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ORIGINAL PAPER

Simultaneous phytoremediation of chromium and phenol by L*emna minuta* Kunth: a promising biotechnological tool

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Abstract The aim of this work was to evaluate the usefulness of *Lemna minuta* Kunth for the simultaneous removal of Cr(VI) and phenol. The impact of these contaminants on plant growth and some biochemical processes have also been discussed for a better understanding and utilization of this species in the field of phytoremediation. The optimal growth conditions and plant tolerance to Cr(VI) and/or phenol as well as removal were determined. Plants exposed to Cr(VI) and phenol were able to efficiently grow and remove both contaminants at high concentrations (up to 2.5 and 250 mg/L, respectively) after 21 days, indicating that they were resistant to mixed contamination. There were no significant differences between chlorophyll, carotene and malondialdehyde content of treated plants with respect to the controls, which would be due to an efficient antioxidant response. *L. minuta* showed a higher biomass than control without contaminant when was exposed to low concentrations of Cr(VI), suggesting an hormesis effect. The main removal process involved in chromium phytoremediation would be sorption or accumulation in the biomass. Moreover, our results suggest that phenol could be used as a donor of carbon and energy by these plants. These findings demonstrated that *Lemna minuta* Kunth might be suitable for treatment of different solutions contaminated with Cr(VI) and phenol, showing a high potential to be used in the treatment of effluents containing mixed contamination.

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Introduction

Pollution of the biosphere has seriously increased in the last decades, mainly by the spills of industrial wastewaters. Commonly, in many of them high levels of heavy metals (such as Chromium) are often coupled with aromatic organic pollutants like phenols (Ontañon et al. 2015).

In particular, when heavy metals reach natural resources like soil and aquatic environments, they accumulate at high concentrations producing deleterious effects on human life health and aquatic biota (Dixit et al. 2015). As they cannot be degraded, they accumulate in water, soil, sediments and living organisms (Rai et al. 2002). Moreover, consumption of such aquatic food stuff enriched with toxic metals may cause health hazards through food-chain magnification. Heavy metals toxicity depends on their redox state. In this sense, Cr(VI) is more toxic than Cr(III) because of its high solubility, availability and mobility in soil as well as through biological membranes (Oliveira 2012). In plants, Cr can promote growth of several species at low concentration although it is not an essential element. However, at high concentration, Cr produces germination inhibition, reduction of seedling growth and development, leaf chlorosis and necrosis besides other physiological and biochemical alterations (Shanker et al. 2009).

In addition, phenol is harmful to several aquatic organisms including anuran amphibians, fishes and crustaceans (Paisio et al. 2009). It is also toxic to humans, inducing carcinogenicity and causing reproductive and developmental harm, neurotoxicity and acute toxicity. In

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plants, phenol produces inhibition of germination, seedling growth and development, and chlorosis, among others (Jha et al. 2013; Zhou et al. 2013).

Based on all these facts, Cr(VI) and phenol removal is of high relevance for a safe environment. In this sense, many biological methods have been proposed to remove these contaminants from water and soil (Chen et al. 2012). Among them, phytoremediation exploits the ability of plants to absorb, accumulate, metabolize, volatilize or stabilize pollutants, including heavy metals and/or organic compounds (Delgadillo-López et al. 2011).

Particularly, phytofiltration is an unconventional wastewater treatment technology which is gaining interest due to its multiple advantages including the fact that it is a cost-effective and environmentally sound alternative. Phytofiltration involves the use of aquatic plants, either floating, submerged or emergent, to remove pollutants from solution, mainly through their root system although in some cases, fronds are also involved directly in the removal process (Olguín and Sánchez-Galván 2012). Aquatic macrophytes play an important role in the structural and functional aspects of aquatic ecosystems by altering water movement regimes, providing shelter to fish and aquatic invertebrates, serving as a food source, and altering water quality by regulating oxygen balance, nutrient cycles and accumulating heavy metals (Sood et al. 2012). The ability to remove contaminants makes them interesting research subjects, especially for the treatment of industrial effluents and sewage waste water.

Taking into account the hazard of Cr(VI) and phenol and the advantages of phytoremediation, the removal of Cr(VI) and phenol using different plant species has been studied (Arora et al. 2006; Weerasinghe et al. 2008). However, these studies are frequently restricted to the evaluation of the removal of each contaminant individually. Therefore, in the present investigation the simultaneous removal of Cr(VI) and phenol using an aquatic plant, *Lemna minuta* Kunth, was performed in order to elucidate the main mechanisms involved and to determine if this plant species can be considered as a biotechnological tool useful for treating effluents or wetlands with mixed contamination.

This research was carried out between years 2014 and 2015 at Departamento de Biología Molecular (FCEFQyN) from Universidad Nacional de Río Cuarto, Río Cuarto (Córdoba), Argentina.

Materials and methods

Plant material and culture conditions

An aquatic plant species was collected from a wetland located in Elena (Córdoba Province, Argentina) (32° 57′ south latitude and 64° 37′ west longitude), which receives input stream from El Barreal river and effluents derived from a tannery. This plant was collected in plastic bottles and then thoroughly washed under gentle running water.

