Evaluation of ecotoxicity of contaminated water for validation of phytoremediation time

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ABSTRACT

Phytoremediation has been used as an alternative for removal of heavy metals in aquatic environments, but plant residence time and toxicity reduction need to be studied. Ecotoxicological bioassays and root anatomic studies were conducted in order to validate the phytoremediation of *Echinochloa crus-galli* L. at three different ages. The experiment was conducted using *E. crus-galli* seeds with processing factorial experimental design (2x3) and four replicates. Cadmium presence and absence (0.8 and 0 mg L^{-1}) at three times (20, 30, and 45 days after germination). Cd levels were quantified by flame atomic absorption spectroscopy on both aerial parts and roots. A bioassay was performed testing both acute and chronic effects using microcrustacean *Daphnia similis* with the purpose of evaluating phytoremediation efficiency. Regardless of biomass, *E. crus-galli* L. can be used for 16 to 19 days for 45 days after germination (DAG), tolerating the phytotoxicity of this metal. The residual solution after phytoremediation had chronic effect on *D. similis,* indicating that the time taken was not sufficient to reduce the toxicity of the solution. Thus, ecotoxicological essays are important tools in evaluating the efficiency of this type of process. While *E. crus-galli* L. is a valuable tool in Cd phytoremediation programs, exposure time must be higher than 19 days to reduce concentrations of this metal in the water to conform to the CONAMA 357/2005 e 430/2011 standards.

Keywords: acute and chronic effect, *Echinochloa crusgalli* L. macrophytes, time of exposure.

Avaliação da ecotoxicidade da água contaminada com cádmio para validação do tempo de fitorremediação

RESUMO

A fitorremediação tem sido utilizada como uma alternativa para remoção de metais pesados em ambientes aquáticos porém o tempo de permanência das plantas e redução da toxicidade precisam ser estudados. Neste sentido, bioensaios ecotoxicológicos e estudos da anatomia radicular foram realizados com o objetivo de validar a fitorremediação de *Echinochloa crusgalli* L. em três tempos após período de germinação. O experimento foi conduzido utilizando sementes de *E. crusgalli* L. com esquema fatorial (2x3) e quatro repetições. Presença e ausência de cádmio (0,8 e 0 mg L^{-1}) em três idades (20, 30 e 45 dias após a germinação). Os níveis de Cd foram quantificados por espectroscopia de absorção atômica com chama nas partes aéreas e raízes das plantas. Bioensaios para avaliar os efeitos agudos e

crônicos frente ao microcrustáceo *Daphnia similis* foi utilizado com intuito de avaliar a eficiência do processo de fitorremediação. Independentemente da biomassa, *E. crusgalli* L. até 45 dias após germinação (DAG) pode ser usado por 16 a 19 dias para remediação tolerando a fitotoxicidade deste metal. A solução residual após a fitorremediação teve efeito crônico sobre *D. similis* indicando que o tempo utilizando não foi suficiente para reduzir o efeito tóxico da solução. Conclui-se que os ensaios ecotoxicológicos são ferramentas importantes na avaliação da eficiência deste tipo de processo. Assim, *E. crusgalli* L. pode ser usado em programas de fitorremediação de Cd em baixas concentrações, porém o tempo de exposição deverá ser maior que 19 dias visando reduzir as concentrações deste metal na água de forma a atender os padrões CONAMA 357/2005 e 430/2011.

Palavras-chave: *Echinochloa crusgalli* L. macrófitas, efeito crônico e agudo, tempo de exposição.

1. INTRODUCTION

The removal of heavy metals from aquatic ecosystems has attracted attention, all around the world because of their pronounced or drastic effects as biosphere contaminants (Ullah *et al.,* 2015). Among heavy metals, cadmium (Cd) is widely found in phosphates and zinc mining. It is also commonly used in rechargeable batteries (Ko *et al.,* 2012) and eventually can reach aquatic bodies. Inhalation by occupational exposure has been historically registered, but the carcinogenic risk of cadmium-contaminated food and water have been ignored by regulatory agencies. The negative impact of Cd comes not only from its toxic effect but also from different accumulative capacities in different aquatic organisms (Ullah *et al.,* 2015). In this context, Qiu and Wang (2016) observed in Daya Bay, a typical subtropical bay in southern China, that higher cadmium concentrations (5.11 $\mu g.g^{-1}$ dw) were found in mollusks (*Veremolpa scabra*) than in shrimp and crab. In addition, according to these authors, higher Cu, Zn and Cd concentrations have been recorded in wild fish than in animals kept in tanks. For example, Ekweozor *et al.* (2017) and Shovon *et al.* (2017) found heavy metals, including Cd in fish tissues above the limits allowed for human consumption according to FAO/WHO, which implies problems for human health. Remediation solutions, such as phytoremediation, need to be evaluated. Fawzy *et al.* (2012) indicate that plants might accumulate Cd in all tissues. In contrast to chemical analyses that identify and quantify the concentrations of toxic substances, the bioassays toxicity test evaluates the effect of substances on the organisms (Costa *et al.,* 2008).

