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Comparative study of the effects of salinity and UV radiation on metabolism and morphology of the red macroalga *Acanthophora spicifera* (Rhodophyta, Ceramiales)

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Abstract

Increase of harmful radiation to the Earth's surface due to ozone depletion results in higher exposure to harmful ultraviolet-B radiation (UV), while fluctuations in seawater salinity may alter water density, ionic concentration, nutrient uptake, and osmotic pressure. This study evaluated the effects of salinity and UV on metabolism and morphology of *Acanthophora spicifera* (M.Vahl) Børgesen. Water with 30 and 37 psu [g(salt) kg^{-1}(sea water)] was used for experiments during 7 d of exposure to UV (3 h per day). We demonstrated that UV treatment predisposed, irrespective of salinity, *A. spicifera* to a decrease in its growth rate and cell viability, as well as affected its morphological parameters. After exposure to PAR + UVA + UVB (PAB), samples showed structural changes and damage, such as increasing cell wall thickness and chloroplast disruption. Our results indicate that UV led to dramatic metabolic changes and cellular imbalances, but more remarkable changes were seen in samples exposed to high salinity.

Additional key words: alga; cell viability; growth rate; metabolites; microscopy.

Introduction

Sun radiation is composed of a continuous spectrum divided by wavelength range, including ultraviolet radiation (UV) (100–400 nm) and visible (PAR) (400–780 nm) (Diffey 2002, Balogh *et al*. 2011). The distribution of electromagnetic radiation on the Earth's surface occurs unevenly since 39% of incident radiation on the Earth is visible light, and 5% of incident radiation is ultraviolet (Balogh *et al*. 2011). Even though UV reaching the Earth's surface is comparatively small, it is the most harmful radiation to organisms. This radiation is subdivided into three different spectral regions: UVA (400–315 nm), UVB (315–280 nm), and UVC (280–100 nm). UVA radiation is the closest to the visible spectrum, and it is not absorbed by ozone. UVB radiation is not completely

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Abbreviations: ANOVA – analysis of variance; BSA – bovine serum albumin; CW – cell wall; C – chloroplast; CC – cortical cells; cyt *c* – cytochrome complex; DM – dry mass; FM – fresh mass; GR – growth rates; HCA – hierarchical clustering analysis; IPCC – Intergovernmental Panel on Climate Change; LM – light microscopy; MCW – methanol:chloroform:water; M – mitochondria; MTP – mitochondrial transmembrane potential; N – nucleus; PAB – PAR + UVA + UVB; P – plastoglobuli; PCA – principal component analysis, psu – practical salinity unit [g(salt) kg^{-1}(sea water)]; ROS – reactive oxygen species; SD – standard deviation; S – starch grains; SC – subcortical cells, TB-O – toluidine blue; TEM – transmission electron microscopy; UVA – UV A radiation; UVB – UV B radiation; UVC – UV C radiation.
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absorbed by O_3 and it is harmful to living organisms which can lead to DNA damage. UVC radiation is extremely harmful, but it is completely absorbed by O_3 (Madronich *et al.* 1992).

Depleted stratospheric ozone layer results in an increase of UVB, thus, allowing harmful radiation to reach the Earth's surface. Very high doses of radiation can affect macroalgae at various levels, such as growth rates (Navarro *et al*. 2010, Pereira *et al.* 2014, Simioni *et al*. 2014, Zao *et al*. 2014), photosynthetic performance (Aguilera *et al*. 1999, Polo *et al*. 2014), physiological responses (Bischof *et al*. 2000, Pereira *et al*. 2014), pigment content (Roleda *et al*. 2004, Navarro *et al*. 2010, Schmidt *et al*. 2010, Borderie *et al*. 2011, Almeida *et al*. 2013, Polo *et al*. 2014), ultrastructural organization (Poppe *et al*. 2003, Holzinger and Lütz 2006, Schmidt *et al*. 2009), and DNA lesions (Mitchell *et al*. 1992, Bischof *et al*. 2002, Heo *et al*. 2010).

Moreover, according to the IPCC 2012, rainfall is expected to be very uneven in South America, including increased rainfall in the southeast and south of Brazil, Paraguay, Uruguay, Argentina, and some regions of Bolivia (Scherner *et al.* 2013), leading to changes in abiotic factors that affect aquatic ecosystems, such as salinity, causing osmotic stress in marine organisms. Salinity is a factor of great importance for the development of algae; macroalgae show different sensitivity under different stressors (Connan and Stengel2011). Fluctuations in seawater salinity may alter water density, ionic concentration, nutrient uptake, and osmotic pressure (Lobban and Harrison 1994, Fong *et al*. 1996), resulting in physiological and biochemical changes in affected organisms. Changes in salinity may affect growth rate (Barthley *et al*. 2013, Baghel *et al*. 2014, Felix *et al*. 2014), morphology (Nejrup and Pedersen 2012, Araújo *et al*. 2014, Mandal *et al*. 2015), and photosynthetic performance (Macler 1988, Jayasankar 2005, Nitschke *et al*. 2014).

UVB has the potential to cause damage to DNA and to the photosynthetic process (Wiencke *et al.* 2000, Heo *et al.* 2010, Gómez and Huovinen 2011, Figueroa *et al.* 2014). Salinity, as well as UV, can generate ROS, causing oxidative damage to DNA (Baltruschat 2008, Heo *et al.* 2008). DNA damage can affect the expression of proteins, such as Rubisco. The Rubisco is a stromal protein that catalyzes the first step of both photosynthetic CO_2 assimilation and photorespiratory pathways (Fukayama *et al*. 2012, Recuenco-Munoz *et al*. 2015). This protein is formed by large and a small subunits. Genes coding for the large subunit constitute the chloroplastic genome, while genes coding for the small subunit constitute the nuclear genome (Recuenco-Munoz *et al*. 2015).

