductive cycle (doubling time of 1–4 days or less) and the easy culture in laboratory conditions are important features that make the duckweed species relevant models for comparative ecotoxicological studies. Therefore, duckweeds have been widely used in toxicity tests of different chemicals and effluents (Landolt, 1986; Wang, 1990; Teisseire and Guy, 2000; Frankart et al., 2002; Pomati et al., 2004; Razinger et al., 2007). *L. minor* is often selected to represent vascular aquatic plants in toxicity tests and, as an example, the EC50 for an exposure of 96 h to Cu was established at 1100 $\mu g\,l^{-1}$ (Wang, 1986).

Different studies in duckweed showed that an excess of copper interferes with respiration, photosynthesis, pigment synthesis and enzyme activity (Teisseire and Guy, 2000; Prasad et al., 2001a; Frankart et al., 2002; Babu et al., 2003).

The abandoned mine of Azere is located in the county of Tábua (Centre of Portugal) in the Southwest part of the uraniferous region of Beiras. Part of the contaminated waters of the mine is stored in two artificial ponds. Besides uranium, these waters exhibit a high level of contamination with trace metals. Among these metals, copper was present in relatively high concentrations. Two species of duckweed, *Lemna minor* L. and *Spirodela polyrrhiza* (L.) Schleiden, were found growing in these ponds and were selected for our study.

In this work we compare the physiological responses to copper exposure and the uptake capacities of the two species of duckweed from the uranium mine with another ecotype of *Lemna minor* L. collected from a non-contaminated pond. The concentrations of copper accumulated in the plants were measured to determine the uptake capacities of each duckweed population. Copper phytotoxicity was assessed through the analyses of several biochemical parameters, namely, concentration of photosynthetic pigments, electrolyte leakage, lipid peroxidation and changes in antioxidant enzymes activities.

## 2. Materials and methods

### 2.1. Plant material

Two species of duckweed, *Lemna minor* L. (Lm(EC1)) and *Spirodela polyrrhiza* (L.) Schleiden (also known as *Lemna polyrrhiza* L.) (SP), were collected from the artificial ponds of the abandoned uranium mine of Azere. The two species of duckweed were the only aquatic species growing in the mine water. Present in equal abundance, they were forming a single heterogeneous colony. A second ecotype of *Lemna minor* L. (Lm(EC2)) was collected from a non-contaminated pond in the Botanical Garden of the University of Coimbra (Portugal). The water from the uranium mine, besides high concentrations of uranium, contained multiple contaminants among which various trace metals, including copper. As presented in Table 1, Cu concentration of the mine water was more than 70-fold higher than the one measured in the water of the Botanical Garden. As a pre-treatment before the experiment, the three populations were cultivated separately for 6 weeks in a 1/10 Hoagland nutrient solution containing 1 mM $NH_4H_2PO_4$, 10 mM $KNO_3$, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, as macronutrients, 46 $\mu$M $H_3BO_3$, 9 $\mu$M $MnCl_2{\cdot}4H_2O$, 0.76 $\mu$M $ZnSO_4{\cdot}7H_2O$, 0.32 $\mu$M $CuSO_4$, 0.55 $\mu$M $H_2MoO_4$, as micronutrients and 78 $\mu$M Fe-EDTA as the iron source. Duckweeds were cultivated under controlled laboratory conditions at a temperature of 25 °C, with cool white fluorescent light (160 $\mu M\,m^{-2}\,s^{-1}$ PAR) and an 16:8 h light:dark cycle. Growth medium (pH = 6) was replaced every 7 days.

### 2.2. Copper treatment

For the Cu-exposure experiment, about 5 g of duckweed material, determined after 5 min blotting on dry tissue paper, was collected and transferred to a 600 ml polypropylene beaker containing 500 ml culture medium supplied with Cu. The range of Cu concentrations was established based on the concentrations of the mine water. Considering that Lm(EC1) and SP were growing well under these conditions of contamination, an enrichment of 25, 50 and 100 $\mu$M of Cu, corresponding, respectively, to 1.65-, 3.3- and 6.6-fold the concentration of the mine water, were selected for the Cu-exposure experiment. For each population, a control without any enrichment in Cu was included in the experiment. Cu treatment was given to the plants as $CuSO_4$ and the incubation was maintained for 72 h. After copper exposure, plants were carefully washed with deionised water before chemical and biochemical analyses. Three replicates were made per Cu treatment and per plant type.

**Table 1**
Trace elements and uranium concentrations of the waters in which duckweed populations were collected.

| | Cu ($\mu g\,l^{-1}$) | Fe ($\mu g\,l^{-1}$) | As ($\mu g\,l^{-1}$) | Cd ($\mu g\,l^{-1}$) | Pb ($\mu g\,l^{-1}$) | Ni ($\mu g\,l^{-1}$) | U ($\mu g\,l^{-1}$) |
|---|---|---|---|---|---|---|---|
| Azere mines water | 960 | 2320 | 110 | 50 | 29 | 122 | 34 |
| Non-contaminated pond water | 13 | 89 | 12 | 12 | 12 | 12 | – |

### 2.3. Copper accumulation

Approximately 100 mg of dry plant tissue samples were weighed, placed in Teflon vessels with 2 ml of $HNO_3$ 65%, and digested under pressure, for 10 h at 150 °C (Heinrichs et al., 1986). After cooling, the samples were diluted with ultrapure water up to 10 ml and stored in polyethylene vessels at room temperature until analysis.

Background copper concentration was determined in the control plants using graphite absorption spectrophotometry (PerkinElmer Model AAnalyst 100) whereas, in exposed plants, Cu was determined in all plant samples using flame AAS.

### 2.4. Photosynthetic pigments estimation

Approximately 120 mg of fresh material was ground in liquid nitrogen and placed in 10 ml of 80% cold acetone for 24 h at 4 °C and in darkness. The contents of chlorophyll a (chl a), chlorophyll b (chl b) and carotenoids were determined after the colorimetric method and the experimental equation described by Lichtenthaler (1987).

### 2.5. Estimation of electrolyte leakage

The electrolyte leakage induced by copper was estimated by measuring the electric conductivity (Pang et al., 2003). After being washed with deionised water several times, 1 g of plant material was transferred into 10 ml of deionised water. The electric conductivity was measured after 4 h at room temperature (EC1). Thereafter, the beakers containing the fronds were placed in a boiling water bath for 15 min and then cooled to room temperature; a second measurement was made as above (EC2). The relative electrolyte leakage was calculated as (EC1/EC2) × 100 and is expressed as the percentage of the total electrolyte content released to the medium.