In the exhaustive search for alternatives to clean-up aquatic environments polluted with cadmium, two major requirements stand out, the simplicity of method efficiency and lower costs (Sharma and Pandey, 2014). According to De Souza and Silva (2019), the phytoremediation process is an excellent biotechnological technique in terms of expense and sustainability, since it aims at improvements with minimum impact to nature. Knowledge of the ideal plant age for higher absorption/accumulation in relation to the period of exposure is a key aspect for better implementation and usage of this technique. Gomes (2012) and De Souza and Silva (2019) emphasize that longer periods of exposure to contaminants are a negative point of this process; however, information about exposure time is largely unknown. Like any other remediation process, the use of plants is designed to reduce contaminant levels to levels that are safe and compatible with human health (Andrade *et al.*, 2007). Singha *et al.,* (2019) elucidate the importance of aquatic macrophytes in facilitating phytoremediation for global sustainability and environmental pollution. Aquatic macrophytes have been used in phytoremediation programs with metals in tropical regions. Their use has shown high rates of heavy metal removal of 85% - 95% for iron, copper, zinc, cadmium, arsenic and chrome with plants of tested species such as *Eichhornia crassipes, Pistia stratiotes, Lemna minor, Echinochloa crusgalli,* among other macrophytes (Pereira *et al.,* 2011; Martelo and Lara Borrero, 2012; Silva *et al.,* 2013). Peng *et al.* (2017) cite that *E. crusgalli* has potential for

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phytoremediation of Cd in soils, but there is no data for water or how much time is required to perform the phytoremediation process.

This work evaluated the cadmium removal capacity of *Echinochloa crusgalli* L. at three different ages (20, 30 and 45 days) to validate the duration of the phytoremediation by chemical analysis and ecotoxicological essays with both acute and chronic exposure by *Daphnia similis* as test-organism. Anatomical modifications will also be evaluated in the presence of the metal.

2. MATERIAL AND METHODS

The experiments were carried out in a greenhouse and *E. crus-galli L. Beauv.* seeds were used. Plants at 20, 35 and 45 days after germination (DAG) were transferred to 2-L trays with modified Hoagland and Arnon 1950 nutritive solution (ionic force 40%) supplemented with 0.8 mg L^{-1} of Cd or, in the negative control, without Cd and incubated for 10 days. During this period, plants were sorted by size and shape to keep the population uniform as the test organism. Figure 1 shows a general design of the methodology used.
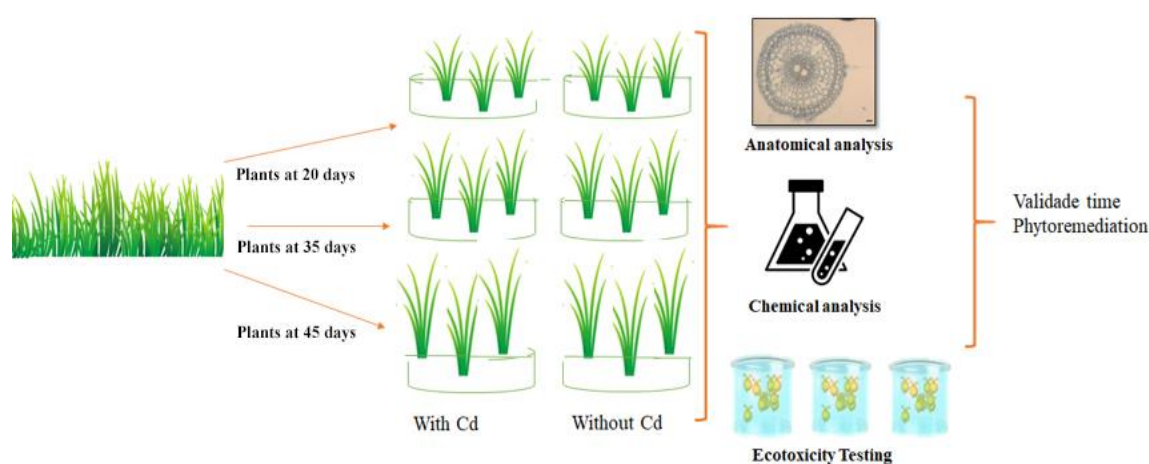
Figure 1. Design of the methodology using *E. crus-galli L. Beauv.*

