



## Salinity effects on photosynthetic pigments, proline, biomass and nitric oxide in *Salvinia auriculata* Aubl.

Efeito da salinidade sobre pigmentos fotossintéticos, prolina, biomassa e óxido nítrico em *Salvinia auriculata* Aubl.

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**Abstract: Aims:** Effects of salt stress on the physiology of *Salvinia auriculata* were investigated. **Method:** Plants were supplemented with 0, 50, 100 and 150 mmol L<sup>-1</sup> NaCl and incubated for 5 days. NO content was evaluated after 2 hours and 5 days. Photosynthetic pigments, proline and nutrients were analyzed after 5 days. **Major Results:** Higher chlorophyll *a* content was observed in plants treated with 50 mmol L<sup>-1</sup>, decreasing in higher NaCl concentrations, while chlorophyll *b* content decreased with increasing NaCl concentrations. Exposure to 50 mmol L<sup>-1</sup> NaCl increased biomass, while higher concentrations caused loss of biomass. Ca, K and Mg decreased with increasing NaCl concentrations, and the Na/K ratio was significantly increased at 150 mmol L<sup>-1</sup> NaCl. Proline increased significantly at 150 mmol L<sup>-1</sup>. Extracellular NO content increased after 2 hours, with significantly higher NO concentrations in roots observed at 50 mmol L<sup>-1</sup>. Decreases in NO content were observed after 5 days. **Conclusions:** The results indicate that moderate salinity induces NO production earlier during incubation, probably associated to signaling for the production of compounds that assist in stress tolerance. At higher concentrations, this tolerance is reduced. This allows for further understanding of the physiological and biochemical mechanisms associated with the adaptation of this macrophyte to saline conditions, which, in turn, affect this species ecology and distribution in coastal areas.

**Keywords:** *Salvinia auriculata*; salinity stress; nitric oxide; chlorophyll; NaCl; macrophyte.

**Resumo: Objetivos:** Efeitos do estresse salino sobre a fisiologia de *Salvinia auriculata* foram investigados. **Metodologia:** As plantas foram expostas a 0, 50, 100 e 150 mmol de NaCl L<sup>-1</sup> e incubadas durante 5 dias. O conteúdo de NO foi avaliado após 2 horas e 5 dias. Pigmentos fotossintéticos, prolina e nutrientes foram analisados após 5 dias. **Resultados Principais:** Observou-se maior teor de clorofila *a* em plantas tratadas com 50 mmol L<sup>-1</sup>, diminuindo em concentrações mais altas, enquanto o conteúdo de clorofila *b* diminuiu com o aumento das concentrações de NaCl. A exposição a 50



mmol L<sup>-1</sup> de NaCl aumento a biomassa, enquanto concentrações mais elevadas causaram perda de biomassa. Ca, K e Mg diminuíram com o aumento das concentrações de NaCl, e a razão Na/K foi significativamente aumentada em 150 mmol L<sup>-1</sup> NaCl. A prolina aumentou significativamente a 150 mmol L<sup>-1</sup>. O conteúdo extracelular de NO aumentou após 2 horas, e diminuiu após 5 dias. Após 2 horas, concentrações significativamente maiores nas raízes foram observadas a 50 mmol L<sup>-1</sup>, enquanto após 5 dias diminuições foram observadas. **Conclusões:** Os resultados indicam que a salinidade moderada induz a produção de NO durante a incubação, possivelmente associada à sinalização para a produção de compostos que auxiliem na tolerância à salinidade. Em concentrações superiores esta tolerância é reduzida. Com isso, é possível compreender melhor os mecanismos fisiológicos e bioquímicos associados a essa adaptação em macrófitas sob condições salinas, que afetam sua ecologia e distribuição em áreas costeiras

**Palavras-chave:** *Salvinia auriculata*; estresse salino; NO; clorofila; NaCl; macrófita.

## 1. Introduction

Salinity is the major environmental factor that limits plant growth and primary productivity in aquatic ecosystems (Moradi et al., 2013). In coastal water bodies, salinity can vary seasonally and can be influenced by changes in water levels, precipitation, evaporation (Schallenberg et al., 2003), hydrological alterations (Howard & Mendelssohn, 1999) and anthropogenic activities (Roache et al., 2006).

Exposure to salinity may cause several morphological, physiological and biochemical changes in plants, due to excess ions and water deficit (Greenway & Munns, 1980; Maskri et al., 2010). The most common effects in plants are toxicity, diminished CO<sub>2</sub> assimilation and enhanced generation of reactive oxygen species (Chawla et al., 2013). Changes in fundamental processes have also been observed, such as growth, photosynthesis, protein synthesis and lipid metabolism (Parida & Das, 2004). High salinity concentrations in plants also generate changes in plant productivity (Doganlar et al., 2010; Hasegawa et al., 2000), nutrient imbalances (Ashraf, 2009), accumulation of osmoprotective compounds, such as proline (Bohnert et al., 1995), and changes in nitric oxide (NO) content (Zhang & Blumwald, 2001).

Na<sup>+</sup> acts on the activation of a wide range of enzymes in plants, is involved in membrane osmosis, and can also replace K<sup>+</sup> in some osmotic and metabolic functions. Cl<sup>-</sup> plays an important role in photosynthesis, enzyme activation, osmotic regulation and cell division (Ashari-Esna & Gholami, 2010). Excessive Na<sup>+</sup> and Cl<sup>-</sup> concentrations affect the absorption of many essential nutrients such as K, Ca Mg and N (Abdallah et al., 2016; Iqbal et al., 2015). This occurs through competitive interactions affecting the ionic selectivity of cell membranes (Stoeva & Kaymakanova, 2008) and photosynthetic activity (Parida et al., 2002), reducing stomata

opening and leading to decreases in intracellular CO<sub>2</sub> (Munns & Tester, 2008).

