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Ni accumulation and its effects on physiological and biochemical parameters of *Eichhornia crassipes*

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ABSTRACT

Ni accumulation and concomitant physiological and biochemical responses were studied in *Eichhornia crassipes*[1] exposed to different concentrations (1, 2, 3 and 4 mg l^{-1} Ni) for 72 h. *E. crassipes*[1] rapidly sorbed Ni during the first 24 h. Ni concentration in roots was significantly higher than in aerial parts. Continuity of the photosynthetic activity was observed by a significant increase of pigment concentrations during the first 48 h, followed by a decrease at 72 h. In aerial parts, MDA concentration increased significantly during first 48 h at the highest Ni concentration exposure. However, was evidently counteracted by the compensatory protective mechanism of increased antioxidant enzyme activities. In roots, a significant increase in enzyme activities of antioxidant system did not prevent lipid peroxidation observed by an increase in MDA concentration at 72 h of exposure. Our results suggest that *E. crassipes*[1] was able to tolerate Ni stress increasing the activity of its antioxidant system at short exposure times and low Ni concentrations studied.

1. Introduction

The free-floating macrophyte *Eichhornia crassipes*[1] has demonstrated high ability to remove metals from water (Delgado et al., 1993; Maine et al., 2006; Hasan et al., 2007; Alvarado et al., 2008; Mishra and Tripathi, 2009; Hadad et al., 2011). For this reason, this species has been used in constructed wetlands for the treatment of effluents containing heavy metal (Liao and Chang, 2004; Maine et al., 2006; Jayaweera et al., 2008; Chunkao et al., 2012; Smolyakov, 2012). However, metal accumulation in plant tissues can affect different cellular components thereby interfering with the normal metabolic functions (Monferrán et al., 2009; Sinha et al., 2009; Thomas et al., 2013; Singh and Pandey, 2011; Deng et al., 2014). Some authors have shown that transient and variable metal-induced oxidative stress was observed in different aquatic macrophytes (Devi and Prasad, 1998; Mishra et al., 2006). This could involve the death of the plant affecting the efficiency of a constructed wetland.

Sharma and Dietz (2009) summarized the strong relationship between metal toxicity, redox homeostasis and antioxidant capacity in plants. Reactive oxygen species (ROS) are commonly generated under different stress conditions, including presence of heavy metals (Arora et al., 2002). ROS have strong oxidizing activities that can attack all types of biomolecules leading to membrane peroxidation, ion leakage, inactivation and damage of proteins and DNA (Mithöfer et al., 2004). In the absence of a protective mechanism, plant structure and function will be severely damaged. However, plants have a complex enzymatic antioxidant system which includes superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT) and glutathione reductase (GR), as well as non-enzymatic molecules as ascorbate and glutathione (Noctor and Foyer, 1998; Halliwell, 2006).

Ni is considered as an essential micronutrient for plants (Eskew et al. 1983; Marschner, 1995; Ragsdale, 1998) since it is a constituent of enzymes involved in N metabolism and biological N fixation, being the central ion in the active center of urease (Watt and Ludden, 1999). Ni uptake in plants is carried out mainly

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[1] Capsule: *E. crassipes* has the ability to adjust their physiology and biochemical responses under Ni stress.

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by root systems via passive diffusion, as well as by active transport (Seregin and Kozhevnikova, 2006; Chen et al., 2009). These different uptake mechanisms vary with plant species and Ni form (Vogel-Mikus et al., 2005). High Ni concentrations can be toxic (Parida et al., 2003; Gajewska and Sklodowska, 2005; Drzewiecka et al., 2012), but the antioxidative enzymatic system can be stimulated under Ni stress, helping plants to tolerate high Ni concentrations (Randhawa et al., 2001; Gajewska and Sklodowska, 2007; Jócsák et al., 2008).

Ecophysiological and biochemical changes in different aquatic plants exposed to Ni have been studied. Maleva et al. (2004) reported that Ni concentrations of 0.025 and 0.25 mg ml^{-1}, affected photosensitization and caused a decrease in photosynthesis rate in floating and submerged plants. Zhao et al. (2008) exposed *Hydrocharis dubia* to Ni concentrations between 0.5 and 4 mM, concluding that Ni was strongly phytotoxic at the highest concentrations studied. Singh and Pandey (2011) reported that *Pistia stratiotes* plants exposed to Ni concentrations between 0.01 and 10 mg l^{-1} showed visible toxicity symptoms at high Ni concentrations and also an increase of antioxidant enzyme activities at low Ni concentrations. Doganlar et al. (2012) reported that Ni concentrations between 0.25 and 16 mg l^{-1} caused oxidative stress in *Lemna gibba*. However, there are scarce reports about Ni effects on *E. crassipes*[1] (Hadad et al., 2009, 2011).

