J. Phycol. **47,** 802–810 (2011)

DOI: 10.1111/j.1529-8817.2011.01006.x

CELL WALL VARIABILITY IN THE GREEN SEAWEED *CODIUM VERMILARA* (BRYOPSIDALES CHLOROPHYTA) FROM THE ARGENTINE COAST[1]

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Cell wall chemistry in the coencocytic green seaweed *Codium vermilara* (Olivi) Delle Chiaje (Bryopsidales, Chlorophyta) is well understood. These cell walls are composed of major amounts of neutral β-(1 → 4)-D-mannans (Mn), sulfated polysaccharides (SPs), which include pyranosic arabinan sulfates (Ar*p*S), pyruvylated galactan sulfates (pGaS), and mannan sulfates (MnS); also minor amounts of *O*-glycoproteins are present. In this study, cell wall samples of *C. vermilara* were investigated with regard to their monosaccharide composition and infrared spectra (using Fourier transform infrared spectroscopy coupled to principal component [FTIR-PC] analysis). Samples from three different populations of *C. vermilara* from the Argentine coast showed: (i) an important variation in the relative arabinan content, which increases from north to south, and (ii) a measurable degree of cell wall variability in the sulfate distribution between the different sulfated polysaccharides, independent of the amount of each polysaccharide present and of total sulfate content. When cell wall composition was analyzed over three consecutive years in a single geographic location, the quantity of Mn and overall sulfate content on SPs remained constant, whereas the pGaS:Ar*p*S molar ratio changed over the time. Besides, similar cell wall composition was found between actively growing and resting zones of the thallus, suggesting that cell wall composition is independent of growth stage and development. Overall, these results suggest that *C. vermilara* has developed a mechanism to adjust the total level of cell wall sulfation by modulating the Ar*p*S:pGaS:MnS molar ratio and also by adjusting the sulfation level in each type of polymer, whereas nonsulfated Mn, as the main structural polysaccharide, did not change over the time or growing stage.

Key index words: **cell wall; *Codium vermilara*; green seaweed; sulfated polysaccharides; temporal and geographic variation; β-mannans**

***Abbreviations:* Ar*p*S, pyranosic arabinan sulfates; FTIR, Fourier transform infrared spectroscopy; MnS, mannan sulfates; pGaS, pyruvylated galactan sulfates; SPs, sulfated polysaccharides; TFA, trifluoroacetic acid**

Seaweeds of the order Bryopsidales (Chlorophyta) are ''giant single-cell'' algae because the habit comprises continuous siphons, which lack cross cell walls, and septa are formed only at the time of differentiation of reproductive structures. Species of *Codium* occur abundantly on hard substrata and in the intertidal zone of temperate and tropical regions of the globe. Overall, this genus comprises ~150 taxonomically accepted species all over the world (Verbruggen et al. 2007). Specifically, *C. vermilara* grows in a restricted area of the Argentine littoral comprising only south of Buenos Aires, Río Negro, and Chubut provinces from 39° to 45° S latitude (Boraso de Zaixso 2004 and this study).

Chemical composition and in situ localization of cell wall polymers in *C. vermilara* were studied in detail previously (Ciancia et al. 2007, Fernández et al. 2010). This green seaweed has an average cell wall composition of ~32% w/w of Mn and ~12% of SPs. SPs comprise three main diverse polysaccharide structures: (i) ~6–8% w/w of Ar*p*S, with 3-linked β-L-Ara*p* 2,4-disulfate units and 3-linked mono- and nonsulfated β-L-Ara*p* residues in smaller quantities (Ciancia et al. 2007, unpublished results); (ii) ~4–6% w/w of pGaS composed of 3-linked β-D-Gal*p* residues with sulfate groups mainly on C-4, and single stubs of 3,4-*O*-(1′-carboxyethyliden)-β-D-galactopyranose units; (iii) low amounts of MnS, with 4-linked β-D-Man*p* units (Table S1 in the supplementary material). In addition, < 1% w/w of hydroxyproline-rich glycoproteins-like (HRGPs-L) epitopes was found in its cell walls (Fernández et al. 2010).

[1]Received 6 July 2010. Accepted 14 January 2011.
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Besides, the spatial arrangement of different cell wall polymers in *C. vermilara* was extensively characterized using monoclonal antibodies and chemical imaging techniques, and a preliminary cell wall model was proposed (Fernández et al. 2010).

The temporal scale of cell wall variability of several green, red, and brown seaweeds has previously been analyzed (Lahaye and Yaphe 1988, Chirapart and Ohno 1993, Moraudi-Givernaud et al. 1993, Oliveira et al. 1996, Givernaud et al. 1999, Orduña-Rojas et al. 2008, Robic et al. 2009, Skriptsova et al. 2010). In most of the cases, variability in cell wall composition and structure was linked to complex environmental exogenous factors as well as endogenous changes in the organisms (e.g., growth and development, morphological change, reproduction, etc.). Although cell wall chemistry of the genus *Codium,* including *C. vermilara,* has been extensively studied (Bilan et al. 2007, Ciancia et al. 2007, Farias et al. 2008, Estevez et al. 2009, Ohta et al. 2009, Fernández et al. 2010), the importance of the variability displayed in the structure of cell wall in relation to different geographic locations and seasons remains unknown until now.

Cell walls are the first layer in the physical contact of plant cells with symbiotic and pathogenic organisms, and this is relevant not only for land plants but also for macroalgae as well (Küpper et al. 2002, Nürnberger et al. 2004). It has been demonstrated that structural differences in SPs of a few brown and red macroalgae may affect the recognition and pathogenic responses against different pathogens (Bouarab et al. 1999, Küpper et al. 2002). Pathogen recognition depends on the detection of oligosaccharides released by pathogen enzymatic action on the cell wall matrix (producing defense elicitors). These oligosaccharides trigger a defensive response based on reactive oxygen responses (ROS) mediated by putative NADPH oxidases, and also in the production of antimicrobial compounds (Weinberger et al. 2001, 2005, 2010). Hence, differences in the structure of cell wall macromolecules could be related to different responses against pathogens.