Biomarkers are used to indicate an exposure to or the effect of xenobiotics present in the environment and in organisms. Biomarkers of exposure provide functional measures of exposure that are characterized at a sub-organism level (Brain & Cedergreen, 2009). The use of species-specific biomarkers have been utilized in a diverse array of studies aimed at assessing organismal, population, or ecosystem health (Ashraf, 2009).

Two main plant metabolites used as biomarkers to salinity stress are proline and nitric oxide (NO). Proline is an osmotic regulator, enzyme denaturation protector and a macromolecule or molecular assembly stabilizer, as well as a nitrogen and carbon source reservoir and/or a hydroxyl radical scavenger in aquatic macrophytes (Bagdi & Shaw, 2013). NO, on the other hand, is a signaling molecule produced as a physiological response in plants under stress conditions (Kausar & Shahbaz, 2013; Lamattina et al., 2003). It is involved in several physiological processes that include germination, root growth, stomatal closing, and adaptive response to biotic and abiotic stresses (Delledonne, 2005; Delledonne et al., 1998; Neill et al., 2008). NO acts as an antioxidant during different stress situations, increasing under NaCl stress. This indicates that nitrosative stress could participate in damage mechanisms produced by abiotic toxic conditions (López-Carrión et al., 2008), although not many studies are available in this regard (Siddiqui et al., 2011).

*Salvinia auriculata* is a freshwater free-floating aquatic macrophyte that, under favorable conditions (e.g. high P and N concentrations), colonizes large water areas in short periods of time (Peixoto et al., 2005). This species is a pollution bioindicator in aquatic ecosystems, since it shows great ability to remove and accumulate various organic and inorganic elements and compounds present in water

and sediment (Henry-Silva & Camargo, 2002; Soares et al., 2008).

In order to improve fishing activities in the northern region of the State of Rio de Janeiro, Brazil, the implementation of artificial sandbars separating coastal lagoons from the sea has become a common practice (Suzuki et al., 2002). However, these sandbar openings can cause radical changes in the physicochemical properties and physical and chemical conditions of these areas, including drastic reductions in water volume and profound changes in the biota, including aquatic macrophytes, such as *S. auriculata*, due to excess brackish water and increased salt water influx (Suzuki et al., 2002). In addition, intrusion processes of saline or brackish water in these coastal ecosystems may cause an indirect salinization process of other water bodies not directly connected to the sea, by groundwater (Gomes et al., 2011).

Thus, the aim of this study is to verify salinity effects on *S. auriculata* by evaluating chlorophyll *a* and *b*, carotenoids, proline and NO content after exposure to different salinity concentrations.

## 2. Material and Methods

### 2.1. Plant material

Approximately 20 kg fresh weight (FW) of *S. auriculata* were sampled from the Jacú Lagoon, a freshwater lagoon in the municipality of Campos dos Goytacazes, Rio de Janeiro, Brazil. At the laboratory, the specimens were washed several times to eliminate solid residues and maintained for five days at 25 °C and 12 hour-photoperiods (100  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) in plastic boxes. Plants were maintained in a nutritive solution containing several trace-nutrients such as K, Mn and Mg, according to Hoagland & Arnon (1950) and Smart & Barko (1985).

### 2.2. NaCl assays

Approximately 50 g FW of *S. auriculata* were transferred to a box containing 3 L of the nutritive solution and supplemented with different NaCl concentrations (0, 50, 100 and 150  $\text{mmol L}^{-1}$ ), in triplicate. These concentrations were chosen after pilot studies in our lab demonstrated that 50  $\text{mmol L}^{-1}$  caused no stress on the samples, while data on concentrations above of 150  $\text{mmol L}^{-1}$  have already been published (Gomes et al., 2011). Samples were incubated for five days in controlled conditions, in 12- hour photoperiods (100  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at 20 °C (dark period) and at 25 °C (light period). NO content was evaluated after 2 hours (since

NO is rapidly formed during signaling) and 5 days. The remaining parameters (photosynthetic pigments, proline and nutrients) were analyzed after 5 days. This limit was set due to the death of most plants when exposed to the highest concentration.

### 2.3. Determination of photosynthetic pigments

Photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and carotenoids) were analyzed, in triplicate, according to Wellburn (1994). *S. auriculata* leaves (0.2 g FW each) were cut into strips and transferred to polypropylene tubes containing dimethylsulfoxide (DMSO). After digestion, 1 mL of each sample was separated and chlorophyll *a*, chlorophyll *b* and carotenoids were measured at 480, 649 e 665 nm, respectively, on a UV-Vis spectrophotometer (model UV-160A, Shimadzu, São Paulo, Brazil). All procedures were carried out in a low-light environment. Values were expressed in  $\text{nmol}\cdot\text{cm}^{-2}$  of dry weight (DW).

### 2.4. Proline quantification

Proline content was determined according to Bates et al. (1973). Leaf and root samples (300 mg FW each, in triplicate), were homogenized in 6 mL of sulfosalicylic acid 3%, at 4 °C. Samples were then transferred to polypropylene tubes, incubated and centrifuged at 5,000 rpm for 20 minutes. Subsequently, 1 mL of the supernatant of each sample was incubated with 1 mL of an acid ninhydrin solution containing 2.5% ninhydrin, 60% phosphoric acid (v/v) and 1 mL of glacial acetic acid (100%) in a boiling water bath (CT 246, Cientec, Belo Horizonte, Brazil) during 1 hour. After incubation, the samples were rapidly cooled on ice and absorbances were determined at 518 nm on a UV-Vis spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan).

