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Copper accumulation by the root and leaf biomass of *Salvinia natans* (L.) All. (Salviniaceae)

Larissa Braun de Souza
Federal University of Mato Grosso

Corresponding author
Franciele de Freitas
Federal University of Mato Grosso
francieledfreitas@gmail.com

Sabrine Lunardi
Federal University of Mato Grosso

Janaina Saiz Cassins Von der Osten
Federal University of Mato Grosso

Rafael Arruda
Federal University of Mato Grosso

Ricardo Lopes Tortorela de Andrade
Federal University of Mato Grosso

Leandro Dênis Battirola
Federal University of Mato Grosso

Abstract. Aquatic plants are often exposed to metal contamination. The study evaluated the bioaccumulation of copper (Cu) ions in aqueous solution by the biomass of leaves and roots of the macrophyte *Salvinia natans*. Plants of *S. natans* were submitted to culture solutions with different concentrations of Cu ions, evaluated at intervals of seven days. The leaf and root samples were separately subjected to atomic absorption spectroscopy with flame atomization to assess the concentration of copper accumulated in its biomass. The results demonstrated a pattern of accumulation dependent on the concentration of the metal in the culture medium and the time of exposure of the plants to the contamination. The accumulation was greater in the biomass of the roots when compared to the leaves. Throughout the experiment, toxicity symptoms were observed in the morphology of plants subjected to all copper concentrations, demonstrating the macrophyte's viability for bioindicating the toxicity of this metal in aquatic environments. A high accumulation of copper ions was obtained both in the biomass of the roots and leaves of the plants, confirming their potential bioaccumulator of Cu. The analysis of biomass suggests an important characteristic of metal compartmentalization by the plant, associating the absorption by the roots and the possible transfer to the leaves. In general, our results show that *S. natans* is an organism with a potential bioindicator and bioaccumulator of Cu and consists of a viable cost-effective option for phytoremediation of aquatic environments contaminated by metals.

Keywords: Aquatic ecosystems; Metals; Phytoremediation; Plants; Treated solutions

Introduction

Bioaccumulation is the process by which living beings absorb and retain certain chemical substances by way of metabolic activity, often explored in phytoremediation strategies (Sooksawat et al., 2013; Rodriguez-Hernandez et al., 2019;

Suman et al., 2018; Eid et al., 2019; Muro-González et al., 2020). In aquatic environments, the process of bioaccumulation of metallic substances by organisms is mainly guided by the quantity and chemical form of the element present in water, sediment or food source (Dummee et al., 2012; Hejna et al., 2019; Jeong et al., 2021).

Biological organisms that have this ability are often composed of agents that have the potential to remedy or indicate environmental impacts caused by the contamination of the environment by different chemical compounds, including heavy metals (Oves et al., 2016; Zhang et al., 2020). An example of success in this regard are aquatic macrophytes (Lunardi et al., 2017; Sun et al., 2019; Al-Homaidan et al., 2020).

Aquatic macrophytes are widely distributed in the tropical regions of the world, where they play different ecological roles, acting essentially as catalysts in the cycling of nutrients and as biological filters of various types of pollutants (Esteves, 1998; Chambers et al., 2008), and also in the dynamics and structuring of habitats supported by ideal climatic conditions (temperature, photoperiod, nutrients, etc.), its high productivity (Thomaz & Cunha, 2010). The potential these plants to remove contaminants, such as heavy metals, is associated with the fact that the constituents of the cell wall of these plants have a binding affinity for the metal ions, allowing for the capture of the elements of the aquatic environment and their accumulation in the biomass (Rodrigues et al., 2016; Freitas et al., 2019). Among the aquatic macrophytes, several species have been prominent in this function, mainly representatives of Salviniaceae (Freitas et al., 2018a, b; Loría et al., 2019; Palacio et al., 2020).

Copper is one of the most common metals in nature, present in living beings as an essential constituent, originating from geological processes or from residues arising from anthropic activities (Casagrande et al., 2018; Holtra & Zamorska-Wojdyła, 2020). It is widely used in agricultural industries, cosmetics, coatings, fungicides, food industry, chemical industry, fuel additives, textile industries, medical industry, paints, plastics, wastewater treatment, and electronics (Rajput et al., 2017).

