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THEMATIC ISSUE

Effects of the presence of nutrients in the removal of high concentrations of Cr(III) by *Typha domingensis*

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Abstract The aim of this work was to study the influence of nutrients on the tolerance, removal efficiency and accumulation of high concentration of Cr(III) by *Typha domingensis*. This species was exposed to the following Cr(III) treatments, arranged in triplicate: 200 mg L^{-1} Cr; 600 mg L^{-1} Cr; 200 mg L^{-1} Cr + 50 mg L^{-1} P + 50 mg L^{-1} N; 600 mg L^{-1} Cr + 50 mg L^{-1} P + 50 mg L^{-1} N; without metal or nutrient additions (control 1); 50 mg L^{-1} P + 50 mg L^{-1} N, without metal addition (control 2). In order to simulate extreme events, the concentrations studied were higher than the concentrations commonly found in constructed wetlands. Cr and nutrient concentrations in water decreased in all treatments along time. Nutrient addition did not affect Cr removal for the two concentrations studied. In tissues, the highest Cr concentrations were observed in roots in all treatments. The mass balance showed that sediment showed the highest accumulation of Cr. Metals caused growth inhibition and a decrease in chlorophyll concentration. Despite these sublethal effects, *T. domingensis* demonstrated that it could accumulate Cr. These results confirm the high tolerance of this species ensuring the survival plant and wetland efficiency in time.

Keywords Nutrients · Metals · Macrophytes · Effluents

Introduction

Constructed wetlands (CWs) have been used for the treatment of industrial effluents, urban and agricultural storm waters, mine waters, etc. (Hammer and Bastion 1989; Kadlec and Knight 1996; Kadlec and Wallace 2009; Maine et al. 2007, 2009; Song et al. 2006; Vymazal and Kröpfelová 2008; Vymazal 2011). Macrophytes have been studied because of their ability for contaminant removal from water and their subsequent use in wetlands constructed for wastewater treatment. They are key components of these systems (Kadlec and Wallace 2009; Vymazal 2011). Emergent macrophytes are able not only to take up contaminants in their tissues but also to influence the biogeochemical cycles of the sediment, due to their capacity to transport oxygen to the rhizosphere influencing the sediment redox conditions (Brix and Schierup 1990; Jacob and Otte 2003). Macrophytes have a remarkable ability to concentrate metals in their belowground tissues at levels that exceed those of the surrounding sediment. The exclusion of metals from aboveground tissues is a metal tolerance strategy (Taylor and Crowder 1983; Kabata-Pendias and Pendias 2011; Hechmi et al. 2014). Macrophytes in their natural habitats are usually exposed to low concentrations of different contaminants. The conditions for plants growing in wetlands constructed for industrial wastewater treatment are completely different since they must have the ability to tolerate high concentrations of several contaminants at the same time.

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The uptake of metals depends on the chemical form present in the system and the life-form of the macrophytes submerged, free-floating and emergent (Chandra and Kulshreshtha 2004). In the case of Cr, although beneficial effects on plants have been reported, its essentiality for plants has not been established (Shanker et al. 2005; Mangabeira et al. 2011). Cr can occur in several oxidation states, although the most stable forms are the trivalent Cr(III) and hexavalent Cr(VI) species in surface waters (Fendorf 1995). In most industrial effluent primary treatments, Cr(VI) is reduced totally or partially to Cr(III). However, Cr(VI) is relatively unstable under most environmental conditions, and converts into the less toxic trivalent form in surface waters, especially in the presence of organic matter (Kadlec and Wallace 2009), as in the case of the studied free water surface wetland. For these reasons, Cr(III) was chosen for this study. Chromium generally appears to accumulate in plant roots and is poorly translocated to the tops (Lyon et al. 1969; Tiffin 1972; Turner and Rust 1971). Cr(III) is taken up passively, being retained by the cation-exchange sites of the cell walls of plants (Skeffington et al. 1976; Shanker et al. 2005).