In this work, we explore the cell wall variability present in samples of *C. vermilara* collected in different geographic locations from the Argentine coast and in different seasons for three consecutive years for one location. The overall cell wall variability in terms of the level of overall sulfation on SPs, the molar ratio between pGaS and Ar*f*S, and finally the Mn levels was studied using chemical methods and using FTIR-PC analysis. In addition, cell walls from apical and basal zones of the thallus were studied in the same way.

MATERIALS AND METHODS

Algal sample. Samples of *C. vermilara* were collected in La Farola (LF, Buenos Aires Province; 39°00′ S, 61°65′ W), San Antonio Oeste (SAO, Rio Negro Province; 40°43′ S, 64°56′W), and Bahia Arredondo (BA, Chubut Province; 45°01′ S, 65°48′ W) at the Argentine coast. Samples were identified according to Boraso de Zaixso (2004). Algal material was composed only of sterile diplophase thalli, as no gametangia structures were observed. Voucher materials for SAO, BA, and LF were deposited in the herbarium of the Museo Bernardino Rivadavia, Buenos Aires, Argentina (SAO collection number 40466; BA collection number 47170; La Farola M3–M10 samples, collection numbers 47171–47178, respectively).

Extraction of the water-soluble polysaccharides (MxE). The dried plants were milled in liquid nitrogen and extracted with H_2O for 24 h at room temperature (20 $g \cdot L^{-1}$), and the residue was separated from the supernatant by centrifugation. The supernatant and the residue were dialyzed and freeze-dried separately, obtaining M3E–M10E and M3R–M8R, respectively.

Chemical analysis. Chemical analyses were performed as described in Ciancia et al. (2007) and Estevez et al. (2009). Total carbohydrate content of extracts was analyzed using phenol–sulfuric acid method (Dubois et al. 1956). In the case of dried seaweed samples, total sugar content was determined using a phenol–sulfuric acid method adapted for insoluble material (Ahmed and Labavitch 1977). Sulfate was determined in three technical replicates, after hydrolysis of the sample, using ionic isocratic chromatography on a Dionex DX-100 apparatus equipped with a Dionex AS4A column (Dionex Corporation, Sunnyvale, CA, USA) and using an Na_2CO_3 1.8 mM, $NaHCO_3$ 1.7 mM solution as eluant. In addition, sulfate content for extracts was determined turbidimetrically, according to Dodgson and Price (1962). To determine the inorganic sulfate content of the dry seaweeds, the method of Thero and Hartiala (1971) was applied, with and without previous hydrolysis of the sample. Results obtained showed that the organic sulfate content was negligible compared with the inorganic sulfate present in these samples indicating that in this case, it is not possible to determine the organic sulfate content using the methods tested here. Sulfate content of samples M3R–M8R was determined after total degradation of the sample. The mixture of gases were separated using gas chromatrography with porapac column in Carbo Erba EA 1108 chromatograph (Milan, Italy), and using thermic detector, sulfate was detected as SO_2.

Monosaccharide composition analysis. To determine the monosaccharide composition using gas chromatography (GC), alditol acetates were obtained by reductive hydrolysis and acetylation (Stevenson and Furneaux 1991) or by hydrolysis with TFA 2M for 2 h at 121°C, reduction with $NaBH_4$, and acetylation (Morrison 1988). Both methods gave similar results, and so only those of reductive hydrolysis are shown in the tables. One to two milligrams of BA, LF, and SAO ($n = 5$ biological replicates), M3–10, and MxE ($n = 2$, biological replicates and $n = 3$ technical replicates) were hydrolyzed in each case.

Model cell wall compounds for FTIR-PC analysis. Extraction of water-soluble polysaccharides from dried plants was carried out as described in Ciancia et al. (2007). The main room-temperature water extract (V1) was stepwise fractionated with 0.125 M potassium chloride producing a precipitate and a soluble fraction, comprising Ar*f*S and pGaS, respectively. Desulfation of arabinan and galactan was carried out using the microwave-assisted method described by Navarro et al. (2007). SMn was obtained from V1 using ionic exchange chromatography, and its structural details will be described elsewhere. An insoluble residue, mostly composed of a nonsulfated Mn was obtained after exhaustive extraction of *C. vermilara* with water (Ciancia et al. 2007).

FTIR-PC analysis. FTIR spectra ($n = 10$) were recorded from 4,000 to 250 cm^{-1} with a 510P Nicolet FTIR spectrophotometer (Madison, WI, USA), using dry samples (1–2 mg) from BA, LF, and SAO collection sites for analyzing geographic

variability and M3-M10 dry milled samples for the analysis of cell wall temporal variability in KBr disks. In addition, freeze-dried extracted samples (MxE) were also analyzed in the same way. Thirty-two scans were taken with a resolution of 4 cm^{-1}. Data collection and processing were performed on the OMNIC 7.2 (Thermo-Nicolet, Madison, WI, USA). Ten spectra from each sample were corrected using automatic subtraction of the background and were then saved in JCAMP.DX format for further analysis. Using win-das software (Wiley, New York, NY, USA), spectra were baseline corrected, normalized, and assessed by principal component (PC) analysis, using the covariance-matrix method (Kemsley 1996). Model cell wall polysaccharides were processed as described above.

RESULTS AND DISCUSSION

To study variability of the different cell wall polysaccharides in the green seaweed *C. vermilara* associated with geographic, temporal, and developmental factors, samples were analyzed using two complementary analytical methods, the monosaccharide composition and FTIR-PC analysis.