Macroalgae are divided into three large groups: green, brown, and red (Bicudo and Menezes 2010). Among them, red macroalgae present the greatest diversity on the Brazilian coast. They are economically the most exploited algae, as they biosynthesize sulphated polysaccharides, carrageenans and agarans (De Reviers 2006, Franceschini *et al.* 2010). *Acanthophora spicifera* (M.Vahl) Børgesen is a red alga belonging to class Rhodophyceae, order Ceramiales, and family Rhodomelaceae. Algae in this division are eukaryotic with phycobiliproteins, chlorophyll (Chl) *a*, xanthophyll, and starch, termed floridean starch grains, as reserve substances (Lang 2006). The alga is native to Florida and the Caribbean and is grown for human consumption owing to its high nutritional values (Horn 2012). In Brazil, it occurs from the State of Maranhão to the Rio Grande do Sul coast (Algabase). This alga is easily found in regions of calm and shallow sea and rocky banks. It is typically attached to hard substrates, such as rocks, basalt ledges, or floating, as a result of its fragile constitution (Fialho 2013).

Acanthophora spicifera is a species with substantial utility, as it is used for consumption in marine ecosystems as a part of the diet for fish, urchins, crabs, and sea turtles (Lang 2006). It is also used in research by the pharmaceutical industry because of its bioactive compounds (Lang 2006). Several compounds of interest have been identified in *A. spicifera* (Wang *et al*. 1998, Zeng *et al.* 2001), which have important antibacterial, antioxidant antiviral, anti-implantation, and anti-fouling activities (Wang *et al*. 1998, Zeng *et al*. 2001, Duarte *et al*. 2004, Lang *et al*. 2006, Ganesam *et al*. 2008, Zakaria *et al*. 2011).

In this study, the aim was driven by the hypothesis that stress from the combination of PAR + UVA + UVB (PAB treatment) radiation and high salinity results in a strong and deleterious effect on the metabolism and morphology of *A. spicifera*. In order to test this theory, we investigated the *in vitro* combined effect of PAB treatment and salinity on the growth rate, cell viability, ultrastructure, total soluble protein, sugar and starch contents in this species.

Materials and methods

Collection sites: The experiments were performed with seaweeds collected at two different environments in Florianópolis (Santa Catarina Island, Brazil): Conceição Lagoon and Sambaqui Beach (Fig. 1S, *supplement available online*). Conceição Lagoon (27º34'S, 48º27'W) is a small coastal lagoon located in the easternmost central part of Santa Catarina, with an approximate area of 19 km^2, connected 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.

Abiotic parameters: Sea water used in the experiments was collected from both sites. Seawater samples (n = 3) of each area were filtered through a filter with 0.45-µm

porosity, stored in plastic vials, and frozen for analysis of dissolved inorganic nutrients. Nitrate and ammonium (Aminot and Chaussepied, 1983), as adapted by Murphy and Riley (1962), and phosphate (Aminot and Chaussepied 1983) concentrations were estimated using a *U–2900* spectrophotometer (*Hitachi*, Japan). Salinity was measured through a refractometer (*Alfakit*, Brazil), and pH was measured using a pH meter (*206–Lutron*, Taiwan).

Algae collected: Tetrasporophytic samples of *A. spicifera* (~ 80 g of total thallus) attached to rocks were collected from both areas (Conceição Lagoon and Sambaqui Beach) in December 2014 during the Southern Hemisphere summer season. Collection was made at the intertidal zone during the low tide in the morning. The specimens were transported at ambient temperature in dark containers to the Plant Cell Biology Laboratory (Federal University of Santa Catarina, Florianópolis, Santa Catarina, Brazil), and macroepiphytes were meticulously eliminated by cleaning with a brush and washing with filtered seawater.

Experimental design: Thalli were collected from two areas (Conceição Lagoon and Sambaqui Beach) and followed for two groups: natural samples, that were collected directly from the environment, without prior treatment and acclimatization, and proceeded for the analyzes; and samples for experiment: PAR and PAR + UVA + UVB (PAB treatment), which were maintained in a culture medium with sterilized sea water from each site enriched with von Stosch enrichment solution at half strength without EDTA (Edwards 1972) and cultivated under laboratory-controlled conditions, including 24 ± 2°C, continuous aeration, 70 ± 10 µmol(photon) 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 sea water was renewed every two days. PAR was measured with a light radiometer (*PMA 2100, Solar Light*, USA) using a *PMA 2132* sensor (*Solar Light*, USA).

After acclimation, thalli [~ 4.0 g of fresh mass (FM) ~ 8 individuals per beaker] of *A. spicifera* were cultivated for seven days in beakers containing 500 mL of respective sterilized sea water, renewed every two days, under two different conditions of radiation: PAR (control) for 12 h per day and PAR + UVA + UVB (PAB treatment) for 3 h per day. Daily integrated irradiances of PAR, UVA, and UVB were 657.40 kJ m^{-2} [70 ± 10 µmol(photon) m^{-2} s^{-1}], 72.03 kJ m^{-2} (6.67 W m^{-2}), and 2.48 kJ m^{-2} (0.23 W m^{-2}), respectively. Eight replicates were used for each experimental group. Culture conditions were the same as those described for the acclimation period. UV was provided through a *Vilber Lourmat* lamp (*VL–6LM*, Marne La Vallée, France) with peak output at 312 nm for UVB and peak output at 365 nm for UVA, as measured with a light radiometer (Model *PMA 2100*), using a *PMA 2107B-UW* underwater UV sensor (UVA + UVB) and a *PMA 2102* (UVB) (*Solar Light*, USA). The different UV treatments were based on the average of daily measurements under natural environmental conditions. The thalli were also subjected to PAB irradiation during 7 d (3 h per day) in a culture room, from 12:00 to 15:00 h, mimicking the period of the highest incidence of UV radiation in the environment.