Nonetheless, the response to toxicity differs substantially according to plant species, growth stage, culture conditions, Ni concentration and exposure time. Hence, the present study purpose is to contribute to a better understanding of the biochemical responses of aquatic macrophytes subjected to Ni stress. For this reason, the aim of this work was to asses Ni accumulation in tissues and its physiological and biochemical effects on *E. crassipes*.[1]

2. Material and methods

2.1. Experimental setup

Young plants of *E. crassipes*[1] and 100 l of water were collected from an unpolluted pond of the Paraná River floodplain. Before the experiment, plants were acclimated in the laboratory under controlled conditions, photoperiod of 11–13 h (light-darkness period), temperature of 24 ± 2 °C, 1400 μmol m^{-2} s^{-1} of photosynthetic photon flux density, and humidity of 50 ± 10%. After 7 days, only young healthy plants, of a uniform size and a similar weight (30–40 g) were selected for experimental purposes.

One plant and two liters of pond water were disposed in plastic experimental pots. Pond water was used to simulate natural aquatic system conditions. The chemical composition of the pond water used in the experiment was (mean ± standard deviation): conductivity = 124 ± 1 μS cm^{-1}; dissolved oxygen (DO) = 7.6±0.10 mg l^{-1}; soluble reactive phosphorus (SRP) = 0.035 ± 0.002 mg l^{-1}; $N–NH4^{+}$ = 0.550 ± 0.019 mg l^{-1}; $N–NO_3^{-}$ = 0.651 ± 0.005 mg l^{-1}; $N–NO_2^{-}$ = 0.008 ± 0.001 mg l^{-1}; Ca^{2+} = 10.3 ± 0.8 mg l^{-1}; Mg^{2+} = 3.8 ± 0.5 mg l^{-1}; Na^{+} = 13.7 ± 1.0 mg l^{-1}; K^{+} = 3.50 ± 0.5 mg l^{-1}; Cl^{-} = 10.6 ± 1.3 mg l^{-1}; SO_4^{2-} = 8.0 ± 1.8 mg nl^{-1}; HCO_3^{-} = 51.7 ± 0.8 mg l^{-1}, Fe = 5 μg l^{-1}, Ni = non detected (Detection limit = 5 μg l^{-1}).

Ni solution (prepared using $NiCl_2 \cdot 6H_2O$) was added to obtain concentrations of 1, 2, 3 and 4 mg l^{-1}. Water pH was maintained between 6.3 and 6.6 to avoid metal precipitation. The study was conducted over 3 days, sampling at periods of 24, 48 and 72 h. Treatments and controls (in the absence of Ni) were performed in triplicate for each sampling periods.

After treatments, plants were harvested, rinsed with distilled water, air dried and separated in aerial parts and roots. Afterwards, samples were frozen using liquid nitrogen for storage at −80 °C, only for enzymatic and lipid peroxidation measurements.

2.2. Ni accumulation and translocation

After separation in aerial parts and roots, samples were dried, ground and digested with a HNO_3:HCl mixture (USEPA, 1994). Ni concentrations were determined by atomic absorption spectrophotometry (PerkinElmer AAnalyst 200). Certified standard solutions were used. Detection limits were 20 μg g^{-1}. Determinations were carried out in triplicate.

Translocation factor (TF) was calculated as the ratio of Ni concentration in the aerial parts to that in the roots (Baker and Brooks, 1989).

2.3. Chlorophyll and carotenoid concentrations

Chlorophyll and carotenoid concentrations were estimated using the procedure described by Arnon (1949). Photosynthetic pigments were extracted from fresh leaves using 80% chilled acetone for 48 h in the dark at 3–5 °C without shaking. A pinch of magnesium carbonate was added to protect and stabilize the pigments. Extracts were filtered and measured by spectrophotometry at 663, 646 and 470 nm. Photosynthetic pigments were reported as chlorophyll *a* (Chl-*a*), chlorophyll *b* (Chl-*b*) and carotenoids. The equations reported by Wellburn (1994) were used to calculate pigment concentrations (mg g^{-1} FW).

2.4. Enzyme extraction and protein determination

Enzyme extracts were prepared from individual plants (not pooled). The following steps were carried out at 3–5 °C and performed according to Pflugmacher and Steinberg (1997) with modifications. After being ground with liquid nitrogen, 0.3 g of material plant (roots and aerial parts) were homogenized with 0.1 M phosphate buffer (pH 6.5), containing 20% (v/v) glycerol, 1.4 mM dithioerythritol (DTE) and 1 mM ethylenediaminetetraacetic acid (EDTA), in a ratio 2:1 v/w. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4 °C and the supernatant (crude extract) was kept stored in separate aliquots at −80 °C until the enzyme analyses were done. Protein concentration in the supernatant was determined according to Bradford (1976) using bovine serum albumin as standard.

2.5. Antioxidant enzyme assays

Catalase activity (*CAT, EC 1.11.1.6*) was measured monitoring H_2O_2 decomposition at 240 nm (Maehly and Chance, 1954). The final volume reaction mixture contained 50 mM phosphate buffer (pH 7.0), 30% H_2O_2 and the crude extract. Guaiacol peroxidase activity (*POD, EC 1.11.1.7*) was assayed using the procedure of Bergmeyer (1974), in which decomposition of hydrogen peroxide by peroxidase occurs, with guaiacol as hydrogen donor. Superoxide dismutase activity (*SOD, EC 1.15.1.1*) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to Beauchamp and Fridovich (1971). Glutathione reductase activity (*GR, EC 1.6.4.2*) was estimated by monitoring the increase in absorbance at 412 nm due to the formation of 2-nitro-5-thiobenzoic acid (TNB) (Smith et al., 1988).