First, the homogeneity of the cell wall composition along the different parts of the thallus of *C. vermilara* was investigated. Hence, monosaccharide analysis ($n = 3$) was carried out on actively growing (apices) and resting regions (bases) of the thalli separately, in a pool of plants ($n = 10$) from LF on the Argentine coast (Fig. 1, A and B; Fig. S1 and Table S1 in the supplementary material). It was assumed that, after subtraction of glucose, which derives from storage polysaccharides, the remaining sugars arise almost exclusively from the cell wall polysaccharides (Chopin and Whalen 1993). This methodology has been applied to red seaweeds (Usov and Klochkova 1992, Usov 1998); therefore, galactose arises from the sulfated galactan, arabinose from the sulfated arabinan, and mannose from mostly neutral mannans (Ciancia et al. 2007, Fernández et al. 2010). Similar results for monosaccharide composition and total carbohydrates content were obtained in apical and basal portions in the same plant (Fig. 1B), being the variability similar to that obtained among different plants ($n = 5$, I–V) of the same population and time of the year (Table S1; Fig. 1, A and B). These results indicate that cell wall composition in *C. vermilara* is highly similar between different parts of the thallus in the same plant, and also between plants in the same population. Based on these facts, for all the following analyses regarding geographic and temporal variability of the cell walls in *C. vermilara*, several plants ($n = 10$) were pooled together.

To test if geographic distribution of different populations has any measurable impact on cell wall composition, two pooled samples ($n = 10$) of *C. vermilara* from three different locations on the Argentine coast, collected during the same season, were analyzed. Figure 1C shows the monosaccharide composition of samples from *C. vermilara* collected from three different geographic localities, LF, SAO, and BA, that comprise a latitudinal area ranging from 39° to 45° S on the Argentine coast (Fig. S1), during the same time of the year (in July, winter in the Southern Hemisphere). These results show that the galactan content remains almost constant, but there is a significant variation of the arabinan content, giving a molar ratio galactose:arabinose of 1.0:1.3 for plants from BA, 1.0:0.7 for those from SAO, and 1.0:0.4 for samples from LF (Fig. 1C). Thus, there is a decrease of relative arabinan content from south to north. Besides, the amount of mannans shows a small, but significant increase in that direction for the three different locations.

FTIR spectroscopy is a sensitive technique used here for the characterization of macromolecules based on the absorption of light caused by the vibration of asymmetrical chemical bonds present in their particular monomeric components (Prado Fernandez et al. 2003). Cell walls of *C. vermilara* are complex composites of a variety of polysaccharides and *O*-glycoproteins (Ciancia et al. 2007, Fernández et al. 2010). FTIR analysis of cell wall polysaccharides using algal biomass as starting material was used previously for the study of red seaweeds (Chopin and Whalen 1993). On the basis of the high amounts of polysaccharide-based cell walls in *C. vermilara* biomass (~44% w/w; Fernández et al. 2010), we decided to assess the noise effect due to low-molecular-weight components (Low-MW) present in the cytoplasm and cell walls of milled samples on the FTIR spectra. Two methanol-extracted samples and nonextracted ones collected from LF showed almost identical FTIR spectra (Fig. S2 in the supplementary material), thus confirming that cell wall polysaccharides are the main macromolecules that contribute to the major absorbances in the region analyzed here (1,500–800 cm^{-1}). The IR region between 1,650 and 1,500 cm^{-1} was excluded from all the analyses, as it contains strong bands at 1,650 cm^{-1} and 1,550 cm^{-1}, characteristic for amide I and II of proteins.

However, FTIR spectra of these cell walls were extremely complex, and statistical procedures such as PC analysis were required for their analysis. PC analysis is a mathematical operation that allows samples to be characterized by their scores on a small number of new variables (PC axes) instead of large numbers of original measurements (here, absorbances), thus summarizing the information and highlighting differences between them in PC loadings (Kemsley 1996, Chen et al. 1998). Based on our previous work (Ciancia et al. 2007, Fernández et al. 2010), extreme structures of cell wall macromolecules in *C. vermilara* comprising a highly sulfated pyranosic arabinan (Ar*p*S), pGaS, and neutral mannans (Mn) that are the major polysaccharides, in addition to a MnS and the chemically desulfated pyranosic arabinan (Ar*p*) and pyruvylated galactan (pGa), generated for this study, were used as model

