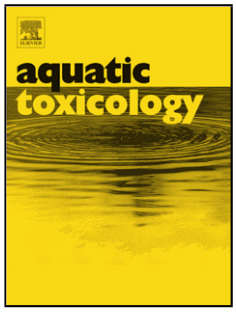

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Effect of daily exposure to Pb-contaminated water on *Salvinia biloba* physiology and phytoremediation performance

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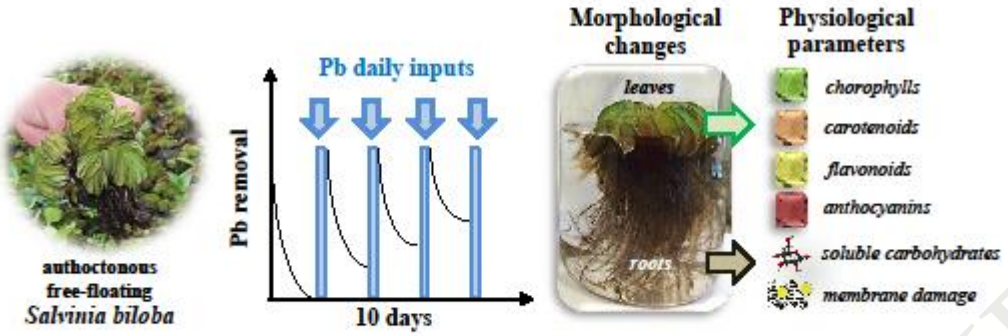
Graphical abstract

Graphical Abstract



ABSTRACT

Lead (Pb) removal from water column was evaluated in batch experiments using naturally occurring *Salvinia biloba* Raddi (*S. biloba*) specimens collected from Middle Paraná River and exposed every 24 h to a fresh discharge of water contaminated with 2.65±0.07, 12.62±0.02 or 30.57±0.01 mg L^{-1} Pb, during 10 consecutive days. *S. biloba* demonstrated a great ability for metal concentration-dependent Pb removal under these stressful conditions. Additionally, Pb toxicity in plants was assessed by the quantification of physiological parameters in root-like modified fronds (named "roots"), and its aerial leaf-like fronds (named "leaves") of submerged *S. biloba*. Photosynthetic (carotenoids, chlorophyll *a*, *b*, and total) and antioxidant pigments (anthocyanins and flavonoids), soluble carbohydrate content, and membrane stability index of both roots and leaves were affected as the metal concentration increased. In general, root deterioration was more pronounced than that in leaves, suggesting a greater implication of the former organs in Pb removal by *S. biloba*. All of these deleterious effects were well correlated with qualitative changes observed at plant phenotype during the assay. In conclusion, *S. biloba* may be considered as a water fern useful in phytoremediation

strategies towards management of residual water bodies contaminated with Pb. In addition, these macrophytes could also be valuable for water biomonitoring contributing to improve risk assessments related to metal presence in wastewaters.

Keywords: biomonitoring, lead accumulation, metal regular discharge, phytoremediation performance, physiological response, *Salvinia biloba*.

1. Introduction

Environmental contamination with metals is a worldwide problem due to increased industrialization and urbanization. Metals are major pollutants of fresh water reservoirs because of their toxic, non-biodegradable, and persistent nature (Azimi *et al*., 2017). Cadmium, lead (Pb), mercury, and arsenic are the toxic metals with high environmental importance; all of them currently appear on the list of the ten chemicals of major public concern to the World Health Organization (WHO, 2017). Among them, Pb is probably the most abundantly distributed element with high molecular weight globally. Important physico-chemical properties such as softness, malleability, ductility, poor conductivity, and resistance to corrosion seem to make difficult to give up its use (Flora *et al*., 2012; Wani *et al*., 2015). Although widespread Pb use has dropped in several countries, it is still used in many industries like car repair, boat building, battery manufacturing and recycling, metal plating and finishing, arms industry, tetraethyl lead manufacturing, among others. Therefore, Pb is frequently present in industrial wastewaters (Arbabi *et al*., 2015). Some methods like precipitation, ion exchange, electrochemical processes and/or membrane processes are commonly applied to the treatment of industrial effluents contaminated with Pb and other metals. However, the

application of such processes is sometimes restricted due to technical or economic constraints (López-Mesas *et al*., 2011).

Free Pb or its derivate compounds present in aquatic environments in sufficient amounts can cause acute or chronic toxicity to aquatic organisms. In addition, a lot of cases of Pb poisoning both in children and adults are still reported, particularly in developing countries (Flora *et al*., 2012; Wani *et al*., 2015; CDC, 2016). The level of toxicity is determined by bioavailability factors such as water chemistry, solubility, salinity, and organic matter content (Cardwell *et al*., 2013). Many of the adverse effects of Pb are reversible once exposure levels (*i.e.*, concentration in the environment) fall. The search for new technologies, preferably clean and inexpensive ones, has directed the attention towards biological systems that can be used to remove Pb from water by natural processes, and trap it where its biodisponibility to pose harmful effects to living organisms is very low (Park *et al*., 2010). In such sense, phytoremediation methods using plant biomass for removing metals from effluents and natural water reservoirs have proven to be efficient and economical, and present high yield (Dhir, 2014; Arbabi *et al*., 2015; Dixit *et al*., 2015). In particular, the use of aquatic plants for wastewater renovation has become an important consideration for communities with limited financial resources. Strategies using macrophyte species highly abundant in the region and exhibiting high growth rates and elevated biomass production are of choice to make phytoremediation technologies sustainable and economically viable (Rai, 2009; Sukumaran, 2013). In addition, these plants have the advantage to be easily removed from water body surfaces.

In Argentina, the ecosystem surrounding the Paraná River is characterized by an extensive wetland area with abundant and diverse aquatic vegetation due to favorable thermal and light regime (Ferreira *et al*., 2011). Therefore, it seems appropriate the

implementation of free-floating plants in lagoons for treating metal-containing effluents (Sukumaran, 2013; Dhir, 2014). Different macrophytes from Middle Paraná River have been widely studied for use in constructed wetlands for wastewater treatment because of their high metal removal capacity (Maine *et al*., 2001, 2004; Hadad *et al*., 2006, 2007; Suñé et al., 2007). In particular, the genus *Salvinia* has shown high growth rate, great capacity to survive under adverse environmental conditions, and proper transition metal removal capacity (Dhir, 2014; Fuentes *et al*., 2014; Prado *et al*., 2016; Leal-Alvarado, 2016; Freitas *et al*., 2018). *Salvinia* sp. has about 20 species found in tropical and temperate regions around the world, among the most common species in our region are *S. auriculata* Aubl., *S. minima* Baker, *S. natans* (L.) All., and *S. biloba* Raddi; however, studies on *S. biloba* with regard to removal of toxic metals from aquatic biota are scarce (Freitas *et al*., 2018). In a recent study from our group, we have demonstrated the high efficiency of *S. biloba* to remove Pb in water samples contaminated with three metal concentrations (4.8±0.3, 9.1±0.4 and 19.6±0.5 mg L^{-1}) at different exposure times (0-24 h) (Tello Zevallos *et al*., 2018). We also evaluated the Pb removal kinetics and demonstrated that surface adsorption was the predominant mechanism for metal elimination in *S. biloba* during the first 24 h of metal exposure. In addition, we also probed the Pb-hypperacumulator capacity of these autochthonous free-floating macrophytes. However, we realize that most studies on metal bioremediation by macrophytes and their toxic effects on plant physiology have been conducted by submitting plants to "one-shot" pollutant exposure; *i.e.*, a single toxicological impact. Nevertheless, there are no reported data describing metal phytoremediation processes in plants repeatedly exposed to successive inputs of the contaminant. Moreover, there is limited information on the effect of Pb on physiological parameters of *S. biloba* and how continuous Pb exposure may affect *S. biloba* capacity to remove the metal from