The collected plant was identified as *Lemna minuta* Kunth (Araceae, Lemneae) based on morphological characteristics and dichotomous key tool. The hydrophyte was maintained in plastic pots containing Hoagland medium (Hgd) (Hoagland and Broyer 1936) diluted ½ [(mg/L): 4 $MgSO_47H_2O$; 1.56 $FeSO_47H_2O$; 1.07 $ZnSO_47H_2O$; 0.14 H_3BO_3; 0.025 $Na_2MoO_47H_2O$; 1.016 $CuSO_45H_2O$; 22.75 KH_2PO_4; 29 KNO_3; 34 $CaNO_3$; 0.64 $MnCl_24H_2O$] and maintained in a glasshouse. They were subcultured in fresh medium each week.

Determination of optimal culture medium and incubation conditions

Culture medium and incubation conditions for the optimum plant growth were determined. For that, *L. minuta* plants were cultivated in sterile Hgd diluted ½ medium, Huebert medium (Hbt) (Huebert et al. 1993), modified Hutner (Htr) diluted 1/10 medium prepared according to Vermaat and Hanif (1998) and tap water (Tw) as control. Plants (0.10 g) were cultivated into plastic containers of 0.15 × 0.10 × 0.45 m containing 0.90 L of each culture medium or Tw. These containers were exposed at three different incubation conditions (Table 1) during 14 days: Growth chamber 1, Growth chamber 2 and glasshouse. Fresh weight (FW) of plants was determined at the end of the experiments. Also, qualitative changes of plants such as pigmentation and tonicity were registered.

Cr(VI) and phenol phytoremediation

Cr(VI) and phenol tolerance screening in solid medium

Cr(VI) and phenol tolerance of *L. minuta* was tested on Petri plates (120 × 25 mm) containing sterile semisolid (0.8% agar) Hgd medium ½. This medium was supplemented with the proper volume of stock solutions of $Cr_2O_4K_2$ (Sigma) to reach final Cr(VI) concentrations of 0.5, 1.0 and 2.5 mg/L and phenol (Merck) in order to treatments with 5, 25 and 50 mg/L as final concentrations. In each plate, two *L. minuta* plants were placed, considering that a plant consists in a single root containing variable frond numbers.

The tolerance assay was done in quadruplicate, and the plates were incubated at controlled conditions in growth chamber 1 during 21 days. Plants growing in Hgd medium (½) without contaminants were considered as controls. The average root number of plants exposed to Cr(VI) or phenol was determined at 7, 14 and 21 days. As indicative of

Table 1 Incubation conditions

Condition	Growth chamber 1	Growth chamber 2	Glasshouse
Temperature (°C)	24 ± 4	25 ± 2	24.5 ± 9
Relative humidity (%)	70	ND	80
Light intensity (μmol m^{-2} s^{-1})	600	60	ND
Photoperiod (light/darkness (h))	16/8	16/8	13/11

ND non-determined

biomass development, the area occupied by the group of the plants was determined using the ImageJ program. From these results, maximum Cr(VI) and phenol concentration tolerated by *L. minuta* was established and they were used for the following assays.

Cr(VI) and phenol removal by L. minuta plants

Two sets of experiments, carried out in parallel, were performed to establish the potential of *L. minuta* to individually remediate Cr(VI) and phenol. For that, plants (0.1 g) were inoculated in Erlenmeyer flasks containing 20 mL of Hgd medium (½) with different Cr(VI) concentrations (1, 2.5, 5, 10 and 20 mg/L) or phenol (5, 25, 50, 100, 150, 200 and 250 mg/L) and incubated in the previously selected growth chamber. Culture media samples were taken at different time intervals. Residual Cr(VI) and phenol were analyzed ("Cr(VI) and phenol quantification" section) until contaminants were not detected. The results were expressed as Cr(VI) and phenol removal efficiency (%) using solutions of each contaminant without plants, as controls. At the end of both experiment set, plants were harvested and FW was registered.

Later, *L. minuta* capability to simultaneously remove these contaminants was also determined. For that, the plants (0.1 mg) were inoculated in Erlenmeyer flasks containing 20 mL of Hgd medium (½) with a fixed Cr(VI) concentration (2.5 mg/L) and phenol at different concentrations (from 25 to 250 mg/L). At the end of the experiment, plant biomass was evaluated. Then, plants were frozen using liquid nitrogen and conserved at −80 °C until their analysis for chlorophyll, carotene and malondialdehyde (MDA) determination.

All experiments were carried out in triplicate.

Lipid peroxidation was determined spectrophotometrically by measuring the concentration of thiobarbituric acid-reactive substances (TBARs), as described by Heath and Packer (1968). MDA ($\varepsilon = 155$ $mM^{-1}cm^{-1}$) at A_{532} and corrected for non-specific turbidity at A_{600} nm.

Chlorophyll *a* and *b* content was determined using acetone as extraction solvent (Dere et al. 1998). The amount of chlorophyll *a*, *b* and carotenes "C" were calculated according to the following formula, and latter expressed mg of chlorophyll per gram of FW tissue.

$$\text{Chl } a \text{ (μmol/L)} = 11.75 \times A_{662} - 2.350 \times A_{645}$$

$$\text{Chl } b \text{ (μmol/L)} = 18.61 \times A_{645} - 3.960 \times A_{662}$$

$$\text{"C"} = 1000 \times A_{670} - 2.270 \times \text{C} \quad \text{"}a\text{"} - 81.4 \times \text{C} \text{ "}b\text{"}/227$$

Cr speciation determination

L. minuta Kunth was grown in Hgd medium (½) with Cr(VI) (2.5 mg/L) and phenol (200 mg/L) in order to determine the mechanisms involved in Cr(VI) removal.

