

CHARACTERIZATION OF CELL WALL POLYSACCHARIDES OF THE COENOCYTTIC GREEN SEAWEED *BRYOPSIS PLUMOSA* (BRYOPSIDACEAE, CHLOROPHYTA) FROM THE ARGENTINE COAST¹

Marina Ciancia^{2,3}

Cátedra de Química de Biomoléculas, Departamento de Biología Aplicada y Alimentos (CIHIDECAR-CONICET), Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina

Josefina Alberghina

Departamento de Ecología Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria-Pabellón 2, 1428 Buenos Aires, Argentina

Paula Ximena Arata

Cátedra de Química de Biomoléculas, Departamento de Biología Aplicada y Alimentos (CIHIDECAR-CONICET), Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina

Hugo Benavides

Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP), Paseo Victoria Ocampo N° 1, Escollera Norte, B7602HSA Mar del Plata, Buenos Aires, Argentina

Frederik Leliaert, Heroen Verbruggen

Phycology Research Group and Center for Molecular Phylogenetics and Evolution, Ghent University, Krijgslaan 281 (S8), B-9000 Gent, Belgium

and *Jose Manuel Estevez*³

Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE-CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, 1428 Buenos Aires, Argentina

Bryopsis sp. from a restricted area of the rocky shore of Mar del Plata (Argentina) on the Atlantic coast was identified as *Bryopsis plumosa* (Hudson) C. Agardh (Bryopsidales, Chlorophyta) based on morphological characters and *rbcl* and *tufA* DNA barcodes. To analyze the cell wall polysaccharides of this seaweed, the major room temperature (B1) and 90°C (X1) water extracts were studied. By linkage analysis and NMR spectroscopy, the structure of a sulfated galactan was determined, and putative sulfated rhamnan structures and furanosidic nonsulfated arabinan structures were also found. By anion exchange chromatography of X1, a fraction (F4), comprising a sulfated galactan as major structure was isolated. Structural analysis showed a linear backbone constituted of 3-linked β -D-galactose units, partially sulfated on C-6 and partially substituted with pyruvic acid forming an acetal linked to O-4 and O-6. This galactan has common structural features with those of green

seaweeds of the genus *Codium* (Bryopsidales, Chlorophyta), but some important differences were also found. This is the first report about the structure of the water-soluble polysaccharides biosynthesized by seaweeds of the genus *Bryopsis*. These sulfated galactans and rhamnans were in situ localized mostly in two layers, one close to the plasma membrane and the other close to the apoplast, leaving a middle amorphous, unstained cell wall zone. In addition, fibrillar polysaccharides, comprising (1 \rightarrow 3)- β -D-xylans and cellulose, were obtained by treatment of the residue from the water extractions with an LiCl/DMSO solution at high temperature. These polymers were also localized in a bilayer arrangement.

Key index words: *Bryopsis plumosa*; cell wall; green seaweed; pyruvylated galactan sulfate; sulfated polysaccharides

Abbreviations: Ara, arabinose; CB, Coomassie Blue; CW, Calcofluor White; Fuc, fucose; Gal, galactose; Glc, glucose; IEC, ion exchange chromatography; Man, mannose; Rha, rhamnose; RR, Ruthenium Red; TBO, Toluidine Blue ortho-chromatic; TFA, trifluoroacetic acid; Xyl, xylose

¹Received 1 April 2011. Accepted 29 August 2011.

²Author for correspondence: e-mail ciancia@agro.uba.ar.

³Research Member of the National Research Council of Argentina (CONICET).

Green seaweeds biosynthesize matrix sulfated polysaccharides with a great variety of chemical structures. They have been classified into two main groups (Percival and McDowell 1981): (i) sulfated glucuronoxylorhamnans and glucuronoxylorhamnogalactans, with negative optical rotation, and (ii) sulfated xyloarabinogalactans, which have positive optical rotation. As example of the first group, the major acidic polysaccharide structure of *Ulva* and *Entoromorpha* species (Ulvales) is a linear sulfated polymer comprising alternating 4-linked β -D-glucuronic acid and α -L-rhamnose units (Lahaye and Robic 2007). On the other hand, seaweeds belonging to the Bryopsidales (Chlorophyta) are supposed to belong to the second group, and only small to trace amounts of uronic acids were detected in some of these samples. The genus *Bryopsis*, as well as *Codium*, belongs to the suborder Bryopsidineae, comprising species generally occurring in temperate, tropical, and subtropical marine waters (Lamb and Zechman 2006, Guiry and Guiry 2011). There is very little information about these polysaccharides. Moreover, only the sulfated polysaccharides from some species of the genus *Codium* (Love and Percival 1964a, Bilan et al. 2007, Ciancia et al. 2007, Farias et al. 2008, Estevez et al. 2009, Ohta et al. 2009) have been studied in detail. It has been shown that *Codium* species biosynthesize sulfated galactans constituted by 3-linked β -D-galactopyranose (β -D-Gal) residues partially sulfated on C-4 and/or C-6, with ramifications on C-6 and important amounts of pyruvate forming mainly five-membered cyclic acetals (*S* configuration) with *O*-3 and *O*-4 of nonreducing terminal β -D-Gal residues. A minor part of pyruvate forms six-membered cyclic acetals with *O*-4 and *O*-6 (*R* configuration) (Bilan et al. 2006). The relative amount of these structural units in the galactan varies with the species and also for different fractions of the same species (Bilan et al. 2007, Farias et al. 2008, Estevez et al. 2009). Besides, a highly sulfated pyranosic arabinan was detected in *C. dwarkense* (Siddhanta et al. 1999) and also in *C. fragile* and *C. vermilara* and characterized (Ciancia et al. 2007, Estevez et al. 2009). Its structure is now under study. In addition, hydroxyproline-rich glycoproteins (HRGPs) have been detected in minor quantities (Estevez et al. 2009, Fernández et al. 2010). A small fraction from *C. fragile* showed a neutral arabinan composed of 3-linked and 5-linked α -L-arabinofuranose residues; it has been suggested that this structure could be part of the carbohydrate moiety of the HRGPs (Estevez et al. 2009).

The temporal scale of cell wall variability of several green, red, and brown seaweeds, in most of the cases, was linked to complex environmental exogenous factors as well as endogenous changes in the organisms (e.g., growth and development, morphological change, reproduction, etc.). In addition, the importance of the variability displayed in the structure of cell wall polysaccharides from *C. vermilara*

and their relative amounts in relation to different geographic locations and seasons has been addressed recently in our group (Fernández et al. 2011). Briefly, samples from three different populations from the Argentine coast showed: (i) an important variation in the relative content of sulfated arabinans, which increases from north to south, and (ii) a measurable degree of cell wall variability in the sulfate distribution between the different sulfated polysaccharides, independently of the amount of each polysaccharide present and of total sulfate content. Overall, these results suggested that *C. vermilara* has developed a mechanism to adjust the total level of cell wall sulfation by modulating the molar ratio of the individual polymers and also by adjusting the sulfation level in each type of polymer, whereas nonsulfated polymers, as the main structural polysaccharides, did not change significantly over the time or growing stage.

Only a recent reference has appeared concerning characterization of the acidic polysaccharides from *Bryopsis* (Song et al. 2010). The hot water extract of *Bryopsis plumosa*, as well as the acidic and alkaline extracts were obtained from culture samples from Qingdao (China). The acidic extract gave glucose as major monosaccharide component. The alkaline and hot water extracts were quite similar, mainly composed of carbohydrates, but also sulfate and proteins. The major sugar components were galactose (Gal), followed by arabinose (Ara), also minor amounts of glucose (Glc), mannose (Man), rhamnose (Rha), and fucose (Fuc) were detected. The only structural feature informed was sulfation on a primary carbon atom, inferred from the infrared spectrum. The best in vitro antioxidant activity was found for the acidic extract (Song et al. 2010).

