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ORIGINAL ARTICLE

Effects of copper and lead exposure on the ecophysiology of the brown seaweed *Sargassum cymosum*

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Abstract The effects of the heavy metals copper (Cu) and lead (Pb) on *Sargassum cymosum* were evaluated by determining uptake capacity, growth rates, photosynthetic efficiency, contents of photosynthetic pigments and phenolic compounds, 2,2-diphenyl-1-picrylhydrazyl radical-scavenging capacity, and morphological and cellular changes. *S. cymosum* was cultivated with Cu and Pb separately and combined at concentrations of 10, 25, and 50 μM for 7 days in laboratory-controlled conditions. Seaweeds under Cu treatment showed the highest biosorption capacity, and growth rates were significantly reduced compared to the control. The photosynthesis/irradiance curves showed alterations in kinetic patterns in the metal-treated samples. Specifically, Cu treatment alone inhibited electron transport rate (ETR) response, while Pb alone induced it. However, samples treated with both Cu and Pb (Cu+Pb) showed inhibition in ETR. The total amount of pigments increased relative to control. Light microscopy showed an increase in phenolic compounds, with physodes migrating towards cortical cells. Scanning electronic microscopy revealed alterations in the typical rough surface of thallus, when compared with control, especially for Pb treatments. Based on these results, it could be concluded that Cu and Pb are stress factors for *S. cymosum*, promoting alterations in seaweed metabolism and stimulating protective mechanisms against oxidative stress. However, the high bioaccumulation capacity of both heavy metals indicates a possible application for *S. cymosum* as a biosorbent agent for contaminated wastewater when metals are in low concentrations.

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Introduction

The coastal environment is a dynamic system widely modified by the impacts of anthropogenic settlement and industrial activities (Melville and Pulkownik 2007; Gouveia et al. 2013). Changes have become evident in water quality, especially by the introduction of potential toxic compounds (Fichet et al. 1998; Martins et al. 2012; Scherner et al. 2013). Among the pollutants, heavy metals play an important role by their participation in biogeochemical cycles, high sediment persistence, and ecological effects (Pagliosa et al. 2006). These heavy metals have also raised major concerns over their toxicity to aquatic flora and fauna (Jothinayagi and Anbazhagan 2009; Rovai et al. 2013).

Among heavy metal pollutants, lead (Pb) and copper (Cu) are particularly toxic and sometimes abundant in different marine environments. Even in small concentrations, these substances have strong effects on marine organisms (Fichet et al. 1998). However, some seaweeds have the ability to uptake relatively high metal concentrations (Mamboya et al. 1999; Hu et al. 2010), thus playing an important role in heavy metal bioaccumulation (Davis et al. 2000). Accordingly, these organisms are considered potential biomonitors, or bioindicators, of water quality and the presence of contaminants (Karez et al. 1994; Melville and Pulkownik 2007).

Pollution resulting from human activities is considered to have high potential for contributing to the decline of natural populations, even the extinction of some species of seaweeds (Thibaut et al. 2005; Martins et al. 2012; Scherner et al. 2013). This increase in pollutants has, in different degrees, caused a shift in the physiognomies of benthic communities (Martins et al. 2012; Scherner et al. 2013), leading to local extinction (Scherner et al. 2012a, 2012b).

At the cellular level, seaweeds possess many mechanisms for retention of potentially toxic metallic ion pollutants from seawater. Seaweeds have well-recognized mechanisms of biosorption, a process by which heavy metals are uptaken in ionic form in aqueous solution. Moreover, such metals can be retained in ionic form through passive ligation with structural components, such as polysaccharides of cell walls and plasma membranes (Romera et al. 2007; Brinza et al. 2009; Jacinto et al. 2009; Kleinübing et al. 2012). Other intracellular mechanisms can also be implemented in the bioaccumulation of these ions, such as blending with cysteine-rich proteins; antioxidant activity of flavonoids, carotenoids, and phenolic compounds; as well as enzymatic machinery that can result in the immobilization of ions in vesicles and vacuoles (Pinto et al. 2003).

Over the last few decades, seaweed studies have been conducted for different biotechnological applications (Fourestand and Volesky 1996; Davis et al. 2000, 2003; Jacinto et al. 2009). Reports have suggested the use of seaweeds in phytoremediation whereby toxic heavy metals are removed from industrial effluents, making use of the metal-sequestering properties of the biomass (Vieira and Volesky 2000).

Brown seaweeds, in particular, are resistant to environmental pollution and can support high pollution concentrations in the environment (Davis et al. 2003; Luna et al. 2010). On the other hand, prolonged exposure could result in metabolic damage, especially in growth rates and photosynthetic efficiency. These alterations could be explained by redirecting energy for defensive pathways, decreasing organelles and synthesis of structural compounds, as well as oxidizing photosynthetic pigments (Collén et al. 2003; Pinto et al. 2003; Polo et al. 2014). Additional structural changes were observed at the cellular level for red seaweeds, with variations in cell-surface patterns (Santos et al. 2014; de Felix et al. 2014).

Distributed in tropical and subtropical zones, the genus *Sargassum* C. Agardh (Phaeophyceae, Fucales) has been recorded with 10 species in Brazilian coastal waters, representing an algal community with the largest biomass in some regions (Széchy and Paula 2000). The species *Sargassum cymosum* C. Agardh is found from Ceará (05°S and 39°W) to Rio Grande do Sul (30°S and 53°W), and it is distributed in lower intertidal and upper subtidal rocky shores, forming dense populations (Fujii et al. 2011). Based on its broad distribution, it is expected that natural populations of *Sargassum* could be affected by the introduction of pollutants and chemicals from the high multiple uses of Brazilian coastal waters.

Therefore, the aim of this study was to evaluate the effects of the heavy metals copper and lead, both separately and combined, on *S. cymosum*, analyzing uptake capacity, growth rates, photosynthetic efficiency, photosynthetic pigments, phenolic compounds, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity, and morphological and cellular changes. It is hypothesized that Cu and Pb alter physiological responses, chemical composition, and morphology and cellular structure of *S. cymosum*, triggering specific metabolic pathways for synthesis of defensive compounds against heavy metal stress, most likely resulting in oxidative damage.

Material and methods

Algal material and laboratory culture conditions

Individuals of *S. cymosum* were collected at Armação do Pântano do Sul Beach (27°44′42″S and 48°30′27″W), Florianópolis, SC, in May, July, and August 2013. The

samples (100 g of total thallus, FW) were manually collected from the lower intertidal and upper subtidal rocky shore and transported in dark plastic bags to the Plant Cell Biology Laboratory (Federal University of Santa Catarina, Florianópolis, SC). Macroepiphytes were meticulously eliminated by cleaning with a brush and washed with filtered seawater. The seawater was mechanically filtered (10 and 20 μM) and sequentially sterilized in current flux irradiated by a UV lamp. Apical portions were maintained in culture medium with filtered seawater plus von Stosch enrichment solution at half strength (VSES/2 without EDTA; Edwards 1972) and cultivated under laboratory-controlled conditions, including ~24 °C, 35 psu, continuous aeration, ~80 μmol photons m^{-2} s^{-1} (fluorescent lamps, Philips C-5 Super 84 16 W/840), and 12 h photocycle (starting at 8 AM), during 7 days for acclimation before experimental treatment with Cu and Pb. Light was measured with a Solar Light PMA 2100 quantameter (Glenside, PA, USA) and a spherical sensor Solar Light 2132 (Glenside, PA, USA).

Experimental setup

Apical thallus portions were selected (~2.0 g FW; two individuals per Erlenmeyer flask) from the acclimated *S. cymosum* and cultivated for 7 days under the same acclimation conditions and experimental treatments with Cu and Pb in Erlenmeyer flasks containing 500 mL of natural sterilized seawater enriched with VSES/2 (without EDTA). Experimental treatments were carried out with control (i.e., no metal addition), Cu, Pb, or Cu+Pb, supplied as $CuCl_2$ and $PbCl_2$ (10, 25, and 50 μM for both individual metal treatments and 10+10, 25+25, and 50+50 for combined metal treatments), totaling four metal treatments. Stock solutions of 500 mL were prepared in Erlenmeyer flasks. Four replicates were made for each experimental group (16 Erlenmeyer flasks). Proposed concentrations were based on recently analyzed data of Rovai et al. (2013) and concentrations of heavy metals observed in natural waters in the study area.