The cadmium concentration used here (0.8 mg L^{-1}) was four times higher than the maximum allowed according to Brazilian legislation CONAMA No. 430/2011 (Conama, 2011) for discharges in water bodies and nearly the maximum tolerated by *E. crus-galli L. Beauv* (unpublished data).

The experimental design was completely randomized (CRD) with 2x3 factorial (2 concentrations x 3 Days After Germination – DAG) and four replicates. Each replicate contained 15 plants. Initially, the normality of data was tested using Shapiro Wilk normality test (p> 0.05). In cases of non-normal data, the $(x + 1)0.5$ transformation were applied. After this, variance analyses were performed using the F test (p <0.05) and the Scott-Knott test was implemented in SISVAR software (Ferreira 2011).

To evaluate the capacity of *Echinochloa crusgalli* L. in removing cadmium at three different DAG, after 10 days of exposure the plants were removed and root samples were selected for anatomy analyses. Their roots were collected and fixed in FAA (formaldehyde, acetic acid and 70% alcohol) and later conserved in alcohol 70° GL. They were cross-sectioned with manual microtome. The sections were stained with a 0.5% toluidine blue mixture, following methods described by Kraus and Arduin (1997). The sections were placed on slides with glycerinated gelatin and covered with coverslips. Three sections of blade were analyzed, one blade was mounted for each experimental replicate, and three measurements per section. The sessions were photomicrographed using an Olympus U-TV1X-2 digital camera coupled to the Olympus CX41 microscope and assisted by Carl Zeiss AxioVision Viewer 4.8 software.

Measurements were performed without software for image analysis.

For each root section, an endoderm index, a count and a sequence of metaxylem calculations, and the Carlquist-IVC Vulnerability Index calculation (Carlquist, 1975) were measured according to Equation 1:

$$IVC = \text{Tracheal Element Diameter (µM) / Number of Tracheal Elements} \qquad (1)$$

The remainder, aerial parts and root system were separated for oven drying at 60°C for 72 hours. For Cd quantification (in µg g^{-1}), about 0.5g of dry material was digested in 10 mL of HNO$_3$, filtered in a rapid-flow quantitative filter and washed with 10 mL of MILLI Q water, according to USEPA (1998). The Cd accumulation and concentration in roots and in aerial parts were evaluated according to Equations 2 and 3:

$$\text{Aerial part} = \text{Cd content in aerial part x dry} - \text{matter weight} \qquad (2)$$

$$\text{Roots} = \text{Cd content in roots x dry} - \text{matter weight} \qquad (3)$$

Quantification of Cd in plants and in the aqueous solutions after phytoremediation in the three phases of development and in the control were performed by flame atomic absorption spectrometry (Agilent Technologies 200 Series).

The Phytoremediation Index (PI) can be understood as the plant's ability to remove a certain amount of toxic element over time. This index is dependent on the relative growth rate (TCR), which represents the dry masses in grams (g), the time in days and biomass accumulation of Cd in milligrams (mg) divided by the amount of dry mass of the plant in grams (g) for each age and proportional to the exposure time, according to Equation 4 (Pereira, 2010):

$$PI = (\text{TCR x metal concentration}) \qquad (4)$$

To determine how many days plants should be kept in the system so that phytoremediation could reach the desired levels, Equation 5 was used - Phytoremediation in Days (PD).

$$PD = (\text{Concentration / PI) / Plant's number} \qquad (5)$$

An acute and chronic bioassay was performed with the microcrustacean *Daphnia similis* Claus (Cladocera, Crustacea) according to Brazilian law Nbr 12713/2016 and Nbr 13.373/2017 (ABNT 2016; 2017). The test consisted of the exposure of 10 females of *D. similis* to the nutrient solution with and without Cd after phytoremediation with *E. crus-galli L.* within the 3 stages of growth: 20, 35 and 45 days after germination (DAG). As control 1, water was used to culture these organisms. The dead / living organisms were counted after 24 h to 120 h of exposure in the acute bioassay and for 10 days in the chronic bioassay. The following variables were quantified: Temperature (T °C), Conductivity (µS.cm^{-1}), Dissolved Oxygen (mg L^{-1}) and pH at the beginning and end of tests.

