J Appl Phycol
DOI 10.1007/s10811-014-0236-3

A sulfated galactan from the mucilaginous sheath of the red filamentous alga *Chroodactylon ornatum* (Stylonematophyceae, Rhodophyta)

Juan M. Cabrera · Carlos A. Stortz · María C. Rodríguez

Received: 15 July 2013 / Revised and accepted: 5 January 2014

Abstract The pseudofilamentous red alga *Chroodactylon ornatum* was grown in f/2 culture medium with the addition of 10, 34, and 100 μM (nominal concentration) cupric sulfate. The bioassays were terminated at two selected end points (days 4 and 12). Growth inhibition, changes in pigment composition, and oxidative stress indicators such as phenolic compounds and lipid peroxidation (dosed as thiobarbituric reactive substances) were observed in cultures with 34 and 100-μM cupric sulfate. Quinacrine (Atebrin) and chlorotetracycline fluorochromes showed abundant vacuoles of acidic content, related with mucilage secretion. Structural analyses by methylation, desulfation-methylation, alkaline treatment, and NMR spectroscopy revealed that the mucilaginous sheath of *C. ornatum* contains a sulfated galactan with a backbone of alternating 3-linked β-D-galactopyranose and 4-linked α-L-galactopyranose moieties, i.e., an agaran. The absence of 3,6-anhydrogalactose and of its precursor unit (α-galactose 6-sulfate) were confirmed. The highly sulfated polysaccharide contained these ester groups on the C-2 and C-4 hydroxyl groups of the 3-linked unit and on C-3 of the 4-linked units. The large proportion of sulfate esterification in this polysaccharide can be related to the extracellular biosorption of copper divalent cation (2.5±0.4 mg per gram of dry weight) and to copper tolerance in bioassays.

Keywords *Chroodactylon ornatum* · Mucilage · Sulfated galactan · Copper bioassays · Biosorption

Introduction

Chroodactylon ornatum (C. Agardh) Basson is a cosmopolitan epiphytic red algae species consisting of false branched pseudofilaments of globose or elliptical cells enclosed in a broad and firm mucilage sheath (Basson 1979). The species has been reported for marine, brackish, and freshwater environments, but its taxonomic delimitation has been frequently hampered by its salinity-dependent polymorphism (Pujals 1961; Lewin and Robertson 1971).

The classification of red algae is now being revisited with increasing data of molecular phylogeny. Basal red algae have been included in at least five recently proposed subclasses: Cyanidophyceae, Rhodellophyceae, Porphyridiophyceae, Compsopogonophyceae, and Stylonematophyceae (Yoon et al. 2006, 2010; Zuccarello et al. 2008; Müller et al. 2010). *Chroodactylon* is included within the Stylenomatophyceae, which comprises both filamentous and unicellular taxa (Yoon et al. 2006, 2010).

The polysaccharides from red macroalgae have received much attention, both due to their academic interest and to the commercial applications of carrageenans and agarans, their main matrix polysaccharides (Stortz and Cerezo 2000). On the other hand, the structure of polysaccharides from red microalgae has received less attention, with the exception of those from the genus *Porphyridium*, thoroughly studied due to its biotechnological applications (Lapidot et al. 2010). Several authors have stated that polysaccharide structures could also provide valuable information for modern taxonomy of these basal Rhodophyta

J. M. Cabrera · C. A. Stortz
Departamento de Química Orgánica-CIHIDECAR, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, 1428 Buenos Aires, Argentina

J. M. Cabrera · M. C. Rodríguez (✉)
Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, 1428 Buenos Aires, Argentina
e-mail: cecirodriguez@qo.fcen.uba.ar

Present Address:
J. M. Cabrera
INIBIOMA – CRUB, Universidad del Comahue, 8400 Bariloche, Argentina

Published online: 23 January 2014

(Usov 2011). In fact, low molecular weight carbohydrates are currently used as chemotaxonomical markers, especially in basal red algae (Karsten et al. 1999, 2003).

To the best of our knowledge, the only previous structural report on the mucilage of *Chroodactylon ornatum* (as *Asterocytis ornata*) is that of Medcalf et al. (1981), who reported that it was constituted of a 3-linked galactan with low sulfate content and devoid of 3,6-anhydrogalactose. Sorbitol was reported as the low molecular carbohydrate (Karsten et al. 1999, 2003).

In red macroalgae, sulfated galactans confer mechanical resistance and protection against dehydration (Kloareg and Quatrano 1988). They have also been ascribed a role in recognition, release, and attachment of reproductive structures (Bouzon et al. 2006). Sulfate and hydroxyl groups in galactans can act as ligands for cations, contributing to algal nutrition (Volesky and Holan 1995). This aspect is the basic principle of the application of red algal galactans as biosorbents in bioremediation protocols (Mehta and Gaur 2005). Biosorption is a passive process which includes several mechanisms such as ion exchange, chelation, adsorption, and diffusion through the cell walls. Adsorption to cell surface ligands occurs passively and rapidly (in few seconds to minutes) and generally contributes to the major part of metal accumulation by algal biomass (Mehta and Gaur 2005). Adsorption and immobilization of copper by algal surfaces contributes to heavy metal tolerance in algae (Lombardi et al. 2002).

Here, we present the composition and structure of the sulfated galactan of the sheath of *C. ornatum* to evaluate its potential role in copper biosorption and tolerance in bioassays.

Materials and methods

Strain stocks of *Chroodactylon ornatum* (UTEX LB 2201) were maintained without agitation in 30‰ salinity f/2 medium (Andersen 2005) under constant illumination (80 μmol photons m^{-2} s^{-1}) at 20 °C.

Bioassays

Bioassays were carried out in Erlenmeyer flasks containing 25 mL of f/2 medium (control) and f/2 medium with the addition of cupric sulfate in order to obtain final nominal copper concentrations of 10, 34, and 100 μM. Initial copper (II) concentrations in the culture media were dosed after Areco and dos Santos (2010). Namely, to a cell-free culture medium (1 mL), 1 mL of sodium diethyldithiocarbamate (DDTC) in ethanol–water (1:1) and 8 mL of 1 mM sulfuric acid were added. The yellow DDTC–copper (II) complex was quantified spectrophotometrically at 440 nm.

The bioassays were carried out in quadruplicate and incubated without agitation under constant illumination by cool fluorescent lights (80 μmol photons m^{-2} s^{-1}) at 20 °C. Every day, flasks were gently hand-agitated. Assays were started with the inoculation of a known algal biomass (expressed as fresh weight). Two end points were selected: 4 and 12 days. At both end points, cells were randomly sampled and viability was confirmed using epifluorescence microscopy. Growth rate was estimated as $(\ln FW_f - \ln FW_i)/t$ where *FW* and *t* refer to fresh weight and end point time, respectively.

In order to estimate water content in *FW* samples, algal aliquots were collected in the same way as for *FW* determination but were oven dried at 40 °C until constant weight.

Pigment composition

Chlorophyll a At least 20 mg (*FW*) of algae were suspended in 1 mL of 96 % ethanol and ultrasonicated in Eppendorf tubes in an ice bath for 2 min. After keeping overnight at 4 °C, pigment content was calculated using the equations of Lichtenthaler (1987) and expressed as $\mu g.mg^{-1}$ *FW*.

