Acta Botanica Brasilica - 31(4): 555-565. October-December 2017.
doi: 10.1590/0102-33062017abb0059

Effects of salinity on the physiology of the red macroalga, *Acanthophora spicifera* (Rhodophyta, Ceramiales)

Débora Tomazi Pereira[1*], Carmen Simioni[1], Elisa Poltronieri Filipin[1], Fernanda Bouvie[1], Fernanda Ramlov[2], Marcelo Maraschin[2], Zenilda Laurita Bouzon[1] and Éder Carlos Schmidt[1]

Received: February 15, 2017
Accepted: May 4, 2017

ABSTRACT

Salinity is an important abiotic factor since it is responsible for the local and/or regional distribution of algae. In coastal regions, salinity changes with prevailing winds, precipitation and tide, and particularly in extreme intertidal conditions. *Acanthophora spicifera* is a red seaweed that occurs in the supratidal region in which changes in abiotic conditions occur frequently. This study evaluated the effects of salinity on the metabolism and morphology of *A. spicifera*. Algae were acclimatized under culture conditions with sterilized seawater for seven days. Experiments used different salinities (15 to 50 psu) for seven days, followed by metabolic analyses. This study demonstrates that extreme salinities affect physiological parameters of *A. spicifera*, such as decrease in growth rate, as well as morphological parameters and concentrations of secondary metabolites. *Acanthophora spicifera* exhibited high tolerance to 25 to 40 psu, with little change in physiology, which favors the occurrence of this species in diverse environments. However, 15, 20, 45 and 50 psu were the most damaging and led to loss of biomass, depigmentation of apices, and the highest concentrations of antioxidant metabolites. The 50 psu treatment caused the greatest changes in general, greatly reducing a biomass and chlorophyll content, and facilitating the presence of endophytes.

Keywords: *Acanthophora spicifera*, microscopy, morphology, physiology, salinity

Introduction

Many abiotic factors are important to aquatic ecosystems, including, for example, temperature, irradiance, nutrients, pH and salinity. Each factor can uniquely affect the metabolism of organisms. Salinity is particularly important, especially for algae, since it is responsible for local and/or regional distribution (Yarish & Kirkman 1990; Nejrup *et al*. 2012). Salinity tends to be a fairly stable abiotic factor in oceanic waters, but in the coastal region where red algae are found, this factor changes with prevailing winds, precipitation, and tides (Lartigue *et al*. 2003; Nejrup *et al*. 2012)we investigate the impact of rapid fluctuations in salinity on short-term net oxygen production and ammonium (NH4+. Since salinity affects osmotic adjustment and turgor pressure regulation, it is an important abiotic factor in the marine environment for the maintenance and survival of algae (Kirst 1990).

According to the IPCC (2012), rainfall is expected to become very irregular in South America. Increased rainfall is expected in southeastern and southern Brazil, Paraguay, Uruguay, Argentina and some regions of Bolivia (Scherner *et al*. 2013), leading to a change in the abiotic factors of aquatic ecosystems, including salinity. Fluctuations in salinity can change the density of water, nutrient uptake, and osmotic

[1] Laboratório de Biologia Celular Vegetal, Departamento de Biologia Celular, Embriologia e Genética, Universidade Federal de Santa Catarina, 88049-900, P. O. Box 476, Florianópolis, SC, Brazil
[2] Laboratório de Morfogênese Vegetal, Departamento de Fitotecnia, Universidade Federal de Santa Catarina, 88049-900, P. O. Box 476, Florianópolis, SC, Brazil

[*] Corresponding author: de.tomazi@gmail.com

pressure in plant cells (Lobban *et al*. 1994; Fong *et al*. 1996), leading to significant physiological and biochemical stress for algae, such as changes in growth rate, photosynthetic performance, morphology, and germination in the green microalga *Nannochloropsis salina* (Bartley *et al*. 2013) and thalli of the red algae *Pterocladiella capillacea* (Felix *et al*. 2014), *Stylonema alsidii* (Stylonematophyceae) (Nitschke *et al*. 2014), and *Kappaphycus alvarezii* (Mandal *et al*. 2015). For red algae spores treated with different salinity, the development of the *Gelidium floridanum* was inhibited under hyposaline conditions, but only delayed under hypersaline conditions (Filipin *et al*. 2016). However, studies of thalli and spores have generally yielded little information about the response of secondary metabolites relative to their antioxidant activity when salinity changes.

Acanthophora spicifera is a red seaweed found in tropical and subtropical regions (Kilar & Mclachlan 1986). It is native to the Caribbean and Florida (Horn 2012), and it is exotic in Brazil, occurring from Maranhão State to Rio Grande do Sul (Fig. 1A). This species occurs in the supratidal region where changes in abiotic factors are constant. Little information about the phenotypic plasticity of this alga is available, but in Florianópolis, Santa Catarina, Brazil (Fig. 1A), it is found in two distinct environments: Conceição Lagoon and Sambaqui Beach. Conceição Lagoon (27°34'S; 48°27'W) is a small coastal lagoon located in the easternmost central part of Santa Catarina, connecting with the sea through a channel. It receives drainage of small freshwater flows and the Capivara River to the north, with consolidated substrate, and it is the biggest lagoon in the region (Souza-Mosimann *et al*. 2011). Sambaqui Beach (27°29'S; 48°32'W) is located in the north bay of Florianópolis. It is part of the channel between the island and the mainland. Analyses performed in our laboratory indicate that Conceição Lagoon waters possesses on average 1.06 ± 0.27 µM NH_4^+ (ammonia), 8.47 ± 0.01 µM NO_3^- (nitrate), 0.17 ± 0.01 µM PO_4^{-3} (phosphate), and 25 practical salinity unit (psu). For Sambaqui Beach, these values were 1.13 ± 0.05 µM NH_4^+, 3.73 ± 0.01 µM NO_3^-, 0.52 ± 0.01 µM PO_4^{-3}, and 30 psu. However, measurements carried out by our research group show that the salinity at this site reached up to 37 psu, in summer. This indicates that this species can survive in quite different conditions, taking into account salinity and soluble nutrients.