### 2.6. Lipid peroxidation

The level of lipid peroxidation products in plants was expressed as 2-thiobarbituric acid reactive materials and was determined by estimation of the relative malondialdehyde (MDA) content (the main aldehyde produced) based on the method of Heath and Packer (1968). We homogenized 500 mg of material in 5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 × *g* for 5 min at 4 °C. For every 1 ml of aliquot (of supernatant), 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added. The mixture was heated at 95 °C for 30 min and then cooled quickly in an ice bath. The resulting mixture was centrifuged at 10,000 × *g* for 15 min at 4 °C and the absorbance of the supernatant was measured at 532 and 600 nm. The non-specific absorbance at 600 nm was subtracted from the absorbance at 532 nm. The concentration of MDA was calculated using the extinction coefficient of 155 $mM^{-1}\,cm^{-1}$.

### 2.7. Protein extraction and quantification

Plant material (500 mg of fronds and roots) was homogenized in 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone (w/v) at 4 °C. The homogenate was filtered through four layers of cheese cloth and centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatant was used to measure the protein content according to Lowry et al. (1951).

### 2.8. Superoxide dismutase activity

The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the reduction of nitroblue tetrazolium (NBT). SOD activity was determined according to the method of Paya et al. (1992), using 0.1 mM hypoxanthine and 0.025 U/ml xanthine oxidase as $O_2^-$ source and 0.1 mM NBT as $O_2^-$ scavenger in $KH_2PO_4$ buffer (pH 7.4). NBT reduction was monitored at 560 nm in a PerkinElmer Lambda 6 spectrophotometer. SOD activity was determined for protein fractions in the presence of 0.025% (v/v) Triton X-100, in 3 ml of assay mixture. SOD activity is the measure of NBT reduction without protein minus NBT reduction with protein. One unit of the activity is the amount of protein required to inhibit the initial reduction of NBT by 50%.

### 2.9. Catalase activity

The CAT activity was measured using a method adapted from Wong and Whitaker (2003). The reaction mixture contained 50 mM of potassium phosphate buffer (pH 7), 10 mM $H_2O_2$ and a suitable aliquot of enzyme in the final volume of 3 ml. Decrease in the absorbance at 240 nm was measured. The molar extinction coefficient was taken as 0.04 $cm^2\,\mu mol^{-1}$. The enzyme activity was determined as μmoles of $H_2O_2$ degraded $min^{-1}\,g^{-1}$ fresh weight (FW) and expressed as units $mg^{-1}$ protein.

### 2.10. Statistical analysis

The different sets of data (electrolyte leakage, lipid peroxidation, photosynthetic pigments, protein content, SOD activity, CAT activity and Cu concentrations accumulated) were compared using one-way ANOVA. When the differences were significant ($P \leq 0.05$), Tukey tests were performed for post-hoc comparisons. For each population, regression curves were constructed in order to obtain dose–response models that explain the variability of Cu concentrations in plants when expressed as a function of Cu treatment concentrations. All the statistical analyses were run using SPSS (version 14.0).

## 3. Results

### 3.1. Copper uptake

The tissue concentrations of copper in control plants were 6.9 ± 0.4; 6.8 ± 0.6; 7.1 ± 0.3 $\mu g\,g^{-1}$ dry weight (DW) for Lm(EC1), SP and Lm(EC2), respectively. Plant copper concentration ranged from 200 to 1700 $\mu g\,g^{-1}$ DW in the different populations and Cu exposure treatments (Fig. 1). In each Cu treatment, Lm(EC2) exhibited significantly higher Cu concentrations than Lm(EC1) and SP (Fig. 1). The three obtained dose–response curves suggest the existence of two different patterns of Cu accumulation. For Lm(EC2), a linear model was the best to explain the relation between Cu concentration in the medium and the Cu accumulated ($r^2 = 0.998$). On the other hand, for the two species from the uranium mine the best fit was obtained with quadratic regression models ($r^2 = 0.999$ and $r^2 = 0.998$ for Lm(EC1) and SP, respectively).

As shown in Fig. 1, the level of Cu accumulated in Lm(EC2) exposed to 25 μM (around 1.6 $mg\,l^{-1}$) reached 400 $\mu g\,g^{-1}$ DW and is similar to the levels of Cu accumulated in Lm(EC1) and SP exposed to 50 μM (around 3.2 $mg\,l^{-1}$).

### 3.2. Macroscopic symptoms of copper phytotoxicity

For Lm(EC1), SP and Lm(EC2), a 72 h exposure to a nutrient solution enriched with 50 and 100 μM of copper induced chlorotic

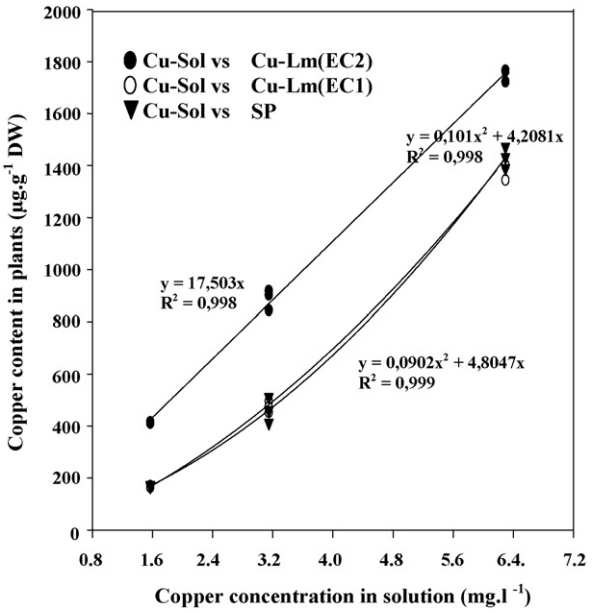


**Fig. 1.** Dose–response curves of Cu concentrations accumulated ($\mu g\,g^{-1}$ DW) in three populations of duckweed as a function of Cu treatment concentrations ($mg\,l^{-1}$). Regression curves and correlation coefficients ($r^2$) are given. Lm(EC1) and Lm(EC2): *Lemna minor* ecotype 1 and 2; SP: *Spirodela polyrhiza*.

symptoms and a depigmentation of the fronds which turned grey. A slight chlorosis after an exposure to a medium enriched with 25 μM of Cu was observed only in Lm(EC2). The severity of the symptoms revealed to be tightly related with copper concentration. For the two highest copper concentrations, more intense macroscopic symptoms were detected in Lm(EC2) which exhibited an obviously higher area of damaged fronds and/or more acute depigmentation symptoms.

### 3.3. Photosynthetic pigments

The effect of copper exposure in photosynthetic pigments depended on the nature of the pigment and the population considered (Table 2). The data expressed as a percentage of the control are presented in Fig. 2. For Lm(EC1), SP and Lm(EC2), a significant concentration-dependent decline of chl a and carotenoid occurred as a consequence of Cu exposure (Fig. 2). Significant differences in chl a and carotenoid content were observed between Lm(EC2) plants exposed to 25, 50 and 100 μM-Cu and the control treatment. For Lm(EC1) and SP plants, a significant decrease in chl a and carotenoids was observed only in the two highest concentrations. The content of chl b seems to be less affected by Cu exposure. Significant differences ($P < 0.01$) in chl b were found in the ecotype Lm(EC1), with plants exposed to 50 and 100 μM-Cu, showing a significantly lower chl b content than controls, and Lm(EC2) plants exposed to 25, 50 and 100 μM-Cu. However, for this ecotype, whatever the concentration of exposure, the decrease induced remained almost constant.