aqueous systems. Therefore, the aim of the present work was to evaluate how daily exposure of *S. biloba* to water contaminated with different metal concentrations affects the plant's performance in Pb removal, and the capacity of this ongoing Pb exposure to weaken physiological vitality from the leaves and roots of *S. biloba*. These data will allow us to determine their tolerance and provide basic information related to the potential use of these locally available macrophytes for long-term water depuration strategies.

2. Materials and Methods

1.1. Collection of macrophytes

Specimens of *Salvinia* sp. were collected manually from a shallow lake at the Middle Paraná River (32°52' 35" S; 60° 40' 33" O) as described by Tello Zevallos *et al*. (2018) (Fig. 1). During collection, the plants were stored at ambient temperature in plastic recipients containing river water. Subsequently, the specimens were transported to the laboratory and cultivated at room temperature (25 ± 2 ºC) under hydroponic conditions in 10 L glass aquaria using a combination of municipal drinking water and lagoon water. No further nutrients or special requirements were added. Taxonomic classification was carried out by the *Instituto de Botánica del Nordeste* (IBONE-CONICET, Universidad Nacional del Nordeste, Corrientes, Argentina) based on morphological characteristics. *Salvinia* sp. specimens were classified as *Salvinia biloba* Raddi (synonym: *Salvinia herzogii* de la Sota) according to De La Sota (1995).

1.2. Pb removal studies

Plants with uniform size and without any visual signs of deterioration (loss of turgor, chlorosis and/or necrosis of leaves) were selected for experimental purposes. The metal

concentrations selected were in accordance with previously reported data (Sánchez-Galván *et al*., 2008; Tello Zevallos *et al*., 2018). Ten grams (10.0 g) of wet biomass were gently rinsed with deionized water during 15 min and placed in 400 mL glass containers with different initial Pb concentrations (2.65± 0.07 mg L^{-1}; 12.62±0.02 mg L^{-1} or 30.57±0.01 mg L^{-1}). Three experimental units were used as replicates for each Pb concentration tested, and the data were reported as mean value ± standard deviation (S.D.). A control group containing *S. biloba* biomass not subjected to Pb exposure was used as test reference. Additionally, two controls were included. One, containing Pb at the same initial concentration as the treatment but without biomass, was used to determine the possible adsorption of the metal onto the surface of the containers. The content of Pb in the water column did not change during the experimental time, indicating that no metal adsorption occurred in the system. Another control, containing biomass in water without Pb, was used as blank of the experiment in which no metal was quantified either in plant or medium. In all cases, pH was adjusted and maintained at 6.0±0.5 with 1 N HNO_3 (Cicarelli, Argentina) in order to avoid $Pb(OH)_2$ precipitation. Every 24 h, samples from water column were taken to determine residual Pb concentration. Immediately after samples were taken, plants were transferred to a new glass container with fresh Pb solution at the same initial metal concentration as before (*i.e.*, 2.65± 0.07 mg L^{-1}; 12.62±0.02 mg L^{-1} or 30.57±0.01 mg L^{-1}). This procedure was aimed to simulate a daily discharge of the contaminant and was performed during 10 consecutive days. In this sense, control plants were maintained during 10 days in daily renewed distilled water, simulating the same procedure as used for the plants exposed to Pb. All experiments were accomplished at an average room temperature of 24±2 ºC under artificial light intensity with a photon flux density of 50 µmol m^{-2} s^{-1} (Osram Dulux L HE, Germany) and 12 h photoperiod. At the end of the

experimental period (10 days), total biomass was removed, separated to submerged root-like modified fronds (named "roots"), and aerial leaf-like fronds (named "leaves"), and finally treated for physiological parameter analysis. Pb quantification was performed by using an atomic absorption spectrophotometer Varian AA240FS (Varian Inc. Palo Alto, USA) as described by Tello Zevallos *et al*. (2018). Additionally, plant material was photographically recorded during the entire assay in order to visually assess phenotypic changes to *S. biloba* biomass related to Pb phytotoxicity.

2.3. Physiological parameters

2.3.1. Quantification of photosynthetic pigments

Photosynthetic pigments (chlorophylls and carotenoids) were determined according to the method described by Leal-Alvarado *et al*. (2016). Fifty mg of fresh biomass (FW) were added with 80% (v/v) acetone (4.5 mL) and incubated 24 h in darkness. The extract was then centrifuged at 13000 x *g* for 10 min. The supernatant was transferred to test tubes, and absorbance was measured at 470, 645 and 663 nm by using a Lambda 25 UV-vis spectrophotometer (Perkin Elmer, USA). Contents of chlorophyll and carotenoid were calculated according to Wellburn's equations (Wellburn, 1994):

$$\text{chl } a \text{ (µg/mL)} = 12.25(A_{663}) - 2.79(A_{646})$$

$$\text{chl } b \text{ (µg/mL)} = 21.5(A_{646}) - 5.1(A_{663})$$

$$\text{chl}_{\text{total}} \text{ (µg/mL)} = \text{chl } a + \text{chl } b$$

$$\text{carotenoids (µg/mL)} = [(1000A_{470} - 1.82[\text{chl } a]) - 85.02[\text{chl } b]]/198$$

Concentrations of photosynthetic pigments were expressed as µg g^{-1} FW.

2.3.2. Determination of antioxidant pigments

Phenolic compounds content was determined according to the method proposed by Bizzo *et al.* (2014). For anthocyanin determination, plant samples (50 mg of FW) were incubated with 5 mL of methanol:HCl (99:1) and kept in the dark for 24 h. The extract was then centrifuged at 13000 x *g* for 10 min. The absorbance of the supernatant was measured at 550 nm using a Lambda 25 UV-vis spectrophotometer (Perkin Elmer, USA). Anthocyanin concentration (nmol g^{-1} FW) was calculated using an extinction coefficient of 33,000 mol^{-1} cm^{-1} (Wanger, 1979).