Acanthophora spicifera presents a red-brownish or greenish color, grows in tufts of 8 to 10 cm and has branched erect branches alternately and abundantly (Fig. 1B) (Cordeiro-Marino 1978). These branches are covered by numerous short papillose branches, which have 4 to 5 short spinescent tips (Cordeiro-Marino 1978) (Fig. 1B). *A. spicifera*

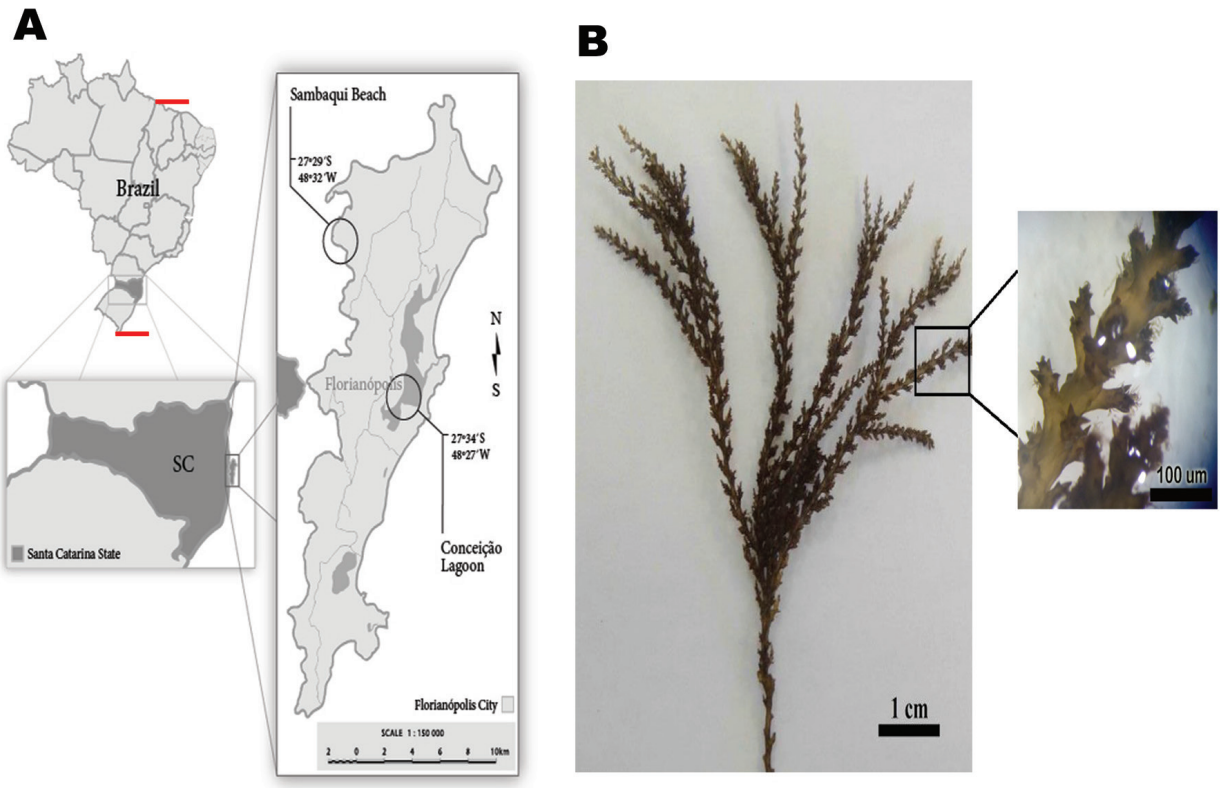

Figure 1. A. Map with the occurrence of *A. spicifera,* from Maranhão State (red line) to Rio Grande do Sul (red line), and the location of both sites on Santa Catarina Island where the seaweed occurs: Conceição Lagoon and Sambaqui Beach. **B.** Macroalga *A. spicifera* collected at Sambaqui Beach with red-brownish color, typical of the species. Stipe branched erect alternating and abundant and covered by numerous short papillose branches, which have four to five short spinescent tips (magnifying).

has a three-phase and isomorphic life cycle. The sexed phase is followed by two asexual generations that produce spores, termed carposporophytic and tetrasporophytic generations (Cordeiro-Marino 1978). Several compounds of interest have been identified in *A. spicifera*, including flavonoids with antibacterial (Seenivasan *et al*. 2012), antitumor (Lavakumar *et al*. 2012), procoagulant and antioxidant activities (Zeng *et al*. 2001). Phenolic compounds with antiproliferative (Murugan & Iyer 2014) and antioxidant activities were also identified (Ganesan *et al*. 2008). This species is also used as a biofilter (Fialho 2013) and as a foodstuff in the human diet (Lang 2007; Zakaria *et al*. 2011). It is also a food source for fish, crabs and green turtles (*Chelonia mydas*) (Lang 2007). *A. spicifera* is also a producer of agarana (Duarte *et al*. 2004), a sulphated polysaccharide of great economic value.

Therefore, this work aimed to determine the effect of salinity on the growth rate, concentration of photosynthetic pigments, as well as structure and concentration of secondary metabolites, phycobiliproteins, carotenoids, phenolics and flavonoids of the macroalga *A. spicifera*. It is anticipated that these data will improve our understanding of the present distribution of this species and allow projection of its future distribution.