3. RESULTS AND DISCUSSION

3.1. Anatomical analysis

Cd and other heavy metals removal through phytoremediation emerges as a sustainable technology for contaminated wastewater (Mahajan and Kausha, 2018). In this experiment, no association was observed between the ages of *E. crus-galli* and Cd concentration in different anatomical parameters. These variables behaved independently the presence of Cd, as in Table 1:

Table 1. Percentage of aerenchyma in cortex, Carlquist vulnerability index (CVI) and endoderm thickness (µm) of *Echinochloa crusgalli* roots in relation to DAG.

Time DAG	Aerenchyma (%)	CVI	Endoderm thickness (µm)
20	24.8 a	14.1 a	10.7 b
35	14.4 b	9.6 a	12.2 a
45	8.4 b	17.6 a	12.2 a
CV (%)	48	51	7.35

* Averages followed by the same letter do not differ statistically from each other (Skott-knott, 5%).

E. crus-galli is considered a weed to several crops from flooded and/or irrigated areas, particularly rice crops. As a strategy for anaerobic conditions, rice developed aerenchyma, which allows the transport of O_2 from the air to the roots. Aerenchyma formation can be constitutive or induced by an environmental factor (Castro *et al.*, 2009). In the present study, there was higher aerenchyma formation in roots of plants at 20 DAG, which might be associated with a strategy for establishment in a flooded environment (as the trays used in our assays). The same occurred at a lower intensity in older plants (Table 1). Starting at 35 DAG, there was a lower number of aerenchyma, which may favor the adaptation of these plants to environments contaminated with heavy metals, since with more cells, more Cd probably could fixate within their walls (Tian *et al.,* 2011; Küpper and Andresen, 2016). The result of CVI of *E. crus-galli* plants for all ages analyzed indicate that the transport of assimilates and nutrients were not affected by the presence of Cd in the solution.

Plants cannot escape fluctuations in nutrient availability and / or toxicity in their environment, so they create endodermal barriers. They are modified in response to a multitude of abiotic stresses (Doblas *et al.*, 2017). Endoderm thickening, as observed in Table 1, may have protective roles for the root system because of lignin impregnation within the cell walls. These barriers can be formed when roots are exposed to high concentrations of potentially toxic elements such as Cd (Lux *et al.,* 2011). According to Silva *et al.,* (2013) and Castro *et al.,* (2009), apoplastic barriers can act as biological filters, avoiding the excessive absorption of Cd following the obligatory symplast path, which reduces metal translocation to the aerial part, thus protecting the photosynthetic system from its toxic effects. The plant`s root is the organ in charge of nutrient acquisition, but at the same time it must provide an efficient boundary against external stresses in order to maintain plant fitness.

3.2. Chemical analysis

The results of the present study indicated that the cadmium in plants showed higher concentration and accumulation in roots than in the aerial parts during 10 days of exposure for all plant ages (20, 30 and 45 DAG) (Table 2).

Table 2. Cd concentration and accumulation in root and aerial part of *Echinochloa crus-galli* L. grown in solution with Cd and phytoremediation in days. Phytorem: Phytoremediation.

Time DAG	[Cd] (mg L^{-1})	Weight of root dry mass (g)	[Cd] in root (µg g)	Cd Accumulation in root (µg g)	Weight of aerial part dry mass (g)	[Cd] in aerial part (µg g)	Cd accumulation in aerial part (µg g)	Phytorem. in days (PD)
20	0	0,413 ±0,04 b	3,3 ±0,3 d	0,01 ±0,003 d	0,520 ±0,04 c	2,3 ±1,7 c	0,01 ±0,001 d	0
35	0	0,416 ±0,04 b	6,2 ±0,3 d	0,02 ±0,003 d	0,850 ±0,04 b	3,1 ±1,7 c	0,02 ±0,001 d	0
45	0	0,503 ±0,04 b	6,4 ±0,3 d	0,03 ±0,003 d	0,916 ±0,04 a	5,4 ±1,7 c	0,04 ±0,001 d	0
20	0,8	0,346 ±0,04 b	313,7 ±9,1 a	0,55 ±0,03 c	0,516 ±0,04 c	51,7 ±1,7 a	0,27 ±0,01 c	19 ±1a
35	0,8	0,466 ±0,04 b	270,3 ±9,1 b	0,83 ±0,03 b	0,796 ±0,04 b	38,9 ±1,7 b	0,35 ±0,01 b	16 ±1a
45	0,8	0,896 ±0,04 a	231,7 ±9,1 c	1,36 ±0,03 a	0,906 ±0,04 a	54,0 ±1,7 a	0,40 ±0,01 a	16 ±1a

