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Effect of concentration and exposure time on copper accumulation in *Eichhornia crassipes* (Mart.) Solms. (Pontederiaceae)

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Abstract: Different factors can influence the absorption and storage of substances in plant biomass. In this study, we evaluated the effect of copper concentration in growth medium and plant exposure time on copper accumulation in *Eichhornia crassipes* (Mart.) Solms. (Pontederiaceae) roots and leaves under controlled conditions. Plants were subjected to four treatments of copper concentrations of 1, 3 and 5 $\mu\text{g}\cdot\text{mL}^{-1}$, with a control treatment of 0 $\mu\text{g}\cdot\text{mL}^{-1}$, and evaluated at seven-day intervals over 21 days. Copper concentration in biomass was analysed by atomic absorption spectroscopy with flame atomisation. The results showed that copper concentration in the growth medium and time of exposure had a significant effect on the amount of copper accumulated by *E. crassipes* roots and leaves, with roots more efficient compared to leaves. It is likely that *E. crassipes* has mechanisms for the translocation of metal from the root system to the leaves. Symptoms of copper toxicity were observed in the vegetative parts of the plants at the end of the experiments. This study demonstrates that *E. crassipes* presents great potential in the absorption and accumulation of copper under laboratory conditions, indicating its effectiveness for applications in phytoremediation processes.

Keywords: Bioaccumulation; aquatic macrophytes; heavy metals; water resources.

Introduction

The mobilisation of heavy metals through human activities and their various applications has led to a wide release of these elements into the environment (Girard, 2013). The increased concentration of these metals in the environment generates harmful effects on human health and the natural components of aquatic and terrestrial ecosystems (Ali et al., 2013). Among metals, copper is consistently found in urban, industrial and agricultural waste (Sodré & Lenzi, 2000). In low concentrations this metal is essential to all living things, however when present above certain limits it becomes potentially toxic (Yruela, 2005). Water is the main source of dispersal of these contaminants in the environment, either by surface runoff or percolation and contact with underground springs. Therefore, knowledge of concentrations as well as the removal of these elements from aquatic environments is extremely important to environmental and public health (Moraes & Jordão, 2002).

Among current methods used in the removal of metals from water, those involving traditional physicochemical processes usually present technical-financial constraints, as well as causing alterations and disturbance to the environment (Ali et al., 2013; Silva et al., 2014). In this sense, phytoremediation is a feasible alternative technique due to a favourable cost-benefit ratio, relatively simple execution and the possible mobilisation of non-biodegradable substances (Agunbiade et al., 2009). This technology is based on the use of plants as natural catalysts for absorption and substance accumulation (Aurangzeb et al., 2014). Biological aspects associated with high and constant productivity, as well as the large capacity for absorption and storage of nutrients are all factors of great interest in the use of aquatic macrophytes for phytoremediation (Henry-Silva & Camargo, 2008; Kumar et al., 2008; Yu & Zhou, 2009).

In general, aquatic macrophytes are used as bioindicators in the detection of environmental changes induced by human activities, as they have

high sensitivity or tolerance to pollution parameters (Espinoza-Quiñones et al., 2005), and are commonly used in the treatment of effluent in aquatic environments due to their ability to accumulate heavy metals through adsorption and desorption processes (Low & Lee, 1990; Fett et al., 1994; Diniz et al., 2005; Lima et al., 2016). Due to these properties, macrophytes have been widely used in controlled experiments to determine the pattern of these processes (e.g. Freitas et al., 2017). Among these species, *Eichhornia crassipes* (Mart.) Solms. (Pontederiaceae) stands out due to its highly productive capability of increasing its biomass by up to 15% per day (Alves et al., 2003), and is therefore considered an important invasive of aquatic environments in tropical and subtropical regions of the world (Holm et al., 1991).

Considering the productive potential and the capacity of *E. crassipes* in retaining and accumulating various substances (Guimarães et al., 2006; Mokhtar et al., 2011; Vitoria et al., 2011), this study evaluated (i) the effect of copper concentration, and (ii) plant exposure time to contaminant in the accumulation of copper in the roots and leaves of *E. crassipes* under laboratory conditions, indicating its possible application as a phytoremediation species in aquatic environments contaminated with copper.