Phycobilipigments Thirty milligrams (*FW*) of algal biomass were resuspended in 1 mL of 0.1-M phosphate buffer pH 7 and ultrasonicated in an ice bath for 2 min. After centrifugation, phycobilipigment (PBP) contents were calculated using the equations of Bermejo Román et al. (2002) and expressed as $\mu g.mg^{-1}$ *FW*.

Lipid peroxidation

Lipid peroxides were dosed as thiobarbituric acid-reactive substances (TBARs) in 20 mg (*FW*) algal aliquots by resuspension in freshly prepared trichloroacetic acid (with and without thiobarbituric acid) (Vavilin et al. 1998). Following heating at 95 °C for 30 min, TBARs were determined by their absorbance at 532 nm. A molar extinction coefficient for the thiobarbituric acid-malondialdehyde complex of 155 mM^{-1} cm^{-1} was used. Results were expressed as nmols of TBARs.mg^{-1} *FW*.

Phenolic compounds

According to Singleton et al. (1999), 20-mg (*FW*) algal samples were suspended in 1 mL of 0.1 M phosphate buffer pH 7. Afterwards, 2.5 mL of 1:10 diluted Folin-Ciocalteau reagent were added followed by 2 mL of 0.5 % Na_2CO_3 solution. Absorbance was read at 765 nm after incubation at 45 °C for 15 min. Gallic acid was used as standard and total phenolic compounds were expressed as microequivalents of gallic acid.

Microscopical observations

Quinacrine dihydrochloride (Atebrin) and chlorotetracycline (CTC) were added to living cells in the growth medium to a

final concentration of 10^{-4} M. After 10 min, the cells were washed in fresh medium and observed in a Leica DMIRE2 Confocal TCS SP2 SE microscope with a 488-nm band excitation filter and a 525–530-nm emission filter. Light microscopy was conducted in a Zeiss Axioskop equipped with differential interference contrast optics.

Statistical analysis

Results from different treatments were compared statistically by the non parametric Kruskal-Wallis test (STATISTICA; StatSoft 1999) followed by a Tukey-Cramer post hoc test. Significant differences were validated using an orthogonal contrast analysis. The significance level was fixed at $\alpha<0.05$.

Polysaccharide extraction and carbohydrate analysis

Cells were disrupted by tip-ultrasonication in an ice bath (repeated 15-s pulses at 20 kHz). The pellet was recovered by centrifugation and freeze-dried. Extraction of the lyophilized material proceeded by mechanical stirring in water at room temperature for 12 h (fraction RT), followed by water at 90 °C (×4) for 12 h (fractions W90-1–4). After each extraction step, the residue was removed by centrifugation and the supernatant was concentrated and thoroughly dialyzed using Spectra/Por tubing with a molecular weight cutoff of 6,000–8,000 Da before freeze-drying. Dialyses were performed for 48 h against running tap water followed by 24 h in a close system against distilled water.

Carbohydrate content was analyzed by the phenol-sulfuric acid method (Dubois et al. 1956) using galactose as standard. Sulfate was measured with the turbidimetric method of Dodgson and Price (1962) after hydrolysis of the samples with 1 M HCl for 4–5 h at 105–110 °C and also by ion chromatography. The content of protein was estimated by the method of Lowry et al. (1951), using bovine serum albumin as standard. Molecular weights were calculated from the reducing power according to the method of Park and Johnson (1949).

Monosaccharide composition was determined by gas liquid chromatography (GLC) of the hydrolyzates, using different procedures: (a) when the presence of 3,6-anhydrogalactose was suspected, by the reductive hydrolysis and acetylation according to Stevenson and Furneaux (1991); (b) in absence of 3,6-anhydrogalactose, the regular hydrolysis of the polysaccharides proceeded with 2-M trifluoroacetic acid (90 min, 120 °C) followed by conversion of the hydrolyzates to their alditol acetates. Aliquots of the hydrolyzates were also converted to the acetylated aminodeoxyalditols using (S)-1-amino-2-propanol and (S)-α-methylbenzylamine, in order to determine their absolute configuration (Cases et al. 1995). GLC was carried out on a Hewlett–Packard 5890A gas chromatograph equipped with a flame-ionization detector. Nitrogen was used as the carrier gas, with a head pressure of 15 psi and a split ratio of 100:1. For alditol acetates, the equipment was fitted with a capillary column 30 m × 0.25 mm i.d. 0.20 μm, SP 2330. Chromatography was carried out (a) isothermally at 220 °C for alditol acetates, (b) from 160 to 210 at 2 °C min^{-1}, then from 210 to 240 at 5 °C min^{-1} for partially methylated alditol acetates. The injector and detector temperature were set at 240 °C. For acetylated aminodeoxyalditols, an Ultra2 column was employed operating at 180 to 220 °C at 4 °C min^{-1} and then from 220 to 270 at 1 °C min^{-1}. A derivatized κ-carrageenan was used as a standard for GLC peak assignations. When necessary, GLC–MS analyses were carried out on a Shimadzu QP 5050 A apparatus working at 70 eV using similar conditions to those described above, but using He as a gas carrier at a flow rate of 0.7 mL min^{-1} with a split ratio of 11:1.

Polysaccharides were converted to the triethylammonium salt or to the pyridinium salt before methylation according to Ciucanu and Kerek (1984) (powdered NaOH in dimethyl sulfoxide-iodomethane). Solvolytic desulfation was carried out by the microwave-assisted procedure of Navarro et al. (2007), using six pulses of 10 s each. The sample was subjected to an in situ methylation (Navarro et al. 2007). The desulfated–methylated product, after hydrolysis, was derivatized to the alditol acetates and monosaccharide composition was analyzed by GLC (or GC/MS) as described above. One-pot alkaline treatment was carried out as described by Navarro and Stortz (2003). The polysaccharide (2 mg) was dissolved in water (0.4 mL) and $NaBH_4$ (1 mg) was added. After 1 h, 5 M NaOH (1.7 mL) was added and the solution heated at 80 °C in a water bath. The cyclization reaction was stopped after 5 h by neutralization with 4 M TFA. The solvent was evaporated-off and the residue derivatized to the acetylated alditols according to Stevenson and Furneaux (1991).

Nuclear magnetic resonance

The nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance II 500 spectrometer at 500.13 (^{1}H) and 125.77 (^{13}C) MHz provided with a 5-mm probe, at room temperature, using ca. 20-mg polysaccharide in 0.4 mL of D_2O. Multiplicity determinations and 2D spectra were obtained using standard Bruker software.