### 3.4. Electrolyte leakage

An increase of the relative electric conductivity (EC) related with Cu concentration, revealing a "concentration-dependent" deleterious impact of Cu on the cell membrane integrity, was observed for Lm(EC1), SP and Lm(EC2), (Table 2). As shown in Fig. 3 (data expressed as a percentage of the control), a significant increase ($P < 0.01$) in the electrical conductivity was detected in the cell membranes of the ecotype Lm(EC2) exposed to all Cu concentrations. For Lm(EC1) and SP, the EC only increased significantly with the 50 and 100 μM Cu-enriched solutions. Moreover, within each Cu treatment, Lm(EC2) always exhibited higher values of relative EC than the ones observed for the species from the Azere mine.

### 3.5. Lipid peroxidation

Mean values of MDA content are given in Table 2. For all the duckweed populations under study, the relative MDA content (expressed as a percentage of the control) in the Cu-treated plants

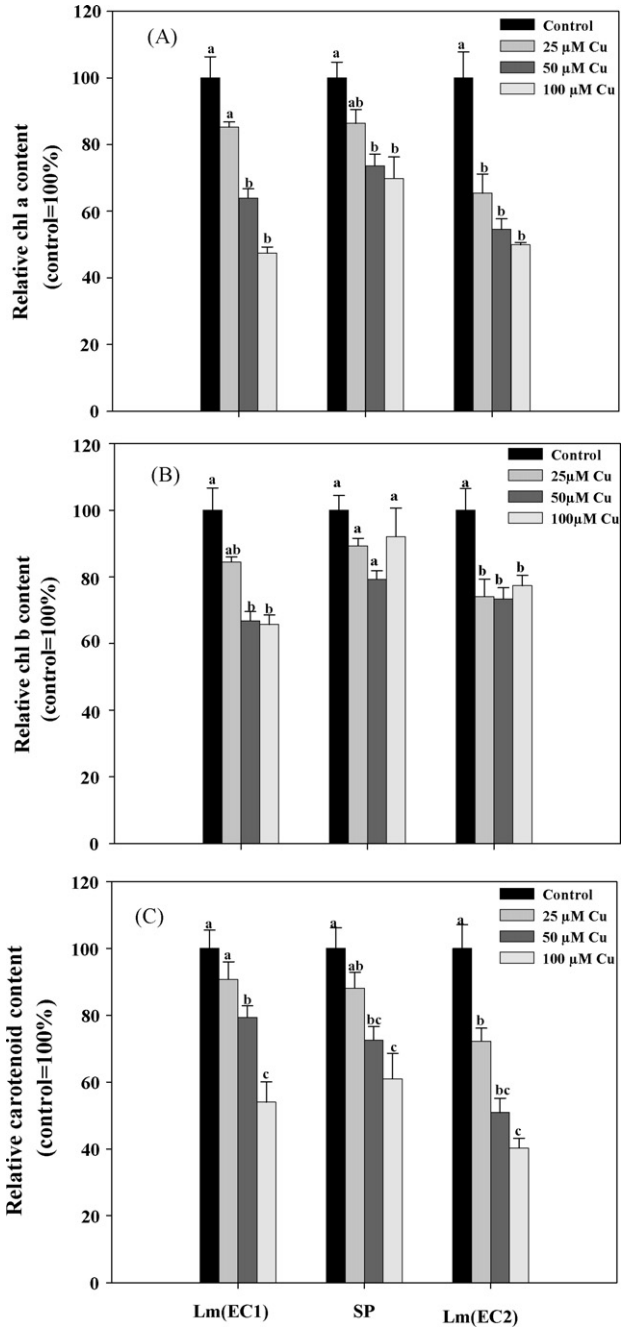


**Fig. 2.** Cu influence on the relative chlorophyll, chl a (A) and chl b (B) and carotenoid (C) contents of Lm(EC1), Lm(EC2) and SP after 72 h of exposure to 0, 25, 50 and 100 μM of Cu. Values are mean + S.E. ($n = 3$). Different letters indicate significantly different values within each population after one-way ANOVA ($P < 0.01$) and Tukey test.

**Table 2**
Mean values of the different physiological parameters measured in Lm(EC1), Lm(EC2) and SP plants after an exposure of 72 h to a medium enriched with 0 (control), 25, 50 and 100 μM of copper.

| | Chl a content (mg g$^{-1}$ FW) | | | Chl b content (mg g$^{-1}$ FW) | | | Carotenoid content (μmol g$^{-1}$ FW) | | | Eletrolyte leakage (% total electrolyte content) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lm(EC1) | Lm(EC2) | SP | Lm(EC1) | Lm(EC2) | SP | Lm(EC1) | Lm(EC2) | SP | Lm(EC1) | Lm(EC2) | SP |
| Control | 0.76 | 0.56 | 0.63 | 0.35 | 0.20 | 0.28 | 0.20 | 0.19 | 0.17 | 14.20 | 11.48 | 10.26 |
| 25 μM Cu | 0.69 | 0.40 | 0.55 | 0.32 | 0.17 | 0.26 | 0.18 | 0.14 | 0.15 | 18.06 | 22.8 | 10.7 |
| 50 μM Cu | 0.54 | 0.35 | 0.47 | 0.27 | 0.17 | 0.24 | 0.16 | 0.10 | 0.12 | 21.37 | 36.33 | 16.72 |
| 100 μM Cu | 0.40 | 0.32 | 0.44 | 0.26 | 0.18 | 0.27 | 0.11 | 0.08 | 0.10 | 41.66 | 54.66 | 62.68 |

| | MDA content (μmol g$^{-1}$ FW) | | | Protein content (mg g$^{-1}$ FW) | | | SOD activity (Units mg$^{-1}$ protein) | | | CAT activity (Units mg$^{-1}$ protein) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lm(EC1) | Lm(EC2) | SP | Lm(EC1) | Lm(EC2) | SP | Lm(EC1) | Lm(EC2) | SP | Lm(EC1) | Lm(EC2) | SP |
| Control | 10.1 | 7.7 | 10.8 | 20.83 | 17.95 | 21.2 | 1.5 | 2.8 | 2 | 14 | 11.98 | 16.1 |
| 25 μM Cu | 12.3 | 11.5 | 13.2 | 19.44 | 17.7 | 20.47 | 1.6 | 3.2 | 2.1 | 14.78 | 10.88 | 16.46 |
| 50 μM Cu | 13.4 | 10.8 | 13.9 | 18.38 | 15.54 | 18.57 | 1.7 | 3.2 | 2.3 | 12.93 | 8.79 | 14.73 |
| 100 μM Cu | 14.1 | 10.2 | 14.7 | 15.96 | 14.53 | 17.15 | 1.9 | 3.4 | 2.5 | 9.26 | 6.18 | 10.28 |

was significantly higher ($P < 0.01$) compared to the respective controls (Fig. 4). However, within the copper treatments there was no significant increase of MDA. In each copper treatment, Lm(EC2) tended to exhibit higher values of relative MDA content than the two species from the Azere mine. For plants exposed to 100 μM-Cu, the highest increment was observed for the ecotype Lm(EC2) with 148% of the control treatment while the values for Lm(EC1) and SP reached 141% and 136%, respectively.

