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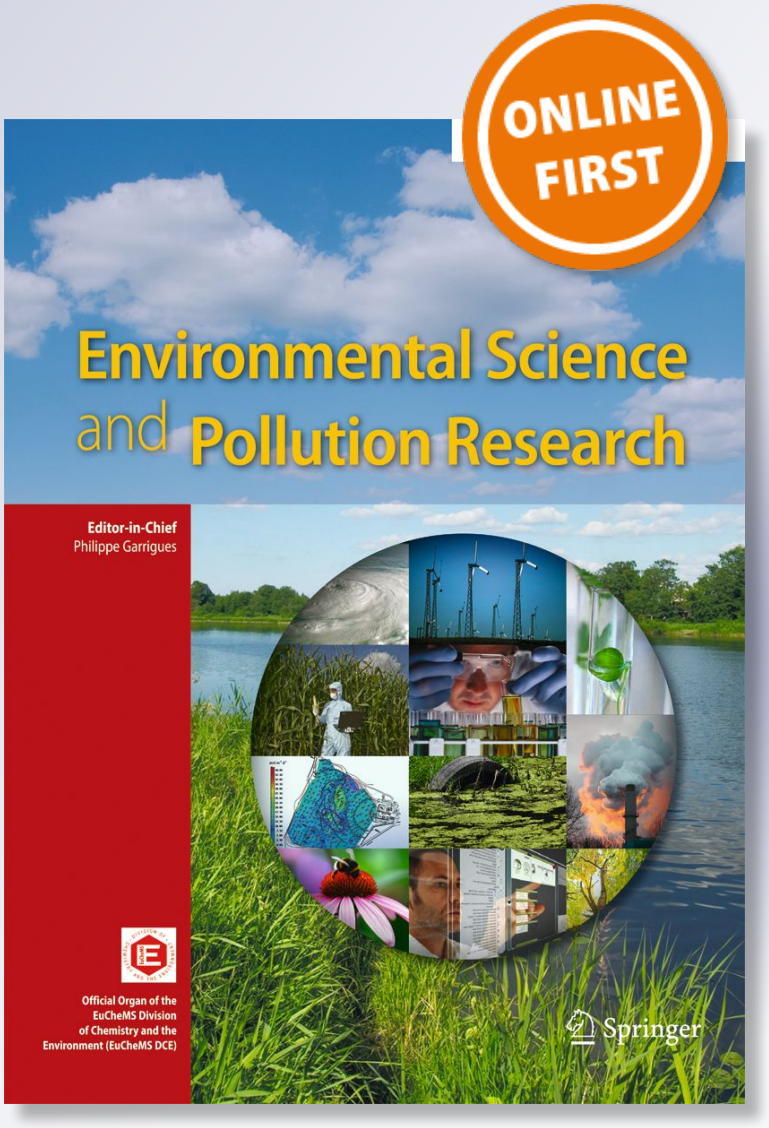

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RESEARCH ARTICLE

Phytoextraction of Pb, Cr, Ni, and Zn using the aquatic plant *Limnobium laevigatum* and its potential use in the treatment of wastewater

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Abstract In order to study the bioaccumulation of Pb, Cr, Ni, and Zn and the stress response, the floating aquatic plant *Limnobium laevigatum* was exposed to increasing concentrations of a mixture of these metals for 28 days, and its potential use in the treatment of wastewater was evaluated. The metal concentrations of the treatment 1 (T1) were Pb 1 μg L^{-1}, Cr 4 μg L^{-1}, Ni 25 μg L^{-1}, and Zn 30 μg L^{-1}; of treatment 2 (T2) were Pb 70 μg L^{-1}, Cr 70 μg L^{-1}, Ni 70 μg L^{-1}, and Zn 70 μg L^{-1}; and of treatment 3 (T3) were Pb 1000 μg L^{-1}, Cr 1000 μg L^{-1}, Ni 500 μg L^{-1}, and Zn 100 μg L^{-1}, and there was also a control group (without added metal). The accumulation of Pb, Cr, Ni, and Zn in roots was higher than in leaves of *L. laevigatum*, and the bioconcentration factor revealed that the concentrations of Ni and Zn in the leaf and root exceeded by over a thousand times the concentrations of those in the culture medium (2000 in leaf and 6800 in root for Ni; 3300 in leaf and 11,500 in root for Zn). Thus, this species can be considered as a hyperaccumulator of these metals. In general, the changes observed in the morphological and physiological parameters and the formation of products of lipid peroxidation of membranes during the exposure to moderate concentrations (T2) of the mixture of metals did not cause harmful effects to the survival of the species within the first 14 days of exposure. Taking into account the accumulation capacity and tolerance to heavy metals, *L. laevigatum* is suitable for phytoremediation in aquatic environments contaminated with moderated concentrations of Cr, Ni, Pb, and Zn in the early stages of exposure.

Keywords Bioaccumulation · Heavy metals · Floating macrophyte · Tolerance · Phytoextraction · *Limnobium laevigatum*

Highlights
- Accumulation of Pb, Cr, Ni, and Zn in roots was higher than in leaves of *L. laevigatum.*
- This species can be considered as a hyperaccumulator of Ni and Zn.
- The toxicity of metals does not represent any danger to the survival of macrophytes.
- *L. laevigatum* is a species of interest for use in phytoremediation of wastewater.

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Introduction

Water is very important to life and the economic development of any region in the world. It is for this reason that pollution of aquatic environments is one of the major problems worldwide, especially for developing countries and emerging economies, with consequent risks for human health through consumption of water and/or contact with contaminants, as well as for the aquatic ecosystem (Smith 2003). The microorganisms, nutrients, heavy metals, organic chemicals, oils, and suspended sediments are the most important pollutants in both freshwater and saltwater. Anthropogenic activities such as mining, agriculture, and industrial plants systematically discharge dissolved pollutants and suspended matter into surface water

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bodies, thereby decreasing the water quality significantly (Wang et al. 2010).

Heavy metals, because of their persistent and non-degradable nature, represent a group of contaminants that once incorporated into the environment are difficult to eliminate. Also, in this group there are many elements considered toxic and extremely dangerous (Hg, Pb, Cd, etc.) due to their transfer and accumulation in different biotic and abiotic compartments (Kunze et al. 2002). While part of the contribution of heavy metals to aquatic ecosystems comes from natural sources, such as lithogenic or geochemical ones, currently the largest contribution of these to aquatic ecosystems is from anthropogenic sources such as storm drains, discharges of industrial effluents, runoff in agricultural and urban land and mining activities, among others. However, in these activities, the contribution of metals is frequently associated with inadequate management of wastewater discharges into surface waters, with waste reaching rivers, streams, and lakes as dissolved or suspended material (Fu et al. 2013). In aquatic environments, heavy metals tend to be combined with mineral substances (carbonates, sulfates, etc.), as well as with organic molecules through phenomena such as ion exchange, adsorption, chelation, and complexation, with these processes explaining why heavy metal accumulation, takes place in the sediments of rivers, lakes, and oceans (Förstner and Wittmann 1981).

In the central region of Argentina, the progressive increase in human activity has contributed systematically to rising emissions of heavy metals, with the consequent deterioration of the environmental quality (Merlo et al. 2011). For some decades, the Argentine people have been aware of the environmental risk posed by the emission, discharge, and disposal of waste containing heavy metals in large quantities. However, despite efforts to comply with environmental legislation, there have been cases where waterways have been used as dumps and discharge effluents have not been treated properly. In addition, heavy metals from agricultural practices also affect water and sediment due to surface runoff (Harguinteguy et al. 2013).

With regard to their effects on living beings, although some metals are essential (as cofactors of metalloproteins and enzymes), at high concentrations they have toxic effects for most life forms (Kabata-Pendias and Pendias 2001). For example, Cu^{2+}, Cr^{3+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} ions are all essential (Kunze et al. 2002) but in great amounts have high toxicity. On the other hand, the nonessentials Hg^{2+}, Cd^{2+}, and Pb^{2+}, which are harmful even in low concentrations, and many metals directly affect biological processes in plants by causing a reduction in growth, inhibition of photosynthesis and respiration, and functional modification of cell organelles (Vangronsveld and Clijsters 1994).