Cr(VI) and total Cr were determined in the medium and in the biomass, after 21 days of incubation, by diphenylcarbazide method ("Cr(VI) and phenol quantification"section) and atomic absorption spectrophotometry (AAS), respectively. AAS determinations were carried out by a specialized laboratory (FARESTAIE, Mar del Plata, Argentina), using a SHIMADZU AA-6800 Analyst. Cr(III) concentration was calculated as the difference between total Cr and Cr(VI).

Cr(VI) and phenol quantification

Cr(VI) was determined at 540 nm after reaction with diphenylcarbazide in acid solution. The reaction mixture contained 500 μL of H_2SO_4 0.2 N, 200 μL of diphenylcarbazide (5 mg/mL), 500 μL of each sample and distilled water to reach a final volume of 5 mL, according to APHA (1995) modified method. The absorbance data were converted to Cr(VI) concentration using a calibration curve from 0 to 10 mg/L, with a r^2 of 0.988.

Residual phenol concentration was spectrophotometrically evaluated according to Wagner and Nicell (2002) using Beckman DU640 spectrophotometer. Briefly, samples of 100 μL were mixed with 700 μL of sodium bicarbonate (pH 8), 100 μL of 4-aminoantipyrine (20.8 mM) and 100 μL of potassium ferricyanide (83.4 mM). After 5 min, absorbance at 510 nm was measured. The absorbance data were converted to phenol concentrations using a calibration curve from 0 to 100 mg/L with a r^2 of 0.995. The values of residual Cr(VI) and phenol obtained were expressed as removal efficiency (%).

Statistical analysis

All experiments were performed in triplicate and repeated twice. Results are presented as the mean and the standard deviation. Data were analyzed using ANOVA, followed by the posteriori Multiple Range test ($p \leq 0.05$), using R (3.1.1) software.

Results and discussion

Determination of optimal culture medium and incubation conditions

Optimization of medium composition and incubation conditions is necessary to achieve the better growth of the selected plant species.

To select the more suitable culture medium, different media (Hgd ½, Hbt and Htr 1/10) were tested. Moreover, the effect of incubation conditions was also evaluated, cultivating *L. minuta* in different growth chambers and in a glasshouse. The aspect of plants is shown in Fig. 1, and tissue biomass obtained at the end of the experiment is shown in Fig. 2.

In Fig. 1 it is possible to observe the higher pigmentation and healthy aspect of plants in Hgd ½ medium than in other media. The images of plants incubated in Htr (1/10) are not shown because there was no growth of plants in this medium outside of the growth chamber used.

In contrast, the highest growth was observed for plants incubated in growth chamber 1 using Hgd (½) medium. Under the mentioned condition, the biomass obtained was seven times higher than that obtained under the other conditions ($p < 0.05$).

These results are in agreement with those of other authors which showed that Hgd medium is suitable to efficiently cultivate aquatic plants (Srivastava et al. 2011).

Hgd (1/2) medium, high light intensity (600 μmol m^{-2} - s^{-1}), 24 ± 4 °C, 70% of relative humidity and a photoperiod of 16/8 h (light/darkness) were more favorable for plant development. Thus, they were selected for the following experiments. It should be noted at this point that the data obtained, about the culture medium and incubation conditions, are important, as descriptive parameters for laboratory maintenance, allowing a growth characterization of the requirements of this native species from Córdoba province.

Determination of Cr and phenol phytoremediation potential

Cr(VI) and phenol tolerance screening in solid medium

Firstly, *L. minuta* Kunth tolerance to Cr(VI) and phenol exposition was evaluated through the root number developed under different concentrations of both contaminants, in order to establish their effects.

A significant reduction in root number average ($p < 0.05$) was observed when the concentration of both contaminants was increased (Table 2).

In particular, when *L. minuta* grew in presence of Cr(VI), root number average increased with the time; however, a significant reduction was observed from 1 mg/L Cr(VI), compared to the control without contaminant ($p < 0.05$). This effect was more pronounced in the highest Cr(VI) concentration (2.5 mg/L) evaluated, for which the root number was reduced around 50% compared with control plants, after 21 days of exposure. Under Cr treatment (2.5 mg/L) *L. minuta* fronds were chlorotic and root length was also lower than control (data not shown).

It has been described that toxicity of inorganic contaminants depends on its concentration, among other variables (Navarro-Aviñó et al. 2007). Moreover, since plant roots are the first organs to come in contact with Cr(VI), their growth is largely affected. The adverse effect of Cr on roots has been demonstrated in other plants, and this metal was strongly accumulated in this organ as insoluble compounds with low translocation to aerial parts of plants (Mallick et al. 2010).

In addition, a significant reduction in root number average of plants growing in 25 and 50 mg/L of phenol was observed compared to control plants ($p < 0.05$), and this effect was more pronounced and statistically significant at 21 days of incubation. However, treated plants showed green fronds in healthy condition similar to control plants, in all phenol concentrations used.

Regarding the lower toxicity observed in *L. minuta* plants growing with phenol compared with those growing with Cr(VI), some researchers have indicated that organic compounds are less toxic to plants since they are less reactive and probably accumulated in the biomass, in comparison with inorganic compounds (Cherian and Oliveira 2005).

When plant growth was estimated through the area occupied by plants in plates containing Cr(VI) or phenol, no statistically significant difference ($p > 0.05$) was detected compared with control plants. These results could indicate that these contaminants could produce toxic effects only in roots, without effect on aerial part of the plants, at least macroscopically.

Cr(VI) and phenol removal by L. minuta plants

Based on the first tolerance screening, *L. minuta* capability to individually remove Cr(VI) and phenol was evaluated.