2.6. Lipid peroxidation

In the plant samples (roots and aerial parts), the level of lipid peroxidation was estimated by measuring the concentration of malondialdehyde (MDA), the major thiobarbituric acid (TBA)-reactive material, as described by Heath and Packer (1968). MDA concentrations were calculated following the equation presented by Hodges et al. (1999).

2.7. Statistical analysis

Data are the mean values of triplicates and are expressed as means ± standard deviation (SD). After verifying the assumptions of normality and homoscedasticity, analysis of variance (ANOVA) was applied to determine whether significant differences existed in chlorophyll and carotenoid concentrations, lipid peroxidation, enzyme activities and metal tissue concentrations (aerial parts and roots) between the different Ni concentrations and exposure times. Duncan's multiple range test was performed to evaluate significant differences between treatments ($p < 0.05$). SPSS Statistics v20.0.0 software was used for all statistical calculations.

3. Results

3.1. Ni accumulation and translocation

Ni concentration in plant tissues (aerial parts and roots) increased both in terms of the exposure times and initial metal concentration in water (Fig. 1). The highest Ni concentrations were determined in roots, being found concentrations 10 times higher than in aerial parts. The highest Ni concentrations in aerial parts and roots were 0.29 ± 0.02 and 3.34 ± 0.26 mg g^{-1} DW, respectively, detected when plants were exposed to 4 mg l^{-1} Ni after 72 h of treatment. The greatest Ni accumulation was observed during the first 24 h both in aerial parts and roots. Ni concentrations in aerial part continued increasing significantly along time. In roots, Ni concentration did not increase significantly along time after the first 24 h, at the two lowest concentrations but continued increasing significantly at the two highest concentrations.

Calculated TFs (between 0.027 and 0.093) showed that Ni was scarcely translocated to the aerial parts (Table 1).

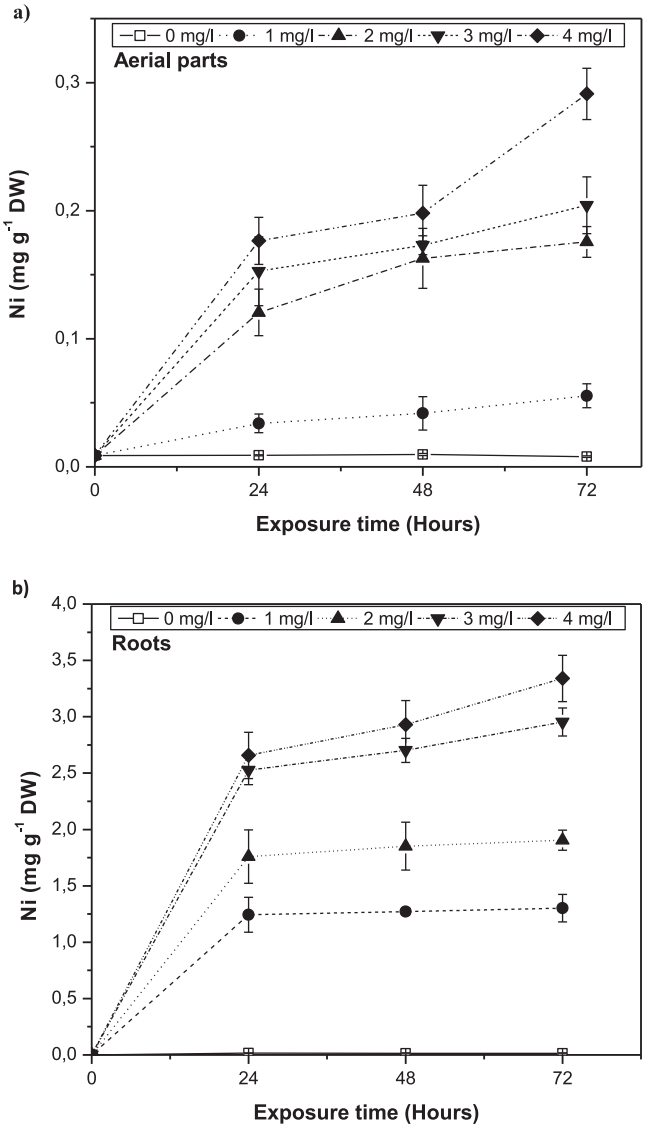

Fig. 1. Ni accumulation in aerial parts and roots of *E. crassipes* at different concentrations and exposure times. All the values are mean of triplicates ± SD.