### 2.5. NO determinations

The NO determinations were carried out in the samples incubated for two hours and 5 days using fluorescence microscopy and fluorometry, according to Tun et al. (2006), with minor modifications. For quantification of endogenous NO by microscopy fluorescence, root segments (10 segments/sample, in triplicate) were incubated with 2 mL of a nutritive solution containing 0, 50, 100 and 150  $\text{mmol L}^{-1}$  NaCl, during two hours in the light (100  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at 25 °C and during 5 days in 12-hour photoperiods at 20 °C in the dark and at 25°C in the light. After the NaCl treatments, the samples were incubated during 1 hour with 15  $\mu\text{mol L}^{-1}$  4,5-diaminofluorescein

diacetate (DAF-FM-DA, Calbiochem, Darmstadt, Germany, a cell-permeable fluorescent dye. Samples were then washed twice with the respective NaCl concentrations and plates were prepared. Samples were visualized using an Axioplan-Zeiss fluorescence microscope (Carl Zeiss, Jena, Germany) with the filter set for DAF-FM-DA excitation at 515 nm and emission at 525 nm. Images were acquired using a digital AxioCam MRC5 camera (Carl Zeiss, Jena, Germany) and the fluorescence intensity of the root tips was determined using the AxioVisionLE software version 4.8 package (Carl Zeiss) as number of pixels per area. All experiments were conducted at least twice.

For quantification of NO release by fluorometry, plants (100 mg FW each sample, in triplicate) were incubated in 3 mL of nutritive solution containing 0, 50, 100 and 150 mmol L<sup>-1</sup> NaCl, during two hours in the light (100 μmol.m<sup>-2</sup>.s<sup>-1</sup>) at 25 °C and during 5 days in 12-hour photoperiods at 20 °C in the dark and at 25 °C in the light. Subsequently, the samples were incubated for 1 hour with 10 μmol L<sup>-1</sup> 4,5-diaminofluorescein (DAF-FM, Calbiochem), a cell-impermeable fluorescent dye to analyze released NO. After incubation, 2 mL of the supernatant were separated and analyzed on a spectrofluorometer (Shimadzu RF-5301, Kyoto, Japan) using a DAF-FM filter set at excitation at 515 nm and emission at 525 nm. The data was presented as relative fluorescence.

## 2.6. Nutrient determinations

Nutrient (Ca<sup>+2</sup>, K<sup>+</sup>, Na<sup>+</sup>, Mg<sup>+2</sup>, Cl<sup>-</sup>, P (phosphate) and N (nitrate)) determinations were conducted according to Malavolta et al. (1997). Samples were collected after the five days of treatment with NaCl and dried at 60 °C during four days. The samples were then grounded to a fine powder and samples of 500 mg DW, in triplicate, were used for the nutrient determinations. Ca<sup>+2</sup>, K<sup>+</sup>, Na<sup>+</sup> and Mg<sup>+2</sup> were analyzed by atomic absorption spectrophotometry (AAS4, Carl Zeiss). Total P content was obtained by complete digestion of the samples (500 mg FW, in triplicate) with nitric acid (100%) and perchloric acid (100%) (1:1, v/v) and subsequent colorimetric quantification using the molybdate method (Malavolta et al., 1997). Cl<sup>-</sup> was determined in aqueous extracts by titration with silver nitrate, also according to Malavolta et al. (1997). N determinations (100 mg DW, in triplicate) were conducted using the methodology proposed by Nessler (Jackson, 1965), by sulfuric digestion and subsequent absorbance readings at

480 nm on a UV-Vis spectrophotometer UV-160A, Shimadzu).

## 2.7. Statistical analyses

With the exception of the extracellular and intracellular NO results, the data were evaluated by a two-way ANOVA ( $p < 0.05$ ) with a *posteriori* Bonferroni post-test. All other data were analyzed using the non-parametric Kruskal-Wallis test ( $p < 0.05$ ) and a retrospective Dunn's test to compare the results between treatments, since data followed a non-normal distribution. All data were analyzed using the GraphPad Prism 4 statistical software package.

## 3. Results

Figure 1 shows results after 5 days of incubation with different NaCl concentrations. Color changes in the plants were observed during the assays, especially at the higher NaCl concentration of 150 mmol L<sup>-1</sup>.

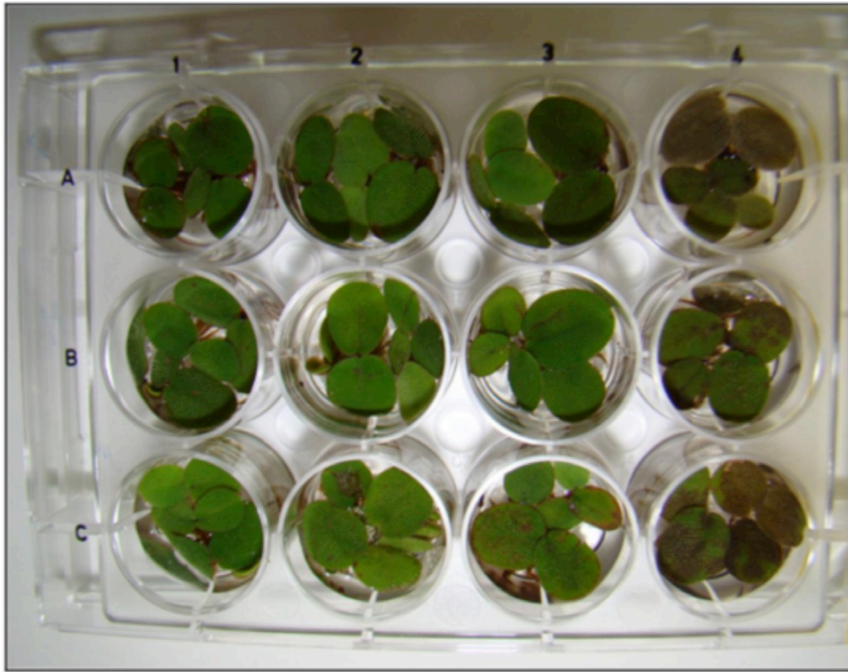
Corroborating the color changes, the different NaCl treatments significantly affected photosynthetic pigment content in *S. auriculata* (Table 1).