It is important to clarify that abiotic controls (without plant) were carried out. In these controls, the concentration of Cr(VI) or phenol remained constant throughout the assay, indicating no significant loss by physical process.

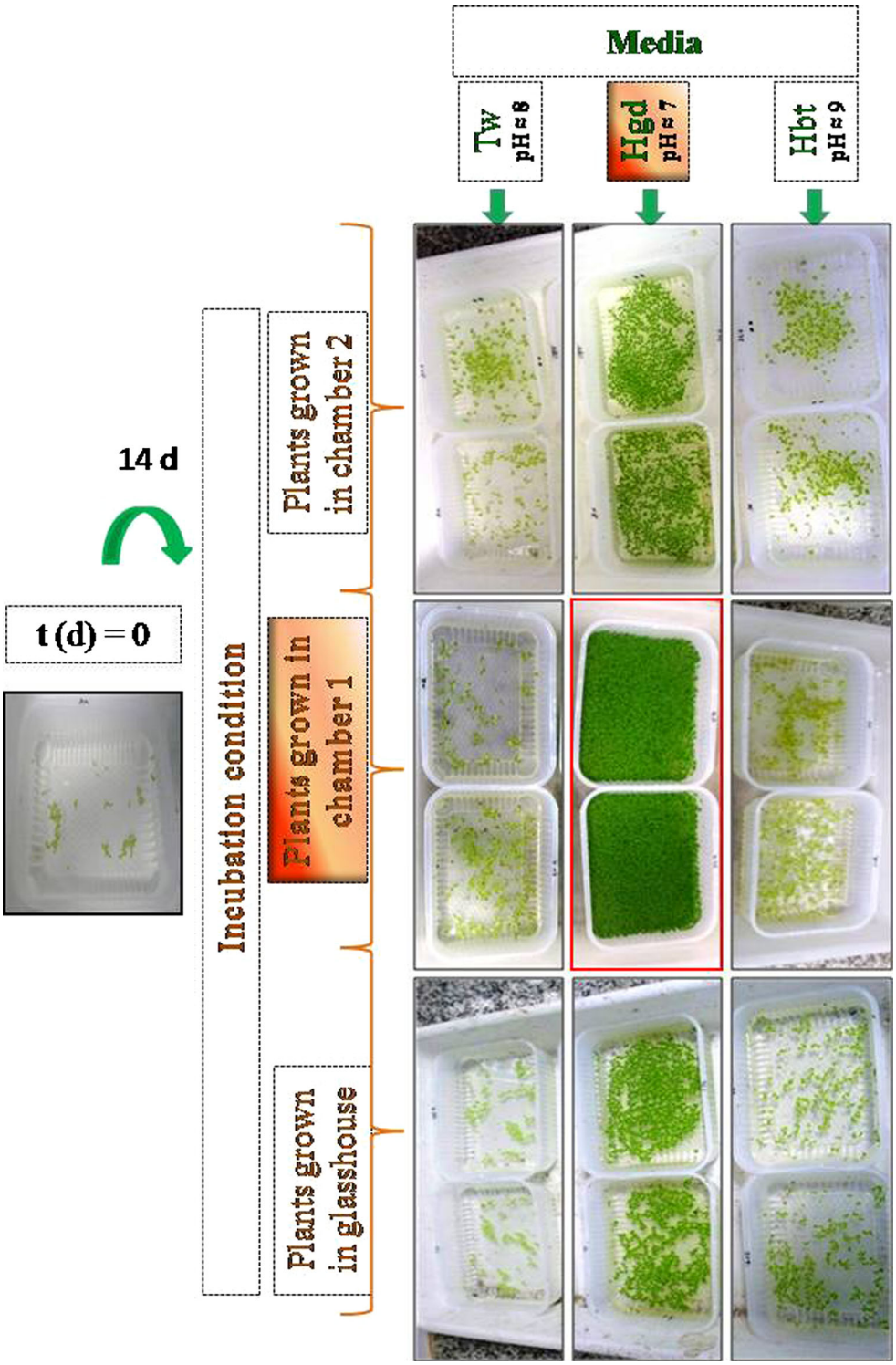

Fig. 1 Plant growth after 14 days of incubation under different culture conditions. Tw: Tap water, Hgd: Hoagland (1/2) medium, Hbt: Huebert (1/10) medium, t (d): Time (days)

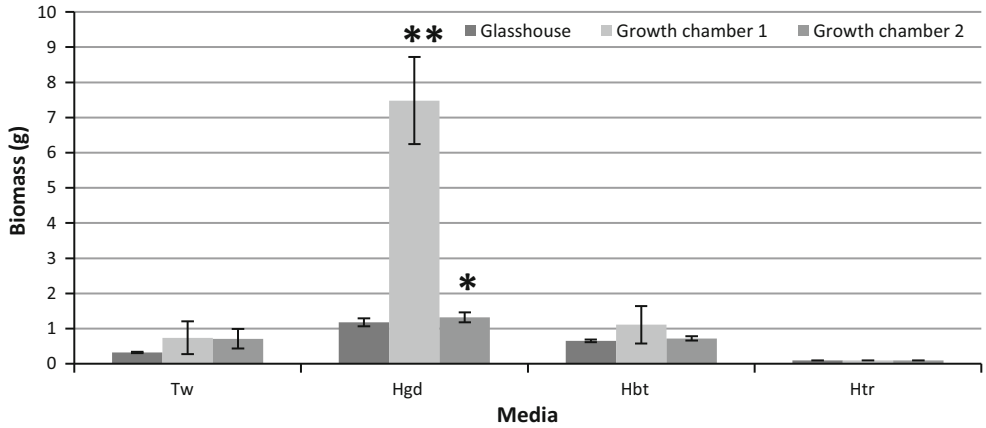

Fig. 2 Effect of culture media and incubation conditions on the biomass of *L. minuta* Kunth plants. *Represents significant differences respect to Tw-Glasshouse and Htr treatments. **Represents significant differences with all other treatments. Tw: Tap water, Hgd: Hoagland medium, Hbt: Huebert medium, Htr: Hutner medium