Different studies have shown that heavy metals can reduce the levels of photosynthetic pigments and cause degradation of chlorophylls (Megateli et al. 2009), with the decrease in chlorophyll content in leaves of plants treated with metals has been attributed to alterations in the synthesis of this pigment, as well as increasing degradation (Somashekaraiah et al. 1992). Some authors have reported that many heavy metals (Hg, Cd, Cu, Ni, Zn, and Pb) displace the central Mg atom of chlorophyll in plants that are stressed by their presence (Prasad and Strzałka 1999), which can affect the structure of the chlorophyll molecule, light absorption, and, consequently, photosynthesis. Reduced chlorophyll content is also related to the interaction of metals such as Cr, Zn, Mg, and Fe, with the functional group -SH of enzymes involved in the synthesis of chlorophyll or, for example, acid inhibition δ-aminolevulinic dehydratase (ALAD) with Pb (Prasad and Strzałka 1999), an enzyme also involved in the biosynthesis of chlorophyll. Other mechanisms related to the decrease of pigments include the impediment of the absorption of essential elements for the synthesis of these pigments or from competition with added metals such as Zn (Wang et al. 2009).

Exposure of plants to heavy metals can also result in an increased concentration of reactive oxygen species (ROS), thereby causing oxidative stress (Apel and Hirt 2004), and since lipid molecules and unsaturated lipids are sensitive to oxidation by ROS, lipid peroxidation induced by heavy metals is an indicator of oxidative stress (Metwally et al. 2005). Malondialdehyde (MDA) is one of the major end products of the oxidation of polyunsaturated fatty acids and is a structural component of cell membranes (Li et al. 2013), i.e., is an indicator of the production of free radicals and its consequent tissue damage (Ozturk et al. 2010). In lipid peroxidation, there is reorganization of the double bonds in unsaturated fatty acids, resulting in the formation of hydroperoxide conjugated dienes (HPCD) (Levin and Pignata 1995), which represents a measure of the degree of disruption of the integrity of the cellular membranes exposed to contaminants.

The degree of toxicity of a metal depends on a number of factors, among which, one of the most important is its bioavailability. This is related to the abundance of the heavy metals in the environment, residence time, and the chemical species found, with other factors including the type of metal-forming compounds (salts, minerals, oxides, etc.), temperature, pH, redox potential, the presence of organic matter, dissolved oxygen levels, and the presence of other compounds in the water that can change their chemical state or react with the metals (Winner and Gauss 1986).

In recent years, much more attention has been paid to the methods for the removal of heavy metals in effluents that are discharged into surface waters, such as exchange, reverse osmosis, precipitation, solvent extraction, membrane filtration, electrochemical treatments, and ab-adsorption, among others (Miretzky et al. 2006). However, these techniques generally have a high cost, making them impractical for large-scale application (Olguín and Sánchez-Galván 2012). Thus, phytoremediation, despite being an unconventional method

for treating wastewater, is gaining interest because it has many advantages including its low cost and the fact that it is environmentally friendly (Cheng 2003). In addition, many studies have shown that the use of aquatic macrophytes in constructed wetlands is an effective method for removing heavy metals from contaminated water (Polechońska and Samecka-Cymerman 2016).

Macrophytes commonly show great plasticity of structures, morphology, and behavior in aquatic systems and obtain nutrients through their roots from the sediment or the water column (Jackson 1998). Heavy metals that are not degraded persist for long periods in the environment and can use the same pathways as the nutrients to be absorbed and accumulated by plants (Choi et al. 2006). Moreover, macrophytes accumulate metals in their leaves, which are translocated from the root to the upper parts of the plant or incorporated from the water (Harguinteguy et al. 2016; Sharma et al. 2015). The amount of accumulation of metals in macrophytes is the result, on the one hand, of their concentrations in the water and, on the other, of the time the organisms are exposed to them. According to Samecka-Cymerman and Kempers (2004), contamination of surface waters can be demonstrated by increased concentrations of heavy metals in aquatic plants, their potential in biomonitoring studies, and phytoremediation. Consequently, in recent years, there has been a growing interest in identifying hyperaccumulators, is detected, as these have a great potential for phytoremediation of contaminated water and soil (Duman et al. 2009). Related to this, Zayed et al. (1998) proposed two criteria for the use of aquatic plants as hyperaccumulators of metals: a concentration of metal in the tissue higher than 0.5% of the dry weight of the plant and a bioconcentration factor (BCF) greater than 1000. Where BCF is the most common index used to describe the ability of plants to concentrate metals from the liquid phase. Another indicator, the translocation factor (TF), describes the mobility of the metals in the plant, as well as the translocation of metals from the root to the aerial organs, with TF high values (>1) showing a high capacity for translocation (Soda et al. 2012).

In aquatic systems, there is generally a close relationship between the concentrations of metals in water and plants. However, their concentrations in the tissues also depend on the rate of incorporation and tolerance to the metal, which vary according to the species (Lafont 2002). A species of aquatic plant can be used in phytoremediation of contaminated waters with metal if it meets the following requirements: tolerant to high levels of the metal to be extracted, can accumulate high levels of it in harvestable parts, has a rapid growth, produces high biomass, and possesses an abundant root system (Garbisu and Alkorta 2001).

An aquatic species of interest that has been mentioned in some studies is *Limnobium laevigatum* (Humb. & Bonpl. Ex Willd.) Heine. This perennial aquatic plant from the Hydrocaritaceae family is distributed in countries in the Neotropics (Cook and Urmi-König 1983), and is a floating macrophyte species, which can also grow in very moist soil areas. The main roots, with long root hairs, have a rapid geotropic growth, while secondary roots are thinner with a lower growth. Its stems are short and from these are born petiolated leaves arranged in a rosette form, typically having a pad in the abaxial leaf surface formed by a parenchymal tissue which can be up to 1 cm thick. This plant has two types of reproduction: sexual, through the production of flowers and seeds, and clonal with the production of new clones (ramets) that form part of the same mother plant until separation (Aponte and Pacherres 2013).

Among native aquatic species, *L. laevigatum* (Humb. & Bonpl. Ex Willd) is a promising plant for phytoremediation of wastewater, due to its rapid rate of population growth and high efficiency (close to 80%) in reducing the chemical oxygen demand (COD) (Murillo Castillo et al. 2012). However, this species has not yet been studied to obtain experimental evidence of exposure to contaminants. As native plant species living in aqueous solutions with high levels of metals may constitute suitable bioaccumulators and tolerant species to use in systems experiencing discharge of industrial effluents, the purpose of the present research was to determine the bioaccumulation of Pb, Cr, Ni, and Zn and the tolerance of a floating macrophyte, *L. laevigatum*, to these metals in a culture media in order to evaluate its potential use in wastewater treatment.

Materials and methods

Obtaining plant material and culture media

Similarly sized individuals of the species *L. laevigatum* were collected at Lake San Roque, near the mouth of Las Mojarras stream. The site was selected by considering its almost pristine characteristics (Harguinteguy et al. 2014) and the abundance of this species. Therefore, the collection of the samples had a low impact on the population density.

The collected plants were washed "in situ" and moved to a greenhouse in plastic containers with water from the river. Once there, they were thoroughly washed under running water to remove any remaining material adhered to the surface. Then, 400 g of fresh weight (FW) of similar-sized individuals were placed in aquariums of 100-L capacity and maintained in hydroponics, with the medium being prepared with running water over a nutrient solution 0.10% *V*/*V* (Aponte and Pacherres 2013) [nutrient solution: 5.8 g L^{-1} de KH_2PO_4, 8.5 g L^{-1} of KNO_3 and 5.3 g L^{-1} NH_4NO_3 according to Franzaring et al. (2007)]. The temperature was maintained between 18 and 22 °C, pH 7–8, and the light source was provided by energy-saving lamps of 85 W with irradiation being higher than 100 μE m^2 s^{-1} and a 14:10 h light: dark

cycle. In addition, submersible pumps (brand Baojie, model BL-200) of recirculating water were utilized in order to generate a water stream to prevent the precipitation of particles at the bottom and/or the deposition of these on the surface of roots and leaves. The plants were maintained under these conditions for 4 weeks for acclimation and also during the exposure time to metal treatments.