Higher chlorophyll *a* content was observed in plants treated with 50 mmol L<sup>-1</sup>, decreasing in plants incubated with higher concentrations (100 and 150 mmol L<sup>-1</sup>). On the other hand, chlorophyll *b* content decreased with increasing NaCl concentrations, and statistically significant differences between controls and plants incubated with 150 mmol L<sup>-1</sup> NaCl were observed (Table 1). The chlorophyll *a* to chlorophyll *b* ratio was higher in plants treated with 50 mmol L<sup>-1</sup> NaCl, with no statistical differences between plants incubated with 100 and 150 mmol L<sup>-1</sup> NaCl. Total chlorophyll content (chlorophyll *a* + chlorophyll *b*) was not statistically different ( $p < 0.05$ ) between treatments. Carotenoid content increased with increasing NaCl concentrations (Table 1), albeit non-significantly. Plant biomass also varied significantly between the NaCl treatments. Plants exposed to 50 mmol L<sup>-1</sup> showed significantly higher FW and DW when compared to the other NaCl concentrations. Loss of biomass occurred in plants submitted to 100 and 150 mmol L<sup>-1</sup> NaCl (Figure 2).

Nutrient content in *S. auriculata* was also affected by the NaCl treatments after 5 days (Table 2). Statistically significant differences between controls and 150 mmol L<sup>-1</sup> NaCl were observed for Ca<sup>+2</sup>, K<sup>+</sup>, Na<sup>+</sup>, Mg<sup>+2</sup> and Cl<sup>-</sup>, as well as

for the Na/K ratio. The nutrients  $\text{Ca}^{+2}$ ,  $\text{K}^{+}$  and  $\text{Mg}^{+2}$  decreased with increasing NaCl concentrations, while N and P showed no significant differences among saline treatments. A significant increase

in the Na/K ratio was observed between controls and plants incubated with  $150 \text{ mmol L}^{-1}$  NaCl, expected due to the increase of  $\text{Na}^{+}$  concentrations in the incubation solutions.  $\text{K}^{+}$  content was the

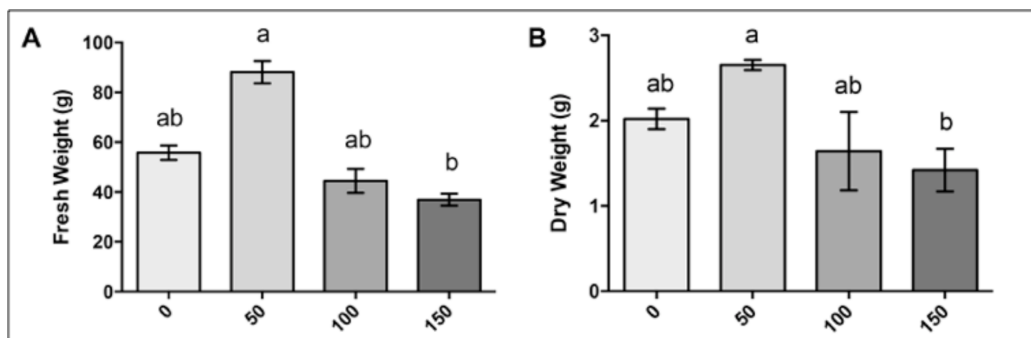


**Figure 1.** Morphological aspects of *S. auriculata* seedlings incubated with 0 (column 1), 50 (column 2), 100 (column 3) and 150 (column 4)  $\text{mmol L}^{-1}$  NaCl in 12-well culture plates, in triplicate (rows A, B and C).

**Table 1.** Photosynthetic pigments analyses in plants of *Salvinia auriculata* incubated during five days with different concentrations of NaCl. Data is displayed as means  $\pm$  standard deviation ( $n=3$ ). All parameters are displayed as  $\text{mg}\cdot\text{g}^{-1}$  DW.

NaCl [ $\text{mmol L}^{-1}$ ]	0	50	100	150
Chlorophyll a	18.17 b $\pm$ 0.9	24.62 a $\pm$ 1.4	21.26 ab $\pm$ 0.6	19.69 ab $\pm$ 0.9
Chlorophyll b	13.38 a $\pm$ 1.7	9.18 ab $\pm$ 1.2	9.18 ab $\pm$ 1.3	8.2 b $\pm$ 0.6
Chlorophyll a/b	1.37 b $\pm$ 0.2	2.68 a $\pm$ 0.2	2.2 ab $\pm$ 0.4	2.51 ab $\pm$ 0.13
Total Chlorophyll	32.77 a $\pm$ 1.3	33.80 a $\pm$ 2.5	29.47 ab $\pm$ 1.2	28.13 a $\pm$ 1.4
Carotenoids	1.83 a $\pm$ 0.4	4.49 ab $\pm$ 0.3	4.63 ab $\pm$ 0.6	5.50 ab $\pm$ 0.3

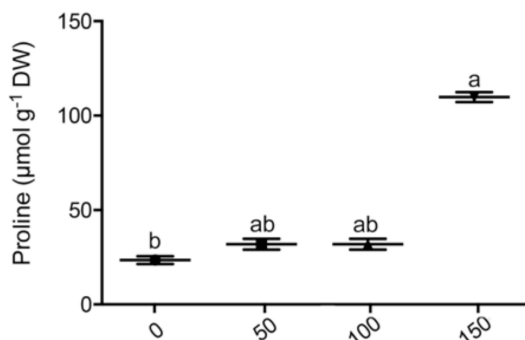
Different letters, for each pigment analysis, indicate statistically significant differences ( $p<0.05$ ) among NaCl treatments by Dunn's test.