### 3.6. Protein content and antioxidant enzyme activities

Protein content decreased with increasing concentrations of Cu with a very similar trend between the three populations under study (Table 2). In Lm(EC1), SP and Lm(EC2), this decrease of the relative protein content (data expressed as a percentage of the control) was significant in plants exposed to 50 and 100 μM-Cu. In plants exposed to 100 μM of Cu, protein content was 20% lower than the respective controls (Fig. 5A).

An increase in SOD activity was observed with increasing Cu treatment concentrations for Lm(EC1), SP and Lm(EC2) (Table 2). The SOD activity in all plants exposed to 50 and 100 μM-Cu was significantly higher than that in the respective controls (Fig. 5B). For the highest Cu concentration and in all plants, the increase in the relative SOD activity was between 20% and 24% higher than that in the respective controls.

The relative CAT activity was significantly reduced in plants from the ecotype Lm(EC2) exposed to 50 and 100 μM-Cu, with the lowest value observed in the plants exposed to 100 μM-Cu (Fig. 5C). For Lm(EC1) and SP, a significant effect of copper on CAT activity was only observed at the highest concentration (100 μM-Cu).

## 4. Discussion

### 4.1. Copper accumulation

Our results clearly showed two different trends of accumulation capacity between the plants from the uranium mine and the *L. minor* from the non-polluted area. Indeed, the dose–response curves of Lm(EC1) and SP might be explained by an evolved capacity of the duckweed plants from the mine to limit the uptake of some trace metal contaminants, like Cu, present on the site. Murphy et al. (1999) reported that the copper tolerance of *A. thaliana* is associated with a rapidly activated release of citrate. Mkandawire and Dudel (2002) reported the exudation of oxalate in *Lemna gibba* when exposed to uranium. The release of organic anions can protect the plants by chelating the trace metal ions in the rhizosphere to form non-toxic complexes. Although in our study we did not determine the organic exudates of duckweed, we can hypothesize that the release of organic anions might be involved in the

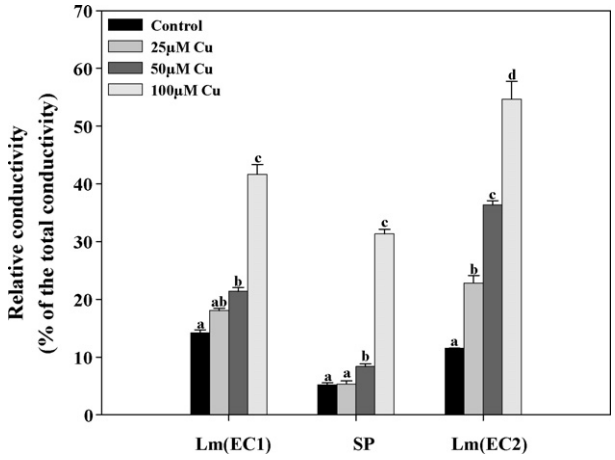


**Fig. 3.** Cu influence on ion leakage (observed as a function of electrical conductivity) in Lm(EC1), Lm(EC2) and SP after 72 h of exposure to 0, 25, 50 and 100 μM of Cu. Values are mean + S.E. ($n = 3$). Different letters indicate significantly different values within each population after one-way ANOVA ($P < 0.01$) and Tukey test.

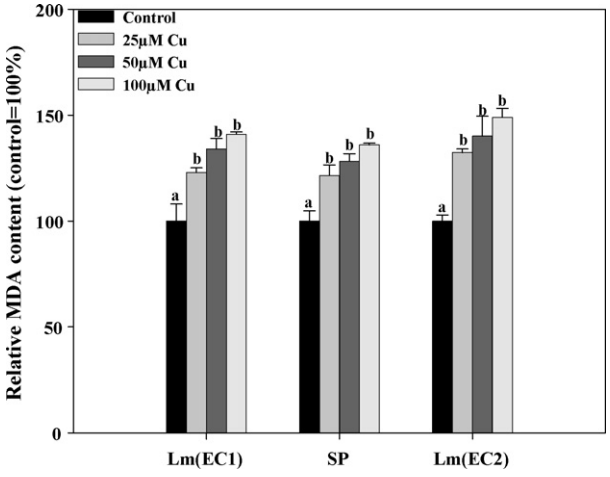


**Fig. 4.** Cu influence on the relative level of lipid peroxidation measured as thiobarbituric acid reactive substances (control = 100%) in Lm(EC1), Lm(EC2) and SP after 72 h of exposure to 0, 25, 50 and 100 μM of Cu. Values are mean + S.E. ($n = 3$). Different letters indicate significantly different values within each population after one-way ANOVA ($P < 0.01$) and Tukey test.