On the other hand, more information is available about the fibrillar components of the cell walls of the Bryopsidales. These seaweeds lack cellulose, or at least this polymer is not the major fibrillar component of the cell wall, and have β -(1 \rightarrow 4)-mannans (Love and Percival 1964b, Mackie 1969, Huizing and Rietema 1975, Chanzy et al. 1984, Kaihou et al. 1993, Ciancia et al. 2007) or β -(1 \rightarrow 3)-xylans (Mackie and Percival 1959, Mackie 1969, Fukushi et al. 1988, Maeda et al. 1990, Yamagaki et al. 1997) instead. In addition, it has been shown that the major fibrillar component of the cell wall can vary in the different life stages (Huizing and Rietema 1975, Wutz and Zetsche 1976, Huizing et al. 1979). Thus, gametophyte microthalli of *Derbesia* spp. biosynthesize xylans, whereas sporophyte macrothalli of the same genus produce mannans. Similarly, gametophyte macrothalli from the genus *Bryopsis* biosynthesize xylans, whereas sporophyte microthalli produce mannans. However, considerable amounts of a glucan were detected in some cases (Wutz and Zetsche 1976, Huizing et al. 1979). Moreover, cellulose was found to comprise <10% (w/w) of the fibrillar material of the cell walls from *Bryopsis*

maxima, being present in an amorphous state, in contrast to the trihelical arrangement of the β -(1 \rightarrow 3)-xylan (Fukushi et al. 1988, Maeda et al. 1990).

In this work, we carried out the morphological and phylogenetic characterization of *Bryopsis plumosa* from Mar del Plata (Buenos Aires, Argentina). Besides, its cell wall polysaccharides were studied, and we were able to isolate and determine the structure of a highly pyruvylated galactan sulfate. Its structure is compared with that of seaweeds from the genus *Codium*.

MATERIALS AND METHODS

Algal sample. Thalli of *B. plumosa* were collected in Mar del Plata, Province of Buenos Aires, Argentina, in December 2008. A voucher material was deposited in the herbarium of the Museo Bernardino Rivadavia, Buenos Aires, Argentina (collection number N° BA47428). Gametophyte thalli of *B. plumosa* were washed with filtered seawater and analyzed for epiphytic and epizoic contaminants in a Nikon AFX-II macroscope (Nikon, Tokyo, Japan). Sporophytic microthalli were not found.

DNA sequencing and clustering. A dry sample of the Mar del Plata population was preserved in silica-gel for molecular analyses. DNA extraction followed Verbruggen et al. (2007). The *rbcL* gene was sequenced as described by Verbruggen et al. (2009a) and the *tufA* gene as described by Verbruggen et al. (2009b). Both sequences were submitted to GenBank (*rbcL*: JQ755422; *tufA*: JQ755423). The *rbcL* sequence was combined with sequences available in GenBank (list of GenBank accession numbers in Fig. 2) and aligned by eye. The alignment was submitted to UPGMA clustering analysis in PAUP, with 100 bootstrap replicates.

LM and histochemistry. For LM semithin sections (~10 μ m) were mounted on glass slides and then observed with a Carl Zeiss Axiolab microscope (Carl Zeiss, Jena, Germany). The staining procedures used in LM histochemical characterization based on Krishnamurthy (1999) were carried out on fixed tissues described before and included (i) Toluidine Blue O (TBO; 0.05% w/v) in 0.1 M HCl at pH 1.0 that stains sulfated polysaccharides (red-purple, γ metachromasia), (ii) Ruthenium Red (RR; 0.001%) in aqueous solution (Luft 1971) for acidic polysaccharides (red), (iii) Calcofluor White (CW; 0.1% w/v) in aqueous solution for detection of β -(1 \rightarrow 3)- and β -(1 \rightarrow 4)-polysaccharides, and (iv) Coomassie Blue (CB; 0.1% w/v) in methanol: water: acetic acid (25:73:2 v/v) that stains proteins. The residues RX2 and DR from the sequential extractions were stained with Lugol's (iodine in 0.5% IK) for α -glycans (brown).

Extraction of the polysaccharides. Thalli of *B. plumosa* were washed with filtered seawater and analyzed for epiphytic and epizoic contaminants in a Nikon AFX-II macroscope.

The selected plants were dried, milled (100 g), and then extracted with EtOH 96% (1,000 mL) for 3 h at room temperature. The residue was separated from the supernatant by centrifugation in a Sigma 4K15 (Niedersachsen, Germany) refrigerated centrifuge (8000g, 10°C) and dried by solvent exchange and finally in vacuo. This treatment was carried out once more. The residue from the ethanol extraction was extracted with H₂O (20 g \cdot L⁻¹) at room temperature for 18 h and centrifuged. The supernatant was dialyzed and freeze-dried, obtaining B1. The residue from the first H₂O extraction was extracted one more time in similar conditions, giving B2. The residue from the second room-temperature water extraction was extracted twice for 3 h with water at 90°C, giving two

extracts. The first one was treated with α -amylase (Knutsen and Grasdalen 1987) to give a purified sample, X1. The residue RX2 (1 g) from the water extraction was extracted with an 8.4% LiCl solution in DMSO (200 mL) at 106°C for 5 h (Flores et al. 2000). The mixture was cooled to room temperature and centrifuged obtaining residue RD and a supernatant SD. Both were dialyzed (molecular weight cut-off 6.0–8.0 kDa) and freeze-dried. DS was treated with α -amylase (Knutsen and Grasdalen 1987).

Ion exchange chromatography (IEC). X1 was chromatographed on DEAE-Sephadex A-25. The sample (100 mg) was dissolved in water and centrifuged, and the supernatant was applied to a column (90 \times 1.5 cm i.d.), previously stabilized in H₂O. The first elution solvent was water, and then it was NaCl solutions of increasing concentration of up to 4 M. Fractions of 4 mL were collected. Finally, the phase was boiled in 4 M NaCl solution. The presence of carbohydrates in the samples was detected by the phenol sulfuric acid method (Dubois et al. 1956); after obtaining blank readings, the eluant was replaced by another with higher concentration of NaCl. The products obtained were dialyzed (molecular weight cutoff 3,500) and freeze dried.

Chemical analysis. The total sugar content was analyzed by PhOH-H₂SO₄ method (Dubois et al. 1956). Sulfate was determined turbidimetrically (Dodgson and Price 1962). The protein content was determined by the method of Lowry et al. (1951). The pyruvic acid content was determined by the method of Koepsell and Sharpe (1952), whereas the percentage of uronic acids was determined according to Filisetti-Cozzi and Carpita (1991). The total carbohydrate content of RX2, DS, and DR was analyzed by a phenol-sulfuric acid method adapted for insoluble material (Ahmed and Labavitch 1977). To determine their monosaccharide composition, the samples (1–3 mg) were dissolved in 100% trifluoroacetic acid (TFA; 37°C, 1 h), followed by dilution of the acid to 80%, heating at 100°C for 1 h, and further dilution to 2M to achieve the regular hydrolysis conditions, reduction with NaBH₄, and acetylation (Morrison 1988). For these same samples, total nitrogen and sulfur were estimated, after total degradation, by conversion to N₂ and SO₂, respectively. The mixtures of gases were separated by gas chromatography with a porapac column in Carbo Erba EA 1108 chromatograph (Milan, Italy), using a thermal detector; the total protein content was expressed using a factor of 5.13 (Lourenço et al. 2002).