**Figure 2.** Fresh weight (A) and dry weight (B) variations of *S. auriculata* incubated in different NaCl concentrations (0, 50, 100 and 150). Different letters indicate statistically significant differences ( $p<0.05$ ) among NaCl treatments by Bonferroni's test. Data is displayed as means  $\pm$  standard deviation ( $n=3$ ).

most affected when compared to the other analyzed nutrients, decreasing significantly with increases in NaCl concentrations.

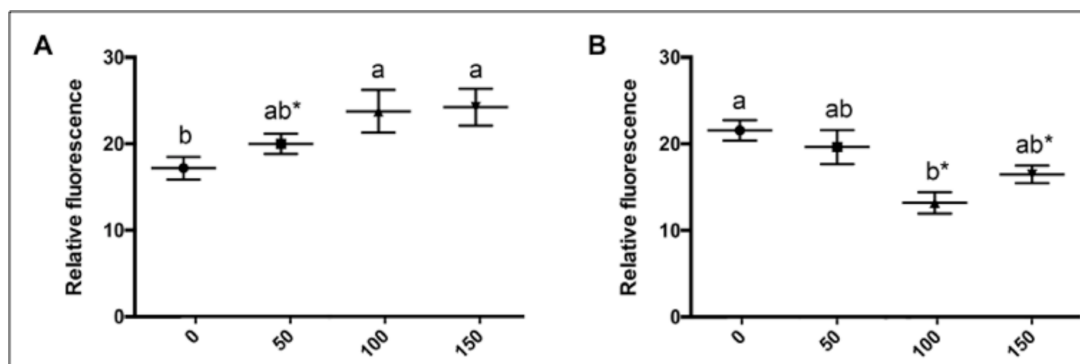
The proline content in *S. auriculata* increased significantly in the 150 mmol L<sup>-1</sup> NaCl treatment compared to controls (Figure 3).



**Figure 3.** Proline content ( $\mu\text{mol.g}^{-1}$  DW) in *S. auriculata* incubated during five days with different NaCl concentrations. The horizontal bars indicate the median values ( $n=3$ ). The vertical bars indicate the minimum and maximum values. Different letters indicate significant differences ( $p<0.05$ ) among NaCl treatments by Dunn's test.

Extracellular NO content increased after 2-hour incubations with NaCl, with significant differences ( $p < 0.05$ ) observed between controls and exposure to 150 mmol L<sup>-1</sup> NaCl (Figure 4A). On the other hand, plants showed decreases in NO releases with increasing NaCl concentrations after 5 days of incubation (Figure 4B), with significant differences observed between controls and plants exposed to 100 mmol L<sup>-1</sup> NaCl.

Intracellular NO results are displayed in Figure 5. After two hours of incubation, significantly higher NO concentrations in *S. auriculata* roots were observed in plants incubated with 50 mmol L<sup>-1</sup> NaCl (Figure 5c and Figure 6A) when compared to the other investigated concentrations (Figure 5). After 5 days, decreases in intracellular NO were observed in roots, with significant differences between the treatments (Figure 5 and Figure 6B). Plants incubated during two hours showed higher induction of NO synthesis (Figure 5 a-h and Figure 6A) when compared to incubation for 5 days (Figure 5i-p and Figure 6B), where lower NO content was observed. Control roots presented significantly lower ( $p<0.05$ ) intracellular NO when compared to the 50 mmol L<sup>-1</sup> NaCl treatment (Figures 5a and 5b, 6A and 6B).