In addition to the problem of high dispersion in the environment, the narrow limit between concentrations considered acceptable and those that are toxic to living beings in general is also a concern (Grigoletto et al., 2012; Santana et al., 2017; Zhou et al., 2018; Luo et al., 2020). In concentrations considered critical for plants, copper promotes leaf chlorosis and causes cytotoxicity and promotes oxidative stress by generating reactive oxygen species (ROS) that are harmful to plants (Kumar et al., 2020). The decline in plant biomass, reduced root growth and chlorosis is the most usual indication in plants under Cu stress (Lange et al., 2017; Freitas et al., 2018a).

Currently, studies are focused on evaluating the cumulative and support potential of plants exposed to large amounts of toxic metals. In the same sense, it is reported that the bioaccumulation process in different plant tissues is often unknown (Muro-González et al., 2020). This study evaluated the bioaccumulation of copper by the biomass of leaves and roots of the macrophyte *Salvinia natans* (L.) All. (Salviniaceae) by varying the initial concentration of the metal in the culture medium and the time of exposure of the plants to the contaminant, in order to its potential use in remediation strategies and treatment of liquid solutions with metals.

Methods

Plant material

Specimens of *S. natans* were obtained in a ponds located in the urban area of Alta Floresta, northern Mato Grosso, Brazil (9°53'54.9"S and 56°03'38.5"W). After collection, the plants were conditioned in thermal boxes and transported to Integrated Laboratory of Chemical Research (LIPeQ) at the Sinop-MT Campus of Federal University of Mato Grosso - UFMT.

The Salviniaceae are comprised of floating aquatic macrophytes, native to South America, which are very productive in these environments (Pitelli et al., 2014; Maria et al., 2018). *Salvinia* are popularly known as moss or water fern, is an annual macrophyte, heterosporate pteridophyte, whose reproduction occurs either by way of spores or vegetatively (Freitas et al., 2018a). This plant has a floating, branched and filamentous stem, with longer than wider leaves, covered with small hairs or papillae, all at the apex placed in regular rows and lying flat on the surface of the water (e.g. Bercu, 2006). The roots are adapted to function as a sponge so as to retain water and assist in the filtration of the nutrients (Pompêo, 2008).

Experimental design

All plants were washed in the laboratory under running water to remove impurities from the collection site. The plants were distributed into 12 containers each containing 30 liters of water with additional nutrients necessary for plants maintenance: 240 mL of Ammonium Carbonate $(NH_4)_2CO_3$ (1 mol.L^{-1}) and 90 mL of Potassium Phosphate Dibasic (K_2HPO_4) (1 mol.L^{-1}). Buffering of pH between 6.5 and 7.0 was carried out by adding Potassium Phosphate Monobasic (KH_2PO_4) (1mol.L^{-1}) to the culture medium. The values of pH values were selected based on the pH of the water from the water bodies where *S. natans* live in the region.

In each container 60 specimens of *S. natans* were placed. 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). Copper sulfate ($CuSO_4$) was used at the concentrations stipulated for the study. 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 N° 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 sets of five subjects of *S. natans* 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. The experiment was carried out in triplicate for each variable evaluated.

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 (H_2SO_4) and Hydrogen Peroxide (H_2O_2) (Hseu, 2004). In each sample 7 mL of H_2SO_4 was added and after one hour, 3 mL of H_2O_2, with subsequent heating at 250 ºC in a digester block. After two hours the samples were taken from the digester block and, after cooling, an additional 4 mL of H_2O_2 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 atomization (Varian model AA140). The standard solution used for the calibration curve was traceable to Specsol's NIST (National Institute of Standards and Technology).

Data 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

Salvinia natans presented an accumulation pattern which was dependent on concentration and time. In general, the higher the concentration of copper in the medium, the higher the accumulation levels in both leaf and root biomass. The results also show a higher copper concentration in the roots of the plants. The highest accumulation value was obtained for the root biomass, in treatment three (5 $\mu g.mL^{-1}$) at 14 days, when the accumulated copper content was 9.570 $\mu g.g^{-1}$, a 290-fold increase in relation to the initial value (0 days = 33 $ug.g^{-1}$). For leaf biomass, the highest value was 2.320 $\mu g.g^{-1}$, at 7 days, corresponding to a 105-fold increase in relation to the initial value (0 days = 22 $ug.g^{-1}$).