For flavonoid content determination, 50 mg of FW were mixed with 5 mL of ethanol:acetic acid (99:1). Then, the samples were gently boiled for 10 min in a water bath at 80 °C. The absorbance was measured at 330 nm using a UV-vis Lambda 25 spectrophotometer (Perkin Elmer, USA). Flavonoid concentration was expressed as absorbance units (AU_{330}) g^{-1} FW.

2.3.3. Soluble carbohydrates

Soluble carbohydrate content was determined according to Bizzo *et al*. (2014). A total of 100 mg of FW was mixed with 80% (v/v) methanol (1 mL) at 70 °C for 30 min. After samples were cooled, 1 mL of the extract was mixed with 1 mL of 5% (v/v) phenol and 5 mL of H_2SO_4 (95 %). Finally, the mixtures were incubated for 1 h at room temperature and the absorbance of the supernatant was measured at 640 nm using a UV-vis Lambda 25 spectrophotometer (Perkin Elmer, USA). Glucose (50 mg) was used as a standard. The concentration of the soluble carbohydrates was expressed as mg g^{-1} FW.

2.3.4. Membrane stability index (MSI)

MSI was determined as a surrogate parameter of cell membrane damage. Briefly, the electrical conductivity of leaves and roots was assessed according to Leal-Alvarado *et*

al. (2016). Fifty mg of FW were rinsed with distilled water, placed in a glass tube containing 10 mL of distilled water, and incubated at 40 ºC for 30 min in a water bath. Then electrical conductivity (E_1) was measured using a conductimeter (EC 215, Hanna Instruments, Romania). Immediately, tubes were boiled in a water bath for 15 min and the electrical conductivity (E_2) was recorded again. MSI was calculated as follow:

$$MSI\ (\%) = [1 - (E_1/E_2) \times 100]$$

1.3. Statistical analysis

Statistical analyses were performed using SigmaStat 3.5 program (Systat Software Inc., USA). Analysis of variance (ANOVA) was used to compare the data obtained from all measured parameters between control and Pb-treated plants. Tukey's honestly significant difference (HSD) *post-hoc* test was applied (95% of confidence level) when necessary ($p < 0.05$).

3, Results and Discussion

3.1. Daily Pb removal performance from aqueous solutions

The efficiency of Pb removal by *S. biloba* was assessed during ten consecutive days of plants exposure to a daily input of water artificially contaminated with 2.65±0.07, 12.62±0.02 or 30.57±0.01 mg L^{-1} Pb. As can be seen in Fig. 2, metal elimination from water column was strongly affected by Pb concentration and exposure time. During the first 24 h, *S. biloba* showed a great capacity to remove Pb, and this behavior was in total agreement with previously reported data (Paris *et al*, 2005; Tello Zevallos *et al*., 2018). Noticeably, even at the highest Pb concentration analyzed (*i.e.*, 30.57±0.01 mg L^{-1}), 86.7±5.1% of the metal was eliminated from water-contaminated samples at the end of the first treatment day. This result correlates well with the decreasing tendency observed in a previous study from our group showing *S. biloba* Pb removal efficiency values of 98.2±0.8%, 96.2±0.5% and 91.2±1.9% for 4.8±0.3, 9.1±0.4 and 19.6±0.5 mg L^{-1} Pb, respectively, during the first 24 h (Tello Zevallos *et al*., 2018). However, at the second consecutive Pb input of 30.57±0.01 mg L^{-1} (days 2), metal removal efficiency falls to around 50%, and at day three (*i.e.*, third consecutive Pb exposure) *S. biloba* phytoremediation capacity reached a *plateau* and stayed at around 10% during the following water Pb-contaminated challenges until the end of the experimental period. In contrast, when macrophytes were exposed to a sustained daily input of 2.65±0.07 mg L^{-1} Pb, plants were extremely efficient in the metal removal maintaining a high elimination performance during the entire sampling period. As shown in Figure 2, during the first eight daily inputs of water contaminated with 2.65±0.07 mg L^{-1} Pb, the percentage of metal removal was over 94% and no significant differences in such performance were observed as compared with the first 24 h. Only after the ninth consecutive metal discharge, Pb removal by *S. biloba* was significantly different

(p<0.05) from day one but it was still high (89.3±3.6%). At the end of the treatment period (10 days), all the macrophytes exposed to a daily discharge of water contaminated with 2.65±0.07 mg L^{-1} Pb still showed a great metal removal efficiency of 87.4±4.6%, similar to that observed for the plants that were exposed for 24 h to a metal concentration of 30.57±0.01 mg L^{-1}. Noticeably, in both cases, total metal (mg Pb) uptake at plant biomass was approximately the same (Fig. 3). However, when *S. biloba* specimens were exposed every 24 h to water contaminated with 12.62±0.02 mg L^{-1} Pb, the percentage of metal removal was maintained above 90% during the first three days showing a moderate but constant decrease from day fourth until the end of the sampling period. At the tenth day, a metal removal efficiency of 29.3±7.9% was calculated. These results suggest that it is possible to find a relationship between the metal input and the exposure time that optimizes metal uptake by plant biomass. As can be observed from Figure 3, a greater extent of metal could be trapped by these macrophytes if the plants were exposed to a daily discharge of water contaminated with Pb at a concentration between 15-20 mg L^{-1} during 10 consecutive days. All of these results demonstrate the great ability of *S. biloba* to eliminate Pb during a repeated exposure to water samples contaminated with the metal, as may be the case for a plant-based wastewater treatment system from a metallurgical industry. In addition, our results support the use of *S. biloba* for phytoremediation of water bodies contaminated by Pb, due to its ability to hyper-accumulate the metal in its tissues (Tello Zevallos *et al*., 2018).

Several authors have reported the remarkable potential for metal phytoremediation of *Salvinia* sp. and have explained that this ability partially depends on plant surface characteristics. In general, all these macrophytes 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 (Sánchez Galván

et al. 2008; Dhir, 2009; Lima *et al*., 2016). Recently, we have reported the presence of different functional groups in *S. biloba* biomass such as carboxyl, phosphate, sulphur, amide, hydroxyl and others suggesting the involvement of these functional groups in metal adsorption (Tello Zevallos *et al*., 2018). In addition, some other studies have been conducted in order to understand the molecular basis of the response of these macrophytes when exposed to metals. Estrella-Gómez *et al*. (2009) demonstrated the relationship between Pb accumulation and the activation of chelation and metal sequestration mechanisms mediated by phytochelatins (PC) in the roots of *S. minima*. PC expression is one of the best documented mechanisms to deal with heavy metals in plants and other organisms. These proteins have the ability to bind toxic metals, forming complexes that are stored in vacuoles and chloroplasts, thus reducing the deleterious effect of metal ions to cells (Mendoza-Cózatl et al., 2006). Moreover, these authors also analyzed the relationship between Pb accumulation and changes in glutathione (GSH) levels in both leaves and roots of *S. minima*, suggesting that an increase in GSH biosynthesis may play an important role in protecting plant leaves from the oxidative damage caused by Pb (Estrella-Gómez *et al*., 2012). More recently, Leal-Alvarado *et al*. (2018) concentrated into the molecular basis of the metal detoxification mechanisms in *Salvinia*. These authors analyzed the expression levels of four genes coding for transporters (*SmABCC*, *SmATPase*, *SmNhaD* and *SmABCG*) in *S. minima* exposed to Pb for 24 h, and demonstrated that those genes showed a rapid and sharp increase in their expression levels (up to 60 times) in just 3-4 hours. In addition, these authors proposed that the above-mentioned genes are also implicated in metal detoxification and accumulation mechanisms, cell homeostatic control, and cell membrane integrity regulation of plants. Although these data represent a first sight scenario of the molecular basis for understanding the mechanism involved in *S. minima*

ability to accumulate and tolerate Pb, it is expected that all aquatic ferns belonging to this genus share similar biosorption mechanisms.