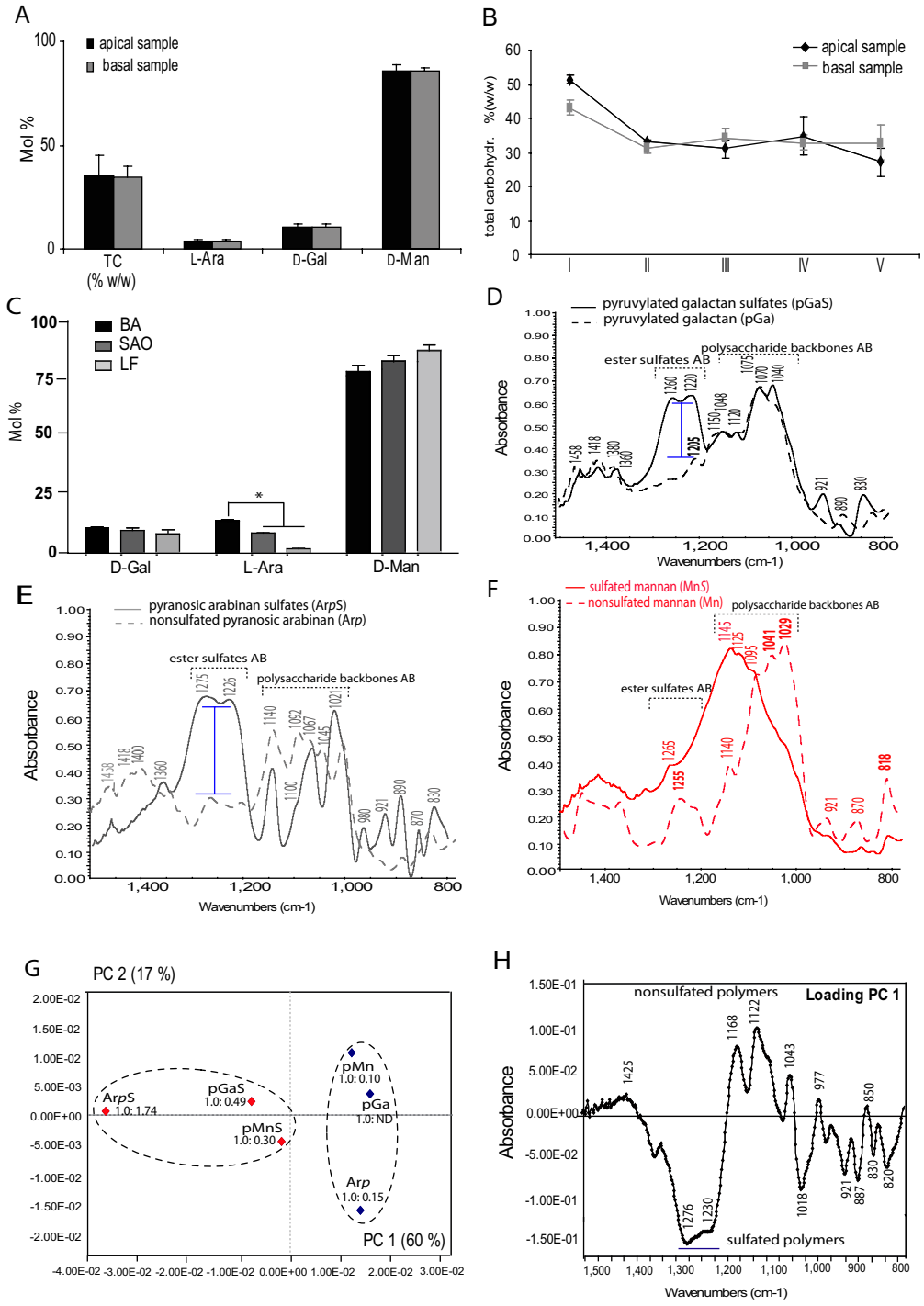

FIG. 1. Monosaccharides composition and Fourier transform infrared spectroscopy coupled to principal component (FTIR-PC) analysis of samples of *Codium vermilara* from three different geographic locations on the Argentine coast. (A) Developmental cell wall variability in a single plant was followed by monosaccharide composition. Chemical analysis of actively growing (apices) and resting (bases) regions of the thallus from different plants of *C. vermilara*. Apical and basal zones of the same plant were analyzed separately ($n = 10$). (B) Total carbohydrate contents of apical and basal zones in different plants ($n = 5$, I–V). (C) Geographic cell wall variability in samples from *C. vermilara* analyzed. (D–F) FTIR spectra of model cell wall polysaccharides isolated from *C. vermilara* (see Table S2 in the supplementary material). (D) FTIR spectra of the pyruvylated galactan sulfate (pGaS) and the nonsulfated pyruvylated galactan (pGa). (E) FTIR spectra of the pyranosic arabinan sulfate (Ar*p*S) and the nonsulfated pyranosic arabinan (Ar*p*). (F) FTIR spectra of the sulfated mannan (MnS) and the nonsulfated mannan (Mn). (G) FTIR-PC analysis of model sulfated and non-low sulfated. PC1 explains 60% of the total variability and discriminates the sulfated polymers (Ar*p*S and pGaS, and MnS) from the desulfated counterparts (Mn, Ar*p*, and pGa). The molar ratio of the predominant sugar:sulfate is indicated to highlight the overall sulfation in each type of polymer (see Table S2). (H) PC1 loading showing the main IR bands that discriminate between model sulfated and non-low-sulfated compounds are associated to asymmetric stretching of the O=S=O ester sulfates (Prado Fernandez et al. 2003).

compounds. Their structures are summarized in Table S2 (see the supplementary material). For each FTIR spectrum of sulfated polymer (Ar*p*S, pGaS, and MnS), the spectra of the nonsulfated counterpart (Ar*p*, pGa, and Mn; Table S2) was carried out (Fig. 1, D–F). The FTIR-PC analysis of the model compounds (Fig. 1, G and H) in the region of 1,500–800 cm^{-1} showed the IR bands at 1,275–1,220 cm^{-1} that are associated to asymmetric stretching of the O=S=O ester sulfates (Prado Fernandez et al. 2003), which clearly discriminate the sulfated polymers (Ar*p*S and pGaS, and MnS) from the non-low-sulfated counterparts (Mn, Ar*p*, and pGa). As expected, bands of ester sulfates are abundant in the sulfated polymers, but almost absent in the non-low-sulfated polymers indicating that the intensity of 1,275–1,220 cm^{-1} bands can be used as a strong indicative of overall sulfation of cell wall polymers in *C. vermilara,* as was shown previously for other types of sulfated polysaccharides (Prado Fernandez et al. 2003). Analysis of FTIR spectra of these model compounds is crucial to interpreting the complex PC loading obtained using FTIR-PC analysis of complex cell walls such as those present in *C. vermilara* and also allowed us to assess the contribution of each sulfated and nonsulfated counterpart polymer to the overall cell wall variability.