Materials and methods

Collection sites, seawater and algal material

The experiments were performed with seaweeds collected at Sambaqui Beach, Florianópolis (Santa Catarina Island, Brazil). Sambaqui Beach (27°29'S; 48°32'W) is located in the north bay of Florianópolis. It is part of the channel between the island and the mainland. Tetrasporophytic samples of *Acanthophora spicifera* (M. Vahl) Børgesen (80 g of total thallus) attached to rocks were collected from Sambaqui Beach in December 2014 during the Southern Hemisphere summer season. Collection was made in the intertidal zone during low tide in the morning. The specimens were transported at ambient temperature in dark containers to the Laboratório de Biologia Celular Vegetal (Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, Brazil, and macroepiphytes were meticulously eliminated by cleaning with a brush and washing with filtered seawater. Thalli were maintained in culture medium with sterilized seawater from the site containing von Stosch medium with $MnCl_2$, EDTA, $NaHPO_4$, $FeSO_4$, biotin, cyanocobalamin, thiamine and nitrate (Edwards 1972) and cultivated under laboratory-controlled conditions, including 24 ± 2 °C, continuous aeration, 70 ± 10 µmol photons $m^{-2}s^{-1}$ (fluorescent lamps, Philips C-5 Super 84 16W/840), and 12 h photocycle (from 08:00 to 20:00) during seven days for acclimation before experimental treatments. The sterilized seawater was renewed every two days. PAR was measured with a Solar Light PMA 2100 quantameter (Solar Light Co., Glenside, PA, USA) and a Solar Light 2132 spherical sensor (Solar Light Co.).

Experimental setup

After acclimation, thalli (± 4.0 g fresh weight (FW) ± 8 individuals per beaker) of *A. spicifera* were cultivated for seven days in beakers containing 500 mL of respective sterilized seawater. Culture conditions were the same as those described for the acclimation period. Low salinities were obtained by addition of distilled water, while high salinities were attained through gradual freezing and thawing of seawater until the final concentration was reached. Four replicates were used for each experimental group (15, 20, 25, 30, 35, 40, 45 and 50 psu). The reference value was 30 psu because this is the salinity observed in the seawater of Sambaqui Beach. Culture conditions were the same as those described for the acclimation period. At the end of the experiments, some of the algae were photographed for analysis of external morphology, and the rest was frozen for cytological and metabolic analysis.

Morphological features

At the end of the experiments, the algae were photographed with a digital camera (Sony Dsc W-620 14.1 Megapixels) for analysis of external morphology.

Growth rate

Growth rates (n = 4) were calculated using the following equation: Growth rates [% day^{-1}] = $[(W_t/W_i)^{1/t} - 1] \times 100$, where W_i = initial fresh weight (FW), W_t = fresh weight after seven days, and t = experimental time in days (Penniman *et al*. 1986).

Photosynthetic pigments

At the end of the experimental period, fresh samples were frozen by immersion in liquid nitrogen and kept at -80 °C until ready for use. All pigments were extracted in quadruplicate for determination of chlorophyll *a* and phycobiliprotein contents.

Chlorophyll *a* was extracted from approximately 1 g dry weight (DW) with 3 mL DMSO at 40 °C for 30 min, using a glass tissue homogenizer (Hiscox & Israelstam 1979; Schmidt *et al.* 2010). The homogenates were centrifuged at 2000 x *g* for 20 min, and pigments were quantified from the supernatant in a spectrophotometer (Hitachi, Model 100-20; Hitachi Co., Japan) at 630, 647 and 664 nm. Chlorophyll *a* concentrations were calculated according to Jeffrey & Humphrey (1975).

Phycobiliproteins were extracted from about 1 g FW ground to a powder with liquid nitrogen and 3 mL 0.05 M phosphate buffer, pH 6.4, at 4 °C in darkness. The

homogenates were centrifuged at 2,000 *g* for 20 min. Levels of allophycocyanin (APC), phycocyanin (PC), and phycoerythrin (PE) were determined by spectrophotometry (Hitachi, Model 100-20; Hitachi Co., Japan) at 498, 615 and 651 nm, and concentrations were calculated using the equations of Kursar & Alberte (1983).

The extraction of carotenoids was performed according to Aman *et al*. (2005). Carotenoids were extracted from samples (1.0 g FW, n=4) using methanol 80 %. After maceration, the samples were kept at rest (1 h) in a darkroom. The recovered extract was centrifuged at 2000 x *g* for 10 min, and total carotenoids were quantified from the supernatant in a spectrophotometer (Hitachi, Model 100-20; Hitachi Co., Japan) at 450 nm.

Total phenolic and flavonoid compounds

Analyses of phenolic compounds were performed using the spectrophotometric method of Folin-Ciocalteau based on Popova *et al*. (2007). Polyphenolics were extracted from frozen samples of 1.0 g DW (n = 4) by using methanol 80 % maintained in a darkroom for 1 h. In a test tube, 300 mL of the extract, 225 mL of Folin, and 2.5 mL of sodium carbonate 2 % were added, followed by incubation at room temperature for 1 h. Absorbance of the reaction mixture was measured at 750 nm by spectrophotometry (Hitachi, Model 100-20; Hitachi Co., Japan). Quantification of the total phenolic compounds was made from the curve standard of gallic acid (50 to 1250 μg dg.mL^{-1} - r^2 = 0.99, y = 0.0106x).

The same extract with methanol 80 % was used to determine flavonoid compounds. Total flavonoid content was determined by the aluminum chloride colorimetric method (Zacarias *et al*. 2007). Briefly, an aliquot of 0.5 mL of extracts was added to 2.5 mL of ethanol and 0.5 mL of 2 % aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$). After incubation at room temperature for 1 h, absorbance of the reaction mixture was measured at 420 nm by spectrophotometry (Hitachi, Model 100-20; Hitachi Co., Japan). The quantification of flavonoids was done from the curve of standard quercetin (700 to 2500 μg.mL^{-1} - r^2 = 0.99, y = 0.0108x).

Light microscopy (LM) and cytochemistry

Thalli samples approximately 5 mm in length were fixed in 2.5 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight following the description in Schmidt *et al*. (2009). Subsequently, the samples were dehydrated in increasing series of aqueous ethanol solutions and infiltrated with Historesin (Leica Historesin, Heidelberg, Germany). Then, sections 5 μm in length were stained with 0.5 % Toluidine Blue (TB-O), pH 3.0 (Merck Darmstadt, Germany), and used to detect acid polysaccharides through a metachromatic reaction (Gordon & McCandless 1973), while Periodic Acid-Schiff (PAS) was used to identify neutral polysaccharides (Gahan 1984). The samples were investigated with an Epifluorescent microscope (Olympus BX 41, Tokyo, Japan) equipped with Image Q Capture Pro 5.1 software (QImaging Corporation, Austin, TX, USA).