The rate of total copper accumulation in root biomass was higher in plants exposed to contamination of 5 $\mu g.mL^{-1}$ (9.570 $\mu g.g^{-1}$ of Cu), followed by plants submitted to contamination of 3 $\mu g.mL^{-1}$ (7.880 $\mu g.g^{-1}$ of Cu), and to a contamination of 1 $\mu g.mL^{-1}$ (840 $\mu g.g^{-1}$ of Cu) (Figure 1). The highest copper accumulation rate in leaf biomass also occurred for plants exposed to contamination of 5 $\mu g.mL^{-1}$ (2.320 $\mu g.g^{-1}$ of Cu), followed by plants exposed to a contamination of 3 $\mu g.mL^{-1}$ (1.920 $\mu g.g^{-1}$ of Cu) and to a contamination of 1 $\mu g.mL^{-1}$ (620 $\mu g.g^{-1}$de Cu) (Figure 1).

The pattern of copper accumulation over time was similar for plants undergoing all treatments (1 $\mu g.mL^{-1}$, 3 $\mu g.mL^{-1}$ e 5 $\mu g.mL^{-1}$), with a considerable increase being registered at the first seven days of experiment (the time when the highest values of accumulation occurred), followed by decay of the accumulation as from the 14th day (Figure 2). The control treatment (0 $\mu g.mL^{-1}$) and the time of 0 days were used only as reference and presented values considered tolerable and expected of copper in the biomass of the plants when it is also being a micronutrient, with variations over the experiment time not being observed.

These results evidenced significant differences between the accumulation of copper by the plants submitted to the different contaminant concentrations, both for the leaves (Two-way ANOVA: $F_{3,97} = 19.62$, $P < 0.001$) and the roots (Two-way ANOVA: $F_{3,98} = 191.39$, $P < 0.001$). The same was observed in relation to the time of exposure of the plants to the metal for the leaves (Two-way ANOVA: $F_{3,97} = 4.96$, $P = 0.003$) and roots (Two-way ANOVA: $F_{3,98} = 54.09$, $P < 0.001$). The interaction between metal concentration factors and exposure time to leaves was (Two-way ANOVA: $F_{8,97} = 2.50$, $P = 0.02$) and roots (Two-way ANOVA: $F_{8,98} = 18.26$, $P < 0.001$).

Throughout the experiment the plants presented typical symptoms of toxicity attributed to the metals, with changes in leaf color (darkening and yellowing), necrotic spots on the surface of the floating leaves, root fragmentation and tissue destruction being visually observed. At the end of the experiment (21 days) all remaining plants presented these types of morphological damage.

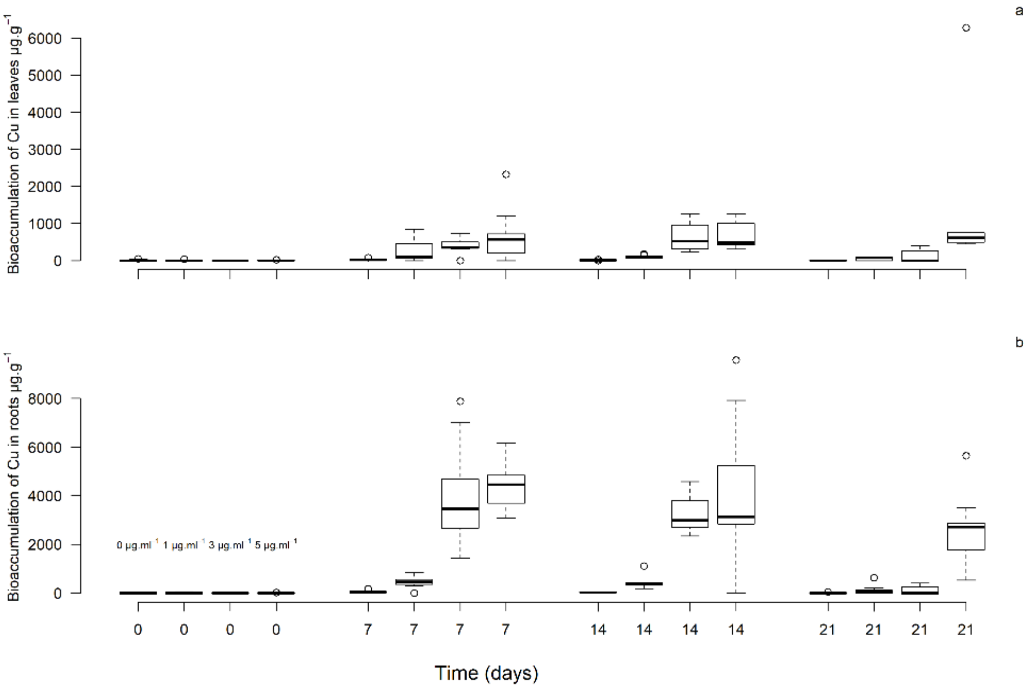

Figure 1. Accumulation of copper (Cu) by the biomass of leaves (a) and roots (b) of the macrophyte *S. natans*, evaluated in different treatments and days of exposure, under laboratory conditions.