Then, the overall variability in the complex samples of *C. vermilara* from three different geographic localities, LF, SAO, and BA, was analyzed using FTIR-PC approach. PC analysis of data from FTIR was displayed in a two-dimensional plot including only the first two PCs (Fig. 2, A and B) and allowed us to visualize the degree of affinity among the samples (collection sites) and the source of variation in each PC loading (specific IR bands). Samples from SAO segregate in Principal Component 1 (PC1), which explains 60% of the total variability, into one defined group, and those from LF/BA segregate into another group (Fig. 2A). Model FTIR spectra in the region of 1,500–800 cm^{-1} of the different polysaccharides were considered as a reference; no attempts were made to assign the bands that differentiate them, in spite of the fact that some bands are known to be associated to certain functional groups (Prado Fernandez et al. 2003). Thus, nonsulfated Mn segregates close to samples from SAO, and model MnS spectrum locates closer to those of LF/BA (Fig. 2A) suggesting that MnS are relatively more abundant in samples from LF/BA and Mn in plants from SAO. On the other hand, IR bands associated with Ar*p*S (Fig. 1E) differentiate SAO/LF samples from BA regarding PC2, indicating a putative increased content of Ar*p*S in SAO/LF. FTIR-PC analysis with the aid of model sulfated and nonsulfated macromolecules suggests that these samples from three different populations present a high variability in nonsulfated/sulfated Mn and also in sulfation levels on Ar*p*S, in spite of their close geographic distribution. These results show that chemical data and FTIR-PC analyses give different and complementary information about the cell wall variability of these samples. That is, the monosaccharide composition indicates the relative proportion of the three different polysaccharide types in the cell walls; on the other hand, the FTIR-PC analysis gives an idea of the relative degree of sulfation of each of these polysaccharides for each group of samples.

The degree of cell wall variability in samples of *C. vermilara* collected in different seasons from three consecutive years in a single population (M3–M10) was also investigated. Chemical analysis showed only a small variation in the galactose:arabinose molar ratio and the amount of mannans fluctuated ~81.4–86.6 mol %. Thus, no significant difference was established between these samples. Using FTIR-PC analysis, it was found that M3–M5 segregate in one group away from M6–M10 along PC1 (Fig. 2C). However, this difference could not be related directly to the specific structure of a model compound. On the other hand, M3–M10 showed a wide distribution with respect to PC2 loadings. A more detailed analysis of the relative monosaccharide composition of these samples showed that the main difference between these two groups is due to small variations in the galactose:arabinose molar ratios that distinguish M3–M5 (with 1.0:040-0.053 molar ratio) from M7–M10 (with 1.0:026–0.034), whereas the mannose content remains almost constant for all of them (81.4–86.6 mol %; Table 1). These results suggest that the relative amount of total galactans and of arabinans, as well as the degree of sulfation of these polymers in the cell walls of *C. vermilara* present small changes over the time, but independently of the seasons.

Overall sulfate levels from SPs were estimated in whole cell walls of *C. vermilara* using several methods (see Materials and Methods), but background noise, including contaminants such as inorganic sulfate, produced important interferences (results not shown). To quantify the level of overall sulfation on SPs (including the cell wall polymers Ar*p*S + pGaS + MnS) and also to reveal if there are any changes in the molar ratio of total cell wall polysaccharides to sulfate in samples obtained during different collection times in three successive years, the samples were extracted with water, and chemical analysis was done in cell wall water-soluble extracts (M3E–M10E). Although total carbohydrate levels varied in M3E–M10E, the molar ratio carbohydrates:sulfate remained almost constant for all of them (1:0.5–1:0.6) (Table 1). On the contrary, galactose:arabinose molar ratio changed notably from 1.0:0.3 (in M7E, M9E, and ME10) to 1.0:0.5–0.6 (in M3E, M4E, M5E, and M8E) to 1.0:1.0 (in M6E), indicating that pGaS:Ar*p*S ratios may vary over the time, as was shown before for whole cell wall in samples M3–M10 (Table 1). Using FTIR-PC analysis, all the samples were very similar in PC1. However, it was