Results

Bioassays

According to colorimetric determination, the actual amount of divalent copper cation in the assay media Cu1-3 was always lower than the nominal one (Table 1). In the control medium, the copper concentration was below the range of detection of the method employed. It should be taken into account that the

Table 1 Growth rates, phenolic compounds, and TBARs dosage at both end points (days 4 and 12) for controls and copper exposed cultures (Cu1-3)

	Nominal Cu^{2+} concentration (μM)	Actual Cu^{2+} concentration (μM)	Growth rate (day^{-1})		Phenols ($\mu mol.mg^{-1}$)		TBARs ($nmol.mg^{-1}$)	
			Day 4	Day 12	Day 4	Day 12	Day 4	Day 12
Control	0.04	<0.1[a]	0.051±0.005 A	0.025±0.007 A	2.18±0.10 A	3.49±0.33 A	0.032±0.003 A	0.025±0.001 A
Cu1	10	4.51±0.71	0.077±0.020 A	0.020±0.002 A	1.68±0.40 A	3.60±0.04 A	0.028±0.001 A	0.036±0.001 B
Cu2	34	30.32±1.88	0.059±0.009 A	0.004±0.002 B	3.03±0.50 B	2.83±0.05 B	0.034±0.007 A	0.046±0.005 C
Cu3	100	77.03±6.06	0.033±0.022 A	0.002±0.001 B	3.32±0.16 B	2.82±0.01 B	0.043±0.006 B	0.046±0.003 C

The values refer to mean ± SD, n=4. Different capital letters indicate significant differences within each column according to non parametric Kruskal-Wallis test (p<0.05). Groups formed were verified using contrast analysis

[a] Colorimetric determination fell below the range of detection of the sodium diethyldithiocarbamate (DDTC) method

concentration of free divalent copper cation responds to a chemical equilibrium dependent on pH, temperature, and culture media components which can act as ligands, partially sequestering the free divalent species from the aqueous solution.

After 12 days of metal exposure, significant growth inhibition was observed for the higher copper concentrations (Table 1). In all cases, even when growth rates were severely inhibited (e.g., Cu2 and Cu3) cells remained viable, as indicated by epifluorescence observations. During the acute period (4 days end point), high values for standard deviations disabled to assign significant differences in growth rates respect the control culture, even when a tendency for reduction was observed in Cu3.

At both end points, phenol dosage in control cultures and Cu1 differed significantly from those in assays Cu2 and Cu3 (Table 1), but after 12-days incubation, cultures exposed to the higher metal concentrations exhibited a decrease in phenols dosage respect the control.

During acute metal exposure, TBARs content indicated oxidative stress for assays Cu3 (Table 1). When prolonging exposure, all the copper-exposed cultures exhibited higher values of this oxidative stress indicator respect to the control. In Cu1, no significant changes with respect to the control cultures were detected in pigment composition at both end points (Table 2). On the other hand, Cu2 and Cu3 reduction in chlorophyll and phycobilipigments was always evident. In all cases, chlorophyll content did not change in each treatment between both end points, but antenna pigment dosage was always lower at day 12. Consequently, the chlorophyll *a*/phycobilipigments ratios were always higher at the second end point. At both end points, a significant difference in this ratio was found among control-Cu1 vs Cu2-Cu3 (Table 2).

Microscopic monitoring of metal effect on chloroplasts showed that the stellate morphology started to become blurry with increasing copper concentration (Fig. 1). Pyrenoids were always present and cell size did not exhibit major changes. Both in control and treatments, several vacuoles (smaller and bigger ones) lay dispersed in those parts of the cells not occupied by the chloroplast (Figs. 1 and 2). The acidic nature of these vacuoles was denoted by intense green fluorescence with quinacrine (Fig. 2a). Overlapping of chlorophyll *a* with acid vacuoles was denoted by yellow fluorescence. In aged or copper-stressed cultures, acidic vacuoles were bigger and were generally located on one of the extremes of the cells. CTC, a marker of membrane-associated Ca^{2+}, revealed several small peripherical cytoplasmic vesicles after quenching unspecific fluorescence in the sheaths with EGTA (Fig. 2b). No polarized distribution of such vesicles could be observed, even in germinating cells. Since staining with toluidine blue at pH 1.5 revealed purple metachromasia of the mucilage, we related the numerous acidic vacuoles with the secretion of a sulfated galactan.

Water content in fresh algal biomass was 84.0±0.1 %. Taking into account this relation, we carried out a preliminary evaluation of the capacity of the biomass as a biosorbent and estimated that after incubation in Cu3 (in the present culture conditions, pH and temperature), 1 g of alga (as dry weight) removed 2.5±0.4 mg of divalent copper cation.

Sheath polysaccharide extraction and composition

After sequential aqueous extraction of the lyophilized alga, products extracted at RT and hot water (W90-1 to -4) were obtained. Their yields and compositions in terms of total carbohydrates, sulfate, protein, molecular weight, and constituent monosaccharides are summarized in Table 3. Only small amounts of material were extracted after the fourth hot-water extraction.

Following the last step of polysaccharide extraction, microscopical examination of the residue showed complete sheath disorganization and only minor quantities of toluidine blue-positive material. Products RT and W90-1 had higher protein contents (Table 3) than those of the following successive extractions. On the whole, these two products differed from the rest, both in their composition and in the molar ratios of their monosaccharide components, whereas W90-2 to 4 showed strong similarity to each other. It is noteworthy that

Table 2 Chlorophyll *a*, phycocyanin (PC), phycoerythrin (PE), allophycocyanin (APC) and chlorophyll *a*: phycobilipigments ratio at both end points (days 4 and 12) for controls and copper exposed cultures (Cu1-3)

	Chl *a* (μg.mg^{-1})		PC (μg.mg^{-1})		PE (μg.mg^{-1})		APC (μg.mg^{-1})		Chl *a*/PBP ratio	
	Day4	Day12	Day 4	Day12	Day 4	Day12	Day 4	Day12	Day 4	Day12
Control	0.17±0.04 A	0.21±0.04 A	1.02±0.40 A	0.76±0.35 A	0.75±0.22 A	0.69±0.38 A	0.73±0.15 A	0.44±0.09 A	0.07±0.004 A	0.11±0.013 A
Cu1	0.20±0.01 A	0.24±0.07 A	1.09±0.36 A	0.59±0.10 A	0.88±0.22 A	0.69±0.12 A	0.73±0.10 A	0.44±0.11 A	0.07±0.009 A	0.13±0.058 A
Cu2	0.09±0.01 B	0.09±0.01 B	0.16±0.10 B	0.07±0.01 B	0.17±0.04 B	0.14±0.01 B	0.23±0.05 B	0.22±0.03 B	0.19±0.028 B	0.21±0.010 B
Cu3	0.10±0.01 B	0.09±0.02 B	0.09±0.03 B	0.04±0.01 B	0.19±0.08 B	0.11±0.01 B	0.23±0.15 B	0.17±0.02 B	0.16±0.007 B	0.28±0.055 B

The values refer to mean ± SD, $n = 4$. Different capital letters indicate significant differences within each column according to non parametric Kruskal-Wallis test ($p<0.05$). Groups formed were verified using contrast analysis

the first hot water extraction had the lower yield. Rising water temperature in the extraction procedure led to higher xylose and glucose content in W90-1 (Table 3), the latter presumably as a consequence of floridean starch solubilization. Additionally, a small percentage of mannose was detected in W90-1. In any case, galactose was the main sugar component throughout the extracted products, being the only monosaccharide in W90-4. All the extracts exhibited a high sulfate ester content (ca. 34 %, except for W90-1, contaminated with non-sulfated polysaccharides, which was 22 %). This product also showed the lowest molecular weight concomitant with lower sulfate content and higher xylose molar percentage and smaller quantities of mannose. The configurational analysis of galactose revealed a nearly 1:1 ratio of D/L-enantiomers, suggesting an agaran structure (Table 3).