Biosorption of Cu and Pb

The concentrations of Cu and Pb in seawater and algal samples were analyzed at the beginning and end of the experiment by inductivity-coupled plasma atomic emission spectrometry (ICP-AES, ARCOS from M/s. Spectro, Germany), using the following analyte line: Cu 324.754 and Pb 220.353 nm plasma view-axial, with a detection limit of 0.001 ppm for Cu and Pb. Algal samples (1 mg FW) were first exposed to Cu and Pb for a period of 7 days. Before beginning the experiment, these samples were washed in distilled water, dried at 65 °C, and digested in concentrated nitric acid. Seawater samples (50 mL) were digested using concentrated nitric acid. Total metal absorption was expressed in percent, calculated based on milligram of Cu and Pb in 500 mL of water by milligram of metals in 1 g FW (de Felix et al. 2014). The Bioconcentration Factor (BCF) was calculated as remaining metal concentration and wet weight algal biomass (expressed in ppm) divided by the initial concentration of metal added in the culture medium (de Felix et al. 2014). All analyses were performed in quadruplicate.

Growth rate

Growth rates (GRs) were calculated using the following equation: $GR\ [\%.day^{-1}]=[(W_t / W_i)^{1/t} - 1]\times 100$, where W_i=initial algal FW, W_t=algal FW after 7 days, and t=experimental time in days (Lignell and Pedersén 1989).

In vivo fluorescence of chlorophyll *a*

Photosynthetic performance was estimated as the in vivo fluorescence of chlorophyll *a* of PSII by using a portable Pulse Amplitude Modulation fluorometer (PAM-2500, Walz, Germany). Maximum quantum yield (Fv/Fm) was measured after adapting the seaweeds to 10 min darkness and calculated following Schreiber et al. (1986). Electron transport rate (ETR) was estimated through photosynthesis-irradiance (*P-I*) curves, irradiating thalli with seven increasing actinic irradiance intensities of photosynthetic active radiation (*E*; 0, 24, 61, 108, 236, 456, and 752 μmol photon m^{-2} s^{-1}) provided by the PAM device and calculated as $ETR=Y(II)\times E\times A\times 0.8$, where Y(II) is the effective quantum yield, A is thallus absorbance, and 0.8 is the fraction of chlorophyll *a* associated with PSII (Platt et al. 1980; Figueroa et al. 2003). Absorbance was determined following Ramus and Rosenberg (1980) and Betancor et al. (2014). The following parameters were determined from *P-I* curves (n=4): (1) maximum ETR (maximal ETR at saturating irradiance), (2) photosynthetic efficiency (α, initial slope of *P-I* curve that indicates the efficiency of electron transport), (3) light saturation (Ik, light intensity approximating onset of photosynthetic saturation), and (4) photoinhibition (β, initial slope of *P-I* curve at the end of the saturation phase) by fitting the *P-I* curves to a hyperbolic tangent model with photoinhibition, following Platt et al. (1980). Additionally, photosynthetic recovery was evaluated by incubating the samples for another 24 h in culture medium with filtered seawater (without the metal) enriched with VSES/2 (without EDTA) and under the same laboratory-controlled conditions.

Pigment analysis

Photosynthetic pigments (chlorophylls *a* and *c*; Chl *a* and Chl *c*) of *S. cymosum* were analyzed from fresh frozen samples (n=4) and kept at −40 °C until ready for use. Chlorophylls were extracted from approximately 0.5 g FW in 3 mL of pure acetone

P.A. (Sigma-Aldrich, St. Louis, MO, USA) for 10 min in ice and light protected. The extracts were then centrifuged for 5 min at 1, 990×*g*, and the pigments were quantified spectrophotometrically at 630, 640, and 664 nm (Hitachi, Model 100–20; Hitachi Co., Japan). Pigment concentrations were calculated according to Jeffrey and Humphrey (1975).

Carotenoids were extracted according Aman et al. (2005) by exhaustive extraction from 0.5 g FW samples (n=4) in 10 mL of pure methanol P.A. (Sigma-Aldrich, St. Louis, MO, USA). The methanolic crude extracts were evaporated to concentrate the extracts. The specific absorbance was determined spectrophotometrically (Hitachi, Model 100–20) at 450 nm. Total carotenoid concentrations were calculated based on β-carotene standard curve (0–300 μg mL^{-1}; y=0.0077x+0.0721, r^2=0.9958).

Phenolic compounds

The analysis of phenolic compounds was made using the spectrophotometric method of Folin-Ciocalteau based on Randhir et al. (2002) and Huang et al. (2005). This method allows the quantification of different phenolic compounds, such as total phenols and hydrolysable tannins, in Phaeophyceae. Phenolic compounds were extracted from 0.5 g FW samples (n=4), using 4 mL 80 % aqueous methanol. The extracts were centrifuged for 10 min at 750×*g*. Aliquots of 50 μL of supernatant crude extracts were added to 180 mL of distilled water, 10 mL of Folin reagent, and 30 mL of sodium carbonate 20 %*w*/*v*, and incubated at room temperature for 1 h. Absorbance of the reaction mixture was measured at 750 nm, using a spectrophotometer (Hitachi, Model 100–20). Phloroglucinol was used as standard at concentrations from 100 to 1,250 μg mL^{-1} (y=0.0004x; r^2=0.999). Additionally, possible UV-absorbing compounds extruded by the algae in the respective treatment were evaluated (n=4) by measuring the spectrum of absorbance (280–380 nm) in the filtered seawater, also utilizing phloroglucinol as standard.

DPPH radical-scavenging capacity

To determine radical-scavenging ability, the method reported by Kim et al. (2002) was used. Briefly, 2.9 mL of DPPH 0.1 mM solution in 80 % aqueous methanol (Sigma-Aldrich, St. Louis, MO, USA) was added to an aliquot of algal extracts (0.1 mL of methanolic extract) and incubated for 30 min. The absorbance of antioxidant reaction was measured at 517 nm using a microplate spectrophotometer (TP Reader NM, Thermoplate). The inhibition percentage (%) was determined (n=3) using the following formula: Inhibition (%)=$((Abs_{Control}-Abs_{Sample}) / Abs_{Control})\times 100$ %, where $Abs_{Control}$ is the absorbance of DPPH control and Abs_{Sample} is the absorbance of the samples. The synthetic antioxidant tert-butyl hydroxytoluene (BHT) was used as positive control.

Light microscopy

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 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 5 μm in length were stained with 0.5 % Toluidine Blue (TB-O), pH 3.0 (Merck Darmstadt, Germany), as described by Schmidt et al. (2012), and investigated with an Epifluorescent microscope (Olympus BX 41) equipped with Image Q Capture Pro 5.1 software (Qimaging Corporation, Austin, TX, USA). Similarities based on the comparison of individual treatments with replicates suggested that the light microscopy (LM) analyses were reliable.

Scanning electron microscopy

For observation under the scanning electron microscope (SEM), samples approximately 5 mm in length were fixed overnight with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose (Schmidt et al. 2009). Samples were dehydrated with ethanolic gradient series, dried on a Critical Point EM-CPD-030 (Leica, Heidelberg, Germany), and then sputter-coated with gold prior to examination as described by Schmidt et al. (2012). The samples (n=4) were examined under SEM JSM 6390 LV (JEOL Ltd., Tokyo, Japan) at 10 kV. Copper and lead were analyzed in the cell wall using SEM (NORAN System 7, Thermo Scientific Instruments) coupled to an energy dispersive X-ray spectrometer (SEM-EDX), without post-fixing the samples in osmium tetroxide or coating with gold.

Data analysis

Data of the different measured parameters were analyzed by bifactorial analysis of variance (ANOVA), considering the metals and concentration as independent variables, and by a posteriori Tukey's test with $p\leq 0.05$. For in vivo fluorescence of chlorophyll *a*, data of exposure/recovery and concentration were considered as independent variables, and the a posteriori Tukey's test was performed with $p\leq 0.05$. All statistical analyses were performed using the Statistica software package (Release 10.0).