3.2. Phenotypic evaluation of Pb phytotoxicity in S. biloba

The phytotoxic effects observed in the biomass of *S. biloba* specimens after 10 days of receiving daily discharges of water contaminated with different Pb concentrations are shown in Figure 4. As can be seen, when the plants were exposed to concentrations of 2.65±0.07 mg L^{-1}, 12.62±0.02 mg L^{-1} and 30.57±0.01 mg L^{-1} of Pb (Fig. 4 B, C and D, respectively) green chlorophyll changes towards darker areas (*i.e.*, leaf chlorosis), loss of turgor (alterations in size and shape), and signs of necrosis (cell death) were observed both in the juvenile fronds and in the most developed ones. In general, a gradual response of *S. biloba* to Pb phytotoxicity could be observed. The external morphological damage, such as the occurrence of necrotized areas on the surface of leaves, increased relative to the increase of metal concentration in the solution. The necrotized areas presented dark brown pigmentation from the edge of the leaf toward the center. At the high Pb concentration of 30.57±0.01 mg L^{-1}, a deposition of opaque material was clearly observed along the middle lamella, giving it a sinuous shape. On the contrary, after 10 days, the control group (Fig. 4 A) showed no apparent changes in its physiological state with respect to its initial condition. In addition, in a stereomicroscopic analysis, it was possible to determine that tissue necrosis was also markedly present in those areas of greatest contact with Pb-contaminated water, indicating that submerged leaves of *S. biloba* are also capable of absorbing water and ions (Wollf *et al*., 2012). The deleterious changes induced by Pb sustained exposure were also especially notorious in the papillae located at leaves epidermis (Fig. 5). Moreover, it was determined that plants exposed to 2.65±0.07 mg L^{-1} Pb showed signs

of phytotoxicity in less than half of their leaf area while the specimens exposed to the higher concentrations showed approximately more than half of the foliar area with damage. This was especially noticeable in plants exposed to 30.57±0.01 mg L^{-1} Pb.

It has been reported that aquatic plants exposed to transition metals show typical symptoms, such as leaf and root necrosis, chlorosis, brownish-red coloration, reduced biomass or reduced yield (Wolff *et al*, 2012; Freitas *et al*., 2018). This pattern is associated with stomatal opening, a reduced nutrient absorption/transportation, and a reduced rate of photosynthesis (Clemens, 2006). According to our observations, *S. biloba* exposed to Pb presented morphological changes proportional to the concentration of metal received. These results support a potential use of this aquatic fern as an ecological indicator of Pb presence in contaminated environments since phenotypic changes induced by the metal were clearly visible and easily quantified.

3.3. Photosynthetic pigments

The contents of chlorophyll and carotenoids are used as trusted indicators of metal toxicity in higher plants. Chlorophylls are the main chloroplast pigments responsible for collecting solar radiation during the photosynthetic process (Gomes *et al*., 2014). Total chlorophyll concentration is a unifying parameter for indicating the effect of specific interventions. However, it is important to record changes in the two components of chlorophyll, chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*). This is due to the fact that metals could affect each component at a different level creating changes in some parts of plant physiology and not in others (Manios *et al*., 2003).

On the other hand, carotenoids are essential to photosynthesis acting as secondary pigments, pro-vitamin factors, and to eliminate free radicals in damaged tissue (Gomes *et al*., 2014). In particular, carotenoids quench excess excitation energy to protect

chlorophyll molecules from oxidative damage and stabilize the lipid bilayer of cell membranes preventing peroxidation caused by reactive oxygen species (ROS) (Ramel *et al*., 2013; Munné-Bosch *et al*., 2013).

In the present study, the photosynthetic pigments were the most affected physiological parameters after daily Pb exposure in *S. biloba*. The amount of chlorophylls (chl *a* and total) and carotenoids in the leaves of plants exposed during 10 consecutive days to a daily discharge of Pb-contaminated water decreased significantly ($p<0.05$) with respect to the control group. This effect was greater as the metal concentration increased (Table 1).

In the roots, photosynthetic pigment content was also significantly affected during the 10 days of daily exposure to the different Pb concentrations analyzed (Table 1). Nevertheless, chl *b* content in either organ did not change statistically, probably due to a faster hydrolysis ratio of chl *a* compared with chl *b* when plants are under stress (Manios *et al.,* 2003). Although, chl *b* is not essential for photosynthesis, it is important for the success of aquatic plants in absorbing light at 425-475-nm regions, where chl *a* poorly absorbs (Tanaka & Tanaka, 2011). The observed reduction in pigment content is consistent with the photographs depicted in Fig. 4, in which plants exposed to the higher Pb concentration showed a marked increase in the darkening and chlorosis of leaves. Our results demonstrate that a repeated Pb exposure interferes with the normal metabolism of *S. biloba*, since both chlorophylls and carotenoids play an important role in capturing sunlight for the photosynthetic processes. Despite this, no plant showed a marked reduction of their biomass as compared with the control group after 10 days of daily Pb exposure. However, when daily Pb exposure was extended from 10 to 44 days, only the untreated *S. biloba* specimens (control group) showed an increase in plant biomass (Castillo Loría, 2017; unpublished master's thesis).