To evaluate its accumulation capacity, *L. laevigatum* was exposed to three treatments with increasing concentrations of mixed solutions of lead (Pb), chromium (Cr), nickel (Ni), and zinc (Zn), and also to a control group (without added metal). The metals concentrations of the treatment 1 (T1) were Pb 1 μg L^{-1}, Cr 4 μg L^{-1}, Ni 25 μg L^{-1}, and Zn 30 μg L^{-1}; of treatment 2 (T2) were Pb 70 μg L^{-1}, Cr 70 μg L^{-1}, Ni 70 μg L^{-1}, and Zn 70 μg L^{-1}; and of treatment 3 (T3) were Pb 1000 μg L^{-1}, Cr 1000 μg L^{-1}, Ni 500 μg L^{-1}, and Zn 100 μg L^{-1}. The metal concentrations of the treatment 1 (T1) corresponded to the guide levels of the quality of surface freshwater in order to protect aquatic life, as established by the National Hazardous Waste Law (Argentina Legislation 1991) [(Argentina Legislation 1991); National Law 24,051)], with the exception of Cr, whose amount used consisted of twice that established by this law to ensure its subsequent detection at the time of quantification. Considering previous qualitative experiments, the values of metals in T3 were established which ensured survival of the plants.

Each treatment was conducted in triplicate, with twelve aquaria being assigned to random treatments and each experiment starting with 400 g of FW individuals of *L. laevigatum*. At the initial time (day 0) and at 7, 14, 21, and 28 days after starting the treatments, about 100 g of plants were harvested from each aquarium. After draining on absorbent paper, various morphological parameters such as the total number of leaves and chlorotic leaves, the number of ramets, and the number of shoots and long roots (Aponte and Pacherres 2013) were measured, and then the stem-leaf roots were separated. Finally, samples were dried using a camera, lyophilized, and stored in darkness until subsequent analysis of the heavy metals and physiological parameters.

Analysis of heavy metals

Quantification of heavy metals

Lyophilized plant material, corresponding to 1 g of dry weight (DW) of stems and leaves 0.6 g DW root, was made into ashes in a muffle at 450 °C for 4 h. After calcining the samples, they were allowed to cool and the ashes were digested with concentrated HNO_3 (Nekrasova et al. 2011) steeping the suspension for 48 h. The solid residue was filtered using a filter paper of 2.5 μm (Scheicher & Schüll, Blauband 589[3], N°: 300210), and the resulting solution was diluted in ultrapure water to a final volume of 10 mL. Finally, the content of Pb, Cr, Ni, and Zn was analyzed by atomic absorption spectrophotometry flame (FAAS), with control samples being analyzed in the same way. The results were expressed in micrograms per kilogram of DW, and quality control was performed with certified material (± uncertainty for the certified value, with 95% confidence) of leaves of Oriental snuff (ICHTJ-CTA-OTL-1) using the same protocol in order to verify the analytical method.

Determination of bioconcentration factor

The BCF was calculated as the concentration of metal in the plant (mg kg^{-1} of DW) with respect to the concentration (mg L^{-1}) of metal in the culture medium (Olguín and Sánchez-Galván 2012), given by:

$$\mathrm{BCF} = \frac{\textit{concentration of metal in the plant}}{\textit{concentration of metal in the solution}}$$

Translocation factor

The TF was calculated by dividing the concentration of metal accumulated in the air tissue of the plant (mg kg^{-1} of DW) by the metal concentration accumulated in the roots (mg kg^{-1} of DW) (Soda et al. 2012):

$$\mathrm{TF} = \frac{\textit{concentration of metal in the leaf and stem}}{\textit{concentration of metal in the root}}$$

Determination of physiological parameters in *L. laevigatum*

Quantification of carotenoids, chlorophylls, and feofitinas

First, 20 mg of plant material was weighed and 10 mL of ethanol at 96% *V*/*V* was added to this. Then, the sample was homogenized at 1000 rpm for 1min, and filtered with filter paper <10 μm and the absorbance of chlorophylls (665 and 649 nm) and carotenoids (470 nm) was measured in the solution. After adding 1 mL of 0.06 M HCl to 5 mL of chlorophyll extract to achieve the formation of phaeophytins, the optical density (OD) was measured at 654 and 666 nm 10 min later using a UV-visible spectrophotometer (Beckman DU 7000, USA). According to Wintermans and De Mots (1965), the concentrations (mg g^{-1} of DW) of carotenoids, chlorophylls *a*, and phaeophytins *a* (Carot, Chl-*a*, and Pheo-*a*) were calculated.

Quantification of hydroperoxide conjugated dienes

The quantification of hydroperoxide conjugated dienes (HPCD) was performed using the ethanol from the extraction of pigments obtained as described in "Quantification of

carotenoids, chlorophylls, and feofitinas". We collected 0.2 mL of ethanol and the OD of a 1/15 diluted solution (ethanol 96% *V*/*V*) was measured at 234 nm in a UV-Visible spectrophotometer (Beckman DU 7000, USA). The HPCD content was calculated using $\varepsilon = 2.65 \times 104\ M^{-1}\ cm^{-1}$ (Levin and Pignata 1995) and results were expressed in millimoles per gram of DW.

Quantification of malondialdehyde

The concentration of malondialdehyde (MDA) was measured using a colorimetric method, with 50 mg of stem leaf and 70 mg of *L. laevigatum* being homogenized (separately) using 2.5 ml of distilled water at 1000 rpm. Next, to each tissue, an equal volume of TBA (2-thiobarbituric acid) was added at 0.5% *W*/*V* in a solution of TCA (trichloroacetic acid) at 20% *W*/*V*. The samples were then incubated at 95 °C for 30 min and the reaction was stopped with an ice bath, filtered with filter paper <10 μm, and the OD at 532 nm was measured, subtracting the nonspecific absorption at 600 nm (Pignata et al. 2002) with a UV-Visible spectrophotometer (Beckman DU 7000, USA). Finally, the MDA content was calculated using the molar extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$ and results were expressed in micromoles per gram of DW.

Statistical analysis

A statistical analysis of the results was performed using the software InfoStat Version 1.1. A comparison between treatments and exposure times was made by using analysis of variance (ANOVA) for two factors to determine whether there were significant differences at a significance level of $p < 0.01$ and a posteriori test of DGC ($p < 0.05$). Assumptions of normality and homoscedasticity were tested using the Shapiro–Wilk and Levene tests, respectively, and the variables that were not normally distributed were fourth-root transformed prior to statistical analysis. For each treatment, chemical determinations were performed in triplicate, using a statistical analysis of the average values.

Results

Analysis of heavy metals in *L. laevigatum*

Accumulation of lead

The accumulation of Pb in leaves and roots of the aquatic plant *L. laevigatum* showed a significant increase with increasing metal concentration for the different treatments during 28 days of exposure (Fig. 1a), and with accumulation of Pb being at least three times higher in root than in leaf. For each treatment, only Pb accumulation in leaves showed significant differences over exposure time (Fig. 1b), and the maximum concentration occurring at 21 days of exposure for treatment 3. The Pb accumulation over exposure time for each treatment showed no significant differences in roots.

Chromium accumulation

The Cr accumulation in leaves and roots of *L. laevigatum* revealed a significant increase with increasing metal concentration for the different treatments during the 28 days of exposure (Fig. 2a), with the accumulation of Cr in root being four times higher than that accumulated in leaf. The accumulation of Cr over exposure time for each treatment revealed significant differences in the leaves of the floating macrophyte (Fig. 2b), with the maximum accumulation being observed at 14 days for treatment 2 and at 21 days of exposure for treatment 3. The Cr accumulation over exposure time for each treatment showed no significant differences in roots.

Accumulation of nickel

The accumulation of Ni in leaves and roots of the floating macrophyte showed a significant increase with increasing metal concentration for the different treatments during the 28 days of exposure (Fig. 3a), with the Ni accumulation being greater in roots than in leaf. The Ni accumulation over exposure time for each treatment showed significant differences in both leaves and roots in the aquatic plant (Fig. 3b, c), and in both cases, the accumulation of Ni increased over time.