Results

Biosorption of copper and lead

After 7 days in culture with Cu and Pb in separate or combined treatments (Cu+Pb), samples of *S. cymosum* showed different

heavy metal biosorption capacity relative to the respective control (Fig. 1). The highest biosorption was obtained for 10 μM Cu (Fig. 1a), followed by 10 μM Pb and Cu+Pb 10 μM (Cu) (Fig. 1b, c), indicating larger biosorption dynamics in monometallic Cu solutions. In general, samples treated with Cu showed higher metal biosorption than samples treated with Pb, while lower biosorption was observed for Cu+Pb treatments.

Metal bioconcentration followed a trend similar to that observed for bioaccumulation. Samples treated with Cu showed higher bioconcentration factors than either Pb or Cu+Pb (Supplementary Fig. 1). The treatment with the highest bioconcentration factor was 10 μM Cu (Supplementary Fig. 1A). For Cu+Pb treatments, Cu showed higher bioconcentration factor than Pb at the same concentration (Supplementary Fig. 1C and D).

Growth rates

After 7 days in culture, *S. cymosum* exhibited significant differences in GRs (Table 1). Samples under 50 μM Cu and 50 μM Cu+Pb treatments presented significant decrease of GR when compared to control. No differences were observed for treatments with Pb. Interestingly, when exposed to Cu+Pb, lower concentrations did not show any statistical differences from control. Otherwise, all treatments with Cu, both separately and combined with Pb (Cu+Pb), showed lower means when compared to control.

In vivo fluorescence of chlorophyll *a*

Treatments with Cu, Pb, and Cu+Pb modified the magnitude of the patterns of photosynthesis/irradiance (*P-I*) curves relative to metal type and concentration, when compared to the respective control (Fig. 2). Cu treatments showed significant photoinhibition of ETR kinetics, and no increase of *P-I* curves under Cu treatment were observed after 24 h of recovery (Fig. 2a, b), except for control. Exposure to Pb treatment increases the ETR curves (Fig. 2c), and no variations were observed after recovery, except for control. Finally, 25 and 50 μM Cu+Pb treatments negatively affected ETR kinetics during exposure (Fig. 2e), while a slight recovery was observed for the same treatments after 24 h (Fig. 2f). An increase in *P-I* control curves was also observed (Fig. 2f).

Heavy metal exposure and recovery treatments showed significant impact on photosynthetic performance (Table 2 and Supplementary Table 1). Among all parameters, ETRmax was most affected by exposure to Cu when compared to the respective control ($p \leq 0.05$), while Pb-treated algae showed no effect for either exposure or recovery period

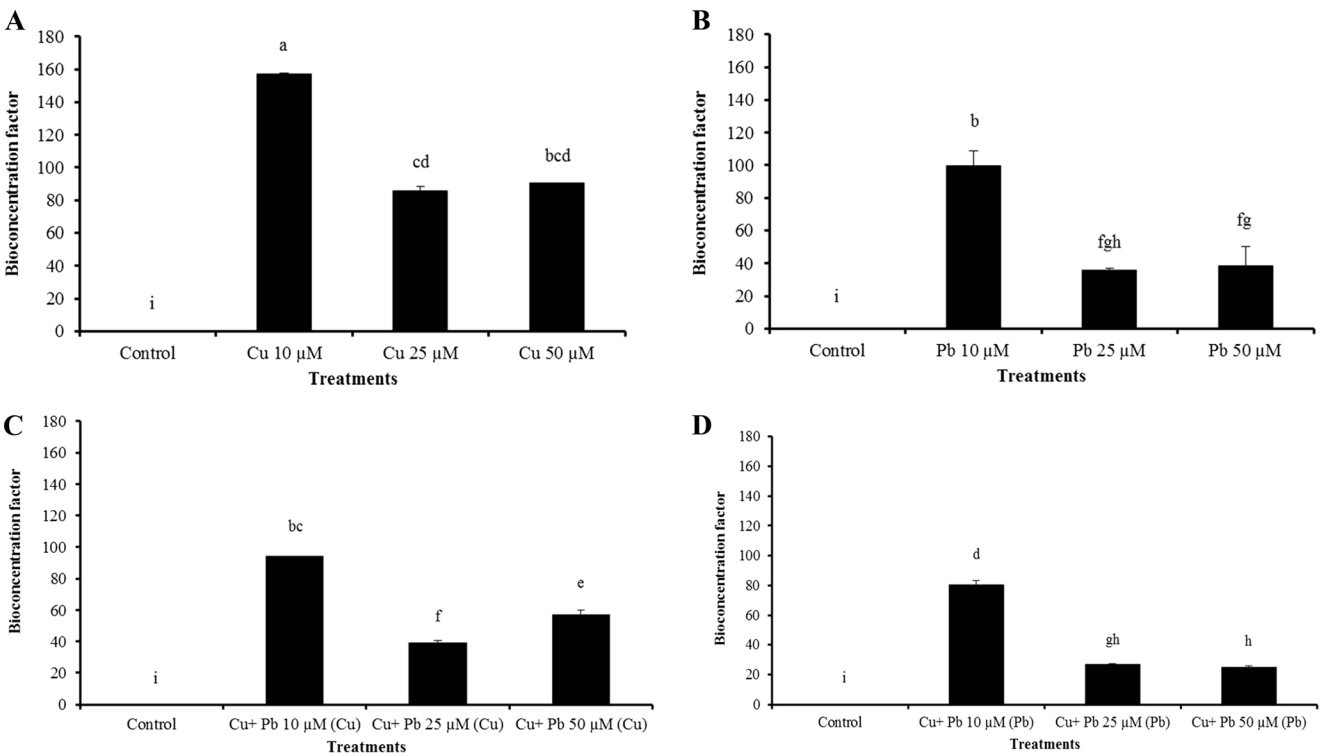

Fig. 1 Heavy metal biosorption of *S. cymosum* after 7 days of exposure to different concentrations (10, 25, and 50 μM) of copper (Cu), lead (Pb), or Cu+Pb. **a** Biosorption of Cu. **b** Biosorption of Pb. **c** Biosorption of Cu with Cu+Pb treatment. **d** Biosorption of Pb with Cu+Pb treatment. *Different lowercase letters* represent significant differences between the treatments, according to bifactorial ANOVA and Tukey's a posteriori test ($p \leq 0.05$). Data are means±SD ($n=4$)

Table 1 Growth rates of *S. cymosum* after 7 days of exposure to different concentrations of copper (Cu), lead (Pb), and Cu+Pb. Different lowercase letters represent significant differences between the treatments, according to bifactorial ANOVA and Tukey's a posteriori test ($p \leq 0.05$). Data are means±SD ($n=4$)

Treatments	Growth rate (% day^{-1})
Control	2.41±0.47a
Cu 10 μM	0.79±0.80abc
Cu 25 μM	0.56±0.97abc
Cu 50 μM	−1.75±1.03c
Pb 10 μM	2.62±1.00a
Pb 25 μM	1.88±1.00a
Pb 50 μM	1.86±0.90a
Cu+Pb 10 μM	1.66±0.57ab
Cu+Pb 25 μM	0.58±0.97abc
Cu+Pb 50 μM	−1.55±2.81bc

(Table 2). During the exposure and recovery periods, photosynthetic efficiency, i.e., alpha parameter, showed a significant reduction for samples treated with the highest concentrations of Cu and Cu+Pb (Table 2). However, the beta parameter only showed significant differences between exposure and recovery for Cu-treated samples (Supplementary Table 1). Finally, Ik was mainly affected during both exposure and recovery periods for samples treated with Cu (Table 2).

Maximum quantum yield (Fv/Fm) showed a wide range of results among treatments (Fig. 3). Cu treatment showed the highest reduction of Fv/Fm parameter, but only at 25 and 50 μM, during both exposure and recovery periods (Fig. 3a). Pb treatments did not result in any statistical differences from Fv/Fm control, either for exposure or recovery (Fig. 3b). For Cu+Pb treatments, only 50 μM Cu+Pb during exposure registered significant reduction of Fv/Fm (Fig. 3c), and total recuperation was observed after recovery (Fig. 3c).