3.2. Effects of Ni on photosynthetic pigments

Photosynthetic pigments concentrations were significantly affected by the different Ni treatments in *E. crassipes*[1] (Fig. 2). Ni produced a significant increase in Chl *a* for concentrations of 3 and 4 mg l^{-1} in the first 24 h of treatment. Comparing with the control, a significant increase in Chl *a* concentrations was observed for all treatments at 48 h. The increases observed in Chl *a* varied between 12.11 and 45.85 % above the control value. Then, Chl *a* concentrations decreased for all Ni treatments at 72 h, not presenting significant differences regarding the control.

Regarding the control, Chl *b* concentration increased significantly for all Ni treatments at 24 h while it presented significant differences only for 2 and 3 mg l^{-1} Ni at 48 h. Increases for more than 50% were observed over the control at 24 (3 and 4 mg l^{-1} Ni) and 48 h (2 mg l^{-1} Ni). Then, at 72 h Chl *b* concentrations decreased for all Ni concentration compared with those obtained for the others times exposure, but without significant differences regarding the control.

Carotenoid concentrations increased significantly for the three highest Ni concentrations at 48 h (Fig. 2). Then, carotenoid concentrations decreased significantly for 2, 3 and 4 mg l^{-1} Ni treatments at 72 h. The lowest Ni concentration did not show significant differences with the control along the experiment, suggesting that this pigment was not affected.

3.3. Effects of Ni on antioxidant enzyme activity

Enzyme activities were affected by Ni exposure in aerial parts and roots (Figs. 3 and 4).

In aerial parts (Fig. 3), CAT activity increased significantly only at 24 h for the two highest Ni concentrations and at 48 h for the two lowest concentrations. The increases observed varied between 15.33 and 44.91% above the control values. A significant decrease of about 35% below the control values was observed after 72 h of exposure, for the two highest Ni concentrations. In aerial parts, there was a significant increase in POD activity with increasing Ni concentration and exposure times, except for 1 mg l^{-1} Ni. The increases observed varied between 87.02 and 420.83% above the control.

SOD activity increased significantly in aerial parts for all treatments, except for 1 mg l^{-1} Ni treatment at 24 h. It is important to note that for the two lowest Ni concentrations, SOD activity

Table 1
Translocation factor for Ni accumulation in plants.

Ni concentrations	Exposure time (h)		
(mg l^{-1})	24	48	72
1	0.027	0.033	0.043
2	0.068	0.093	0.092
3	0.060	0.062	0.072
4	0.066	0.068	0.087

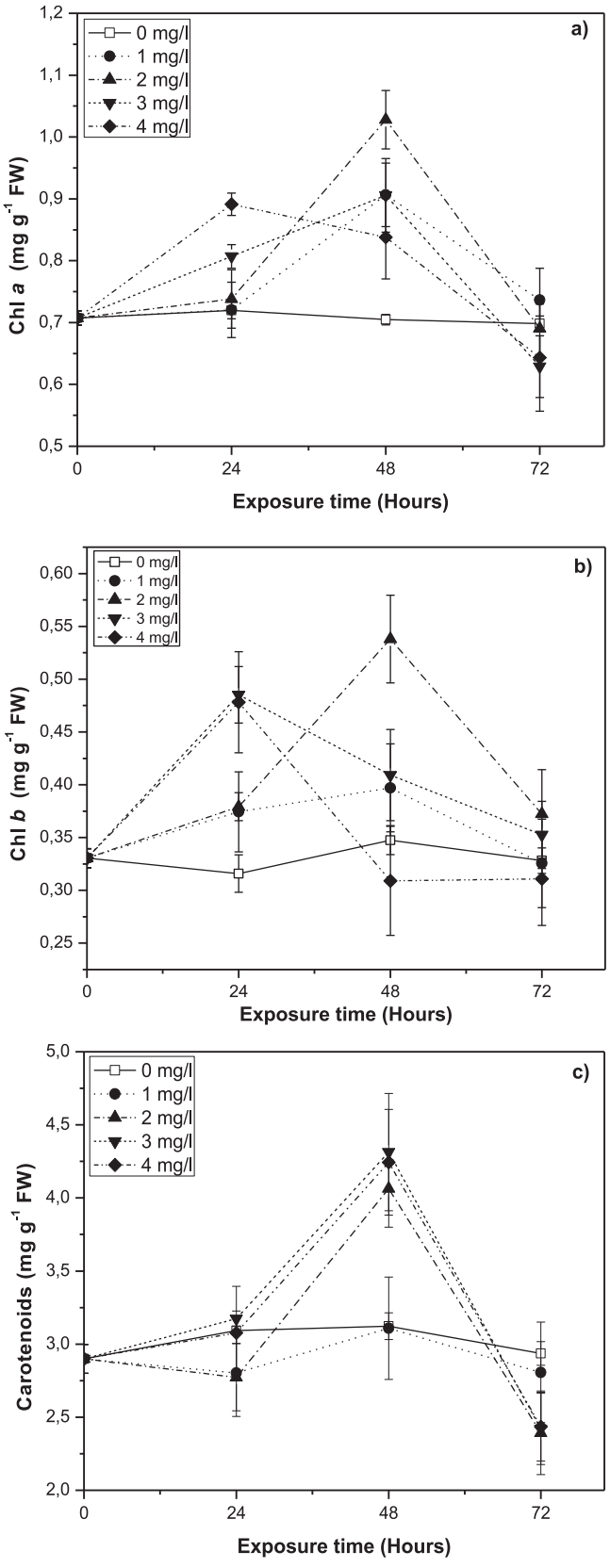

Fig. 2. Effects of Ni on photosynthetic pigments in the leaves of *E. crassipes*. All the values are mean of triplicates ± SD.