Pb excess in plant tissues affects photosynthesis by a series of multiple factors, including disruption of chloroplast organization; synthesis inhibition of chlorophylls, plastoquinone and carotenoids; blocking of the electron transport chain; inhibition of Calvin cycle enzyme activity and inhibition of CO_2 uptake as a result of stomata closing (Gomes *et al*., 2014). Moreover, Gautam *et al*. (2011) suggested that chlorophyll synthesis inhibition in the presence of Pb occurs by a reduction of the activity of the 5-aminolevulinic acid dehydratase (ALAD), a key enzyme in the route of chlorophyll synthesis. In addition, oxidative stress is a well-known feature of transition metal physiological effects in plants (Fryzova *et al*., 2018). ROS are produced in the chloroplast, either as byproducts of the reduction of molecular oxygen or as a result of excitation in the presence of highly energized pigments, causing marked changes in the composition of the thylakoid membrane where the photosynthetic pigments are deposited (Singh *et al*., 2010; Pourrut *et al*., 2011; Fryzova *et al*., 2018). Other authors have also shown the negative impact of Pb on the concentration of photosynthetic pigments in *Salvinia* species. Dhir *et al*. (2011) determined that *S. natans* exposed for 48 h to 35 mg L^{-1} of Pb showed a clear decrease in the content of chlorophyll and carotenoids on plants leaves. Estrella-Gómez *et al*. (2009) reported that Pb accumulation caused more damage in roots than in leaves due to a decrease in roots carotenoids content. Our results correlate well with these findings since carotenoids in roots of *S. biloba* were, in general, more affected than in leaves. However, at the highest Pb concentration evaluated (30.57±0.01 mg L^{-1}) a similar reduction (approx. 60%) in carotenoid content was observed in both organs (Table 1). These differences in pigment susceptibility between roots and leaves have been observed in other phytoremediation plants suggesting that most of the metals entering the plants are stored in the root cells in order to restrict metal translocation to aerial organs, thus protecting the

photosynthetic tissues (Rai *et al*., 2004; Fuentes *et al*., 2014; Prado *et al*., 2016). More recently, Leal-Alvarado *et al*. (2016) investigated the effect of Pb on the physiological processes that affects photosynthesis in roots and leaves of *S. minima* Baker exposed to 40 µM $Pb(NO_3)_2$ (approx. 8 mg L^{-1} Pb) during 24 h. These authors concluded that Pb exposure first decreases the photosynthetic rate by damaging the root membrane, and then inducing stomata closure at plants leaves that impairs CO_2 availability. However, no changes at chlorophylls and carotenoids levels in leaves of *S. minima* were observed in 24 h of Pb exposure. Therefore, our results highlight the significance to perform repetitive metal exposition trials to identify additional physiological changes in response to Pb, and how these changes could affect the photosynthetic apparatus of plants as a biomarker of physiological damage, thus becoming an important tool for selection of tolerant macrophytes for phytoremediation purposes.

3.4. Antioxidant pigments

As observed in Table 1, the concentration of anthocyanins in *S. biloba* leaves after 10-day exposure to water contaminated with Pb at different concentrations does not show significant differences. In addition, flavonoids in leaves of *S. biloba* did not change after 10 days of stressing exposure to 2.65±0.07 and 12.62±0.02 mg Pb L^{-1} of Pb. Nevertheless, the concentration of flavonoids in fronds of *S. biloba* exposed during 10 days to a daily discharge of water containing 30.57±0.01 mg L^{-1} of Pb significantly decreased with respect to the unexposed control specimens (Table 1). Fini *et al*. (2011) suggested that in plants subjected to severe/prolonged stresst, the very conditions that lead to the inactivation of antioxidant enzymes can also upregulate the biosynthesis of

antioxidant flavonoids, which suggests flavonoids constitute a secondary ROS-scavenging system.

Phenolic compounds are essential in the defense mechanisms of plants against stress caused by biotic and abiotic agents (Sgherri *et al*., 2003, Posmyky *et al*., 2009, Tolrá *et al*., 2009; Caretto *et al*., 2015). These molecules can act as metal ion-chelating agents, preventing the generation of ROS induced by the action of different metals (Michalak, 2006). In addition, numerous reports indicate that these compounds could be involved in metal tolerance described for *Salvinia* sp. Bizzo *et al*. (2014) reported an increase in the amount of total phenolic compounds in individuals of *S. auriculata* exposed for 48 h to an abiotic stress induced by the presence of Cu(II). In a recent study, Prado *et al*. (2016) observed that the amount of total soluble thiols was strongly increased in leaves of *S. rotundifolia* and *S. minima* after 7 days of receiving only an input of Cr(VI). In our study, no increase in antioxidant pigment content in *S. biloba* leaves was observed, possibly because of the more severe Pb exposing condition caused by the daily input of metal-contaminated water.

On the other hand, in roots, the phytotoxic effect of Pb was higher than in leaves (Table 1). In this case, the amount of anthocyanins and flavonoids significantly decreased at all metal concentrations tested ($p<0.05$) after 10 days of sustained Pb exposure. These results may be associated with the observed decrease in carotenoid content described above (Table 1), suggesting that roots of Pb-exposed *S. biloba* plants accumulated a high metal concentration that was harmful to root cell homeostasis. Altogether, these facts suggest that *S. biloba* displays a coordinated and differential response to Pb in leaves and roots, where GSH could also play an important role in protecting leaves from the detrimental effects of Pb in a similar way as reported for *S. minima* (Estrella-Gómez *et al*. 2012).

3.5. Soluble carbohydrates

The accumulation of soluble carbohydrates in *S. biloba* after 10 days of a daily input with water contaminated with different Pb concentrations showed dissimilar behavior depending on the organ of the plant analyzed. In the leaves, a decreasing tendency in carbohydrate amount was observed as Pb concentration increased (Table 1). In particular, when *S. biloba* specimens were daily exposed during 10 days to a discharge of water contaminated with 30.57±0.01 mg L^{-1} of Pb, their leaves showed a marked reduction of more than 70% in the concentration of soluble carbohydrates with respect to the control plants (Table 1). Dhir *et al*. (2011) reported that the presence of heavy metals in the environment affects the production of soluble carbohydrates in *Salvinia*, resulting in a decrease in their photosynthetic capacity. Additionally, the decrement in soluble carbohydrates in *S. biloba* leaves may be associated with a Pb-induced closure of stomata that impairs CO_2 availability in a similar way as reported for *S. minima* (Leal-Alvarado *et al*., 2016). These observations correlate with the above-mentioned reduction in total chlorophyll amount, especially that of chl *a* and of ratio between chl*a* and chl *b* that are both important parameters in order to estimate the effect of an environmental event in plants (Manios *et al*., 2003).

In contrast, in the roots of *S. biloba* exposed to Pb-contaminated water, the content of soluble carbohydrates equally increased for all metal concentrations tested (Table 1). These results might be due to a reduction in carbohydrate utilization as a consequence of a lower rate in carbon assimilation caused by the marked reduction in the concentration of photosynthetic pigments (*i.e.*, chlorophylls and carotenoids) in these organs. Similar phenomena were reported by other authors in the roots of macrophytes phylogenetically related to *Salvinia* sp. when those plants were exposed to metals. Wilson and Al-Hamdani (1997) observed a significant increase in the accumulation of

sucrose, starch, and total nonstructural carbohydrates in *Azolla caroliniana* grown in the presence of Cr(VI). More recently, Prado *et al*. (2010) reported an increase in sucrose and fructose content in the submerged fronds of *S. minima* after 6 days of exposure to 5 mg L^{-1} and 10 mg L^{-1} Cr(VI).