Growth rate (GR) was calculated using the following equation (n = 4):

$$GR\ [\%\ per\ day] = [(F_t/F_i)^{1/t} - 1] \times 100$$

where F_i – initial FM, F_t – FM after seven days, and t – experimental time in days (Penniman *et al*. 1986).

Cell viability was measured on the last day of the experiment using thalli of *A. spicifera* [250 mg(FM), n = 4] by the reduction of 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT*, Sigma-Aldrich*, St. Louis, MO, USA) to formazan by cellular enzymes, where MTT is reduced to water-insoluble purple formazan, depending on the viability of the cells. The concentration of formazan was determined spectrophotometrically (*Multileitora Infinite M200, Tecan*, Switzerland) since it is proportional to the amount of active mitochondria and, therefore, to the amount of living cells. Algal segments were carefully washed with sterile sea water and incubated with 30 µL of MTT stock solution (0.17 g L^{-1}) in a test tube with sterilized sea water (3 mL of final volume) at 37°C for 3 h. Algal fragments were then washed in distilled water and transferred to a test tube containing 1.0 mL of dimethylsulfoxide (*Merck*, Darmstadt, FRG) (Mosmann 1983, Mendes *et al*. 2013). The specific absorbance was determined with a *Tecan* microplate spectrophotometer (*Infinite M200 PRO*, Männedorf, Switzerland) at 570 nm. Cell viability was expressed as a percentage, considering the absorbance of natural samples as 100%.

Light microscopy (LM) and cytochemistry: Thalli samples of control and PAB treatment, approximately 5 mm in length, were fixed in 2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight following the method of 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 of 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 and McCandless 1973). In order to characterize cortical and subcortical cells, as well as cell walls of *A. spicifera*, ten cells on ten different glass slides (n = 100) stained with TB-O were evaluated and measured. 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).

Ultrastructure analysis (transmission electron microscopy, TEM): Four replicates were made for each experimental group; two samples per replication were then examined by TEM under *JEM 1011* (*JEOL Ltd*., Tokyo, Japan) at 80 kV. Similarities based on the comparison of individual treatments with replicates (four grids per treatment) suggested that the ultrastructural analyses were reliable. For observation under TEM, ten samples each of control and PAB treatment, approximately 5 mm in length, were fixed overnight with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) with 0.2 M sucrose (Schmidt *et al*. 2009). The material was post-fixed with 1% OsO_4 for 4 h, dehydrated in acetone gradient series, and embedded in *Spurr*'s resin. Thin sections were stained with aqueous uranyl acetate followed by lead citrate.

Total soluble sugar and starch: The extraction of total soluble sugars was performed according to Shannon (1968). Briefly, an aliquot of 1.0 g(FM) (n = 4) was extracted with 2 mL of methanol:chloroform:water (MCW) (12:5:3) and centrifuged at 4,000 rpm for 5 min. The supernatant was collected, and the pellet was re-extracted using 2 mL of MCW. One part of chloroform and 1.5 parts of water were added for each four parts of supernatant, followed by centrifuging at 4,000 rpm for 5 min, yielding two phases. The upper aqueous phase was collected and dosaged using anthrone 0.2% (w/v) (Umbreit and Burris 1964). Starch extraction was performed according to McCready *et al*. (1950). The pellets used in the total soluble sugar extraction were ground with 30% (v/v) perchloric acid and centrifuged at 4,000 rpm for 5 min. The supernatant was collected, and the precipitate was extracted again, as specified above. The extract was centrifuged, and the supernatants of both extractions were pooled and analyzed according to Umbreit and Burris (1964), using 0.2% (w/v) anthrone. The sugar and starch concentrations were expressed in mg g^{-1}(dry mass, DM), calculated using D-glucose as standard.

Total soluble protein: Total soluble protein was extracted according to the method of Choo *et al*. (2004) and Barros *et al*. (2006), from frozen samples of 0.3 g(FM) (n = 4) by using an extraction buffer (50 mM phosphate buffer, pH 7, 1 mM EDTA; 1mM DTT; 1 mM PMSF; 1% PVP; 0.25% *Triton X-100*) in the proportion of 0.3 g(FM) to 0.600 mL of the buffer. The solution was mixed with an *Ultra–Turrax* homogenizer and centrifuged (4,000 rpm at 4°C) for 30 min. The total soluble protein contents were expressed in mg g^{-1}(DM), after being determined according to Bradford (1976), using a Bradford solution (*Sigma*) BSA as standard.

Statistical analyses: The data passed the *Shapiro-Wilk*'s normality test and all samples were within normal range. Afterwards, the data were analyzed by bifactorial analysis of variance (*ANOVA*) and *Tukey*'s *a posteriori* test. All samples were compared to each other (natural, PAR and PAB treatment). The two independent factors were salinity at either 30 or 37 psu and irradiation treatment (natural samples, PAR and PAB treatment), considering $p \leq 0.05$. Bifactorial statistical analyses were performed using the *Statistica* software package (*Release 10.0*). Data were also tested by principal component analysis (PCA) and hierarchical clustering analysis (HCA). Graphical design was carried out using scripts written in *R language* (*v. 3.1.1*). The data and HTML reports were generated from the analysis (using *R Markdown* features) to determine similarities of physiological variables were analyzed in the present work.