Methylation analysis. The polysaccharide (5–10 mg) was converted into the corresponding triethylammonium salt (Stevenson and Furneaux 1991) and methylated according to Ciucanu and Kerek (1984). The sample was dissolved in dimethylsulfoxide; finely powdered NaOH was used as base. For SD, an 8.4% LiCl solution in DMSO was used, and the reaction was carried out at 55°C; the mixture was sonicated before addition of NaOH. Iodomethane was used as methylation agent. The methylated samples were derivatized to the alditol acetates in the same way as the parent polysaccharides.

Gas chromatography (GC). GC of the alditol acetates were carried out on a Hewlett Packard 5890A gas-liquid chromatography (Avondale, PA, USA) equipped with a flame ionization detector and fitted with a fused silica wall coated open tubular column (0.25 mm i.d. \times 30 m) with a 0.20 μ m film of SP-2330 (Supelco, Bellefonte PA, USA). Chromatography was performed: from 200°C to 230°C at 1°C \cdot min⁻¹, followed by a 30 min hold for alditol acetates. For the partially methylated alditol acetates, the initial temp. was 160°C, which was increased at 1°C \cdot min⁻¹ to 210°C and then at 2°C \cdot min⁻¹ to 230°C. N₂ was used as the carrier gas at a flow rate of 1 mL \cdot min⁻¹, and the split ratio was 80:1. The injector and detector temperature was 240°C.

GC-mass spectrometry (MS). GC-MS of the methylated alditol acetates was performed on a Shimadzu GC-17A gas-liquid chromatography equipped the SP-2330 interfaced to a

GCMSQP 5050A mass spectrometer (Kyoto, Japan) working at 70 eV. The total flow rate was $7 \text{ mL} \cdot \text{min}^{-1}$, and the injector temperature was 240°C . Mass spectra were recorded over a mass range of 30–500 amu.

NMR spectroscopy. The 500 MHz ^1H NMR, proton decoupled 125 MHz ^{13}C NMR spectra, and two-dimensional NMR experiments [heteronuclear multiple quantum coherence (HMQC) and correlation spectroscopy] were recorded on a Bruker AM500 at room temperature, with external reference of TMS. Samples were exchanged in 99.9% D_2O (0.5 mL) four times. Chemical shifts were referenced to internal acetone (δ_{H} 2.175, δ_{CH_3} 31.1). Two-dimensional NMR experiments were recorded in DMSO for DS.

RESULTS AND DISCUSSION

Samples of *Bryopsis* sp. were collected in a restricted area of the rocky shore of Mar del Plata located at $38^\circ 00' \text{ S}$, $57^\circ 33' \text{ W}$, on the Atlantic coast of Argentina. In the area, this seaweed is usually found in sheltered habitats or in habitats with low wave action. It is distributed from the lowermost part of the midlittoral zone to the few upper meters of the subtidal (Olivier et al. 1970, Sar et al. 1984). It is also observed in deep tide pools at the upper midlittoral, forming a characteristic fringe between 30 and 80 cm depth. The species is epilithic growing on both hard rocky and sedimentary slit substrates. Less commonly, it is epiphytic on coralline rhodophytes (*Corallina officinalis*, *Jania* sp.) and epizoid on mussels (*Brachydontes rodriguezii*). The temporal distribution of the species covers all the annual cycle, showing a seasonal variation with two periods of maximum abundance, one in early summer and another in autumn. During these periods, it is common to observe an extension in its zonal distribution to the middle levels of the intertidal in not much exposed areas (H. Benavides, pers. comm.).

Boraso de Zaixso (2004) reviewed the species of *Bryopsis* recorded for the Argentine coast. Papenfuss (1964) reported the presence of *B. plumosa* for the Atlantic littoral from the coast of Uruguay to Tierra del Fuego. However, no study about species of this genus particularly in the coast of Buenos Aires province, north of the Argentine Atlantic littoral has been reported. There is only a reference to the genus, as *Bryopsis*, in this area in a technical survey (Parma et al. 1987), so studies of the phylogeny and morphology of this seaweed for this particular location have not been published before. Morphological characters of the sample collected in Mar del Plata (Argentina) are identical to those corresponding to *Bryopsis plumosa* (Agardh 1823, Harvey 1858, Setchell and Gardner 1920, Collins 1928, Feldmann 1937, Taylor 1937, Koster 1941, Fritsch 1956, Yale Dawson et al. 1964, Coll Cunillera 1976, Medlin 1984) comprising dark to mid-green thalli forming dense tufts, from 3.5 to 10 cm in length (Fig. 1a). Gametophyte thallus is uniaxial, with delicate feather-like branches or pinnae decreasing gradually towards the apex beginning from a rhizoidal holdfast. The main

axis is rarely dichotomous, of 0.4 mm in diameter, with first-order branches located at the upper 1/3–2/3 portion, and irregularly branched rhizoids at the lower end. All branches are regularly and distichously arranged in two rows on opposite sides of the central filament (Fig. 1b). Each pinna is constricted; it shows symmetrically rounded margins at the base and has an obtuse to rounded apex (Fig. 1c). However, because morphological identification of morphologically simple algae can be misleading, we have also generated *rbcL* and *tufA* barcodes to characterize the Argentine taxon. A UPGMA dendrogram of the *rbcL* alignment showed that the sequence from the Argentine taxon is identical to that of two sequences of *B. plumosa* from France and Australia, and a sequence of *B. vestita* from New Zealand (Fig. 1d, BV = 99). The analysis also showed that the species *B. plumosa*, as morphologically defined, is polyphyletic (arrows in Fig. 1d). Therefore, our *rbcL* and *tufA* DNA barcodes will

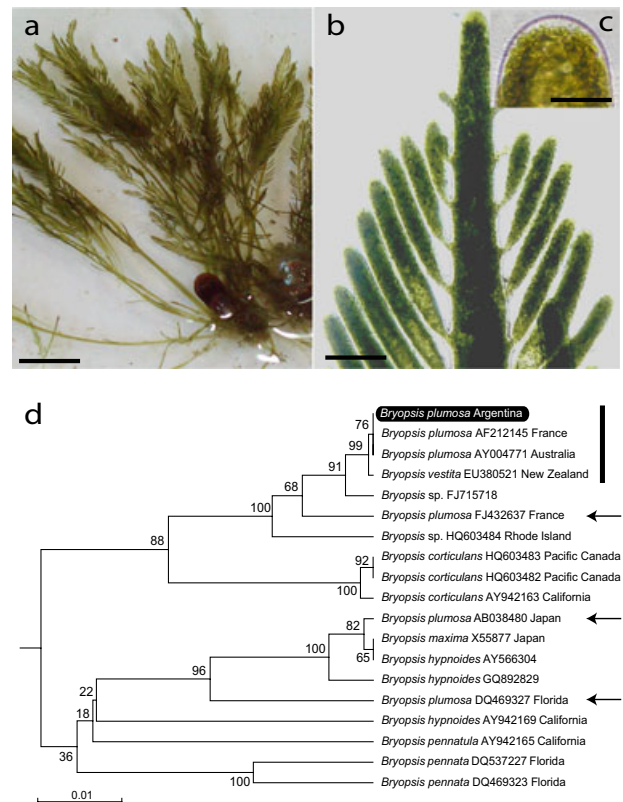


FIG. 1. (a) General aspect of the thallus of *Bryopsis plumosa*. Scale bar = 1 cm. (b) Gametophyte thallus is uniaxial with delicate feather-like branches or pinnae decreasing gradually toward the apex. Scale bar = 1 mm. (c) Coenocytic structure of a single utricle cell. Scale bar = 200 μm. (d) A UPGMA dendrogram of the *rbcL* alignment showed that the sequence from the Argentine taxon is identical to that of two *B. plumosa* sequences from France and Australia and a *B. vestita* sequence from New Zealand. The species *B. plumosa*, as morphologically defined, is polyphyletic (arrows). UPGMA, unweighted pair group method with arithmetic mean.

continue characterizing the strain we have used in the light of future taxonomic changes that may be carried through to resolve this taxonomic conundrum. Only the *rbcL* alignment was analyzed in this study, because a larger set of reference sequences is presently available for this gene in GenBank. However, a *tufA* barcode was also generated because this gene has recently been proposed as the most suitable barcoding gene for Ulvophyceae (Saunders and Kucera 2010) and data availability for this gene can be anticipated to grow drastically over the coming years.