Statistical analyses

The data passed the Shapiro-Wilk normality test, and all samples were within normal range. Afterwards, the data were analyzed by unifactorial Analysis of Variance (ANOVA) and Tukey *a posteriori* test. All samples were compared, using salinity as the independent factor and using a cutoff of $p \leq 0.05$. Different letters indicate significant differences according to ANOVA and Tukey's test ($p \leq 0.05$). The statistical analyses were performed using the Statistica software package (Release 10.0).

Results

Morphological features and growth rates

After seven days of exposure to different salinities, *A. spicifera* presented changes in external morphology. After treatment of 15 psu, the seaweed presented no significant changes, maintaining a greenish brown stipe typical of the species (Fig. 2A). With 20 psu, a depigmentation rendered the stalk yellowish with few branches (Fig. 2B). Treatment with 25 psu led to a slightly depigmented stalk with some new branches (Fig. 2C). Results of the 30 psu treatment were similar to those with 20 psu, leaving a yellowish stalk, but large, new branches (Fig. 2D). After treatments of 35 and 40 psu, the stalks were depigmented with some new branches (Fig. 2E, F). At 45 and 50 psu, no new branches were observed, and the stipe had slight depigmentation (Fig. 2G, H). Thus, at salinities of 20, 25, 30, 35, and 40 psu, external morphological responses were nearly identical, including the presence of new branches and depigmented stipe with a yellowish coloration. On the other hand, in salinities of 15, 45 and 50 psu, the responses showed a slight depigmentation of the stalk and no growth of new branches. All treatments presented a depigmentation of the stipe, but this was already expected because the intensity of PAR in the culture room was lower than that found in the field. The data took this into account, and did not confound the treatment results.

The treatments were significantly different (Fig. 3), with positive growth rates for treatments of 15 to 35 psu and negative growth rates for 40 to 50 psu. Significantly higher growth rate was observed in the sample exposed to 25 psu, with a growth of 3.60 % per day, while the lowest growth rate, statistically, was found in the sample exposed to 50 psu, with a growth rate of 0.36 % per day.

Photosynthetic pigments

Quantification of photosynthetic pigments is

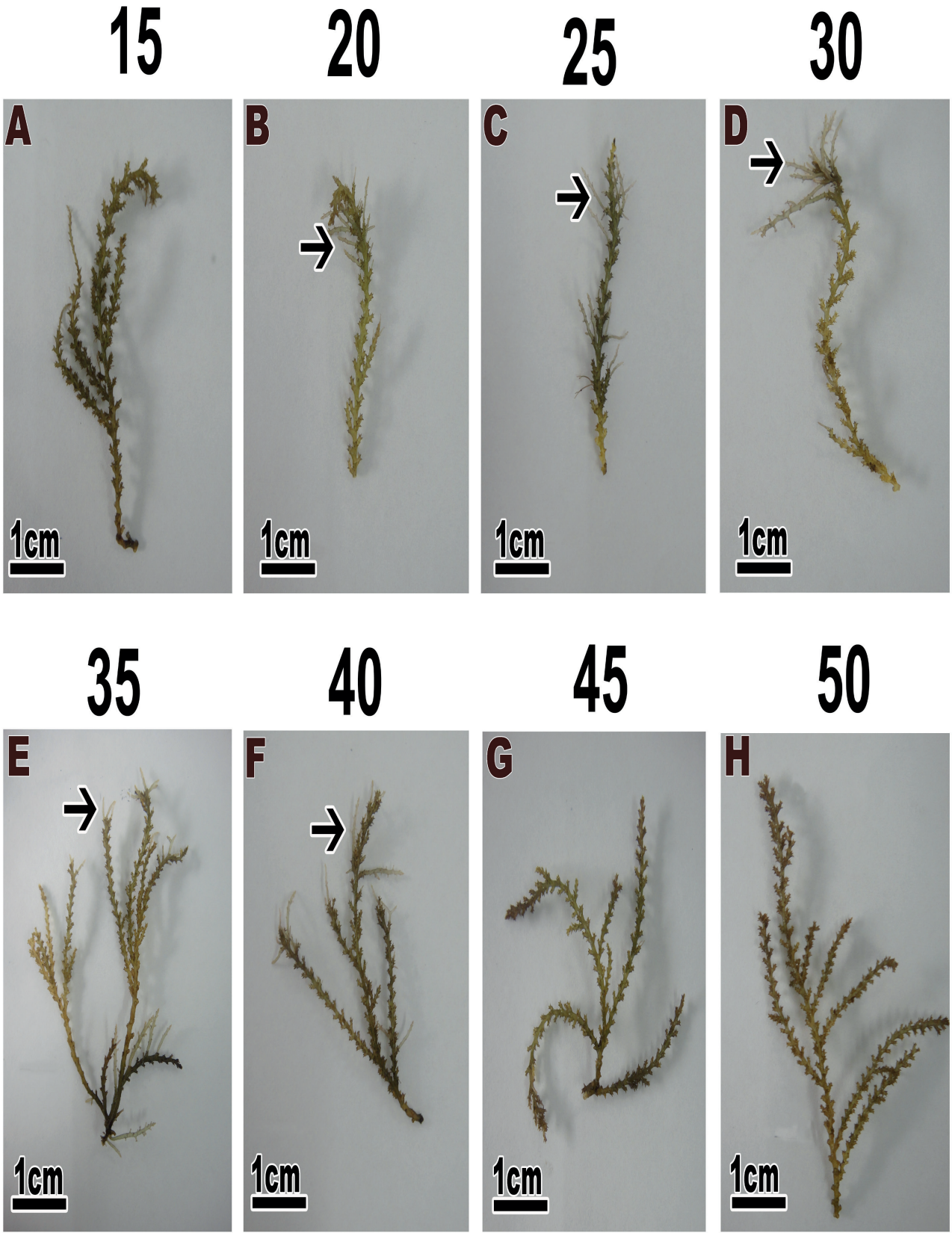

Figure 2. Morphological features of *A. spicifera* treated with 15, 20, 25, 30, 35, 40, 45 and 50 psu. A, G, H: Note that responses showed a slight depigmentation of the stalk, when compared with the seaweed in the field (Fig. 1), and no growth of new branches. B-F: Observe the presence of new branches (arrows) and depigmented stipe with a yellowish color. Please see the PDF version for color reference.