3,6-Anhydrogalactose was not detected in any of the products by the reductive hydrolysis procedure. Moreover, the absence of 3,6-anhydrogalactose precursor units (6-sulfated units) was confirmed by alkaline treatment. No cyclization was observed even after 16-h alkaline treatment.

Structural analyses of W90-4

Further structural analyses were carried out with W90-4, since it looks as the more homogeneous (galactose only) of the samples, and its lower molecular weight might have been an aid for solubilization. Despite this, the sulfated galactan of W90-4 was poorly soluble in DMSO, even after retrieval of the triethylammonium salt. This resulted in a substantial undermethylation in the attempt of permethylating, as suggested by the high percentage of non-methylated galactose (Table 4, W90-4 TAS) and the nearly absent trimethylated derivatives. The enhanced solubility of the pyridinium salt allowed methylation, leading to a significant reduction in molar percentages of non-methylated galactose and 2-*O*-methylgalactose and an increase in 2,3,6-tri-*O*-methylgalactose (Table 4, W90-4 PS); 6-*O*-methylgalactose increases (19 %), and 2,6-di-*O*-methylgalactose (44 %) is the main component of this permethylated derivative.

Solvolytic desulfation caused a reduction in sulfate content of W90-4 from 34 to 10 %. After desulfation (W90-4 D-PS), nearly equal amounts of 2,3,6- and 2,4,6-tri-*O*-methylgalactose appear, indicating the typical alternating 1,3/1,4-galactan structure of red seaweed galactans (Table 4, W90-4 D-PS) (Stortz and Cerezo 2000). The small amounts of remaining methylated sugars in the desulfated-methylated product arise from undermethylation and from the presence of sulfate groups not removed by the desulfation procedure (about one third of the original sulfate content remains after desulfation).

Analyzing back the W90-4 PS sample (Table 4), after enantiomeric analysis of the main units and considering that L-galactose units should be 4-linked, whereas D-galactose units should be 3-linked, as expected from the agaran backbone

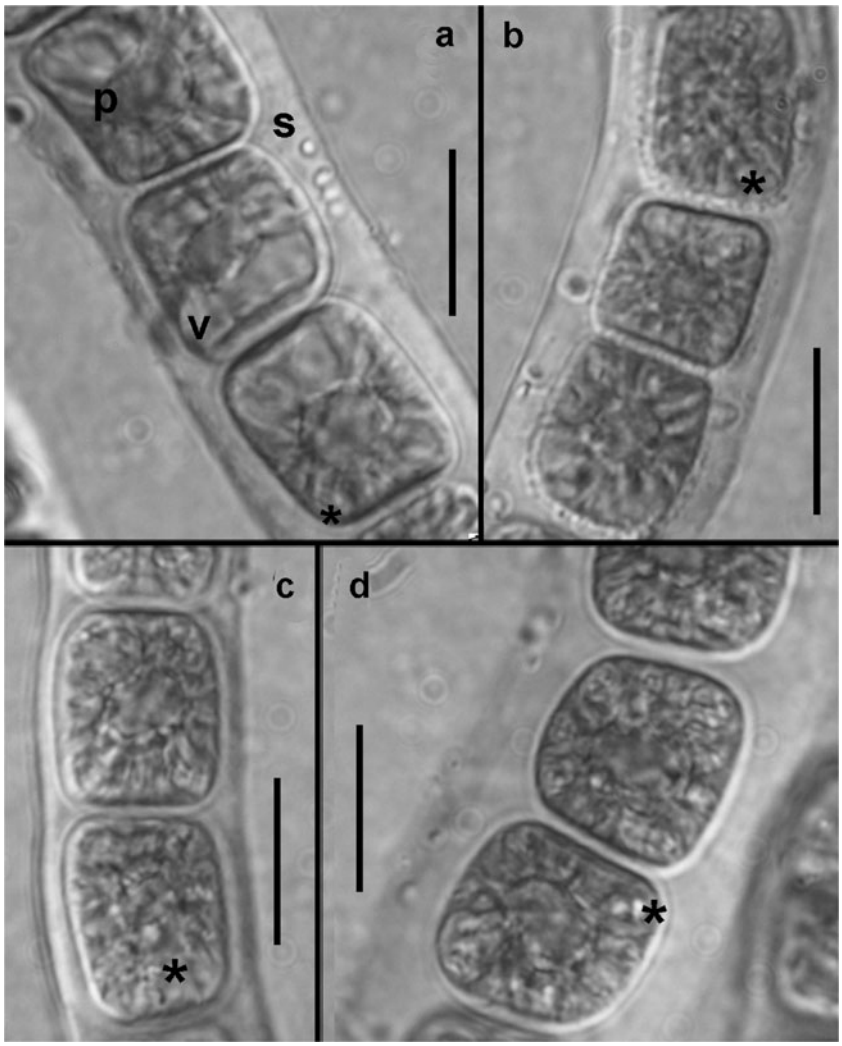


Fig. 1 Nomarski micrographs of filaments of *Chroodactylon ornatum* exposed to different divalent copper cation concentrations. **a** control culture; **b** assay Cu1; **c** assay Cu2, and **d** assay Cu3. All filaments exhibit a prominent mucilage sheath (*s*) and central pyrenoid (*p*) and small secretion vesicles (*asterisk*). In some cases, prominent vacuoles (*v*) are evident. *Scale bar* represents 10 μm

structure (Stortz and Cerezo 2000), the following rationale can be applied: one third of 2,6-di-*O*-methylgalactose, half of 2-*O*-methylgalactose (3 %) and the whole 6-*O*-methylgalactose and 2,4,6-*O*-methylgalactose belonged to the D-series (Table 4). This implies that the 3-linked units appear sulfated in O-4 (38 %), disulfated on O-2 and O-4 (48 %), with small amounts