Zinc accumulation

The accumulation of Zn in *L. laevigatum* leaf showed a significant increase with increasing metal concentration for the different treatments during the 28 days of exposure (Fig. 4). In root, the accumulation of Zn rose with increasing concentration in the culture medium, with the exception of treatment 3 (Fig. 4) where a decreased level of accumulation of the metal was observed. Zn accumulation, over exposure time for each treatment, showed no significant differences in both root and leaf (data not shown).

Bioconcentration factor and translocation factor

In Fig. 5, the BCF in leaf and root and the TF of *L. laevigatum* are shown, evaluated in treatment 2 ($0.07\ mg\ L^{-1}$), where equal concentrations were used for all the metals studied. The analysis carried out after 14 days of exposure in order to emphasize the differences between metals and to reduce variation due to the exposure time.

The BCF showed that the concentration of all the metals studied in root and the concentrations of Ni and Zn in leaf were over a thousand times greater than the concentrations

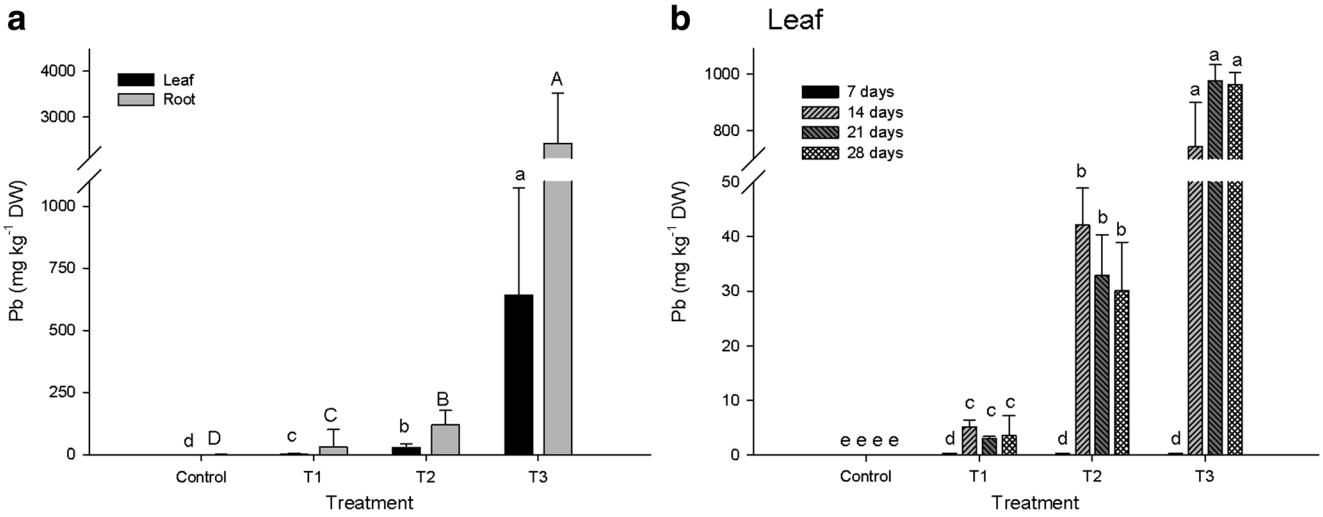

Fig. 1 Accumulation of Pb in *L. laevigatum* for different concentrations of metal during the 28 days of exposure (**a**) and Pb accumulation in leaf during the exposure time for each treatment (**b**). Significant differences ($p < 0.05$) according to the DCG test are shown with *lowercase letters* for leaf and *uppercase letters* for roots

of these in the culture medium, with the highest values of BCF, both in leaf and in root, being observed for Ni and Zn. In all cases, the plants were more bioconcentrated in root than in leaf.

The TF was always lower than one, for all metals studied (Fig. 5). However, the value of TF of 0.42 calculated for Pb was significantly higher than that observed for Cr, Ni, and Zn in the translocation from root to leaf.

Effects of a mixture of Cr, Pb, Ni, and Zn on the physiological parameters in *L. laevigatum*

The synthesis of photosynthetic pigments revealed a decrease of nearly to 30% after treatment of the higher metal concentrations (treatment 3) and close to 20% in the content of chlorophyll *a*, phaeophytin *a,* and carotenoids in leaves after 21 days of exposure (Fig. 6a, b). Oxidation of chlorophylls to phaeophytins showed a significant increase in treatment 3 after 21 days of exposure (Fig. 6a, b).

A significant increase in the levels of malondialdehyde (MDA) was observed in root in treatments 1 and 2 (Fig. 7a), with a significant increase noted in the early stages of exposure and until 28 days (Fig. 7b). Furthermore, the levels of hydroperoxide conjugated dienes (HPCD) showed a significant increase in leaf after 28 days of exposure (Fig. 7c). However, no differences were observed in the synthesis of the lipid membrane peroxidation products (MDA and HPCD) in leaves, or in the levels of HPCD in root for the different treatments (data not shown).

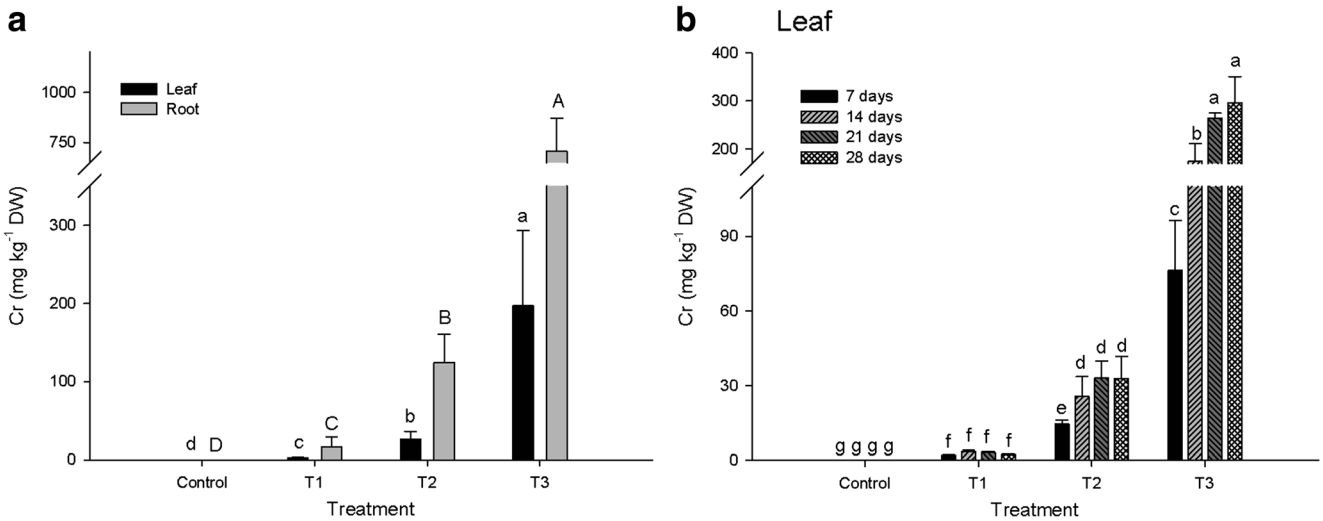

Fig. 2 Accumulation of Cr in *L. laevigatum* for different concentrations of metal during the 28 days of exposure (**a**) and Cr accumulation in leaf during the exposure time for each treatment (**b**). Significant differences ($p < 0.05$) according to the DCG test are shown with *lowercase letters* for leaf and *uppercase letters* for roots