Methods

Study area: Specimens of *E. crassipes* were obtained from fish ponds located along the banks of natural water bodies within the rural area of Alta Floresta, northern Mato Grosso, Brazil (9°53'54.9"S and 56°03'38.5"W). The climate in this region is warm, humid and tropical (AW) under the Köppen classification, characterised by high rainfall summers and dry winters. Samples were collected in July 2014. After collection, the plants were conditioned in thermal boxes and transported to the Integrated Laboratory of Chemical Research (Laboratório Integrado de Pesquisas Químicas – LIPEQ) at the Sinop-MT Campus of the Federal University of Mato Grosso (Universidade Federal de Mato Grosso – UFMT)

Eichhornia crassipes is a free floating macrophyte native to South America, and can reproduce sexually or by stolons. The recommended pH of culture water for plant growth is between 6 and 8, with temperature between 25°C and 28°C, although the species can survive over a wide temperature range (e.g. Oliveira, 2012). This macrophyte is distributed in practically all water bodies in Brazil, with yield values of up to 1,000 kg.ha⁻¹.d⁻¹ (Costa, et al., 2000).

Laboratory procedures: All plants were washed in the laboratory under running water to remove impurities from the collection site. The plants were then distributed into 12 plastic containers each containing 30 litres of water with additional nutrients necessary for plant maintenance [(240 mL of Ammonium Carbonate (NH₄)₂CO₃ (1 mol.L⁻¹); 90 mL

of Potassium Phosphate Dibasic (K₂HPO₄) (1 mol.L⁻¹)]. Buffering of pH between 6.5 and 7.0 was carried out by adding Potassium Phosphate Monobasic (KH₂PO₄) (1mol.L⁻¹) to the culture medium.

Twelve specimens of *E. crassipes* were placed in each container, totalling 144 plants evaluated. These were divided into four treatments (T1, T2, T3 and T4), with each treatment differing in copper concentration (T1 = 1 µg.mL⁻¹ copper, T2 = 3 µg.mL⁻¹ copper, T3 = 5 µg.mL⁻¹ copper, and T4 = 0 µg.mL⁻¹ copper as the control treatment). The copper concentrations used in each treatment were defined based on the maximum value allowed (MPV = 0.009 mg/L Cu) in aquatic ecosystems, established by Brazilian Law, Resolution No. 357 March 17, 2005, National Council of the Environment (Conselho Nacional do Meio Ambiente – CONAMA, 2005), with all treatments being above the maximum value, thus characterising a contaminated environment. The entire experiment was conducted under laboratory conditions, with monitoring and control of water pH (6.5 and 7.0) using a portable pH meter and automatic light control (12 hours light/day).

Removal of plants for chemical analyses occurred at intervals of 0, 7, 14 and 21 days of exposure to the different contaminant concentrations (T1, T2, T3 and T4). At each of these seven-day intervals, three individuals of *E. crassipes* from each of the treatments were taken for analysis. After the containers were removed, the plants were lightly washed under running water then placed into a drying oven at 70°C for seven days. After drying, the roots and leaves of the plants were crushed separately. The zero-day exposure time treatments were analysed upon arrival from the field in order to maintain the original conditions of their natural environment.

For the individual digestion of root and leaf samples, 0.2 grams of dry mass from each sample was used. The digestion consisted of a mixture of sulfuric acid (H₂SO₄) and Hydrogen Peroxide (H₂O₂) (Hseu, 2004). In each sample 7 mL of H₂SO₄ was added and after one hour, 3 mL of H₂O₂, with subsequent heating at 250 °C in a digester block. After two hours the samples were taken from the digester block and, after cooling, an additional 4 mL of H₂O₂ was added. After the biomass digestion process, the samples were diluted with distilled water and a 25 mL solution was obtained, with the present copper content then determined by atomic absorption spectroscopy with flame atomisation (Varian model AA140). The standard solution used for the calibration curve was traceable to Specscol's NIST (National Institute of Standards and Technology). In total, 288 *E. crassipes* biomass samples were analysed, 144 from the leaves and 144 from the roots.