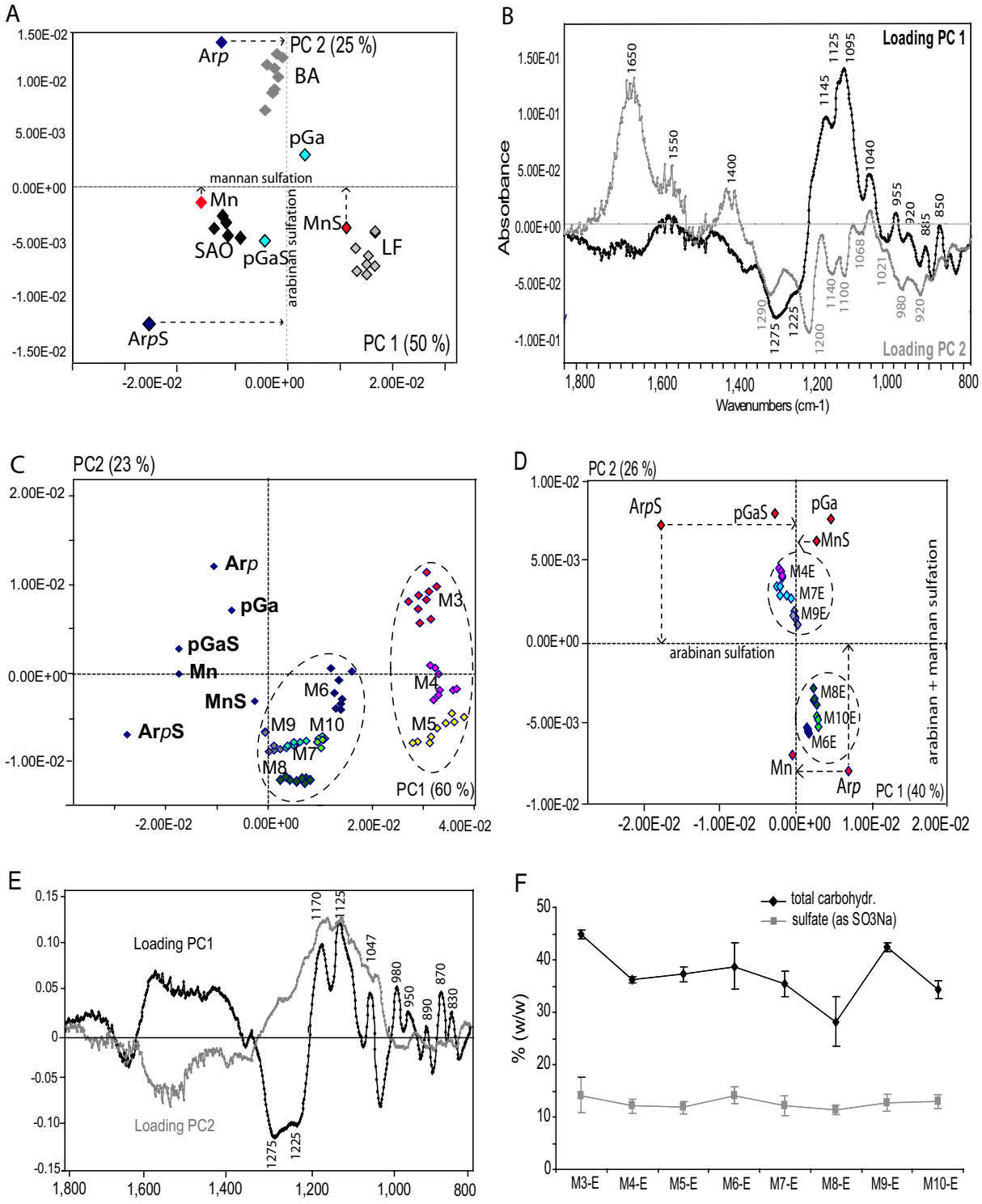

FIG. 2. Geographic and temporal cell wall variability on samples of *C. vermilara* studied using Fourier transform infrared spectroscopy coupled to principal component (FTIR-PC) and monosaccharides analysis. (A) Geographic cell wall variability in samples of *C. vermilara* from three different geographic locations on the Argentine coast analyzed using FTIR-PC. Biplot PC1-PC2 shows the separation of Bahia Arredondo (BA)/La Farola (LF) from samples of San Antonio Oeste (SAO) in PC2. Based on model spectra, PC1 clearly discriminates geographic samples based on level of sulfate groups on galactan polymers (pGa-pGaS) and PC2 groups samples based on arabinan/mannan sulfate content (Ar*p*-Ar*p*S and Mn-MnS). Midinfrared spectra were analyzed using the covariance-matrix approach for PC analysis (Kemsley 1996). (B) Loadings of PC1 and PC2, where major IR bands are shown. (C) Samples obtained from different seasons in three consecutive years in a single population LF were analyzed using FTIR-PC. PC1 explains 69% of the total variability and differentiates M3–M5 from M6–M10 samples based on arabinose:galactose molar ratio (see also Table 1). (D) FTIR-PC analysis of water-soluble cell wall extracts (MxE). (E) Major IR bands in loadings of PC1 and PC2 are shown. (F) Ratio between total carbohydrates and sulfate contents in samples MxE remains constant over the time ($n = 5$).

TABLE 1. Chemical analysis of the whole cell walls (M3–10) and cell wall extracts (M3E–M10E) from *Codium vermilara*.

Sample	Collection time	TC[1] %	Monosaccharide composition (mol %)				Gal:Ara Molar ratio
			D-Gal	L-Ara	D-Man	D-Xyl + L-Rha + L-Fuc	
M3	Nov 07	20.0 ± 2.0	11.2	5.9	82.8	ND	1.0:0.53
M4	Dec 07	13.2 ± 0.8	10.5	4.3	85.2	ND	1.0:0.40
M5	Mar 08	14.1 ± 1.1	10.3	4.9	84.9	ND	1.0:0.47
M6	Jul 08	19.5 ± 1.7	9.7	3.7	86.6	ND	1.0:0.38
M7	Dec 08	20.0 ± 1.1	11.1	3.8	85.1	ND	1.0:0.34
M8	May 09	21.9 ± 1.8	14.0	4.6	81.4	ND	1.0:0.32
M9	Sep 09	18.8 ± 2.8	11.3	2.9	85.9	ND	1.0:0.26
M10	Nov 09	19.7 ± 3.3	12.5	3.7	83.8	ND	1.0:0.30

Extract	Collection time	TC:SO_3Na Molar ratio	Monosaccharide composition (mol %)				Gal:Ara Molar ratio
			D-Gal	L-Ara	D-Man	D-Xyl + L-Rha + L-Fuc	
M3-E	Nov 07	1.0:0.5	48.5	30.3	13.2	8.0	1.0:0.6
M4-E	Dec 07	1.0:0.5	52.2	29.4	9.5	8.9	1.0:0.6
M5-E	Mar 08	1.0:0.5	53.1	31.3	7.9	7.7	1.0:0.6
M6-E	Jul 08	1.0:0.5	40.7	39.1	8.9	13.1	1.0:1.0
M7-E	Dec 08	1.0:0.5	64.9	17.6	8.2	9.3	1.0:0.3
M8-E	May 09	1.0:0.6	55.8	25.1	9.6	9.5	1.0:0.5
M9-E	Sept 09	1.0:0.5	55.9	14.1	8.7	21.4	1.0:0.3
M10-E	Nov 09	1.0:0.6	58.6	18.9	14.4	8.1	1.0:0.3

[1]TC, total carbohydrates after discounting glucose; ND, not detected.

possible to distinguish samples M6E, M8E, M10E from M4E, M7E, M9E in PC2 (Fig. 2D). PC2 loading that comprises 23% variability in the overall data set showed a relatively higher sulfation in both Ar*p*S/MnS types of polymers in M4E, M7E, M9E than in M6E, M8E, M10E samples (Fig. 2E). FTIR-PC analysis confirmed that cell wall variability is associated to changes in levels of sulfation in specific macromolecules (Fig. 2, E and F), as was shown before for whole cell walls, although the level of sulfation was constant. The residues from the water extraction procedures were dialyzed and freeze-dried (M3R–M8R), and the sulfate content was determined. With these data and those obtained from M3E–M8E, the overall degree of sulfation in the samples was estimated in the range of 3.7%–5.1% (w/w). This finding also confirms that the overall level of sulfation in the cell walls remains quite constant over time in *C. vermilara*, at least for a single collection site (LF).