Rosa *et al*. (2009) found that different conditions of environmental stress can induce the mobilization of soluble sugars where they are required for maintenance of the osmotic homeostasis of cells, since these compounds not only act as structural cellular constituents, but also as intracellular signaling molecules involved in the regulation of metabolic processes associated with growth and survival of plants. As we have shown, *S. biloba* roots have been highly affected by sustained Pb-exposure, so it is also possible that the stress caused by metal accumulation produced an increase in the amount of soluble carbohydrates as a protective mechanism, since these organs play a very active role in nutrient management.

3.6. Membrane stability index (MSI)

Lead ions are known to induce membrane peroxidation, membrane damage, and increased electrolyte leakage (Singh *et al*., 2010; Leal-Alvarado *et al*., 2016). In this study, cell membrane stability for control and metal-treated plants was measured from root and leaf tissues using the membrane stability index (MSI) as a surrogate marker of Pb phytotoxicity. As can be seen in Table 1, the leaves from the control plants and *S. biloba* specimens daily exposed to Pb concentration of 2.65±0.07 mg L^{-1} and 12.62±0.02 mg L^{-1} showed MSI (%) values of approximately 70% after 10 days of treatment. These observations suggest that, in these metal concentrations, there was no increased cellular membrane dysfunction due to Pb stress at *S. biloba* leaves. However, when the plants were exposed to the highest concentration of 30.57±0.01 mg L^{-1} there

was a sharp decrease in the MSI (%), suggesting that a significant membrane damage had occurred.

Conversely, in roots, the toxic effect of metal was present at all Pb concentrations tested with respect to control group, even at the lowest concentrations (Table 1). Our data indicate that Pb did cause a structural damage and alterations in *S. biloba* membrane properties at both organs that may be associated with changes of the membrane proteins or lipid composition caused by the metal presence (Fuentes *et al*., 2014). However, as we have already discussed for other physiological parameters, the impact of Pb toxicity was different between roots and leaves, being higher in the roots. The difference in MSI (%) values between leaves and roots suggests a more active participation of the latter organs in capturing and storing the metal. In this regard, Hoffman *et al*. (2004) determined that of the 88% of the Pb removed by *S. minima* from water samples artificially contaminated with 20 mg L^{-1} of metal in 24 h, 75% was accumulated in the roots while only 13% was accumulated in the leaves. More recently, Leal-Alvarado *et al*. (2016) analyzed the proportion of Pb incorporated in *S. minima* after 24 h of exposure to a solution of 40 μM $Pb(NO_3)_2$ (aprox. 8 mg L^{-1} Pb), determining that roots accumulated six times more metal than leaves. In addition, these authors observed that the rate of electrolyte release in leaves was lower than in roots reflecting that, due to the greater accumulation of Pb in the roots, the damage at cell membrane level was higher. In general, during the first stages of a phytoremediation process, the metal ions are adsorbed and incorporated into the roots of the plants where they are stored through the formation of complexes with amino acids, binding to peptides/proteins, and/or sequestered in vacuoles. These mechanisms restrict the translocation of metals to higher organs, and protect the tissues of the leaves, particularly the photosynthetic cells, from the damage caused by the accumulation of metals. Therefore, it is feasible to assume

that the decrease in the efficiency of metal removal by plants is mostly linked to the loss of the integrity of their roots due to the high metal accumulation. However, when the metal exposition is more aggressive (*i.e.*, persistent inputs of high metal concentration), translocation and accumulation of metals on leaves seem to be fastest-pushed with the concomitant damage to leaves cells, as can be assumed from our results.

In summary, all these data show the great potential of *S. biloba* species to be used in the sanitation of waters polluted with Pb through phytoremediation strategies. Overall, the results presented here suggest that the incorporation of this macrophyte in the design of industrial effluent treatment systems could be a successful strategy in favor of removing metals.

3. Conclusions

At the present study, autochthonous *S. biloba* macrophytes obtained from Middle Paraná River (Argentina) were analyzed in order to evaluate Pb removal performance under sustained and stressing metal exposure conditions for phytoremediation purposes. *S. biloba* plants seem to have high long-term Pb tolerance and a good metal uptake capacity that depends on metal concentration of the medium. Low daily Pb input levels allowed plants to maintain a high efficiency in metal removal performance from water during 10 days. However, total metal uptake by *S. biloba* may be maximized at higher Pb concentrations partially compromising the metal removal capacity of the plants. The accumulation of Pb in plant tissues induced evident alterations in leaf morphologies, supporting the potential use of this aquatic fern as an ecological indicator of Pb presence in contaminated environments. Additionally, some physiological and biochemical parameters were more sensitive to the metal entering the tissues than others. In general, levels of photosynthetic and antioxidant pigments, soluble carbohydrates, and

membrane integrity showed significant changes in plants exposed to Pb-contaminated water in both roots and leaves of *S. biloba*. The roots were more implicated in the pollutant removal process and, in such sense, the more affected by metal exposure. The sensitivity of plants to metals depends on an interrelated network of physiological and molecular mechanisms such as: (i) uptake and accumulation of metals through binding of extracellular exudates and cell wall constituents; (ii) efflux of metals from cytoplasm into vacuoles; (iii) complexion of metal ions inside the cell; (iv) induction of antioxidative enzymes; and (v) modification of plant metabolism to the rapid repair of damaged cell structures in order to adapt and survive in adverse environments. The results presented in this paper demonstrate that *S. biloba* bears immense potential to accumulate and withstand high levels of Pb presented in water and possesses efficient detoxification mechanisms, including cellular antioxidants which play a major role in protecting photosynthetic machinery against oxidative stress. Furthermore, our results also suggest that selection of suitable aquatic macrophytes for potential application in long-term phytoremediation strategies might require an additional focus on the relationship between sustained toxicity of metals and the primary metabolism of the considered plant species. In addition, owing to features such as high biomass production under natural conditions, *S. biloba* proves to be a robust free-floating aquatic macrophyte with a great value for phytoremediation of Pb-contaminated water bodies.

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Highlights

Figure 1. Location of macrophyte collection in the present study. Specimens of *Salvinia* sp. were manually collected from a shallow lake located at the Middle Paraná River in front of Rosario city, Province of Santa Fe, Argentina.