At the cell wall level, two fibrillar layers (fl) and one amorphous layer (al) can be distinguished (Fig. 2a). To show the *in situ* localization of fibrillar polysaccharides in the cell walls of *B. plumosa*, Calcofluor White (CW) staining (Krishnamurthy 1999) was used and two distinct layers were detected (Fig. 2b). Besides, the presence of negatively charged sulfate groups was revealed with TBO at pH = 1 (Fig. 2c) (Krishnamurthy 1999) and RR (Fig. 2d) stainings, showing two external layers, one close to the apoplast and the other, to the cytoplasm. This arrangement of fibrillar and sulfated polysaccharides in the cell wall seems partially different to that found in *Codium* spp. (Estevez et al. 2009, Fernández et al. 2010), in which the fibrillar

domain was divided in two layers, while sulfated polysaccharides formed a middle amorphous layer. Cell wall proteins were visualized in the amorphous layer and in the upper fibrillar layer (Fig. 2e).

To study the cell wall of *B. plumosa*, water-soluble polysaccharides were isolated (Fig. S1 in the supplementary material) giving two major fractions, one at room temperature (B1) and another one at 90°C, both of them in low yields (Table 1). Analysis of the major room temperature water extract, B1, showed similar quantities of carbohydrates and proteins, and also considerable amounts of sulfate. The major monosaccharide constituent was Gal, followed by Ara, and small amounts of other sugars together with a significant amount of pyruvic acid. On the other hand, the major hot water extract contained Glc as main carbohydrate component. By treatment with α -amylase a purified product, X1, was isolated, which contained Gal and Ara in important quantities, but also Rha, and minor amounts of other sugars (Table 1). In addition, X1 was fractionated by anion exchange chromatography giving 10 fractions (F1–F10, Table S1 in the supplementary material) with increasing sulfate content. An incipient separation according to the monosaccharide composition was also observed. F1 is enriched in Rha, F3 in Ara, whereas in F4–F10, Gal is the major monosaccharide component. Yields of these fractions were very low, with the exception of fraction F4.

Methylation analysis of B1 gave mainly Gal and Ara partially methylated monosaccharides (Table 2). The galactose derivatives showed a pattern very similar to that found by methylation analysis of galactans from *Codium* (Ciancia et al. 2007, Estevez et al. 2009). The ^{13}C NMR spectrum of B1 was complex (Fig. 3a). In the anomeric region, two main signals were present at 104.6 and 103.7 ppm, which could correspond to C-1 of β -D-Gal units. No signals were detected at lower fields suggesting the absence of α -Ara in the furanose form. A peak at 26.0 ppm, which correlated with the signal at 1.41 ppm in the HMQC spectrum (not shown), as well as the peak at 101.7 ppm, which appeared only in the ^{13}C NMR spectrum (C-2 of the pyruvic acetal in *R* configuration), confirmed the presence of pyruvylated units forming a 6-membered cycle with O-4 and O-6 of the β -D-Gal units (Bilan et al. 2006), giving a ratio Gal:pyruvate of 3:1 (Table 3). Thus, 1/3 of the galactose units in the galactan backbone were pyruvylated. On the other hand, the methylation pattern showed that at least most of the sulfate was also substituting the galactose units, indicating the presence of galactose sulfated on C-4 or C-6, or both positions. However, a certain degree of substitution with side chains could be ruled out. Besides, $\sim 14\%$ of the 3-linked Gal units was not substituted (Table 2). These data showed that the galactan in B1 has structural features similar to those found in *Codium* extracts, the most important difference is the presence in B1 from *B. plumosa* of the 3-linked

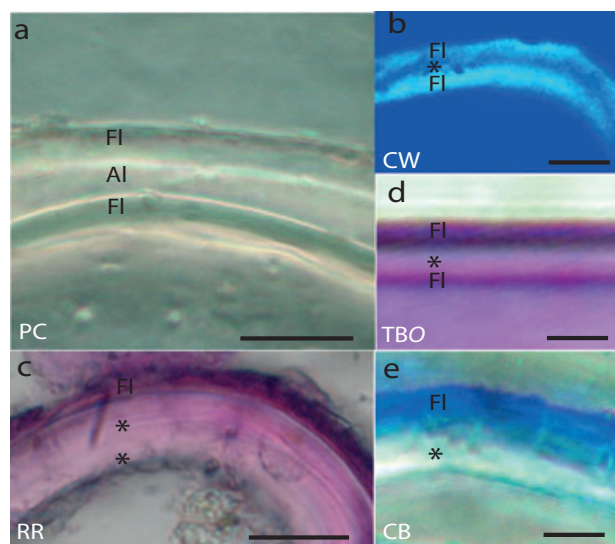


FIG. 2. *In situ* distribution of cell wall polymers. (a) Double two fibrillar (FI) and amorphous (AI) cell wall layers of the utricle in longitudinal section observed with light-directed and phase contrast (PC) microscopy. (b) Distribution of (1 \rightarrow 3)- β -D-xylans and cellulose in cell walls of *Bryopsis plumosa* by Calcofluor White (CW) staining. Scale bar = 10 μm . Absence of labeling is denoted by an asterisk (*). (c) Localization of sulfated polymers in the cell wall from *B. plumosa* assessed by histochemistry. Negatively charged sulfate groups present in the sulfated polysaccharides were labeled with Toluidine Blue O (TBO) at pH = 1. Scale bar = 10 μm . (d) Negatively charged polymers location (sulfated and pyruvylated polymers) with Ruthenium Red (RR). Absence of labeling is denoted by an asterisk (*). (e) Cell wall proteins visualized with Coomassie Blue (CB). Scale bar = 10 μm . Absence of labeling is denoted by an asterisk (*).

TABLE 1. Yields and analyses of the cell wall fractions isolated from *Bryopsis plumosa*.

Yield ^a %	Total carbohydr. %	Sulfate (SO ₃ Na) %	Proteins ^b %	Monosaccharide composition (mols %)							
				Rha	Fuc	Xyl	Ara	Man	Gal	Glc	
Water-soluble cell wall fractions											
B1 ^c	0.8	33.9	12.5	32.0	3.6	1.9	4.7	14.8	5.9	63.8	5.4
X1 ^d	1.3	21.4	4.7	nd	17.4	4.1	6.5	28.3	6.9	24.0	9.8
F4	0.2	nd	9.1	nd	8.6	4.5	17.2	13.6	5.8	42.7 ^b	7.7
Fibrillar cell wall fractions											
RX2	62.3	40.0	7.7	28.2	–	–	46.8	3.5	tr.	3.8	45.9
DS	29.5	56.9	1.6	13.9	–	–	65.8	2.8	tr.	1.8	29.6
DR	29.7	8.1	5.8	41.0	–	–	26.9	11.7	2.0	11.8	47.7

Ara, arabinose; Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Xyl, xylose.

^aPer 100% of milled seaweed.

^bNitrogen × 5.13 (Lourenço et al. 2002).

^cSmall amounts of pyruvic (3.7%) and uronic (2.5%) acids were detected.

^d2.3% of pyruvic acid and 5.6% of uronic acids were found.

TABLE 2. Methylation analysis of cell wall fractions isolated from *Bryopsis plumosa*.