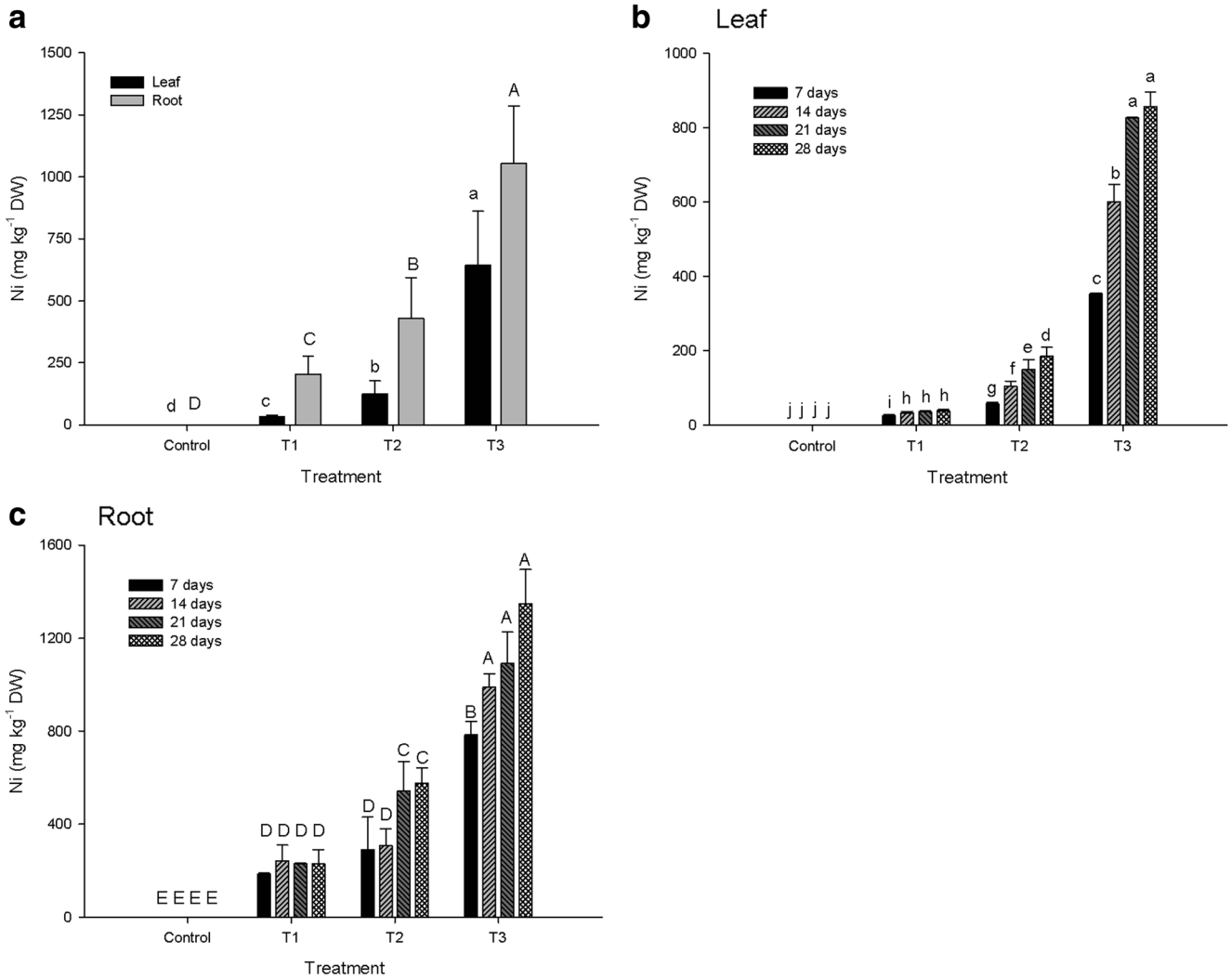

Fig. 3 Accumulation of Ni in *L. laevigatum* for different concentrations of metal during the 28 days of exposure (**a**) and Ni accumulation in leaf during the exposure time for each treatment (**b**) and Ni accumulation in root during the exposure time for each treatment (**c**). Significant differences ($p < 0.05$) according to the DCG test are shown with *lowercase letters* for leaf and *uppercase letters* for roots

Effects of a mixture of Cr, Pb, Ni, and Zn on the morphological parameters in *L. laevigatum*

All the morphological parameters measured reflected differences between treatments and between different times of exposure to metals (Table 1) and the phenological state of the macrophyta *L. laevigatum* throughout the experiment for different concentrations of Cr, Ni, Pb, and Zn (supplementary material Fig. A.1).

Longer roots were observed in the control and treatment of lower metal concentrations. The number of leaves on the plant, number of ramets, and the number of leaves in the ramets increased in treatment 2, while in treatments 1 and 3 these values did not differ from control. Plants in treatments of higher concentrations showed an increase in the number of chlorotic and necrotic leaves, whereas buds revealed a decrease with increasing concentration of heavy metals with the lowest number being observed for treatment 3.

With respect to the exposure time, the root length, number of ramets, and the number of buds were lower after 21 days, with the number of leaves on the ground and ramets being greatest between 21 and 28 days. The number of chlorotic and necrotic leaves increased until 21 days, without variations recorded in these parameters in the later stages of exposure.

Discussion

Accumulation of the heavy metal, BCF, and TF

The accumulation of metals in the macrophyte *L. laevigatum* was concentration dependent, coinciding with other aquatic plants of free-living and floating leaves (Hadad et al. 2011; Mishra and Tripathi 2008; Veselý et al. 2011). Due to its

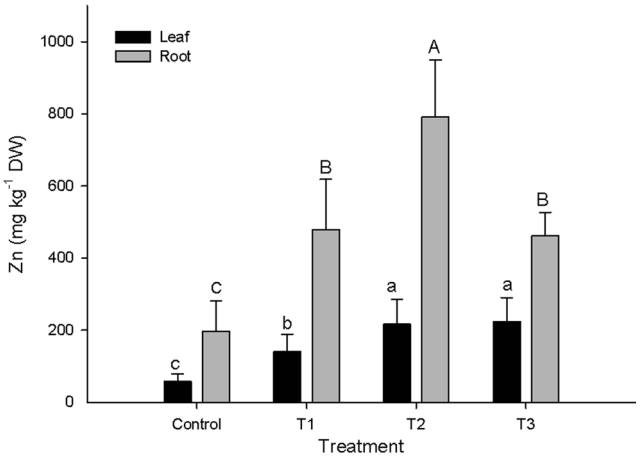

Fig. 4 Accumulation of Zn in *L. laevigatum* for different concentrations of metal during the 28 days of exposure. Significant differences ($p < 0.05$) according to the DCG test are shown with *lowercase letters* for leaf and *uppercase letters* for roots

association with human activity, the toxicity of Pb and Cr has been studied extensively in many plants to assess tolerance to these metals (Andra et al. 2009) and the potential for accumulation, in order to select species suitable for phytoremediation of contaminated environments (Gupta and Chandra 1994). The accumulation of Pb and Cr in *L. laevigatum* was higher than that observed by Veselý et al. (2011) in *Salvinia auriculata, Salvinia minima* and *Azolla filiculoides* and by Mishra and Tripathi (2008) in *Eichornia crassipes* and *Spirodela polyrrhiza*. Overall, the average accumulation of Cr and Pb in this study was four times higher in the root than in the leaf, with this difference being greater than that observed for Pb in *Vallisneria spiralis*, which accumulated around two times more in root than in leaf (Gupta and Chandra 1994), but lower than that observed for Cr in *Salvinia herzogii* and *Pistia stratiote,* which was found to accumulate eight times more in root than in leaf (Maine et al. 2004).

Metal uptake by the root system of plants can be enhanced by the role of these metals as micronutrients (Assunção et al. 2003), as may have occurred in the uptake and bioaccumulation of Ni and Zn in the present study. In *L. laevigatum,* it was found that Ni accumulation in the root was twice as high as that in leaf, with Zn accumulation being three times higher in roots. The values for Ni in this study were higher than those observed in *Nasturtium officinale*, where after being exposed to 1 mg L^{-1} for a week were not significantly different from the control (Duman et al. 2009), and similar to those observed in *Elodea canadensis*, when exposed to 0.5 mg L^{-1} accumulated 700 mg kg^{-1} (Maleva et al. 2009). Here, *L. laevigatum* in the first week managed to accumulate 800 mg kg^{-1} root. In the case of Zn, *L. laevigatum* accumulated higher concentrations than those observed in *P. stratoites* and *S. polyrrhiza* at a concentration of 1 mg L^{-1} Zn after 15 days of exposure (Mishra and Tripathi 2008), but similar in those reported in *Ceratophyllum demersum* after being exposed to 3 mg L^{-1} Zn for 15 days (Umebese and Motajo 2008). After reaching a maximum accumulation of Zn (treatment 2), a significant decrease in the levels of accumulation of this metal in *L. laevigatum* was then observed, which could have been due to competition in the absorption of metals in the culture medium, as it has been demonstrated that the presence of high concentrations of Pb^{2+} prevents the entry of cations Zn^{2+} into the root system (García Vargas 2007).

