Environmental Toxicology

Effect of pH on Cr(III) Accumulation, Biomass Production, and Phenolic Profile in 2 Salvinia Species

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Abstract: We analyzed the effect of pH on Cr(III) accumulation, biomass production, and phenolic profile of Salvinia rotundifolia and Salvinia minima plants grown in the presence of increasing concentrations of CrCl3. Biomass accumulation, metal tolerance index, and photosynthetic pigment contents indicate that Salvinia rotundifolia seems to be more tolerant of Cr(III) than S. minima at different pHs. Increased metal accumulation by Salvinia species under increasing pH could be explained by changes of the protonation status of cell wall functional groups because both the highest and the lowest pH values used in the present study were outside of the levels at which Cr(III) species start to precipitate. The metal translocation factor indicates that in buffered conditions S. rotundifolia tend to retain more Cr(III) in lacinias than S. minima, probably through the involvement of insoluble phenolics. The results of the present study could be useful to the management of solution pH to maximize the removal of Cr(III) by aquatic plants. Environ Toxicol Chem 2019;38:167–176. © 2018 SETAC

Keywords: pH; Biomass production; Cr(III) accumulation; Salvinia species; Phenolic compounds

INTRODUCTION

Chromium (Cr) is a redox-dynamic transition metal with many industrial uses. It can exist in different oxidation states, the most stable forms being trivalent (Cr(III)) and hexavalent (Cr(VI)), which are commonly found in the environment (Zayed and Terry 2003). However, the hexavalent form can be reduced quickly by soil/water organic matter in the pH range 4.5 to 7.5 (Bartlett and Kimble 1976). In addition, the redox potential of the pair Cr(VI)→Cr(III) is high, and only a few oxidants occurring in soils and/or aquatic systems are able to oxidize Cr(III) to Cr(VI) (Kota et al. 2000). Therefore, Cr(III) is generally considered to be the stable form in equilibrium with most soil/water systems (Losi et al. 1994). Although Cr(III) appears to be the stable oxidation state in aquatic systems, fluctuations of water physicochemical characteristics such as pH, temperature, dissolved organic matter, and redox potential can affect its speciation and availability (Losi et al. 1994). Among the factors that affect the speciation and availability of Cr(III), pH emerges as the primary control, with cationic species being more soluble under acidic conditions (pH < 7) and anionic species more soluble under alkaline conditions (pH > 7). Major aqueous species of Cr(III) are Cr$^{3+}$ and Cr(OH)$^{2+}$, which occur at pH ≤ 4.5; Cr(OH)$_2$$^{-}$ and Cr(OH)$_3$(aq), which occur at pH ≥ 5.5; and polyhydroxyl species Cr(OH)$_4$$^{-}$, Cr$_2$(OH)$_2$$^{4+}$, Cr$_3$O$_4$(OH)$_4$$^{3-}$, Cr$_4$(OH)$_5$$^{5-}$, and Cr$_2$O$_2$(OH)$_2$$^{4-}$, which occur at high alkaline pHs (Remoundaki et al. 2007).

In rivers, lakes, and lagoons the levels of Cr(III) normally range from >1 to 40 μg L$^{-1}$, whereas in the oceans is much lower with a mean value of 0.3 μg L$^{-1}$ (Santonen 2009). However, because of excessive use of Cr(III) compounds in several industrial applications, environmental contamination with this metal has gained substantial attention worldwide. In most polluted waters the concentration of Cr(III) ranges from <1 to 44 mg L$^{-1}$, but levels as high as 80 mg L$^{-1}$ have also been found in paper mill effluents (Santonen 2009). The trivalent form is regarded by diverse clinical reports as an essential micronutrient for humans and other animals (Levina and Lay 2008; Shadreck and Mugadza 2013), but plant physiologists still debate whether Cr(III) has beneficial or deleterious effects on plants (Prado et al. 2015). In this context, it has been assumed that Cr(III) at low concentrations can be beneficial to plants, although it is highly toxic at high concentrations (Singh et al. 2013). However, increasing evidence indicates that Cr(III) causes more toxic than beneficial effects on both aquatic and terrestrial plants (Karuppananandam and Kumariah 2008; González et al. 2015; Lukina et al. 2016).
Even in some lower and higher plants, such as Pseudokirchneriella subcapitata, Chlorella kessleri, and Salsola kali, Cr(III) seems to be more toxic than Cr(VI) (Gardea-Torresdey et al. 2005; Vignati et al. 2010). Beyond the beneficial or detrimental effects of a determined metal, plants have developed different strategies to avoid the excessive movement of metals in their tissues (Emanverdian et al. 2015). Mechanisms to cope with heavy metals include the binding of metal to cell walls, complexion with secondary metabolites, and vacuolar sequestration, among others (Tong et al. 2004). Soluble phenolics have been recognized as efficient heavy metal complexing molecules (Michalak 2006; Borowska et al. 2018), and there is increasing evidence linking their accumulation with the exposure of plants to heavy metals (Kováčik et al. 2009; Careto et al. 2015). In addition, different studies have shown that both structural polymerized phenolics (lignin) and cell wall–associated, non-structural polyphenols (tannins) increase in plants exposed to heavy metals (Pawlak-Sprada et al. 2011).