The *S. natans* plants presented high accumulation of copper ions in both their roots and leaves, evidencing a pattern of accumulation dependent on the concentration of the metal in the culture solution associated with the time of exposure of the plants to contamination. The relationship between the accumulation of the metals and their concentration in the medium is known and has been described for the macrophyte *Lemna aequinoctialis* Welw. (Lemnaceae) (Pio et al., 2013) and *Salvinia biloba* Raddi (Salviniaceae) (Freitas et al., 2018a), in which variations in the total absorbed occurred mainly due to the difference in concentration of the pollutant in the culture medium. Espinoza-Quiñones et al. (2005) pointed out that high values accumulated by *Salvinia* sp. were reached with an increase in the time of exposure of the plants to the contamination, in other words, culture time.

The greater accumulation of copper recorded on the seventh day of exposure of the plants to the contaminant, especially in concentrations of de 3 µg.mL^{-1} and 5 µg.mL^{-1} of Cu, may be associated with the fact that an excessive concentration of an ion in the medium causes its absorption to take place preferentially, in relation to a specific ion present in a lower concentration. This result characterizes competition as to root absorption between the contaminant present in an excessive manner and the nutrients present in the solution at normal concentrations (Pilon-Smits, 2005).

The root cells of these plants can regulate the entry of some elements of higher demand, but which may be toxic in excessive quantities (Kerbauy, 2004). This relationship was evidenced by Hu et al. (2009), when they described that the macrophytes *Sagittaria sagittifolia* L. (Alismataceae) and *Potamogeton crispus* L. (Potamogetonaceae) under the same concentration of ions, absorbed larger amounts of Cu in relation to Pb and Cd, probably due to copper being essential to plants as micronutrients. The acquisition of Cu by the roots is similar to the mechanism involved for the uptake of Fe and suggests that its uptake from roots involves reductive Cu (I) uptake mechanisms at root cell surface from Cu (II) (Kumar et al., 2020).

The greater accumulation of copper ions by the roots of *S. natans* was expected considering that this plant is classified as a floating free macrophyte and its root system maintains direct contact with the ions of metals dissolved in the water and have, in general, a larger area of abortion than the leaves (Barros & Henares, 2015). In the study with leaves, stems and roots of *Potamogeton pectinatus* L. exposed to different concentrations of Cu, it was observed that in all treatments in the root biomass there was a higher concentration of the metal (Costa et al., 2018). In addition to efficient roots, species of *Salvinia* present modified submerged leaves, adapted to the absorption of elements dissolved in the medium, acting in an associated manner on the roots (Sculthorpe, 1967). Although it is known that in plants the copper tends to accumulate in the root tissue and can be transferred to the buds (Yang et al., 2015).