Table 2 Root number average of *L. minuta* Kunth plants growing with different Cr(VI) and phenol concentrations

Concentration (mg/L)		Root number average (±SD)		
		Time (days)		
		7	14	21
Cr(VI)	0	15 ± 3	31 ± 7	41 ± 10
	0.5	16 ± 2	26 ± 4*	31 ± 9
	1	10 ± 3*	24 ± 4*	19 ± 6*
	2.5	9 ± 3*	20 ± 7*	18 ± 5*
Phenol	0	15 ± 3	31 ± 7	41 ± 9
	5	15 ± 4	28 ± 4	28 ± 9
	25	12 ± 3	23 ± 7	22 ± 3*
	50	9 ± 2*	22 ± 4	25 ± 1*

* Represents significant differences with respect to controls without contaminants ($p < 0.05$)

For low Cr(VI) concentrations (1–5 mg/L), high removal efficiencies were observed, which varied between 75 and 85%, after 21 days of exposure. Contrarily, when the contaminant concentration increased to 10 and 20 mg/L, removal efficiency was drastically reduced (20 and 10%, respectively), after 18 days of treatment (Fig. 3).

The ability of *L. minuta* to remove phenol was also studied (Fig. 4). The results showed that plants were capable of removing phenol until 250 mg/L with an efficiency of 100%, after 11 days. Moreover, plants did not evidence macroscopic adverse toxic effects.

In these experiments, biomass was also determined (Table 3) and it could be observed that FW of Cr(VI)-exposed plants significantly increased (under 5 mg/L treatment) compared with control plants ($p < 0.05$). However, when higher Cr(VI) concentrations (10–20 mg/L) were used, biomass was similar to that reached by control plants ($p > 0.05$).

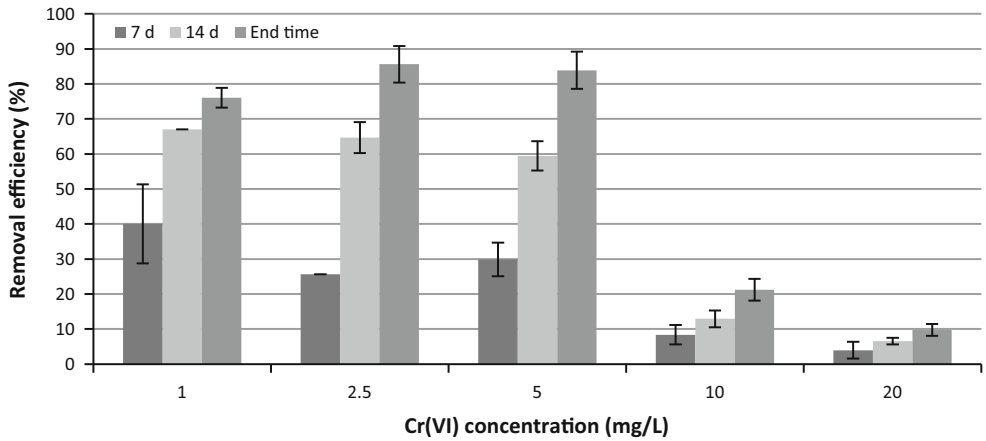

Fig. 3 Cr(VI) removal efficiency (%) of *L. minuta*, growing with different Cr(VI) concentrations

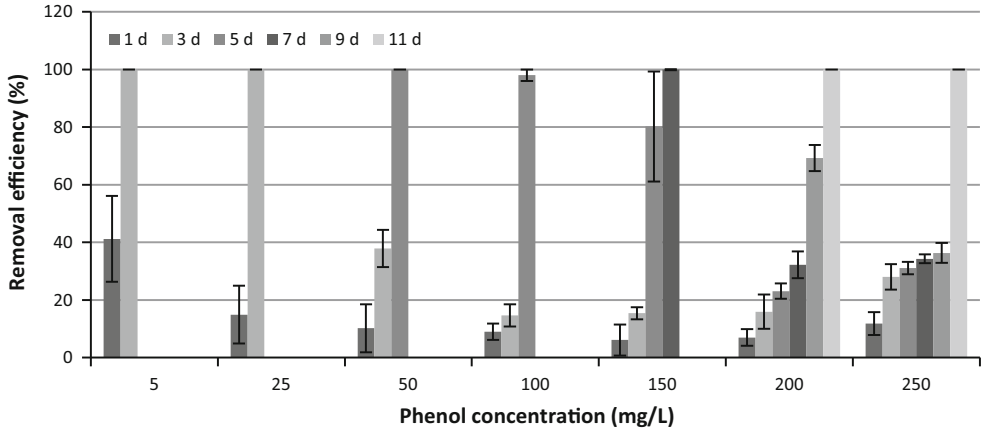

Fig. 4 Phenol removal efficiency (%) of *L. minuta*, growing with different phenol concentrations

On the other hand, FW of phenol-exposed plants showed no significant difference when phenol concentrations between 5 and 100 mg/L were used ($p > 0.05$). However, biomass data were significantly higher ($p < 0.05$) when these plants were treated with these phenol concentrations and exposed for more time (21 days, data not shown), clearly showing a stimulating effect of the contaminant on plant growth. Contrarily, this effect was not observed for phenol concentrations from 150 to 250 mg/L at experimental end time.