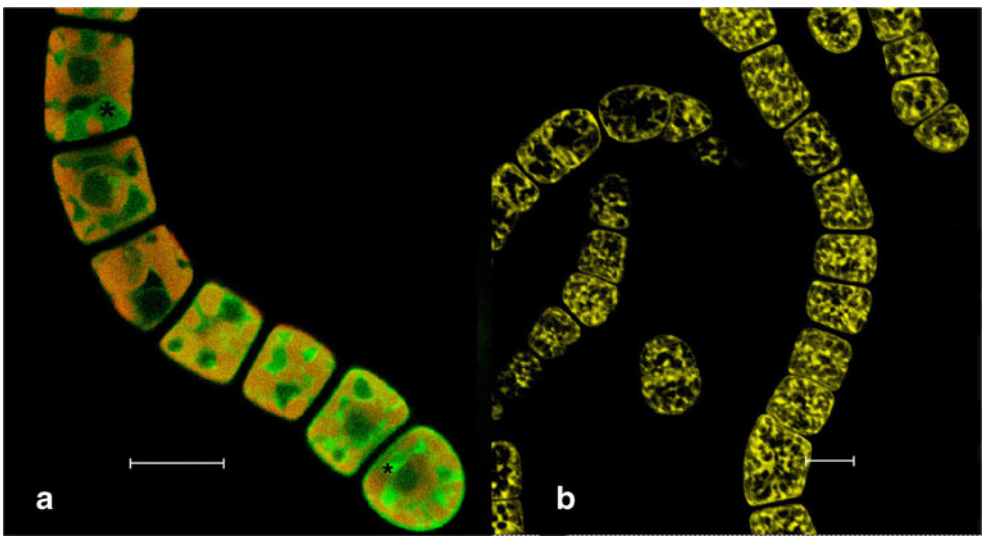


Fig. 2 Confocal microscopy of control cultures. **a** Stained with quinacrine hydrochloride. *Asterisks* indicate quinacrine stained vacuoles of different sizes; **b** Stained with CTC-EGTA. Several small vacuoles are denoted as *bright small areas* all over the cell surface. *Scale bars* = 10 μm

Table 3 Yields and analyses of the products extracted from the sheaths of *Chroodactylon ornatum*

	RT	W90-1	W90-2	W90-3	W90-4
Yield (%)[a]	12.3	3.5	10.3	10.3	10.2
Carbohydrates (%)	30±10[b]	29±6	37±7	35±4	38±4
Protein (%)	21±1	25±2	7±1	4±3	3±2
Sulfate (as % $NaSO_3$)	33±2	22±1	35±1	35±10	34±5
Molecular weight (kDa)	20.9±1.2	10±0.1	51.6±6.4	64.9±9.8	27.3±6.2
Component sugars (mol/100 mol)					
D-Xyl	3±1	10±5	3±1	2±1	1
D-Gal	48±1	38±1	50±1	46±1	50±4
L-Gal	45±1	32±1	43±1	48±1	49±4
Glc	4±1	17±4	4±2	4±1	Tr[c]
Man	–	3±1	–	–	–

[a] For RT yield from liophylized alga; for the remaining products yield of extraction from insoluble residue of RT

[b] The values refer to mean ± SD, $n = 2$

[c] Traces (less than 1 %)

of non-sulfated or 4,6-disulfated units (Table 4, Fig. 3). On the other hand, half of 2-*O*-methylgalactose, two thirds of 2,6-*O*-methylgalactose and the whole 2,3,6-*O*-methylgalactose corresponded to the L-series, indicating that the 4-linked units might be either non sulfated (36 %) or sulfated in O-3 (58 %), with small amounts of products disulfated in O-3 and O-6 (Table 4, Fig. 3).

The ^{13}C-NMR spectrum of W90-4 (Fig. 4), as well as HSQC experiments showed four main anomeric signals, which can be ascribed to 3-linked β-Gal non 2-substituted units (103.7 ppm for ^{13}C/4.50 ppm for ^{1}H), 3-linked β-gal units substituted in C-2, and/or C-2 and C-4 (100.9/4.47), 4-linked α-L-Gal units (100.8/5.28) and 4-linked α-L-Gal 3-sulfated units (100.0/5.29). These assignments were helped by comparison with literature data (Santos et al 1992; Farías et al 2000; Kolender and Matulewicz 2002).

Table 4 Methylation analyses of product W90-4 as triethylammonium salt (TAS), pyridinum salt (PS), and after desulfation (D-PS)

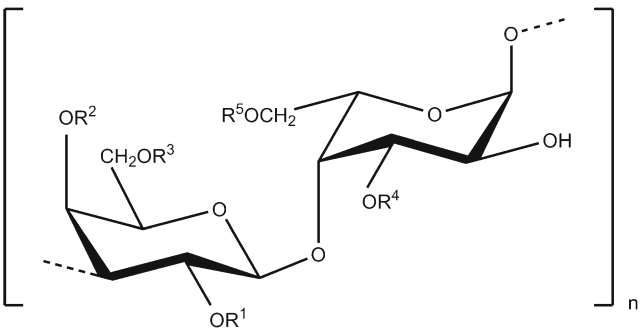
Position of *O*-Me	W90-4 TAS	W90-4 PS	W90-4 D-PS	Linkage analyses	
				→3)α-D-	→4)α-L-
2,4,6-Gal[a]	2	3	33	3	
2,3,6-Gal	2	18	29		18
2,6-Gal	41	44[b]	6	15	29
6-Gal	8	19[b]	2	19	
2,3-Gal		2	8		
2,4-Gal		2	8		
2-Gal	14	6[b]	3	3	3
3 or 4-Gal	1	2	1		
Gal	29	6	4		
Glc		–	5		

[a] 2,4,6-Gal = 2,4,6-tri-*O*-methylgalactose, etc.

[b] Enantiomeric analyses of partially methylated sugars with chiral α-methylbenzylamine indicated the following D/L ratios: 2,6-Gal = (1:2); 2- Gal = (1:1), whereas 6-Gal belonged entirely to the D series

Discussion

The red alga *Chroodactylon ornatum* exhibits, after 12 days of exposure to copper, a growth inhibition much more evident than at day 4 for the higher metal concentrations. An increment in toxicity is expected the longer the exposure period (Eklund and Kautsky 2003). Growth reduction is a very sensitive parameter in algal bioassays, making possible to record it even before other stress signals such as changes in pigment content, oxygen evolution, or photosynthetic efficiency (Brown and Newman 2003). In our assays, oxidative stress symptoms appeared from the acute stage, while reduction in growth rate was somewhat delayed. Yet, it cannot be discarded that high standard deviation of fresh weight values for 4-day cultures masked the deleterious effect on growth



$R^1 = R^2 = SO_3^-$, $R^3 = H$, 48%
$R^1 = R^3 = H$, $R^2 = SO_3^-$, 38%
$R^1 = H$, $R^2 = R^3 = SO_3^-$, 7%
$R^1 = R^2 = R^3 = H$, 7%

$R^4 = SO_3^-$, $R^5 = H$, 58%
$R^4 = R^5 = H$, 36%
$R^4 = R^5 = SO_3^-$, 6%

Fig. 3 Average structure for the diads of 3-linked and the 4-linked galactose units in the sulfated galactan of *Chroodactylon ornatum* (fraction W90-4)