Fluctuations of pH are considered to be a very important parameter that directly influences plant growth. Changes of pH can alter the availability of essential nutrients and trace metals and even cause direct effects on primary and secondary metabolism at extreme pH values (Lager et al. 2010; Radić et al. 2016). Thus, in heavy metal–polluted waters plant metabolism becomes affected by both heavy metal and pH (Saygideger et al. 2004). A very important question regarding plant–water–metal interaction is how much the solution pH affects metal uptake and plant metabolism. In this way, knowledge of how the solution pH affects plant growth and the amount of Cr removed by the plant becomes of primary importance to maximize the phytoremediation of Cr(III)-polluted aquatic systems.

In living plants, Cr(III) can be retained on the cell wall through cation exchange interactions or sequestered into the vacuoles of epidermal and cortical cells (Mangabeira et al. 2011). Thus, we hypothesized that in Salvinia species grown in buffered solutions of Cr(III) significant changes in physiological parameters related to plant growth, metal accumulation, and synthesis of secondary metabolites induced by both heavy metal and solution pH must occur. The aim of the present study was to analyze in plants of Salvinia rotundifolia and Salvinia minima grown at different pH values 1) Cr(III) accumulation, metal tolerance index, and metal translocation factor; 2) the content of photosynthetic pigments and biomass production; and 3) the accumulation of soluble and insoluble phenolics. Uniform plants with fully expanded floating leaves (fronds) and root-like submerged leaves (lacinias) were selected from both species and transferred to plastic trays containing either unbuffered (tap water) or buffered (Mcllvaine buffer) Cr(III) solution. The Mcllvaine buffer (citrate-phosphate buffer) was used because of its broad pH range. To avoid the excessive increase of phosphate and citrate anions in treatment solutions, the molarity of buffer was relatively low (10 mM). On the other hand, we did not use zwitterionic N-substituted aminosulfonic acids, usually known as Good’s buffers, because these buffers can complex Cr(III) and interact with biological systems (Ferreira et al. 2015). The Mcllvaine buffer was prepared using tap water to get an aquatic environment similar to the origin of plants and to avoid posttransplanting stress (Table 1). In addition, plants were not cultivated in Hoagland’s solution, to avoid chelation and/or ion competition between Cr(III) species and Hoagland ions for binding sites of the cell wall (Prado et al. 2010). Buffered and unbuffered Cr(III) solutions (5 and 20 mg L\(^{-1}\)) were prepared by stepwise dilution of a Cr(III) stock standard solution either with Mcllvaine buffer or with tap water. A Cr(III) stock standard solution was prepared at a concentration of 500 mg L\(^{-1}\) by dissolving the appropriate amount of pure CrCl\(_3\) \(\times\) 6H\(_2\)O (analytical grade) in ultrapure water. Selected pHs were 4.0, 6.0, and 7.6 because these are the most frequently found in heavy metal–polluted industrial effluents and acid mine drainages (Shi 2009).