Results

Abiotic parameters: The seawater used for acclimation and experiments was respectively collected from Conceição Lagoon and Sambaqui Beach and sterilized with UVC radiation. The following results were obtained:

	[µM(NH_4^+)]	[µM(NO_3^-)]	[µM (PO_4^{-3})]	psu
Conceição Lagoon	1.06 ± 0.27	8.47 ± 0.01	0.17 ± 0.01	30
Sambaqui Beach	1.13 ± 0.05	3.73 ± 0.01	0.52 ± 0.01	37

Morphological features and growth rate (GR): Morphological features of natural samples of *A. spicifera* collected from 30 psu (Conceição Lagoon) and 37 psu (Sambaqui Beach) are shown in Fig. 1*A*,*B*, respectively. Both natural algal samples showed a typical brownish red color and cylindrical thallus with numerous small brightly colored branches around the central axes. PAR-treated algae had elevated size, growth of new branches (Fig. 1*C*,*D*), yellowish red color, and slight depigmentation. However, PAB-treated algae showed a reduced amount of branches and more depigmentation (Fig. 1*E*,*F*), when compared to PAR, as well as shapeless and uncompact thallus consistency. Additionally, algae treated with 37 psu and PAB showed partial necrosis beyond depigmentation (Fig. 1*F*), most likely related to significant mass loss (0.40% per day).

After 7 d of experimental treatment with different radiation (PAR and PAB), the GRs of *A. spicifera* from 30 psu and 37 psu were analyzed by two-way *ANOVA*. We observed that salinity and treatment, separately, influenced the growth rate, while the interaction between these factors was not significant (Table 1S, *supplement available online*). GRs showed statistical differences between treatments and groups (Fig. 2). Specifically, algae samples from 30 psu grew by rate of 5.65% per day under the PAR regime, similar to GRs of 30 psu-algae samples

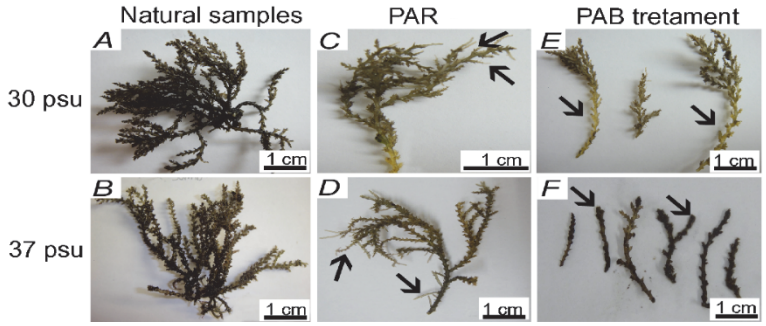

Fig. 1. Morphological features of *Acanthophora spicifera* from 30 psu (Conceição Lagoon) and 37 psu (Sambaqui Beach), including natural samples (*A*,*B*), PAR-treated samples (*C*,*D*), and PAB-treated samples (*E*,*F*). *A*,*B*: The branches with brownish red pigmentation characteristic of *A. spicifera*. *C*,*D*: The formation of new branches (*arrows*). *E*,*F*: Alterations in lateral branches, necrosis (*arrows*), and depigmentation.

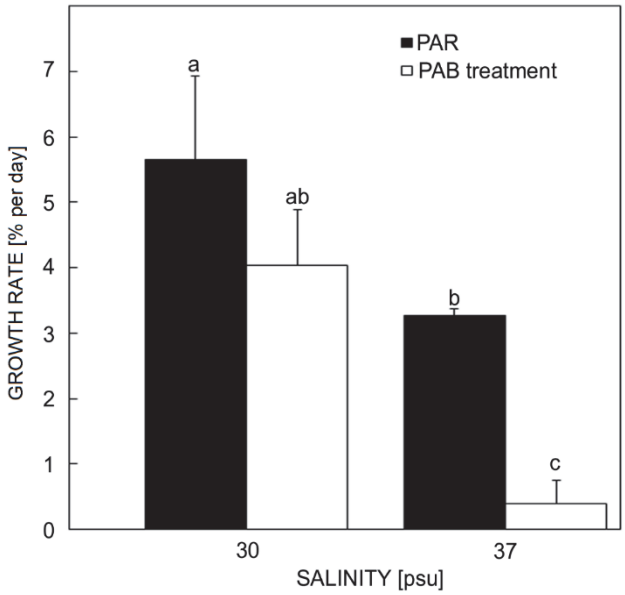

Fig. 2. Growth rate of *Acanthophora spicifera* exposed to 30 psu and 37 psu after seven days of exposure (3 h per day) to PAR and PAB treatments (n = 8; mean ± SD). *Different letters* indicate significant differences according to bifactorial *ANOVA* and *Tukey*'s test.

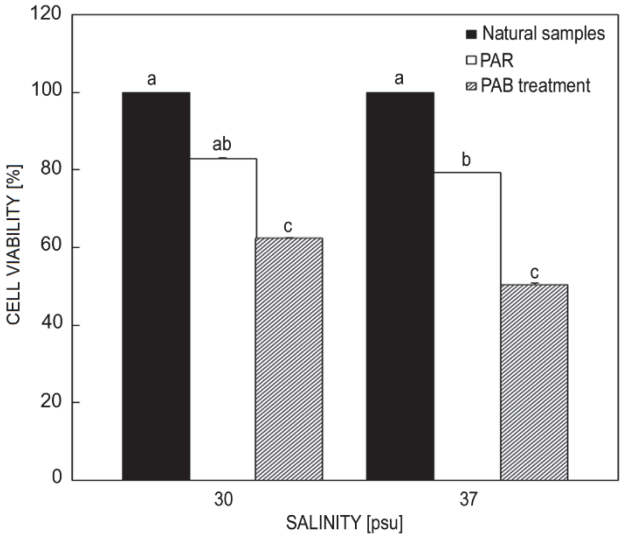

Fig. 3. Cell viability of *Acanthophora spicifera* exposed to 30 psu and 37 psu by MTT assay of natural samples and samples treated with PAR and PAB treatment for seven days (3 h per day) (n = 4; mean ± SD). *Different letters* indicate significant differences according to bifactorial *ANOVA* and *Tukey*'s test.

treated with PAB (4.04% per day). The 37 psu-samples reached GRs of 3.27% and 0.40% per day after PAR and PAB treatments, respectively, indicating statistical significant differences.