Statistical analysis: To determine any significant mean differences in the accumulation of copper between treatments (T1, T2, T3 and T4) over time (0, 7, 14 and 21 days), an Analysis of Variance

for two factors (two-way ANOVA) was used. Both effects "days of experiment" and "pollutant concentration" were considered fixed effects. Accumulation of the pollutant throughout the experiment was represented by a scatter plot, with the Y axis representing copper accumulation in the plants, and the X axis representing the ordinal variable "days of experiment". To evaluate accumulation as a function of time, a non-parametric trend line was fitted. All models and figures were constructed in R environment (Core Team, 2014).

Results and Discussion

The pattern of copper accumulation in the roots and leaves of *E. crassipes* was similar, however, when considering the total concentrations obtained, we observed the highest values in the roots (Figure 1). When evaluating the rate of total copper accumulation in leaf biomass, it was observed that the greatest accumulation occurred within plants exposed to the 5 $\mu\text{g.mL}^{-1}$ concentration (2,823 $\mu\text{g.g}^{-1}$ of Cu), followed by plants exposed to the 3 $\mu\text{g.mL}^{-1}$ concentration (1,770 $\mu\text{g.g}^{-1}$) (Figure 1a). The lowest accumulation values were observed in the 1 $\mu\text{g.mL}^{-1}$ treatment, ranging between 7 $\mu\text{g.g}^{-1}$ and 480 $\mu\text{g.g}^{-1}$ (Figure 1a). Control samples (0 $\mu\text{g.mL}^{-1}$) showed no oscillation.

Copper accumulation in leaf biomass over time (0 to 21 days) was higher in plants exposed to the 5 $\mu\text{g.mL}^{-1}$ Cu concentration (466 times higher than the control), followed by the 3 $\mu\text{g.mL}^{-1}$ concentration (354 times the control value). The lowest accumulated value was obtained in plants exposed to the 1 $\mu\text{g.mL}^{-1}$ concentration (108 times the control) (Figure 2). The accumulation of copper by *E. crassipes* leaves varied significantly between

copper concentrations (Two-Way ANOVA: $F_{3,128} = 161.97$; $p < 0.001$), as well as time of exposure (Two-Way ANOVA: $F_{3,128} = 148.53$; $p < 0.001$). The interaction between the two variables – copper concentration and time of exposure – was also significant (Two-Way ANOVA: $F_{9,128} = 32.54$; $p < 0.001$).

In relation to copper accumulation in the roots of *E. crassipes*, higher values were recorded for the 5 $\mu\text{g.mL}^{-1}$ concentration (8,180 $\mu\text{g.g}^{-1}$) (Figure 1b). For the roots of plants subjected to the 3 $\mu\text{g.mL}^{-1}$ concentration, there was little variation in absorption after the first 7 days of exposure, presenting an accumulated value of 5,258 $\mu\text{g.g}^{-1}$. Accumulation in the roots of plants exposed to the 1 $\mu\text{g.mL}^{-1}$ concentration was 1,145 $\mu\text{g.g}^{-1}$ (Figure 1b). For total time of plant exposure to contaminants, accumulation of Cu by the roots was higher in plants subjected to the 5 $\mu\text{g.mL}^{-1}$ concentration (790 times the value of the control), followed by the 3 $\mu\text{g.mL}^{-1}$ concentration (185 times the value of the control), then the 1 $\mu\text{g.mL}^{-1}$ concentration (161 times the value of the control) (Figure 2).

Significant variation was shown in accumulation by *E. crassipes* roots between different copper concentrations (Two-Way ANOVA: $F_{3,128} = 44.45$; $p < 0.001$), and between the number of days of exposure (Two-Way ANOVA: $F_{3,128} = 34.41$; $p < 0.001$). As observed in the accumulation of copper in leaf biomass, the interaction between pollutant concentration and days of exposure was also significant (Two-Way ANOVA: $F_{3,128} = 9.48$; $p < 0.001$). It was observed that at the end of the 21 days of the experiment, the remaining plants presented signs of necrosis and tissue destruction in all evaluated treatments.