Monosaccharide ^a	B1 ^b	X1 ^c	F4 ^{b,d}	DS	Major structural units
2,3,4-Rha	–	1	–	–	Rhap(1 →
3,4-Rha	–	4	–	–	→ 2)Rhap(1 →
2,4-Rha	2	3	8	–	→ 3)Rhap(1 →
2-Rha	–	5	–	–	→ 3,4)Rhap(1 →
Rha	–	3	–	–	→ 2,3,4)Rhap(1 →
2-Fuc	–	1	–	–	–
2,3,4-Xyl	–	5	2	–	Xylp(1 →
2,4-Xyl	4	3	–	41 ^c	→ 3)β-D-Xylp(1 →
Xyl	–	1	–	8	–
2,3,5-Ara	5	4	–	3	Araf(1 →
3,5-Ara	16 ^c	17 ^c	2	10	→ 2)Araf(1 →
2,3-Ara	–	3	–	–	→ 5)Araf(1 →
3-Ara	4	–	3	–	→ 2,5)Araf(1 →
2,3,4,6-Man	–	4	–	–	–
2,3,6-Man	–	2	–	–	–
3-Man+Man	–	2	–	–	–
2,3,4,6-Gal	–	1	–	–	–
2,4,6-Gal	8	2	36 ^c	1	→ 3)β-D-Galp(1 →
2,3,4-Gal	–	1	–	–	–
2,6-Gal	10	6	3	3	→ 3)β-D-Galp 4S(1 →
3,6-Gal	2	–	–	4	–
2,3-Gal	–	2	–	–	–
2,4-Gal	9	5	29 ^c	–	→ 3)β-D-Galp 6S(1 →
6-Gal	1	2	–	–	–
2-Gal	27 ^c	9 ^c	16	–	→ 3)β-D-Galp 4,6 PyT(1 → → 3)β-D-Galp 4,6 S(1 →
Gal	–	3	–	–	–
2,3,6-Glc	3	5	–	30 ^c	→ 4)GlcP(1 →
Glc	11	4	–	–	–

Ara, arabinose; Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Xyl, xylose.

^aMethylated at the positions indicated.

^bMols %.

^cTraces (<1%) of 2,3,4-Xyl; 2,3,4,6-Gal; 6-Gal; 2,3,4,6-Man; 2,3,6-Man; Rha were detected in B1.

^dSmall amounts of monomethylated sugars were detected.

^ePercentages significant for the structure of the sample are shown by bold.

4,6-*O*-(1'-(*R*)-carboxy)ethylidene-β-D-galactopyranose (4,6-Pyr-β-D-Gal) units in the galactan chain, instead of 3,4-*O*-(1'-(*S*)-carboxy)ethylidene-β-D-galactose terminal units, which predominated in the former

galactans (Bilan et al. 2007, Ciancia et al. 2007, Farias et al. 2008, Estevez et al. 2009, Ohta et al. 2009). It is important to note that this difference could have great consequences in the galactan structure and conformation, as that of *Codium* is a highly ramified structure, whereas there is no evidence of ramification, at least with Gal units in B1.

An important peak in the gas-liquid chromatogram of methylated B1 was identified by GC-MS as 2,4,6-tri-*O*-acetyl-3,5-di-*O*-methylarabitol (Table 2). However, no peaks were detected in the anomeric region of the ¹³C NMR spectrum corresponding to those reported for α-L-arabinofuranosyl residues. Whether this was due to the fact that this structure comprises β-L-arabinofuranose residues (anomeric signals at ~103 ppm, Bock and Pedersen 1983) or the anomeric signal of the α-L-arabinofuranose residues was shifted down-field by the influence of the C-2 substitution, or both, is under study. These facts could make the anomeric signal of these units appear in the same region of the spectrum as those of the galactan. Nevertheless, it is clear that the arabinan moiety was mostly composed by nonsulfated units. It is not known yet if this arabinan structure formed an arabinogalactan, constituting side chains of the galactan backbone described before, or if they are two independent polysaccharides.

Methylation analysis of X1 showed a great diversity of structural units (Table 2). However, it is clear that the galactan and the arabinan moieties have structures similar to those described for B1. Besides, the different rhamnose derivatives in this product suggest the presence of a sulfated rhamnane structure. Sulfated rhamnans have been previously isolated from some species of the green seaweeds *Monostroma*, *M. nitidum*, and *M. latissimum* Wittrock (Ulotrichales, Chlorophyta) (Mao et al. 2008, 2009, Zhang et al. 2008) and from *Gayralia oxysperma* (Ulvales, Chlorophyta) (Cassolato et al. 2008). These polysaccharides have mainly 2-linked, but also 3-linked and 4-linked α-L-Rha units partially sulfated; besides, they have single stubs of uronic acid

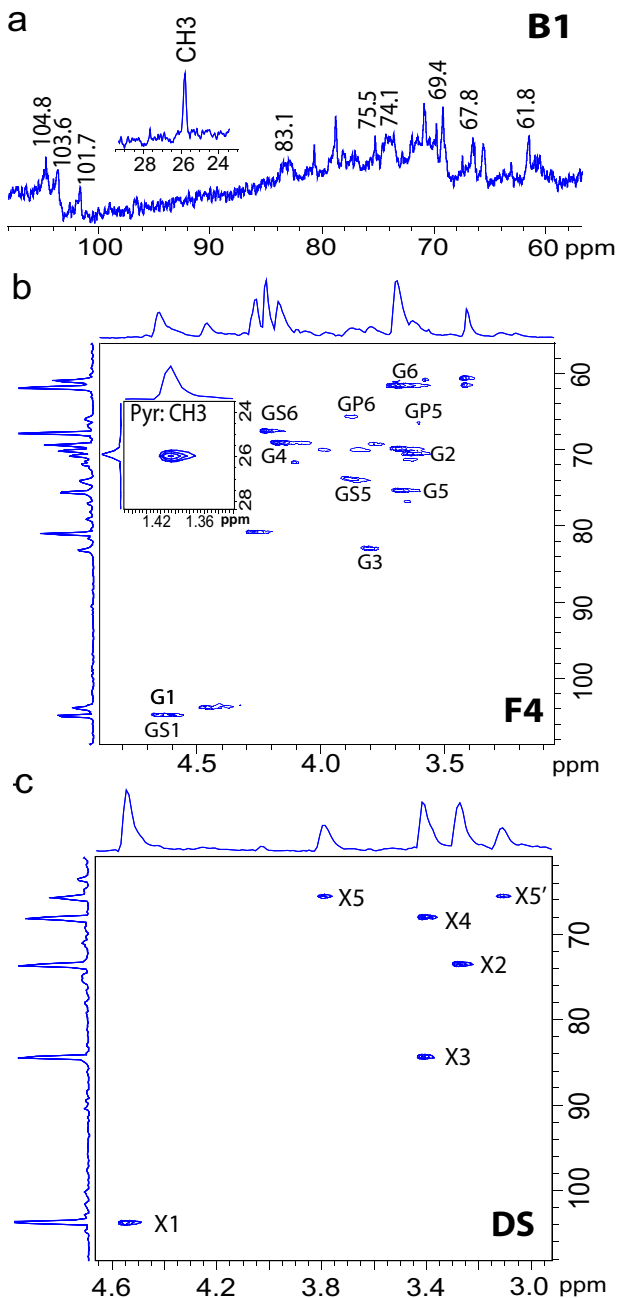


FIG. 3. Spectroscopic analysis. (a) NMR ^{13}C spectrum of B1. (b) Bidimensional HMQC spectrum of F4 [G1-G6, signals corresponding to 3-linked β -D-galactose units; GS1-GS6, signals corresponding to 3-linked β -D-galactose 6-sulfate units; and GP1-GP6, signals corresponding to 3-linked 4,6-*O*-(1'-*R*-carboxy)ethylidene- β -D-galactopyranose units] (c) Bidimensional HMQC spectrum of DS showing the signals of (1 \rightarrow 3)- β -D-xylose units (X1-X5/5'). HMQC, heteronuclear multiple quantum coherence.

residues as side chains. The methylation pattern of Rha units in X1 suggests a similar rhamnan structure; moreover, 5.6% of uronic acid was also detected in this sample. If this structure is confirmed, it will be interesting to analyze the taxonomic significance of the presence in *Bryopsis*

plumosa of small quantities of a rhamnan similar to the predominant sulfated polysaccharides in species of *Monostroma* and *Gayralia*. On the other hand, the important amount of terminal xylose (Xyl) units suggest that they constitute single stubs linked to one of the polysaccharide structures of this fraction.