**TABLE 1: Physicochemical parameters of the tap water used in the growth of Salvinia species**

| Parameter                                      | Value (mg L\(^{-1}\)) |
|------------------------------------------------|------------------------|
| pH                                             | 7.0–7.2                |
| Electrical conductivity (µS cm\(^{-1}\))       | 200                    |
| Dissolved oxygen (mg L\(^{-1}\))               | 3                      |
| Total dissolved solids (mg L\(^{-1}\))         | 100                    |
| Turbidity (nephelometric turbidity units)      | <1                     |
| As (µg L\(^{-1}\))                             | 0.2                    |
| Cu (µg L\(^{-1}\))                             | 2.2                    |
| Fe (µg L\(^{-1}\))                             | 12.0                   |
| Mn (µg L\(^{-1}\))                             | 0.2                    |
| NH\(_4\) (mg L\(^{-1}\))                      | <0.02                  |
| NO\(_3\) (mg L\(^{-1}\))                      | <0.05                  |
| NO\(_2\) (mg L\(^{-1}\))                      | 9.0                    |
| HPO\(_4\) (mg L\(^{-1}\))                     | <0.2                   |
| HCO\(_3\) (mg L\(^{-1}\))                     | 50.0                   |
| SO\(_4\) (mg L\(^{-1}\))                      | 11.0                   |
| Cl (mg L\(^{-1}\))                             | 12.0                   |
| Ca\(^{2+}\) (mg L\(^{-1}\))                    | 10.0                   |
| Mg\(^{2+}\) (mg L\(^{-1}\))                    | 7.0                    |
| Na\(^{+}\) (mg L\(^{-1}\))                     | 20.0                   |
| K\(^{+}\) (mg L\(^{-1}\))                      | 5.0                    |
| Cr (µg L\(^{-1}\))                             | <1.1                   |
| Ag\(^{+}\) (µg L\(^{-1}\))                     | 0.05                   |
| Pb (µg L\(^{-1}\))                             | 2.3                    |
| Hg (µg L\(^{-1}\))                             | <0.01                  |
| Cd\(^{2+}\) (µg L\(^{-1}\))                    | 2.5                    |
| CN (µg L\(^{-1}\))                             | <0.001                 |
| Zn\(^{2+}\) (µg L\(^{-1}\))                    | 3.6                    |
| Hardness (mg CaCO\(_3\) L\(^{-1}\))            | 112.0                  |

*Data are the average of the last 10 yr and were provided by the Servicio Provincial de Agua Potable y Saneamiento, Tucumán, Argentina.*
For each species and each Cr(II) concentration (buffered and unbuffered), 3 plastic trays (10 × 8 × 4 cm) containing 20 plants (~35 g fresh wt) and 150 mL of treatment solution each were prepared. Trays were transferred to a growth chamber for 7 d under controlled conditions: 200 μmol m⁻² s⁻¹ light intensity, 12:12-h dark: light cycle, 80% relative humidity, and 25/20 ± 1 °C day/night temperature. We chose 7 d as the treatment period because preliminary tests carried out in our laboratory showed that S. rotundifolia and S. minima plants were able to grow and stay healthy in tap water without nutrient supply for at least 9 d (Prado et al. 2010). Every 2 d, trays were either supplemented with tap water or McIlvaine buffer to compensate for the water loss (evapotranspiration) and maintain the initial volume. The pH of treatment solutions was monitored with a digital pH meter (Adwa AD1000). Measurements of pH were made at 0 d (immediately after transferring plants to treatment solutions) and at 3, 5, and 7 d after starting the experiment. To assess the effect of pH on metal availability, the Cr(III) concentration of each treatment solution was determined colorimetrically at 0, 3, 5, and 7 d.

At the end of the experiment (7 d of Cr(III) treatment) plants were harvested, washed with running tap water to remove the metal attached to plants, rinsed with distilled water, and blotted with paper towels. Ten blotted plants were divided in fronds and lacinias, weighted to obtain the fresh weight, and stored at −20 °C for chemical determinations. Fronds and lacinias from another 10 plants were dried at 80 °C in a hot air oven until constant weight (usually 48 h). After recording the dry weight, fronds and lacinias were ground for Cr(III) determination.

**Colorimetric determination of Cr(III)**

In buffered and unbuffered solutions Cr(III) was determined colorimetrically following the procedure of Memon et al. (2005) with minor modifications. Briefly, to sample solution (0.1 mL) was added 0.5 mL concentrated H₂SO₄ and 0.25 mL of 20 mM KMnO₄. Then it was heated without boiling (~45 °C) for approximately 15 min for complete oxidation of Cr(III) to Cr(VI). The solution was cooled, and 2.5% NaNO₂ solution was added dropwise to reduce the excess of KMnO₄ (decolorize the pink solution). After that, 0.1 mL of 0.5% 1,5-diphenyloxazole (DPC) reagent (2.5 g DPC dissolved in 50 mL acetone; the solution was prepared fresh daily) was added and made up to 2 mL with distilled water. After standing for 20 min, the absorbance was measured at 540 nm against a reagent blank. A control reaction without KMnO₄ was also performed. In the absence of KMnO₄ the absorbance value was 0, indicating that no spontaneous oxidation of Cr(III) occurred in buffered and unbuffered solutions. The reliability of the colorimetric method was checked by a calibration curve made from CrCl₃ × 6H₂O standard solution in the range of 0.2 to 30 mg L⁻¹ Cr(III) concentration. The standard deviation of the calibration curve was 0.0025, which indicated a good fit of data within an error limit <2%. To test whether the McIlvaine buffer interferes with the DPC assay, a standard curve was also made from a Cr(III) standard solution prepared in 10 mM McIlvaine buffer. No differences in absorbance values were observed between 2 standard solutions.