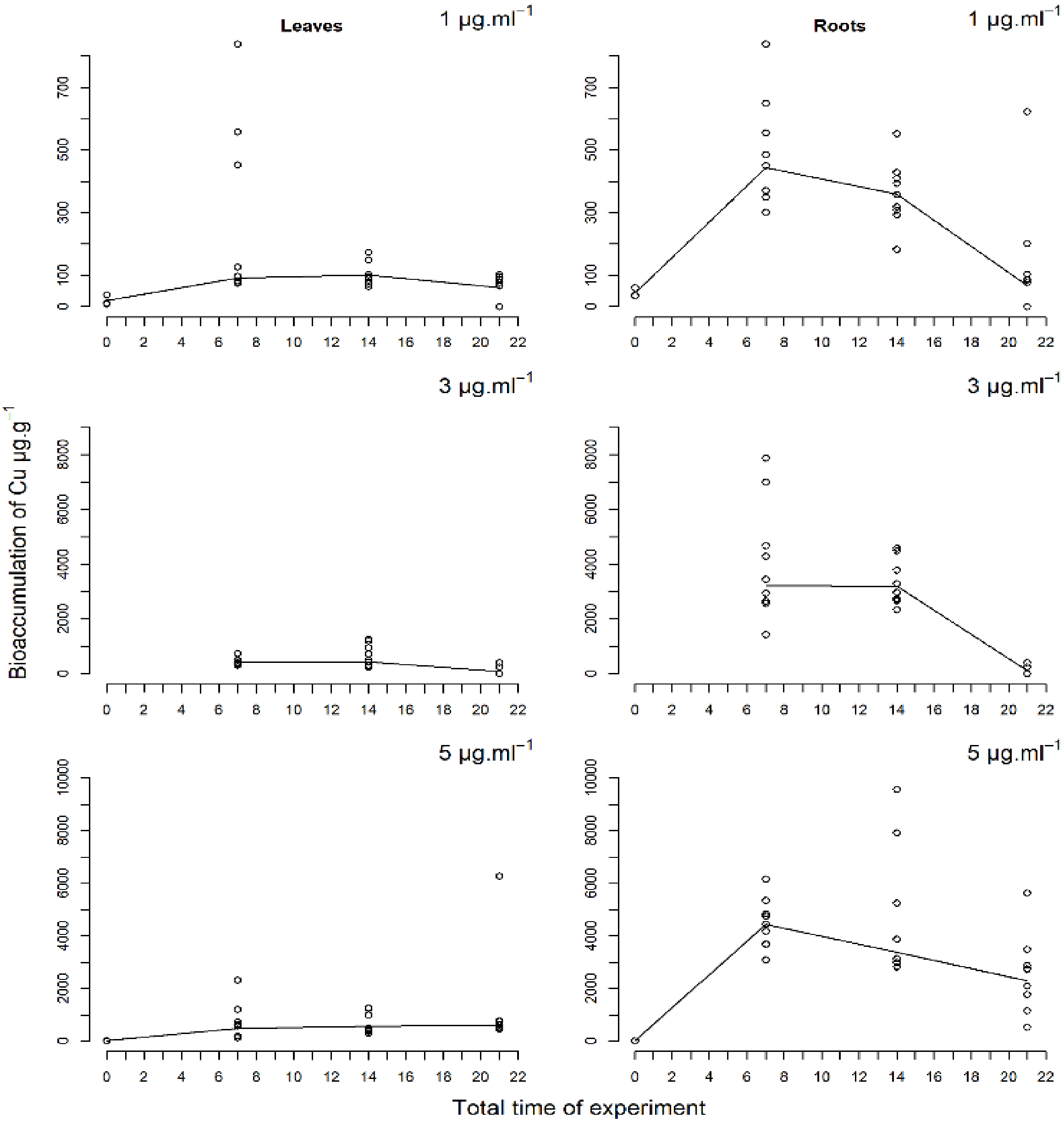

Figure 2. Time variation in copper (Cu) accumulation by *S. natans* leaves and roots in different treatments (concentrations), applied under laboratory conditions.

In the macrophytes the physiological differences between species in terms of capture and storage of nutrients have important implications for the effects and residence time of the metal in the plant, consequently, they should be considered in the treatment systems that use these plants. Wolff et al*.* (2012) discuss the dynamics of cadmium accumulation between the floating and submerged leaves in *Salvinia auriculata* Aubl. (Salviniaceae) as an important factor in the minimization mechanism of toxic effects of the metal on the plant. *Salvinia* in general can present higher values of accumulation when compared with other species of macrophytes, as they have their leaves submerged which helps in this process (e.g. Oliveira et al., 2001; Guimarães et al., 2006; Molisani et al., 2006). Our results show the same trend, evidencing that in *S. natans* the capacity of distribution and compartmentalization of the metal in the biomass of roots and leaves influences the capacity of copper accumulation, which is an important mechanism for phytoremediation.

As from the first week of the experiment, *S. natans* specimens demonstrated visual morphological modifications related to the effects of copper toxicity on plants. Among these alterations, we observed changes in pigmentation (foliar chlorosis), necrotic spots on the leaf surface, especially on the edges, and fragmentation of part of the roots. Shaw et al*.* (2004) cite these symptoms among the easiest to evidence in metal phytotoxicity along with senescence and abscission of the leaves. These symptoms are not considered specific for copper toxicity, being described for other macrophytes exposed to different metals (Mendes et al*.,* 2009; Wolff et al*.*, 2012; Pereira et al., 2012).