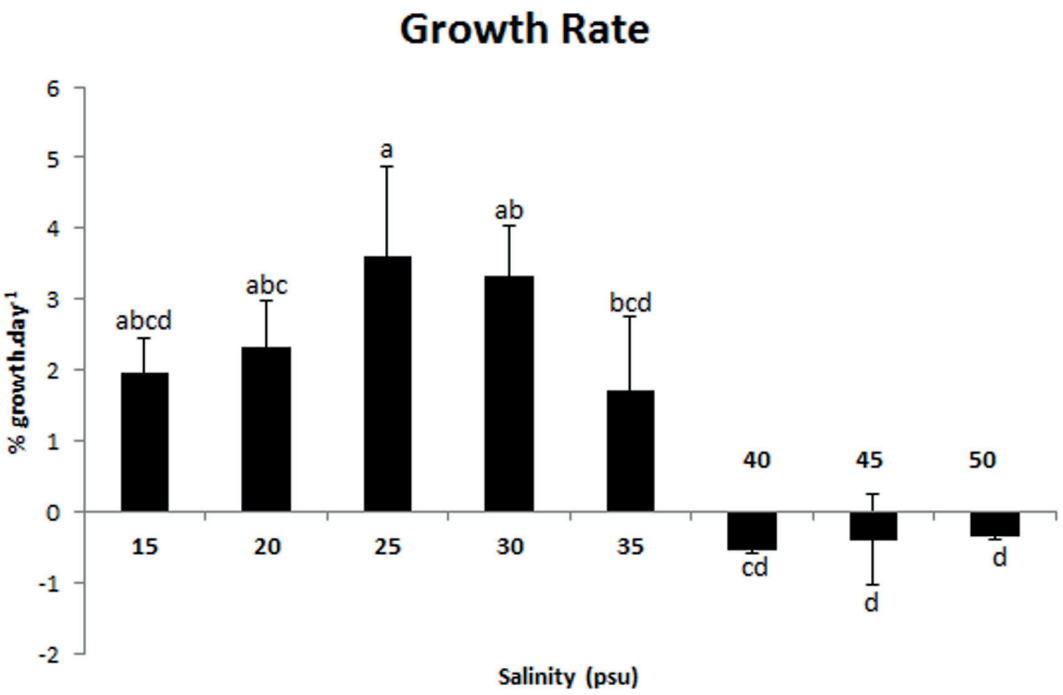

Figure 3. Growth rates (% day^{-1}) of *A. spicifera* exposed to 15, 20, 25, 30, 35, 40, 45 and 50 psu for a period of seven days (n = 4; mean ± SD). Different letters indicate significant differences according to unifactorial analysis of variance and Tukey's test ($p \leq 0.05$).

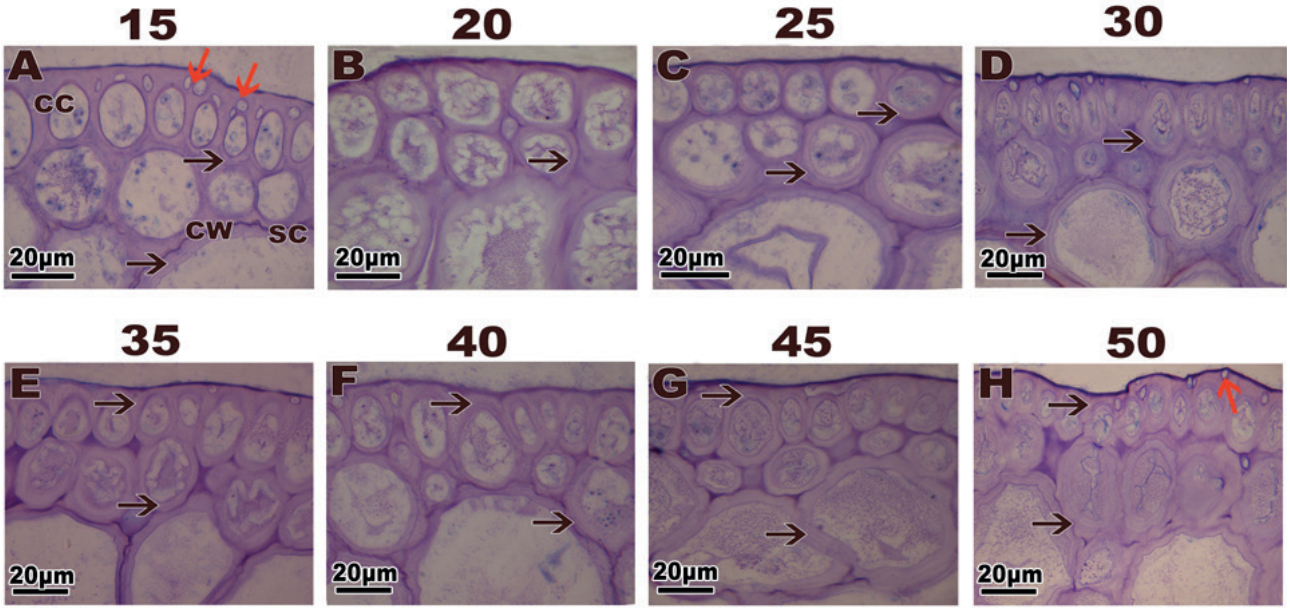

Figure 4. Light microscopy of transversal sections of *A. spicifera* exposed to 15, 20, 25, 30, 35, 40, 45 and 50 psu for a period of seven days and stained with TB-O. Note the metachromatic reaction of cell wall (CW), cortical cells (CC) and subcortical cells (SC). Note that CC and SC showed a reduction in cell volume and an increase in CW thickness in all salinities (black arrows), while greater thickening was observed in the 45 and 50 psu samples. Note the presence of endophytes (red arrows) in 15 and 50 psu samples. Please see the PDF version for color reference.