* Averages followed by the same letter in the column do not differ statistically from each other (Skott-knott, 5%). Transformed data.

Plants at 45 days when grown in cadmium solution presented higher weight of dry root matter compared to plants without metal solution. This result demonstrates the tolerance of these plants at low concentrations of Cd. Ezaki *et al.* (2008) also observed that at concentrations below 0.1mM of Cd and Cu, *Echinochloa crus-galli* showed tolerance, not changing the relative length of the roots. The higher dry-mass weight of the 45-day Cd treatment contributed to the higher accumulation of Cd in the root. The weight of aerial part dry mass increased over time, contributing to the accumulation of Cd in aerial parts over time. This root accumulation can also be explained by endoderm thickness on the 35- to 45-day-old plants which act as apoplastic barriers in the roots protecting the aerial part. Similar results were also observed by De Maria *et al.,* (2013) with sunflowers, concluding that in the initial growth phase, the first most probable response of the plant was metal accumulation (with the production of phytochelatins that immobilize Cd in cells), whereas later, plants reduce and/or block the absorption and translocation of the toxic metal. Lux *et al.,* (2011) confirmed that cadmium-concentrations are often higher in roots than in aerial parts. Regarding time, Gomes *et al.,* (2014) observed that efficiency in reducing mercury (Hg) using young *Typha domingensis* (10 cm of the water column) is dependent on the time of exposure. The authors found that at less than 5 days, 90% of Hg had been removed from the water column. This result can be explained by the biomass difference between *T. domingensis* and *E. crus-galli* macrophytes. It was observed that at all ages of *E. crus-galli*, there was 25% absorption, which indicates inadequate exposure time, since it did not reach the maximum limit allowed for the release of effluent by Brazilian Legislation (0.2 mg L^{-1} of Cd). This fact may be due to the occurrence of saturation of the binding sites of this metal in *E. crus-galli* roots (Table 2). This same result was found by Oliveira *et al.,* (2001) when exposing *Eichhornia crassipes* to solution with different Cd concentrations. The authors observed that above 1 g L^{-1}, saturation of the binding sites of this metal in the roots plants was observed, since the increase in the Cd content in roots was only 7.3%, even with a two-fold increase in the concentration of this element in the nutrient solution. Bindu *et al.,* (2010) evaluated the phytoremediation capacity of *Colocasia esculenta* at different Cd concentrations and observed that at the same concentration over time there was an increase in the Cd removal percentage. Plants were evaluated at 5, 10, 15 and 20 days of exposure, obtaining 30.5%, 34.5%, 40.5% and 41% of removal, respectively, at a Cd concentration of 2 mg L^{-1}. In the present study, since *E. crus-galli L* had smaller biomass compared to *C. esculenta* and no rhizome, the presented removal percentage was around 25% at 10 days of exposure.

The calculation in phytoremediation days was performed because of the lack of this information in the literature. This information is useful for companies that wish to employ phytoremediation as an alternative to remove Cd from their contaminated wastewaters. It was found that values between 16 and 19 days (estimated by Phytoremediation in Days (PD), regardless of plant age, would be sufficient time for remediation of the solution containing Cd at the test concentration (Table 1). *E. crus-galli* L. has potential for Cd phytoremediation at low concentrations of Cd in solution; however, in our experiment, Cd concentrations in the solution remained higher than that allowed by Brazilian law. It is possible to conclude that *E. crus-galli* L with higher biomass, that is, with greater DAG, would be more likely to survive for 16 to 19 days, tolerating the phytotoxicity of this metal (Table 1). According to Fernandes (2014), at Cd concentrations of up to 1 mg L^{-1} in solution, this plant did not present morphological and physiological damage (non-published data). However, in order to achieve this result, it is necessary to increase root biomass, which is responsible for assimilation.