**Biomass production and metal tolerance index**

For biomass production another set of experimental trays was made. Immediately after putting the plants in treatment solutions, half of the plants (X₁) were harvested, whereas the remaining plants (X₂) were harvested at the end of the experiment. In both cases, harvested plants were rinsed with distilled water to remove the metal attached to the plants and dried at 80 °C until constant weight. Biomass accumulation corresponds to the difference between X₂ (dry wt) and X₁ (dry wt) and was expressed as milligrams per plant dry weight. The metal tolerance index was calculated as the ratio between the dry weight of Cr-treated plants and the dry weight of Cr-untreated plants and expressed as a percentage (Reisinger et al. 2008).

**Photosynthetic pigments**

Photosynthetic pigments (chlorophyll a [Chla], Chlb, Chl [a+b], and carotenoids) were measured at the end of experiment. Briefly, frond samples (0.1 g fresh wt) were added with 2 mL of dimethyl sulfoxide and incubated for 12 h at 45 °C in darkness (Chappelle et al. 1992). Chlorophyll and carotenoid concentrations were calculated from absorbance values at 664, 648, and 472 nm using the Wellburn equations (Wellburn 1994). Concentrations of photosynthetic pigments were expressed as milligrams per gram fresh weight.

**Cr accumulation and metal translocation factor**

Oven-dried, floating and submerged leaves of Cr-untreated and Cr-treated plants were digested in concentrated HNO₃ at 115 °C for 15 min following the US Environmental Protection Agency (1994) 3051 protocol. Determination of Cr was carried out by atomic absorption spectrophotometry (Perkin-Elmer 373), and Cr content was expressed as micrograms per gram dry weight. A blank of HNO₃ was also measured to ensure the correctness of metal quantification. The accuracy of the sample preparation method was ascertained by adding Cr(III) reference solutions (5 and 20 mg L⁻¹) to samples. Reference solutions were prepared from a 1000 mg L⁻¹ Cr(III) stock solution (Certipur™; Merck) by stepwise dilution made with 0.1% v/v HNO₃. The overall recovery associated with the digestion process was found to be in the range 90 to 95%. The error of metal determinations, based on variation in replicate analyses (n = 2) on the same sample, was 10% or lower. In Cr-untreated samples, the content of Cr(III) was below the detection limit. The metal translocation factor was calculated as the ratio between the Cr concentration in fronds and the Cr concentration in lacinias (Ton et al. 2015).

**Soluble and insoluble phenolics**

Soluble phenolics were extracted with ethanol 96% according to Swain and Hillis (1959) with minor modifications.
Briefly, frond and lacinia samples (1.0 g fresh wt) were extracted with 3 mL 96% ethanol, incubated in darkness at room temperature for 48 h, and centrifuged at 3000 g for 5 min. Supernatants were recovered and used for soluble phenolic determination. Aliquots of supernatants (0.1 mL) were added with 0.2 mL (1:1, v/v) of diluted Folin-Ciocalteu reagent and 1.8 mL of distilled water. After standing at room temperature for 2 min, 0.8 mL of 7.5% Na₂CO₃ was added and left standing again at room temperature for 5 min. Next, the absorbance was read at 760 nm against a reagent blank using a UV visible spectrophotometer (Hitachi U-2800A). Precipitates from soluble phenolic extraction were washed twice with 2 mL ethanol 96% and centrifugation at 3000 g for 5 min. Washed precipitates were dried at 37°C for 48 h and used to obtain cell wall-bound insoluble phenolics. Extraction of insoluble phenolic was adapted from Assabgui et al. (1993). Dried samples (0.5 g) were hydrolyzed with 2 mL of 2 N NaOH in a water bath at 60°C for 60 min. After cooling, solutions were slowly acidified to pH2 with 5 N HCl and solubilized insoluble phenolics were extracted with ethyl acetate. Following ethyl acetate, fractions were taken near dryness under a stream of N₂ gas and dissolved in 0.5 mL of 96% ethanol. Solubilized insoluble phenolics were determined using the Folin-Ciocalteu reagent, as described. Concentrations of soluble and insoluble phenolics were determined using a standard curve made with pure phenol and expressed as milligrams of phenol equivalent per gram fresh weight.