Previously, it has been suggested that there are strong directional selection forces in seawater environments for organisms to synthesize strongly negatively charged polysaccharides and glycoproteins in their cell walls and intercellular matrices (Aquino and Landeira-Fernandez 2005), such as those present in cell walls from *C. vermilara* (Ar*p*S, pGaS, and MnS). Like other plant and algal cell wall polysaccharides (e.g., pectins, alginates, and red seaweed galactans), it is highly possible that SPs are synthesized in the Golgi apparatus and exported to the wall in secretory vesicles (Tveter-Gallagher et al. 1984). The complex assembly and remodeling of sulfated and other types of negatively charged polysaccharides involves enzymes, such as pectin methyl esterase (e.g., pectins in land plants), alginate-C5-epimerase (e.g., alginates in brown seaweeds), and D-/L-galactose-6-sulfurylases (e.g., carrageenans and agarans in red algae), that confer these polysaccharides the ability to make viscous solutions in situ only when the polymer was secreted into the walls, but not before. The overall sulfate levels on the cell walls would be sensed in an accurate way by the cells, as this could have an enormous impact on the degree of hydration, mechanical, and ionic regulation to maintain the cellular homeostasis (Kloareg and Quatrano 1988).

As overall sulfation levels in cell walls from *C. vermilara* appear to remain constant, it is tempting to hypothesize that this seaweed has developed a putative regulatory mechanism to sense and control the total sulfate levels in its cell walls. This mechanism would comprise modulation of the synthesis, degradation of overall SPs (including Ar*p*S, pGaS, and MnS), and regulation of the expression and activity of Golgi-localized sulfohydrolases (SHs), enzymes in charge of removing sulfate groups from polysaccharides. These enzymes were first described for galactans in red seaweeds (Lawson and Rees 1970, Wong and Craigie 1978), but they were not investigated in green seaweeds. The regulatory mechanism would also involve regulation of putative sulfotransferases (STs), enzymes in charge of transferring sulfate groups to the polysaccharide backbones (Tveter-Gallagher et al. 1984, Wu and Gretz 1990) in any of these three cell wall polymers. At the same time, nonsulfated Mn with a highly regular β-(1 → 4) backbone as the main structural polysaccharide in the cell walls of *C. vermilara* does not change over the time of the year or in the different developmen-

tal stages analyzed here, although a small, but significant variability was observed with latitude. This observation is consistent with a putative mechanical role proposed for Mn, as there is no cellulose or any other kind of ''fibrillar'' β-(1 → 4)-polysaccharide to fulfill this crucial function.

The authors gratefully acknowledge N. E. Sottile, F. D. Sottile, D. F. Sottile, and P. M. Sottile for collecting some of the samples of *C. vermilara* from La Farola; G. Soriano for sending the sample from Bahia Arredondo; and C. Carrasco for that from San Antonio Oeste. Funding provided by FONCYT (PICT 2006-0983) and National Research Council of Argentina, CONICET grant (PIP 2010-071) to J. M. E; University of Buenos Aires (UBACyT G048), CONICET (PIP 559), FONCYT (PICT 2008-0500) to M. C.

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.

Aquino, R. S. & Landeira-Fernandez, A. M. 2005. Occurrence of sulfated galactans in marine angiosperms: evolutionary implications. *Glycobiology* 15:11–20.

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.

Boraso de Zaixso, A. L. 2004. Marine Chlorophyta of Argentina. *Hist. Nat. (Segunda Ser.)* 3:95–119.

Bouarab, K., Potin, P., Correa, J. & Kloareg, B. 1999. Sulfated oligosaccharides mediate the interaction between a marine red alga and its green algal pathogenic endophyte. *Plant Cell* 11:1635–50.

Chen, L., Carpita, N. C., Reiter, W.-D., Wilson, R. H., Jeffries, C. & McCann, M. C. 1998. A rapid method to screen for cell-wall mutants using discriminant analysis of Fourier transform infrared spectra. *Plant J.* 16:385–92.

Chirapart, A. & Ohno, M. 1993. Seasonal variation in the physical properties of agar and biomass of *Gracilaria* sp (*chorda* type) from Tosa Bay, southern Japan. *Hydrobiologia* 260/261:541–7.

Chopin, T. & Whalen, E. 1993. A new and rapid method for carrageenan identification by FTIR diffuse reflectance spectroscopy directly on dried, ground algal material. *Carbohydr. Res.* 246:51–9.

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.

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 for the determination of sugars and related substances. *Anal. Chem.* 28:350–6.

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 coencocytic 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.

Fernández, P. V., Ciancia, M., Miravalles, A. B. & Estevez, J. M. 2010. Cell-wall polymer mapping in the coenocytic macroalga *Codium vermilara* (Bryopsidales, Cholorphyta). *J. Phycol.* 46:456–65.

Givernaud, T. E. L., Gourji, A., Mouradi-Givernaud, A., Lemoine, Y. & Chiadmi, N. 1999. Seasonal variation of growth and agar composition of *Gracilaria multipartite* harvested along the Atlantic coast of Morocco. *Hydrobiologia* 398/399:167–72.

Kemsley, E. K. 1996. Discriminant analysis of high-dimensional data: a comparison of principal components analysis and partial least squares data reduction methods. *Chemometrics Intelligent Lab. Syst.* 33:47–61.

Kloareg, B. & Quatrano, R. S. 1988. Structure of the cell wall of marine algae and ecophysiological functions of the matrix polysaccharides. *Oceanogr. Mar. Biot. Annu. Rev.* 26:259–315.

Küpper, F. C., Müller, D. G., Peters, A. F., Kloareg, B. & Potin, P. 2002. Oligoalginate recognition and oxidative burst play a key role in natural and induced resistance of sporophytes of Laminariales. *J. Chem. Ecol.* 28:2057–80.