Methylation analysis of F4 showed major amounts of 3-linked β -D-Gal units, partially substituted on C-6, possibly with sulfate, although a certain degree of substitution with side chains could not be ruled out. Small amounts of arabinan and rhamnan structures were detected (Table 2). The ^{13}C NMR spectrum of F4 (not shown) showed three signals in the anomeric region at 104.9, 103.6, and 101.7 ppm (Table 3). The latter signal, as well as that at 26.0 ppm indicated the presence of pyruvic acid acetals, as found for B1. HMQC spectrum (Table 3) showed the presence of two major units, namely, 3-linked β -D-Gal and β -D-Gal sulfated on C-6. All the signals corresponding to the nonsulfated units are very clear in the spectrum and are in agreement with previous assignments (Bilan et al. 2007). It was previously shown that sulfation of C-6 of a Gal unit produces a shift of the carbon signal of around 6 ppm to lower fields, and that of H-6 in 0.5 ppm, whereas that of C-5 is shifted around 1.5 ppm upfield (Ruiz Contreras et al. 1988). Signals of C-5/H-5 and C-6/H-6 of the 6-sulfated units were assigned based on these data; the remaining signals of these units would give similar displacements to those of the nonsulfated units. Smaller signals corresponding to C-5/H-5 and C-6/H-6 of 3-linked 4,6-Pyr- β -D-Gal residues were also detected (Chiovitti et al. 1997) (Fig. 3b). It is worth noting that the galactan structures present in B1, X1, and F4 have similar structural units, but in different amounts, which could be related to their solubility behavior. Comparison of data from B1 and F4 allowed us to speculate that the signals at 103.6/4.46, 80.8/4.26, and 61.0/3.41 present in the spectra of both samples (Fig. 3) correspond to C-1/H-1, C-4/H-4, and C-5/H-5 of 2-linked β -L-arabinofuranose units (Bock and Pedersen 1983).

F2 (~60% Ara) was also subjected to methylation analysis; however, the small amount of sample just allowed to identify 3,5-di-*O*-methylarabinose as the only Ara derivative, confirming that a 2-linked furanosidic non-sulfated arabinan is part of the system of polysaccharides biosynthesized by this seaweed. The only other peaks detected in the chromatogram corresponded to nonmethylated Xyl and Gal derivatives, indicating that these structures were not methylated under the usual conditions. No nonmethylated Ara was present.

The residue obtained after the sequential water extraction (RX2) still contains an important amount of carbohydrates, mainly Xyl and Glc, which should be part of the fibrillar moiety of the cell wall and represents 62% w/w of the milled seaweed (Table 1). A strongly chaotropic medium (8.4% LiCl/

TABLE 3. NMR signal assignments (ppm) of the cell wall polysaccharides isolated from *Bryopsis plumosa*.

Structural unit	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5,5'	C-6/H6,6'
3-linked β -D-Gal	104.9/4.65	70.7/3.63	83.1/3.80	69.4/4.17	75.5/3.67	61.8/3.69
3-linked β -D-Gal 6S					74.1/3.86	67.8/4.22
3-linked β -D-Gal 4,6-Pyr ^a					66.5/3.67	65.9/3.86
2-linked β -L-Araf ^b	103.6/4.46			80.8/4.26	61.0/3.41	
3-linked β -D-Xyl	105.1/4.63	74.5/3.44	86.9/3.55	68.7/3.62	66.3/3.28, 3.93	

Ara, arabinose; Gal, galactose; Xyl, xylose.

^aSignals corresponding to the acetal: C-1:177.0, C-2:101.7, and C-3:26.0/H-3:1.41 (*R* configuration).

^bSpeculative assignments.

DMSO) at high temperature, which was reported to solubilize cellulose (Flores et al. 2000) was used for further extraction of RX2, giving the final residue DR, which still remained insoluble, and DS, soluble in these conditions (Table 1). This procedure extracted most of the remaining carbohydrates, DR had only 8% of carbohydrates, but major amounts of protein. On the other hand, DS was constituted by ~57% of carbohydrates, mainly Xyl and Glc. Methylation analysis of DS (Table 2) showed that 3-linked xylans and 4-linked glucans were the two major components of this cell wall extract. The small amount of nonmethylated Xyl was attributed to low solubility of the sample in the methylation reaction medium, although this reaction was carried out at 55°C; harder conditions resulted in degradation of the sample (results not shown). HMQC spectrum of DS in DMSO (Fig. 3c) showed only the signals due to the 3-linked xylan, assigned based on data from Yamagaki et al. (1997) (Table 3). This was interpreted to be due to low solubility of cellulose in the solvent. In addition, although some α -glucans could still be present in RX2, no Lugol staining (for α -glucans as storage polysaccharide) was found in DS (Fig. S2 in the supplementary material), indicating that in this sample, Glc arises mostly from cellulose. Moreover, treatment of DS with α -amylase did not change significantly its monosaccharide composition (results not shown).

CONCLUSION

The seaweed of the genus *Bryopsis* present in Mar del Plata (Buenos Aires, Argentina) is *B. plumosa*. The fibrillar as well as the acidic polysaccharides showed a bilayer arrangement in the cell wall. These fibrillar polysaccharides are a (1 \rightarrow 3)- β -D-xylan and cellulose, as previously found for another species of this genus. *B. plumosa* showed a mixed xylan/cellulose cell wall closer to that of *Caulerpa* species, whereas other phylogenetically closer seaweeds, like *Codium* and *Acetabularia* species, have mannan-rich, cellulose-devoid cell walls (Chanzy et al. 1984, Maeda et al. 1990, Dunn et al. 2007). The acidic polysaccharides, soluble in water, have a great structural heterogeneity. However, it was possible to detect putative sulfated rhamnan structure and furanosidic 2-linked arabinan structure, and to determine the

structure of a linear galactan highly pyruvylated and also partially sulfated mainly on C-6 of the 3-linked β -D-Gal units. All the coenocytic organisms studied until now have developed distinct cell walls based on a great diversity of polysaccharides.

These results contribute to the present knowledge about cell walls from green seaweeds of the Bryopsidales, which is quite scarce, taking into account the wide variety and geographical distribution of these seaweeds.

This work was supported with grants from University of Buenos Aires (UBACyT 2008–2010, G048), National Research Council of Argentina, CONICET (PIP 559-2009 to M. C. and PIP 071-2010 to J. E.), and the National Agency for Promotion of Science and Technology, ANPCYT (PICT 2008-0500 to M. C.). F. L. and H. V. are postdoctoral fellows of the Research Foundation – Flanders.