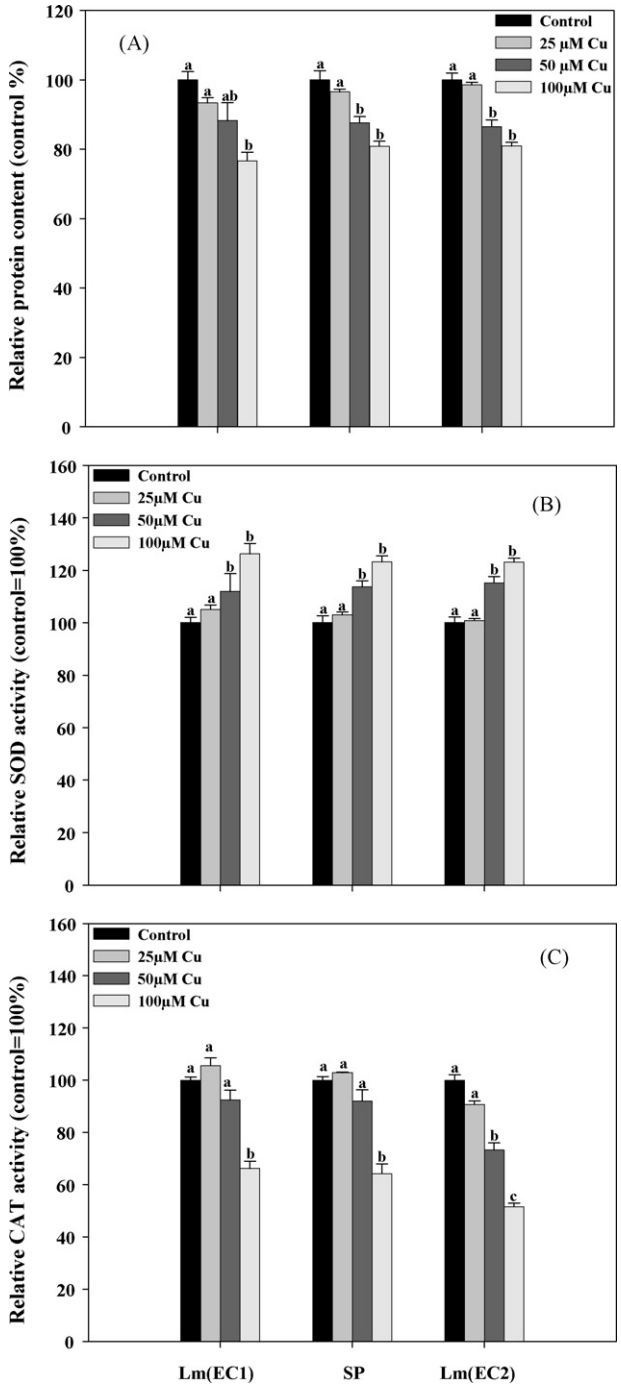


**Fig. 5.** Cu influence on the relative protein content (A), SOD (B) and CAT (C) activities (control = 100%) in Lm(EC1), Lm(EC2) and SP after 72 h of exposure to 0, 25, 50 and 100 µM of Cu. Values are mean + S.E. ($n = 3$). Different letters indicate significantly different values within each population after one-way ANOVA ($P < 0.01$) and Tukey test.

capacity of the species collected from the uranium mine to slow down the uptake of Cu, and therefore, delay the toxic effects of Cu excess. Regarding Cu uptake, Razinger et al. (2007) reported that *L. minor* exposed to 10 µM of Cu for 24 h could accumulate concentrations in a range of 300 and 800 µg $g^{-1}$ DW. These values are higher than the ones we obtained in plants exposed to the lowest Cu concentration (25 µM), whatever the population considered. These different findings may be due to the different habitats where the plants were collected.

### 4.2. Macroscopic symptoms of Cu phytotoxicity and photosynthetic pigments

Toxic effects of Cu were evident from the chlorosis symptoms that the Lm(EC2) fronds exhibited already after 72 h of exposure to 25 µM-Cu. When all populations under study were exposed to 50 and 100 µM-Cu, the intense depigmentation led to a greying aspect of the fronds. Although not measurable statistically, the differences in the intensity of the visible damages between the two duckweeds from the mine (Lm(EC1) and SP) and from the non-contaminated pond (Lm(EC2)) revealed a stronger impact of the metal on Lm(EC2). The analysis of the photosynthetic pigments confirmed the Cu-induced damages observed at the macroscopic level, with the existence of differences between the populations under study. Although all the plants suffered a decrease of the chl a and carotenoid content when compared to the respective controls, Lm(EC2) was more sensitive to Cu toxicity than the duckweeds from the mine, showing a significant decrease of chl a and carotenoid after exposure to the lowest Cu treatment (25 µM). When related to Cu accumulated, these decreases in chl a and carotenoids were observed when the concentrations accumulated in all plants reached 400 µg $g^{-1}$ DW (corresponding to a Cu-concentration of exposure of 25 µM.$l^{-1}$ for Lm(EC2) and 50 µ.$l^{-1}$ for Lm(EC1) and SP). This value seems to be the threshold concentration above which the deleterious impact of Cu on photosynthetic pigments exceeds the protective capacity of repairing systems of the plant.

The accumulation of Cu in plants often induces important metabolic disturbances and particularly chlorophyll degradation (Van Assche and Clijsters, 1990; Rama Devi and Prasad, 1998; Prasad et al., 2001b; Küpper et al., 2002; Chatterjee et al., 2006; Li et al., 2006; Perales-Vela et al., 2007). The loss of photosynthetic pigment content is generally due to the direct peroxidative breakdown of pigments and chloroplast membrane lipids by the reactive oxygen species (Sandamann and Böger, 1980). At lower Cu concentrations (sub-µM), the central $Mg^{2+}$ ion of the chlorophyll is replaced by $Cu^{2+}$ (Küpper et al., 2002). At higher concentrations (µM and mM), in addition to this replacement, $Cu^{2+}$ can inhibit the synthesis of δ-aminolevulinic acid, a precursor of cholorophylls and the protochlorophyllide reductase that catalyzes the reductive formation of chlorophyllide from protochlorophyllide during biosynthesis of chlorophylls (Stiborová et al., 1986; Van Assche and Clijsters, 1990). Finally, as demonstrated in the case of *A. thaliana* exposed to Cd (Herbette et al., 2006), it appears that copper may also affect the photosynthesis through genetic down regulation. In our study, the exact mechanism leading to photosynthetic pigments loss remains to be elucidated.

The present results also demonstrated a slower degradation of chl b indicating that chl a and carotenoids are more sensitive than chl b to Cu damage. As proposed in previous studies, the slower degradation of chl b could suggest the involvement of photoxidative mechanisms in Cu-induced damages (Wieckowski and Waloszek, 1993; Prasad et al., 2001a).

### 4.3. Lipid peroxidation and membrane leakage

Lipid peroxidation is a free radical mediated process (Slater, 1984a,b) and is considered to be the best measure of damage caused by increasing reactive oxygen species (ROS) production (Halliwell, 1991; Rao et al., 1995). Although Cu interferes with a number of physiological processes, the primary target of Cu toxicity is probably the cell membrane (De Vos et al., 1989, 1993). Copper can act as an efficient generator of toxic oxygen species that will induce severe lipid peroxidation and generate lipid radicals and large quantities of reactive aldehydes like MDA (Halliwell and Gutteridge, 1984; Aust et al., 1985; Girotti, 1985; Weckx and Clijsters, 1996).

Our results showed a gradual increase in MDA content which was tightly related with Cu concentrations in the water. The increase of MDA was significant even after an exposure to the lowest Cu treatment (25 μM) observed in the three populations of duckweeds under study. In parallel to lipid peroxidation, our results also showed a gradual increase in electrical conductivity (EC) which also appeared to be tightly related to Cu concentrations, and was more pronounced for Lm(EC2) plants. As already observed for photosynthetic pigments, the increase in EC is observed when the concentrations accumulated in the plants reach 400 $\mu g\, g^{-1}$ DW. An increase in ion leakage through membrane is often described as a consequence of membrane lipid peroxidation (Saelim and Zwiazek, 2000; Dash and Mohanty, 2002). Moreover, like other class B metals, Cu binds strongly to S- and N-containing ligands and proteins. As a consequence, disulfide bridges are formed which lead to a change in the properties of the ionic membrane channels and leakage of ions. Our results support the assumption that an exposure to 25, 50 and 100 μM of Cu has a deleterious effect on the cell membranes of the plants exposed and that this deleterious effect is dependent on the ecotype.