Later, simultaneous Cr(VI) and phenol removal assays were studied. A fixed Cr(VI) concentration (2.5 mg/L) and different phenol concentrations (2.5–250 mg/L) were used in these experiments. The results are shown in Fig. 5.

Cr(VI) and phenol removal increased with time, reaching 100% of phenol removal in all the studied combinations. In addition, Cr(VI) removal efficiency increased from 75 to 95% as phenol concentrations changed from 25 to 250 mg/L, reaching the maximum removal when 250 mg/L of phenol was added to the medium.

Table 3 Biomass of Cr(VI) and phenol-exposed plants

Treatment	Concentration (mg/L)	Harvest time (days)	Fresh weigth ± SD (mg)
Control	0	3	161.3 ± 9.7
		5	174.7 ± 10.2
		7	304.3 ± 8.9
		11	439.9 ± 4.4
		18	446.4 ± 34.4
		21	366.1 ± 54.6
Cr(VI)	1	21	315.5* ± 41.8
	2.5	21	429.8* ± 20.7
	5	21	445.6* ± 50.0
	10	18	186.3 ± 12.6
	20	18	114.4 ± 13.0
Phenol	5	3	174.4 ± 16.4
	25	3	162.7 ± 4.2
	50	5	176.9 ± 7.4
	100	5	180.2 ± 0.8
	150	7	268.9 ± 41.0
	200	11	352.5 ± 26.4
	250	11	307.2 ± 24.5

* Represents significant difference with control without contaminant ($p < 0.05$). Harvest time varied based on complete contaminant removal

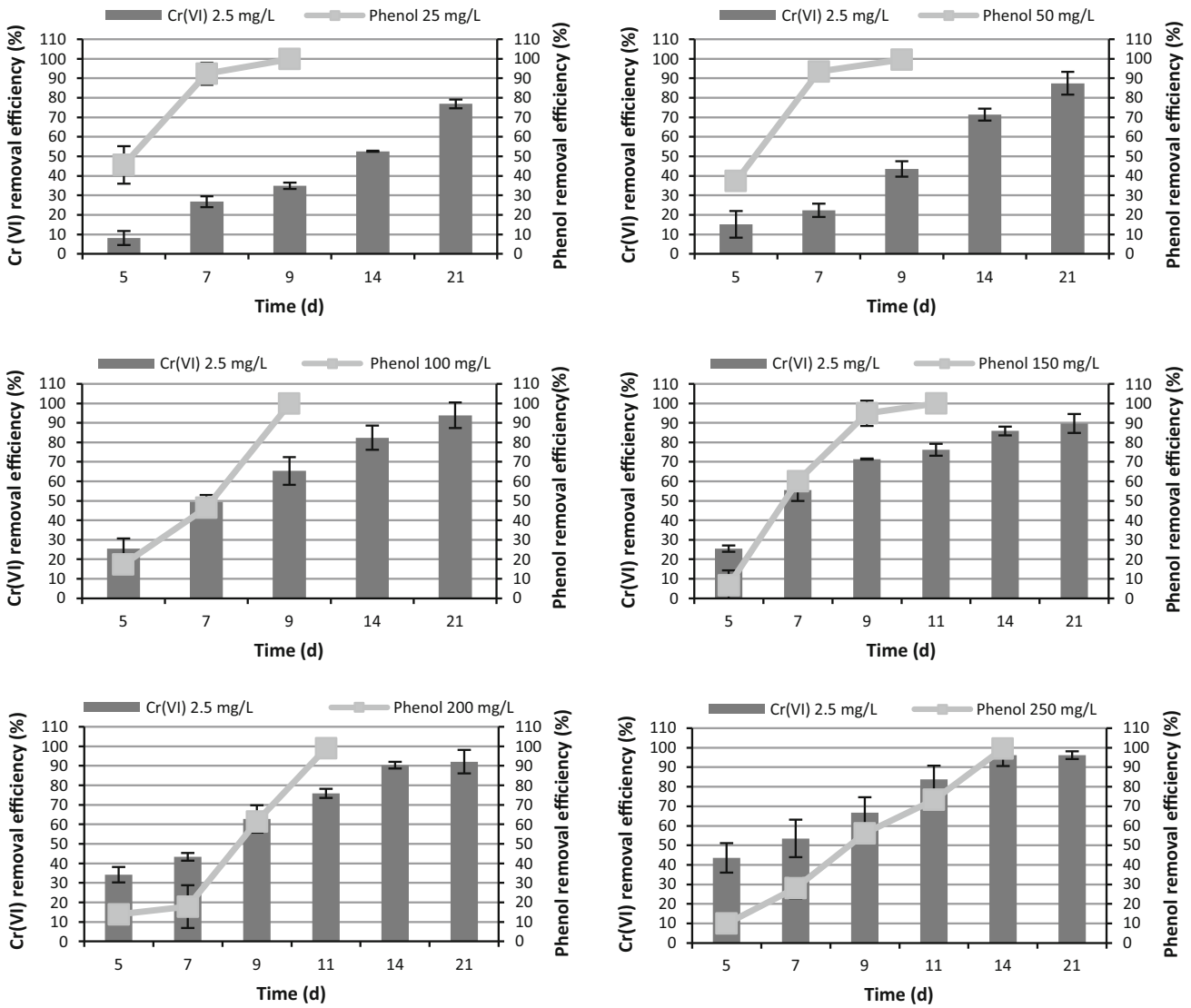

Fig. 5 Cr(VI) and phenol removal efficiency (%) of *L. minuta*, growing in presence of 2.5 mg/L Cr(VI) and different phenol concentrations