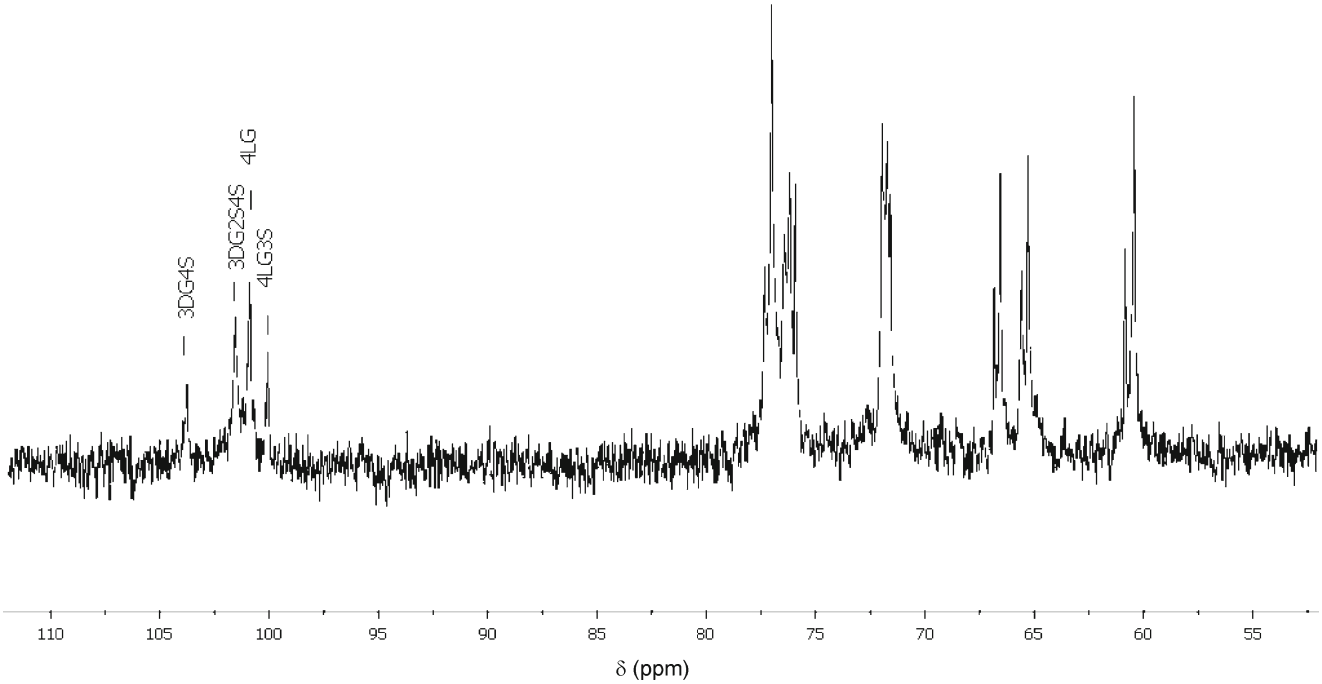


Fig. 4 ^{13}C NMR (125 MHz) spectrum of W90-4. The nomenclature of Miller (1999) is used to identify the units to which the four anomeric carbons belong

rates. Copper is transported across the plasmalemma by a protein transporter of the CTR family (Blaby Haas and Merchant 2012). Metal excess induces the active metal pumping-out of the cell or its inclusion within vacuoles. But metal disposal, together with oxidative stress buffering mechanisms, are energy consuming and ultimately provoke growth reduction.

Oxidative stress symptoms were evident from the first end point in *C. ornatum* in Cu2 and Cu3, namely lipid peroxidation, increased phenol content, and altered pigment composition. Phenolic compounds act as free radical scavengers, reducing agents, and metal chelators, and thus effectively inhibit lipid oxidation. In fact, phenolic compounds dosage increased together with TBARs dosage. Nevertheless, a distinction may be drawn between both end-points. In the acute phase, phenolic compounds increased only in Cu2 and Cu3. However, for these treatments, chronic oxidative stress did not result in further increase in phenolic compounds. In fact, the dosage was lower than in Cu1. This is in agreement with previous reports which indicate that phenolic compounds are effective free radical scavengers in the acute stage of oxidative stress both in cultures (Sztrum et al. 2012) and in natural populations (Ratkevicius et al. 2003; Contreras et al. 2005).

As previously reported for *Gracilaria lemaneiformis* (Xia et al. 2004) there are no major changes in pigment content up to 10-μM Cu. Reduction in pigments in the acute phase is always stronger for PBP. Similar results were reported by Küpper et al. (2002) for *Antithamnion plumula*, with phycobiliproteins disappearing at a faster rate than chlorophyll at 30-μM Cu. Phycobilisomes serve the major light-harvesting function for PS II (Gantt 1990). Down-regulation of PSII acts a protective mechanism against oxidative stress (Hader et al. 2002). Such down-regulation could operate via reduction of both chlorophyll *a* and associated antenna pigments, as observed for the higher copper concentrations in *C. ornatum*. After 12 days of exposure to metal, the ratio chlorophyll *a*/PBP decreases. The relative increase in PBP might be related with ROS scavenging as well as with the antioxidant properties of soluble phycobiliproteins (Romay et al. 2003).

In bioassays, as those performed herein, algal biomass may affect copper toxicity either by changing availability through direct absorption or adsorption or by producing chelates which complex the metal. The sulfated galactan in W90-4 (and presumably in the other products) extracted from the sheath of *C. ornatum* confers negative charge to the cell surface providing sites for the binding of metal cations. The polysaccharide is delivered to the sheath by secretion vesicles of acidic nature as revealed by staining with quinacrine and CTC. We attribute the delayed effect on growth rate to metal extracellular binding in the mucilage sheaths. Note that preliminary biosorption evaluation indicated that when exposed to 100 μM (nominal concentration) *ca.* 65 % of the initial metal cation was removed from the culture solution. Comparing these data (on dry weight basis), the values of copper removal for *C. ornatum* are ca. 25 % of those obtained for *Chondrus crispus* and *Asparagopsis armada* (Romera et al. 2007). Taking into account that these authors, unlike our assays, used an optimum pH for metal complexation, this microalga exhibited an

interesting metal removal capacity. Furthermore, *C. ornatum* is euryhaline and can be employed, for example, in algal mats for estuarine waters.

Heavy metals have been reported to bind to the cell wall and the surrounding mucilage, with only small amounts taken into the cytosol (Diannelidis and Delivopoulos 1997; Andrade et al. 2004). In fact, encapsulated algal cell walls are commercialized as metal biosorbents under the trademark AlgaSorb® (Mehta and Gaur 2005). However, when metal concentration is too high, cell wall polysaccharides do not prevent copper from reaching the cytoplasm. For example, in *Enteromorpha flexuosa* no Cu was detected in cells exposed to 50 μg Cu L^{-1}, while Cu signal became detectable in cells exposed to 250 μg Cu L^{-1} (Andrade et al. 2004). Intracellular copper accumulation was also revealed by accumulation of electron dense granules in various algal vacuoles from copper mine areas (Correa et al. 1999; Leonardi and Vasquez 1999), where copper concentrations reach up to 6 mg L^{-1} (Castilla 1996) (similar to that in Cu3).

Products extracted from the mucilage are very similar, although some differences can be pointed out. Yields are approximately 10 % in all cases, except for W90-1, which is considerably lower (3.5 %). On the whole, W90-1 seems to be heterogeneous, consisting of an agaran fraction together with, at least, floridean starch. Lower galactose and sulfate contents also suggest heterogeneity; for instance, the presence of 10 % xylose does not rule out a xylan or a skeletal galactan substituted with xylose side chains. Yet, further fractionation to obtain more homogeneous fractions and structural analyses on this product are necessary to confirm its composition and structure.