Chen et al. (2010) studied the influence of Cr(III) speciation on Cr phytoremediation by *Ipomoea aquatica* in a hydroponic experiment. This species was exposed to three concentrations of Cr. EDTA and chlorides were added to enhance Cr accumulation in tissues. Hegazy et al. (2011) studied the ability of *T. domingensis* to uptake and accumulate Al, Fe, Zn and Pb because these metals exceeded the limits indicated by the Egyptian environmental regulations. *T. domingensis* accumulated high concentration of metals in root tissues and could consider as a potential species to be used in phytoremediation to remove metal pollutants from contaminated wastewater. The same was observed by Mojiri (2011) who studied the phytoremediation of metals by *T. domingensis* from municipal wastewater. Unlike other authors, in our work, extremely high concentrations of Cr(III) were used in the presence of nutrients to enhance the tolerance of *T. domingensis*. These concentrations are not usually found in constructed wetland.

Nutrients and metals play an important role in the growth and metabolism of plants. However, they produce toxic effects at high concentrations (Kabata-Pendias and Pendias 2011). Di Luca et al. (2013); Hadad et al. (2007); and Mufarrege et al. (2010) studied the interactions between metal accumulation in tissues of floating macrophytes and the nutrient concentrations in surrounding water. These authors reported that nutrient addition enhances the metal tolerance of floating macrophytes and would, therefore, enable the development of vegetation in constructed wetlands. Moreover, Göthberg et al. (2004) proposed to add nutrients to attenuate the metal accumulation in *Ipomea aquatica* and increase their tolerance.

In our work, the emergent macrophyte *T. domingensis* was chosen because it was the dominant macrophyte in a wetland constructed for the treatment of effluents of a metallurgical industry (Maine et al. 2009, 2013). Cr(III) and nutrients (P and N) were studied for being found in treated effluents at this constructed wetland. In order to simulate extreme events, the concentrations studied were higher than the concentrations commonly found in constructed wetlands. The aim of this work was to study the influence of nutrients on the tolerance, removal efficiency and accumulation of high concentration of Cr(III) by *T. domingensis*.

Materials and methods

Experimental design

T. domingensis, water and sediment were collected from an unpolluted pond near Santa Fe City, Argentina. Only healthy plants of a uniform size and weight were selected. The plants were pruned for their transport to the greenhouse.

Plastic reactors were disposed outdoors under a semi-transparent plastic roof. Each plastic reactor of 10 L capacity contained two plants and 4 kg of sediment. After 15 days of acclimation, the plants were pruned again to a height of approximately 20 cm and contaminant solutions (5 L) were added to the reactors to obtain treatments with water concentrations of:

Cr200:	200 mg L^{-1} Cr
Cr600:	600 mg L^{-1} Cr
Cr200+Nut:	200 mg L^{-1} Cr + 50 mg L^{-1} P + 50 mg L^{-1} N
Cr600+Nut:	600 mg L^{-1} Cr + 50 mg L^{-1} P + 50 mg L^{-1} N
Control 1:	without metal and/or nutrient addition
Control 2:	50 mg L^{-1} P + 50 mg L^{-1} N, without metal addition

The solutions were prepared using water from the sampling site and $CrCl_3 \cdot 6H_2O$. Solutions were prepared with pond water. Water pH was maintained between 5.4 and 5.8 to avoid metal precipitation during the experiment. Water level in the reactors was maintained by adding water from the sampling site. Nutrient concentrations were 50 mg L^{-1} of P and N (added as H_2KPO_4 and NH_4NO_3, respectively). Temperature ranged from 21.1 to 31.3 °C during the experimental period. The experiment lasted 30 days, and it was performed in triplicate. In each reactor, water was sampled at 0, 8, 24 y 48 h; 5, 10, 20 y 30 days.