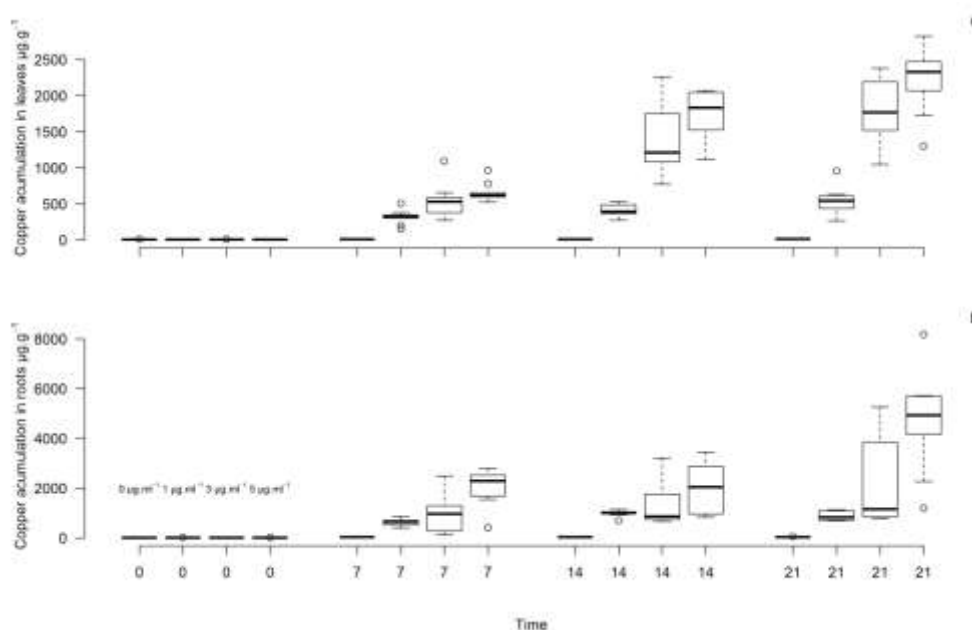


Figure 1. Accumulation of copper in leaves (a) and roots (b) of *E. crassipes* over the 21 days of the experiment according to the analyses carried out at 0, 7, 14 and 21 days with copper concentrations of 1, 3 and 5 $\mu\text{g}\cdot\text{mL}^{-1}$.

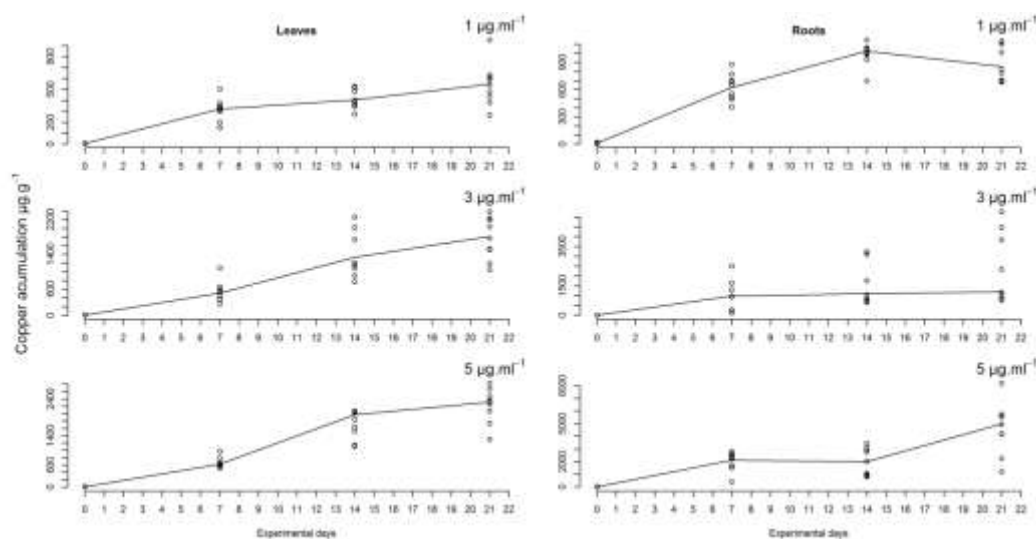


Figure 2. Variation in the rate of copper accumulation by *E. crassipes* throughout the experiment, separated by different concentrations and location of accumulation (leaves and roots).