MTT assay for cell viability: Cell viability of *A. spicifera* was assessed by MTT assay after seven days of exposure to PAR or PAB. Statistical differences were observed in cell viability between natural samples and PAR and PAB samples from both salinities (Fig. 3). Specifically, it was observed that cell viability was affected by the individual treatments, as well as the interaction between salinity and irradiation treatments and that the effects were statistically significant (Table 1S). Algae cultivated under PAB showed 37.4 and 49.4% lesser cell viability than natural samples from 30 and 37 psu, respectively.

Morphological description: In natural samples of *A. spicifera* from 30 and 37 psu, the cortical region could be characterized by the formation of 1–2 cortical layers. Cortical cells were small, from 2.1 to 9.5 µm in diameter and from 2.6 to 9.25 µm in length, and surrounded by a thick cell wall from 0.15 to 1.1 µm. The subcortical cells were larger than the cortical cells and more vacuolated (Fig. 4*A,B*), with sizes varying from 7–34 µm in diameter and 7–26.9 µm in length and cell wall thickness varying from 0.4–1.5 µm (Fig. 4*A,B*).

Samples cultivated with PAR and both salinities showed cortical cells with a wide range of diameters from 2.25 to 10.5 µm and lengths from 2.5 to 9.7 µm, as well as cell wall thickness, ranging from 0.10–0.90 µm (Fig. 4*C,D*). Subcortical cells measured 6.8–36 µm in diameter and 6.9–27 µm in length, and cell wall thickness was measured from 0.35 to 1.7 µm (Fig. 4*C,D*).

A. spicifera samples from both salinities cultivated under PAB showed a reduction in cell volume and cell wall thickness (Fig. 4*E,F*). Cortical cells were measured between 2.7 and 6.1 µm in diameter and from 1.9 to 6.8 µm in length. Cell wall thickness was measured between 0.5 and 2.35 µm (Fig. 4*E,F*). Subcortical cells were measured from 5 to 27 µm in diameter and from 4.4 to 19 µm in length (Fig. 4*E,F*). The cell wall of subcortical cells revealed an increase in thickness from 0.6 to 4.3 µm (Fig. 4*E,F*).

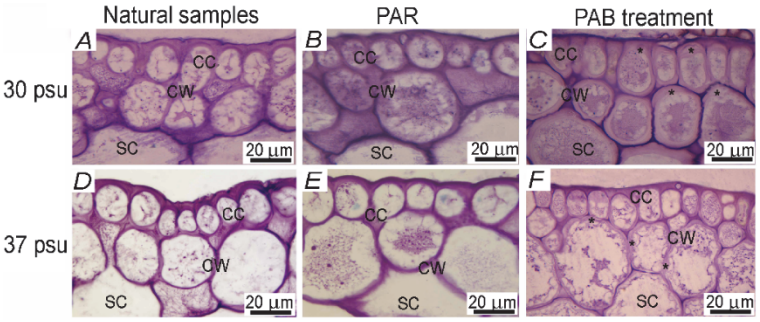

Fig. 4. Light microscopy of transversal sections of *Acanthophora spicifera* exposed to 30 psu and 37 psu natural samples (*A*,*B*), algae exposed to PAR (*C*,*D*), and algae exposed to PAB treatment (*E*,*F*) stained with TB-O. The metachromatic reaction of cell wall (CW), cortical cells (CC), and subcortical cells (SC). PAR and PAB treatment samples showed metachromatic reaction in the CW similar to that of natural samples. The CC and SC showed reduction in cell volume and increase in CW thickness in PAB-treated algae (*C,F* asterisks), and the cytoplasm of CC and SC, was denser than that of natural and PAR samples, with metachromatic granulations (*E*,*F*).

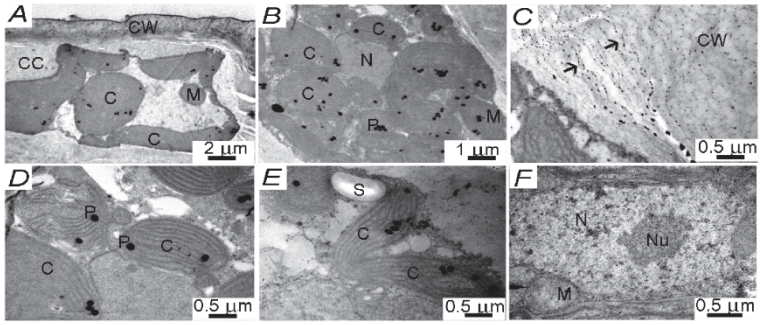

Fig. 5. Transmission electron microscopy of natural and PAR samples (control) of *Acanthophora spicifera* exposed to 30 psu (*A,C,F*) and 37 psu (*B,D,E*). *A*: Cell overview with a compact cortical cell (CC) filled with cellular organelles, such as chloroplast (C) and mitochondria (M), surrounded by cell wall (CW). *B*: Cell with many chloroplasts (C), plastoglobuli (P), nucleus (N), and small mitochondria (M). *C*: Detail of fibers (arrows) organized in the cell wall (CW). *D*: Chloroplasts (C) assumed the typical internal organization of red algae with many plastoglobuli (P). *E*: Chloroplasts (C) with the presence of starch grains (S), the reserve material in algae. *F*: Interphase nucleus (N) and condensed chromatin in the nucleolus (Nu), as in mitotic prophase.