Sulfated galactans in red macroalgae have a linear backbone built up of alternating 3-linked β-D-galactopyranose and 4-linked α-galactopyranose residues. If the latter is in the L-configuration, the galactan belongs to the agar group. The 1:1 ratio D/L galactose indicates that the polysaccharides obtained by sequential aqueous extraction from *C. ornatum* match those encountered in macroalgae, and that they have to be classified as agarans. In both agarans and carrageenans, 4-linked residues may be present, in part or completely, as 3,6-anhydro derivatives. The formation of 3,6-anhydrogalactose residues in vivo proceeds by an enzymatic simultaneous elimination of a sulfate group and a proton of the 3-OH group from 4-linked α-galactose 6-sulfate residues (Genicot-Joncour et al. 2009). Similar transformation in vitro may be readily effected by alkaline treatment of the polysaccharide (Navarro and Stortz 2003). The sulfated agaran in W90-4 of *C. ornatum* lacks both 3,6-anhydrogalactose and the 6-sulfated precursor units, in agreement with the preliminary results reported by Medcalf et al (1981). These authors have reported a non sulfated, 3,6-anhydrogalactose-containing galactan for another Stylonematal alga, *Stylonema alsidii*.

Except for product W90-1, the polysaccharides contained high proportions of sulfate groups (ca. 35 %). Methylation and desulfation-methylation analyses on W90-4 indicated that hydroxyl group on C-3 of the 4-linked units and C-2 and C-4 in the 3-linked unit of the diad were highly substituted with sulfate esters. This pattern of sulfation for the 3-linked unit is unusual and to the best of our knowledge, it has not been previously reported for agarans. The presence of 3-linked β-D-galactose units sulfated on C-2 and C-4 in substantial amounts has only been reported for carrageenans from *Tichocarpus crinitus* (Byankina Barabanova et al. 2013) and *Plocamium costatum* (Miller 1999), which was actually a hybrid D/L-galactan with a different general structure.

Both the methylation analysis and the NMR spectra indicate a 1:1 ratio of 4-linked L-galactose units and 3-linked D-galactose units, as expected for an agaran structure. This differs from the previously proposed structure (a 3-linked galactan) for the mucilage of this species (as *Asterocytis ornata*) reported by Medcalf et al. (1981).

It is noteworthy that the agaran from *C. ornatum* differs considerably from the heteropolysaccharides isolated from *Porphyridium* or *Rhodella* (Gloaguen et al. 2004; Genicot-Joncour et al. 2009). In these microalgae, glucose, D- and L-galactose, xylose, glucuronic and galacturonic acids have been identified as major monosaccharide constituents. Further structural studies of polysaccharides in other Stylenomatophyceae are needed, but the presence of an agaran in this species could be estimated as a chemotaxonomical marker. Structural features show certain similarities with the porphyrans in Bangiophyceae (Usov 2011).

This is the first report of the structure of an agaran in Stylenomatophyceae, with a highly unusual sulfation pattern on the 3-linked unit (3DG2S4S). The high sulfation content of this polysaccharide suggests its relationship with copper tolerance in bioassays, and opens the possibility of further studies, assessing the biosorbent capacity of this alga or its isolated polysaccharides.

Acknowledgments This work was funded by grants from UBA (W-759) and CONICET (PIP 0559/10). JMC is a Research Fellow of the National Research Council of Argentina (CONICET), and CAS is a Research Member of the same institution.

References

Andersen RA (ed) (2005) Algal culturing techniques. Elsevier Academic Press, San Diego

Andrade LR, Farina M, Amado Filho GM (2004) Effects of copper on *Enteromorpha flexuosa* (Chlorophyta) *in vitro*. Ecotoxicol Environ Safety 58:117–125

Areco MM, dos Santos Afonso M (2010) Copper, zinc, cadmium and lead biosorption by *Gymnogongrus torulosus*. Thermodyn Kinet Stud Colloids Surf B: Biointerfaces 81:620–628

Basson PW (1979) Marine algae of the Arabian Gulf coast of Saudi Arabia (second half). Bot Mar 22:65–82

Bermejo Román R, Alvárez-Pez JM, Acién Fernández FG, Molina Grima E (2002) Recovery of pure B-phycoerythrin from the microalga *Porphyridium cruentum*. J Biotechnol 93:73–85

Blaby Haas CE, Merchant SS (2012) The ins and outs of algal metal transport. Biochim Biophys Acta 1823:1531–1552

Bouzon ZL, Ouriques LC, Oliveira EC (2006) Spore adhesion and cell wall formation in *Gelidium floridanum* (Rhodophyta, Gelidiales). J Appl Phycol 18:287–294

Brown M, Newman JE (2003) Physiological response of *Gracilariopsis longissima* (Rhodophyceae) to sublethal copper concentrations. Aquat Toxicol 64:201–213

Byankina Barabanova AO, Sokolova EV, Anastyuk SD, Isakov VV, Isakov VV, Glazunov VP, Volod'ko AV, Yakovleva IM, Solov'eva TF, Yermak IM (2013) Polysaccharide structure of tetrasporic red seaweed *Tichocarpus crinitus*. Carbohydr Polym 98:26–35

Cases MR, Cerezo AS, Stortz CA (1995) Separation and quantitation of enantiomeric galactoses as their mono-*O*-methylethers as their diastereomeric 1-deoxy-1-(2′-hydroxypropylamino)-alditols. Carbohydr Res 269:333–341

Castilla JC (1996) Copper mine tailing disposal in Northern Chile rocky shores: *Enteromorpha compressa* (Chlorophyta) as a sentinel species. Environ Monit Assess 40:171–184

Ciucanu I, Kerek F (1984) A simple and rapid method for the permethylation of carbohydrates. Carbohydr Res 131:209–217

Contreras L, Moenne A, Correa JA (2005) Antioxidant responses in *Scytosiphon lomentaria* (Phaeophyceae) inhabiting copper enriched coastal environments. J Phycol 41:1184–1195

Correa JA, Castilla JC, Ramirez MA, Varas M, Lagos N, Vergara S, Moenne A, Roman D, Brown MT (1999) Copper mine tailings and their effects on marine algae in Northern Chile. J Appl Phycol 11:57–67

Diannelidis BE, Delivopoulos SG (1997) The effects of zinc, copper and cadmium on the fine structure of *Ceramium ciliatum*. Mar Environ Res 44:127–134

Dodgson KS, Price RG (1962) A note on the determination of the ester sulphate content of sulphated polysaccharides. Biochem J 84:106–110

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Analyt Chem 28:350–356

Eklund BT, Kautsky L (2003) Review on toxicity testing with marine macroalgae and the need for method standardization—exemplified with copper and phenol. Mar Pollut Bull 46:171–181

Farías WR, Valente A, Pereira MS, Mourao PA (2000) Structure and anticoagulant activity of sulfated galactans. J Biol Chem 275: 29299–29307

Gantt E (1990) Pigmentation and photoacclimation. In: Cole KM, Sheath RG (eds) Biology of the Red Algae. Cambridge University Press, Cambridge, pp 203–219