- Agardh, C. A. 1823. *Species algarum rite cognitae, cum synonymis, differentiis specificis et descriptionibus succintis. I. (Fucoideae, Florideae, Ulvoideae)*. ex officina Berlingiana, Greifwald, Lundae (Lund, Sweden), i + 531 pp.
- Ahmed, A. E. R. & Labavitch, J. M. 1977. A simplified method for accurate determination of cell wall uronide content. *J. Food Biochem.* 1:61–365.
- Bilan, M. I., Vinogradova, E. V., Shashkov, A. S. & Usov, A. I. 2006. Isolation and preliminary characterization of a highly pyruvylated galactan from *Codium yezoense* (Bryopsidales, Chlorophyta). *Bot. Mar.* 49:259–62.
- Bilan, M. I., Vinogradova, E. V., Shashkov, A. S. & Usov, A. I. 2007. Structure of a highly pyruvylated galactan sulfate from the pacific green alga *Codium yezoense* (Bryopsidales, Chlorophyta). *Carbohydr. Res.* 342:586–96.
- Bock, K. & Pedersen, C. 1983. Carbon-13 nuclear magnetic resonance data for monosaccharides. *Adv. Carbohydr. Chem. Biochem.* 41:27–66.
- Boraso de Zaixso, A. L. 2004. Marine Chlorophyta of Argentina. *Hist. Nat. (Seg. Ser.)* 3:95–119.
- Cassolato, J. E. F., Noseda, M. D., Pujol, C. A., Pellizzari, F. M., Damonte, E. B. & Duarte, M. A. R. 2008. Chemical structure and antiviral activity of the sulfated heterorhamnan isolated from the green seaweed *Gayralia oxysperma*. *Carbohydr. Res.* 343:3085–95.
- Chanzy, H. D., Grosrenaud, A., Vuong, R. & Mackie, W. 1984. The crystalline polymorphism of mannan in plant cell walls and after recrystallisation. *Planta* 161:320–9.
- Chiovitti, A., Bacic, A., Craik, D. J., Munro, S. L. A., Kraft, G. T. & Liao, M.-L. 1997. Cell-wall polysaccharides from Australian red algae of the family Solieriaceae (Gigartinales, Rhodophyta): novel, highly pyruvated carrageenans from the genus *Callophycus*. *Carbohydr. Res.* 299:229–43.
- Ciancia, M., Quintana, I., Vizcarguenaga, M. I., Kasulin, L., Dios, A., Estevez, J. M. & Cerezo, A. S. 2007. Polysaccharides from the

- green seaweeds *Codium fragile* and *C. vermilara* with controversial effects on hemostasis. *Int. J. Biol. Macromol.* 41:641–9.
- Ciucanu, I. & Kerek, K. 1984. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* 134:209–17.
- Coll Cunillera, J. F. 1976. Algas marinhas bentónicas do Uruguai. Thesis, Departamento de Botânica do Instituto de Biociências de Universidad de Sao Paulo, Brasil, 191 pp.
- Collins, F. S. 1928. *The Green Algae of North America and Suppl.* G. E. Stechert and Co., New York, 400 pp.
- Dodgson, K. S. & Price, R. G. 1962. A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochem. J.* 84:106–10.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. 1956. Colorimetric method of determination of sugars and related substances. *Anal. Chem.* 28:350–6.
- Dunn, E. K., Shoue, D. A., Huang, X., Kline, R. E., MacKay, A. L., Carpita, N. C., Taylor, I. E. P. & Mandoli, D. F. 2007. Spectroscopic and biochemical analysis of regions of the cell wall of the unicellular 'mannan weed', *Acetabularia acetabulum*. *Plant Cell Physiol.* 48:122–33.
- Estevez, J. M., Kasulin, L., Fernández, P. V., Dupree, P. & Ciancia, M. 2009. Chemical and *in situ* characterization of macromolecular components of the complex cell walls from the coenocytic green alga *Codium fragile*. *Glycobiology* 19:212–22.
- Farias, E. H. C., Pomin, V. H., Valente, A. P., Nader, H. B., Rocha, H. A. O. & Mourao, P. A. S. 2008. A preponderantly 4-sulfated, 3-linked galactan from the green alga *Codium isthmocladum*. *Glycobiology* 18:250–9.
- Feldmann, J. 1937. Les algues marines de la Cote des Alberes. I–III. Cyanophycées, Chlorophycées, Phaeophycées. *Rev. Algol.* 9:141–329 + lám.
- Fernández, P. V., Ciancia, M. & Estevez, J. M. 2011. Cell wall variability in the green seaweed *Codium vermilara* (Bryopsidales, Chlorophyta) from the Argentine coast. *J. Phycol.* 48:802–10.
- Fernández, P. V., Ciancia, M., Miravalles, A. B. & Estevez, J. M. 2010. Cell wall polymer mapping in the coenocytic macroalga *Codium vermilara* (Bryopsidales, Chlorophyta). *J. Phycol.* 46:556–65.
- Filiseti-Cozzi, T. M. C. C. & Carpita, N. C. 1991. Measurement of uronic acids without interference from neutral sugars. *Anal. Biochem.* 197:157–62.
- Flores, M. L., Cerezo, A. S. & Stortz, C. A. 2000. Studies on the skeletal cell wall of the cystocarpic stage of the red seaweeds *Iridaea undulosa* B. Part II. Fractionation of the cell wall and methylation analysis of the inner core-fibrillar polysaccharides. *Int. J. Biol. Macromol.* 27:21–7.
- Fritsch, F. E. 1956. *The Structure and Reproduction of the Algae I.* Cambridge University Press, Cambridge, UK, 791 pp.
- Fukushi, Y., Otsuru, O. & Maeda, M. 1988. The chemical structure of the D-xylan from the main cell wall constituents of *Bryopsis maxima*. *Carbohydr. Res.* 182:313–20.
- Guiry, M. D. & Guiry, G. M. 2011. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. <http://www.algaebase.org> (accessed 8 June 2011).
- Harvey, W. H. 1858. *Nereis boreali-americana* III. Chlorospermeae. *Smithson. Contrib. Knowledge* 10:1–140 14 pls.
- Huizing, H. J. & Rietema, H. 1975. Xylan and mannan as cell wall constituents of different stages in the life-histories of some siphonous green algae. *Br. Phycol. J.* 10:13–6.
- Huizing, H. J., Rietema, H. & Sietsma, J. H. 1979. Cell wall constituents of several siphonous green algae in relation to morphology and taxonomy. *Br. Phycol. J.* 14:25–32.
- Kaihou, S., Hayashi, T., Otsuru, O. & Maeda, M. 1993. Studies on the cell-wall mannan of the siphonous green algae, *Codium latum*. *Carbohydr. Res.* 240:207–18.
- Knutsen, S. H. & Grasdalen, H. 1987. Characterization of water extractable polysaccharides from Norwegian *Furcellaria lumbricalis* (Huds) Lamour. (Gigartinales, Rhodophyceae) by IR and NMR spectroscopy. *Bot. Mar.* 30:497–505.
- Koepsell, H. J. & Sharpe, E. S. 1952. Microdetermination of pyruvic and α -ketoglutaric acids. *Arch. Biochem. Biophys.* 38:443–9.
- Koster, J. 1941. Quelques observations sur les *Bryopsis* du Golfe de Naples. *Blumea* 4:225–358.
- Krishnamurthy, K. V. 1999. Transmission electron microscopic cytochemistry. In Krishnamurthy, K. V. [Ed.] *Methods in Cell Wall Cytochemistry*. CRC Press, Boca Raton, Florida, pp. 177–226.
- Lahaye, M. & Robic, A. 2007. Structure and functional properties of ulvan, a polysaccharide from green seaweeds. *Biomacromolecules* 8:1765–74.
- Lamb, D. W. & Zechman, F. W. 2006. Phylogenetic analyses of the Bryopsidales (Ulvophyceae, Chlorophyta) based on RUBISCO large subunit gene sequences. *J. Phycol.* 42:669–78.
- Lourenço, S. O., Barberino, E., De-Paula, J. C., da S. Pereira, L. O. & Lanfer Marquez, U. M. 2002. Amino acid composition, protein content, and calculation of nitrogen-to-protein conversion factors for nineteen tropical seaweeds. *Phycol. Res.* 50:233–41.
- Love, J. & Percival, E. 1964a. The polysaccharides of the green seaweed *Codium fragile*. part II. The water-soluble sulphated polysaccharides. *J. Chem. Soc.* 1964:3338–45.
- Love, J. & Percival, E. 1964b. The polysaccharides of the green seaweed *Codium fragile*. part III. A β -1,4-linked mannan. *J. Chem. Soc.* 1964:3345–50.
- Lowry, O. H., Rosenbrough, N. J. & Farr, A. L. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* 193:265–75.
- Luft, J. H. 1971. Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. *Anat. Rec.* 171:347–68.
- Mackie, W. 1969. The degree of polymerization of xylan in the cell wall of the green seaweed *Penicillus dumetosus*. *Carbohydr. Res.* 9:247–9.
- Mackie, I. M. & Percival, E. 1959. The constitution of xylan from the green seaweed *Caulerpa filiformis*. *J. Chem. Soc.* 1959:1151–6.
- Maeda, M., Fukushi-Fujikura, Y. & Otsuru, O. 1990. Cellulose in the cell wall of the siphonous green alga, *Bryopsis maxima*. *Carbohydr. Res.* 201:91–9.
- Mao, W.-J., Fang, F., Li, H.-Y., Qi, X.-H., Sun, H.-H., Chen, Y. & Guo, S.-D. 2008. Heparinoid-active two sulfated polysaccharides isolated from marine green algae *Monostroma nitidum*. *Carbohydr. Polym.* 74:834–9.
- Mao, W., Li, H., Li, Y., Zhang, H., Qi, X., Sun, H., Chen, Y. & Guo, S. 2009. Chemical characterization and anticoagulant activity of the sulfated polysaccharide isolated from *Monostroma latissimum* (Chlorophyta). *Int. J. Biol. Macromol.* 44:70–4.
- Medlin, L. 1984. Short note on changes in the abundance and occurrence of six macroalgal species along the Texas coast of the Gulf of Mexico. *Contrib. Mar. Sci.* 27:85–91.
- Morrison, I. M. 1988. Hydrolysis of plant cell walls with trifluoroacetic acid. *Phytochemistry* 27:1097–101.
- Ohta, Y., Lee, J.-B., Hayashi, K. & Hayashi, T. 2009. Isolation of sulfated galactan from *Codium fragile* and its antiviral effect. *Biol. Pharmacol. Bull.* 32:892–8.
- Olivier, S. R., Bastida, R. & Torti, M. 1970. Las comunidades bentónicas de los alrededores de Mar del Plata (Argentina). *Actas IV Congreso Latinoamericano de Zoología (Caracas, Venezuela)* 11:559–93.
- Papenfuss, G. F. 1964. Problems in the taxonomy and geographical distribution of Antarctic marine algae. In Carrick, R., Holdgate, M. & Prevost, J. [Eds.] *Biologie Antarctique*. Hermann, Paris, pp. 155–60.
- Parma, A., Pascual, M. & Sar, E. 1987. Clave para el reconocimiento de los géneros de algas macrofitas del intermareal rocoso bonaerense. *Ser. Tec. Didáct. Mus. La Plata* 15:1–27.
- Percival, E. & McDowell, R. H. 1981. Algal walls. Composition and biosynthesis. In Tanner, W. & Loewus, F. A. [Eds.] *Encyclopedia of Plant Physiology*, 13B vol. Springer, Berlin, pp. 277–316.
- Ruiz Contreras, R., Kamerling, J. P., Breg, J. & Vliegthart, J. F. G. 1988. ^1H - and ^{13}C -n.m.r. spectroscopy of synthetic monosulphated methyl α -D-galactopyranosides. *Carbohydr. Res.* 179:411–8.
- Sar, E., Pascual, M. & Parma, A. 1984. Consideraciones ecológicas sobre las algas del litoral rocoso bonaerense. *Rev. Mus. La Plata (Nueva Ser.) Secc. Bot.* 13:143–7.