Within the analyzed the ages of *E. crusgalli* (20, 35 or 45 DAG), the exposure time of 10 days was not sufficient to verify differences in the cadmium removal from the solution. For DAG times of 20, 35 and 45,   Cd concentration in the solution (mg L^{-1}) was 0.60 ±0.02; 0.57 ±0.02; 0.63 ±0.02, respectively. The control was 0.09 ±0.02 mg L^{-1}.

3.3. Ecotoxicity testing

In relation to the ecotoxicological assays, no acute effect after 48 hours of exposure of *D. similis* was observed in the solution after phytoremediation. However, at this residual concentration (0.6 mg L^{-1} of Cd), exposed for longer time (10 days), chronic effects were observed (Figure 2). Although the addition of the nutrient solution has caused a considerable increase in the conductivity values, this alteration was not enough to provoke an osmotic imbalance in *D. similis*, but the effect observed was due to the presence of residual cadmium.
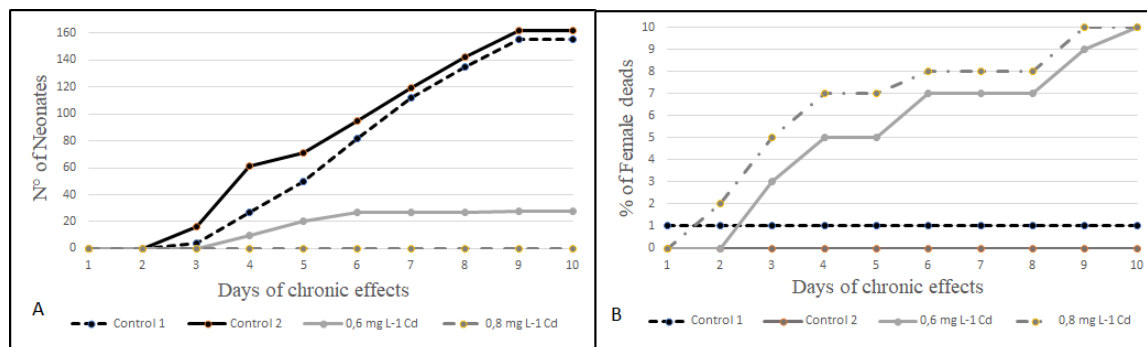
Figure 2. Results of ecotoxicological assays. *Daphnia similis* survival at different exposure times in solution with and without Cd after phytoremediation with *Echinochloa crusgalli* L. for 10 days. Legend: Control 1 - Cultivation water; Control 2 - Hoagland and Arnon   nutritive solution (1950) without Cd; [0.6 mg L^{-1} Cd] - Nutritive Solution with Cd after 10 days (Onset); [0.8 mg L^{-1} Cd] - Hoagland and Arnon Nutritive Solution (1950) with Cd (Ending).

For females of *D. similis* exposed for 10 days at 0.6 mg L^{-1} Cd concentration (after phytoremediation), there was an effect on reproduction, with low production of newborns and non-reproduction in those exposed to 0.8 mg L^{-1} Cd (initial concentration) (Figure 2a). These results are corroborated by the low survival rate of females (Figure 2b). Phytoremediation using *E. crus-galli* reduced the toxicity by only 25%. Penha *et al.* (2014) observed similar data when exposing *Girarda* sp. to 0.8 mg L^{-1} CdSO$_4$ for 96 hours, with only 35% survival. The results of ecotoxicological tests corroborate those obtained in the present study, in which this effluent cannot be released into aquatic ecosystem according to Brazilian Resolution 430/2011, since toxicity has not been fully removed, remaining above the legal threshold.

4. CONCLUSIONS

Our data indicate that exposures of *E. crus-galli* for periods longer than 19 days may be required to reduce the concentrations of Cd in water to levels that comply with Brazilian legislation. Under these conditions, ecotoxicological tests were important tools for evaluation of the efficiency of the process. However, this kind of evaluation is not common to phytoremediation studies.

Independent of DAG, *E. crus-galli* reduces around or up to 25% Cd toxicity in the contaminated solution. For this, this species can be used in phytoremediation programs of Cd with other plants.

As a characteristic of aquatic macrophytes, the species used adapts to different environmental conditions, a fact that was observed after root anatomy analysis.

5. ACKNOWLEDGMENTS

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