Pigments, total phenolic compounds, and antioxidant activity

The content of photosynthetic pigments (chlorophylls *a* and *c* and total carotenoids) in *S. cymosum* was significantly influenced by exposure to (1) different heavy metal treatments and (2) increasing heavy metal concentrations (Fig. 4a–c). A general increasing trend of pigment contents was observed under metal treatment when compared to control; however, no clear pattern of influence was identified. Organisms treated with 10 μM Cu and 25 μM Pb showed the highest levels of pigments, analyzing both Cu and Pb separately (Fig. 4a–c).

Phenolic compounds presented significant variation between treatments relative to control (Fig. 4d). With the exception of 10 μM Pb, all treatments increased total phenolic contents, irrespective of metal concentration.

A significant effect of heavy metals on DPPH radical-scavenging capacity was also observed for all treatments. The results showed increase on DPPH, by several-fold, for Cu, Pb, and Cu+Pb treatments compared to control. However, no distinct trend could be identified between the metals and their effect on DPPH radical-scavenging capacity and no relationship between the metals and DPPH radical-scavenging capacity could be established relative to concentration (Fig. 4e). Even if 10 μM Cu treatment induced DPPH antioxidant activity compared to control, it was lower than all other metal treatments.

Seawater spectrum of absorbance (280 to 380 nm) was evaluated to analyze the presence of UV absorbing compounds extruded from the seaweeds. Cu treatments showed the highest increase in absorbance, and an increasing absorbance was noted at increasing metal concentration (Fig. 5a). In contrast, a reduction in absorbance was observed for Pb treatments (Fig. 5b). For treatments with Cu+Pb, the presence of Cu appears to have increased seawater absorbance at increasing concentrations of Cu+Pb (Fig. 5c). These measurements were coincident with the seawater coloration of culture medium, as observed 7 days following heavy metal exposure when Cu treatments, in particular, showed a change from an initial colorless appearance (Supplementary Fig. 2).

LM observations and cytochemistry

Control sample of *S. cymosum* stained with TB-O showed a metachromatic reaction in the cell walls, indicating the presence of sulfated acidic polysaccharides (Fig. 6a). Staining of metal-treated samples showed a reaction in the cell wall similar to that observed for control sample (Fig. 6b–j). In the cytoplasm of control cortical cells, dark blue and yellow physodes were observed (Fig. 6a). For metal-treated samples, the cytoplasm of cortical cells showed an increasing quantity of physodes (Fig. 6b–j). Physodes migrated to the cell surface of all metal-treated samples with higher chromatic reaction corresponding to increasing metal concentration (Fig. 6b–j).

SEM observations

Observations under SEM showed the rough surface of the thallus of *S. cymosum* control with an equally rough surface of cortical cell walls (Fig. 7a). For treatments of Cu at 10 and 25 μM, the samples showed a gradual increasing roughness of surface (Fig. 7b–j); however, the same pattern was not

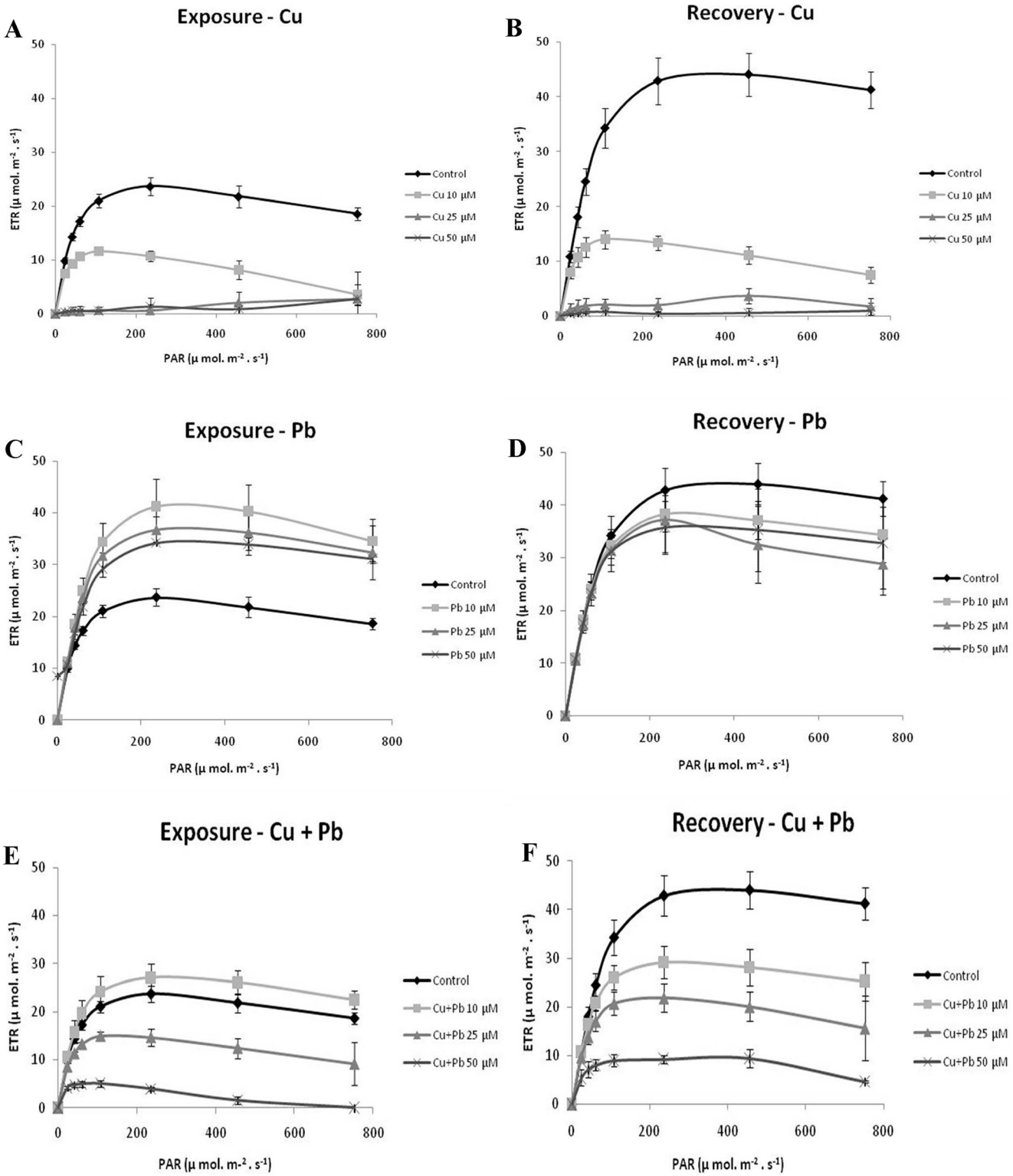

Fig. 2 Curves of electron transport rate (ETR) at crescent PAR levels. Photosynthesis/irradiance (*P-I*) curves of *S. cymosum* after 7 days of exposure to copper (Cu), lead (Pb), or Cu+Pb, followed by recovery after 24 h. **a** Cu treatments during exposure. **b** Recovery of Cu. **c** Pb treatments during exposure. **d** Recovery of Pb. **e** Cu+Pb treatments during exposure. **f** Recovery of Cu+Pb

observed for treatments at 50 µM Cu (Fig. 7d). On the other hand, treatments with Pb and Cu+Pb (Fig. 7e–j) showed a reduced roughness of surface, most likely associated with heavy metal absorption.