At the beginning and at the end of the experiment, the Cr concentrations in plants and sediment were determined. Plants were sampled and separated into roots, rhizomes, and submerged and aerial parts of leaves. Sediment was sampled using a 3-cm-diameter PVC corer and stored at 4 °C until analysis.

Plant study

Plant height was measured, and the external appearance of plants was observed daily, to detect possible senescence.

Chlorophyll concentration was measured at the beginning and at the end of the experiment. Relative growth rate (RGR) (cm cm^{-1} day^{-1}) was calculated in each treatment considering initial and final plant height, according to Hunt (1978):

$$\text{RGR} = \frac{\ln H_2 - \ln H_1}{T_2 - T_1}$$

where H_1 and H_2 are the initial and final plant height (cm), respectively, and $(T_2 - T_1)$ is the experimental period (days).

Chemical analysis

Conductivity was assessed with an YSI 33 model conductimeter, pH with an Orion pH meter and DO with a Horiba OM-14 portable meter. Water samples were filtered through Millipore membrane filters (0.45 µm) for nutrient determinations. Chemical analysis was performed following APHA (1998). Cr concentrations were determined in water samples by atomic absorption spectrometry (PerkinElmer AAnalyst 200).

Soluble reactive phosphorous (SRP) and ammonium were determined throughout the experiment. SRP was determined by the colorimetric molybdenum blue method (Murphy and Riley 1962) (UV–Vis PerkinElmer Lambda 20). Ammonium and nitrate was determined by potentiometry (Orion ion-selective electrodes, sensitivity: 0.01 mg L^{-1} of N, reproducibility: ±2 %). Nitrite was determined by coupling diazotation followed by a colorimetric technique, and COD was determined by the open reflux method.

The plants were washed with tap and distilled water, and subsequently oven-dried at 60 °C for 48 h. Dried plant samples were ground and digested with a $HClO_4$:HNO_3:HCl (5:3:2) mixture.

Sediment samples were digested in the same way as plant samples. Plant and sediment digests were analyzed for Cr by atomic absorption spectrometry (PerkinElmer, AAnalyst 200). These determinations were carried out in triplicate.

Cr amounts (mg) were estimated by multiplying Cr concentration in plant tissues, sediment (mg g^{-1} dry weight) or in water (mg L^{-1}) by mass (g dry weight) or volume (L).

Chlorophyll was extracted with acetone for 48 h in cold darkness (3–5 °C). The percentage of transmittance of the extracts at 645 and 665 nm was recorded with a spectrophotometer UV–Vis in order to calculate chlorophyll *a* concentration (Westlake 1974).

QA/QC

All glassware was pre-cleaned and washed with 2 N HNO_3 prior to each use. $CrCl_36H_2O$ used to prepare metal solution was of analytical grade. Certified standard solutions were used. Replicate analyses (at least ten times) of the samples showed a precision of typically less than 4 % (coefficient of variation). Detection limits were 30 µg g^{-1} for Cr for sediment and plant tissues. The H_2KPO_4 and NH_4NO_3 used to prepare nutrient solution were of analytical grade. Replicate analyses (at least ten times) of the samples showed a precision of typically less than 4 % (coefficient of variation).

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine whether significant differences existed among treatments in relative growth rate and chlorophyll *a* concentrations. Two-way ANOVA (factors: treatments and plant tissues) was performed to determine whether significant differences existed in metal concentrations in water, sediment and plant tissues, and metal tissue amounts (aerial parts, submerged parts of leaves, rhizomes and roots) and nutrient concentration in water. Duncan's test was used to differentiate means where appropriate. In all comparisons, a level of $p < 0.05$ was used.