### 4.4. Protein content and antioxidant enzyme activities

In our study, a Cu concentration-dependent decline of protein content was observed for the three populations of duckweed. As suggested by Romero-Puertas et al. (2002) in the case of Cd, one hypothesis that could explain this protein content decrease is an enhancement of the proteolytic activity. The oxidative alteration of the proteins can be the first signalling step leading to protein degradation (Stadtman, 1990). Therefore, an increase in the proteolysis rate could be related to the oxidizing properties of Cu.

Parallel to the decrease in protein content, our results also showed an increase in the relative SOD activity as the level of Cu exposure rises. As for protein content, the impact of Cu on SOD activity was observed to be similar between Lm(EC1), SP and Lm(EC2). The increase of SOD activity is a consequence of an increased production of active oxygen species (Rama Devi and Prasad, 1998) and it may happen through the activation of latent SOD (Doke et al., 1994) or the increased expression of genes encoding SOD (Bowler et al., 1992). Several studies on various species already reported such an increase in the activity of this enzyme after an exposure to Cu (Van Assche and Clijsters, 1990; Chongpraditnum et al., 1992; Weckx and Clijsters, 1996; Rama Devi and Prasad, 1998; Srivastava et al., 2006). However, enzyme activity is expressed as units of activity per gram of protein. In our study, due to the fact that protein content decreased with Cu exposure, we may also consider the possibility that the increase in SOD activity could partly result from a relative increase of this enzyme inside the pool of proteins.

Although SOD blocks $O_2^-$ radicals, the enzyme does not provide complete protection to the cell since $H_2O_2$ emerges as a product of its functioning (Meloni et al., 2003). $H_2O_2$, a precursor of the highly reactive hydroxyl radical will be destroyed by catalases (CAT) and peroxidases. In peroxisomes and cytosol, catalases play a key role in controlling the level of hydrogen peroxide and numerous stress factors were reported to stimulate this enzyme (Foyer et al., 1994).

The fast mobilization of CAT was considered by Weckx and Clijsters (1996) as a cellular adaptation to cope with $H_2O_2$ overproduction generated by cupric ions. However, in our study CAT activity was lower with increasing concentrations of Cu. This can be related to the sensitivity of the enzyme to the $O_2^-$ radicals produced under Cu stress as it is known that the enzyme activity can be inhibited by increased levels of $O_2^-$ (Cakmak, 2000). Alternatively, the loss in CAT activity might also be ascribed to the degradation caused by induced peroxisomal proteases (Sandalio et al., 2001). Other studies have reported that a decrease in CAT activity in plants under Cu stress might be due to the replacement of total or partial Fe from the active sites (Agarwala et al., 1977; Luna et al., 1994). If we consider the rapid drop of CAT activity when plants are exposed to 50 and 100 μM of Cu, we can assume that a threshold for Cu tolerance has been exceeded and that this threshold is lower for Lm(EC2) than for Lm(EC1) and SP. For Lm(EC1) and SP, the deleterious impact of Cu was observed after an exposure to 100 μM that corresponds to 1400 $\mu g\, g^{-1}$ DW of Cu in plants, whereas for Lm(EC2), CAT activity decreased after an exposure to 50 μM corresponding to a Cu concentration of 850 $\mu g\, g^{-1}$ DW. Teisseire and Guy (2000) reported an enhancement of CAT activity in *L. minor* fronds exposed for 24 h to a range of 0 to 10 μM of Cu. The authors also demonstrated that when the duration of exposure to 1.6 μM of Cu exceeded 24 h, CAT activity started to decrease. In our study, the absence of a significant CAT activity enhancement might be due to the fact that our lower concentration of exposure is above 10 μM (from 25 to 100 μM) or that our time of exposure exceeds 24 h (about 72 h).

## 5. Conclusions

The uranium mine duckweeds (Lm(EC1) and SP) tolerate exposures to higher levels of Cu than the *L. minor* ecotype (Lm(EC2)) from a non-polluted area. Our results strongly suggest a more severe impact of Cu on Lm(EC2) than on the uranium mine duckweeds regarding the levels of lipid peroxidation, electric conductivity and photosynthetic pigments. When related to the quantity of copper accumulated there is a threshold of 400 $\mu g\, g^{-1}$ DW above which a deleterious impact of copper is observed, especially in electric conductivity and photosynthetic pigments, whatever the duckweed species or ecotype. This threshold is only reached after an exposure to a higher concentration in the case of the uranium mine plants. *L. minor* and *S. polyrrhiza* from the uranium-polluted area have developed mechanisms to cope with metal toxicity. On the contrary, we did not observe any significant differences of SOD and CAT activity in response to copper exposure between the duckweeds from a contaminated and non-contaminated area. The antioxidant metabolism is commonly viewed as a good indicator of the exposure to pollutants and an important protective mechanism. Apparently these two enzymes are not involved in the tolerance to copper exhibited by the two duckweed species from the uranium mine. The combined results of the antioxidant metabolism and copper accumulation suggest that the higher Cu tolerance of the uranium mine species is mainly based on the development of protective mechanisms to limit the metal uptake.

Finally, it seems interesting to highlight that, although Lm(EC1) and SP are two distinct species with different genotypes, they exhibit a very similar physiological behaviour regarding Cu tolerance and uptake capacities. On the contrary, *L. minor* from the non-contaminated area revealed a much lower tolerance to the metal than the *L. minor* from the uranium mine confirming that they are two different ecotypes. These findings strongly emphasise the importance of the environmental pressure in the evolution and differentiation of new ecotypes and their importance in phytoremediation projects.

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## References


Agarwala, S.C., Bisht, S.S., Sharma, C.P., 1977. Relative effectiveness of certain heavy metals in producing toxicity and symptoms of iron deficiency in barley. Can. J. Bot. 55, 1299–1307.

Andrade, L.R., Farina, M., Amado Filho, G.M., 2004. Effects of copper on *Enteromorpha flexuosa* (Chlorophyta) *in vitro*. Ecotoxicol. Environ. Saf. 58, 117–125.

Aust, S.D., Morehouse, L.A., Thomas, C.E., 1985. Role of metals in oxygen radical reactions. Free Rad. Biol. Med. 1, 3–25.