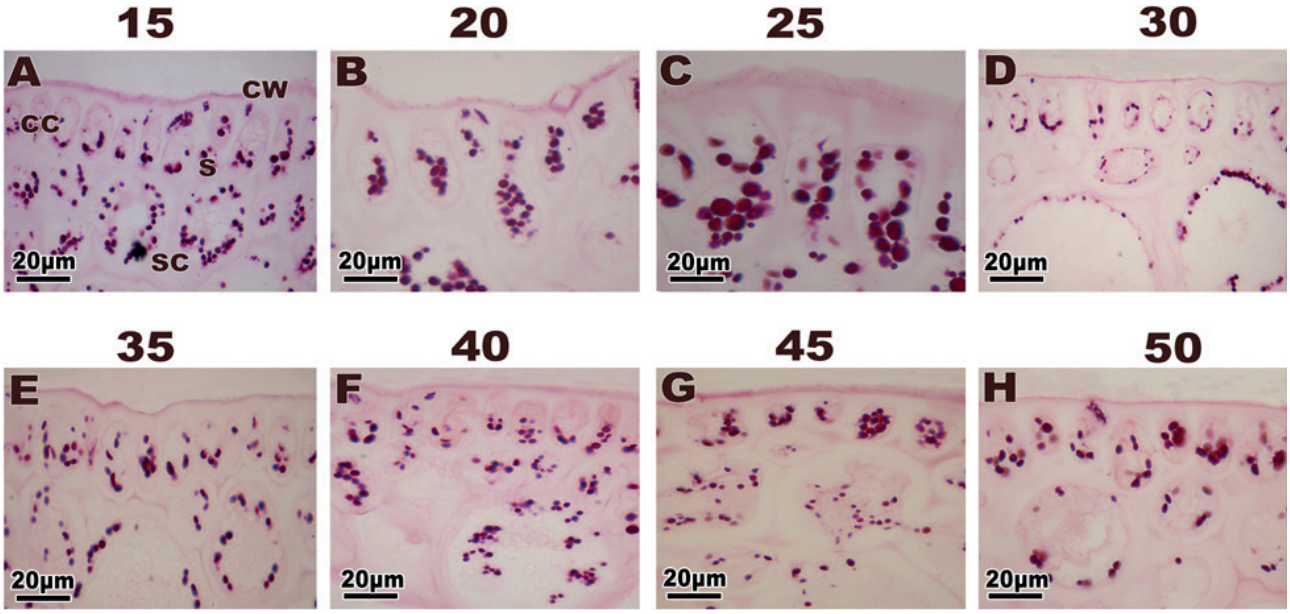

Figure 5. Light microscopy of transversal sections of *A. spicifera* exposed to 15, 20, 25, 30, 35, 40, 45 and 50 psu for a period of 7 days and stained with PAS. Note the reaction with the cellulose compounds present in the cell wall (CW) and the presence the starch grains (S). Note the large amount of starch observed in 15, 20 and 25 psu samples. In contrast, the amount of starch grains in the 30 psu sample was very small, while the 35, 40, 45 and 50 psu samples presented a larger amount of starch grains when compared to the sample treated with 30 psu.

summarized in Table 1. After seven days of experimentation, the concentration of chlorophyll *a* presented significant differences (Tab. 1). The 40 psu sample had the highest concentration of chlorophyll *a*, with a mean of 73.54 ug/g dry mass, while the 50 psu sample showed the lowest concentration of the pigment, with a mean of 56.07 ug/g dry mass. Samples of 15, 25 and 35 psu were statistically the same as those with higher pigment concentrations, followed by samples of 40 psu with a decrease of 3.28 %, 6.08 % and 4.97 %, respectively, when compared to the 40 psu sample.

For APC, the highest concentration was observed for the sample treated with 15 psu, and the lowest concentration was observed in the 30 psu sample with 93.84 and 31.50 ug/g dry mass, respectively. The highest concentrations of PC and PE were found in the 20 psu samples with 110.15 and 206.94 ug/g dry mass, respectively. The lowest concentrations were observed in the 30 psu samples with a concentration of 57.54 and 128.01 ug/g dry mass, respectively.

The highest concentration of total carotenoids was observed in the sample treated with 50 psu with a concentration of 379.09 ug/g dry mass. The lowest concentration was observed in the 30 psu sample with 245.38 ug/g dry mass. For all other samples, concentrations of APC, PC, PE, as well as total carotenoids, were close to the highest concentration of the analyzed pigment, which demonstrates a large decline in the concentration of these pigments in the sample treated with 30 psu.

Phenolic and flavonoid compounds

The concentration of total phenolics and flavonoids was significantly affected by salinity (Tab. 2). The highest concentrations of phenolics and flavonoids were observed in the 50 psu sample, with a concentration of 4.57 and 2.01 ug/g of dry weight, respectively. On the other hand, the lowest concentrations of these secondary metabolites were observed in samples treated with 30 psu at 3.07 and 1.35 µg/g of dry weight, respectively.

LM observations and cytochemistry

Samples of *A. spicifera* treated with 15, 20, 25, 30, 35, 40, 45 and 50 psu and stained with Toluidine Blue (TB-O) showed a metachromatic reaction in the cell walls (CW), indicating the presence of sulfated acidic polysaccharides, such as sulfated agarans (Fig. 4A-H). The 15 psu sample presented a medium thickening of the cell wall (black arrows) in both cortical (CC) and subcortical (SC) cells, with endophytes (red arrows) (Fig. 4A). Samples treated with 20 to 40 psu also showed a thickening of the cell wall in CCs and SCs (arrows), but no endophytes were present (Fig. 4B-F). However, the samples exposed at 45 and 50 psu presented very thick cell walls when compared to all other samples (Fig. 4G, H). Especially, in the 50 psu sample, a significant disorganization in the format of the

Table 1. Contents of photosynthetic pigments (ug/g dry weight) of chlorophyll *a* (Chl *a*), phycobiliproteins (APC: allophycocyanin, PC: phycocyanin, PE: phycoerythrin) and total carotenoids of *A. spicifera* exposed to 15, 20, 25, 30, 35, 40, 45 and 50 psu for a period of seven days (n = 4; mean ± SD). Different letters indicate significant differences according to bifactorial analysis of variance and Tukey's test ($p \leq 0.05$).