Results and discussion

Metal and nutrients removal from water

The chemical composition of the water from the sampling site (a typical unpolluted pond of the zone) used in the experiment was (mean ± standard deviation): pH = 6.7; conductivity = 215 ± 1 µS cm^{-1}; dissolved oxygen (DO) = 6.60 ± 0.10 mg L^{-1}; soluble reactive phosphorous (SRP) = 0.016 ± 0.002 mg L^{-1}; $NH_4^+ = 0.773 \pm 0.005$ mg L^{-1}; $NO_3^- = 0.570 \pm 0.005$ mg L^{-1}; NO_2^- = non-detected (detection limit = 5 µg L^{-1}); $Ca^{2+} = 9.7 \pm 0.1$ mg L^{-1}; $Mg^{2+} = 2.7 \pm 0.2$ mg L^{-1}; $Na^+ = 30.1 \pm$

0.5 mg L^{-1}; $K^+ = 11.1 \pm 0.5$ mg L^{-1}; Fe = 0.292 ± 0.005 mg L^{-1}; $Cl^- = 9.6 \pm 1.0$ mg L^{-1}; $SO_4^{2-} = 6.8 \pm 1.0$ mg L^{-1}; total alkalinity = 101.2 ± 1.2 mg L^{-1}; Ni (mg L^{-1}) = ND (detection limit 5 μg L^{-1}); Zn (mg L^{-1}) = ND (detection limit = 5 μg L^{-1}); and Cr = non-detected (detection limit = 5 μg L^{-1}). The studied constructed free water surface is located in this area.

Figure 1 shows Cr removal percentages from water over time. Cr concentration in water decreases, in all treatments with final removal of 99.9; 99.9; 99.7 and 99.9 % for Cr200, Cr200+Nut, Cr600 and Cr600+Nut., respectively. Chromium removal is consistent with other works from laboratory experiments (Delgado et al. 1993, Maine et al. 2004; Mufarrege et al. 2015). During the first 20 days of the experiment, the treatments Cr200 and Cr200+Nut showed removal percentages significantly higher than the obtained in the treatments Cr600 and Cr600+Nut. The treatment Cr600+Nut showed a significantly higher Cr removal percentage than Cr600, at the day 20. Although it is expected that nutrient enrichment improves the removal of Cr from water by increasing biomass production and metabolism, at the end of the experiment, the addition of nutrients did not affect the removal of Cr in water for the both concentrations studied (Fig. 1). The same result was obtained by Hadad et al. (2007), who reported a fast decrease in Cr water concentration in treatments with *S. herzogii* without differences between metal and nutrient-enriched treatments.

Figure 2 shows SRP, $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ removal percentage from water. Final removal of SRP was: 98.06, 99.99 and 81.43 % for Cr200+Nut, Cr600+Nut and control 2, respectively. Final removal of $N\text{-}NH_4^+$ was: 97, 96.9 and 93.9 % for Cr200+Nut, Cr600+Nut and control 2, respectively. Final removal of $N\text{-}NO_3^-$ was: 99.9, 99.9 and 99.9 % for Cr200+Nut, Cr600+Nut and control 2, respectively. SRP, $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentrations in water decreased with time in the nutrient-enriched treatments. SRP removal percentages along time were not significantly different between Cr200+Nut and Cr600+Nut treatments. The lowest SRP removal was observed in control 2. Regarding $N\text{-}NH_4^+$, Cr200+Nut showed the highest removal during the first 10 days, while $N\text{-}NO_3^-$ showed the highest removal during the first 5 days in control 2 treatment. Cr600+Nut treatment showed the lowest removal of $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ during the first 10 day. At the end of the experiment, significant differences between the $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ removal were not observed. Ammonium is a source of nitrogen that is readily transported by metabolic systems located at the macrophyte plasmalemmas (Bishop and Eighmy 1989), while protein membrane carriers mobilize P as fast as they can. However, when there is an excess of phosphate, the transport velocity is limited by the saturation of their capabilities of transport (Bonilla 2008). Cr exposure enhanced ammonium final removal from water (Fig. 2).