It is important to note that the time for total phenol removal increased when both contaminants were added to the culture media. It is well known that metal uptake and accumulation exert a toxic effect related to inhibition of root cell division and elongation, tissue death and inability to absorb water and other substances (Teixeira et al. 2013). This deleterious effect on the development of this vital organ could reduce the absorption of phenol, affecting its removal. Our studies support this hypothesis because *L. minuta* plants growing in presence of Cr(VI) showed lower root length (data not shown) and a reduced number of roots at this Cr concentration, despite that maximum removal efficiencies (100%) were reached.

Contrarily, it is remarkable the positive effect that phenol addition produced on Cr(VI) removal, reaching values of 10% higher compared with *L. minuta* plants only exposed to Cr(VI). Thus, these results showed a stimulating effect of phenol on Cr(VI) removal. This could be explained by physiological responses induced under phenol exposure which could counteract the stress that Cr(VI) produces on plants, such as antioxidant response. In this response, glutathione production could in turn participate in Cr(VI) removal since the formation of glutathione–chromium complexes has been described as a detoxification mechanism (Guttmann et al. 2008; Kart et al. 2016).

As it is shown in Fig. 6, the biomass of plants growing in presence of both contaminants was lower than that of controls with each contaminant separately. However, it is notable that they reached biomass values higher or similar to the control without contaminants. Thus, these results

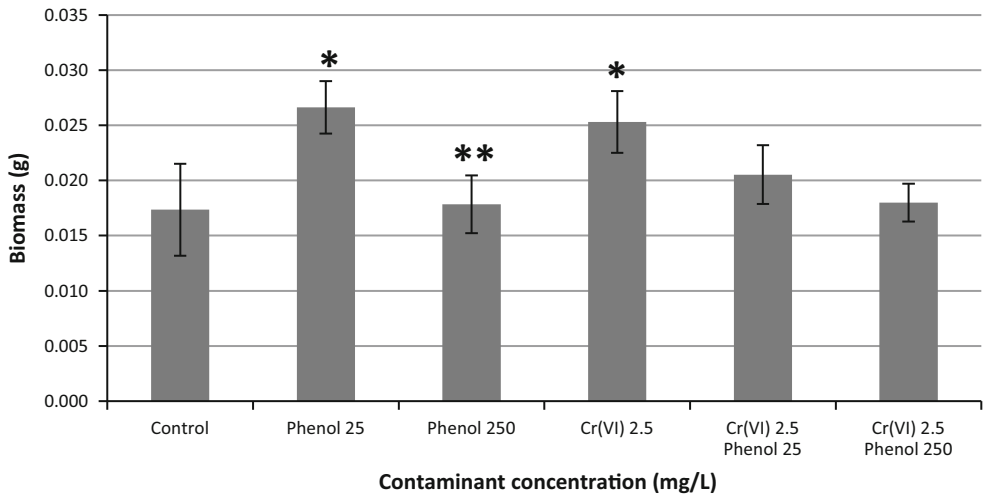

Fig. 6 Effect of Cr(VI) and phenol on biomass (g) of *L. minuta.* *Represents significant differences with respect to control, phenol 250 mg/L and Cr(VI) 2.5 mg/L-phenol 250 mg/L treatments. **Represents significant statistic differences with Cr(VI) 2.5 mg/L-phenol 25 mg/L

show that *L. minuta* plants were resistant to mixed contamination, being capable not only to survive in these conditions, but also to have a normal growth.

Notably, plants exposed to phenol (25 mg/L) during 21 days showed significantly higher growth that the uncontaminated control ($p < 0.05$). It is well known that plants can polymerize organic compounds and then compartmentalize pollutant metabolites in the "bound" residue fraction of plant cell walls (Talano et al. 2010). However, if this process would be occurred in the studied plant with *L. minuta*, no changes in biomass would be expected. In this context, we suggest that phenol could be used as carbon and energy source as it is accepted for bacteria. For that, enzymes that catalyze the opening of phenolic ring should be present. Similarly, Jha et al. (2013) indicated that *Helianthus annuus* hairy roots metabolized phenol by *orto* cleavage metabolic pathway similar to that was also described for bacteria, since some intermediates were found such as *cis*,*cis*-muconic acid and fumaric acid.

As it can be seen in Fig. 6, phenol (250 mg/L) did not promote growth compared with control plants, which could be attributed to the toxicity produced by this high concentration in plant metabolism. In this regard, it has been demonstrated that phenol treatment is frequently associated with the inhibition of plant growth and an increase of oxidative stress (Harvey et al. 2002).

L. minuta plants exposed to Cr(VI) (2.5 mg/L) showed a higher biomass than the corresponding control, which can be attributed to an hormesis effect. Poschenrieder et al. (2013) defined to hormesis as the stimulated phase in growth response that is induced by low concentrations of toxic metal ions without evidence of the underlying mechanisms. Moreover, they indicated that the production of reactive oxygen species and, consequently, the induction of antioxidant system are key mechanisms for hormesis effect induced by metals.