Table 2 Photosynthetic parameters ETRmax, Alpha, Beta, and Ik for *S. cymosum* after 7 days of exposure to different concentrations of copper (Cu), lead (Pb), and Cu+Pb, followed by recovery after 24 h. Different lowercase letters represent significant differences between the treatments, including comparisons between exposure and recovery datasets from the same metal treatment, according to bifactorial ANOVA and Tukey's a posteriori test ($p \leq 0.05$). Data are means±SD ($n=4$)

Treatments	ETRmax	Alpha	Beta	Ik
Exposure				
Control	26.22±2.21b	0.49±0.01a	0.01±0.00b	53.08±3.02ab
Cu 10 μM	15.51±0.66bc	0.39±0.02a	0.02±0.00b	39.28±0.79b
Cu 25 μM	4.17±0.34c	0.33±0.00ab	0.51±0.00a	11.70±0.00b
Cu 50 μM	0.93±0.00c	0.09±0.00bc	0.00±0.00b	0.00±0.00b
Recovery				
Control	50.18±8.99a	0.56±0.08a	0.01±0.01b	91.25±25.54a
Cu 10 μM	17.15±2.53bc	0.44±0.08a	0.01±0.00b	39.39±7.96b
Cu 25 μM	3.43±2.55c	0.07±0.07bc	0.00±0.00b	57.59±20.38ab
Cu 50 μM	50.86±0.0a	0.03±0.03c	0.62±0.14a	9.62±0.00b
Exposure				
Control	26.22±2.21a	0.49±0.01a	0.01±0.00a	53.08±3.02a
Pb 10 μM	50.46±7.03a	0.57±0.05a	0.02±0.00a	87.20±4.42a
Pb 25 μM	42.27±2.18a	0.55±0.04a	0.01±0.00a	73.68±8.45a
Pb 50 μM	33.98±7.40a	0.49±0.09a	0.01±0.00a	68.77±8.66a
Recovery				
Control	50.18±8.994a	0.56±0.08a	0.02±0.00a	91.25±25.54a
Pb 10 μM	37.00±10.778a	0.54±0.08a	0.01±0.00a	66.84±12.73a
Pb 25 μM	46.20±9.142a	0.55±0.07a	0.02±0.00a	83.77±17.71a
Pb 50 μM	40.29±5.308a	0.57±0.04a	0.01±0.00a	70.15±13.50a
Exposure				
Control	26.22±2.21bcd	0.49±0.01a	0.01±0.00a	53.08±3.02ab
Cu+Pb 10 μM	19.96±6.62bcde	0.53±0.05a	0.00±0.00a	38.20±16.77b
Cu+Pb 25 μM	17.84±0.29cde	0.46±0.04a	0.01±0.01a	38.25±3.30b
Cu+Pb 50 μM	7.62±1.48e	0.25±0.04b	0.02±0.00a	30.90±10.21b
Recovery				
Control	50.18±8.99a	0.56±0.08a	0.01±0.01a	91.25±25.54a
Cu+Pb 10 μM	32.15±4.19b	0.56±0.02a	0.01±0.00a	57.31±5.46ab
Cu+Pb 25 μM	26.43±1.63bc	0.49±0.07a	0.01±0.01a	54.71±11.63ab
Cu+Pb 50 μM	11.65±5.67de	0.26±0.12b	0.01±0.00a	28.07±1.21b

Discussion

In the present study, *S. cymosum* exposed to Cu, Pb, or Cu+Pb treatments showed different physical and metabolic responses associated with defense mechanisms against heavy metal toxicity. These results showed dose-dependent responses. The ability of *S. cymosum* to retain divalent Cu and Pb cations was evaluated based on analyses of biosorption, i.e., the retention of metals in the cell wall through passive ligation with structural components, and bioaccumulation as it relates to intracellular metabolism, i.e., the ability to incorporate metal in metabolic pathways (Davis et al. 2003). For biosorption capacity, *S. cymosum* showed higher retention of Cu than Pb, either separately or combined. The retention of divalent cations has been studied in different genera of brown seaweeds, such as *Sargassum* sp., *Padina* sp., *Laminaria* sp., *Bifurcaria* sp., *Fucus* sp., *Macrocystis*, *Lessonia*, and *Durvillaea* (Sheng et al. 2004; Freitas et al. 2008; Huovinen et al. 2010). Other authors suggested high retention of heavy metals in seaweeds, such as copper, zinc, lead, and cadmium, but without differentiation between efficiency of bioaccumulation and biosorption and without considering the alkaline pretreatment of biomass to maximize the viability of ligand sites in alginate of cell walls (Romera et al. 2007; Alahverdi and Savabieasfahani 2012). Nevertheless, *Sargassum* species have been recognized for their highly efficient bioaccumulation capacity for heavy metals (Jothinayagi and Anbazhagan 2009). Considering the possible applications in industrial water treatments and ecological remediation of coastal seawater, these results could be considered economically important (Vijayaraghavan et al. 2009; Luna et al. 2010).

GRs of *S. cymosum* were altered by treatments with Cu and Pb, and compared with control samples, exposure to heavy metals generally reduced GRs. These results indicate changes in metabolism based on the increased use of energy for the production of antioxidant compounds at the cellular level with

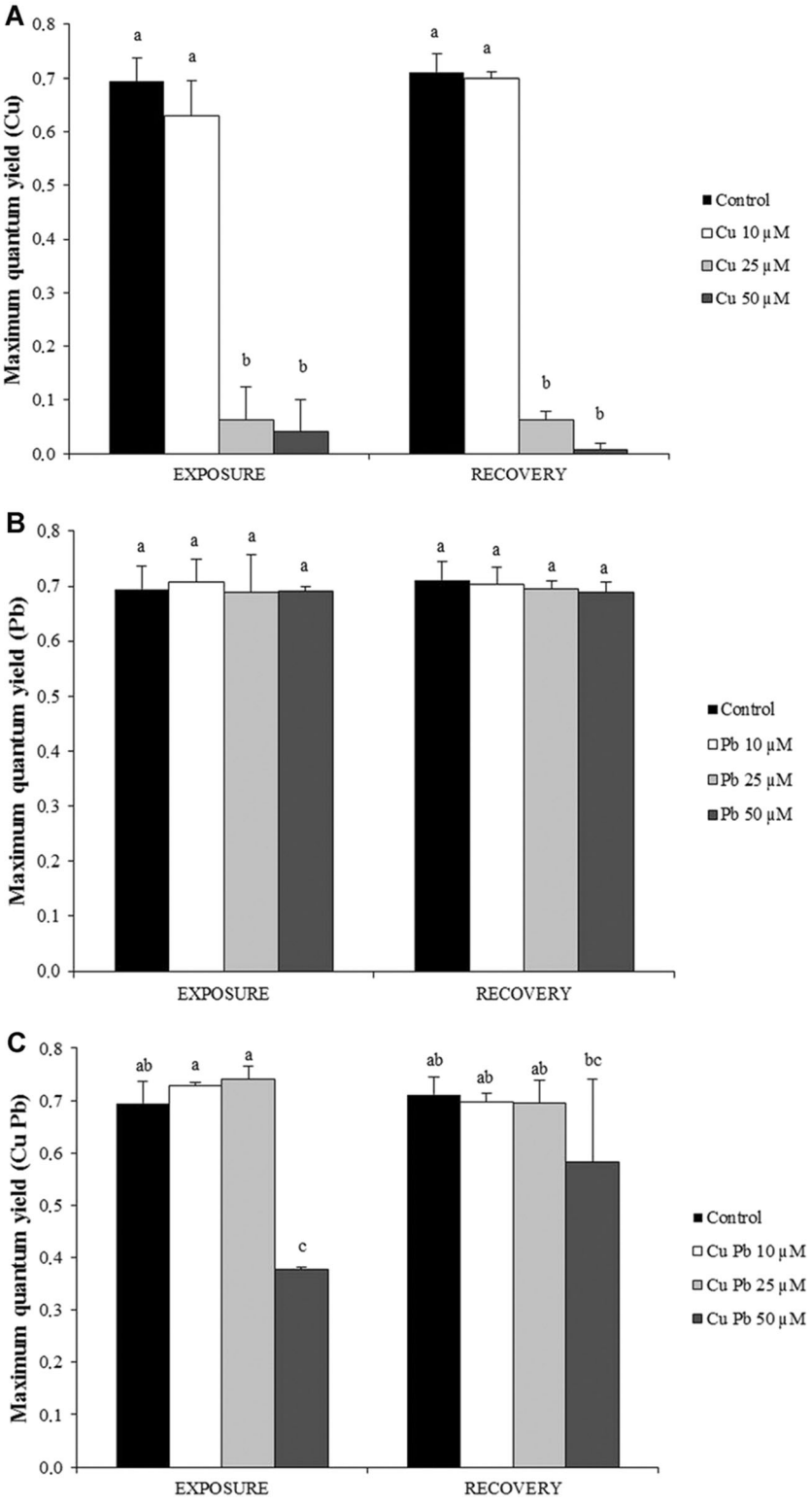

Fig. 3 Maximum quantum yield (Fv/Fm) of *S. cymosum* after 7 days of exposure to different concentrations of copper (Cu), lead (Pb), or Cu+Pb, followed by recovery after 24 h. **a** Fv/Fm for Cu exposure and recovery. **b** Fv/Fm for Pb exposure and recovery. **c** Fv/Fm for Cu+Pb exposure and recovery. *Different lowercase letters* represent significant differences between the treatments, according to bifactorial ANOVA and Tukey's a posteriori test ($p \leq 0.05$). Data are means±SD ($n=4$)

the corresponding diversion of this same energy output from metabolic pathways that would otherwise be employed in the synthesis of proteins and cellular organelles, so necessary for cellular duplication and growth (Santos et al. 2014). Nonetheless, treatment of 10 μM Pb resulted in the highest GR among all treatments, indicating tolerance for this metal at low concentration. On the other hand, Cu treatments showed negative GRs, based on the loss of physodes and blanching of apical portion. Previous studies of red seaweeds exposed to Cu and Pb also