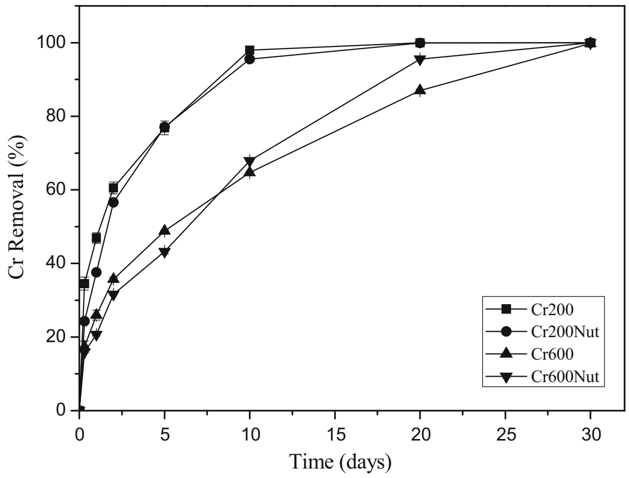

Fig. 1 Cr removal percent from water along time ($n = 6$)

Metal concentration in tissues and sediment

The chemical composition of the sediment used in the experiment was: organic matter (OM) = 4.7; pH = 7.67; Eh = 280 mV (Ag/AgCl); Cr = 0.014 mg g^{-1}; Ni (mg g^{-1}) = 0.018; and Zn (mg g^{-1}) = 0.02.

Figure 3 shows Cr concentrations in plant tissues and sediment. Cr concentrations in sediment were significantly lower than in plant tissues, with exception of Cr600+Nut treatment. The highest Cr concentrations were observed in roots in all treatments. It was widely reported that metals are accumulated in root tissues as a tolerance strategy (Taylor and Crowder 1983; Stoltz and Greger 2002; Sinha and Gupta 2005; Hadad et al. 2007, 2011; Nilratnisakorn et al. 2007; Chandra and Yadav 2010; Mufarrege et al. 2010, 2015; Vymazal 2011; Hechmi et al. 2014). The presence of nutrients influenced Cr accumulation in tissues. In treatments Cr200+Nut and Cr600+Nut, submerged parts of leaves showed significantly higher concentrations than the obtained in Cr200 and Cr600 treatments. This immobilization in submerged tissues allowed the plants to tolerate high Cr concentrations. In the case of emergent macrophytes, metals can be taken up from the sediment by plant roots or by the submerged parts of leaves in direct contact with water (Mufarrege et al. 2015). Maine et al. (2001, 2004) and Suñe et al. (2007) reported that floating macrophytes can take up metals by roots or by leaves that are in direct contact with water. The study of metal accumulation in different tissues of leaves of emergent macrophytes was not found in the literature. Other studies have reported that Cr is readily absorbed by plant roots but little is translocated to the aerial parts unless very high