- Saunders, G. W. & Kucera, H. 2010. An evaluation of *rbcL*, *tufA*, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. *Cryptogam. Algal.* 31:487–528.
- Setchell, W. A. & Gardner, N. L. 1920. The marine algae of the Pacific coast of North America II. Chlorophyceae. *Univ. Calif. Publ. Bot.* 8:156–63.
- Siddhanta, A. K., Shanmugam, M., Mody, K. H., Goswami, A. M. & Ramabat, B. K. 1999. Sulphated polysaccharides of *Codium dwarkense* Boergs. from the west coast of India: chemical composition and blood anticoagulant activity. *Int. J. Biol. Macromol.* 26:151–4.
- Song, H., Zhang, Q., Zhang, Z. & Wang, J. 2010. In vitro antioxidant activity of polysaccharides extracted from *Bryopsis plumosa*. *Carbohydr. Polym.* 80:1057–61.
- Stevenson, T. T. & Furneaux, R. H. 1991. Chemical methods for the analysis of sulphated galactans from red algae. *Carbohydr. Res.* 210:277–98.
- Taylor, W. R. 1937. *Marine Algae of Northeastern Coast of North America*. University of Michigan Press, Ann Arbor, Michigan, 427 pp.
- Verbruggen, H., Ashworth, M., LoDuca, S. T., Vlaeminck, C., Cocquyt, C., Sauvage, T., Zechman, F. W., et al. 2009a. A multi-locus time-calibrated phylogeny of the siphonous green algae. *Mol. Phylogenet. Evol.* 50:642–53.
- Verbruggen, H., Leliaert, F., Maggs, C. A., Shimada, S., Schils, T., Provan, J., Booth, D., et al. 2007. Species boundaries and phylogenetic relationships within the green algal genus *Codium* (Bryopsidales) based on plastid DNA sequences. *Mol. Phylogenet. Evol.* 44:240–54.
- Verbruggen, H., Tyberghein, L., Pauly, K., Vlaeminck, C., Van Nieuwenhuyze, K., Kooistra, W. H. C. F., Leliaert, F. & De Clerck, O. 2009b. Macroecology meets macroevolution: evolutionary niche dynamics in the seaweed *Halimeda*. *Glob. Ecol. Biogeogr.* 18:393–405.
- Wutz, M. & Zetsche, K. 1976. Zur Biochemie und Regulation des Heteromorphen Generationswechsels der Grünalge. *Planta* 129:211–6.
- Yale Dawson, E., Acleto y, C. & Foldvik, N. 1964. The seaweeds of Peru. *Nova Hedwigia Beih.* 13:1–111.
- Yamagaki, T., Maeda, M., Kanazawa, K., Ishizuka, Y. & Nakanishi, H. 1997. Structural clarification of *Caulerpa* cell wall β -(1 \rightarrow 3)-xylan by NMR spectroscopy. *Biosci. Biotechnol. Biochem.* 61:1077–80.
- Zhang, H.-J., Mao, H.-J., Fang, F., Li, H.-Y., Sun, H.-H., Chen, Y. & Qi, X.-H. 2008. Chemical characteristics and anticoagulant activities of a sulfated polysaccharide and its fragments from *Monostroma latissimum*. *Carbohydr. Polym.* 71:428–34.

Supplementary Material

The following supplementary material is available for this article:

Figure S1. Sequential extraction of polysaccharides from *Bryopsis plumosa*.

Figure S2. RX2 and DS stained with Lugol. Scale bar = 50 μ m. Although some staining occurs for RX2, no difference was observed between the stained [Lugol (+)] and unstained [Lugol (-)] samples for DS.

Table S1. Yields and analysis of the fractions obtained by ion exchange chromatography of XI on DEAE Sephadex A-25.

This material is available as part of the online article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.