increased during the first 48 h, and then it remained almost constant. While for the two highest Ni concentrations, SOD activity increased outstandingly during the first 24 h, decreasing thereafter. The increases varied between 49.99 and 228.86% above the control values. GR activity showed a significant increase for all Ni treatments after 48 h of exposure. Then, for the two lowest Ni concentrations the GR activity remained almost constant, being significantly lower than those of two highest Ni treatments at the end of the experiment.

In roots (Fig. 4), CAT activity for the two lowest Ni treatments did not show significant differences with the control along time. While a significant increase was observed in CAT activity at the first 24 h for the two highest Ni concentrations, followed by a decrease in the enzyme activity. At 72 h, CAT activity decreased significantly by more than 20% below the control. POD and SOD activities increased significantly regarding the control for all Ni concentrations and exposure times. The POD activity increased between 47.13 and 205.11% above the control. While, SOD activity increased between 30.67 and 201.77% above the controls. No significant changes were observed during the first 48 h for GR activity in roots. A significant increase was observed for the two highest Ni concentrations at 72 h.

3.4. Effects of Ni on lipid peroxidation

The effects of Ni on MDA concentration are presented in Fig. 5. In aerial parts a significant increase was observed in the first 48 h, for the two highest concentrations, while MDA at the two lowest concentrations did no presented significant differences with the control during the first 48 h. MDA concentrations in aerial parts decreased significantly at 72 h at all treatments.

In roots, a significant decrease in lipid peroxidation was observed during the first 48 h, except for the highest Ni concentration. MDA concentrations increased significantly at 72 h for all the treatments, not presenting significant differences with the control at the two lowest Ni treatments. However, the two highest Ni concentrations were significantly higher than the control, being observed a rise in lipid peroxidation with the increasing Ni concentrations.

4. Discussion

In agreement with literature, *E. crassipes*[1] plants showed a significantly higher Ni concentration in the roots than in the aerial parts (Turnquist et al., 1990; Zhu et al., 1999; Soltan and Rashed, 2003; Hadad et al., 2009, 2011; Hammad, 2011). Both in aerial parts and roots, the greatest Ni accumulation occurred in the first 24 h. Then, Ni concentration in roots continued increasing slowly at the two highest Ni concentrations. This rapid initial Ni uptake followed by a slow phase is in agreement with that described by Turnquist et al. (1990), who found that *E. crassipes*[1] subjected to Ni exposure exhibited two uptake phases, suggesting that at the highest Ni concentrations the ion-exchange sites at the roots (diffusion process) were quickly saturated causing the uptake process to be slowed (Turnquist et al., 1990).

TFs values indicated a poor Ni translocation. It has been widely reported that metals were accumulated in root tissues as a tolerance strategy (Taylor and Crowder, 1983; Sinha and Gupta, 2005; Hadad et al., 2009, 2011; Mufarrege et al., 2010; Vymazal, 2011; Hechmi et al., 2014). In addition, Seregin and Kozhevnikova (2006) reported that Ni retention in roots may be due to sequestration in the cation exchange sites of the walls of xylem parenchyma cells and immobilization in the root cell vacuoles. Metal confinement in the roots is known to be a primary defense process in order to protect the photosynthetic apparatus in the leaves (Drazkiewicz and Baszynski, 2005).