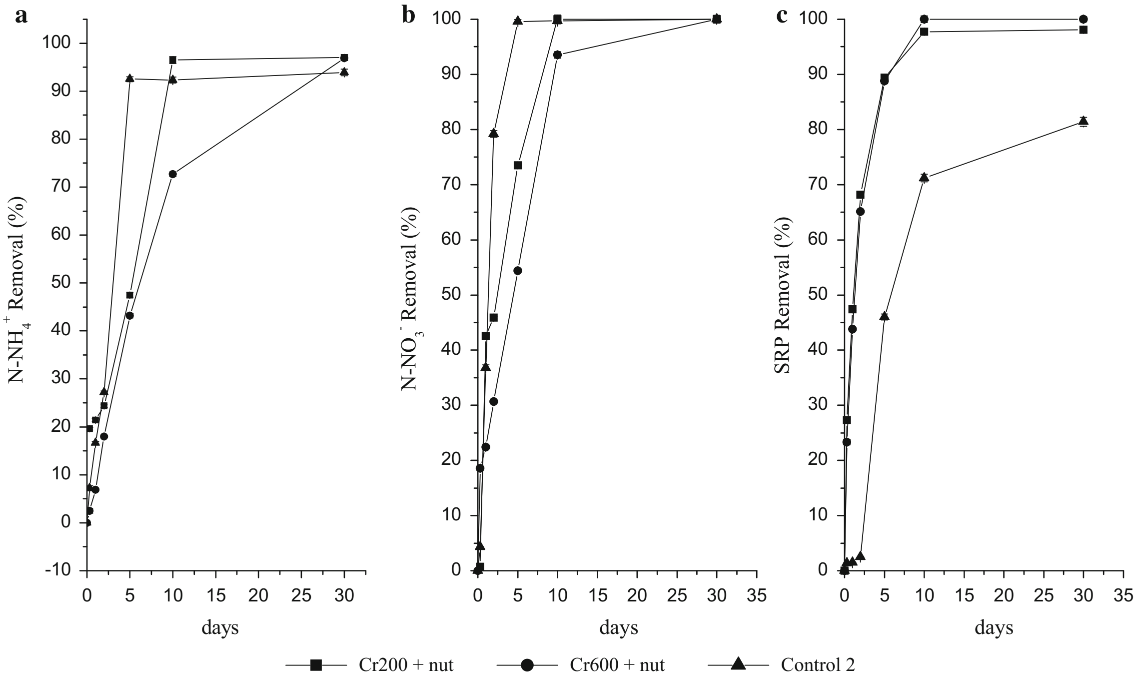

Fig. 2 **a** Ammonium (N-NH$_4^+$), **b** nitrate (N-NO$_3^-$) and **c** soluble reactive phosphorous (SRP) removal percentage from water ($n = 6$)

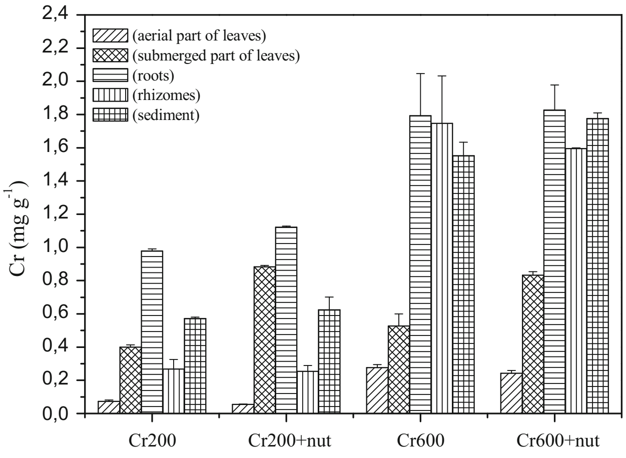

Fig. 3 Final concentrations of Cr (mg g^{-1}) in roots, rhizomes, aerial parts of leaves, submerged parts of leaves of *T. domingensis*, and sediment ($n = 6$). *Bars* represent standard deviations

concentrations of Cr are present in the rooting media (Dekock 1956; Lyon et al. 1969; Tiffin 1972; Turner and Rust 1971). However, Hunter and Vergnano (1953) reported that even in cases of Cr toxicity, high concentrations of Cr were not necessarily found in plant tops. Binding positively charged toxic metal ions to negative charges in the cell walls of the roots, metal-phosphate and metal-phytate formation, and chelation to phytochelatins followed by accumulation in vacuoles have been invoked as mechanisms to reduce metal transport and increase metal tolerance (Chaney 1993; Loneragan and Weeb 1993; Göthberg et al. 2004). Cr(III) does not utilize any specifics membrane carrier and hence enters into the cell through simple diffusion. The diffusion is possible only after the formation of appropriated lipophilic ligands (Chandra and Kulshreshtha 2004). The fact that Cr accumulated in roots and rhizomes allowed plants to tolerate the Cr toxicity and protect the aerial parts of leaves.