Figure 1

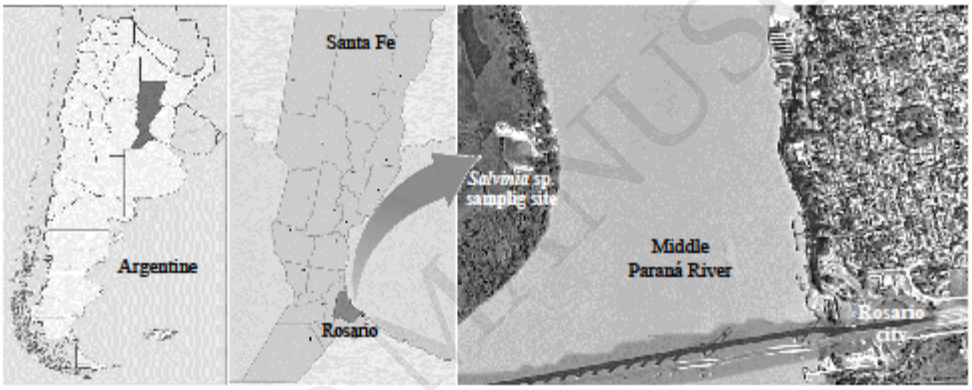

Figure 2. Daily performance of Pb removal daily performance by *S. biloba* specimens exposed every 24 h to a fresh discharge of water contaminated with different Pb concentrations (2.65±0.07 mg L^{-1}, 12.62±0.02 mg L^{-1} and 30.57±0.01 mg L^{-1}) during 10 consecutive days. Data are mean ± SD of three independent experiments (n = 3). Different letters represent statistically significant differences (p<0.05).

Figure 2

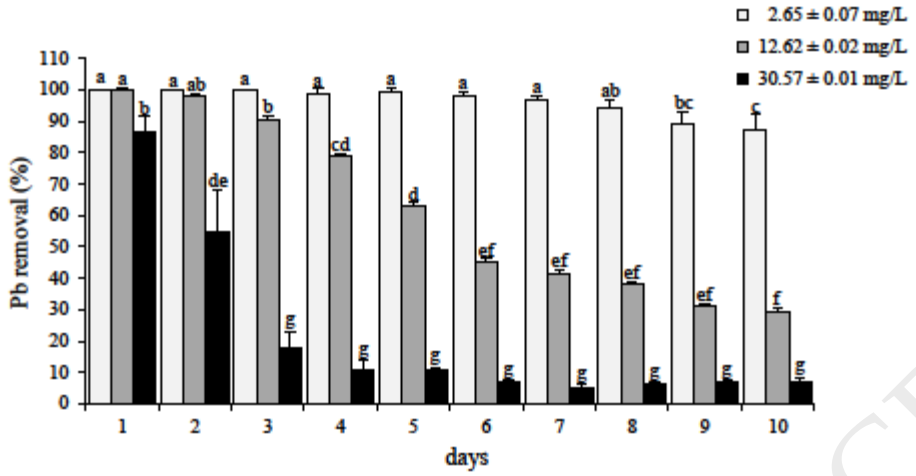

Figure 3. Total Pb uptake (mg) response by *S. biloba* biomass in response to daily input of Pb in different concentrations (in mg L^{-1}). As an example, lines over shadowed planes compare the first input day, where the amount of Pb uptake increases as concentration does, and the last input day (day 10), where a maximum in the total metal uptake is developed at a medium concentration (*e.g.*, around 15-20 mg L^{-1}/day) as a competition between metal concentration and physiological damage.

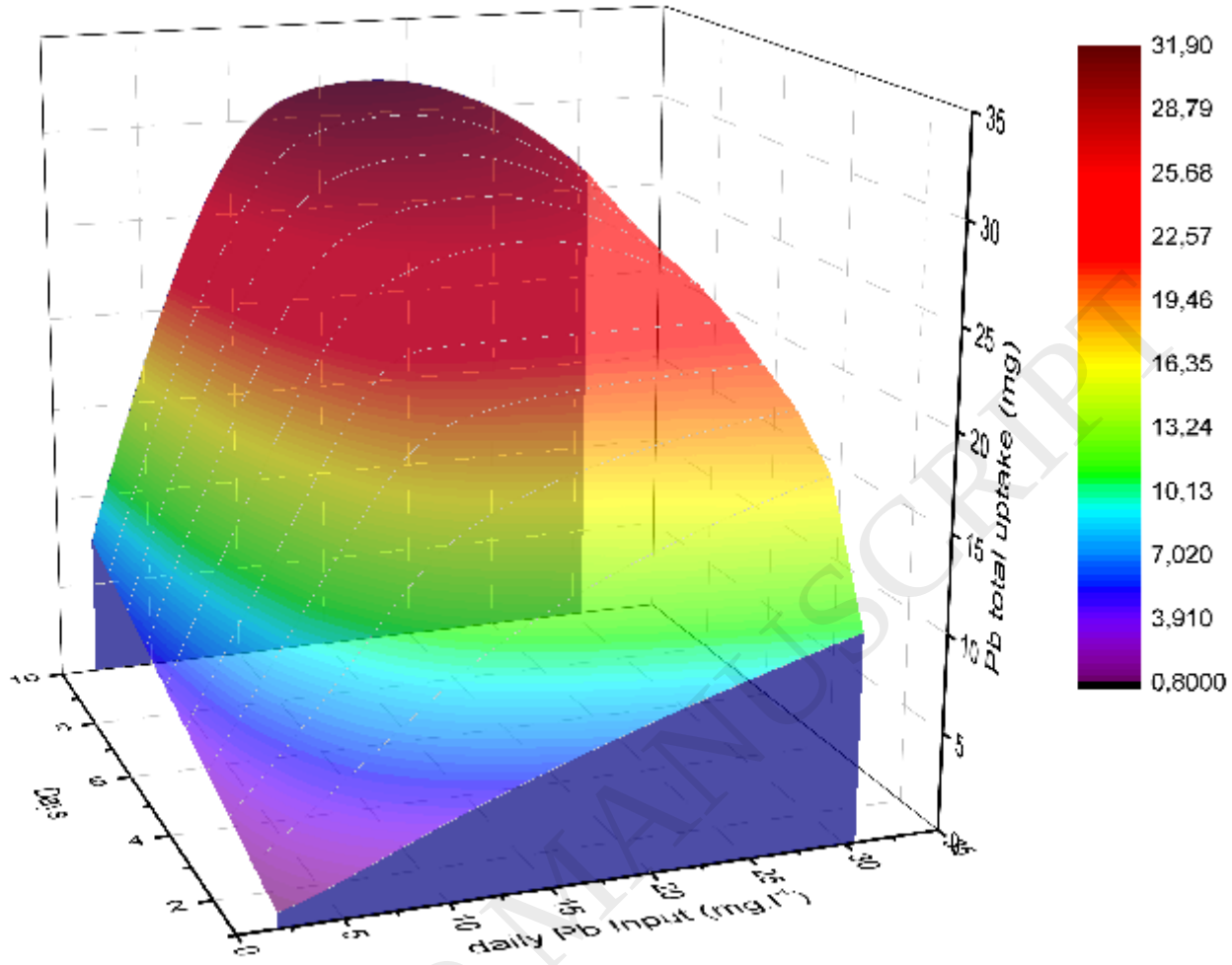

Figure 4. Phenotypic changes observed in *S. biloba* specimens after 10 days of receiving a daily discharge of water contaminated with different Pb concentrations. A) Control, B) 2.65±0.07 mg L^{-1}, C) 12.62±0.02 mg L^{-1}, and D) 30.57±0.01 mg L^{-1}. Arrows indicate some representative changes in leaves (chlorosis and signs of necrosis) described in the text.