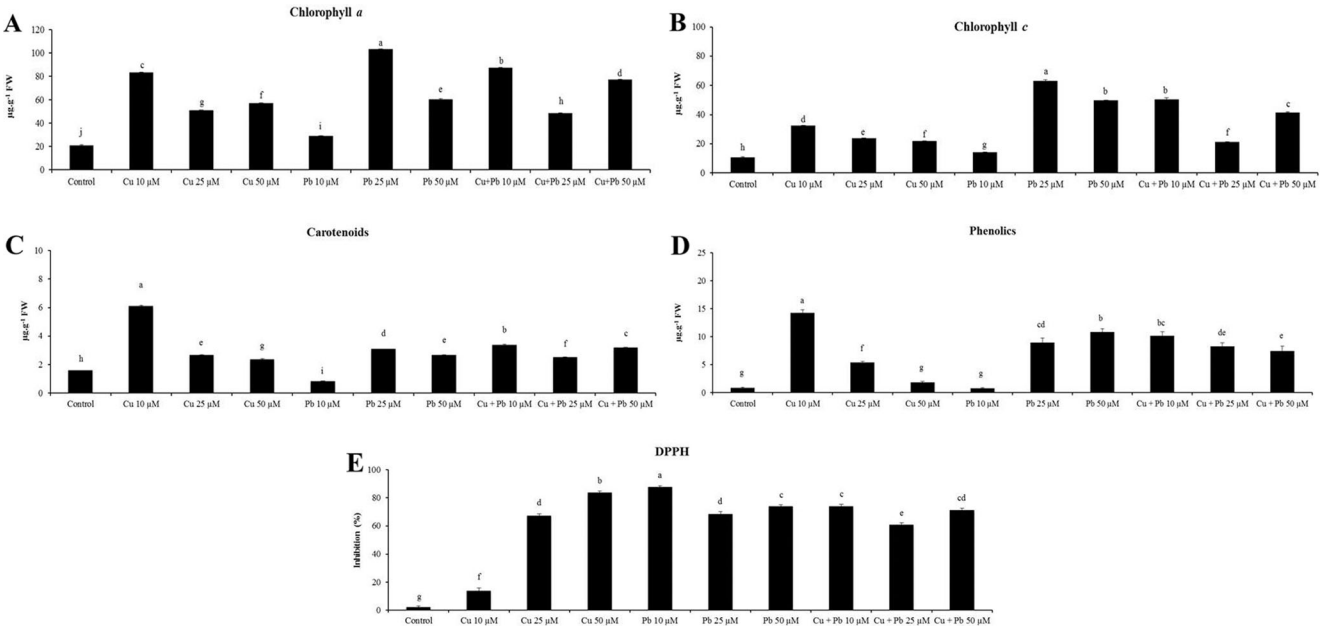

Fig. 4 Pigment contents and antioxidant activity of *S. cymosum* after 7 days of exposure to different concentrations (10, 25, and 50 μM) of copper (Cu), lead (Pb), or Cu+Pb. **a** Concentration of chlorophyll *a* (μg g^{-1} FW). **b** Concentration of chlorophyll *c* (μg g^{-1} FW). **c** Concentration of carotenoids (μg g^{-1} FW). **d** Concentration of phenolic compounds (μg g^{-1} FW). **e** Concentration of DPPH radical-scavenging capacity (inhibition %). *Different lowercase letters* represent significant differences between the treatments, according to bifactorial ANOVA and Tukey's a posteriori test ($p \leq 0.05$). Data are means±SD ($n=4$)

found a reduction of GRs along with damage to the apical portion (Gouveia et al. 2013).

Samples of *S. cymosum* treated with heavy metals showed divergent effects on photosynthetic performance when compared with control sample. Cu-treated samples had a stronger reduction of ETR curves, even after the 24 h of recovery, whereas Pb-treated samples showed increasing ETR responses during exposure, as well as maintenance of high ETR levels during recovery. Cu+Pb treatments showed an ETR reduction at 25 and 50 μM, but slight increase in ETR during recovery. Based on these results, it can be concluded that Cu significantly reduces photosynthetic efficiency in *S. cymosum* samples, despite the increasing response of photosynthetic contents. Other authors have reported the negative effect of Cu on the photosynthetic efficiency of the brown seaweeds *Ascophyllum nodosum*, *Fucus vesiculosus*, and *Fucus serratus* (Nielsen and Nielsenb 2010; Connan and Stengel 2011). The effect of high concentrations of Pb on photosynthesis has been described as increasing the proton gradient on thylakoid membranes and possibly maintaining the total electron flux and photosynthetic process (Kastori et al. 1998). Indeed, while results of maximum quantum yield (Fv/Fm) evidenced photoinhibition effects, with a stronger reduction observed for Cu treatment, without signs of recovery, Pb treatments, in contrast, seemed to exert a photoprotective effect, and any differences were observed either exposure or recovery.

Studying the influence of heavy metals on photosynthetic activity, researchers found a light-induced reduction in the photosynthetic capacity of the brown seaweed *Padina boergesenii* exposed to Cu (Mamboya et al. 1999) and *F. vesiculosus* exposed to Cd (Brinza et al. 2009). Nevertheless, the results of the present study indicated a certain degree of tolerance of *S. cymosum* to Pb exposure and could be considered that this pollutant carry on minor impact natural populations than occasioned to Cu exposure.

In this study, differences in chlorophyll *a* and *c* content were observed as a consequence of heavy metal effect. Cu and Pb treatments have been shown to have greater positive influence on photosynthetic pigments in all concentrations. Toxic compounds, such as heavy metals, promote oxidative stress, and excessive production of reactive oxygen species (ROS) results in damage to cellular structures (Dummermuth et al. 2003). *S. cymosum* showed a high capacity to synthesize photosynthetic pigments, making it possible for chlorophyll *c* to act as a photoprotector of the photosynthetic apparatus. Variations in chlorophyll *a* content could be related to metabolic biosynthesis of antioxidant compounds, as found in other seaweeds, in which chlorophyll contents were reduced in the presence of toxic compounds, as a consequence of interference with the biosynthesis of enzymes and chloroplasts (Han et al. 2008; Santos et al. 2012). To explain the increased Chl *a* content in *S. cymosum*, as determined in the present study, it was hypothesized that this seaweed is able to preserve the capacity of chlorophyll synthesis independently of antioxidant synthesis pathways. Notwithstanding this increase in photosynthetic pigments, it is suggested that normal photosynthetic efficiency could not be maintained, representing an