Statistics

For all determinations at least 3 replicates were done, and 2 independent experiments were performed. Data are presented as the mean of replicates, and bars represent the standard error (SE). Data were subjected to a one-way analysis of variance to confirm the variability of data and the validity of results. The Tukey test was performed to determine significant differences between treatments and between species ($p \leq 0.05$).

RESULTS

Stability of Cr(III) solution and metal availability

No changes in Cr(III) concentrations of both buffered and unbuffered solutions were observed at 0, 3, 5, and 7 d of the experimental period, indicating that no precipitation of Cr(III) species occurred in our experimental condition (data not shown). In a previous study carried out in our laboratory, no significant difference was found in Cr(III) concentration determined either by the DPC method or by atomic absorption spectrophotometry (Prado 2012).

Biomass and metal tolerance index

The biomass of Salvinia species was differently affected by Cr(III) and pH (Figure 1). The biomass of $S$. rotundifolia increased with Cr(III) treatment under all assayed pHs, but the higher increment occurred at pH 6.0. Maximum increases were 15.0% (pH 4.0), 38.3% (pH 6.0), and 18.3% (pH 7.6) and occurred at the highest metal concentration. However, in unbuffered plants the biomass accumulation was not affected significantly by the metal treatment. By contrast, in buffered plants of $S$. minima the biomass decreased with Cr(III) treatment. Maximum decreases of 26.9% (pH 4.0), 33.6% (pH 6.0), 35.6% (pH 7.6), and 11.3% (unbuffered plants) were observed in the presence of 20 mg L⁻¹ Cr(III). The tolerance index was higher in $S$. rotundifolia than in $S$. minima plants in buffered and unbuffered conditions (Figure 2). The tolerance index values in $S$. rotundifolia ranged between 103.8 and 178.2%, whereas in $S$. minima they ranged between 73.3 and 83.3%. At the end of the experimental period, both species showed healthy-looking plants without visible...
situations for each growth condition and each Cr(III) concentration (p ≤ 0.05).

**Photosynthetic pigments**

Photosynthetic pigments were significantly higher in *S. rotundifolia* fronds than in *S. minima* ones, but the accumulation pattern was differentially affected by Cr(III) and solution pH. Under all pH values the presence of Cr(III) increased both chlorophyll and carotenoids in *S. rotundifolia*, whereas in *S. minima* significant decreases occurred (Table 2). Maximum increases of photosynthetic pigments in *S. rotundifolia* were 58.1% (Chl a), 36.0% (Chlb), 50.1% (Chl[a+b]), and 43.8% (carotenoids) and were observed at pH 6.0 in the presence of 20 mg L⁻¹ Cr(III). Maximum decreases in *S. minima* fronds were 18.9, 27.8, 20.9, and 21.0% and occurred at pH 7.6 under the highest metal concentration. In unbuffered plants of both *Salvinia* species, photosynthetic pigments were not significantly affected by Cr(III) treatment. The Chl(a+b) to carotenoids ratio of buffered plants ranged between 6.10 and 7.65 in *S. rotundifolia* and between 5.51 and 6.47 in *S. minima*, whereas in unbuffered plants it ranged between 7.02 and 7.08 in the former and between 5.51 and 5.68 in the latter (Table 2).