LM observations and cytochemistry: Natural samples of *A. spicifera*¸ in the both salinities, stained with TB-O showed a metachromatic reaction in the cell walls, indicating the presence of sulfated acidic polysaccharides, such as sulphated agarans (Fig. 4*A,B*). Stained PAR samples, from the both salinities, showed metachromatic reaction in the cell wall similar to that observed in natural samples (Fig. 4*C,D*) and PAB-treated samples (Fig. 4*E,F*). Both cortical and subcortical cells of PAB-treated samples showed a reduction in cell volume (Fig. 4*E,F*) and an increase in cell wall thickness (Fig. 4*E,F*, asterisks). However, during the analyzes of measurements of cell size and cell wall thickness in LM, was verified the cytoplasm of cortical and subcortical cells was denser, with metachromatic granulations, compared to natural and PAR samples (Fig. 4*E,F*).

TEM observations: When observed by TEM, natural and *A. spicifera* PAR samples of both salinities showed no general ultrastructural differences (Fig. 5*A–F*). Cortical cells showed a vacuolated cortical region filled with nuclei, small mitochondria (Fig. 5*A*), numerous chloroplasts (Fig. 5*B*), and starch grains (Fig. 5*E*), all surrounded by a thick cell wall with very well-organized fibers (Fig. 5*C*). Chloroplasts assumed the typical internal unstacked organization of red algae, *i.e.*, evenly spaced thylakoids (Fig. 5*D,E*). Electron-dense lipid droplets described as plastoglobuli were observed between the thylakoids (Fig. 5*B,D*). Small mitochondria were present in association with chloroplasts and nucleus (Fig. 5*A,B,F*). The nucleus and nucleolus were bulky, occupying a large volume of the cell (Fig. 5*F*).

In contrast, after exposure to PAB for seven days, *A. spicifera* samples from the two salinities underwent ultrastructural changes, including cortical cells with irregular shape (Fig. 6*A,B,C*) and increasing cell wall thickness (Fig. 6*D,E,F*). In addition, chloroplasts showed visible changes in ultrastructural organization, including irregular morphology and disrupted thylakoids (Fig. 6*G,H*). A few floridean starch grains were observed in the cytoplasm (Fig. 6*I*).

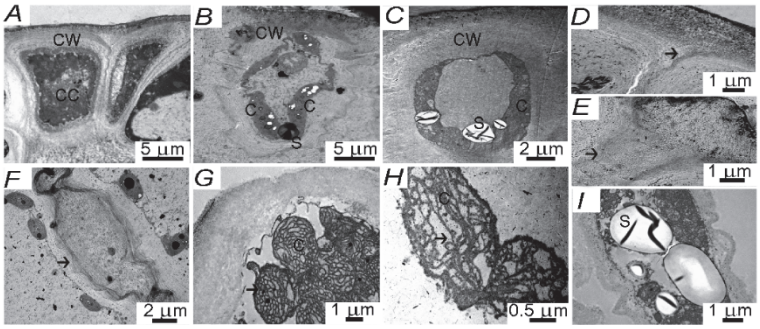

Fig. 6. Transmission electron microscopy of PAB treatment samples of *Acanthophora spicifera* exposed to 30 psu (*B,D,E,G*) and 37 psu (*A,C,F,H,I*). *A*: Cell wall (CW) of cortical cells (CC) with large thickening. *B*: Cell wall (CW) thickness causes deformation and reduction of cell size. *C*: Presence of large starch grains (S) in the chloroplasts (C). *D*, *E*, *F*: Disorganized and corrugated fibers of cortical cell walls (arrows). *G*, *H*: Disrupted thylakoids within the chloroplast (C) (arrows) and irregular morphology. *I*: The number of starch grains (S) is increased in the cell.

Table 1. Contents of total soluble sugars, starch, and total soluble protein of *Acanthophora spicifera* in natural samples and treated samples after 7-d exposure to PAR and PAB treatments, exposed to 30 psu and 37 psu (n = 4, mean; ± SD). *Letters* indicate significant differences according to bifactorial *ANOVA* and *Tukey*'s test.

Salinity	Treatment	Soluble sugars [mg g^{-1}(DM)]	Starch [mg g^{-1}(DM)]	Protein [mg g^{-1}(DM)]
30 psu	Natural samples	0.68 ± 0.07^{b}	$0.22 \pm 0..2^{ab}$	0.56 ± 0.03^{ab}
	PAR	0.85 ± 0.08^{b}	0.16 ± 0.02^{cd}	0.38 ± 0.12^{c}
	PAB treatment	0.76 ± 0.18^{b}	0.13 ± 0.07^{d}	0.44 ± 0.09^{bc}
37 psu	Natural samples	0.79 ± 0.14^{b}	0.26 ± 0.01^{a}	0.54 ± 0.04^{abc}
	PAR	2.26 ± 0.61^{a}	0.17 ± 0.01^{bcd}	0.40 ± 0.06^{bc}
	PAB treatment	0.43 ± 0.06^{b}	0.19 ± 0.03^{bc}	0.66 ± 0.08^{a}