Figure 4

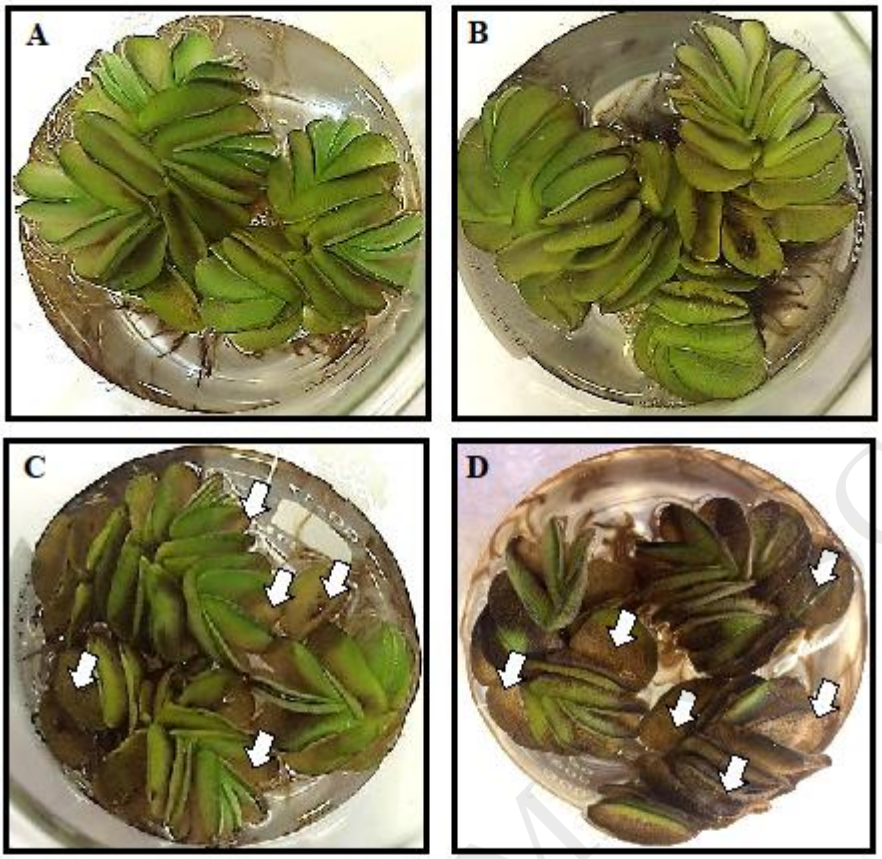

Figure 5. Representative photographs showing structural damage observed (arrows) in the trichomes ("egg-beater" hairs) located on the leaves of *S. biloba* after 10 days of receiving a daily discharge of water contaminated with 30.57±0.01 mg L^{-1} Pb (right) compared to the leaves of control plants (left). Image was taken at x10 magnification.

Figure 5

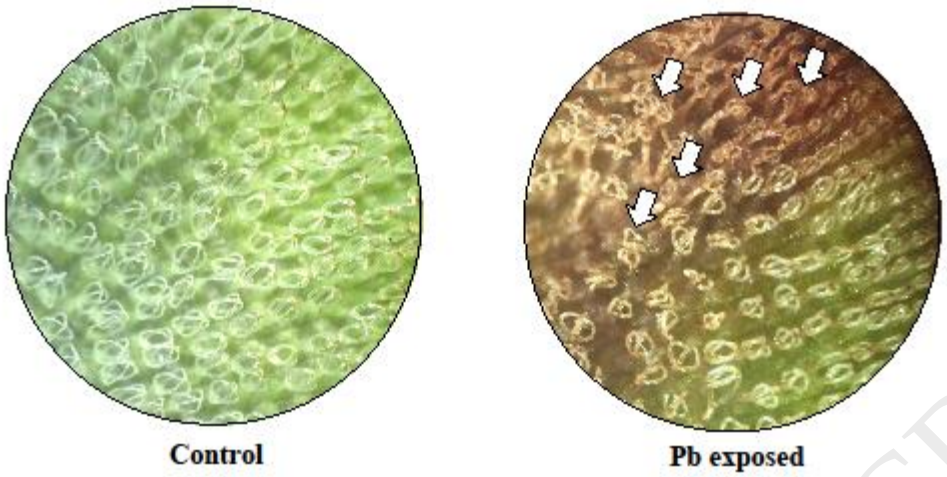

Table 1. Photosynthetic and antioxidant pigment content, soluble carbohydrate concentration, and membrane stability index in *S. biloba* specimens after 10 days of receiving a daily discharge of water contaminated with different Pb concentrations. Data are mean of three replications ± SD (n = 3).

Table 1

Leaves					**Roots**			
Physiological parameter			**Pb (mg L-1)**			**Pb (mg L-1)**		
Control	**2.65±0.07**	**12.62±0.02**	**30.57±0.01**		**Control**	**2.65±0.07**	**12.62±0.02**	**30.57±0.01**
Chl*a* (µg g-1 FW)	317 ± 24a	247 ± 8b	222 ± 21bc	198 ± 15c	33 ± 7a	21± 9b	19± 3bc	13 ± 7c
Chl*b* (µg g-1 FW)	109 ± 25a	83 ± 22a	80 ± 26a	113 ± 28a	10 ± 8a	6 ± 9a	8 ± 6a	7 ± 3a
Chl*total* (µg g-1 FW)	426 ± 31a	330 ± 10b	302 ± 27b	311 ± 28b	43 ± 12a	27 ± 10b	27 ± 13b	20 ± 6b
Carotenoids (µg/g FW)	78 ± 5a	72 ± 6ab	64 ± 4b	31 ± 2c	14.4 ± 5.4a	7.3 ± 2.6b	6.5 ± 1.7b	5.8 ± 1.4b
Anthocyanins (nmol -1 FW)	246 ± 37a	212 ± 45a	185 ± 23a	209 ± 17a	87 ± 11a	66 ± 2b	64 ± 25b	35 ± 9b
Flavonoids (AU_{330} g-1 FW)	32 ± 5a	32 ± 5a	32 ± 3a	12 ± 3b	8.4 ± 2.4a	2.9 ± 0.7b	2.7 ± 0.4b	2.6 ± 0.8b
Soluble carbohydrates (mg g-1 FW)	160 ± 30a	146 ± 30ab	126 ± 22b	46 ± 6c	18 ± 8a	45 ± 20b	41 ± 6b	45 ± 6b
MSI (%)	70 ± 5a	73 ± 2a	67± 11a	17 ± 10b	58 ±7a	41 ± 10b	43 ± 2b	30 ± 21b
* Different letters at same row represent statistically significant differences ($p < 0.05$)								