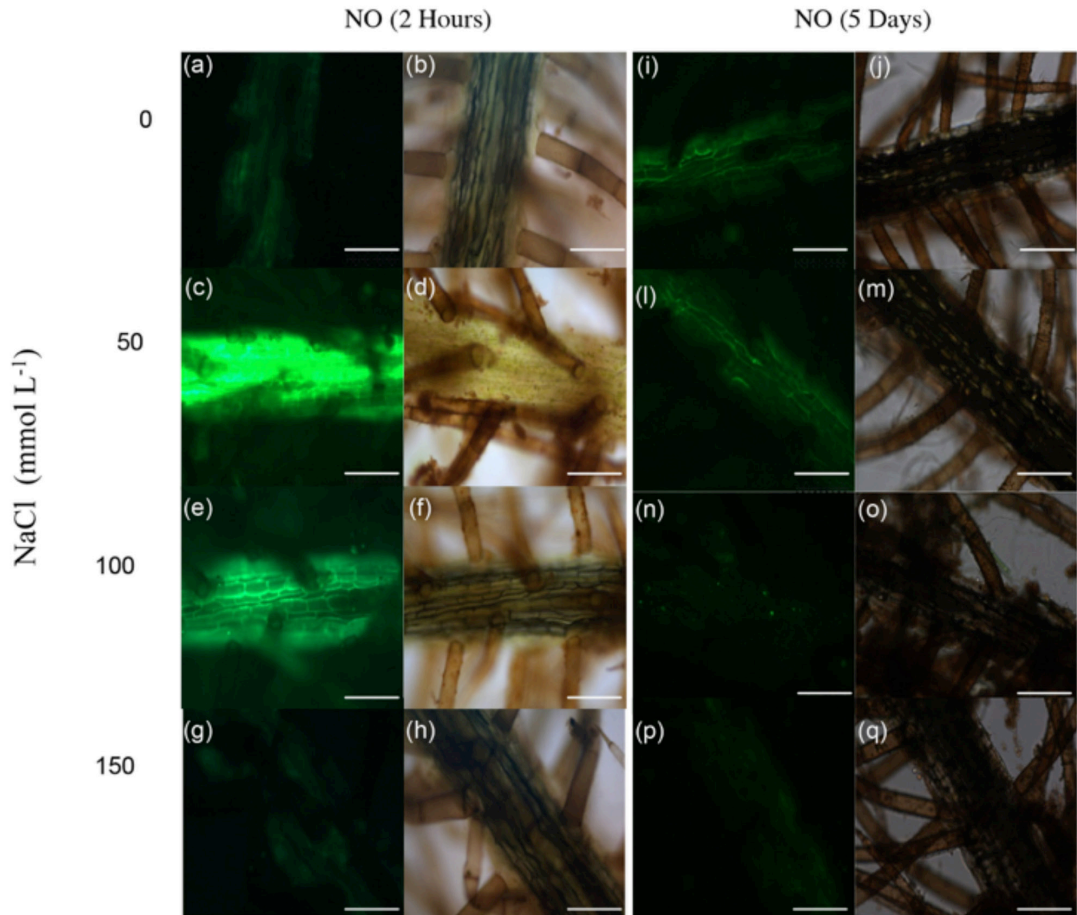


**Figure 4.** Extracellular NO content in plants of *S. auriculata* incubated during two hours (A) and five days (B) with different NaCl concentrations. The horizontal bars indicate the median values and the vertical bars indicate the minimum and maximum values ( $n=3$ ). Different letters indicate significant differences ( $p<0.05$ ) for the NaCl treatments by Bonferroni's test, while asterisks compare significant differences between the two hours and five-day treatments.

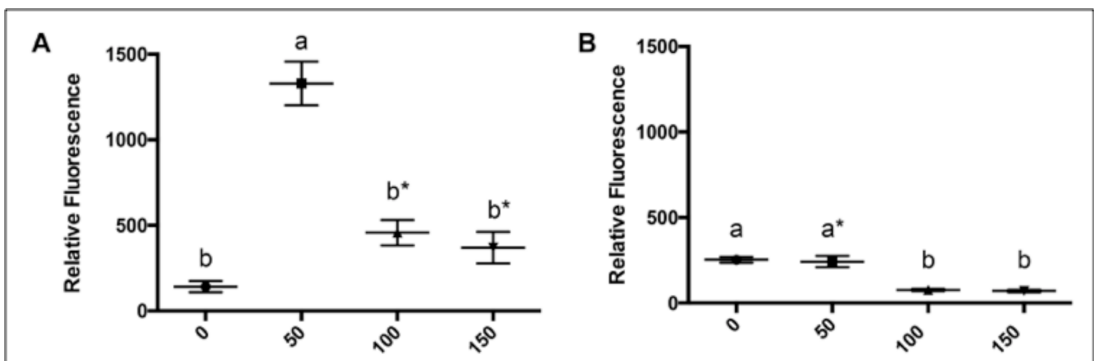
**Table 2.** Nutrient content ( $\text{mg.g}^{-1}$  DW) in *Salvinia auriculata* incubated during five days with different NaCl concentrations. Data is displayed as means  $\pm$  standard deviation ( $n=3$ ).

NaCl [mmol L <sup>-1</sup> ]	P	N	Ca	K	Mg	Na	Na/K	Cl
0	3.84 $\pm$ 0.28 <sup>a</sup>	27.15 $\pm$ 0.46 <sup>a</sup>	11.32 $\pm$ 1.09 <sup>a</sup>	22.41 $\pm$ 0.99 <sup>a</sup>	3.16 $\pm$ 0.46 <sup>a</sup>	6.52 $\pm$ 0.53 <sup>a</sup>	0.29 $\pm$ 0.01 <sup>a</sup>	1.84 $\pm$ 0.22
50	3.56 $\pm$ 0.21 <sup>a</sup>	28.17 $\pm$ 1.18 <sup>a</sup>	7.08 $\pm$ 0.15 <sup>ab</sup>	18.19 $\pm$ 1.37 <sup>ab</sup>	1.73 $\pm$ 0.09 <sup>ab</sup>	26.78 $\pm$ 0.95 <sup>ab</sup>	1.44 $\pm$ 0.14 <sup>ab</sup>	4.14 $\pm$ 0.26
100	3.48 $\pm$ 0.27 <sup>a</sup>	27.91 $\pm$ 0.84 <sup>a</sup>	6.03 $\pm$ 0.39 <sup>ab</sup>	7.92 $\pm$ 0.95 <sup>ab</sup>	1.06 $\pm$ 0.06 <sup>ab</sup>	41.84 $\pm$ 1.90 <sup>ab</sup>	4.81 $\pm$ 0.64 <sup>ab</sup>	4.67 $\pm$ 0.35
150	3.40 $\pm$ 0.29 <sup>a</sup>	27.94 $\pm$ 0.66 <sup>a</sup>	5.05 $\pm$ 0.57 <sup>b</sup>	3.95 $\pm$ 0.41 <sup>b</sup>	0.81 $\pm$ 0.09 <sup>b</sup>	44.33 $\pm$ 0.72 <sup>b</sup>	11.51 $\pm$ 1.12 <sup>b</sup>	5.70 $\pm$ 1.13

Different letters, for each nutrient, indicate statistically significant differences ( $p<0.05$ ) among NaCl treatments by Dunn's test; CV = Coefficient of variation.



**Figure 5.** Intracellular NO contents by fluorescence microscope in roots of *S. auriculata* plants incubated during two hours (a-h) and five days (i-g) in different NaCl concentrations. Bar = 200  $\mu\text{m}$ .