Mass balance

To estimate the extent of metals accumulated in each compartment (sediment and different plant tissues), Cr amounts (mg) were estimated. The treatments Cr200+Nut showed that the submerged part of leaves accumulated higher amount of Cr than Cr200 treatment. In Cr600 treatments, Cr amounts were significantly higher in rhizomes and roots than in Cr600+Nut. Mufarrege et al. (2015) studied *T. domingensis* ability to accumulate and tolerate single- and multi-metal treatments. These authors found that this species accumulates high concentration of metals in submerged parts of leaves in all treatments to which it was exposed, probably because these tissues were in direct contact with the experimental solution. In our work, despite the high concentrations determined in plant tissues, plants were not an efficient compartment for Cr accumulation in comparison with sediment (Fig. 4). The sediment was the main compartment accumulator of Cr, followed by the roots and rhizomes. Previous works showed that the sediment is the main accumulation

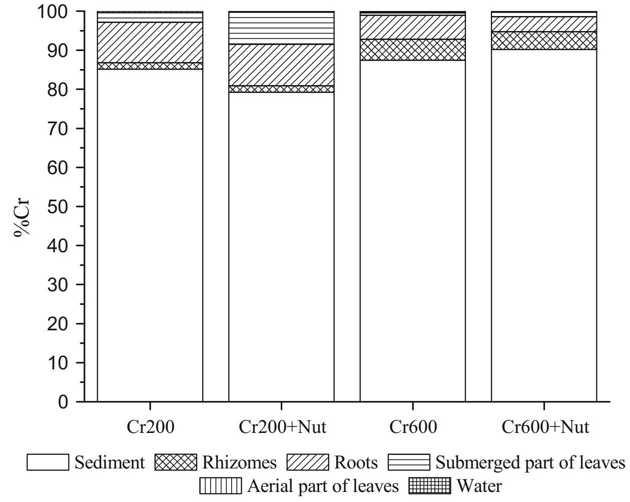

Fig. 4 Cr total amounts (expressed in %) in water, sediment and tissues of *T. domingensis* along time

compartment of metals (Maine et al. 2009). Nevertheless, the advantage of macrophytes is the possibility of being harvested, which leads to important removal rates of contaminants in short periods of time. Besides, macrophytes are involved in the sediment biogeochemistry through oxygen transport from the aerial parts to the rhizosphere, enhancing metal accumulation in sediment (Barko et al. 1991; Sorrell and Boon 1992; Clothier and Green 1997; Sundby et al. 2005).

Plant tolerance

The relative growth rates were positive in all treatments (Fig. 5a). The Cr200+Nut treatment showed a significantly higher RGR than the obtained in the Cr200 treatment, indicating a positive effect of nutrient addition. However, in the treatments Cr600 and Cr600+Nut, the highest toxicity was observed not only represented by the lowest relative growth rate, but also represented by the absence of new shoots, indicating a null vegetative propagation. In Cr600 and Cr600+Nut treatments, despite the nutrient addition, significant differences between the treatment with only Cr addition and the treatments with Cr and nutrients were not observed in the relative growth rate. The control with nutrient addition (control 2) showed the highest growth rate. P is a required element for the growth of plants, taking part in the photophosphorylation and carbon assimilation in the photosynthesis (Zhou et al. 1993). However, when the nutrient concentrations reach a threshold value, they become first inhibitory and afterward toxic. Under P and N stress, most plants showed slow growth rates and low root: shoot ratios (Britto and Kronzucker 2002; Tylova et al. 2008; Jampeetong and Brix 2009; Di Luca et al. 2011). Despite the high P and N concentrations studied, they enhanced *T. domingensis* growth and chlorophyll concentration in the control without Cr addition.