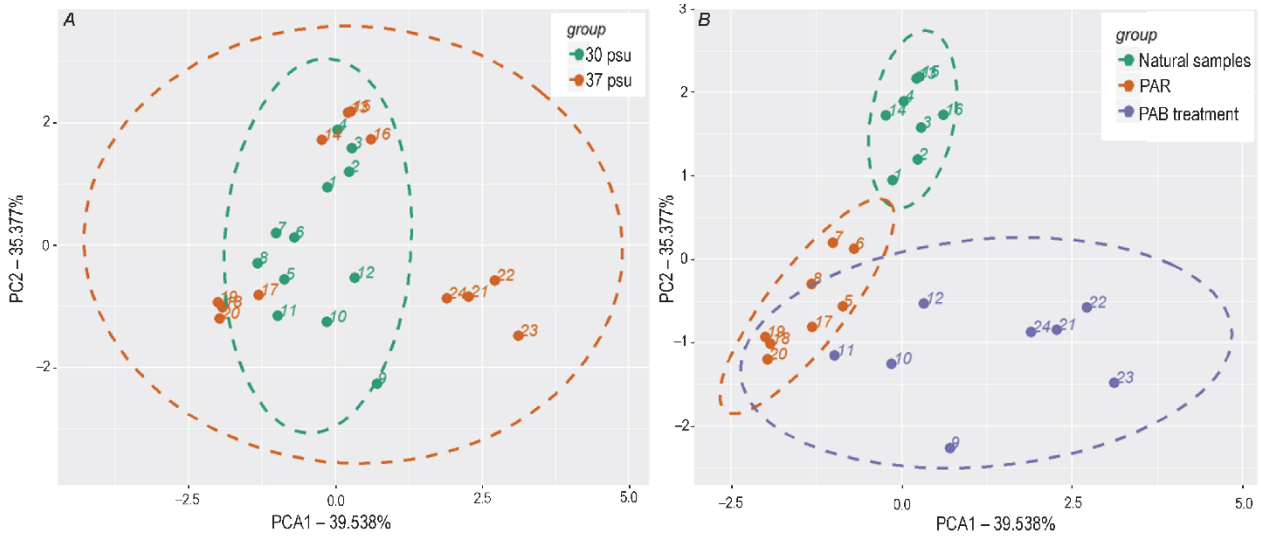

Fig. 7. *A:* PCA of physiological variables taking into account salinity of *Acanthophora spicifera*. *B*: PCA of physiological variables taking into account treatment (natural, PAR, and PAB treatment). The numbers, such as group labels, are defined as follows: 1, 2, 3, 4: natural samples 30 psu; 5, 6, 7, 8: PAR samples 30 psu; 9, 10, 11, 12: PAB treatment samples 30 psu; 13, 14, 15, 16: natural samples 37 psu; 17, 18, 19, 20: PAR samples 37 psu; and 21, 22, 23, 24: PAB treatment samples 37 psu.

Soluble sugars, starch and proteins contents: The content of total soluble sugars, starch and soluble protein varied significantly among the natural samples and samples treated with UV radiation (Table 1). The soluble sugar content of samples exposed to 37 psu and PAR increased compared to that of natural samples and other

treatments (Table 1). Salinity, treatment, and their interactions were significant for soluble sugar (Table 1). Starch contents decreased in samples exposed to both salinities, PAR and PAB treatment, when compared to the two natural samples (Table 1). Salinity and treatment were significant for starch, while interaction between the factors proved to be statistically insignificant (Table 1S). Protein contents significantly increased in the samples exposed to 37 psu and treated with PAB (Table 1). Salinity, treatment, and their interaction were significant cofactors for proteins (Table 1S).

Multivariate analysis: Principal components analysis (PCA) was used to inspect the set of analyzed variables and to search for patterns related to the treatments tested, showing a good discriminating power (Fig. 7). Taking into account salinity as a discriminating factor (Fig. 7*A*), we noticed that the two groups (30 and 37 psu) overlapped, with the exception of samples exposed to UV and 37 psu, showing that salinity was not a factor that differentiated samples exposed to PAR and natural samples. On the other hand, when we performed PCA, taking into account natural, PAR and PAB treatment groups as discriminating factors (Fig. 7*B*), we noted the formation of three large groups in which samples exposed to PAB and 30 psu overlapped with samples exposed to PAR for both salinities, showing that samples exposed to PAB and 37 psu were the most differentiated ones. PCA1 and PCA2 together were able to explain 74.9% of the sample variance.

Discussion

Acantophora spicifera samples exposed to 30 and 37 psu and to PAB treatment showed important changes in morphology, cellular organization, and physiological responses. Specimens cultivated at salinity of 37 psu and PAB irradiation exhibited the most dramatic changes when compared to samples at 30 psu. Strikingly, physical and chemical seawater parameters analyzed from the two areas showed important differences. Sambaqui Beach showed high concentrations of phosphate, potential N limitation, and salinity of 37 psu, whereas Conceição Lagoon showed higher concentrations of nitrogen, no N limitation, and salinity of 30 psu. Concentrations of major dissolved inorganic nutrients can support the growth and metabolic responses of macroalgae (Chow *et al*. 2001, Costa 2006, Dailer *et al*. 2012, Rojas *et al*. 2013). Thus, the elevated growth rates of *A. spicifera* from Conceição Lagoon (30 psu) can be supported by previous nutritional status, as Chow (2012) has proposed. In contrast, *A. spicifera* samples from Sambaqui Beach (37 psu) were more susceptible to exposure under laboratory-controlled PAB treatment, and a greater reduction in growth rate was observed after the UV treatment. Lesser availability of nitrate, as suggested seawater parameters of samples from the Sambaqui Beach, may explain this response (Cronin and Lodge 2003, Jormalainen *et al*. 2003, Navarro *et al*. 2014, Polo *et al*. 2015). It is known that UV has the potential to penetrate to ecologically significant depths in aquatic systems and that turbidity contributes to the depth which can be reached (Pérez Rodrígues *et al.* 1998, Wiencke *et al.* 2000). However, sea water at both beaches had similar turbidity; therefore, this factor should not influence the results obtained.