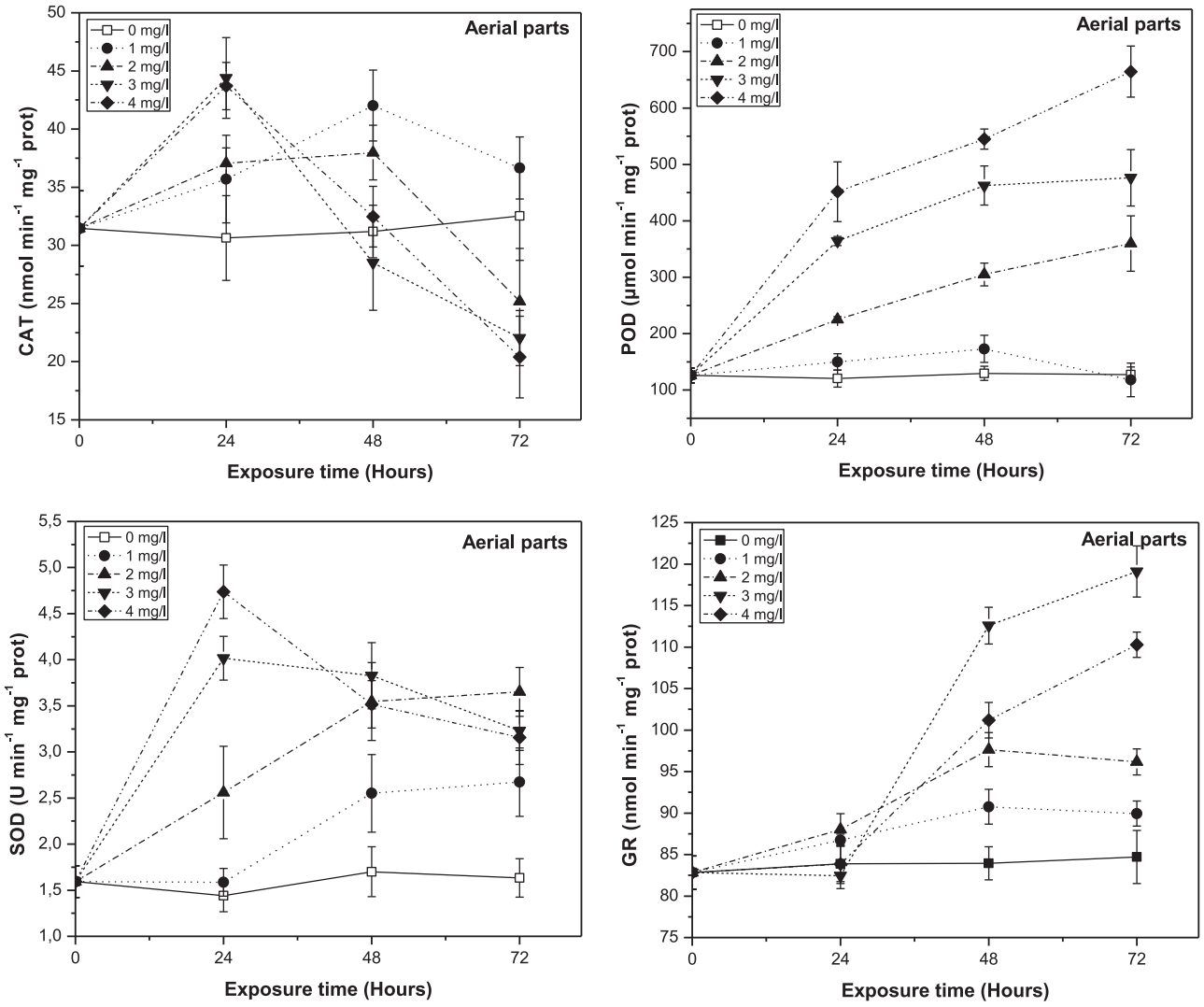

Fig. 3. Effects of Ni on antioxidant enzyme activities of *E. crassipes*, in aerial parts. All the values are mean of triplicates ± SD.

Chlorophyll concentration in plants is a good toxicity indicator for different metals (Burton et al., 2004; Kolotov et al., 2004). The impairment of the photosynthetic machinery in plants exposed to an excess of Ni is reported in the literature (Seregin and Kozhevnikova, 2006). Even so in our study, chlorophyll concentrations presented significant increases regarding the control at 24 and 48 h of exposure, decreasing at 72 h but without showing significant differences with the control. A similar response was observed by Gopal et al. (2002), who reported that at Ni concentration of 5.87 mg l^{-1} or below, *Vigna radiata* plants showed a positive growth and an increase in photosynthetic pigment concentrations. In the same sense, the increase in the concentration of photosynthetic pigments may be due to a strongly improvement in urea-N assimilation by the Ni supplement, because chlorophyll is an N-containing compound (Baker, 1989). Carotenoids also showed a significant increase at 48 h, followed by a significant decrease at 72 h. This response was not surprising, considering that carotenoids are essential components of the photosynthetic apparatus as an antenna pigments but also as essential protectors against the photooxidative damage, acting as scavengers of free radicals to avoid lipid peroxidation (Hou et al., 2007). The carotenoid concentration decrease observed at 72 h might be an outcome of increase oxidative degradation of these pigments by the imposed oxidative stress.

It is known that when Ni concentrations rise above a tolerable limit, the metal can induce the generation of ROS in plants (Boominathan and Doran, 2002) and therefore it can be generate oxidative stress, as evidence suggests in the present work and others (Rao and Sresty, 2000; Gonnelli et al., 2001; Gajewska et al., 2006). Discrepancies between data concerning the response of plant antioxidant enzymes to Ni stress may be explained by differences in plant tolerance, varying experimental conditions as well as the Ni concentrations used. According to literature, information concerning the antioxidant defense responses of *E. crassipes*[1] to heavy metals treatment is available (Odjegba and Fasidi, 2007; Narang et al., 2008a,b; González et al., 2014; Gupta, 2014; Malar et al., 2014; Puzon et al., 2014). In our study, the antioxidant enzymes showed variable responses to different Ni concentrations and exposure time. A generalized significant increase in CAT, POD, SOD and GR activities was observed. Enhancement of the antioxidant enzyme activities observed in *E. crassipes*[1] in response to Ni exposure has also been reported in crop plants such as *Zea mays* (Baccouch et al., 1998, 2001) and *Oryza sativa* (Maheshwary and Dubey, 2009) and in the