Salinity (psu)	Chl a	APC	PC	PE	Total Carotenoids
15	71.13 ± 0.72^{b}	93.84 ± 0.89^{a}	97.20 ± 0.24^{b}	190.84 ± 0.71^{b}	287.93 ± 16.44^{cd}
20	65.75 ± 0.55^{c}	92.56 ± 01.24^{b}	110.15 ± 0.21^{a}	206.94 ± 0.70^{a}	283.44 ± 14.63^{d}
25	69.07 ± 0.62^{b}	68.73 ± 0.35^{c}	93.63 ± 0.52^{c}	184.46 ± 0.69^{c}	306.56 ± 3.95^{c}
30	61.65 ± 1.34^{d}	31.50 ± 0.54^{g}	57.54 ± 0.14^{h}	128.01 ± 0.65^{f}	245.38 ± 8.08^{e}
35	69.89 ± 0.48^{b}	63.04 ± 0.16^{d}	90.75 ± 0.39^{d}	176.74 ± 0.70^{d}	269.62 ± 3.97^{de}
40	73.54 ± 1.14^{a}	52.57 ± 0.37^{e}	74.02 ± 0.20^{f}	190.88 ± 0.42^{b}	336.21 ± 6.56^{b}
45	63.73 ± 0.79^{c}	48.97 ± 0.29^{f}	63.94 ± 0.68^{g}	143.86 ± 0.99^{e}	280.49 ± 13.13^{d}
50	56.07 ± 0.43^{e}	62.21 ± 0.48^{d}	88.95 ± 0.78^{e}	192.03 ± 0.35^{b}	379.09 ± 8.92^{a}

Table 2. Total phenolics and flavonoids (ug/g dry weight) of *A. spicifera* exposed to 15, 20, 25, 30, 35, 40, 45 and 50 psu for a period of seven days (n = 4; mean ± SD). Different letters indicate significant differences according to bifactorial analysis of variance and Tukey's test ($p \leq 0.05$).

Salinity (psu)	Total Phenolics	Total Flavonoids
15	3.20 ± 0.65^{cde}	1.41 ± 0.28^{cd}
20	3.42 ± 0.36^{cd}	1.50 ± 0.16^{c}
25	3.76 ± 0.20^{c}	1.65 ± 0.08^{c}
30	3.07 ± 0.10^{f}	1.35 ± 0.04^{e}
35	3.32 ± 0.14^{ef}	1.46 ± 0.06^{de}
40	3.97 ± 0.46^{b}	1.75 ± 0.20^{b}
45	3.49 ± 0.28^{de}	1.53 ± 0.12^{c}
50	4.57 ± 0.29^{a}	2.01 ± 0.12^{a}

cells can be seen, as well as the presence of endophytes (red arrows) (Fig. 4H).

Samples stained with Periodic Acid-Schiff (PAS) showed a reaction with the cellulose compounds present in the cell wall, and in the cytoplasm, a positive reaction to starch grains was observed (Fig. 5A-H). In samples of 15, 20 and 25 psu, a large amount of starch grains (Fig. 5A-C) was observed. On the other hand, in the 30 psu sample, the amount of starch grains was very small (Fig. 5D), whereas the 35, 40, 45 and 50 psu samples presented a larger amount of starch grains (Fig. 5E-H) when compared to the 30 psu sample, but a smaller amount when compared to the 15, 20 and 25 psu samples.

Discussion

Samples of the red alga *A. spicifera* exposed to 15, 20, 25, 30, 35, 40, 45 and 50 psu showed important physiological responses. Since the intensity of PAR in the culture room was lower than that found in the field, all treatments presented a depigmentation of the stipe. According to Fenchel & Straarup (1971), with greater depth of water, less ingress of light can be expected, resulting in lower concentrations of chlorophyll *a* and phycocyanin in the sediment. Thus, since light intensity is reduced, algae can conserve energy by producing fewer pigment molecules. In the present study with less available light, pigments were diminished in specimens cultivated under 15, 45 and 50 psu, and these were the same specimens that presented most difference relative to external morphology, i.e., absence of new stalks. The tetrasporangia of *A. spicifera* occur at the base of short branches, leaving the stipe a little lighter in color compared to that in the infertile region (Cordeiro-Marino 1978). In treatments of 20 to 40 psu, a depigmentation was perceived in the center of the stalk, but with growth of new branches. In addition to PAR intensity, this depigmentation may indicate that the algae are trying to propagate the species in the form of spore release, showing that the reproductive cycle could be maintained normally in these salinities. In addition, the growth rate was negative in samples treated with 40, 45 and 50 psu, but positive in the 15 psu sample which, however, did not present new branches. This result may have resulted from cell wall thickening in this treatment, as seen in the TB-O in LM, which can lead to weight gain. Fluctuation in salinity leads to cellular stress in algae, in turn causing membrane leakage of ions and electrolytes, pH changes, solute crystallization and pro¬tein denaturation (Collén & Davison 1999; Bischof & Rautenberger 2012; Karsten & Holzinger 2012; Kumar et al. 2012). These events then cause changes in many different physiological processes with the corresponding accumulation of reactive oxygen species (ROS) (Collén & Davison 1999; Bischof & Rautenberger 2012; Karsten & Holzinger 2012; Kumar *et al.* 2012). It is also well known that hypersaline stress is the most harmful to algae since it involves water deprivation. This is different from hyposaline stress where osmotic shock promotes cellular hydration (Kumar *et al.* 2014). With turgor pressure reduced by high salinity, cell division is halted. This

can explain the negative growth rate at the higher salinities (40, 45 and 50 psu). This was also verified in Hypnea cervicornis (Ding *et al*. 2013) and S. alsidii (Nitschke *et al*. 2014).