Babu, T., Akhtar, S., Tariq, A., Lampi, M., Tipuranthakam, S., Dixon, D.G., Greenberg, B.M., 2003. Similar stress responses are elicited by copper and ultraviolet radiation in the aquatic plant *Lemna gibba*: implication of reactive oxygen species as common signals. Plant Cell Physiol. 44, 1320–1329.

Baker, A.J.M., Brooks, R.R., 1989. Terrestrial higher plants which hyperaccumulate metallic elements—a review of their distribution, ecology and phytochemistry. Biorecovery 1, 81–126.

Bowler, C., Montagu, M.V., Inze, D., 1992. Superoxide dismutase and stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 83–116.

Cakmak, I., 2000. Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. New Phytol. 146, 185–205.

Chatterjee, C., Sinha, P., Dube, B.K., Gopal, R., 2006. Excess copper-induced oxidative damages and changes in radish physiology. Commun. Soil Sci. Plant Anal. 37, 2069–2076.

Chongpraditnum, P., Mori, S., Chino, M., 1992. Excess copper induce a cytosolic Cu. Zn superoxide dismutase in soybean root. Plant Cell Physiol. 33, 239–244.

Clijsters, H., Cuypers, A., Vangrosveld, J., 1999. Physiological responses to heavy metals in higher plants: defence against oxidative stress. Zeitschrift für Naturforschung 54, 730–734.

Cook, C.M., Kostidou, A., Vardaka, E., Lanaras, T., 1997. Effects of copper on the growth, photosynthesis and nutrient concentrations of *Phaseolus vulgaris* plants. Photosynthetica 34, 179–193.

Dash, S., Mohanty, N., 2002. Response of seedlings to heat stress in cultivars of wheat: growth temperature-dependent differential modulation of photosystem 1 and 2 activity, and foliar antioxidant defense capacity. J. Plant Physiol. 159, 49–59.

De Vos, C.H.R., Schat, H., Vooijs, R., Ernst, W.H.O., 1989. Copper induced damage to the permeability barrier in roots of *Silene cucubalus*. J. Plant Physiol. 135, 165–169.

De Vos, C.H.R., Schat, H., De Waal, M.A.M., Vooijs, R., Ernst, W.H.O., 1991. Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant *Silene cucubalus*. Physiol. Plantarum 82, 523–528.

De Vos, C.H.R., Ten Bookum, W.M., Vooijs, R., Schat, H., Dekok, L.J., 1993. Effect of copper on fatty acid composition and peroxidation of lipids in the roots of copper tolerant and sensitive *Silene cucubalus*. Plant Physiol. Biochem. 31, 151–158.

Dewez, D., Geoffroy, L., Vernet, G., Popovic, R., 2005. Determination of photosynthetic and enzymatic biomarkers sensitivity used to evaluate toxic effects of copper and fludioxonil in alga *Scenedesmus obliquus*. Aquat. Toxicol. 74, 150–159.

Doke, N., Miura, Y., Sanchez, L., Kawakita, K., 1994. Involvement of superoxide radical in signal transduction: responses to attack by pathogens, physical and chemical shocks and UV irradiation. In: Foyer, C., Mullineaux, P.M. (Eds.), Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press, Boca Raton, FL, pp. 177–198.

Foyer, C.H., Lelandais, M., Kunert, K.J., 1994. Photooxidative stress in plants. Physiol. Plantarum 92, 696–717.

Frankart, C., Eullaffroy, P., Vernet, G., 2002. Photosynthetic responses of *Lemna minor* exposed to xenobiotics, copper, and their combination. Ecotoxicol. Environ. Saf. 53, 439–445.

Girotti, A.W., 1985. Mechanisms of lipid peroxidation. Free Rad. Biol. Med. 1, 87–95.

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 Verlag, Berlin, pp. 1–27.

Halliwell, B., Gutteridge, J.M.C., 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219, 1–14.

Halliwell, B., 1991. Reactive oxygen species in living systems: source, biochemistry and role in human disease. Am. J. Med. 91 (Suppl. 3C), 14–22.

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.

Heinrichs, H., Brumsack, H.J., Loftfield, N., König, N., 1986. Verbessertes Druckaufschlussystem für biologische und anorganische Materialien. Z. Pflanzenernähr. Bodenk. 149, 350–353.

Herbette, S., Taconnat, L., Hugouvieux, V., Piette, L., Magniette, M.L.M., Cuine, S., Auroy, P., Richaud, P., Forestier, C., Bourguignon, J., Renou, J.-P., Vavasseur, A., Leonhardt, N., 2006. Genome-wide transcriptome profiling of the early cadmium response of *Arabidopsis* roots and shoots. Biochimie 88, 1751–1765.

Küpper, H., Šetlik, I., Spiller, M., Küpper, F.C., Prášil, O., 2002. Heavy metal-induced inhibition of photosynthesis: targets of *in vivo* heavy metal chlorophyll formation. J. Phycol. 38, 429–441.

Landolt, E., 1986. The Family of Lemnaceae: A Monographic Study. Vol. 1, vol. 71. Ver Geobotan Institute ETH, Stiftung Rubel, Zurich, pp. 1–566.

Lewis, M.A., 1995. Algae and vascular plant tests. In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology: Effects, Environmental fate, and Risk Assessment, 2nd ed. Taylor and Francis, Washington, DC, pp. 135–169.

Li, M., Hu, C., Zhu, Q., Chen, L., Kong, Z., Liu, Z., 2006. Copper and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in the microalga *Pavlova viridis* (*Prymnesiophyceae*). Chemosphere 62 (4), 565–572.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382.

Logani, M.K., Davies, R.E., 1980. Lipid oxidation biological effects and antioxidants—a review. Lipids 15, 485–495.

Lowry, O.H., Rosenberg, N.J., Farr, A.L., Randal, R.J., 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265–275.

Luna, C.M., Gonzalez, C.A., Trippi, V.S., 1994. Oxidative damage caused by an excess of copper in oat leaves. Plant Cell Physiol. 35, 11–15.

Ma, M., Zhu, W., Wang, Z., Witkamp, G.J., 2003. Accumulation, assimilation and growth inhibition of copper on freshwater alga (*Scenedesmus subspicatus* 86.81 SAG) in the presence of EDTA and fulvic acid. Aquat. Toxicol. 63, 221–228.

Maksymiec, W., 1997. Effect of copper on cellular processes in higher plants. Photosynthetica 34 (3), 321–342.

Marschner, H., 1995. Mineral nutrition of higher plants, 2nd ed. Academic Press, London.

Mazhoudi, S., Chaoui, A., Ghorbal, M.H., El Ferzani, E., 1997. Response of antioxidant enzymes excess copper in tomato (*Lycopersicon esculentum* Mill). Plant Sci. 127, 129–137.

Meloni, D.A., Oliva, M.A., Martinez, C.A., Cambraia, J., 2003. Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ. Exp. Bot. 49, 69–76.