**Cr accumulation and metal translocation factor**

The accumulation of Cr in lacinias and fronds of buffered and unbuffered plants of *S. rotundifolia* and *S. minima* increased under increasing Cr(III) concentration, but the amount of accumulated metal was different at all assayed pHs. Independently of the organ and *Salvinia* species, the highest metal accumulation occurred at pH 7.6 (Figure 3A and B). At both the organ and whole-plant levels, the accumulation of Cr(III) was significantly higher in *S. rotundifolia* than in *S. minima*. It is noteworthy that the accumulation of metal in the former was significantly lower in buffered plants, whereas in the latter it was lower in unbuffered plants. In buffered and unbuffered *S. rotundifolia* fronds the amount of accumulated Cr(III) ranged from 0.08 to 1.3 mg g⁻¹ dry weight, whereas in *S. minima* fronds it ranged from 0.07 to 0.43 mg g⁻¹ dry weight. In lacinias accumulated metal varied between 0.21 and 11.3 mg g⁻¹ dry weight in the former and between 0.20 and 1.33 mg g⁻¹ dry weight in the latter. Based on the value of pH, the maximum accumulation of Cr in lacinias of plants grown in the presence of 5 and 20 mg L⁻¹ Cr(III) showed a different pattern between the 2 *Salvinia* species (Figure 4). In buffered and unbuffered plants the translocation factor was significantly lower in *S. rotundifolia* than in *S. minima*, except at pH 6.0 in the presence of 20 mg L⁻¹ Cr(III) concentration. Values of translocation factor ranged between 0.09 and 0.45 in *S. rotundifolia* and from 0.20 to 0.52 in *S. minima*, respectively (Figure 5).

**Soluble and insoluble phenolics**

Both soluble and insoluble phenolics were significantly higher in *S. rotundifolia* than in *S. minima*, but the accumulation pattern was differently affected by both Cr(III) concentration and solution pH. Comparing with unbuffered plants, soluble and insoluble phenolics in buffered plants of both species showed an increasing accumulation in the presence and absence of Cr(III), but this was more marked for insoluble phenolics (Figure 6A–D). The highest increases of soluble phenolics in buffered plants were noticeable in fronds: 131.6% in the presence of 5 mg L⁻¹ Cr(III) and pH 6.0 (*S. rotundifolia*) and 110.5% at pH 7.6 in the absence of metal (*S. minima*). In unbuffered plants maximum increases of soluble phenolics in fronds and lacinias were 37.4 and 51.7% (*S. rotundifolia*) and 24.4 and 133.3% (*S. minima*), respectively (Figure 6A and C). Regarding insoluble phenolics, the most significant increases in both species were observed in lacinias of buffered plants: 2.3-fold (0 mg L⁻¹ Cr(III) and pH 7.6) in *S. rotundifolia* and 7.3-fold (20 mg L⁻¹ Cr(III) and pH 6.0) in *S. minima*. In unbuffered plants insoluble phenolics increased by 174.4 and 46.3% in fronds and lacinias of *S. rotundifolia* and 9.3 (fronds) and 39.2% (lacinias) in *S. minima* (Figure 6B and D).

**DISCUSSION**

The trivalent Cr(III) is considered an essential element for animals but has never been recognized as an essential element for plants (Shadreck and Mugadza 2013). However, in a few plants it can stimulate some physiological processes such as growth, photosynthesis, and carbohydrate metabolism (Paiva et al. 2009; Gomes et al. 2017). The increase of biomass is usually used as an indicator of the photosynthetic activity of plants exposed to heavy metals (Azevedo et al. 2005). The present data showed increases of biomass and photosynthetic pigments in plants of *S. rotundifolia* exposed to Cr(III) in buffered solutions, whereas in *S. minima* plants significant decreases were observed (Figure 1 and Table 2). The biomass accumulation is a key indicator of the health of plants in heavy metal–polluted sites.
Table 2: Effect of Cr(III) on Chla, Chlb, Car, and Car to Chla ratio in Salvinia rotundifolia and Salvinia minima fronds after a 7-d cultivation period under buffered and unbuffered conditions.