**Figure 6.** Intracellular NO content in *S. auriculata* roots incubated during two hours (A) and five days (B) in different NaCl concentrations. The vertical bars indicate the minimum and maximum values ( $n=3$ ). Different letters indicate significant differences (Two-way ANOVA,  $p<0.05$ ) among NaCl treatments by Bonferroni's test, while asterisks compare significant differences between two hours and five day treatments.

#### 4. Discussion

$\text{Na}^+$  and  $\text{Cl}^-$  are known to cause injuries to plant leaves (Kozłowski, 1997). In the present study, total chlorophyll pigment content decreased with increasing NaCl concentrations, resulting in decreased photosynthetic rate (Table 1). These

results corroborate other studies, that indicate that plants subjected to increased salinity show decreased photosynthetic pigments (Aghaleh et al., 2009; Centritto et al., 2003; Chaves et al., 2009; Jampeetong & Brix, 2009; Netondo et al., 2004). In addition, increases in chlorophyll *a*, carotenoids and the chlorophyll *a*/chlorophyll *b* ratio with

increasing salinity were also observed, whereas chlorophyll *b* content decreased (Table 1). These results also corroborate previous studies indicating that NaCl stress has more effects on chlorophyll *b* than chlorophyll *a* (Houimli et al., 2010). This implies in an increase in the chlorophyll *a*/chlorophyll *b* ratio, since the first step of chlorophyll *b* degradation results in its conversion into chlorophyll *a* (Fang et al., 1998) and therefore, a decrease in total chlorophyll content (Pinheiro et al., 2008). This is also in accordance to other studies, such as those by Upadhyay & Panda (2005), that observed the same regarding *Salvinia molesta* (Mitchell) and *Pistia stratiotes* (Linn) subjected to NaCl salinity. Total chlorophyll content was also higher in plants incubated with 50 mmol L<sup>-1</sup> NaCl compared with the other NaCl treatments, suggesting that this concentration can stimulate *S. auriculata* development and growth. This would suggest that this species would be able to broadly colonized coastal oligohaline wetlands, due to its adaptability to higher salinity conditions via physiological and biochemical changes.

Carotenoids are important pigments that can also act as antioxidants (Edge et al., 1997), protecting plasma membrane lipids from oxidative stress generated in plants exposed to salinity (Falk & Munné-Bosch, 2010). The data from the present study also corroborate with other data reported for other species, such as rice specimens (*Oriza sativa* L.) subjected to salinity also showing increases in carotenoid content (Misra et al., 1997).

A biomass increase was observed in a study with *Phragmites karka* (Retz.) exposed to 100 mmol L<sup>-1</sup> NaCl, with leaf osmotic adjustment conducted primarily by balance of inorganic solutes (K<sup>+</sup> and Na<sup>+</sup>), while soluble sugar and proline content remained unchanged (Abideen et al., 2014). In another study, *Salicornia europaea* growth and net photosynthetic rate were stimulated, rather than inhibited, by 100-400 mmol L<sup>-1</sup> NaCl (Lv et al., 2012). In *S. auriculata*, 50 mmol L<sup>-1</sup> exposure induced higher FW and DW (Figure 1), suggesting that this concentration affects biomass accumulation in this species, while lower biomasses were observed at 100 and 150 mmol L<sup>-1</sup>.

Regarding nutrient content, Na<sup>+</sup> and Cl<sup>-</sup> showed increases, while Ca<sup>2+</sup>, K<sup>+</sup> and Mg<sup>2+</sup> reduced with increasing NaCl concentrations (Table 2). Osmotic stress induced by high salinity (NaCl) has been shown to affect the absorption and translocation of Ca<sup>2+</sup> in plants (Lee & Liu, 1999), as observed herein. Similarly, *Eichhornia crassipes* (Mart.)

and *Pistia stratiotes* (Linn.) maintained under salt stress conditions also showed decreased Ca<sup>2+</sup> content (Niaz & Rasul, 1998) in high salinity conditions. Salinity can also affect K<sup>+</sup> absorption and transport in plants (Shabala & Cuin, 2008; Xu et al., 2010). In the present study, the decreases in K<sup>+</sup> content compared to the controls probably occurred as a consequence of the antagonistic relationship between same-charged ions, as well as the competition between Na<sup>+</sup> and K<sup>+</sup> during nutrient absorption (Niaz & Rasul, 1998; Niu et al., 1995). Consequently, the Na<sup>+</sup>/K<sup>+</sup> ratio also increased with increasing salinity, corroborating other reports (Jampeetong & Brix, 2009). This decrease in K<sup>+</sup> content and increase of the Na<sup>+</sup>/K<sup>+</sup> ratio in the cytosol is characteristic of Na-induced toxicity (Maathuis & Amtmann, 1999). According to Esteves & Suzuki (2008), excess salinity also reduces Mg<sup>2+</sup> absorption by plants, corroborated herein. Decreases in Mg<sup>2+</sup> content have also been described in other species, such as *E. crassipes* and *P. stratiotes* (Niaz & Rasul, 1998), *Hydrilla verticillata* (Rout & Shaw, 2001), *Typha domingensis* (Esteves & Suzuki, 2008) and *S. natans* (Jampeetong & Brix, 2009) subjected to different salinity concentrations.

A slight decrease in P content was also observed, although with no significant difference between salinity treatments (Table 2). This suggests that salinity stress in this species may be associated with decreased P content, although the mechanism by which NaCl influences P absorption is not fully elucidated (Silva et al., 2008) and further NaCl exposure assays are warranted.