Lahaye, M. & Yaphe, W. 1988. Effects of seasons on the chemical structure and gel strength of *Gracilaria pseudoverrucosa* agar (Gracilariaceae, Rhodophyta). *Carbohydr. Polym.* 8:285–301.

Lawson, C. J. & Rees, D. A. 1970. An enzyme for the metabolic control of polysaccharides conformation and function. *Nature* 227:390–3.

Moraudi-Givernaud, A., Givernaud, T., Morvan, H. & Cosson, J. 1993. Annual variations of the biochemical composition of *Gelidium latifolium* (Greville) Thuret et Bornet. *Hydrobiologia* 260/261:607–12.

Morrison, I. M. 1988. Hydrolysis of plant cell walls with trifluoroacetic acid. *Phytochemistry* 27:1097–101.

Navarro, D. A., Flores, M. L. & Stortz, C. A. 2007. Microwave-assisted desulfation of sulfated polysaccharides. *Carbohydr. Polym.* 69:742–7.

Nürnberger, T., Brunner, F., Kemmerling, B. & Piater, L. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol. Rev.* 198:249–66.

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.

Oliveira, E. C., Saito, R. M., Santos Neto, J. F. & Garófalo, G. M. C. 1996. Temporal and spatial variation in agar from a population of *Pterocladia capillacea* (Gelidiales, Rhodophyta) from Brazil. *Hydrobiologia* 326/327:501–4.

Orduña-Rojas, J., García-Camacho, K. Y., Orozco-Meyer, P., Ríosmena-Rodríguez, R., Pacheco-Ruiz, I., Zertuche-González, J. A. & Meling-López, A. E. 2008. Agar properties of two species of Gracilariaceae from the Gulf of California, Mexico. *J. Appl. Phycol.* 20:169–75.

Prado Fernandez, J., Rodríguez Vazquez, J. A., Tojo, E. & Andrade, J. M. 2003. Quantitation of κ-, ι, and λ-carrageenan by mid-infrared spectroscopy and PLS regression. *Anal. Chim. Acta* 480:23–37.

Robic, A., Sassi, J. F., Dion, P., Lerat, Y. & Lahaye, M. 2009. Seasonal variability of physicochemical and rheological properties of ulvan in two *Ulva* species (Chlorophyta) from the Brittany coast. *J. Phycol.* 45:962–73.

Skriptsova, A. V., Shevchenko, N. M., Zvyagintseva, T. N. & Imbs, T. I. 2010. Monthly changes in the content and monosaccharide composition of fucoidan from *Undaria pinnatifida* (Laminariales, Phaeophyta). *J. Appl. Phycol.* 22:79–86.

Stevenson, T. T. & Furneaux, R. H. 1991. Chemical methods for the analysis of sulphated galactans from red algae. *Carbohydr. Res.* 210:277–98.

Thero, T. & Hartiala, K. 1971. Method for determination of the sulfate content of glycosaminoglycans. *Anal. Biochem.* 41:471–6.

Tveter-Gallagher, E., Cogburn, J. N., Mathieson, A. C. & Schiff, J. A. 1984. Localisation of carrageenan and incorporation of $^{35}SO_4^{-2}$ into sulphur amino acids in the red alga *Chondrus crispus*. *Hydrobiologia* 116/117:488–92.

Usov, A. I. 1998. Structural analysis of red seaweed galactans of agar and carrageenan groups. *Food Hydrocoll.* 12:301–8.

Usov, A. I. & Klochkova, N. G. 1992. Polysaccharides of algae. 45. Polysaccharide composition of red seaweeds from Kamchatka coastal waters (northwestern Pacific) studied by reductive hydrolysis of the biomass. *Bot. Mar.* 35:371–8.

Verbruggen, H., Leliaert, F., Maggs, C. A., Shimada, S., Schils, T., Provan, J., Booth, D., et al. 2007. Species boundaries and phylogenetic relationship within the green algal genus *Codium*

(Bryopsidales) based on plastid DNA sequences. *Mol. Phylogenet. Evol.* 44:240–54.

Weinberger, F., Guillemin, M. L., Destombe, C., Valero, M., Faugeron, S., Correa, J. A., Pohnert, G., Pehlke, C., Kloareg, B. & Potin, P. 2010. Defense evolution in the Gracilariaceae (rhodophyta): substrate-regulated oxidation of agar oligosaccharides is more ancient than the oligoagar-activated oxidative burst. *J. Phycol.* 46:958–68.

Weinberger, F., Leonardi, P., Miravalles, A., Correa, J. A., Lion, U., Kloareg, B. & Potin, P. 2005. Dissection of two distinct defense-related responses to agar oligosaccharides in *Gracilaria chilensis* (Rhodophyta) and *Gracilaria conferta* (Rhodophyta). *J. Phycol.* 41:863–73.

Weinberger, F., Richard, C., Kloareg, B., Kashman, Y., Hoppe, H. G. & Friedlander, M. 2001. Structure-activity relationships of oligoagar elicitors toward *Gracilaria conferta* (Rhodophyta). *J. Phycol.* 37:418–26.

Wong, K. F. & Craigie, J. S. 1978. Sulfohydrolase activity and carrageenan biosynthesis in *Chondrus crispus* (Rhodophyceae). *Plant Physiol* 61:663–6.

Wu, Y. & Gretz, M. R. 1990. Stable isotope labelling method for studies of saccharide metabolism in *Agardhiella subulata*. *Hydrobiologia* 260/261:595–600.

Supplementary Material

The following supplementary material is available for this article:

Figure S1. Geographic distribution of *Codium vermilara* and collection points in Argentina.

Figure S2. Fourier transform infrared (FTIR) spectra of MeOH-extracted and nonextracted samples M6 and M7 from La Farola, LF (Argentina).

Table S1. Chemical analysis of actively growing (apices) and resting (bases) regions of the thallus from different plants of *Codium vermilara* collected in December 2007 (LF, M4).

Table S2. Chemical characterization of model cell wall polymers used in FTIR-PC analysis.

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