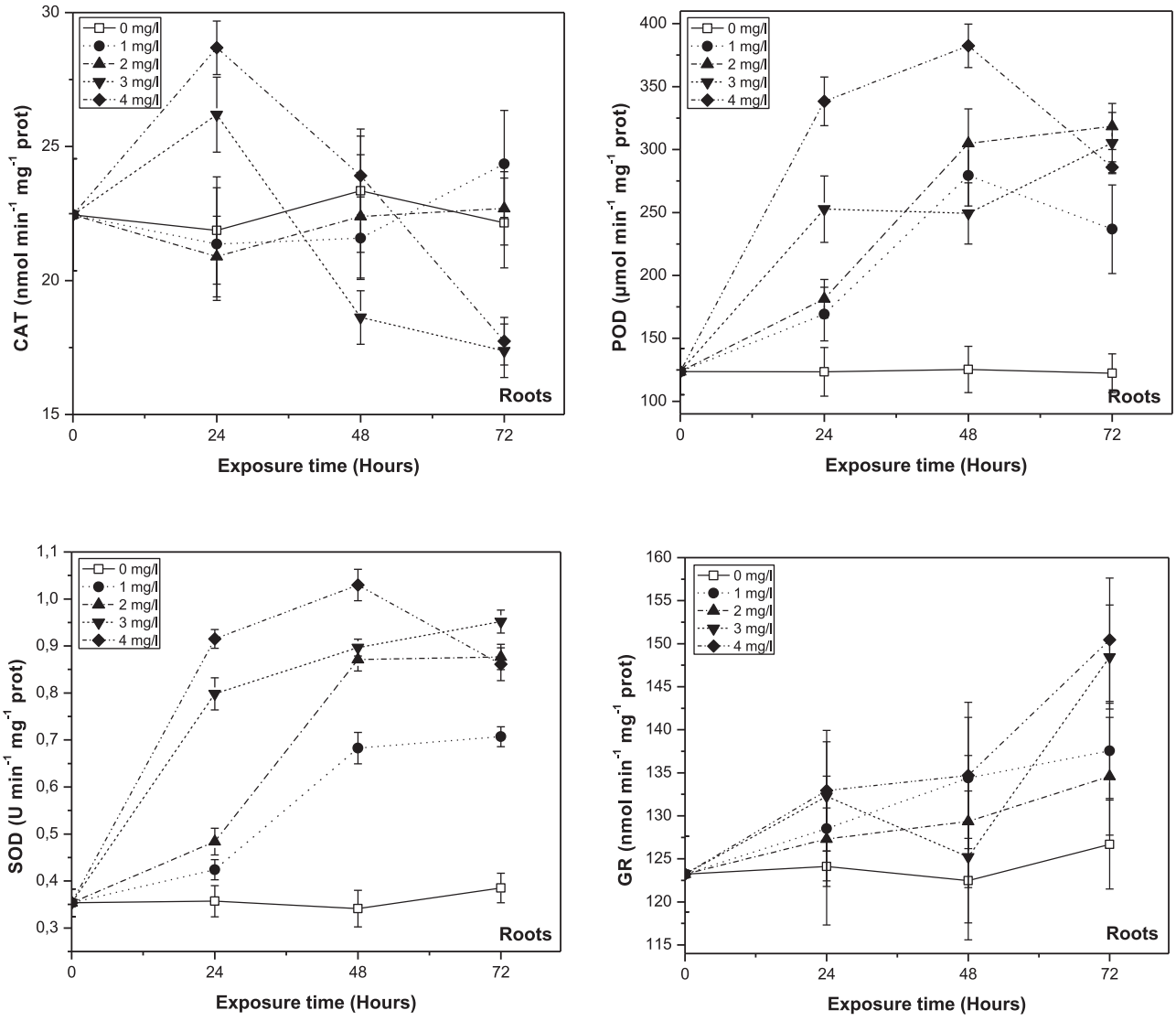

Fig. 4. Effects of Ni on antioxidant enzyme activities of *E. crassipes*, in roots. All the values are mean of triplicates ± SD.

macrophytes *Hydrilla verticillata* (Sinha and Pandey, 2003), *Nasturtium officinale* (Duman and Ozturk, 2010), *P. stratiotes* (Singh and Pandey, 2011) and *L. gibba* (Doganlar et al., 2012). It is important to note that CAT had a significant decrease in activity both in aerial parts and roots for the highest Ni concentrations at the end of the experiment. Decline in CAT activity might be attributed to excess production of ROS, probably by inactivating the enzyme-bound to heme group (Willekens et al., 1997).