Some responses from *A. spicifera* exposed to PAB treatment gave evidence of the dramatic effect on biological performance, including, for example, depigmentation, reduction in the formation of new branches and cellular necrosis, especially for samples treated with 37 psu. This response could be related to delayed cell division, resulting from the formation of pyrimidine dimers induced by UV radiation (Poll *et al.* 2002, Fidantsef and Britt 2012, Biever *et al.* 2014) and supported by low amounts of nitrogen in sea water (Sutherland *et al.* 1996).

Cellular viability was also altered in *A. spicifera* exposed to UV. According to Berridge *et al*. (2005), tetrazolium dye reduction is dependent on NAD(P)H–dependent oxidoreductase enzymes largely in the cytosolic compartment of the cell; therefore, reduction of MTT and other tetrazolium dyes depends on cellular metabolic activity arising from NAD(P)H flux. In response to UV, recent studies showed that mitochondria and chloroplasts produced reactive oxygen species (ROS) that can damage the cellular components (mitochondrial enzymes, membranes) and inhibit cellular metabolic activity (Nawkar *et al*. 2013). According to Yao *et al*. (2004), different stress conditions, such as salinity, temperature, pH, and nutrients (Takagi 2006, Ifeanyi *et al*. 2011, Devi *et al*. 2012), cause changes in mitochondrial transmembrane potential (MTP), releasing cytochrome complex (cyt *c*) into the intermembrane mitochondrial space, which activates caspase activity and, subsequently, causes cell death. As reported by Mittler *et al*. (2011), ROS can act as secondary signaling molecules, as well as a cell-damaging agent. ROS-mediated signaling is a complex mechanism, and striking a balance between ROS-producing enzymes and the oxidation–reduction states of various antioxidants depends on the nature of the individual ROS class (De Tulio 2010). In plants, elevated concentrations of ROS cause oxidative damage resulting from disruption of metabolic activities and increasing activity of membrane-localized NADPH-oxidase in response to UV (Kalbina and Strid 2006).

The internal morphology of *A. spicifera* treated with PAB was altered compared with natural and PAR-treated samples under the both salinities, showing irregular contour of cortical and subcortical cells and increasing cell wall thickness. The cell wall of *A. spicifera* natural samples cultivated with PAR and PAB reacted positively to TB-O staining, indicating the presence of sulphated

polysaccharides. When analyzed by TEM, samples of *A. spicifera* exposed to PAB treatment revealed increasing cell wall thickness, which can be interpreted as a physical defense mechanism against exposure to UV.

The chloroplasts of natural samples and PAR-treated *A. spicifera* assumed the typical internal unstacked organization of red algae, showing evenly spaced thylakoids. In contrast, the chloroplasts of cells exposed to PAB treatment showed significant structural changes, including irregular morphology and disruption of the thylakoids. These changes in *A. spicifera* could have resulted from the toxic effect of UV, a phenomenon which promotes a stress condition that results in the formation of ROS (Mallick and Mohn 2000, He and Häder 2002, Poppe *et al.* 2002, 2003, Gill and Tuteja 2010), causing peroxidation and destabilization of thylakoid membranes (Poppe *et al.* 2002, 2003, Gill and Tuteja 2010), leading, in turn, to a reduction in growth rates and photosynthetic pigment contents.

Floridean starch grains represent the storage material of red algae (Lang 2006). Upon exposure to UV, the concentration of reserves decreased in samples treated at both 30 and 37 psu, when compared to natural samples. This decrease can be related to an alteration in the starch biosynthetic pathway in the Calvin cycle, possibly through degradation pathway activation. The degradation pathway can be used to activate the biosynthesis of defense compounds, such as production of cell wall components, as well as thickening of cell walls, as seen in LC and TEM (Schmidt *et al*. 2012).

Red algae have phycobiliproteins as accessory pigments, assisting Chl in the uptake of light energy (Franceschini *et al.* 2010). As the intensity of the PAR in the culture room was lesser than the intensity of the PAR of the environment, fewer pigments were needed to absorb the light energy. This explanation may be the explanation for the decrease in phycobiliproteins observed in the samples exposed to PAR at both salinities, when compared to natural samples. On the other hand, proteins related to antioxidant defense, such as catalase and superoxide dismutase (Maia *et al.* 2012, Batista *et al.* 2015), may be synthesized as a defense in samples exposed to saline stress, as seen in rice (Lin and Kao 2001), tomato (Mittova *et al.* 2004), corn (De Azevedo *et al.* 2006), and cowpea beans (Cavalcanti *et al.* 2007), and UVB stress, as verified in *Arabidopsis thaliana* (Linnaeus) Heynhold (Rao *et al.* 1996) and soybean (Li *et al.* 2012).

Conclusion: The present study demonstrated that UV radiation adversely affected many physiological parameters in the studied species, irrespective of salinity, such as decrease in growth rate and cell viability, as well as morphological parameters, such as disruption of thylakoids and cell wall thickening. After exposure to PAB treatment for 3 h a day for 7 d, samples showed structural changes and damage, such as increasing cell wall thickness and chloroplast disruption. In addition, after exposure to PAB treatment, cell viability decreased to 37.4 and 49.4% compared to natural samples from 30 psu and 37 psu, respectively. In view of different abiotic conditions at the two collection points, it is important to note that the Conceição Lagoon samples (30 psu) exhibited fewer negative changes, overall, when compared to the Sambaqui Beach samples (37 psu), possibly because Conceição Lagoon waters offered a greater availability of nutrients, benefiting samples from this place during the experimental period, presenting a greater tolerance to UV. Thus, our results clearly indicated that UV radiation in *A. spicifera* led to the failure of protective mechanisms, causing dramatic metabolic changes and cellular imbalances, but with more remarkable changes seen in the nutrient-deficient and high-salinity samples.

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