Eichhornia crassipes showed good performance in the removal of copper from the growth medium, and in the accumulation of this copper in the roots and leaves. We observed that this accumulation was proportional to contaminant concentrations in the growth medium, where the plants exposed to the highest concentrations of copper had a higher absorption rate. This was recorded for both leaf and root biomass. Levels of metal present within plant biomass were also positively related to time of exposure - over the 21 days of the experiment the longer the plants were in contact with the contaminated medium the more metal was absorbed. These results confirm our assumption that *E. crassipes* has considerable potential in the accumulation of metals (Guimarães et al., 2006; Oliveira et al., 2001).

The effects of increased contaminant concentration and availability in the medium, as well as the longer exposure time of macrophytes to contaminants are known. Oliveira et al. (2001) observed that *E. crassipes* removed a significantly higher amount of cadmium from medium when compared to *Salvinia auriculata* Aubl. (Salviniaceae) with this difference increasing as exposure time to cadmium was increased. Hu et al. (2010) reported that the rate of absorption of cadmium, copper and lead by *Sagittaria sagittifolia* L. (Alismataceae) and *Potamogeton crispus* L. (Potamogetonaceae) was dependent upon the increase of metal concentration in the medium, in both plant roots and leaves. Naaz et al. (2013) argue that the absorption kinetics of copper and zinc by *E. crassipes* under similar concentrations and exposure time have resulted in antagonistic behaviours in metal absorption by plants, indicating that other factors such as pH may

influence storage mechanisms of these metals in plant biomass. However, our results show that the relationship between contaminant concentration in the medium and exposure time of plants were determinant factors in total absorption levels obtained. It can be inferred that time is a key factor in the absorption process as it is difficult to control in phytoremediation processes, considering plant physiological mechanisms and their relationship with the absorption and accumulation of metals (Grant et al., 1998; Souza et al., 2009; Yabanli et al., 2014).

The higher accumulation of copper in the roots of *E. crassipes* in relation to the leaves was expected, mainly because it is a free-floating species of macrophyte. These plants have the ability to adsorb materials suspended in water through their root system before these materials settle into the sediment (Matagi et al., 1998). In addition, the aerial parts of the plants do not remain in full contact with the medium, as opposed to the root system (Oliveira et al., 2001). Similar results showing higher accumulation of metals in the roots of macrophytes were obtained in laboratory studies of arsenic absorption using *S. auriculata* and *E. crassipes* (Guimarães et al., 2006). The same was observed in a natural system where higher concentrations of mercury were found in the roots of floating and rooted macrophytes (Molisani et al., 2006).

The cell wall of the roots maintain direct contact with metal ions dissolved in the medium, with this contact related to the plant's increased filtration capacity, and consequently an increased tolerance to contaminants (Rodrigues et al., 2016). An example of this mechanism was presented by Ribeiro et al. (2015) when he associated the highest accumulation of lead (Pb) in *Echinodorus*

grandiflorus Rich. ex Engelm. (Alismataceae) to the thickening of the plant's apoplastic root barriers, and considered this adaptation as a tolerance strategy to the metal. In general, the mechanisms of substance absorption and accumulation by plants are governed by rhizofiltration processes (Kara et al., 2003; Pio et al., 2013).

Copper accumulation in the leaves of *E. crassipes* was continuous over the 21-day experiment, a behaviour that was not observed for the roots. In this species, due to the structures that favour its flotation in water and the distribution of its leaves, even in natural conditions, leaf contact with the culture solution is small and therefore the participation of the leaves in ion absorption from the nutrient medium is reduced (Oliveira et al., 2001). Oliveira et al. (2001) observed that approximately 90% of the metal absorbed by *E. crassipes* was concentrated in the roots of the plants, suggesting that a high translocation rate to the leaves does not occur. Differing results were presented by Mangabeira et al. (2004), who identified the presence of deposits in apoplastic areas through histological cuts, indicating the occurrence of cadmium translocation to other compartments of the plant despite the greatest accumulation of the metal occurring in the roots. The translocation of lead (Oliveira, 2012) and glyphosate (Martins et al., 2009) into tissues of *E. crassipes* is still reported.