MDA is the principal product of lipid peroxidation when plants are under oxidative stress, and it is often used as an indicator of the extent of oxidative damage (Chen et al., 2009). In the present study, MDA concentration increased significantly in aerial parts at the first 48 h and for the two highest Ni concentrations. It could be due to that aerial parts are more sensitive to ROS increase caused by Ni concentration increase in this tissue. However, the observed significant increase in the antioxidant enzyme activities resulted in a decrease of the oxidative damage evidenced by a decrease in MDA concentration at the end of the experiment. This response was also observed in the presence of Pb in aerial parts and roots of *E. crassipes*[1] (Malar et al., 2014).

In roots, a significant decrease in MDA concentration was observed at first 48 h which indicated that a protective antioxidant enzymatic mechanism operated during the first hours at the lowest Ni concentrations. This is in concordance with the increase in the antioxidant enzyme activities observed. At the two highest Ni concentrations the continuous metal accumulation in roots (Fig. 1) maintained an elevated ROS concentration, producing oxidative cell damage in agreement with the increase in MDA concentration at the end of the experiment (Fig. 5).

Most similar studies used an enriched culture medium, such as Hoagland's solution. It is also important to highlight, that the present study was performed using pond water as medium, simulating growth conditions of plants in natural environments. In our experiment, plants were grown under sub-optimal growth conditions regarding the classic mineral Hoagland's solution (Hoagland and Arnon, 1950). Poschenrieder et al. (2013) established that test plants growing in suboptimal growth conditions can uptake low concentrations of toxic ions contributing to stress alleviation. This could explain why in some cases our results are not in agreement with other authors.

According to the obtained results, increased photosynthetic pigment concentration and enzyme activities indicated the ability of *E. crassipes*[1] to tolerate moderate Ni concentrations.

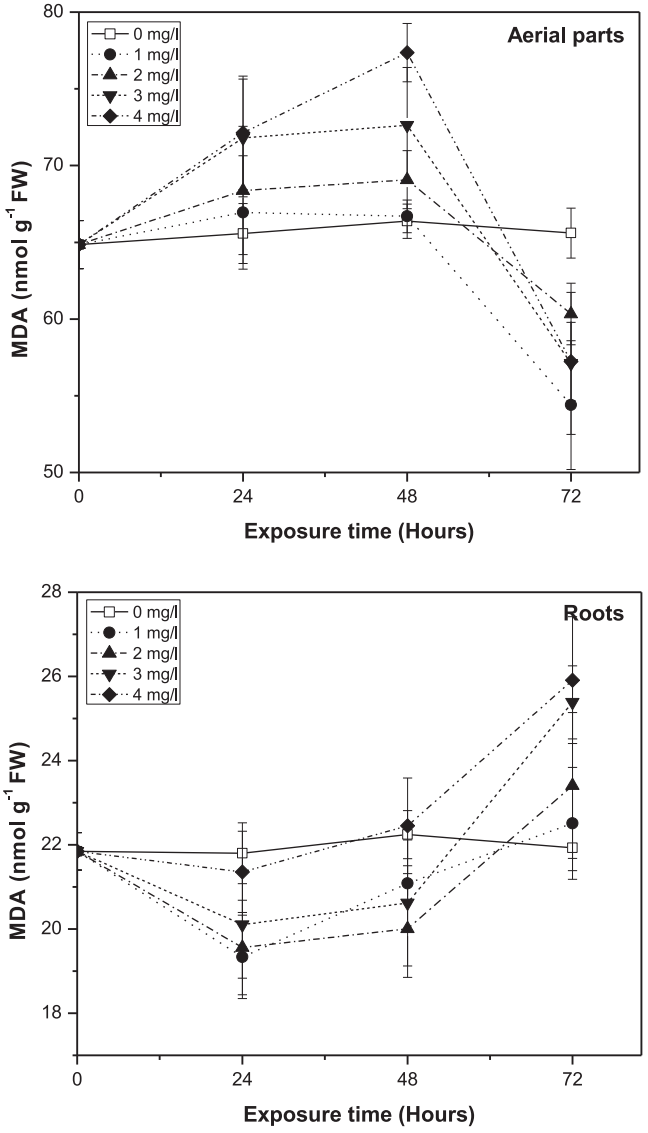

Fig. 5. Effects of Ni on lipid peroxidation of *E. crassipes*, in aerial parts and roots. All the values are mean of triplicates ± SD.

5. Conclusions

- Higher Ni accumulation was observed in roots than in aerial parts.
- In aerial parts and roots, Ni accumulation occurred mainly during the first 24 h.
- An increase in MDA concentrations in aerial parts and in roots were observed for the two highest Ni treatments at the end of the experiment, indicating oxidative damage.
- Rapid increase in antioxidant defense response was observed at all Ni treatments, ensuring redox homeostasis. Further work is needed to understand the total antioxidant defense mechanism in response to Ni concentrations, exposure times and plant species.
- Under Ni stress *E. crassipes*[1] showed ability to adjust their physiology, maintaining the photosynthetic activity in all treatments.
- *E. crassipes*[1] tolerated all Ni treatments during short exposure times by stimulation of its antioxidant enzymatic defense system.

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