Regarding the exposure time, in *L. laevigatum* leaves, very low amounts of Pb were accumulated in the first exposure stage, but which significantly increased in the later stages. For Pb and Cr in root, the maximum accumulation was reached in the first exposure step (data not shown), with similar situation being observed for Cr in roots of *S. herzogii* and *P. stratiote*, where Cr uptake was a rapid process that occurred mostly during the first 24 h, and only increased moderately during the rest of the exposure process (Maine et al. 2004).

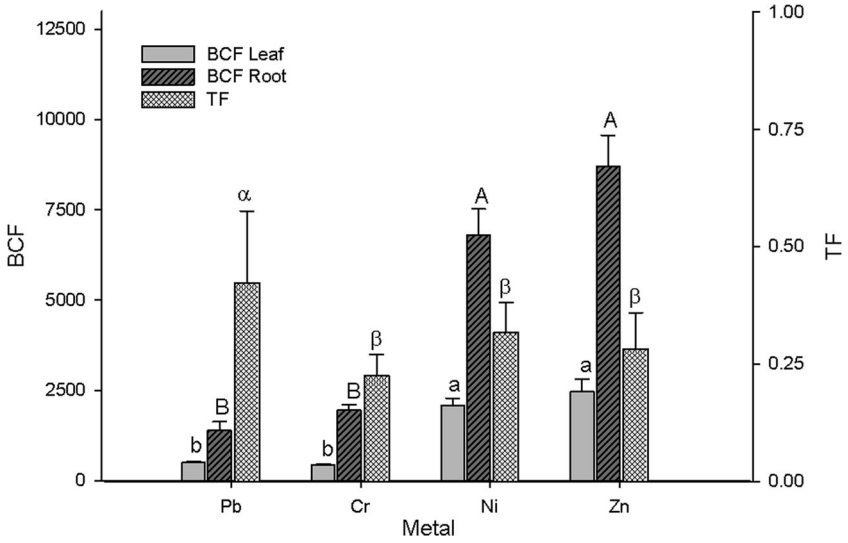

Fig. 5 Bioconcentration factor (BCF) in leaf and root and translocation factor (TF) of *L. laevigatum* evaluated in treatment 2 (0.07 mg L^{-1}) after 14 days of exposure. Significant differences between the BCF of each of the metals are shown in *lowercase le*tters for leaf and *uppercase letters* for root. Significant differences for TF are shown with *Greek letters*

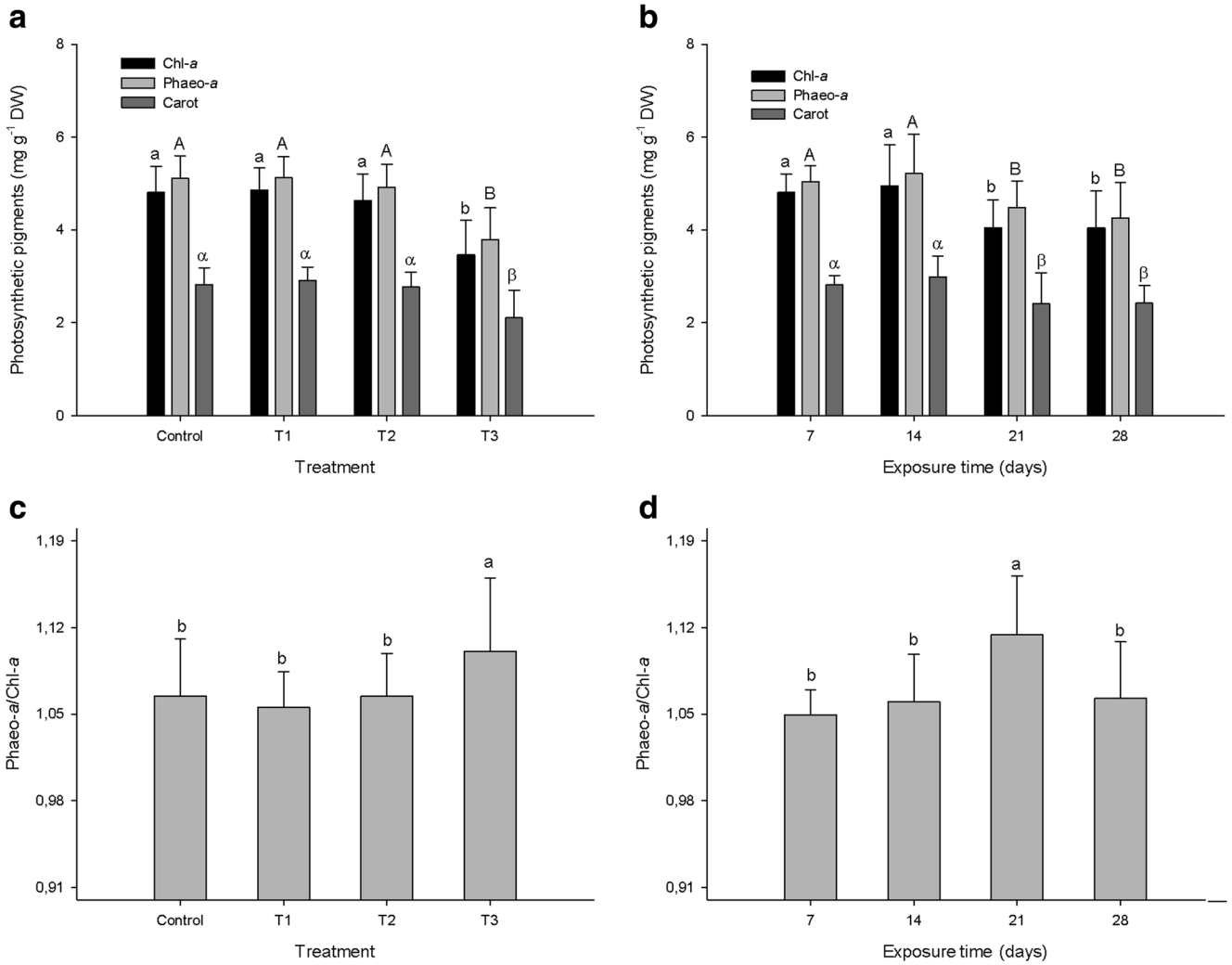

Fig. 6 Concentration of photosynthetic pigments in *L. laevigatum* for different treatments during the 28 days of exposure (**a**) and for different exposure time (**b**) and degradation of chlorophylls for different treatments during the 28 days of exposure (**c**) and for different exposure time (**d**). Values are shown as mean concentration ± SD (n = 12). Significant differences ($p < 0.05$) according to the DCG test are shown in *lowercase letters* for chlorophyll *a* (Chl-*a*) *uppercase* for phaeophytin *a* (Phaeo-*a*) and *Greek letters* for carotenoids (Carot)

The maximum concentration of Ni, both in leaves and in roots, was at 28 days of exposure in the treatment 3. A similar behavior was observed in *E. crassipes*, where the maximum concentration was measured in the intermediate stages of exposure (Hadad et al. 2011). The accumulation of Zn during the exposure time only showed significant differences in *L. laevigatum* roots, with the maximum recorded at 28 days. A similar behavior was also observed in the floating macrophyte *E. crassipes*, where accumulation in the leaf did not vary after 24 h, in contrast to that occurring in the root, where the maximum accumulation was observed in the later stages of exposure (Hadad et al. 2011).