Chlorophyll *a* is the main photosensitizing pigment of red algae (Franceschini *et al*. 2009; Suggett & Prášil 2010), and the lowest concentration of this pigment was observed in the 50 psu sample. With little chlorophyll, the photosynthetic rate tends to decrease, explaining, in turn, the low growth rate in this sample. This decrease in chlorophyll content was most likely associated with the degenerative process of pigment biosynthesis as a compensation mechanism to maintain the availability of carbon compounds for the synthesis of antioxidant compounds, as observed in *Palmaria palmata* (Holzinger & Lütz 2006)experimental designs to investigate this issue are manifold and the target organisms are extremely diverse. Data are included from the prokaryotic cyanobacteria, haptophytes, diatoms, brown algae to green algae (fresh water, snow algae and marine species and *Phycodrys austrogeorgica* (Poppe *et al*. 2003) stressed by ultraviolet radiation. At high salinities, the chlorophyll concentration was also low in *K. alvarezii* (Araújo *et al*. 2014).

Allophycocyanin, phycocyanin and phycoerythrin have three main functions in red algae. They first serve as accessory photosynthetic pigments capturing different wavelengths for the realization of photosynthesis (Suggett & Prášil 2010). Next, they promote photoprotection. To accomplish this, phycobiliproteins are organized in a complex antenna in the thylakoid membrane where APC occupies the center above chlorophyll, followed by PC and PE at the terminus (Parmar *et al*. 2013). This arrangement assists in the process of photoprotection of chlorophyll *a* by preventing the intensity of light and radiation from reaching this important pigment (Watanabe & Ikeuchi 2013; Kaňa *et al*. 2014). Finally, APC, PC and PE serve as antioxidants to prevent oxidative stress (Cano-Europe *et al*. 2010; Kumar *et al*. 2010). The lowest concentrations of these three accessory pigments were verified in the 30 psu treatment (Tab. 1). Because it is an ideal salinity, the seaweed does not suffer from stress, thereby reaching a homeostasis that allows the algae to achieve a photosynthesis rate with its chlorophyll, while, at the same time, reducing the need for large amounts of pigments to aid in the capture of light energy. Plants produce ROS continuously as by-products of aerobic metabolism, but when they are under some stress, the amount of ROS tends to increase. Therefore, the plant needs to recruit its antioxidant agents to avert possible oxidative damage (Apel & Hirt 2004; Takahashi & Badger 2011). Since the seaweed at 30 psu represents its normal, homeostatic state, it produces little ROS and does not require the production of antioxidant agents, such as phycobiliproteins. On the other hand, the largest concentrations of phycobiliproteins were observed in the samples with lower salinities (15 and 20 psu), demonstrating that these treatments do, indeed, cause stress by the increased requirement for light and antioxidants (phycobiliproteins, carotenoids, phenols and flavonoids) when compared with the 30 psu treatment (Tabs. 1, 2), even though the algae still retain the capacity to protect this mechanism. This increase in the concentrations of phycobiliproteins under stress was also verified in *Aglaothamnion uruguayense* under ultraviolet radiation (Ouriques *et al*. 2017). However, UVR stress is different from that under high salinities that place seaweeds under even greater stress, which then forces the reduction of this defense pathway in order to channel energy toward more important defense mechanisms, such as the production of carotenoids, phenols and flavonoids.

Carotenoids, phenols and flavonoids are part of the secondary metabolism of red algae and serve as antioxidants (Zeng *et al*. 2001; Apel & Hirt 2004; Ganesan *et al*. 2008; Kottuparambil *et al*. 2012). All three metabolites were in higher concentrations in the samples treated with 50 psu, but lower concentrations in those treated with 30 psu, the latter repeating the pattern and motif found with phycobiliproteins. As previously seen, hypersalinity stress is much stronger when compared hyposalinity; therefore, the former causes greater cellular disorganization requiring the deployment of antioxidants. The same results were found in Dunaliella salina (Chlorophyta) (Marin *et al*. 1998), *Scenedesmus* sp. (Chlorophyta) (Aburai *et al*. 2015), and *P. capillacea* (Schmidt *et al*. 2015).

Upon hypersaline stress, a thickening of the cell wall was also confirmed to restrict the loss of water to the external environment. The sulfated agarans found in the cell wall of *A. spicifera* is a hydrophilic polysaccharide (Vasconcelos *et al*. 2015); thus, cell wall thickening also attracts and stores water. In addition, cells in 50 psu samples were weakened, favoring the presence of endophytes in the cell wall. Endophytes were also observed in samples treated with 15 psu, indicating that this salinity leaves the cell in a fragile condition. The composition of the host cell wall seems to determine the degree of endophyte infectivity, and infection is more common when a host cell is weak (Correa & McLachlan 1991). Although the causal mechanism of endophyte penetration is unknown in the tetrasporophyte, its presence did indicate some change, perhaps a loss of defenses, in these algae at 15 and 50 psu.

Red algal starch is known as "florid starch", and it is an extraplastidial starch product (Reviers 2006; Franceschini *et al*. 2009). The lowest amount of starch was verified in the 30 psu sample, while samples at all other salinities had a large amount of this material. Siaut *et al*. (2011) showed that carbohydrate accumulation in *Chlamydomonas reinhardtii* (Chlorophyta) increased in response to immediate saline shock, corroborating the results of the present work.

Therefore, it can be concluded that the red seaweed *A. spicifera* has high tolerance to salinities of 25, 30, 35 and 40 psu, with little change in morphology and physiology, thus favoring the expansion of this species in diverse environments. However, salinities of 15, 20,

45 and 50 psu were the most damaging and led to loss of biomass and depigmentation of apices. The 50 psu treatment caused the greatest changes overall, greatly reducing biomass and chlorophyll and facilitating the presence of endophytes, thus indicating that high, more than low, salinity interferes with algae metabolism. In sum, for environments which show higher salinity, the results from this study show that this species could have limited distribution in the future, or become seriously threatened, which would, in turn, have pronounced ecological consequences.

Acknowledgements

The authors would like to acknowledge the staff of the Multiuser Laboratory of Biology Studies (LAMEB), Federal University of Santa Catarina, Florianópolis, SC, Brazil, for the use of their light microscopy. The authors acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) for the financial support. Zenilda L. Bouzon is a CNPq fellow.

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