The processes of translocation and compartmentalisation of metals in macrophytes are known (Fett et al., 1994; Grant et al., 1998; Souza et al., 2009; Yabanli et al., 2014). However, for some species, such as *E. crassipes*, this mechanism is not fully elucidated. Campanelli et al. (2010) point out that the translocation of metals to the leaves occurs when the concentration of metals in the roots approaches its limit, which would serve as a protection strategy for the photosynthetic functions of the plant. Translocation mechanisms are associated with chelation, where after metals are bound to chelators, they are transported to intracellular structures such as vacuoles, where they accumulate and their reactivity and toxic effects on plants is reduced (Santos et al., 2011).

Based on the significant presence of copper in leaf biomass, the results of our study suggest the occurrence of a translocation process between the root system and the vegetative part of *E. crassipes*. This result is confirmed by the decline or stabilisation of copper uptake in roots, while concentrations in leaf biomass increase over time (Mokhtar et al., 2011). The translocation mechanisms are related to the species's tolerance to the metals and, in this respect, the results of this study indicate that *E. crassipes* presents potential for application in the phytoremediation of environments contaminated by copper. More detailed studies of the morphology and physiology of *E. crassipes* are required in order to determine the translocation mechanism of copper to other parts of the plant.

During the experiment *E. crassipes* showed symptoms of degradation, most probably due to copper toxicity. Among the symptoms presented were necrosis points in the leaves, petioles and foliar bulbs; darkening of tissues; stagnation in growth and disintegration of part of the tissues at the end of the experiment (21st day). Many of these symptoms are associated with plant oxidative stress, which is related to the formation of reactive oxygen species, among them the radical superoxide (O_2^-), hydroxyl (HO^*) and hydrogen peroxide (H_2O_2) (Alves et al., 2003). Mendes et al. (2009) verified morphological changes in *E. crassipes* caused by mercury, due to its toxic effects on plant growth. Pereira et al. (2014) assert that anatomical and physiological changes in *E. crassipes* are associated with lead tolerance, allowing an increase in CO_2 capture capacity and hydraulic conductivity. Other species of macrophytes present similar symptoms related to metal toxicity (Peixoto et al., 2005; Wolff et al., 2012, Guimarães et al., 2012; Freitas et al., 2017).

Although *E. crassipes* showed visible symptoms of toxicity in this study, the metal was accumulated incrementally throughout the experiment, indicating mechanisms of adaptation to the high concentrations of this contaminant in the environment. Control treatment plants also presented these symptoms to a lesser extent, suggesting that acclimatisation factors or even senescence of older plants may have contributed to these results, although copper toxicity is attributed the greater contributor in this regard.

Eichhornia crassipes is a macrophyte utilised for several purposes, and can be a good source of sequestering metals present in the water, since, as observed, the high copper content found in the leaves and roots demonstrates the species' potential to be used in phytoremediation, recovery of water resources and effluent polishing. The results show that the storage and distribution of copper in the biomass of roots and leaves of this species most probably occurs due to its tolerance strategies to the metal. In addition to the plant's resistance to high levels of copper in the growth medium, the mechanisms involved in its translocation may have important implications in the length of time the metal stays within the plant, and the eventual feedback into the aquatic environment.

Conclusion

The aquatic macrophyte *E. crassipes* showed efficacy as a copper accumulator at different concentrations over time. The concentration of copper and time of plant exposure to the contaminant are determinant variables in the efficacy of the species in accumulating the metal. The root system was the area with the greatest accumulation of the metal, however, it is possible to infer that there is a high translocation rate of the metal from the root system to the leaves, considering the high concentrations of copper

obtained in its biomass. The plants showed moderate sensitivity to the metal and *E. crassipes* can be recommended as a phytoremediate of copper in aquatic environments.

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