The BCF, which is the capacity of the plant to bioconcentrate metals from the medium, should have a value greater than 1000 in order to consider a plant to be a good accumulator of metals (Zayed et al. 1998). In this study, the BCF revealed that the concentration of Cr, Pb, Ni, and Zn in root and the concentration of Ni and Zn in leaves exceeded over a thousand times the concentrations of these in the culture medium. Moreover, in leaf and root, the highest values observed for BCF were for Ni and Zn, and in all cases, plants had higher concentrations in root than in leaf. These results showed that *L. laevigatum* is a very good accumulator of Ni and Zn and has a moderate capacity for Cr and Pb. Thus, it could be used as a potential phytoextractor for these metals in contaminated waters. For Pb, *L. laevigatum* showed levels of BCF of 1386.12 in root and 499.55 in leaf, with the extraction of Pb, being more efficient than that observed by Singh et al. (2010), who used concentrations of 2 mg L^{-1} Pb and reported a BCF of 582 in *Najas indica*. However, the BCF of *L. laevigatum* was similar to that observed by Wang et al. (2002), who found levels of 624 in the leaf and 1217 in the root of *E. crassipes*. In the case of Cr, the BCF in leaf and root was 440 and 1780 respectively in *L. laevigatum,* higher than

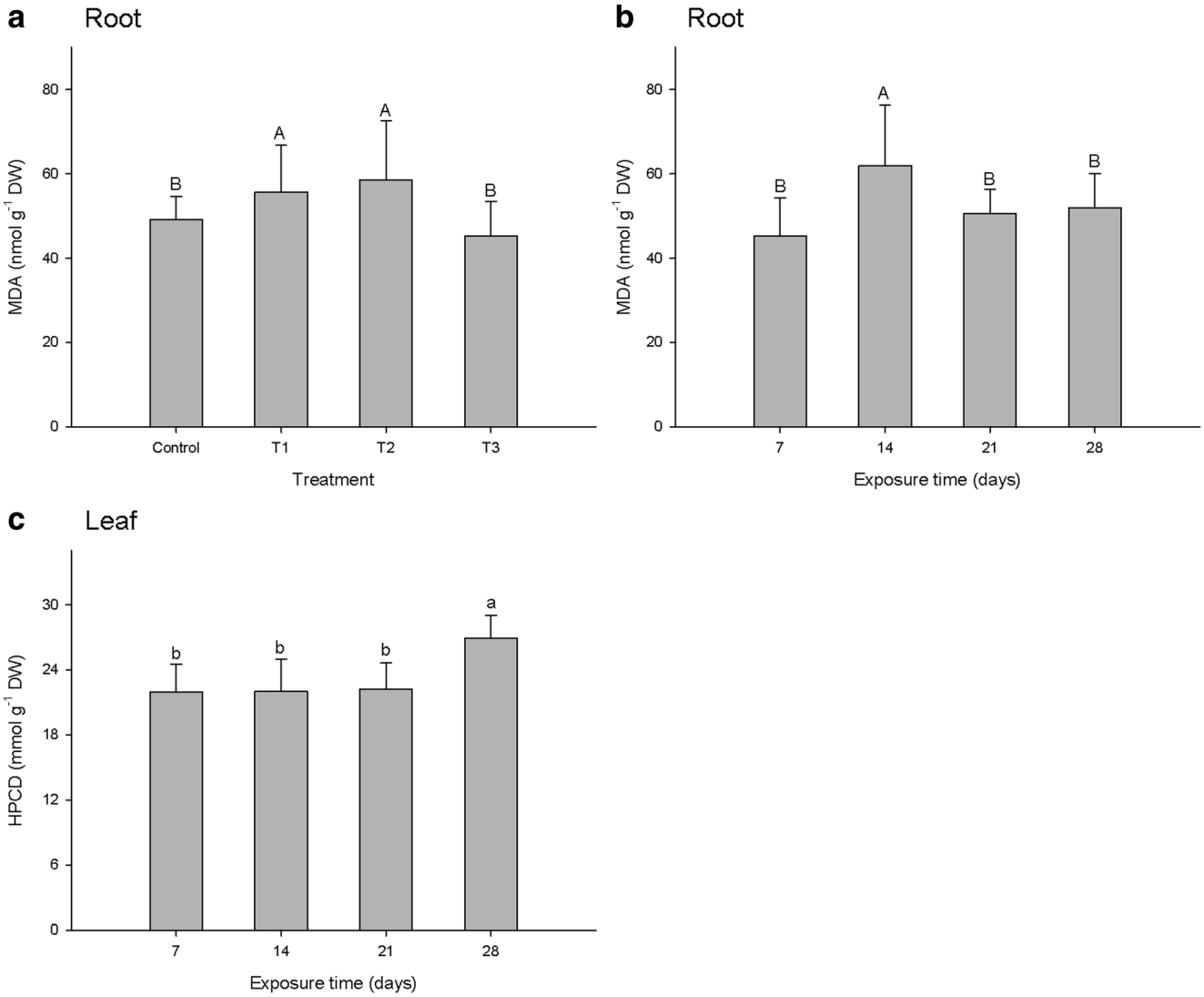

Fig. 7 Levels of malondialdehyde (MDA) in root of *L. laevigatum* for different treatments during the 28 days of exposure (**a**) and for different exposure time (**b**) and of hydroperoxide conjugated dienes (HPCD) in leaf for different exposure times (**c**). Values are shown as mean concentration ± SD (n = 12). Significant differences ($p < 0.05$) according to the DCG test are shown in *lowercase letters* for MDA and *uppercase letters* for HPCD

that observed in *E. crassipes* for the leaf (with a BCF of 160) but lower than that found for the root, where the BCF reached a value of 5600 (Hadad et al. 2011). Moreover, *L. laevigatum* revealed accumulation values higher than those reported by Zurayk et al. (2001), who found that at concentrations of 1 mg kg^{-1} of Cr, *Nasturtium officinale* and *Veronica beccabunga* had BCF values <1000, both in root and in the aerial part of the plant. For Ni, *L. laevigatum* showed BCF levels >1000, being 2000 for leaf and 6800 in root. These results were higher than those found by Liao and Chang (2004) in *E. crassipes* (BCF values of 1370 in leaf and 2200 in root), when exposed to Ni concentrations similar to those of this study. The BCF of Zn for the aquatic plant was 3300 and 11,500 in leaf and root, respectively, being more efficient for the extraction of Zn than *E. crassipes*, which had values of 1500 in leaf and 5800 in root (Yapoga et al. 2013), or than *C. demersum*, which showed very low BCF values of 104 and 138, respectively, in leaf and root (Umebese and Motajo 2008).

The TF is an indicator of the ability of the plant to transport the metals from the roots to the leaves with TF levels greater than 1 indicating a high transfer of metals (Soda et al. 2012). However, in *L. laevigatum,* the TF for all metals was less than 1, which indicated a low transfer of metals from root to leaf. A similar situation was observed for the transfer of Cr and Ni in *N. officinale* and *V. becabunga* (Zurayk et al. 2001) and also the translocation of Pb and Zn in *E. crassipes* (Liao and Chang 2004). During exposure to the mixture of metals, *L. laevigatum* showed a greater capacity for translocation of Pb than for Cr, Ni, and Zn, with similar results being observed in plants of *Lemna minor*, where levels of Pb translocation were

Table 1 Mean values ± SD (n = 12) of the morphological parameters analyzed for different times of exposure to increasing concentrations of heavy metals. An ANOVA analysis was performed to determine significant differences between treatments, exposure time and interaction. Significant differences ($p < 0.05$) according to the DCG test are shown in *lowercase letters*

		Root length	Number of leaves	Number of chlorotic and necrotic leaves.	Number of ramets	Number of leaves in the ramets	Buds
Treatment	Control	12.05 ± 1.38 a	9.39 ± 0.58 b	1.19 ± 0.35 d	1.63 ± 0.59 b	2.76 ± 0.92 b	6.05 ± 1.00 a
	T1	11.62 ± 0.92 a	9.81 ± 1.32 b	1.87 ± 0.96 c	1.84 ± 0.67 b	3.03 ± 0.83 b	5.75 ± 0.81 a
	T2	10.53 ± 2.07 b	10.72 ± 0.83 a	3.24 ± 1.75 b	2.36 ± 0.76 a	4.12 ± 1.08 a	6.41 ± 0.74 a
	T3	9.51 ± 3.38 b	9.99 ± 1.79 b	4.22 ± 1.74 a	1.56 ± 0.86 b	2.98 ± 1.34 b	5.11 ± 1.21 b
	ANOVA	*	**	***	***	***	**
Exposure time (days)	7	12.05 ± 0.93 a	8.95 ± 0.33 b	1.12 ± 0.30 c	1.28 ± 0.05 c	2.57 ± 0.67 b	5.97 ± 0.39 a
	14	12.74 ± 0.9 a	10.88 ± 0.35 a	2.36 ± 0.25 b	2.59 ± 0.09 a	4.13 ± 0.17 a	6.81 ± 0.25 a
	21	9.86 ± 0.49 b	10.76 ± 0.27 a	3.34 ± 0.79 a	2.19 ± 0.25 b	3.74 ± 0.32 a	5.67 ± 0.17 b
	28	9.06 ± 0.74 b	9.31 ± 0.18 b	3.70 ± 0.40 a	1.32 ± 0.11 c	2.44 ± 0.44 b	4.87 ± 0.32 c
	ANOVA	***	***	***	***	**	***
Interaction	ANOVA	ns	*	ns	*	ns	ns

ns not significant

*Significant at the 0.05 probability level, **significant at the 0.01 probability level, ***significant at the 0.001 probability level

significantly higher than those observed for Ni, Zn, and Cr (Kastratović et al. 2015).