Mkandawire, M., Dudel, G.E., 2002. Uranium attenuation from tailing waters by floating macrophyte Lemna gibba L. In: Merkel, J.B., Planer-Friedrich, B., Wolkersdorfer, C. (Eds.), Uranium in the Aquatic Environment. Springer, Berlin, pp. 623–630.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405–410.

Murphy, A.S., Eisenger, W.R., Shaff, J.E., Kochian, L.V., Taiz, L., 1999. Early copper-induced leakage of $K^+$ from *Arabidopsis* seedlings is mediated by ion channels and coupled to citrate efflux. Plant Physiol. 121, 1375–1382.

Pang, J., Chan, G.S.Y., Zhang, J., Liang, J., Wong, M.H., 2003. Physiological aspects of vetiver grass for rehabilitation in abandoned metalliferous mine wastes. Chemosphere 52, 1559–1570.

Paya, M., Halliwell, B., Hoult, J.R.S., 1992. Interactions of a series of coumarins with reactive oxygen species. Biochem. Pharmacol. 44, 205–214.

Perales-Vela, H.V., González-Moreno, S., Montes-Horeasitas, C., Cañizares-Villanueva, R.O., 2007. Growth, photosynthetic and respiratory responses to sub-lethal copper concentrations in *Scenedesmus incrassatulus* (*Chlorophyceae*). Chemosphere 67, 2274–2281.

Pomati, F., Netting, A.G., Calamari, D., Neilan, B.A., 2004. Effects of erythromycin, tetracycline and ibuprofen on the growth of *Synechocystis* sp. and *Lemna minor*. Aquat. Toxicol. 67, 387–396.

Prasad, M.N.V., Malec, P., Waloszek, A., Bojko, M., Strzalka, K., 2001a. Physiological responses of *Lemna trisulca* L. (duckweed) to cadmium and copper bioaccumulation. Plant Sci. 161, 881–889.

Prasad, M.N.V., Greger, M., Smith, B.N., 2001b. Aquatic Macrophytes. In: Prasad, M.N.V. (Ed.), Metals in the Environment: Analysis by Biodiversity. Marcel Dekker, New York, pp. 259–288.

Rama Devi, S., Prasad, M.N.V., 1998. Copper toxicity in *Ceratophyllum demersum* L. (Coontail), a free floating macrophyte: response of antioxidant enzymes and antioxidants. Plant Sci. 138, 157–165.

Rao, G.N., Runge, M.S., Alexander, R.W., 1995. Hydrogen peroxide activation of cytosolic phospholipase A2 in vascular smooth muscle cells. Biochim. Biophys. Acta 1265, 67–72.

Razinger, J., Dermastia, M., Drinovec, L., Drobne, D., Zrimec, A., Koce, J.D., 2007. Antioxidative responses of duckweed (*Lemna minor* L.) to short-term copper exposure. Environ. Sci. Pollut. Res. 14 (3), 194–201.

Reinheckel, T., Sitte, N., Ullrich, O., Kuckelkorn, U., Davies, K.J.A., Grune, T., 1998. Comparative resistance of 20S and 26S proteasome to oxidative stress. Biochem. J. 335, 637–642.

Romero-Puertas, M.C., Palma, J.M., Gomez, M., del Rio, L.A., Sandalio, L.M., 2002. Cadmium causes the oxidative modification of proteins in pea plants. Plant Cell Environ. 25, 677–686.

Saelim, S., Zwiazek, J.J., 2000. Temperature effects on gas exchange, growth and membrane integrity in high temperature-acclimate *Xylia xylocarpa* seedlings. J. Plant Physiol. 156, 380–385.

Sandalio, L., 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.

Sandamann, G., Böger, P., 1980. Copper-mediated lipid peroxidation processes in the photosynthetic membranes. Plant Physiol. 66, 797–800.

Scandalios, J.G., 1993. Oxygen stress and superoxide dismutases. Plant Physiol. 101, 7–12.

Scandalios, J.G., 2002. The rise of ROS. Trends Biochem. Sci. 27, 483–486.

Slater, T.F., 1984a. Free-radical mechanisms in tissue injury. Biochem. J. 222, 1–15.

Slater, T.F., 1984b. Overview of methods for detecting lipid peroxidation. Methods Enzymol. 105, 283–293.

Srivastava, S., Mishra, S., Tripathi, R.D., Dwivedi, S., Gupta, D.K., 2006. Copper-induced oxidative stress and responses of antioxidants and phytochelatins in *Hydrilla verticillata* (L.f.) Royle. Aquat. Toxicol. 80, 405–415.

Stadtman, E.R., 1990. Metal ion-catalyzed oxidation of proteins: biochemical mechanisms and biological consequences. Free Rad. Biol. Med. 9, 315–325.

Stiborová, M., Doubravová, M., Brezinová, A., Friedrich, A., 1986. Effect of heavy metal ions on growth and biochemical characteristics of photosynthesis of barley (*Hordeum vulgare* L.). Photosysnthetica 20, 418–425.

Teisseire, H., Guy, V., 2000. Copper-induced changes in antioxidant enzymes' activities in fronds of duckweed (*Lemna minor*). Plant Sci. 153, 65–72.

Vajpayee, P., Rai, U.N., Ali, M.B., Tripathi, R.D., Kumar, A., Singh, S.N., 2005. Possible involvement of oxidative stress in copper-induced inhibition of nitrate reductase activity in *Vallisneria spiralis* L. Bull. Environ. Contam. Toxicol. 74, 745–754.

Van Assche, F., Clijsters, H., 1990. Effects of metals on enzyme activity in plants. Plant Cell Environ. 13, 195–206.

Wang, W., 1986. Toxicity tests of aquatic pollutants by using common duckweed. Environ. Pollut. Ser. B 11, 1–14.

Wang, W., 1990. Review: literature review on duckweed toxicity testing. Environ. Res. 52, 7–22.

Weckx, J.E.J., Clijsters, H.M.M., 1996. Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. Physiol. Plant 96, 506–512.

Welch, R.M., Norvell, W.A., Schaefer, S.C., Shaff, J.E., Kochian, L.V., 1993. Induction of iron (III) and copper (II) reduction in pea (*Pisum sativum* L.) roots by Fe and Cu status: does the root-cell plasmalemma Fe(III)-chelate reductase perform a general role in regulating cation uptake? Planta 190, 555–561.

Wieckowski, S., Waloszek, A., 1993. Choroplast pigment photobleaching and its effect on low-temperature fluorescence spectra of chlorophyll in greening cucumber cotyledons. Photosynthetica 29, 509–520.

Wong, D.W.S., Whitaker, J.R., 2003. Catalase. In: Whitaker, J.R., Voragen, A.G.J., Wong, D.W.S. (Eds.), Handbook of Food Enzymology. Marcel Dekker, New York, USA, pp. 389–401.

Yruela, I., 2005. Copper in plants. Braz. J. Plant Physiol. 17, 145–156.