Ammonia showed a small progressive increase with increasing of NaCl concentrations, although no significant differences were observed. Decrease in nitrogen uptake in roots have been attributed to the ionic antagonism between NH<sub>4</sub><sup>+</sup> and Na<sup>+</sup> (Naidoo, 1987) or Cl<sup>-</sup> and NO<sub>3</sub><sup>-</sup> (Parida & Das, 2004). On the other hand, Jampeetong & Brix (2009) observed an increase in N content in the leaves and a decrease in the roots of *S. natans* at concentrations of 50, 100 and 150 mmol L<sup>-1</sup> NaCl.

Several studies have shown proline synthesis and accumulation in response to salt stress (Ahmad et al., 2012; Cheng et al., 2013; Singh et al., 2015). This compound acts in the protection of cellular components by dehydration, maintaining the membrane structure and acting as a free radical scavenger (Hasegawa et al., 2000). Proline synthesis and accumulation was corroborated herein, since proline content in *S. auriculata* increased significantly with salinity. This has also been observed for *S. natans* (Jampeetong & Brix, 2009).



NO is a free, gaseous, lipophilic compound with high power diffusion through membranes (Crawford, 2006). It is a versatile cell flag that plays important roles in physiological processes in animals and plants, acting against oxidative stress (Lamotte et al., 2005). Studies show that NO can prevent the oxidative damage caused by salt stress on leaves (Haihua et al., 2002) and root rot (Shi et al., 2007). In our study, the NaCl treatments affected the levels of intra and extracellular NO, suggesting that salinity stimulates the release of NO from *S. auriculata* roots, decreasing endogenous NO levels (Figure 6) at the higher NaCl concentrations.

Salt stress has been shown to induce a rapid increase in NO levels in plants soon after salt exposure, since this compound is involved in salt tolerance (Zhang et al., 2006; Zhao et al., 2004, 2007). In the present study, 50 mmol L<sup>-1</sup> NaCl in *S. auriculata* seems to be a moderate concentration, allowing for increases in NO concentrations after 2 hours. This, in turn, can be related to activation of physiological adjustments, such as biomass (Figure 2) and chlorophyll *a* and carotenoid content (Table 1), enabling the plants to grow in saline conditions and tolerate the salt stress.

According to Beligni & Lamattina (2001), protection of chlorophyll mediated by NO may be due to NO ability to eliminate reactive oxygen species produced in plants under stress. Thus, it can be suggested that the higher endogenous NO after two hours of incubation with 50 mmol L<sup>-1</sup> NaCl observed in the present study may have acted as a signal, stimulating the preservation/biosynthesis of photosynthetic pigments through reduction of reactive oxygen species after five days. In comparison, higher NaCl concentrations, lead to plant chlorosis after 5 days. This suggests that after five days NO content is not due to an induced signal caused by salt stress, but a morphological and physiological response of the plant to stress conditions, including yellowing and bronze staining with increasing NaCl exposure (Figure 1). These results emphasize the importance of analyzing NO content in roots just after salinity exposure (i.e. 2 hours), to identify salinity effects on NO signaling during early stress induction compared to the end of salinity stress.

Studies have shown that NO is also associated with leaf expansion in several species. The increases observed in *S. auriculata* FW and DW associated to higher NO content have been demonstrated in other studies, resulting in leaf expansion, for example (An et al., 2005; Beligni & Lamattina, 2001; Neill et al., 2008).

Regarding NO diffusion, NO can also diffuse into the cell from production sites, such as the mitochondria, to other regions of the cell, which can then produce an effect by interaction with target proteins (Freschi, 2013). It is also possible that NO can diffuse out of the cell through the plasma membrane to adjacent cells to stimulate its effect (Neill et al., 2008). In the present study, higher concentrations of NO in the extracellular medium were observed (Figure 5a) when compared to the intracellular environment (Figure 6A) after two hours of incubation. These results suggest that there may have been transport from the intracellular to extracellular medium in the higher NaCl treatments (100 and 150 mmol L<sup>-1</sup>) after two hours of incubation. Equilibrium between the production and maintenance of NO within the cell is an important factor for cellular signaling in *S. auriculata*, and herein, the best adaptation was observed in plants exposed to 50 mmol L<sup>-1</sup> NaCl.

High salt concentrations can also induce damage in the structure of the plasma membrane of cells through changes in fundamental constituents, such as Ca<sup>+</sup>, as discussed previously (Schapire et al., 2008). This element plays an essential role in the processes that preserve the structural and functional integrity of the membranes and regulate transport and ion selectivity in plants (Shoresh et al., 2011). In this sense, changes in the structure of the plasma membranes induced in higher concentrations of NaCl may also have contributed to the increased extracellular release of NO in *S. auriculata* after two hours of incubation. Studies carried out with the addition of an exogenous NO donor, demonstrated that NO may increase plant tolerance to salinity by increasing DW, reducing oxidative damage, and maintaining a high Na<sup>+</sup>/K<sup>+</sup> ratio in the cytoplasm (Shi et al., 2007; Zhang et al., 2006). Thus, it has been suggested that NO acts as a signal for salt tolerance, causing increased Na<sup>+</sup> secretion (Chen et al., 2010).

## 5. Conclusions

At 50 mmol L<sup>-1</sup> NaCl exposure, *S. auriculata* induced NO production early in the stress process, which is probably associated to signaling to produce compounds that assist in stress tolerance, whereas at 150 mmol L<sup>-1</sup> this tolerance is reduced. Thus, it seems that *S. auriculata* can develop very well in environments with moderate salinity (50 mmol L<sup>-1</sup>) such as oligohaline environments, as is the case of the study area, that can show excess brackish water and increased salt water influx due to the

implementation of artificial sandbars in the region. This, in turn, may directly influence this species distribution in the coastal areas of Northern Rio de Janeiro. Thus, these results are fundamental in understanding the physiological and biochemical mechanisms associated with the adaptation of this macrophyte to saline conditions, which, in turn, affect this species ecology.

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