| Cr(III) (mg·L⁻¹) | S. minima (mg·g⁻¹·dry wt) | S. rotundifolia (mg·g⁻¹·dry wt) | Car/(Chla+Car) |
|------------------|-----------------------------|---------------------------------|-----------------|
|                  | Chla | Chlb | Car | (Chla+Car) | Chla | Chlb | Car | (Chla+Car) |
| Buffered (pH 4.0) | 0.114 ± 0.014 | 0.289 ± 0.051 | 0.072 ± 0.015 | 1.343 ± 0.070 | 0.821 ± 0.039 | 0.398 ± 0.070 | 0.082 ± 0.039 | 1.326 ± 0.053 |
| 5.0               | 1.076 ± 0.025 | 0.483 ± 0.033 | 0.080 ± 0.015 | 1.872 ± 0.075 | 0.284 ± 0.039 | 0.080 ± 0.015 | 1.872 ± 0.075 | 0.284 ± 0.039 |
| Buffered (pH 6.0) | 0.124 ± 0.023 | 0.329 ± 0.092 | 0.098 ± 0.015 | 0.958 ± 0.039 | 0.582 ± 0.036 | 0.284 ± 0.039 | 0.082 ± 0.039 | 1.326 ± 0.053 |
| 5.0               | 1.076 ± 0.025 | 0.483 ± 0.033 | 0.080 ± 0.015 | 1.872 ± 0.075 | 0.284 ± 0.039 | 0.080 ± 0.015 | 1.872 ± 0.075 | 0.284 ± 0.039 |
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| 5.0               | 1.076 ± 0.025 | 0.483 ± 0.033 | 0.080 ± 0.015 | 1.872 ± 0.075 | 0.284 ± 0.039 | 0.080 ± 0.015 | 1.872 ± 0.075 | 0.284 ± 0.039 |
| Buffered (pH 8.0) | 0.124 ± 0.023 | 0.329 ± 0.092 | 0.098 ± 0.015 | 0.958 ± 0.039 | 0.582 ± 0.036 | 0.284 ± 0.039 | 0.082 ± 0.039 | 1.326 ± 0.053 |
| 5.0               | 1.076 ± 0.025 | 0.483 ± 0.033 | 0.080 ± 0.015 | 1.872 ± 0.075 | 0.284 ± 0.039 | 0.080 ± 0.015 | 1.872 ± 0.075 | 0.284 ± 0.039 |
| Unbuffered        | 0.114 ± 0.014 | 0.289 ± 0.051 | 0.072 ± 0.015 | 1.343 ± 0.070 | 0.821 ± 0.039 | 0.398 ± 0.070 | 0.082 ± 0.039 | 1.326 ± 0.053 |
| 5.0               | 1.076 ± 0.025 | 0.483 ± 0.033 | 0.080 ± 0.015 | 1.872 ± 0.075 | 0.284 ± 0.039 | 0.080 ± 0.015 | 1.872 ± 0.075 | 0.284 ± 0.039 |

Data are means of 3 replications. Significant differences between species (S. minima and S. rotundifolia) at different pHs are indicated by different letters (A, B, C). Different lowercase letters at each row indicate significant differences between treatments (p<0.05).
binding sites (Ardestani et al. 2014). According to this model, the binding of Cr(III) to the cell wall is based on metal speciation and competitive binding between Cr(III) species and H\(^{+}\) of cell wall functional groups, commonly called “biotic ligands.” Acid pHs increase the solubility and availability of Cr(III) species (e.g., Cr\(^{3+}\) and CrOH\(^{2+}\)) but also increase the concentration of free H\(^{+}\) in root surrounding solution, leading to increased competition between Cr(III) species and free H\(^{+}\) for cell wall binding sites (Song et al. 2014). As the pH is increased, the concentration of free H\(^{+}\) decreases and the surface charge density of the root cell wall becomes negative (Chathuranga et al. 2013) so that more cationic species (e.g., Cr[OH]\(^{2+}\) and Cr[OH]\(_{2}\)\(^{+}\)) and even polyhydroxyl species (e.g., Cr\(_{2}[OH]_{2}\)\(^{4+}\) and Cr\(_{4}[OH]_{4}\)\(^{5+}\)) can bind to plant roots. In this context an increased accumulation of Cr(III) in Salvinia plants is expected to occur under alkaline conditions. Agreeing with this assumption, the present study showed the highest accumulation of Cr(III) in lacinias of both Salvinia species at pH 7.6 (Figure 3A and B). However, the trend of Cr accumulation on lacinias as a function of the solution pH was different in both Salvinia species (Figure 4). This fact can reflect the presence of different protonizable groups in the cell walls of both Salvinia species or that there is a species-specific synthesis and release into the rhizosphere of Cr(III) complexing compounds such as organic acids, amino acids, and phytosiderophores (Hinsinger 2001). In agreement with this assumption, it has been demonstrated that in growing maize plants organic acids contribute to the mobilization of Cr(III) by converting it into a labile, organically bound form, enhancing its availability to the plant (Srivastava et al. 1999). In addition, it has been demonstrated that the presence of heavy metals can trigger aquatic plants to change the pH of root surrounding solution, which may affect both rhizosphere metal bioavailability and plant metal uptake (Javed and Greger 2011). Further in-depth studies are nevertheless required to investigate the binding of Cr(III) species to the cell wall of Salvinia species to identify the extent of participation of their respective binding sites.