In *Salvinia* species, the symptoms of toxicity related to aerial parts of plants are highlighted (Wollf et al., 2009). Necrosis and chlorosis were observed on leaves of *Salvinia minima* Baker (Salviniaceae) on the second day of exposure to arsenic (Guimarães et al., 2012). Casagrande et al*.* (2018) observed effects similar to those of this study on the external morphology in plants of S. biloba, mainly in the leaves, to which the authors correlated the rupture of the limbus and increase of the aerenchyma in the concentrations of 0.05 and 0.2 of mercury (Hg), respectively, confirmed in internal morphological analysis. The most common action of copper toxicity on plants is the inhibition of the formation of photosynthetic pigments, resulting in direct effects on plant productivity (Jung et al., 2015). Accordingly, Loría et al. (2019) the contents of chlorophyll and carotenoids are used as trusted indicators of metal toxicity in higher plants.

Freitas et al. (2018a) observed high mortality and morphological damage in *Salvinia biloba* plants, cultivated in different solutions with copper, in all evaluated treatments. Specifically for *S. natans*, the exposure to chromium and zinc caused a change in its photosynthetic potential due to the reduction of the efficiency of the enzymes involved in chlorophyll biosynthesis, reduction of iron availability and the formation of chlorophylls substituted with metal (Dhir et al., 2008). Holtra & Zamorska-Wojdyla (2014) when analyzing the performance of *S. natans* on copper absorption found that the effects of the metal on plant morphology varied among the treatments, according to the initial concentrations of the metal.

The determination of accumulation time and the effects of the metal on the metabolism of the plants are of great importance for the advances in the application of phytoremediation, as the decomposition of the plants makes the retained elements in their tissues once again available in the environment (Freitas et al., 2018a). *Salvinia* has a high rate of absorption, probably due to the greater participation of its leaves (floating and submerged) in the accumulation processes. However, the effect of the metal toxicity on the physiological mechanisms of the plant can compromise the growth capacity and accelerate degradation (Yruela, 2005).

Accordingly, Holtra et al*.* (2010) in studies with *S. natans* on the accumulation of boron, during five days of experiment, suggest that a longer period of exposure of the plants to the contamination would lead to a better understanding of this process, as well as the resulting changes in the biological material. During 21 days, we observed that the time of maximum absorption of copper by *S. natans* occurred until the seventh day of the experiment, in which the biomass of leaves and roots presented the highest content of the metal. From this period onwards there was a continuous reduction in accumulation levels, probably due to the general reduction of the metabolism of the plants (Oliveira et al., 2001).

The capacity to remove high amounts of toxic substances is a very important aspect of phytoremediation. Some plant species may be considered hyperaccumulating when they accumulate values over 1.000 $\mu g.g^{-1}$ of metal in relation to the dry mass of the plant tissue (Visoottiviseth et al., 2002), as well as those that tolerate, accumulate and translocate high levels of metals between the biological compartments such as roots and leaves (Kumar et al., 2020). Thilakar et al. (2012) suggest that *S. natans* be classified as a hyperaccumulating macrophyte, supporting high amounts of toxic metals in its biomass, and can be used for the remediation of aquatic environments. This relationship may be associated with the presence of different functional groups such as phosphonates, carboxylates, amide and hydroxide groups in the *S. natans* biomass, important constituents that allow for binding with trace metals (Lima et al., 2016).

In general, *Salvinia* sp. have a great specific surface area, which is rich in carbohydrates, proteins, lipids, and molecules containing carboxyl groups that are involved in the first step of the biosorption mechanism (Loría et al., 2019). Our results demonstrate that in a short period of time, *S. natans* accumulates elevated amounts of copper, reaching values of up to 9.570 $\mu g.g^{-1}$ and 2.320 $\mu g.g^{-1}$ of copper in the biomass of roots and leaves, respectively. The metal toxicity affected the normal development of the plants, however, it did not prevent metal accumulation throughout the experiment. The morphological symptoms shown by plants in response to the elevated presence of copper can be used as ecological indicator of aquatic environments polluted by this metal.

Conclusion

Our results support the use of *S. natans* as a potential phytoremediation agent of Cu. Additionally, macrophytes are highly productive representing a good economic and environmental cost-benefit as a biological material for the treatment of liquid solutions with metals.

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