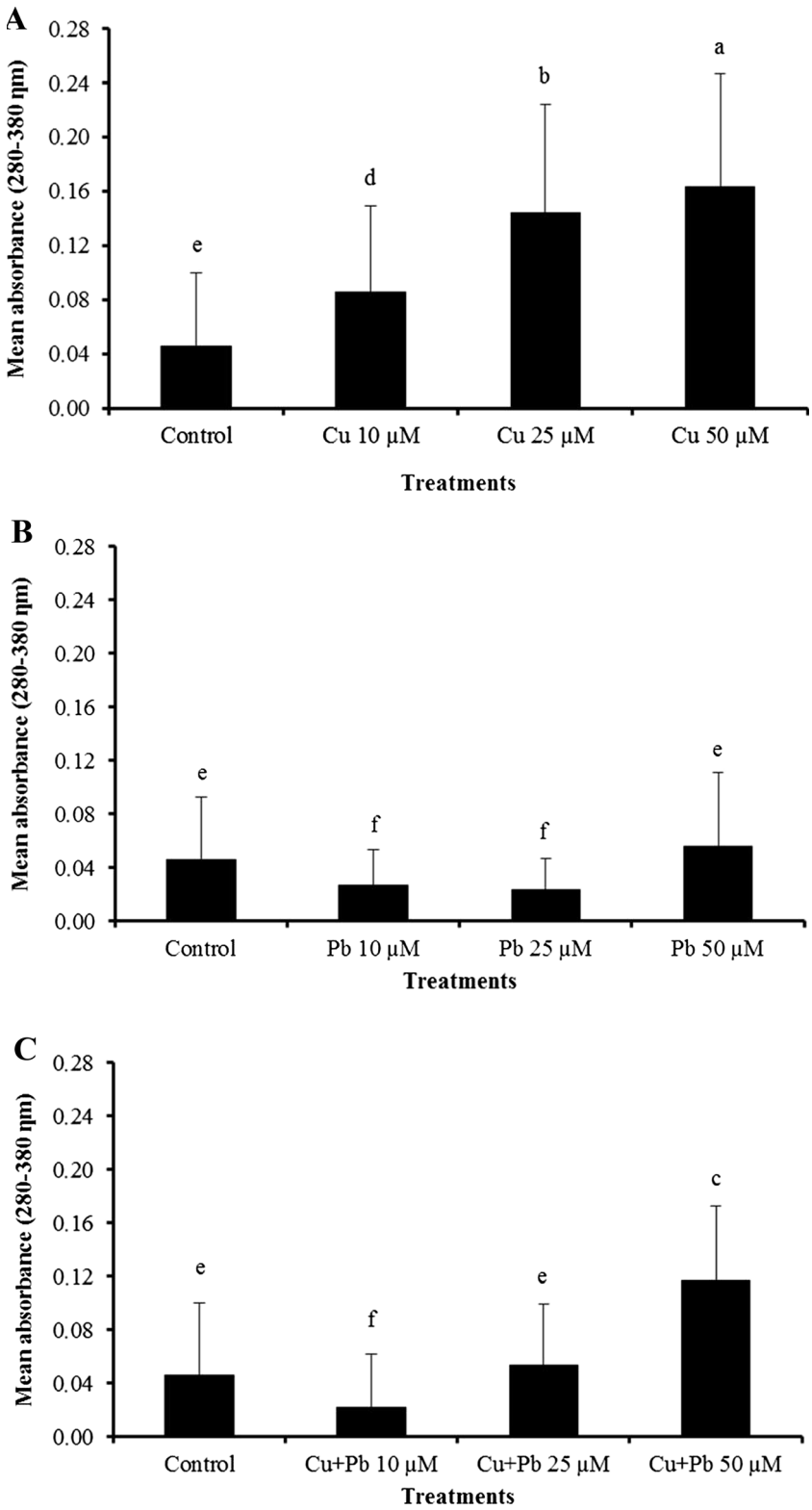

Fig. 5 Water absorbance average at 280–380 nm of *S. cymosum* after 7 days of exposure to different concentrations (10, 25, and 50 µM) of copper (Cu), lead (Pb), or Cu+Pb. **a** Absorbance for Cu treatments. **b** Absorbance for Pb treatments. **c** Absorbance for Cu+Pb treatments. *Different lowercase letters* represent significant differences between the treatments, according to bifactorial ANOVA and Tukey's a posteriori test ($p \leq 0.05$). Data are means±SD ($n=4$)

ecological disadvantage by the high degree of energy expended for synthesis of chlorophylls. Similar behavior was observed by Nielsen and Nielsen (2005) who reported the high concentrations of Cu-induced chlorophyll contents of *F. serratus*, resulting in photoinhibition.

Our results evidenced an increase of carotenoids under metal treatment, a compound of low molecular weight and recognized as cellular defense against ROS (Collén et al. 2003), as well as phenolic compounds, another antioxidant substance against ROS stress. This suggests that Cu and Pb

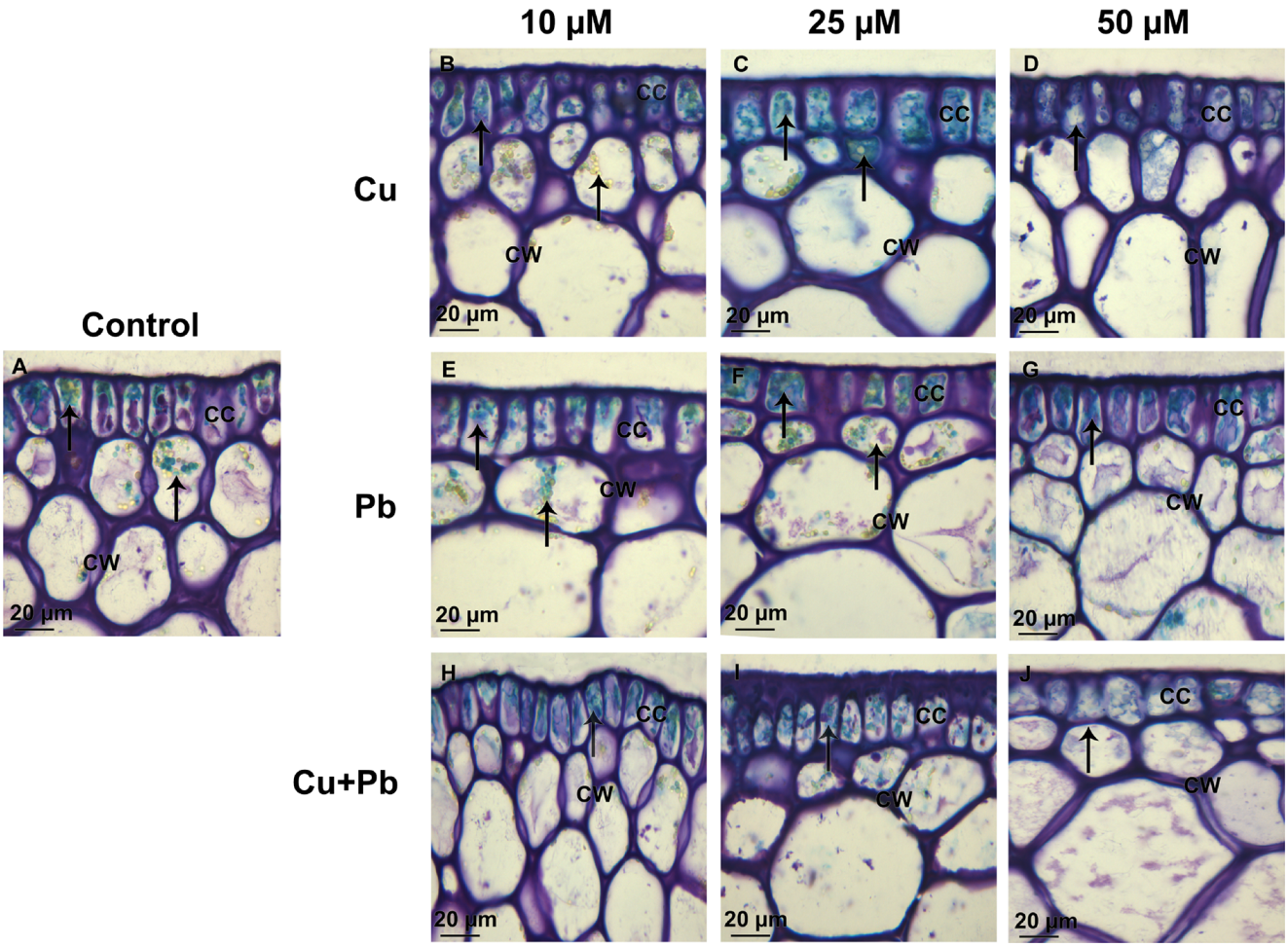

Fig. 6 After 7 days of exposure to different concentrations of copper (Cu), lead (Pb), or Cu+Pb, light microscopy was performed on *S. cymosum* samples stained with TB-O. **a** Control treatment. Observe cortical cells (*CC*) showing metachromatic reaction in the cell walls (*CW*), indicating the presence of acidic polysaccharides and some physodes in blue and yellow (*arrows*). **b**–**d** Cu treatments. Observe the increasing quantity of physodes in metal-treated samples and their migration toward cortical cells (*arrows*). **e**–**g** Pb treatments. **h**–**j** Cu+Pb treatments. Note the presence of physodes (*arrows*)

exposure induces biosynthesis of carotenoids and phenolic compounds as an antioxidant defense. The level of effect was different between Cu than Pb, supporting our hypothesis that Pb at low concentrations does not exert the same toxic effects over *S. cymosum* as Cu. Phenolic compounds are present in brown seaweeds as an major antioxidant mechanism against oxidative stress, and such compounds have been reported to prevent UV radiation damage, as well as damage from the toxicity of heavy metals (Dummermuth et al. 2003; Balboa et al. 2013).