Genicot-Joncour S, Poinas A, Richard O, Potin P, Rudolph B, Kloareg B, Helbert W (2009) The cyclization of the 3,6-anhydro-galactose ring of iota-carrageenan is catalyzed by two D-galactose-2,6-sulfurylases in the red alga *Chondrus crispus*. Plant Physiol 151: 1609–1616

Gloaguen V, Ruiz G, Morvan H, Mouradi-Givernaud A, Maes E, Krausz P, Strecker G (2004) The extracellular polysaccharide of *Porphyridium* sp.: an NMR study of lithium-resistant oligosaccharidic fragments. Carbohydr Res 339:97–103

Hader D, Lebert M, Sinha RP, Barbieri ES, Helbling EW (2002) Role of protective and repair mechanisms in the inhibition of photosynthesis in marine macroalgae. Photochem Photobiol Sci 1:809–814

Karsten U, West JA, Zuccarello GC, Nixdorf O, Barrow KD, King RJ (1999) Low molecular weight carbohydrate patterns in the Bangiophyceae (Rhodophyta). J Phycol 35:967–976

Karsten U, West JA, Zuccarello GC, Engbrodt R, Yokoyama A, Hara Y, Brodie J (2003) Low molecular weight carbohydrates of the Bangiophycidae (Rhodophyta). J Phycol 39:584–589

Kloareg B, Quatrano RS (1988) Structure of the cell walls of marine algae and ecophysiological functions of the matrix polysaccharides. Oceanogr Mar Biol Ann Rev 26:259–315

Kolender AA, Matulewicz MC (2002) Sulfated polysacchride from the red seaweed *Georgiella confluens*. Carbohydr Res 337:57–68

Küpper H, Etlík I, Spiller M, Küpper FC, Práil O (2002) Heavy metal-induced inhibition of photosynthesis: targets of *in vivo* heavy metal chlorophyll formation. J Phycol 38:429–441

Lapidot M, Shrestha RP, Weinstein Y, Arad Malis S (2010) Red microalgae: from basic know-how to biotechnology. In: Seckbach J, Chapman D (eds) Red algae in the genomic age. Springer, Netherlands, pp 205–225

Leonardi PI, Vasquez JA (1999) Effects of copper pollution on the ultrastructure of *Lessonia* spp. Hydrobiologia 398(399):375–383

Lewin RA, Robertson JA (1971) Influence of salinity on the form of *Asterocytis* in pure culture. J Phycol 7:236–238

Lichtenthaler H (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 148:350–382

Lombardi AT, Vieira AH, Sartori LA (2002) Mucilaginous capsule adsorption and intracellular uptake of copper by *Kirchneriella aperta* (Chlorococcales). J Phycol 38:332–337

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Medcalf DG, Brannon JH, Scott JR, Allen GG, Lewis J, Norris RE (1981) Polysaccharides from microscopic red algae and diatoms. Proc Int Seaweed Symp 8:582–588

Mehta SK, Gaur JP (2005) Use of algae for removing heavy metal ions from wastewater: progress and prospects. Crit Rev Biotechnol 25: 113–152

Miller IJ (1999) Further evaluation of the structure of the polysaccharide from *Plocamium costatum* with the use of set theory. Hydrobiologia 398/399:385–389

Müller KM, Lynch MD, Sheath RG (2010) Bangiophytes: from one Class to six; where do we go from here? In: Seckbach J, Chapman D (eds) Red algae in the genomic age. Springer, Netherlands, pp 241–259

Navarro DA, Stortz CA (2003) Determination of the configuration of 3,6-anhydrogalactose and cyclizable α-galactose 6-sulfate units in red seaweed galactans. Carbohydr Res 338:211–218

Navarro DA, Flores ML, Stortz CA (2007) Microwave-assisted desulfation of sulfated polysaccharides. Carbohydr Polym 69:742–747

Park JT, Johnson MJ (1949) A submicrodetermination of glucose. J Biol Chem 181:149–151

Pujals C (1961) Algunas observaciones sobre *Asterocytis ornata* (C. Ag.) Hamel, Rodoficea nueva para Argentina. Darwiniana 12:365–377 (in Spanish)

Ratkevicius N, Correa JA, Moenne A (2003) Copper accumulation, synthesis of ascorbate and activation of ascorbate peroxidase in *Enteromorpha compressa* (L.) Grev. (Chlorophyta) from heavy metal-enriched environments in northern Chile. Plant Cell Environ 26:1599–1608

Romay C, Gonzalez R, Ledon N, Remirez D, Rimbau V (2003) C-phycocyanin: a biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. Curr Protein Pept Sci 4:207–216

Romera E, González F, Ballester A, Blázquez ML, Muñoz JA (2007) Comparative study of biosorption of heavy metals using different types of algae. Bioresour Technol 98:3344–3353

Santos JA, Mulloy B, Mourao PA (1992) Structural diversity among sulfated α-L-galactans from ascidians (tunicates). Eur J Biochem 204:669–677

Singleton VL, Orthofer R, Lamuela-Raventós RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In: Lester P (ed) Methods in Enzymology. Academic, London, pp 152–178

Statistica 5.5 (1999) Statsoft Inc, Tulsa, Oklahoma, USA

Stevenson TT, Furneaux RH (1991) Chemical methods for the analysis of sulphated galactans from red algae. Carbohydr Res 210:277–298

Stortz CA, Cerezo AS (2000) Novel findings in carrageenans, agaroids and 'hybrid' red seaweed galactans. Curr Top Phytochem 4:121–134

Sztrum AA, Sabatini SE, Rodríguez MC (2012) Isocitrate lyase activity and antioxidant responses in copper stressed cultures of *Chlamydomonas reinhardtii* (Volvocales, Chlorophyceae). Phycologia 51:135–143

Usov A (2011) Polysaccharides of red algae. Adv Carbohydr Chem Biochem 65:115–217

Vavilin D, Ducruet JM, Matorin DN, Venediktov PS, Rubin AB (1998) Membrane lipid peroxidation, cell viability and Photosystem II activity in the green alga *Chlorella pyrenoidosa* subjected to various stress conditions. J Photochem Photobiol B 42:233–239

Volesky B, Holan ZR (1995) Biosorption of heavy metals. Biotechnol Prog 11:235–250

Xia JR, Li YJ, Lu J, Chen B (2004) Effects of copper and cadmium on growth, photosynthesis, and pigment content in *Gracilaria lemaneiformis*. Bull Environ Contam Toxicol 73:979–986

Yoon HS, Müller KM, Sheath RG, Ott FD, Bhattacharya D (2006) Defining the major lineages of red algae (Rhodophyta). J Phycol 42:482–492

Yoon HS, Zuccarello GC, Bhattacharya D (2010) Evolutionary history and taxonomy of red algae. In: Seckbach J, Chapman D (eds) Red algae in the genomic age. Springer, Netherlands, pp 25–42

Zuccarello GC, West JA, Kikuchi N (2008) Phylogenetic relationships within the Stylonematales (Stylonematophyceae, Rhodophyta): Biogeographic patterns do no apply to *Stylonema alsidii*. J Phycol 44:384–393