Secondary metabolites have been recognized as efficient heavy metal–protecting compounds (Skórzynska-Polit et al. 2004; Michalak 2006). Among secondary metabolites, phenolic compounds can cope with heavy metals either externally by binding the metal to the cell wall or internally by complexing the metal in the cytoplasm and/or by its sequestration inside the

**FIGURE 3:** Accumulation of Cr(III) in buffered and unbuffered fronds and lacinias of *Salvinia rotundifolia* (A) and *Salvinia minima* (B) plants after a 7-d cultivation period. Data are mean ± standard error of 3 replications (n = 6). For each *Salvinia* species different lowercase and uppercase letters on bars indicate significant differences in metal accumulation by fronds and lacinias, respectively (p ≤ 0.05). nd = no detected.

**FIGURE 4:** Relationship between pH value and accumulated Cr(III) in lacinias of *Salvinia rotundifolia* and *Salvinia minima* plants growing during 7 d at different pHs. Data are mean ± standard error (SE) of 3 replications (n = 6). Vertical bars show the SE.
vacuole (Skórzyńska-Polit et al. 2004). In addition, phenolic compounds can act as antioxidant molecules against reactive oxygen species generated by heavy metal–induced oxidative stress (Michalak 2006). It is known that low and high pHs affect the synthesis and accumulation of secondary metabolites in plants (Schmitzer and Stampar 2010). Increases, decreases, and even no changes of phenolics induced by acid or alkaline pHs have been reported for different plant species (Hawrylak-Nowak 2008; Zhang et al. 2014). However, available data reveal that the effect of pH on the dynamic of phenolic accumulation in heavy metal–exposed plants is not always clear, and contradictory results have been reported (Pal’ove-Balang et al. 2012; Radić et al. 2016). Agreeing with these findings, the present data showed significant differences in the accumulation pattern of soluble and insoluble phenolics in fronds and lacinias of Cr-exposed *S. rotundifolia* and *S. minima* plants growing at different pHs (Figure 6A–D). In both species, insoluble phenolics were significantly higher than soluble phenolics in buffered and unbuffered conditions. The lower content of soluble phenolics observed in lacinias of both species could indicate an increased polymerization of phenolic compounds into the cell wall matrix.

**FIGURE 5:** Metal translocation factor of buffered and unbuffered *Salvinia rotundifolia* and *Salvinia minima* plants after a 7-d cultivation period. Data are mean ± standard error of 3 replications (*n* = 6). Different lowercase letters on bars indicate significant differences for each *Salvinia* species. Different uppercase letters indicate significant differences between species for each growth condition and each Cr(III) concentration (*p* ≤ 0.05).

**FIGURE 6:** Effect of pH and Cr(III) concentration on the content of soluble and insoluble phenolics of buffered and unbuffered *Salvinia rotundifolia* (A, B) and *Salvinia minima* (C, D) plants after a 7-d cultivation period. Data are mean ± standard error of 3 replications (*n* = 6). For both soluble and insoluble phenolics, different letters on bars indicate significant differences between floating and submerged leaves for each growth condition (*p* ≤ 0.05).
in order to immobilize more Cr(III) at the root level. In this regard, we have demonstrated an increased deposition of insoluble phenolics (i.e., lignin) in the roots of Cd-treated citrus rootstocks (Podazza et al. 2016). Nevertheless, a decreased content of soluble phenolics in lacinias could also indicate either "in situ" reduced synthesis or reduced transport from fronds. By contrast, the highest content of soluble phenolics found in the fronds of Salvinia plants could be also related to the cytosolic complexation of Cr(III) species translocated from lacinias. Supporting this assumption, previous reports have shown the complexation of Cr(III) ions with different plant phenolics (Sun et al. 2008; Panwar and Memon 2014). On the other hand, the high content of insoluble phenolics occurring in fronds of Cr-exposed and Cr-unexposed plants probably can be related to the high content of lignin normally present in the cell wall of floating leaves of aquatic ferns such as Azolla and Salvinia (Leterme et al. 2009), rather than the binding of Cr(III) ions. Further studies to provide insight into the pH-dependent mechanisms controlling the accumulation of phenolic compounds in Cr-exposed Salvinia plants are in progress.

The present results reveal that the solution pH affects the growth and Cr(III) uptake by Salvinia species, which indicates that the management of solution pH has important implications for maximizing the removal of heavy metals by aquatic macrophytes and the economic success of the phytoremediation process.

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**Data Accessibility**—Data, associated metadata, and calculation tools are available from the corresponding author (rosamd@csnat.unt.edu.ar).

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