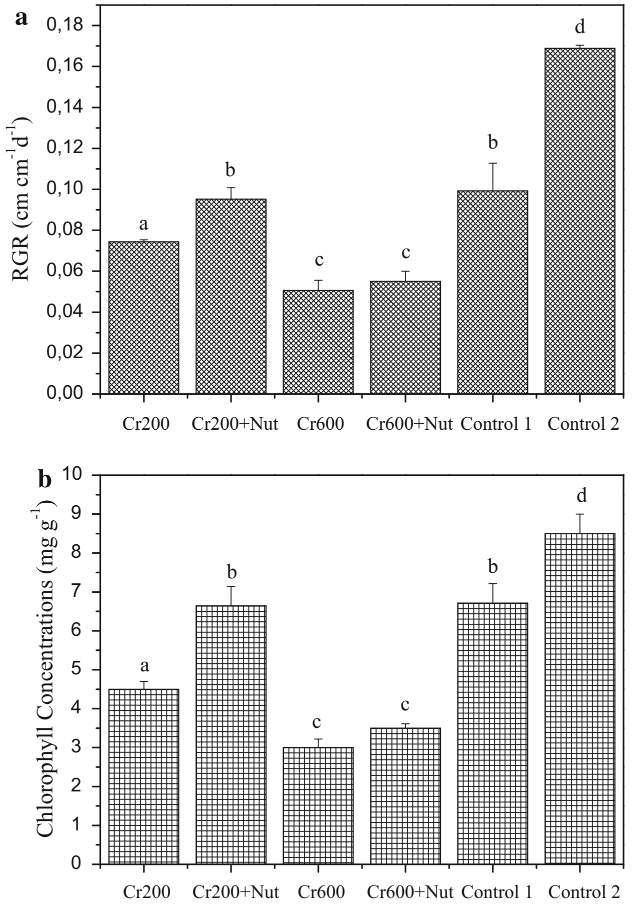

Fig. 5 **a** Relative growth rates ($n = 9$) and **b** final chlorophyll a concentrations (mg g^{-1} d.w.) ($n = 6$). *Different letters* represent statistically significant differences among the treatments. *Bars* represent standard deviations

The chlorophyll concentration (Fig. 5b) showed the same trend that the relative growth rate, being the treatment with nutrient addition which had significantly higher concentrations than those without nutrients. However, Cr600 and Cr600+Nut showed the chlorophyll concentration significantly lower than other treatments. Symptoms of chlorosis were observed in these treatments. Because plants avoid the potential effects of high metal concentrations on the photosynthetic tissues, restriction to shoot translocation is believed to be the strategy of metal tolerance for non-hyperaccumulators. When large quantities of Cr are accumulated by plants, it may cause various types of damage at morphological and ultrastructural levels (Heumann 1987). Rebechini and Hanzely (1974) reported changes in chloroplast fine structure due to lead toxicity in *Ceratophyllum demersum*.

It has been shown that metals accumulation is responsible for the decrease in total chlorophyll concentration

(Abdel-Basset et al. 1995; Manios et al. 2003). However, the capacity to accumulate metals in the aboveground plant tissues represents a central point for the suitability of the plants for metals phytoextraction (Salt and Krämer 2000). The amount of metals accumulated in the aerial parts may vary during the growing season as a consequence of the inherent growth dynamics of the plant, as well as in response to variations in the metals levels and availability in the surrounding water and soil (Larsen and Schierup 1981; Hardej and Ozimek 2002).

Conclusion

The extremely high Cr concentrations were studied, and *T. domingensis* was tolerant to this metal.

Metal caused growth inhibition and a decrease in chlorophyll concentration in plants due to that not shown signs of senescence in all treatments. The nutrient addition improved tolerance of the plants in the Cr200 treatment, but not in Cr600 treatment. Despite the growth inhibition observed at 600 mg L^{-1} Cr, this metal was removed from water.

These results could be useful to be applied in the design and management of wetlands constructed for the treatment of industrial effluents with Cr. During a treatment of industrial and sewage effluents in a combined way, the nutrients will improve the plant growth. Despite the sublethal effects registered, *T. domingensis* demonstrated that it could survive during an event of an accidental dump of extremely high Cr concentrations, which can occur in a constructed wetland. These results confirm the high tolerance of this species ensuring the plant survival and wetland efficiency to continue operating.

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