Nowadays, many plant species are being investigated to determine their potential and effectiveness for phytoremediation process, especially those with high growth rate such as aquatic macrophytes (Rezania et al. 2016). Between them, there are several studies that supported the fact that *Lemna* species shows an exceptional capability for remediation of heavy metals and metalloids as well as organic compounds, even surpassing that of algae and other aquatic macrophytes (Chaudhary and Sharma 2012). In this sense, plants belonging to *Lemna* genus have been used to recover heavy metals from contaminated environment for over 30 years, being *L. minor* and *L. gibba* the most representative species of *Lemna* genus used for phytoremediation assays (Guimaraes et al. 2012). Particularly, our results shows *L. minuta* Kunth plants have shown high efficiency to remediate phenol and chromium even in simultaneous, which it is not frequently considered; despite the mixture of pollutants is the prevailing situation in nature. Thus, this less explored *Lemna* species could be considered as a promising environmental phytoremediator.

However, it is important to remark that aquatic plants could also be used for wastewater treatment in constructed wetlands, involving also in this case their association to microbial community (Vymazal 2007). Thus, with the contribution of microorganisms, involving their own and different metabolic processes from those of used by plants,

CW system allow to gather phytoremediation and bioremediation properties for the improvement of organic and inorganic pollutants removal from contaminated water (Truu et al. 2015).

From all the above results, it is possible to suggest that *L. minuta* plants could use phenol as donor of carbon and energy and that an antioxidant response would be triggered by both contaminants, allowing an efficient phytoremediation process. However, these results must be confirmed with other studies such as detection of specific intermediates of the biodegradation process and molecules associated with oxidative stress. As an approach of that MDA content in plants exposed to phenol and Cr(VI) was evaluated.

Malondialdehyde and chlorophyll/carotenes determination MDA content was determined because it is a product of the lipid peroxidation of cell membrane, indicating oxidative damage. Contents of chlorophylls and carotenes were also measured in order to estimate the effect of the contaminants on plant physiology.

It was observed that there were no significant differences ($p > 0.05$) between MDA content, *Ca*, *Cb*, *Ca*/*Cb* relationship and carotenes of the treated plants with respect to control plants.

Similarly, Ibáñez et al. (2012) and Teixeira et al. (2013) did not find differences in chlorophyll, carotenes and MDA content in different plant species treated with Cr(VI) or phenol. However, they evaluated the effects of each contaminant individually, which may not reflect a synergistic or additive effect of both pollutants when they are added simultaneously. In this context, the fact that *L. minuta* plants did not show changes in these variables could be interpreted as that Cr(VI) and phenol do not produce toxic effects that affect photosynthesis and that, if oxidative stress occur, it is ameliorated by antioxidant system.

Cr speciation determination

To assess the final localization of the metal, *L. minuta* plants were placed in Hgd ½ were supplemented with Cr(VI) and phenol (200 mg/L). Total content of Cr, Cr(III) and Cr(VI) in the liquid medium and biomass was determined.

Cr was found mainly in the plant biomass (99.94%), while the remaining 0.06% was found in the liquid medium, after 21 days. Most Cr was found as Cr(VI) (more than 90%), both in the biomass and in the liquid medium.

The obtained results indicated that the reduction of Cr(VI) to Cr(III) was almost negligible. Thus, the main phytoremediation process of chromium by *L. minuta* would be sorption or accumulation in the biomass.

In this regard, Badr and Fawzy (2008) indicated that the bioremoval technique using aquatic plants contains two uptake processes: (1) an initial fast, reversible, metal-binding processes (biosorption) and (2) a slow, irreversible, ion-sequestration step (bioaccumulation). The metals removal mechanisms, through biosorption or bioaccumulation, are diverse and have been deeply discussed by several authors (Tobin et al. 1984; Veglio and Beolchini 1996; Wang et al. 1996). However, regarding Cr(VI) removal by aquatic plants and other macrophytes, both processes have been observed (Hossain et al. 2005; Chakraborty et al. 2014; Dan et al. 2016; Jena et al. 2016). Thus, bioaccumulation and/or biosorption could be the mechanisms responsible for Cr removal by *L. minuta*. However, these mechanisms have not been elucidated in this work and represent an interesting perspective of this study.

Conclusion

Lemna minuta Kunth was able to remove Cr(VI) and phenol with high efficiency. When plants were exposed to these pollutants they grew normally, producing a biomass similar or higher than control plants without exhibiting symptoms of toxicity. Plants exposed to phenol were able to produce more biomass that uncontaminated plants, after a long adaption period (21 days), suggesting that this contaminant could be used as carbon source. Moreover, Cr(VI) stimulated the plant growth at low concentrations, thus an hormesis effect was clearly evidenced. During simultaneous exposition to both contaminants, plants did not show evidence of damage on photosynthetic processes neither on lipids from cell membrane.

Sorption and/or accumulation into the biomass would be the main process involved in Cr(VI) removal.

Therefore, *Lemna minuta* Kunth could be suggested as an suitable candidate for the phytoremediation of wastewater contaminated with Cr(VI) and phenol, such as those derived from tanneries. However, a more exhaustive investigation about its ability to remove other contaminants and to reduce chemical and biochemical oxygen demand could contribute to establish its potential use for the treatment of diverse industrial effluents. These studies as well as those that could elucidate the mechanisms used by the plants to remove Cr(VI) and phenol are being underway in our laboratory.

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