Effects of a mixture of Cr, Pb, Ni, and Zn on the physiological parameters in *L. laevigatum*

L. laevigatum was able to tolerate the mixture of metals of Pb, Cr, Ni, and Zn in treatment 2 (Pb 70 μg L^{-1}, Cr 70 μg L^{-1}, Ni 70 μg L^{-1} and Zn 70 μg L^{-1}) without showing any significant changes in the photosynthetic pigments within the first 14 days of exposure. For concentrations of 1 mg L^{-1} of Pb and Cr, 0.5 mg L^{-1} of Ni and 0.1 mg L^{-1} of Zn, the concentrations of the photosynthetic pigments chlorophyll *a*, pheophytin *a* and carotenoids decreased significantly after 21 days of exposure. Similar results were previously found in *N. indica,* where a decrease of pigments was recorded, with increasing Pb concentration after 7 days of exposure (Singh et al. 2010). Also with a decrease of 50% of chlorophylls and of 13% in carotenoids in *S. minima* was recorded, compared to the control, after being exposed for 1 week at concentrations of 1 mg L^{-1} of Cr (Nichols et al. 2000), and in *E. canadensis*, a decrease higher than 30% was observed in the levels of chlorophylls and carotenoids after 5 days growing in a culture medium with 2.93 mg L^{-1} Ni (Maleva et al. 2009). In *Egeria densa* and in *Myriophyllum aquaticum* were previously found significant differences from the control for the chlorophyll content, after being exposed for 7 days to 0.15 mg L^{-1} of Zn (Harguinteguy et al. 2015). However, *L. laevigatum* showed low levels of degradation of chlorophylls (phaeophytin *a*/chlorophyll *a*), at low and moderate concentrations of the culture medium for the first exposure times. These results in contrast with those recorded by Megateli et al. (2009) in *Lemna gibba* and Harguinteguy et al. (2015) in *E. densa* and *M. aquaticum*, who reported higher levels of chlorophyll degradation in culture mediums with Zn, Pb, and Ni.

Lipid peroxidation in plants is a consequence of oxidative damage caused by the by-products of numerous metabolic pathways in different cellular compartments. Under stress caused by heavy metals, lipid peroxidation can be initiated by oxidation of polyunsaturated fatty acids of the membranes, forming compounds such as MDA (Wang et al. 2009). In this study, *L. laevigatum* showed a rise in the production of MDA in root, with increasing metal concentrations in the culture medium. Similar results were observed in *Triticum aestivum,* when exposed to concentrations of 0.05 mg L^{-1} Cr and 0.065 mg L^{-1} Zn. Also, some macrophytes have been reported to have increased MDA levels at concentrations higher than those used in the present study: in *Hydrilla verticillata*, the MDA concentration was higher with respect to the control at concentrations of 10 mg Zn L^{-1} (Wang et al. 2009); in *N. indica*, an increase of MDA was registered for a concentration of 0.2 mg L^{-1} Pb (Singh et al. 2010); in *M. aquaticum* and *E. densa*, an increase of MDA was observed with respect to control for concentrations of 0.15 and 1 mg L^{-1} Ni, 1 mg L^{-1} Zn, and 5 mg L^{-1} Pb (Harguinteguy et al. 2015). However, in *L. laevigatum*, the concentration of MDA decreased in the treatment at the highest concentration, in agreement with the fact that in species of macrophytes such as Ceratophyllum, Hydrilla, and Wolffia, a decrease of MDA was also recorded in treatments with high concentrations of many metals (Dhir et al. 2004; Gupta and Chandra 1994). These latter results were difficult to interpret.

In the first week, no increase in MDA was recorded. The highest MDA was revealed after 14 days of exposure, similar to that which was reported in *Hydrilla verticillata* with respect to the control at concentrations of Ni, 1.5 mg L^{-1} after 6 days of exposure (Sinha and Pandey 2003).

The results of our study could be showing tolerance of *L. laevigatum* to the degradation of lipid membranes in early stages of exposure to metals. Related to this, Sinha and Pandey (2003) proposed that inhibition of MDA in early exposure times could be due to a stimulation of the reducing capacity of the plant tissue through an increase of the content of sulfhydryls, which protects the membrane from oxidative attack of free radicals of oxygen.

Effects of a mixture of Cr, Pb, Ni, and Zn on morphological parameters in *L. laevigatum*

L. laevigatum showed no significant difference in root length at low concentrations of metals in solution compared to the control, although a reduction in root length was observed for higher concentration treatments (treatments 2 and 3), similar to that found by Hadad et al. (2011), who reported a decrease in root length in *E. crassipes* when exposed to 1 mg L^{-1} of Cr, Ni, and Zn. This reduction of the root length in *L. laevigatum* may be associated with the effects of metals on the specific tissue of apical development. For example, during exposure of *Zea mays* to Pb, Eun et al. (2000) found accumulation of Pb in the root meristem tissue, which caused changes in the microtubule organization and a significant decrease in root length.

An increased number of chlorotic and necrotic leaves in *L. laevigatum* is a symptom of heavy metal toxicity (Prasad and Strzałka 1999). Here, for these leaves in the treatments of moderate concentrations of metals and during the early periods of exposure, no differences were found respect to control. Furthermore, this species showed more tolerant ability than that reported by Paiva et al. (2009) for *E. crassipes* when exposed to Cr, and by Khellaf and Zerdaoui (2009) in *Le. gibba* plants, exposed to Ni and Zn. In both studies, visible symptoms of chlorosis and necrosis were found at low concentrations of metals and at shorter periods of exposure than used in the present study.

Given the optimal conditions for the spread of *L. laevigatum* (Aponte and Pacherres 2013), the present study found that the number of leaves, ramets, leaves in ramets, and buds of aquatic plant were highest for moderate concentrations of metals (treatment 2) and 14 days of exposure, which could reflect a survival strategy and vegetative propagation of the species as a result of increased stress conditions due to the presence of heavy metals in the culture medium. At higher concentrations of metals, the spread of *L. laevigatum* was found to be lower. These latter results are similar to those reported for *Vallisneria americana*, where a decrease was observed in the number of leaves, ramets, and leaves in ramets when grown in metal contaminated sites (Doust et al. 1994).

Conclusion

By and large, the accumulation capacity of Pb, Cr, Ni, and Zn in *L. laevigatum* was higher with increasing concentrations of the metals in the culture medium and after greater exposure time. The floating macrophyte studied presented higher bioaccumulations of Pb, Cr, Ni, and Zn in the root than in the leaves. Moreover, the concentrations of Ni and Zn in leaf and root exceeded by over a thousand times the concentrations of those in the culture medium. Thus, this species can be considered as a hyperaccumulator of these metals. The changes and effects in the morphological and physiological parameters did not appear to cause harmful effects to the survival of the species during exposure to moderate concentrations of the mixture of metals, in the intermediate stages of exposure.

Given its storage capacity and level of tolerance, *L. laevigatum* is a species of interest for use in phytoremediation of water contaminated with moderate levels of Pb, Cr, Ni, and Zn and in the early stages of exposure.

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