Another antioxidant mechanism evaluated for *S. cymosum* was DPPH radical-scavenging capacity. All metal treatments showed significantly higher antioxidant activity by several-fold, except for 10 µM Cu. These results are different from those observed for carotenoids and phenolic compounds because even treatments with low concentration of carotenoids or phenolics showed exceptionally high levels of DPPH scavenging potential. This difference can be explained by the additional antioxidant defense triggered under metal stress than carotenoids and phenolic compounds synthesis pathways. As a monitor of chemical reactions involving radicals, it is possible that the DPPH assay measures oxidant reactions not associated with the antioxidant potential of carotenoids and phenolic compounds. High radical-scavenging capacity was also observed by Dummermuth et al. (2003) who reported on the arctic seaweed *Polysiphonia arctica* exposed to oxidative stress of ROS, such as hydrogen peroxide. In general, the present results indicate that each metal triggered a different antioxidant defense and that heavy metal concentrations could affect *S. cymosum* in different ways.

Observations under LM identified higher concentration of physodes in cortical cells of metal-treated samples compared with control algae. Brown seaweeds store phlorotannins inside physodes, and these substances are responsible for intracellular heavy metal detoxification (Toth and Pavia 2000). Seaweed exudates have also been found to chelate heavy metals in water near surface, helping to decrease their toxic effects (Gledhill et al. 1999). Previous studies with

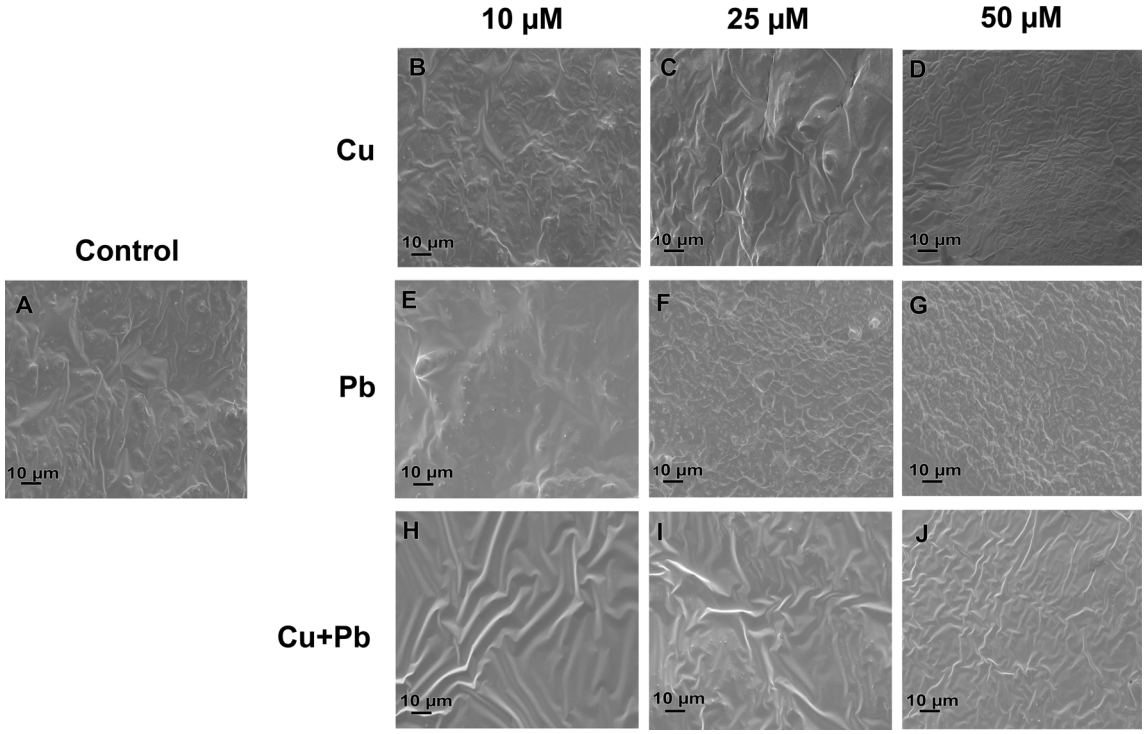

Fig. 7 Scanning electron microscopy (SEM) of the surface of *S. cymosum* thallus after 7 days of exposure to different concentrations of copper (Cu), lead (Pb), or Cu+Pb. **a** Control treatment. Note the roughness of cell wall surface of cortical cells. **b**–**d** Cu treatments. Note the increasing cell wall surface roughness at 10 and 25 µM. **e**–**g** Pb treatments. **h**–**j** Cu+Pb treatments. Observe the reduction of cell wall surface of thallus with increasing heavy metal concentration

S. cymosum registered the migration of phenolic compounds into the physodes toward the cortical cells as a cellular mechanism to protect against stress (Polo et al. 2014). Higher extruded compounds could be corroborated by the brownish-yellow color of seawater medium at the end of the experiment, with higher UV absorbance detected for Cu treatments. Otherwise, for *A. nodosum*, Toth and Pavia (2000) did not find a close relationship between the increase of physodes and increasing concentrations of metal. Nonetheless, it is well documented that most species of brown algae increase their production of phlorotannins as a defense mechanism (Ragan and Glombitza 1986; Toth and Pavia 2000). Thus, the cortical migration of phenolics via physodes is probably related to the extrusion of phenolic compounds as a defense mechanism of *S. cymosum* against oxidative stress, as has been observed for UV radiation (Shoenwaelder 2002, 2008; Polo et al. 2014).

SEM analysis showed changes on the thallus surface of *S. cymosum* after exposure to heavy metals. Different from the results obtained in LM, Pb and Cu+Pb treatments showed the greatest interference, with loss of surface roughness when compared with control. These results could be associated with the retention of metals on the cortical cell wall. On the other hand, it is possible that affinity with the polysaccharides of the cell wall resulted in changes in their structural conformation by alterations on ligand sites and spatial rearrangement, thus increasing the capacity for metal uptake, as observed in this work (Santos et al. 2014). Pinto et al. (2003) also report structural changes induced by heavy metal on seaweeds, allowing channel proteins to enter the cell by active transport that could, in turn, reflect changes in surface conformation of the cell wall. Studies with *Gracilaria domingensis* (Gouveia et al. 2013) showed alterations on thallus surface under SEM observations, with increasing superficial roughness. While these results differed from those of the present study, they do corroborate the effect of heavy metal on this typical thallus surface characteristic.

In sum, the results of the present study lead to the conclusion that the heavy metals Cu and Pb are toxic to *S. cymosum* and that Cu alone has greater toxicity than Pb alone in terms of pigments and photosynthetic efficiency. Analysis showed limited growth, but increasing photosynthetic pigments, caused by biosynthesis of nonenzymatic antioxidants as a response to metal stress. This was evident with light microscopy analysis, in which an increasing concentration of physodes on the cortical cell was observed, probably as a defense against the bioaccumulation of heavy metals. Moreover, reduction in maximum quantum yields indicated possible damage to the photosynthetic apparatus and low capacity of recuperation. Because of physiological changes, *S. cymosum* could be considered as a good bioindicator by its reactive properties relative to heavy metal stressors in the environment and by its sensitivity to pollutants. Furthermore, understanding the defense

mechanisms of such species makes it easier to predict and study the consequences of introducing pollutants into natural environments. Nevertheless, *S. cymosum* showed a high capacity of bioaccumulation and biosorption for these heavy metals. Results like these could stimulate new studies and perspectives for the application of *Sargassum* spp. as natural biosorbents for wastewaters, despite the stronger defense mechanisms against oxidative stress developed by this seaweed.

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